COMPUTER SIMULATION OF METAL-ION EQUILIBRIA IN BIOCHEMICAL SYSTEMS : MODELS FOR BLOOD PLASMA

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by

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The only way of real advance in biology lies in the taking as our starting point, not the separated parts of an organism and its environment, but the whole organism in its actual relation to environment, and defining the parts and activities in this whole in terms implying their existing relationships to the other parts and activities.

J.B.S. Haldane.
(cited in ref. 378)

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SUMMARY

This thesis describes an investigation, by computer simulation, into the nature of the metal ion binding to low molecular weight ligands in blood plasma. A successful attempt is made to accommodate the effects of metal protein binding on the computed distribution that is obtained. An evaluation of the results is undertaken. The value and some applications of the knowledge arising from this kind of study are examined.

The collection, assembly and processing of the data is described. A computer program is written to cope with the very large equilibrium systems that are simulated. The experimentally determined values for the formation constants of the metal ion ligand complexing reactions in the biofluid are found in the literature. These are corrected whenever they are not applicable to physiological conditions of temperature and ionic strength. Where no experimental values were available, formation constants for complexes that seemed likely to be important were estimated using certain types of chemical trend.

The results of the blood plasma model may be summarized as follows. Copper and ferric iron are found to exist exclusively as ternary complexes except that the copper dihistidinato complex is important. With copper, these ternary complexes always involve histidine whilst citrate plays an analogous role in the ferric complex formation. Calcium, magnesium and manganese do not appear to exist as ternary complexes. With these three cations the bicarbonate species predominate although the binding is weak, as a consequence of the

relatively high ligand concentration in plasma. Zinc and lead form both binary and ternary complexes. The ternary zinc cysteinate citrate complex is found to account for a significant percentage of the low molecular weight complex fraction of this metal. This result is in contrast to those of previous models.

C O N T E N T S

APPENDICES

CHAPTER ONE

INTRODUCTION

1.1 Motivation for the research.

This thesis describes an investigation, by computer simulation into the nature of the metal ion binding to low molecular weight ligands in blood plasma. The study is motivated by the large number of biochemical systems which depend on metal ion participation and by the central and pervading role that is played by blood in mammalian physiology. Attention is focused on this biofluid because it has been the subject of extensive research and so there exists in the literature not only a substantial background of information but also much of the necessary data. However, it is emphasised at the outset, that the kind of analysis developed for plasma may in principle, also be applied to other physiological solutions.

The primary object of this research lies in the elucidation of the equilibrium distribution of the complexes formed in multi-component biosolutions by the transition metal ions copper(II), iron(III), lead(II), manganese(II) and zinc(II). These metals all occur in plasma in very small quantities. Their low concentrations *in vivo* are indicative of their catalytic function and are unrepresentative of their considerable biological importance. Total concentrations in normal human plasma fall between 50 and 0,1μM (534) which means that, in general, experimental measurements are difficult and that, as yet, there is no practical method of determining the free metal ion concentrations. In addition, the problems with experimental assessment of the equilibrium concentrations are heightened both by the large number of potential ligands that occur in biofluids and by the fact that probes which interact chemically with the system

are likely to upset the very distribution one is attempting to monitor. For these reasons, simulation of the metal ion ligand equilibria in biofluids using high-speed computers constitutes the only current method of achieving the proposed objective.

Metal ions in biofluids occur in a number of distinctly separate fractions each of which is characterized by the type of chelation involved. Apart from the non-chelated, free metal ions themselves, metals are bound both to low molecular weight ligands and to proteins. Further, the protein bound amount may be divided into two : a fraction in which the metal is loosely held and in labile equilibrium with other similar ions in solution and the remainder which is tightly bound and therefore not exchangeable (see, for example, 552). To all intents, the latter non-exchangeable metalloproteins do not have any direct or dynamic part to play in the strong competition for the relatively scarce transition elements and thus, are of little immediate pertinence to the present study. However, the other protein fraction includes the large percentage of the bound but labile metal ions in the equilibrium pool and as such is likely to determine, at least in part, the concentrations of the low molecular weight complexes in the fluid. As the metal protein interactions are at present not sufficiently well characterized to incorporate them in a simulation of the whole system, this raises a fundamental obstacle which must be overcome or circumnavigated before a realistic picture of the detailed distribution of metal ions in physiological solutions can be constructed. A significant portion of this work is consequently directed as establishing means whereby the effect of labile metal protein complexes on the rest of the

equilibrium system can be accommodated.

Although the low molecular weight fraction of metal ion complexes is small in contrast to the amount of metal bound to protein, its significance *in vivo* should not be underestimated. There is a clear need for a detailed knowledge of the equilibrium concentrations of these complexes because, expecially in the case of the transition metals where the free ion concentration is always very small, complexes of low molecular weight ligands play an important background role in many vital biochemical and physiological processes (see, for example, 534, 507). They are likely to be involved as intermediates when metal ions are inserted into or removed from metalloenzymes or carrier proteins. There is a growing body of evidence which implicates them in the transfer of some metal ions across membranes. In addition, chelation is an effective means of keeping essential metals in solution and it can also be exploited in nature as part of a procedure for altering the potential of certain redox couples. In fact, the concentrations of low molecular weight complexes provide a foundation not only for the metal ion chemistry that takes place in the biofluid itself but also, less directly, for the reactions and equilibria which are set up within cells supported by the medium.

Finally, once the normal equilibrium state of the low molecular weight fraction has been successfully simulated, the stresses which are imposed on the system by metal ion poisoning, deficiency or by drugs can also be investigated. This may throw light on the mechanisms whereby some of these imbalances exert their effects and consequently suggest ways of improving current therapy or of reducing undesirable side-effects.

1.2 The biological role of metal ions.

It is not easy to keep the biological significance of the transition metals in true perspective. This is well illustrated in the literature where those reports which deal specifically with metal ions *in vivo* tend to emphasise their indispensible role whilst elsewhere this is often completely neglected. The polarization of viewpoints has also been intensified by attempts to correct the inadequate impression which has been created by a predominance of organically-orientated investigations (543, 536). So, before focusing on the detailed biochemical mechanisms in which metal ions constitute an essential part, it is as well to consider their overall position in the life process. Hopefully, this will, to some extent, compensate for the bias which is almost inherent in a review of this subject and if, by reflecting that life is indeed chiefly 'organic', it highlights the imposing array of auxillary services that the transition metals provide, then it will have served a useful purpose.

Of all that is known about the chemistry of life, little can be more impressive than the fact that the vast majority of the very considerable number of compounds employed in living systems consist of less than half a dozen elements. The molecules which can be constructed from only carbon, hydrogen, oxygen and nitrogen are infinite both in number and variety and it is primarily this flexibility which makes a chemical life feasible. These four elements account for more than 99 percent of the atoms in a human body (301). However, in spite of all the organic possibilities, all biosystems employ to a greater or lesser extent a generous

selection of inorganic elements. These are used because they provide a wide range of additional chemical facilities. The importance of this inorganic contribution centres on the ability of most of these elements to exist in aqueous solution as stable ions. The transition metals in particular possess two additional features which have a key role to play in the overall biochemical scheme. These are the capacity to bond, co-ordinately and with a particular stereochemistry, to electron donors (especially oxygen and nitrogen) and the ability to set up easily reversible electron transfer systems. These features are by no means the prerogative of the transition metals, but their special usefulness arises because their reaction energies are often moderate enough for them to participate in cyclic processes (619) and because the ranges of properties which the series exhibits makes it easier to meet exact chemical requirements. Thus, the prominence of metals arises largely because without them, the full organic potential could not be biologically realized.

A consideration of the pathways by which primary organic metabolites are interrelated and synthesised into the diverse and often sophisticated compounds required for biological growth, reveals that very many chemical reactions take place *in vivo* under conditions that can truly be described as astonishingly mild. It is no wonder that prior to Wohler's preparation of urea, popular scientific belief held that organic materials could only be constructed under the influence of a so-called 'life force'. In terms of the ordinary reactions conducted in laboratories, it is not only difficult to imagine how all the life processes are so unerringly accomplished and the various syntheses so precisely controlled but it is also

hard to comprehend how so many transformations take place with apparently minimal energy of activation. Today, with hindsight, it is clear that all living organisms have at their disposal a large number of catalytic mechanisms embodied, for the most part, in their enzyme activity. These catalytic properties together with the various energy transfer and storage systems provide the basic tools employed in metabolism. It is in both these sets of biochemical processes that the transition metals are intimately involved.

Throughout the scientific disciplines, generalization has proved an invaluable aid in understanding natural order so the fundamental trends in the biochemical properties of metal ions need to be considered before concentrating on individual systems. The classification of cations in a biological sense is not a recent innovation for the pattern which emerges from the grouping of elements in the periodic table is too clear to be obscured. During the last decade, however, the concept of a fine delineation that emphasises biological features has been advanced, primarily by R.J.P. Williams (2, 95, 100, 73, 50). Using several selected characteristics, he has stressed the differences between the various types of cation whilst simultaneously pointing to the graduation of properties between them. The criteria used are essentially Pearson's hard and soft acid and base (HSAB) principles (35, 100, 86, 87, 101) but these are applied in conjunction with the generally observed biochemical nature of the metal ion in question. A particularly characteristic property called 'mobility' by Williams, describes the ease with which the ion is able to move about *in vivo*. Table 1.1 summarises the picture which is produced (50) and with some additional emphasis

TABLE 1.1.

BIOLOGICAL CLASSIFICATION OF CATIONS.

CATION	FUNCTION	HSAB PREFERENCE	COMPLEX STABILITY	MOBILITY OF IONS
SODIUM POTASSIUM	Charge Carriers	Hard oxygen anions	Weak	Fast
CALCIUM MAGNESIUM	Structure formers and trigger mechanisms	Hard, oxygen anions	Moderate	Moderate
IRON COPPER COBALT	Redox catalysts	Inter-mediate nitrogen ligands	Strong	Slow
ZINC	Super-acid catalyst	Inter-mediate nitrogen/ sulphur ligands.	Strong	Slow

on the analagous trends within the transition series itself, provides a helpful background to the variation in the biological roles played by the individual elements. In strong contrast to the Group IA and IIA elements, the transition metals act predominently as redox catalysts and they are rather immobile *in vivo* because they form strong complexes, preferentially with nitrogen and sulphur donors. Zinc is somewhat exceptional in that the d^{10} ion is not prone to a change of its valence state and so catalytic action is usually confined to its Lewis-acid nature. No doubt, unique abilities in this regard have promoted the evolutionary selection of this metal in spite of the fact that it is far scarcer than less familiar and less used elements such as zirconium, vanadium, titanium and strontium (293). Manganese, at the opposite end of the first series, behaves more like calcium and magnesium. Consequently, its biochemistry is often more in line with these Group IIA cations than that of the other transition metals (95).

1.2.1 The essential elements.

The transition metals are not the only 'trace' inorganics necessary for plant and animal life although they are amongst the more abundant. There are, in total, twenty five elements presently regarded as essential and these are listed in Table 1.2 (294, 301, 196, 601). It can be seen that most of these appear in characteristically minute quantities and the current interest in trace element mechanisms follows from the recognition that importance is not *a priori* reflected in biological concentration. The major effects produced by small

amounts of metal, in particular, have profound implications as far as nutrition, therapeutics and pollution are concerned. It is only by understanding the processes involved and the consequences of disturbing the equilibrium balance that damage to delicate life mechanisms may be foreseen and thus avoided. Attempts to define the set of indespensible elements have been largely motivated by the desire to ascertain the minimal nutritional requirements needed for good health. These have, however, also set the stage for investigation into the mechanisms whereby specific elements exert their vital properties. The ultimate criterion which determines whether a given element is essential, is of course, the detrimental effect on growth that is produced by the exclusion of the substance from every potential source and the reversal of such symptoms once the deficiency is corrected. Often, it is only when the function of an *in vivo* process is impaired that it draws attention to itself. Thus, the understanding of the normal role of trace elements is often aided by the disruption of their physiology.

Recently, very sophisticated techniques have been devised to test the need for various elements and these efforts have been rewarded in that the list of essentials which has been produced is now likely to be close to completion. The attainment of this objective could hitherto never have been claimed with certainty because of the rapid rise in the practical difficulties associated with diminishing concentrations. Those experiments in which the growth of rats is retarded when they are subjected to a stringent trace element isolation (301) illustrate the problems; although the control animals are fed the same ultra-pure diet, they remain

healthy because they can pick up sufficient quantities of the screened mineral from their surroundings. However, there are good grounds for suggesting that there is a limit to the number of elements that mammalian life requires and that this will not be many more than the twenty five already known (nickel, aluminium and boron have been suggested as essentials but their status is still uncertain). The most pertinent factor is the evolutionary disadvantage suffered by a species which is dependent on scarce commodities (2, 301, 72). Hence, the availability of an element must be weighed against its chemical usefulness. It is certainly no coincidence that the composition of the human body has some striking parallels with the composition of seawater and the earth's crust (294, 301). Also, solubility can limit biological utilization by preventing sufficient trace element uptake (2). Furthermore, the strong binding of nickel and chromium in the minerals of basic magmas has been used to account for the dearth of these elements in living systems (548). On the other hand, once a metal ion has been absorbed, chelation tends to prevent it being excreted so most organisms accumulate a broad spectrum of trace and ultratrace elements. Shell-fish, for example, are particularly prone to collecting heavy metals. Because nuclides such as polonium are about one million times more concentrated in the digestive glands of the rock-lobster, somewhat less than 7 kg of this material is officially regarded in South Africa as a radioactive source! (604, 605).

TABLE 1.2

THE ESSENTIAL ELEMENTS

ELEMENT	GRAMS PER 70 KG MAN	PERCENT OF TOTAL NUMBER OF ATOMS		
		IN MEN	IN SEAWATER	IN EARTH'S CRUST
1. OXYGEN	45000	25,5	33	47
2. CARBON	13000	9,5	0,001	0,2
3. HYDROGEN	7000	63	66	0,2
4. NITROGEN	2000	1,4	-	-
5. CALCIUM	1700	0,31	0,006	3,5
6. PHOSPHORUS	700	0,22	-	-
7. POTASSIUM	250	0,06	0,006	2,5
8. CHLORINE	115	0,03	0,33	-
9. SULPHUR	100	0,05	0,17	-
10 SODIUM	70	0,03	0,28	2,5
11. MAGNESIUM	40	0,01	0,033	2,2
12. IRON	4,3	-	-	4,5
13. FLUORINE	2,6	-	-	-
14. ZINC	2,3	-	-	-
15. COPPER	0,10	-	-	-
16. MANGANESE	0,013	-	-	-
17. SELENIUM	0,013	-	-	-
18. IODINE	0,011	-	-	-
19. MOLYBDENUM	0,009	-	-	-
20. CHROMIUM	0,003	-	-	-
21. COBALT	0,002	-	-	-
22. BROMINE	-	-	0,0005	-
23. SILICON	-	-	-	28
24. TIN	-	-	-	-
25. VANADIUM	-	-	-	-

1.2.2. The mechanisms of the biological activity of transition metal ions.

A detailed review of the biomechanisms in which transition metals are an intrinsic part would require a very lengthy exposition and is beyond the scope of this work. The field is growing rapidly and its development is well recorded in a number of collected reports, the most recent and comprehensive of which are 'Inorganic Biochemistry' (1973), edited by Eichhorn, G.L. and the series on 'Metal ions in biological systems' edited by Sigel, H. Here only an outline will be presented to indicate the substantial role of transition elements *in vivo* and to communicate several of the more fundamental ideas behind the simulation of the metal ion-ligand equilibria in biofluids. Evaluation of the metal complex concentrations in physiological media and a knowledge of how these concentrations are altered, either by accident or by design, may contribute considerably towards understanding the overall function of metal-protein systems. The inhibition of enzyme activity by heavy metal poisoning is today well known. Also, in the reversed sense, several investigations have concerned themselves with the effect of chelating agents on systems that are known to be metal ion dependent (154, 346, 347, 343, 549). In fact, it is the delicate balance of metal concentrations upon which optimum health depends (534). Furthermore, the perturbation of these concentrations, within the normal limits, activate and inhibit particular metabolic sequences and thereby constitute the metal ion contribution to the incredibly complicated system of physiological feedback controls.

Mention of the role that enzymes play in the biochemical synthesis of organic compounds has already been made. It seems no exaggeration to claim they are the key to life process; almost every metabolic step is catalysed by one enzyme or another (551). Their remarkable activity has been intensely investigated for many years and it appears, primarily, to be a consequence of the interplay between a number of their features rather than any particular one individually. The potential for modification in macromolecules generally, provides a variety which simply cannot be matched by smaller systems. This is reflected in the astonishing range of highly specific tasks that enzymes perform. Their diversity is exploited in two ways : the structure of the protein creates a geometrical environment in which ordinary chemical functions attain extraordinary properties (73) and the combination of groups, available from a broad spectrum of permutations, ensures that the individual parts are able to react together in a most synergistic manner (339). More specifically, a lowering of the activation energy for the desired reaction is accomplished by imposing a series of constraints on the catalysed mechanism. Amongst these are the introduction of strain in the ground state complex, the stabilization of intermediates, the immobilization of atoms involved in the bond breaking and making process and the advantageous positioning of high reactive functions capable of attacking normally inert sections of the substrate molecule (487, 372). In addition, the active site needs to be highly selective because catalytic efficiency must be high for one set of reactions but low for all others (534). Breslow has pointed out that the organisation of the site and the reactive groups must preclude the possibility of the enzyme 'biting' other parts of itself (372).

FIGURE 1.

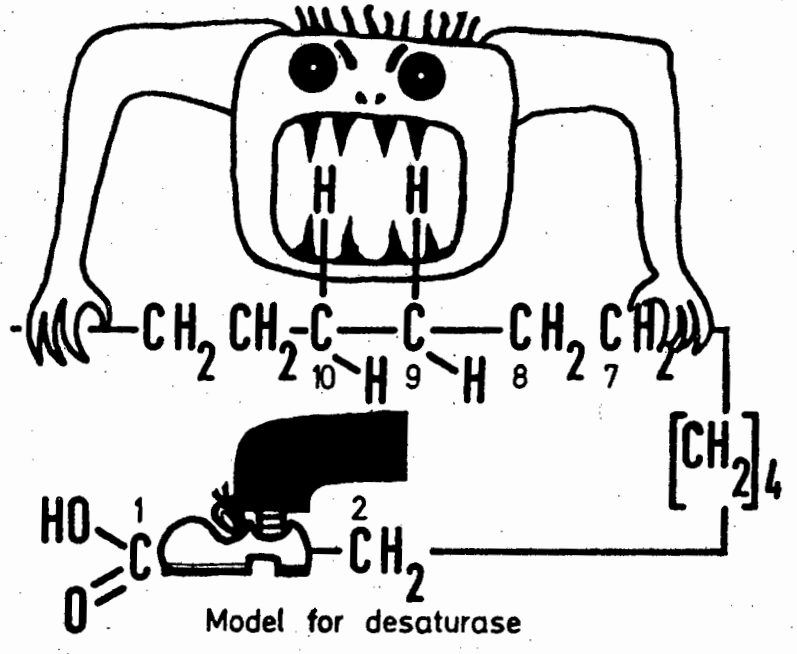


Figure 1 *A whimsical representation of the factors involved in selective enzymatic oxidation*

† from 372

Both selectivity and reactivity have been associated with another feature of proteins, namely, the fact that they exist as polyelectrolytes with definite regions of opposite hydrophylic and charge nature (339, 73). These changes produce unusual states of solvation (possibly similar to the boundary between two phases) which can enhance the general hybridization of properties and thereby create a chemical system completely without analogy in the realm of homogenous solvents and low molecular weight reagents. A sobering perspective of these phenomenal catalytic powers is provided in a theory of their evolution that has been advanced by Black to describe their contribution to the origin of life (562). This picture is well balanced by Mildvan when he notes that 'Before ascribing this combination of techniques to any undue wisdom of Mother Nature or her various male consorts, we must recall that she has had 10^9 years to evolve enzymes while man has had but 10^2 years to comprehend and duplicate them. Thus, like ordinary mortals, the forces of nature plod slowly along by trial and error occasionally making and order of magnitude advance, occasionally a mistake resulting in the disappearance of a species' (487).

Although the trend has certainly appeared, the division between 'organic' and 'inorganic' has not been as extreme in the study of enzymology as in so many other biochemical areas. The reasons for this are mainly twofold; most classes of enzymes have at least one member which is metal ion dependent (73) and the investigations into these complicated systems have been enormously aided by the fact that metals in the active centres provide a most convenient focus for a whole host of experimental probes. Since the metal ion is so physically and chemically distinct from the bulk

of protein surrounding it, monitoring the variations which occur in its environment are facilitated by its presence. In this way metals have been instrumental in revealing much of what is known about the general mechanism of enzyme action (552, 545, 609, 561). Apart from the measurements which can be made on the properties of the naturally occurring ion, substitution of one metal for another is a powerful experimental technique which has only partially been exploited. Furthermore, a recent innovation extends available methods by synthesising ligands with the specific objective of labelling metal-containing enzymes with radioactive isotopes. The investigators forsee that 'The preparation of chelating agents whose complexes can interact, in some selected manner, with biological macromolecules could make possible several new applications of metal ions as probes of biological systems' (614).

Evaluated in terms of the special requirements for enzyme activity, the transition cations are remarkably well endowed with suitable chemical properties. No undue coincidence is implied by this because no doubt these attributes directed evolution's course. It is nevertheless true that the transition metals' biological importance is an outcome of the usefulness of a combination of their features. As they are most effective co-ordinators, their ability to bind several donors simultaneously enables them to transform bimolecular to unimolecular reactions. Also, the independence of their inner and outer co-ordination spheres can be utilized for catalytic purposes (544, 487). Both their capacity to provide a reversible electron sink-and-source and their stereochemical constraints (which can be changed with oxidation state and other factors) are ideal.

While chelation of low molecular weight ligands is able to enhance their reactivity (338), macromolecules can exploit this effect to the full. So, it is not surprising to find that a broad range of metal-enzyme mechanisms have been described in terms of quite detailed aspects of the chelation at the active centre (581, 574, 456, 601).

For operational convenience, metal-enzyme interaction is classified on the basis of binding stability. Those proteins which hold the metal ion very tightly give rise to the so-called metalloenzymes which, typically, have sufficiently high binding constants to prevent the ion being extracted during the isolation and purification of the substance (349, 147). This property causes a stoichiometric relation between the metal and the protein and has led Vallee and Coleman to deduce, 'that this unique association has specific biological significance' (552). The roles of haemoglobin, chlorophyll and vitamin B_{12} are good examples. On the other hand, numerous labile metal-protein interactions occur with no specific or exclusive affinity for certain ion types and these are denoted by the term 'metal-enzyme complex'. The chief role of the metal in such systems is often just to stabilize a special conformation of the protein (552, 363). In general, these enzymes may be activated by a series of different metal ions. A reversible, competitive association with the metals is set up between the proteins and other ligands in solution. The equilibrium is easily displaced by powerful chelating agents which are therefore capable of inhibiting enzyme activity (552, 154, 346, 347). The re-activation which follows the replacement of the metal in question underlines the nature of the

metal's role and points to one of the possible means of controlling metabolic catalysis *in vivo*. Mildvan has suggested that metalloenzymes and metal-activated enzyme complexes can be distinguished by a stability constant value of 10^8 M^{-1} (574). This dividing line, however, is somewhat arbitrary and it is important to remember that the 'stability of metal-containing proteins, like that of simpler metal-ligand complexes, may be expected to span a considerable and continuous range of values depending of the number of donors and the geometry of the binding site' (552). Although the stoichiometry of metal-activated enzyme systems is not apparently constant (as in the case of metalloenzymes), it has been noted that in their functional state they also have a 1:1:1 ratio with respect to metals, enzyme sites and bound substrates (574). The enzymes in blood plasma which are activated or inhibited by metal ions appear in Table 1.3 (576, 577, 552).

Two additional aspects of metal ions in biological roles are worthy of specific mention although they are an integral part of the topics on energetics and synthesis which have been discussed. The first is the wide-spread formation of adducts of biologically important gaseous molecules by metals supported in a protein matrix. The phenomenon includes nitrogen fixation processes in which attachment of the molecule precedes its catalytic reduction and oxygen binding by haemoglobin or similar systems where reversibility is the essence of the reaction (582, 546, 541, 593, 401, 624, 483). The tremendous import of such processes on physiology is difficult to describe adequately. The second aspect which requires some mention, concerns the party played by metal ions in the structure and replication of the nucleic acids. Eichhorn and his co-workers have been amongst the

TABLE 1.3

ENZYMES IN BLOOD PLASMA WITH AN ACTIVITY THAT IS ALTERED BY METAL IONS

ENZYME	Metal activators	Catalytic activity	Metal inhibitors
Alcohol dehydrogenase	Zn^{++}	Alcohols into aldehydes	
Aldolase	Zn^{++}; Fe^{++}; Mn^{++}; Co^{++}	Hexose diphosphate into triose phosphate	Cu^{++}; Ag^{+}
Amylase		Hydrolysis of starch glucosidic bonds	Heavy metals
Alkaline phosphatase	Zn^{++}; Mg^{++}	Hydrolysis of orthophosphonic monoesters	
Arginase	Mn^{++}; Co^{++}; Ni^{++}	Arginine into Ornithine	
Ceruloplasmin	Cu^{++}	Oxidation reactions	
Glycylglycine peptidase	Mn^{++}	Hydrolysis of glycylglycyl glycine	
Glutamade-oxalacetate trans aminase		Amino group transfer	Heavy metals
Glutamate-pyruvate trans aminase		Amino group transfer	Heavy metals
Lactic dehydrogenase	Zn^{++}	Lactate into pyruvate	
Leucine aminopeptidase	Mn^{++}; Mg^{++}	Hydrolysis of peptides with free amino on N-terminal LEU.	
Lipase		Hydrolysis of dicarboxylic acids (fat)	Heavy metals
Malic dehydrogenase	Zn^{++}	Malate into oxalo acetate	
Phosphoglucomutase		Glucose-1-phosphate to Glucose-6-phosphate	Cu^{++}; Zn^{++}; Pb^{++}; Hg^{++}.
Plasma amine oxidase	Cu^{++}	Amino oxidation	

most instrumental in formulating the evidence which shows that 'virtually every step in the utilization of the genetic code for the eventual production of the proteins specified by the code is governed in some way by the presence of metal ions' (585), (542, 584), (231, 283). Possibly the most striking information in this regard is (a) the selectivity exhibited by certain ions which ensures that only deoxynucleotides as opposed to ribonucleotides are incorporated in DNA synthesis, (b) the ability of divalent ions to stabilize the DNA helix and (c) the role played by copper and zinc cations in the reversible unwinding and rewinding of the DNA and, in particular, the proper alignment of base pairs which is brought about by chelation.

1.2.3 Mineral metabolism and homeostasis.

Blood, as the chief distributor of nutrients, enjoys a prominent position in the scheme of metabolic activity and it merits attention amongst other reasons, simply because it is such a vital link in the processes of absorption, utilization and disposal. In many instances, this fluid acts not only as a transporter but also as a storage reservoir so it is appropriate to consider both these properties and specifically, to summarise the metabolism of the essential transition metals in this context.

The word 'homeostasis' embodies a variety of ideas (602, 603). The initial impression conveyed is almost invariably associated with the constancy exhibited by living systems, particularly with

regard to concentrations. In fact, this does reflect a fundamental connotation in that the well-being of an organism depends on limited fluctuations in its 'internal environment'. Actually, the control of this constancy is more to the point. Furthermore, the control applies not only to concentrations but also to factors such as the organism's body weight, temperature, fluid volumes, blood pressure, respiration rate and so on. Anything which has a bearing on the status of the 'internal environment' and which is subject to regulation is included. Moreover, no absolute constancy is necessarily implied because many homeostatic controls involve a systematic variation of the parameter in question. If control is regarded as messages which positively alter a set of characteristics of a receiver, then homeostasis may be seen as a cybernetic feedback which, at the most elementary level, links some kind of sensor to an organ which is capable of affecting the change required to return the parameter to its proper level. This demands that variation in the parameter is likely or essential and that self regulation is really the maintenance of a steady state. In terms of concentration, which is our primary concern, the controlling device must be able to alter the input or output of the substance either by directly influencing the supply or removal or by manipulating the amount stored in some kind of internal reservoir.

As far as transition metal homeostasis is concerned, a complicated and interrelated system has evolved because no universal process is capable of regulating the whole spectrum of essential elements and because trace quantities are particularly subject to severe disturbances as they lack any type of 'concentration buffering'.

Without a fairly advanced form of protection, inadvertent ingestion of small amounts of contaminant could easily result in poisoning. On the other hand, even with the most effective means of avoiding excretion, natural losses of skin, sweat, blood, hair and so on would soon cause general deficiencies if the body's reserves were not regularly replenished. So, it is clear that a very fine balance between supply and demand must be set up and maintained. Uptake of metal ions can be controlled by the secretion of chelators into stomach and intestinal fluids. Losses may be restricted by metal-binding once the ion has been absorbed. Excessive quantities may also be rejected by trapping the metal in mucosal cells thereby ensuring it is lost when the cell is finally sloughed. The differences in transition metal solubility, which are caused by the variation in pH along the gastro-intestinal tract, can also be exploited. Moreover, when natural losses prove insufficient, activation of excretion processes in organs such as the kidneys provides an additional resort. Excretion is, in fact, the predominant means of homeostatic adjustment for cations except iron. It has been shown to be controlled, in some cases, by hormones produced in various, distant sensor glands (563). In spite of this knowledge, much remains to be learned about such controls. It is easily appreciated that an understanding of both regulators and the sensors involved would be advanced by an improved perception of the equilibrium or near-equilibrium conditions under which they operate.

Although the characteristics of the transition metals and proteins have been described in an attempt to show how the extraordinary powers of enzymes are achieved, the specific effects

of these systems on animal physiology and the actual biochemical processes in which they are involved have yet to be considered. Many of the individual aspects will be presented in the summaries of copper, iron, manganese and zinc metabolism which follow but before concentrating on these, a broader perspective can be obtained by examining the role which they all play in connective tissue synthesis. The term 'connective tissue' means that bodily material which joins together the primary epithelial, muscle and nervous tissues (618). The cells producing it may secrete either the solid of cartilage and bone or the fibrous materials used in the construction of collagen, elastin, tendons and skin. Only recently has the participation of trace elements in this area of metabolism become regarded as a general phenomenon with each of the most biologically important transition metals individually involved (447). However, there now seems little doubt that they all have their own particular part to play, probably by their participation in at least one essential enzymic contribution. Both zinc and manganese are involved in the control of calcification processes (479, 436), whilst cobalt is implicated in lipid metabolism and copper in the formation of both collagen and elastin (480). Iron is required for the hydroxylation of lysine and proline in protocollagen synthesis (482) and it has long been known that zinc is essential for rapid healing of wounds (616, 617). Both skeletal and postural defects associated with a reduction in tissue mucopolysaccharide content have been attributed to manganese deficiency which impairs at least two different enzyme systems (481). In general, an inadequate supply of any of the above mentioned minerals eventually leads to connective tissue disorders and in turn, to the variety of pathologies which such

breakdowns imply.

Finally, there follows a short metabolic outline for each of the transition metals which contribute significantly to the pool of low molecular weight complexes in blood plasma. The reader is referred to a selection of comprehensive accounts on the metabolism of the other essential minerals (553, 620, 371, 571, 572, 573, 527, 528).

a) Copper (403, 567, 191, 596, 371, 587, 608, 610, 146, 530, 553, 620, 478, 633)

Due to uncertainty that the copper found in various organisms was possibly just a contaminant, this metal was not recognised as an essential element until the early nineteen twenties. Today, the established minimum requirement for a human being is about two milligrams per day and due to widespread occurence of the mineral it is difficult to consume a balanced diet which does not fulfil these requirements. Consequently, natural cooper deficiency has never been demonstrated to occur in human beings.

Absorption of copper occurs predominantly in the stomach and upper intestine. This process is usually about 30 percent efficient. There is evidence for both active and passive transport through the intestinal membrane. As certain ligands, especially amino acids, increase absorption it is likely that chelation is involved and it is possible that such complexes are absorbed intact.

Once the metal enters the bloodstream, it is transported by serum albumin to the liver where it is largely incorporated into ceruloplasmin. The degradation of this metalloprotein is possibly involved in the synthesis of cytochrome oxidase but little else is known about its catabolism except that ultimately some of its copper is found in the bile. No doubt, much of the non-ceruloplasmin copper *in vivo* is complexed to protein and peptides. As is often the case with heavy metals, copper tends to accumulate in specific organs rather than appear evenly throughout the body tissue. The liver, brain, heart and kidneys contain, in decreasing order, the highest concentrations.

Whenever copper deficiency is induced either artifically, by genetic defect or other diseases it is associated with a pernicious anaemia in all the species examined. The exact function of the metal in this regard is not known but is thought to involve mobilization of iron from body stores, possibly by oxidase conversion of the ferrous to the ferric state.

Important copper-containing enzymes include ceruloplasmin, cytochrome oxidase, monamine oxidase, tyrosinase, laccase, superoxide dismutase, uricase and ascorbic acid oxidase. Copper is essential in the formation of both elastin and collagen where it is involved in the cross-linking of chains and so deficiency results in defective connective tissue, bone disorders, lack of pigmentation and cardio-vascular failure. It has long been known that copper is essential for the normal keratinization of skin.

Excretion of copper is almost exclusively via the faeces and negligible quantities appear in urine. Bile is involved in the homeostatic control of this metal and half of the copper therein is bound to proteins other than ceruloplasmin.

b) Iron (566, 553, 419, 103, 295, 468, 396, 485, 597, 405, 593, 138, 507, 344, 424, 400, 590, 174, 175, 442, 443, 444, 458, 459, 620, 297, 298, 420, 522, 464, 386, 613, 625, 589, 632, 441, 323, 172, 401, 661).

If the theme propounding the essentiality of many transition elements has created the impression that all carry an equal priority, then this perspective should be corrected by even the shortest account of iron metabolism. That this metal is paramount amongst the trace elements is clear whether assessment is made in terms of quantity or with regard to the number and consequence of the functions for which it is physiologically responsible. This is well expressed by Neilands when he wrote 'Among those elements essential to life, iron enjoys a status of extraordinary importance. It is involved in storage and transport of oxygen, in electron transport, in the metabolism of N_2 and H_2, in the reduction of ribotides to deoxyribotides (precursors of DNA), in oxidation and hydroxylation of a host of inorganic and organic metabolites and, finally, in the decomposition or utilization of hydrogen peroxide'. It is no wonder that he is drawn to the conclusion that 'Life, in any form, without iron is in all likelihood impossible' (103).

The homeostatic control of iron is quite different from all the other essential transition elements principally because there is, except in circumstances of the most chronic overload, negligible physiological ability to excrete the metal. This is probably an evolutionary consequence of the fact that only about one fifth of the dietary intake can actually be absorbed. Because such an amount only just replaces the natural loss, (a little through the faeces, and the

rest in blood, sweat, tears and so on) there is obvious difficulty in avoiding negative iron balance. As a matter of concern, iron deficiency anaemia has been said to be second only to protein malnutrition in the number of people it affects. Lack of iron depresses growth, causes a reduced resistance to infection and as a consequence of iron's contribution to the body's energy supply processes, produces fatigue, listlessness and palpitation upon exertion. Thus, this mineral needs to be very carefully husbanded. In stark contrast to the fate of the porphyrin moeity from haemoglobin, the metal is removed in the protein degradation and returned to the storage pool. The critical nature of the position is illustrated by the difference in the daily minimum requirements of adults : whilst men need about 1,0 mg/day and can usually satisfy this demand, pre-menopausal women need 1,5 mg/day and are often iron deficient.

As the homeostatic mechanism for iron does not lie with excretion, investigation into the process and control of absorption has been both determined and intense. However, other than establishing that absorption does indeed regulate the induction of the metal, according to iron status, little has been unequivocably discovered that may suggest exactly how this is managed. Absorption of iron takes place throughout the intestine but mainly in the duodenum and many studies have shown that the principal factor involved is simply that of iron solubility. Anything which tends to make the iron more soluble thereby preventing hydroxide precipitation as the pH rises upon exit from the acid stomach, will promote iron absorption. So, ferrous forms are preferred to ferric. Furthermore, both chelators and reducing agents are almost always beneficial. Ascorbic acid is

well established for its enhancement in both these regards. Alternatively, substances, which form insoluble salts such as phosphate, oxalate and phytate (from cereals) depress absorption, as do excessive quantities of citrate which encourage polymerization. Returning to factors which could regulate the process, no humoral substances whose effect is not attributable to indirect action, have been identified. Moreover, the hypothesis that the phenomenon is controlled by conformational changes, induced by saturation, in the plasma iron-binding protein, transferrin, has failed to be verified *in vivo*. Suggestions which assign the control to substances secreted into the intestinal milieu have produced a fair measure of confusion and much controversy. Such secretions have been claimed to regulate passage of iron through the intestinal membrane by chelating the metal whilst it is rendered soluble in the acid content of the stomach. Whether this binding enhances or inhibits absorption, however, is, in the face of contradictory assertions, unclear and governance by such means is currently thought to be unlikely. More attractive is the concept that iron remains solubilized, if not by co-ordination to low molecular weight-ligands, then by binding to a mucopolysaccharide which can carry the metal to the mucosal surface. In any event, the macromolecular ligand is unable to penetrate the membrane. Hence, iron must be released either to acceptor sites on the intestinal brush borders or to small chelators such as citrate, ascorbic acid or low molecular weight sugars.

As soon as the iron enters the bloodstream, it is rapidly bound into the primary transport form, transferrin. This metal-protein complex has great stability because the free iron concentration must

be kept very low to prevent precipitation and bacterial growth. Actually, the co-ordination is of sufficient strength to prevent iron exchange even between transferrin molecules unless the swop is mediated by a powerful ligand such as citrate. As yet, the exact mechanism of physiological extraction from this avid protein is not known but is likely to involve one or more of the following alterations : (i) reduction of the metal (ferrous ion is bound only weakly, if at all), (ii) transfer to low molecular weight adducts and (iii) conformational changes of the protein. Whether ligands are involved or not, it is evident at this stage that the investigation into both the absorption and transport processes would benefit from any information pertaining to the concentrations of low molecular weight iron complexes *in vivo*.

Most of the transferrin iron is delivered to the erythroid marrow for haemoglobin synthesis but a significant fraction is transported to the liver for storage and other purposes. At the marrow, the iron is transferred into reticulocytes which show a marked preference for the iron-laden transferrin over the apoprotein. Radioactive studies have shown that absorbed metal can be incorporated in haemoglobin within a mere four hours and that a complete dose is utilized within a week. It is this production of red blood corpuscles which is first affected by iron deficiency. Indeed, the reduced concentration of haemoglobin in erythrocytes is a principal indication of this common condition. Haemoglobin normally comprises just less than seventy percent of the body's total iron whilst myoglobin, the oxygen binder in muscle tissue, holds about three percent. Most of the remaining iron is stored as ferritin, a macromolecule consisting of a relatively large inner sphere of ferric hydroxyphosphate polymer

surrounded by protein. Chelating agents have also been implicated in the transfer of iron to and from the ferritin reservoir.

c) Manganese (553, 568, 481, 462, 620, 257)

The metabolism of copper, iron and zinc have all been far more extensively investigated and are much better understood than that of manganese. Even today, knowledge of manganese in physiology exhibits a noticeable dearth of unifying principles. With the single exception of skeletal defects, manganese deficiency symptoms are relatively difficult to induce. This state of affairs is due, in large measure, to the ability of other ions, particularly magnesium, to substitute for this transition element with only a small deterioration in biochemical efficiency. By no means does this imply that manganese is a superfluous mineral (it was recognised as essential about 1930) but the systems in which it participates can often continue operating with an alternative metal ion activator.

The normal intake of manganese is about five milligrams per day. Precisely where absorption best takes place is still not clear but the locality is certainly not as specific as it is with iron. Excretion provides the principal means of homeostatic control. It seems well established that much of the metal that is returned to the intestine is reabsorbed and subsequently again excreted. This cyclical procedure underlies many of the experimental difficulties which are encountered with this mineral. Excretion takes place largely by way of the bile. Hence, manganese in urine is usually negligible. Most mammals have a particularly high tolerance towards overdoses of the metal because excretion is both quick and thorough. This efficiency introduces its own disadvantages, however, because the excretory process, although it can be moderated, cannot be curtailed

completely so any cut-off in the dietary supply brings on the symptoms of deficiency.

Manganese exists chiefly as the divalent ion but provided the trivalent form is stabilized by some complexation, it also appears *in vivo* and has an important role to play. Concentrations of the mineral are highest in bone, pituitary gland, mammary glands and liver, in that order. The state in plasma is rather unclear but some manganese is definitely bound to transferrin. Most of the remainder is associated with another β_1-globulin or serum albumin. The chief enzyme systems in which it participates are pyruvate carboxylase (for CO_2 fixation), arginase, isocitrate dehydrogenase and glycosyl transferase. This last enzyme is responsible for an essential step in mucopoly-saccharide synthesis and it is this which causes the connective tissue defects which are brought on by manganese deficiency. Apart from the skeletal abnormalities mentioned, other effects which arise from deprivation of this metal are the development of congenital ataxia and a loss of balance due to defects in the construction of the inner ear.

d) Zinc. (553, 293, 569, 349, 529, 479, 362, 289, 371, 620, 5, 555, 616, 617).

The occurrence of zinc in living material is quite universal and whilst this in itself pointed to the metal's essentiality, general acknowledgement of its indispensibility to life was not achieved until 1934 because the widespread appearance also made this difficult to prove. The minimum daily requirement is still not well established as deficiency is highly unlikely to develop in persons receiving an average diet. The figure is probably upward of five milligrams per day compared with a normal daily intake of between ten and fifteen milligrams. Death from zinc deficiency has never been observed although severe ill-effects which commonly include lesions of the skin, skeletal defects, reproductive organ abnormalities, dwarfism and hypogonadism are often associated with a general malnutrition. Accordingly, these have been noted particularly in the Middle East.

Absorption of the metal occurs chiefly in the distal portion of the small intestine. Once again, complexation to low molecular weight ligands has an important function because zinc solubility drops sharply as it moves from an acid to a neutral environment. Amino acids, peptides and some synthetic chelating agents increase absorption in contrast to phosphate and phytate which form insoluble compounds with the metal ions.

In blood, about eighty percent of the zinc is held in the erythrocytes (almost all of this is contained in carbonic anhydrase), fifteen percent occurs in the plasma and approximately three percent in the leucocytes. There is considerable exchange of the metal

between the red blood corpuscles and the plasma pool.

The bones (which are the major storage locality), tissues of the eye, the prostate gland and skin all exhibit high concentrations of zinc.

The remarkable acceleration in the healing of wounds which follows zinc administration is well known, and has been attributed to the metal's role in connective tissue synthesis. Zinc has long been regarded as an integral part of insulin although this is no longer accepted implicitly because of the impossiblity of an *in vivo* investigation in the absence of the metal. The number of zinc metalloenzymes and metal-activated enzyme complexes is legion, amongst the more important being carboxypeptidase, carbonic anhydrase, alkaline phosphatase, uricase, arginase, enolase and the alcohol malate and glutamate dehydrogenases.

Most of the excretion of zinc is in the faeces although significant amounts are lost in sweat and skin. Somewhat less than ten percent of the dietary intake appears in urine. Clearance rates vary widely from one organ to another. Homeostatic control resides primarily with excretion but there is evidence that regulation is also imposed upon absorption in spite of the fact that the absorption mechanism probably does not involve an active transport.

1.2.4 Transition metals and disease.

In their discussion about the implications of trace metals in human diseases, McCall *et al.* have commented that the 'trace metals are more diverse, more interdisciplinary, and, often, more misunderstood than any other group of constituents essential to biological function' (478). Now, although this is, indeed, applicable to all trace mineral biochemistry, it is particularly pertinent in the context of metabolic malfunction. Whilst an enormous volume of data has been collected on the fluctuations of metal concentrations *in vivo* that occur with a host of illnesses, it is difficult to co-ordinate this information and to formulate hypotheses that may suggest how and why these concentration changes take place. The authors mentioned above, go on to say that trace metals 'have been implicated in diseases since it was first shown that metals were an integral part of metabolism' and that there 'are few diseases of any consequence which are not accompanied by changes in the concentration of one or more trace metals in some tissues or body fluids'. Yet, even today, very few diagnostic techniques based on trace metal imbalance have been developed. Whilst there are statistically significant increases of copper and to a lesser degree, decreases of zinc with most pathological conditions, a large number of patients in each category exhibit values that are well within the normal ranges (631). The picture is further complicated by the treatment of diseases with drugs that may alter metal ion distributions by chelation (478).

Before the implications of the trace metal involvement in human diseases can begin to be appreciated, a great deal must still

be learned about the normal metabolic interrelationships that exist between trace metals. Once again, there is very little theory upon which to hang a good deal of experimental observation. In fact, only a few principles have emerged. Reports of antagonistic and sympathetic relationships between different metals abound in the literature. For example, excess dietary zinc can induce a copper deficiency which then upsets the metabolism of iron and thereby causes anaemia (567). Also, the effects of copper and other heavy metal excesses can be, at least partially, alleviated by zinc supplementation (569, 343). The inter-relationships of iron with other metals have been the most investigated (596, 523, 626, 627). The link between copper and iron metabolism is most important as it involves haemoglobin biosynthesis; the ferroxidase activity of ceruloplasmin releases iron from the body stores (547). The action of heavy metal poisons is believed to be due, in part, to the replacement of benevolent ions from protein and enzyme systems. Some interaction between different metals is attributable to competition for common intestinal absorption pathways (see, for example, 627). Also, it appears that the ratio between two metal concentrations in living systems is often more important than their absolute values (606).

Although transition metal concentrations *in vivo* are commonly observed to change during many pathological conditions, it is often difficult to establish the extent to which an imbalance is a 'cause' rather than an 'effect' of the disease and its symptoms. Nevertheless, for the purposes of this discussion, one may distinguish three situations in spite of the fact that there is actually a graduation between them. The first is the most clear-cut. This is because it is the extreme situation of poisoning or deficiency which

is imposed by an unbalanced diet. Obviously, such circumstances are ultimately responsible for all the symptoms which accompany them. The second is similar to the first in that the effects of poisoning or deficiency are also induced but in this case, these are due to a defect in the primary homeostatic control of a metal ion. The overall result is still, clearly, too much or too little of the metal in question. The third situation also entails alterations in the concentrations of metal ions as a consequence of the disease but it differs from the others in that the source of the disorder does not necessarily involve metal ions directly. The imbalances result from the disturbance of many related physiological reactions. Furthermore, the changes are less inclined to pivot on a single type of metal ion and the direction of the stress is not so pronounced because concentration upsets are more of an 'effect' than a 'cause' of the disease. A knowledge of metal ion participation in sicknesses belonging to the third category is likely to yield the richest reward because those cases in which there is a more direct metal involvement are relatively rare. At the same time, the very fact that the trends are less definite means that they are less consistent and harder to monitor, systemize and understand.

Cancer is an excellent example of a disease in the third category. This notorious and intensively studied disease is almost invariably associated with high zinc concentrations and low copper contents (293). Cancer cells, therefore, have a greater need for exogenous zinc than their normal counterparts (638). They usually show elevated potassium levels, reduced calcium concentrations and lower pH (638). Furst outlined the relationship between metals, ligands and cancer in 1963 (620). Since then, chemical carcinogenesis

as well as cancer therapy has been increasingly associated with both metals and ligands. Most non-metallic carcinogens are potential chelating agents which may, for example, inhibit enzyme systems and thereby initiate or promote the malignancy. On the other hand, many therapeutic agents are also potential metal ion binders and may be effective because they are able to transport the ions in question into or out of the neoplastic cell (320). There are few transition metals that are not potentially capable of inducing cancer (637) but this should be viewed in light of the fact that many widely used materials, some as innocent as glucose, have been made to produce malignancies in test animals. Conversely, a range of metal anti-tumour compounds is well known (72). Some platinum complexes, especially, are spectacularly effective in certain cases although these 'are not likely to be a panacea for all cancers' (320). They are believed to proceed by way of interaction with nuclear DNA. In this regard, stereo specificity has proved to be an important and intriguing factor. Other chelates, especially some involving copper, are also thought to exert their selective cytotoxic activity by inhibition of DNA synthesis (638). The investigation into the mechanisms whereby metals and ligands encourage or suppress carcinoma has now reached the stage where the actual design of drugs has crossed the threshold of feasibility. D.R. Williams has reveiwed metal ions and amino acids in this context (82). Such a coalition between chemistry and medicine is a powerful defence against disease and so it is hoped that the present research project can contribute towards this goal. There are many possible applications. For example, one may investigate the nature of the actual complex of the metal which exists *in vivo*. It should not be forgotten that in biofluids many species will be sufficiently labile to undergo modification, by ligand

substitution. Thus, the administered compound is not necessarily the active form.

Other diseases in which metal ion concentrations are characteristically disturbed but which cannot be classified as an expression of metal deficiency or poisoning are numerous. Representative examples include Diabetes, Encephalitis, Hodgkin's disease, Parkinson's syndrome, Rheumatoid Arthritis and Schizophrenia (294, 494, 491, 489, 490, 191, 293, 470). This does not mean that metal ion intake cannot influence the condition. On one hand, heavy metal poisoning can initiate or intensify the symptons (e.g. in Parkinson's syndrome (294)) whilst in different circumstances, metals and metal complexes may alleviate the condition (e.g. copper armbands and bangles are popularly thought to be beneficial in many Rheumatic illnesses and although this is difficult to substantiate scientifically there is a growing body of evidence in support of the contention (e.g. 493)). Rheumatoid arthritis, in fact, serves as a good reminder of the complicated interplay of metal ion reactions in physiology which must be unravelled before systematic chemical therapy can begin to succeed. Conflicting opinions as to the cause, treatment and the role of metal ions in this disease abound (see, for example, 469, 492, 495, 496). In this regard, consider the relationship between iron and copper metabolism. Few signs of Rheumatic affliction are more characteristic than anaemia (471). The cause of this is an inability to utilize iron body stores. This is bewildering because ceruloplasmin levels are often raised and, as mentioned, iron mobilization is one of the vital functions of the metalloenzyme (472, 497, 494). It is also tempting to speculate about the connection between copper-orientated therapy and the ceruloplasmin increase but the same increase

is commonly observed during most inflammatory diseases so a direct link is by no means certain (Meyers, O.L., private communication).

Attention may now be focused on those pathological conditions which are associated with actual metal poisoning and deficiency. In addition to cases which arise from an unsatisfactory metal intake, there is a number of physiological disorders which specifically interfere with the normal inorganic homeostasis control processes (474). Apart from the fact that a physiological malfunction may affect only a limited part of a mineral's metabolism and thus deplete or poison only a few body compartments, the consequences of too much or too little metal *in vivo* are obviously largely independent of the cause. For this reason, these two categories will now be considered together.

As outlined in Section 1.2.3, insufficiency of essential metals deactivates enzyme systems and thus, impairs the growth of a large variety of body tissues. This factor is primarily responsible for the specific physiological defects observed in metal deficiency diseases. Menkes's kinky hair disease, for example, arises from a genetic defect which prevents the proper absorption of copper. It causes arterial degeneration, cerebral degeneration, hypothermia, peculiar hair changes and bone lesions (622, 357, 403, 628, 6). Fortunately, there is no counterpart of this condition known in either zinc or manganese metabolism (474). Iron deficiency and the anaemia that results, have been dealt with in some detail (see, Section 1.2.3 and additional references 598, 599, 600). In all cases of deficiency, it is clearly desirable to be able to raise the bodily or compartmental metal concentrations. Section 3.2.4 indicates a possible means whereby the simulation of metal ion distributions in biofluids can indicate how low molecular weight complexes could be set up to bypass defective

absorption pathways and introduce metal directly into the bloodstream.

There is no doubt that a combination of effects contributes to the consequence of metal ion poisoning, whether this is the result of excessive intake or physiological disorder. The disruption of enzyme systems by malevolent replacement of essential types of metal ion is probably the most serious factor. It certainly underlies a large number of the symptoms which arise from this kind of biochemical trauma. The strong affinity for many biochemical sites by metal ions in general may severely interfere with numerous delicate organic processes whenever these must take place in the presence of high concentrations of the offending element. This could be over and above those instances when an essential metal ion is lost by competitive substitution. Many reviews detail the toxic effects of the whole range of trace elements (for two of the more recent, consult 343,549). For example, mercury, cadmium and lead all interfere with cerebral pyruvate metabolism and simulate thiamine deficiency (343). It is also known that lead toxicity interrupts the synthesis of both haem and globin; not surprizingly, this leads to anaemia (343). Under chronic conditions, all metals tend to be deposited in certain organs. This is obviously lethal unless arrested and medically reversed. The toxicity of excess metal ion has also been suggested to be due, at least in part, to the alterations which they can induce in cellular membranes. Changes in function and permeability are produced, especially by copper (623).

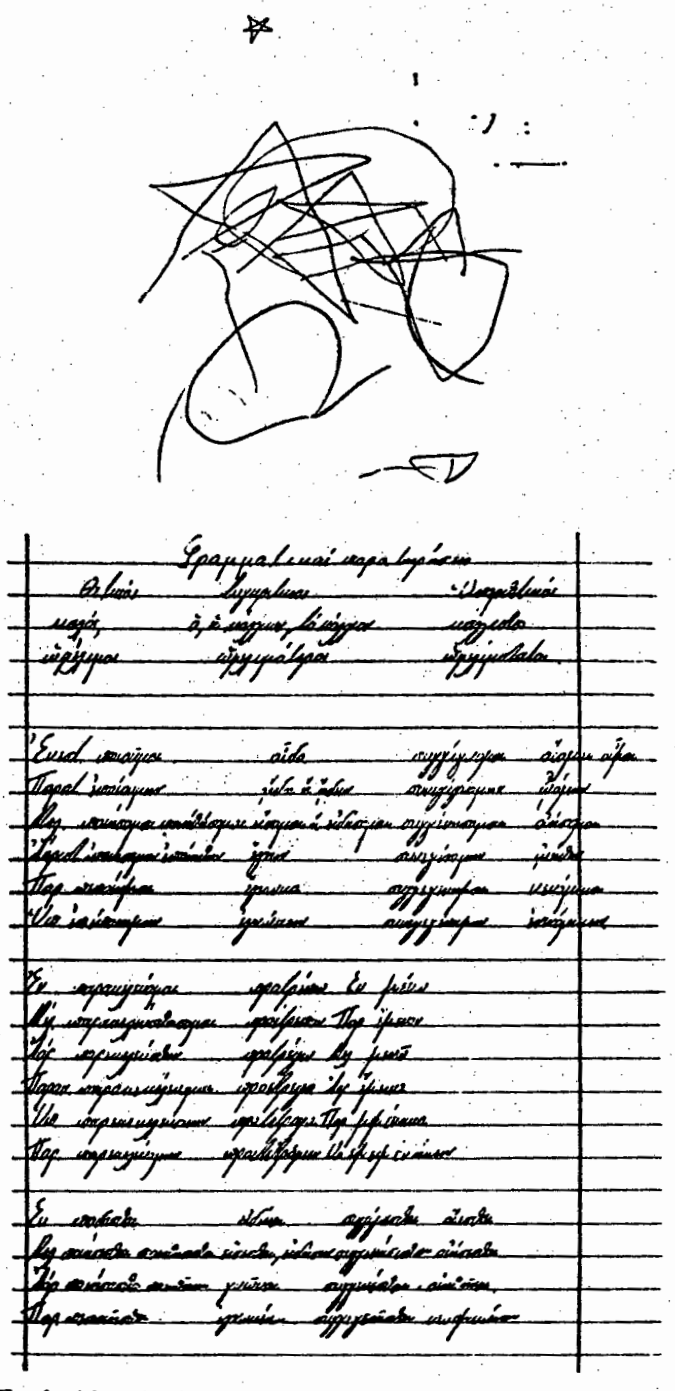
As far as severe metal overload is concerned, it is sufficient to consider just two of the more important conditions which belong to this pathological category. They serve as good illustrations

of the type of problem which may arise. The first of these diseases is particularly pertinent in the Southern African context as it is ubiquitous amongst the Bantu peoples of this subcontinent. It is called Siderosis (468). It differs from other iron overload conditions, such as idiopathic haemochromatosis, in that it is not brought about by some abnormality in mucosal behaviour. The overload results from gross excesses of dietary iron; between 100 and 200 mg per day are commonly ingested. This is to be compared with a daily requirements of about 1,5 mg and a normal Western diet which contains somewhat less than 20 mg per day (386). The iron stems from cooking pots and drums which are used to prepare food and an alcoholic, home-brewed 'beer'. The staple carbohydrate diet makes matters worse because the polysaccharides, by complexation, keep the metal solubilized in the relatively alkaline intestinal fluid and appear to transport the metal directly through the mucosal barrier. Once the iron binding capacity of the plasma transferrin is exceeded, the metal begins to be deposited in a number of organs, particularly the liver and urinary excretion increases markedly (405). It is estimated that the majority of Bantu suffer from Siderosis and that over 20 percent are severly affected (468). It is noteworthy that the normal, protective mucosal blocks are not infallible and, in fact, are clearly overwhelmed. This suggests that iron deficiency anaemia, a far more frequent occurence, may possibly be corrected by a therapeutic strategy based on a knowledge of how the mucosal mechanism is by-passed.

The other example of metal overload which is to be considered, is Wilson's disease. Here, copper levels are elevated as a result of an inability to discharge the metal from the liver (191). This syndrome, otherwise known as hepatolenticular degeneration, has been the subject of many hypotheses but to date, none of these can be

accepted as proven (403). Like most other inborn errors of metabolism, this rare disease is inherited in a recessive manner (341). Although a complete set of symptons is not common to every case of Wilson's disease, the very large majority of victims exhibit a variety of neurological abnormalities (such as tremor, dystonia and ataxia) with cirrhosis of the liver and less often, cartilage disorders (341). Most patients are about twenty years old when the condition is first detected although this is quite variable (191). The uncontrolled disease is lethal and sometimes swift but once again, this is subject to little uniformity (530). Death usually results from haemorrhage and liver failure. Copper concentrations are then invariably found to be elevated in the brain and the liver. The kidneys also usually show increased copper levels whilst plasma values are reduced as a secondary effect of the characteristically low ceruloplasmin concentrations (191). Failure to incorporate copper into this metalloprotein is a most significant aspect of the disease but the exact reason for this and its actual role in the consequences of the disease are still unclear. The presence of a protein with increased copper binding capacity could explain most clinical observations; it would appear that the defect must occur prior to both the excretion of copper and its incorporation into ceruloplasmin (403). This hypothesis has recently been supported by evidence for such a copper binding macromolecule but this has still to be confirmed (640). Although rare, Wilson's disease has been vigorously investigated (see, for example, 341, 191, 352). In this regard, it provides a perfect example of the contribution which research into physiological disorders can make towards the understanding of both normal and abnormal mineral metabolism (341).

FIGURE 2



Handwriting of N.Y., a patient with Wilson's disease: (upper) after one year of intermittent treatment with BAL, and (lower) again after 26 months of treatment with D-penicillamine HCl, 1800 mg daily.

† from 342

Today's growing concern with pollution is certainly a most powerful factor educating the man in the street towards some awareness of the biological implications of the heavy metals. In particular, the toxicity of many non-essential trace elements has been emphasized, sometimes to a point of hysteria. It has been primarily described in terms of industrial waste and the contamination of consumables. Whilst there is no denying the seriousness of both these instances, a better balance would recognize that all metals, even the most essential, are lethal in high concentration. Moreover, it would be wise to reflect that cases of acute poisoning represent only one side of the problem. 'Still unknown is the influence that small amounts of such substances have on man when ingested or inhaled over a prolonged period of time. Yet, this is probably the most important aspect of trace substance exposure' (549). One of the most frightening thoughts about this type of long term intake concerns the mental and psychological consequences. Metals tend to concentrate in all body organs and the brain is no exception. Metals are implicated in a number of intellectual disorders and in general behaviour (see, for example, 473, 513, 430, 432). A short consideration of lead, alone, will suffice to illustrate the point. Bryce-Smith has questioned the adequacy of modern food regulations pertaining to this metal (431). He claims that lead causes brain damage and produces a hyperactivity with a predisposition towards delinquent behaviour. This is supported by a study of lead blood levels of prison inmates compared with those of normal, city-dwelling compatriots (430, 432). These and other similar results merit deep concern, especially when once focuses on the high lead concentrations which have been found to accumulate in the blood of people residing close to some motorways in Britain (634).

Before leaving the topic of pollution and heavy metal toxicity, it is difficult to refrain from commenting on those individuals who, discontented with general background concentrations, wilfully expose themselves to considerable additional amounts of cadmium, nickel and other trace metal poisons. Cigarette tobacco contains relatively high concentrations of these substances. So, even moderate smokers annually inhale several times the amount of nickel required to induce tumours in rats (549). Inhalation, in fact, is a most efficient means of absorbing trace metals.

Although in this discussion little mention has so far been made of the role of medical chelating agents, it is clearly often desirable to administer a drug in order to remove unwanted metal ions. Some such compounds in everyday use are deferoxamine and penicillamine. The former helps in cases of iron overload (402) whilst penicillamine is the currently accepted treatment of Wilson's disease (342). In fact, the overall development of therapeutic ligands has not been neglected (7, 402, 236, 534, 536, 537, 188, 229) but until recently there has been a distinct lack of systematic approach. Progress has been largely a matter of trial and error, although occasionally compounds were suggested by analogy. Thus, even today, there are relatively few medical chelating agents in use and these tend to exhibit general rather than specific binding properties. For example, BAL (2,3 - dimercaptopropanol alias British Anti-Lewisite) was originally developed to treat the toxic effects of Lewisite (an arsenical mustard gas used in World War I) but has since been administered quite widely to counteract a variety of heavy metal poisons. Unfortunately, both EDTA (ethylenediamine-

tetra acetic acid) which is usually taken as its daughter compound calcium disodium edetate and penicillamine are also non-selective. These drugs are all well established and therefore enjoy an advantage because the risk inherent in the use of any drug is obviously greatest when it is first introduced. However, it should not be difficult to appreciate that a search for new ligands, especially for those which are able to discriminate between different metal ions, could produce some substantial improvements. Indiscriminant ligands tend to remove essential ions such as zinc in addition to the toxic ones. Moreover, many chelating agents are themselves poisonous, particularly in high concentrations. So, a systematic drug design must attempt to overcome both these limiting factors. In this regard, a number of principles are being increasingly recognised. Hard and soft acid and base properties must be exploited (534, 224). Whenever possible, drugs should be built from non-toxic sub-units such as amino acids which are harmless after the compound has been biologically degraded. Efforts to understand and simulate the binding sites of nature's metal-carrying macromolecules are potentially most rewarding. Both of the last two principles have been embodied in the synthesis of a peptide with which it is hope to improve upon penicillamine in the treatment of Wilson's disease (641). The tripeptide mimics the copper binding site of serum albumin and therefore, should be able to compete effectively with the protein *in vivo*. It is expected that the copper complex will be small enough to cross the biological membrane in the kidneys and hence, be excreted. Another development with much potential is the construction of cyclic molecules with selective ion binding in the interior of the ring (398). By matching the size of any chosen metal ion to the internal diameter

of such a ligand one hopes to ensure that it will discriminate in a pre-determined manner. Furthermore, the lessons which can be learned from medical chelating agents already in use should not be overlooked. For instance, in the search for powerful ligands it is easy to neglect the fact that unless ternary complexation formation is possible, the chelating agent may be unable to extract the ion from its macromolecular transporting agent. It has been suggested that the existance of a ternary complex is a necessary stage in the migration of copper from serum albumin to the penicillamine complex (269). If this is indeed the case, both denticity and ligand bulkiness are factors which should be taken into consideration. Finally, the performance of metal chelating agents in biofluids may be evaluated by computer simulation. This is considered in some detail in Section 3.2.4.

1.3 Blood and metal ion transport.

A comparison of the number of pages devoted to a synopsis on many of the body fluids by that excellent publication Documenta Geigy 'Scientific Tables' (577) clearly reveals the superiority of blood over the other fluids as far as accumulated knowledge is concerned. The sheer bulk of the data that has been collected about blood reflects both on its importance and on the relative ease with which it may be sampled and studied. The biological functions of this fluid are many and varied (618, 606). A means of supplying nutrients and removing wastes is inherently necessary whenever organs and tissues are physically separate. Thus, the movements of metabolites throughout the body is, obviously, a most fundamental concern. Bloods' role in respiration, alone, for example, requires countercurrents of oxygen and carbon dioxide. The homeostatic function of blood involves a variety of controls in addition to general service as a storage reservoir. These include acid-base homeostasis, body fluid balances and temperature regulation. Blood is also a key factor in the body's defence against infection.

Whole blood consists of a pale, straw-yellow fluid called plasma in which the platelets and the red and white blood corpuscles are suspended (618). The suspended particles are called 'the formed elements' of the blood. The platelets are cytoplasmic particles without a nucleus and are involved in the blood clotting process. The red blood corpuscles or erythrocytes also have no nucleus; their chief constituent is haemoglobin. They transport oxygen from the lungs to the tissues where it is used. On the other hand, the white cells or leucocytes do have a nucleus. They are primarily concerned

with the protection of the body from infection. Healthy adult males usually have a whole blood volume of about 5 litres, approximately one third of which is occupied by the formed elements (577, 618). Blood plasma consists of just over 91 percent water and about 7 percent protein (618). The total protein concentration of plasma is between 7 - 7,5g/100ml (606). Inorganic salts and low molecular weight organic metabolites constitute most of the small plasma fraction remaining.

There are three types of plasma protein in mammals (606, 618). The first is albumin, which accounts for over half of the total. This is a multipurpose macromolecule but is especially responsible for the maintenance of the osmotic pressure between the tissues and the blood vessels. Next, there are the globulins. These also have more than one function. They are responsible for the transport of lipids, hormones and sugars. Many of the γ-globulins are antibodies. The third type of plasma protein is fibrinogen. This particularly elongated macromolecule is a major contributor in the coagulation of blood. This coagulation is actually a most fascinating and complicated process which involves many so-called 'blood clotting factors' including some α- and β-globulins, as well as calcium ions. Blood serum is the liquid which is exuded from blood during the coagulation. It differs from plasma in that the fibrinogen is converted to fibrin during the clotting and is thus removed from the fluid (608). The relative sizes and shapes of the plasma proteins are shown in Figure 4 (from 618).

1.3.1 Metal binding proteins in blood plasma.

Proteins are the major transporting agents for metal ions in blood plasma. The protein-bound metal ion fraction is insignificant only for sodium and potassium. The percentage of these alkali metals attached to macromolecules is very small not only because their binding is weak but also as a consequence of their relatively high plasma concentrations (575). With calcium and magnesium, the metal ions complexed to protein are often in lower concentration than the free ion but, especially for calcium, the magnitudes of both these fractions are similar (577). In the case of the transition metals, however, proteins bind the very large proportion of these elements in plasma. The fraction of cations bound to low molecular weight ligands is invariably small. This is particularly true of the transition elements copper, iron, lead, manganese and zinc, with which this thesis is concerned. Nevertheless, Laurell has pointed out that even though a certain type of cation affinity may predominate, this does not exclude the possibility of the smaller fractions 'possessing a much higher specific activity in certain biochemical processes and thereby being of great physiological importance' (575). On the other hand, the disparity between the various metal ion fractions of plasma is of concern because the behaviour of the low molecular weight fraction may easily be dominated by metal-protein interaction. The protein bound metal fraction thus merits further consideration.

The protein which is most likely to control or seriously affect the low molecular weight distribution of most metals in blood plasma is albumin. This is not only because it has a relatively high concentration. Serum albumin has the least specific cation

affinity of all the important metal-binding proteins. Apart from anything else, it has a high net negative charge at physiological pH (190). In fact, the interaction of serum albumin with a whole range of metals has been experimentally investigated (see, for example, 22, 23, 24, 28, 31, 32, 33, 160, 178, 445, 446). It is potentially, a most powerful binding agent for the large majority of biologically important metals. As it contains numerous, 'hard', carboxylate groups, it is particularly well suited to co-ordinate the ions of calcium and magnesium, both of which appear in much larger concentrations than the transition metals. This protein is thus a dominating factor in the percentage of alkaline-earth ions in plasma which are protein bound. However, it also possesses, at its amino-terminal end, a copper-binding site of great importance in copper metabolism (31, 173, 177, 178, 180, 181, 190). It is this site which carries the metal, after it has been absorbed into the bloodstream, to the liver for incorporation into ceruloplasmin. In addition to serum albumin's role as a transporting agent for essential ions, it also has been demonstrated to be able to moderate the toxic effects of metals (623). Moreover, it also binds a number of organic metabolites which would otherwise be difficult to keep in aqueous solution. The most important of these are bilirubin and the fatty acids; the free concentration of these hydrophobic molecules is maintained at about 1/5000 of their total plasma value by binding to serum albumin (190). Plasma tryptophan, three quarters of which is also attached to albumin protein, is the only amino acid markedly affected in this manner (556, 239). Serum albumin, with a molecular weight of some 65 000 daltons, is formed in the liver (190). It is assembled from free amino acids by the normal ribosome mechanism for protein construction (190). 'The synthetic machinery performs the remarkable feat of selecting

and linking all 575 amino acid residues of albumin within about one minute' (190). The albumin concentration in blood serum is some 42g/l or about 650μM.(190, 577).

With the exception of a normally very small quantity of haemoglobin which is firmly bound to a protein called haptoglobin (575, 644), almost all the iron in plasma is bound to transferrin. The percentage of this metal which is not attached to macromolecules is so minute that many authors have claimed that transferrin holds the entire plasma store of iron (see, for example, the discussion about this in reference 169). A consideration of the ferric hydroxide solubility product shows that at physiological pH, the free Fe^{3+} ion concentration cannot exceed about 10^{-16}M. (560). So, the need for a specific carrier to maintain iron in a soluble and atoxic form is quite clear. In fact, to prevent bacterial growth, even lower free ferric ion levels must be achieved (99). It is thus not surprizing that transferrin binds this metal with an affinity that leaves little for distribution amongst the other iron chelating agents in plasma. The biological significance of transferrin has meant that this β_1-globulin has received much experimental attention (589, 485, 99). It has a molecular weight of about 90 000 daltons (353). With its concentration of about 30μM. (575, 200), transferrin is the most predominant β-globulin in plasma. Titration data, various spectrophotometric techniques, optical rotatory dispersion studies, the effects of chemical modification and electron spin resonance measurements have all been combined to throw light on the metal binding structure of the protein. Co-ordination by three tyrosyl residues, two to four nitrogen ligands and one bicarbonate ion appear to be involved in

the reaction with Fe^{3+} at each binding site. Two of the nitrogen donors are believed to belong to imidazole residues. There has been much controversy about the role of bicarbonate in the transferrin iron-binding (179, 409, 410) but the evidence seems to favour attachment of this inorganic species to the protein rather than directly to the metal (179, 589). There are two ferric ions bound by each transferrin molecule but the equivalence of the binding sites and thus of the two binding constants is also controversial (485, 589, 404). A number of workers have attempted measurements and although their exact magnitudes vary, both values are very large. Under physiological conditions of pH and CO_2 tension, these lead to an apparent equilibrium constant of 5.10^{23} M^{-1}. (162, 611). Transferrin, in fact, binds most of the transition metals but these are all easily displaced by ferric ion (27, 184). Even ferrous ion is only weakly co-ordinated (463). This fact suggests a means whereby the iron may be liberated from transferrin *in vivo*. A simple mechanism is necessary because there is no evidence for any special enzymic process for this dissociation in body tissue (99). Other possible means of release are listed in Section 1.2.3. The importance of chelation in iron metabolism particularly in the removal and exchange of iron from transferrin, is also discussed therein.

Neither the nature nor the concentration of all the metalloproteins in plasma are known. This is for the most part due to the very small abundances of some of these substances. Whereas the more important metalloproteins of copper, iron and zinc have been extensively characterized, spectrographic and chromatographic work by Himmelhoch *et al.* indicate that metalloproteins of, at least, manganese and nickel

FIGURE 3.

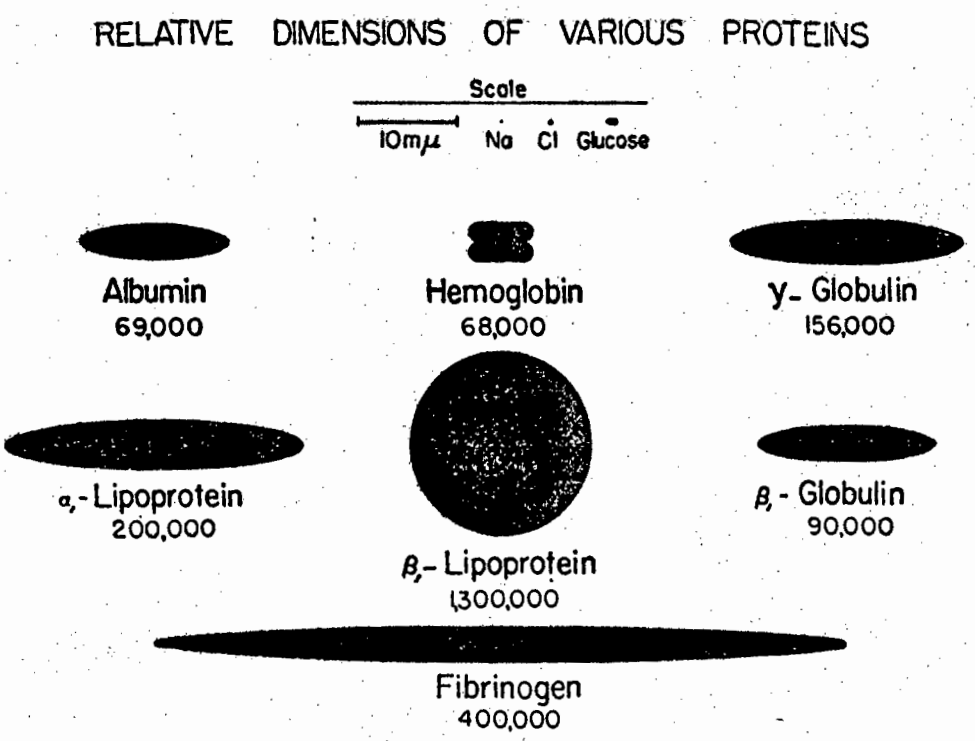


Diagram showing relative dimensions and molecular weights of blood proteins. The molecules of albumin, globulin and fibrinogen are elongated bodies of about the same diameter but of very different lengths according to their molecular weights (E. J. Cohn (1945), *Sci. in Progr.* 4, 319).

† from 618

also exist (559). They point out that, in addition, there may be other metal-binding proteins containing the three first-mentioned cations. For example, they note the possibility of an iron-binding protein in addition to transferrin.

The major copper-containing metalloprotein in plasma is ceruloplasmin (144, 133). This protein with a molecular weight of about 150 000 daltons, contains between six and eight copper atoms per molecule, half of which are in the cuprous state and cannot be oxidized without denaturation (580, 147). Some reports of other copper-binding proteins have appeared but remain to be confirmed (645, 640). Zinc in blood plasma is known to be consistently attached to albumin, transferrin and an α_2-macroglobulin (557, 345). Immunoglobulin G also appears to bind this cation (557). However, approximately two thirds of serum zinc is associated with albumin. This 'loosely-bound' fraction represents the primary transport form (646, 345). The state of manganese and lead in plasma is less clear. Both these metals are certainly bound by albumin (28, 155, 332). Manganese, particularly, can occupy some of the unfilled iron-binding sites of transferrin (184, 99). However, general interaction with the multitude of donor groups in proteins will ensure that all cations in blood will associate, albeit to a limited extent, with the whole spectrum of plasma proteins (see, for example 225).

1.3.2 The low molecular weight complexes in plasma.

Although most of the transition metal ions in blood plasma are protein-bound, it is chemically necessary that the macromolecules

compete for these cations with all the low molecular weight ligands in the biofluid. Hence, the proteins participate in an equilibrium which must exist between the free metal ions and the full variety of possible complexes. The free ion concentration and the low molecular weight complex fraction may be very small. Indeed, with a number of the transition elements, if not all of them the concentrations concerned are below the level of analytical detection. Nevertheless, the small complex fraction and more especially, the distribution of the metal ion amongst the various low molecular weight chelators are of great interest, as outlined in Section 1.1.

Great effort has been expended in numerous studies designed to demonstrate, experimentally, the existence of the low molecular weight metal complex fraction. Many attempts have even been made to elucidate its nature. Neumann and Sass-Kortsak initiated this movement (135). Consequently they have often been quoted in support of other experiments and in various arguments concerning the low molecular weight fraction. These workers showed that a substantial number of amino acids compete effectively with serum albumin for the binding of copper. They proposed that the amino acid bound fraction had a physiological role in the biological transport of the metal. They also suggested that under normal conditions histidine would be the amino acid primarily involved in the formation of mixed amino acid copper complexes in the serum. Now, whilst it is not intended to dispute their findings, it is important at this stage to point to the hazards which militate against attempts to draw physiological conclusions from this type of experiment, especially if these are in any way quantitative. The investigators mentioned measured the percentage

of radioactive copper in the supernatant fluid after ultracentrifugal sedimentation of the protein and the copper protein complex. They showed that this percentage was dramatically diminished in the absence of serum amino acids. Thus, these ligands are responsible for most of the copper which 'escaped' ultracentrifugal sedimentation. Hence, they are implicated in the formation of any low molecular weight fraction. However, whilst care tas taken to utilize physiological concentrations of the amino acids in the experiment, the nature of the technique requires considerably larger than physiological doses of copper. In fact, the copper to albumin molar ratio used, only produces the pertinent effect convincingly when it is ranged upward of 0,5 (this represents approaching saturation of the specific copper binding site of the protein!). The applicable plasma ratio is actually less than 0,002 (see 144 for the non-ceruloplasmin copper and 190 for the albumin concentration). It is clear that under the extreme experimental conditions, the copper distribution amongst low molecular weight ligands need not necessarily be anything like their physiological counterpart. This criticism is applicable to a number of similar studies (for copper: 280; for zinc: 140; for iron: 131, 200, 169). In spite of these difficulties, there is no doubt that the transition metal ions not bound to protein will be largely co-ordinated to low molecular weight ligands, probably as ternary complexes.

In much of the earlier work on metal complex equilibria in plasma, the presence of low molecular weight complexes was ignored. This omission calls the interpretation of many experiments into doubt because the investigators did not take into account all the equilibria

that could be disrupted. Perrin (369) cites the 'typical experiment where plasma was dialysed against large volumes of buffer' (his example is reference 133). He notes that 'amino acids would be progressively removed by diffusion across the cellophane membrane until only the protein remained to bind exchangeable copper; such an experiment does not provide information about the nature of metal complexes in the original plasma'. In fact, this sort of comment is applicable, to differing extents, to many experimental techniques which are applied to the study of metal ions in physiological solutions. Biological investigators, in general, would be well advised to consider the degree to which equilibrium disruption may influence their results. Furthermore, this is pertinent to a number of reported studies which have been concerned with the type of low molecular weight complexes that are formed in plasma. Unless exact physiological conditions are set up and strictly maintained, one may easily upset the equilibrium and alter the concentrations one is attempting to measure. This explains the discrepancies found between certain theoretical simulations and reported experimental studies (369, 340, 166). In fact, there is no valid comparison. However, whilst complexes produced under pseudo- or non-physiological conditions are not necessarily the same as those formed *in vivo*, investigations such as those referenced (340, 166) can yield a valuable insight into the real situation provided they are interpreted with caution.

One of the most interesting insights into the biological role of low molecular weight complexes has been described by Osterberg (319). It concerns the biological specificity of copper and zinc ions. His calculations reveal that the mixed amino acid complexes of copper

may account for the zinc metal-activated enzyme systems which occur in physiological solutions. A superficial appraisal could easily lead to the conclusion that zinc would compete ineffectively with copper ions. Indeed, the latter metal is certainly capable of forming very strong complexes so some means is required to ensure that enzyme sites are reserved for zinc. Osterberg demonstrates that most protein binding of copper would not take place in the presence of histidine and other amino acids because the mixed ligand complexes would be formed preferentially. Thus, many protein sites would be left available for zinc occupation. Osterberg also shows that the zinc mixed ligand complexes are incapable of a role analagous to that of the copper compounds. This and similar types of calculation may be facilitated by accurate computer simulations of the low molecular weight distribution of cations in biofluids.

1.4 Models.

In the sense employed in this thesis, a model is anything which simulates a real system. It is a construction which attempts to imitate reality by representation of the system's various parts and of the interrelationships which are manifest between those parts. Models come in a variety of forms. Sometimes they are real but they may also be imaginary. The models of science are often mathematical; the rigorousness of a mathematical formulation helps to avoid both errors and ambiguities. Models have an important role to play in mankind's efforts to describe the physical universe. In fact, they are an integral part of the scientific method. This philosophy requires that hypotheses, drawn up in the light of observation, be used to make predictions which are then confirmed or refuted by further experiment. In this context, the behaviour of the model constructed on the basis of the hypotheses and manipulating the early observations as data, constitutes the prediction. Typically, the model is subjected to a new set of circumstances. If it reacts towards them in the same way as the real system, the hypotheses are to some extent substantiated. If it does not, they must be rejected or modified. By repeated application of this procedure involving extensive re-evaluation in terms of a broadening horizon of experimental observation, the hypotheses become accepted. Finally, many complementary hypotheses are fashioned into a single broad explanation. This is called a theory. The entire scientific method depends on an ability to predict future observations. Furthermore, these observations should not be a simple re-arrangement of the old data By manipulating the constraints on a model it is often possible to

generate predictions which are by no means obvious because they depend on the relationships which exist within the system. These, then, enable one to design new experiments that test both theories and hypotheses extensively.

One of the foremost applications of computers in science today is model testing. During the last two decades, these machines have been increasingly recognised as an essential scientific tool. In the field of chemistry alone, computerization has revolutionized many areas of research - N.M.R. studies, X-ray crystallography and *ab initio* molecular orbital calculations, to mention just a few. The idea that computers are merely a convenience has been steadily replaced by an awareness that their advantages of speed, calculation reliability and vast data storage capacity endow them with capabilities which cannot be equalled. The ease with which computer models can be varied illustrates this unique potential. In the pre-computer age, the enormous quantity of work which simulation often required precluded the thorough analysis which models can now receive. A good example of this, applied in the determination of formation constants, is the recent PSEUDOPLOT program (317). Essentially, this provides a convenient means of evaluating one's postulated choice of species that exist in solutions under potentiometric investigation. The new program creates an alternative to the lengthy graphical normalized curve fitting procedures as a means of detecting oligonuclear complexes in metal ion-ligand solutions. Another example which displays how effectively computers may supplement graphical methods as far as chemical model selection is concerned is the series of studies by Ulmgren and Wahlberg on ascorbate complexation (234, 233, 235, 448) using the LETAGROP VRID program (42, 46, 47).

1.4.1 Physiological models.

There have been many contributions to the development of the current philosophy on models of living systems. However, two sources in particular must be acknowledged for their part towards the following discussion. In 1973, a conference was held on modelling techniques in animal science (475). This outlined a number of potential applications of systems analysis. It also included a comprehensive account of the simulation of biological systems at the level of biochemistry and physiology (476). However, the most direct attack on physiological simulation has been conducted under the auspices of the Rand Corporation, California (378, 220, 380, 381, 281). De Haven, especially, has detailed the fundamentals of chemical thermodynamic application to models of *in vivo* processes (281). Some of the more important aspects follow:

1) It is taken as axiomatic that all the physical laws apply to biological systems. These laws may not all be known or even in a form that is easily applicable to the complexity of life processes.

2) Even a rigorous model may appear inconsistent and at odds with reality if it 'is being stressed in an inappropriate fashion, i.e., it is being asked questions it was not designed to answer' (281).

3) No model can create new information. It can only reflect old information in an economic, effective and enlightening way. Both this and the previous point are applicable to models in general but are entered here because these issues are easily obscured in complicated physiological simulations.

4) A most important concept is that of body compartments. All chemical systems may be regarded as a finite number of homogenous phases. This is also true of a physiological entity (378, 281). A phase is defined in terms of chemical composition, temperature and pressure and need not be physically continuous. 'The idea of a phase as defined by Gibbs is identical with the concept of compartment as used by many physiologists to designate a body space' (281). Thus, although the cells exist individually in blood, it is legitimate to designate a 'red cell compartment'. Plasma is the body compartment treated in this thesis.

5) Modelling compositional aspects of physiological systems is likely to be easier and more reliable than energetic simulations. The measurement of substance is almost invariably less difficult than the measurement of energy. It is also easier to keep track of matter; energy transformations on the other hand are often quick, diverse and hard to detect.

6) Although no biological system is ever in true equilibrium, they often approach and sometimes attain a steady state. Moreover, 'to achieve high efficiencies of energy conversion, most biological systems operate near to reversible equilibria' (369). Thus, to assume that equilibrium exists is often justifiable. It has the added advantage that any need to involve kinetics is removed. The theory of kinetics is less well developed than that of thermodynamic equilibrium and the mathematical treatment is also more difficult (281).

7) Finally, it is necessary to comment on that mythical target of so many models - the so-called 'standard man'. Whilst it is true that the set of 'normal' values does not necessarily represent any of the individuals upon which they are based, this problem is experienced by all investigators concerned with living organisms. It does not appear unreasonable to infer that the properties of the 'standard man' will reflect those of at least the majority of persons concerned. This is particularly so because most biological variation is continuous. Furthermore, averaged data permits the development of models which may in time be applied to simulate and thereby solve the problems of specific individuals.

In reply to those who criticize the whole concept of simulating physiological solutions, this author cannot express a more cogent argument than that presented by Iversen in his discussion on a model for homeostasis (603).

> 'Many biologists of the old school react strongly against the use of mathematical models for interpreting biological phenomena, arguing that all biology is so fantastically complex that any model is bound to be an oversimplification of the grossest kind. On reflection it will be evident, however, that *a good simplification is a big advantage* - as a matter of fact it is a precondition for a model being at all serviceable. If a map included all the details really present, it would be quite useless..... If we succeed in constructing mathematical models that include the main features of the structure of a biological system, such simplification will not rule out the possibility of finding the biological laws. On the contrary, the model offers us the opportunity to understand the main points of what goes on in biology.'

1.4.2 Previous simulations of metal ion distributions in biofluids.

The well-behaved and uncomplicated nature of metal ion equilibria in aqueous solution is reflected by the large proportion of physiological simulations in the literature which are concerned with metal ion systems. Furthermore, apart from models of metal-protein interactions (for example, 526, 381) and a few studies on compartmental electrolyte distributions (439, 220), most of the simulations mentioned above focus on the distribution of the metal ion amongst the low molecular weight complexes it can form in the biofluid.

In spite of the wide variety of biofluids in which metal ion equilibria are important, for the most part only two of them, natural waters and blood, have been simulated. The concern with natural waters has motivated a number of investigations. In fact, this has led to the determination of formation constants with a host of ligands (see, for example, 434 and 524, 512 and the preceding parts of this series). Moreover, Sillén's classical simulations of sea water pioneered modelling of metal ion distributions in biofluids generally. Sillén's work (see 519) is a striking application of the scientific method. In particular, it demonstrates the value of models. He showed that the control of pH in his sea water model was primarily effected by the reactions between micas and kaolinite. This was in stark contradiction to the widespread belief that bicarbonate buffering was responsible for the constancy of the sea's pH. However, subsequent research into the chemical properties of the clay minerals has substantiated his ideas. Today, they are thought to be quite acceptable. Bicarbonate may have some local buffering action (653). It certainly facilitates equilibration (519). Otherwise, it acts more

as an indicator than as a buffer (653 and 519 with the references therein). Other models of natural waters include a simulation of 'all the principal inorganic chemical species in Lake Keystone, Okla.' (246) and a study of the metal ion equilibria in drinking water contaminated with nitrilotriacetic acid (244). The concentration of this ligand (NTA) is on the increase in natural waters because it is being used in commercial detergents as a replacement for polyphosphates. The 'heavy metal binding sites' in river water have also been recently investigated (535).

Whilst De Land has set up a very successful mathematical model of blood biochemistry (380), his simulation was designed to investigate the gross chemical properties of this biofluid and so it included no transition metal ion equilibria. The investigation into the computation of the distribution of metal ions among mixtures of complexing agents in blood plasma has been pioneered by Perrin and developed by him in a series of published studies which date back to 1965 (3, 286, 158, 369). Since then, Perrin has concentrated on expanding the model's size and eliminating some of the many difficulties which are inherent in this type of physiological simulation. He has been especially concerned with two metal ions, copper and zinc, although recently he has included some calcium and magnesium ion reactions (369). A similar trend may also be discerned in his choice of ligands; earlier models contained only 16 amino acids (286) but this number has been steadily enlarged. In his latest work, 22 amino acids in the presence of copper and zinc were considered to form 217 complex species (369). The results of this study were not materially different from previous computed distributions (158). They have been set out in Table 3.1. Another model, the one with magnesium and calcium ions, contained 147

species. Copper and zinc were included as usual, along with the more important amino acids and some additional inorganic and organic ligands. At the same time, Perrin attempted to take some account of the metal protein interactions in plasma. Unfortunately, his efforts did not meet with much success. For example, the model predicted negligible formation of the copper-albumin complex (369). Also, the computed estimate of the calcium distribution between albumin and globulin is in complete disagreement with that found by direct measurement. Indeed, a comparison of the results in reference 369 with those reported in reference 136 (and other cited therein), shows that the ratios in question are reversed. Incorporation of protein equilibria in simulations such as these is, at present, extraordinarily difficult. This subject is discussed at greater length in Section 2.2. Two other models for metal ion reactions in blood plasma have been published. Giroux and Henkin re-investigated the competition for zinc among amino acids in the presence of serum albumin (164). The major zinc ligands of low molecular weight that they found were essentially the same as those suggested by Perrin although, as they pointed out, Perrin had used an inaccurate value for the total plasma zinc. Using a practical equilibrium constant they had formulated and measured, they found that about two percent of the exchangeable zinc in plasma was not bound to albumin. This value is in agreement with the percentage of ultrafiltratable zinc in normal human serum as measured by Prasad and Oberleas (140). Finally, Brandegard and Osterberg have simulated the calcium ion reactions in blood plasma (525). They set up an eight component model that included proteins, carbonate, phosphate, sulphate, amino acids and organic oxy-acids as ligands.

Their findings are of considerable interest and appear to be reasonably realistic. They do, however, neglect the formation of calcium bicarbonate which, in spite of the undoubtedly weak association, is likely to exist in significant concentration due to the relatively high concentrations of bicarbonate and free calcium ions (see Section 3.2). This omission thus introduces a distortion into their results.

1.5 Objectives of the research.

Perrin's conclusions have far-reaching medical implications. He has, for example, investigated the effects of penicillamine treatment on the copper distribution in the plasma of persons suffering from Wilson's disease (369). However, criticism that his results may be unrealistic is justified on the grounds that his model is too simple. Just over 200 species must be a small fraction of the total number of possible complexes that can form from copper and zinc in a biofluid that contains as many ligands as plasma does. Moreover, the metals he has concentrated on are only two of about a dozen biologically important cations occuring in blood. Whilst there are good reasons for assuming that a model can be rigorous without including all these metal ions (see Section 3.3), a number of questions remain unanswered. For example, what would the consequences be of including the complexing reactions of manganese in the model? Manganese does not as a rule complex very strongly but for this reason may not be associated with proteins to the same extent as the other transition metals, leaving it free to compete more effectively for low molecular weight ligands. Furthermore, the full consequences of protein binding, to anions as well as cations, ought to be taken into account.

The present work is concerned with the construction of a very large model for computing the distribution of metal ions among the low molecular weight ligands in blood plasma. This involves over 50 of the more important ligands and the several thousand species which arise from complexation between these ligands and the metal ions Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+}. A strong emphasis

on the formation of ternary mixed ligand complexes is regarded as a pre-requisite. An attempt is made to accommodate the effects of protein binding and it is hoped that this will enable the simulation to reflect more accurately than earlier models, the complicated interrelationships which stem from the competitive metal ion equilibria in plasma.

Accordingly, the objectives of the research are stated as follows:

1). The acquisition of formation constants.

The model needs as many formation constants as it is to contain complex species. A large fraction of these remain to be determined experimentally. This is especially true of mixed ligand complexes. Moreover, fewer have been measured under conditions appropriate to blood plasma. Methods of estimating missing values are therefore investigated; also, the influence of temperature and ionic strength on the magnitude of various formation constants are considered. Selected means of estimating and correcting formation constants are applied and a large data base is thus accumulated.

2). The development of a computer program capable of handling the very large system which is envisaged.

At present no program exists which could be satisfactorily applied to the present problem. Both the computer core storage requirements and the time needed to reach a solution are substantial. A program

that can cope with both these difficulties, therefore, is developed.

3). Construction of the model.

This entails setting up the formation constants in a self-consistent fashion. It also involves selection of components and their representative concentrations. A major task in the construction of this or any model is the search to find and remove aspects which have no counterpart in reality. For example, care is taken not to overlook erroneous relationships which are so easily generated amongst thousands of similar but legitimate reactions. In the plasma model, this commonly concerns the simulated chelation by a ligand which is known, by experiment, to oxidize or reduce the metal ion in question. Furthermore, omission of the anomalous complex is not necessarily the best resort because the redox potential may be altered in the biofluid by other ligands (158).

4). Evaluation of the consequences of model segmentation.

It is clearly impossible to include every metal ion interaction in the simulation. So, the effects of segmenting the model are investigated. Whilst it is essential to ensure that no important aspect of the system is omitted, it is also pertinent to determine which parts do not play a dominating role and can thus be removed. Such an analysis includes a comparison of the results obtained by Perrin (158, 369) and those produced by the extended model of this thesis.

5). Investigation into the applications of the model.

The importance of metal ions *in vivo* has been emphasized in this introduction and many applications which require a knowledge of the metal ion distribution amongst low molecular weight ligands have been noted. In this connexion, investigations are made (i) into the effects on the computed distribution of metal ion poisoning or deficiency, (ii) into the corresponding effects of administered therapeutics and (iii) into possible applications to problems concerning the transport of metal ions through biological membranes.

CHAPTER TWO

THE ACQUISITION OF FORMATION CONSTANTS

2.1 Metal-ligand equilibria.

The title of this section covers an extremely broad subject, the details of which have been presented in numerous books and articles (see, for example, 367, 364, 368, 365, 656, 654, 655). It is not intended to dwell here on the vast majority of these considerations. Rather, the purpose of this section is to cover those aspects which have a direct bearing on the equilibrium reactions set up between metal ions and ligands in plasma. It is hoped to highlight those thermodynamic principles upon which depend the formation constants to be used and thus, also, the computed distribution of the metal ions among the low molecular weight ligands in the biofluid.

Consider a mixture of metal ions and ligands in aqueous solution. These components come into a competitive chemical equilibrium with the manifold complexes that can be formed from them. In general, there will exist for each type of metal ion with each ligand a series of complexes created by successive co-ordination of ligand moeities to the central cation. This 'step-wise' formation is limited by a saturation of the metal ion and thus terminates with 'a fully co-ordinated species'. The maximum number of ligands which may be attached to the metal ion is a characteristic of the properties of both components. The step-wise complexes will, on the one hand, incorporate only one kind of ligand. Alternatively, 'mixed ligand complexes' will be produced and in fact, are favoured statistically. Further, polynuclear complexes with two or more metal ions, be they similar or not, can also exist. Finally, it

should not be forgotten that the ligands can themselves appear in a number of protonated and deprotonated forms.

The concentrations of all the complex species in the solution depend upon the equilibrium constants of their formation reactions. The computation of these concentrations in a general context is dealt with in Section 3.1.1. For the time being, a more limited approach will simplify the following discussion. Consider the formation of a complex $M_pL_qH_r$. A list of symbols used in this section and their meanings is provided in Appendix 5.1. We have the formation reaction, which we always write in terms of the free concentrations of each component:

$$pM + qL + rH \rightleftharpoons M_pL_qH_r$$

The thermodynamic equilibrium constant is then given by

$$t\beta_r = \frac{\{M_pL_qH_r\}}{\{M\}^r\{L\}^q\{H\}^r} \qquad .....(2.1)$$

The braces indicate activities. Now, letting S represent the complex species and f the activity coefficient, expression 2.1 may be reformulated

$$t\beta_{pqr} = \frac{[M_pL_qH_r]}{[M]^p[L]^q[H]^r} \cdot \frac{f_S}{f_M^p.f_L^q.f_H^r} \qquad .....(2.2)$$

Provided the activity coefficients, f, are fixed, expression 3.2 can be reduced to a 'concentration' formation constant, β_{pqr}, as follows:

$$\beta_{pqr} = \frac{[M_p L_q H_r]}{[M]^p [L]^q [H]^r} \qquad .....(2.3)$$

The analogous expression 3.1 is the general statement of equation 2.3. Note also, that the step-wise formation constants are the products of each individual equilibrium constant for the successive additions of ligands. For example,

$$\beta_{130} = K_1 . K_2 . K_3$$

$$\text{where } K_1 = \frac{[ML]}{[M][L]} \; ; \; K_2 = \frac{[ML_2]}{[ML][L]} \; ; \; K_3 = \frac{[ML_3]}{[ML_2][L]}$$

Several aspects of metal ion equilibria that are relevant to biofluids can now be examined.

1) Co-ordination sites which are left unfilled by ligand components will be occupied by water molecules. These moeities are not reflected in this thermodynamic treatment because as the pure solvent, their activity is taken to be unity.

2) Hydroxyl ion co-ordination may be regarded in the same light as any other ligand attachment. However, for reasons arising from (1) above, their binding is analogous to a deprotonation of the

complex. This treatment is unable to distinguish between the two alternatives because the complexes, although structurally quite different, are stoichiometrically equivalent. Both effects may be expressed by decrementing the hydrogen ion index, r. Thus, this subscript can assume negative values.

3) In aqueous solution, electron donors are not exclusively available to metal ions. Competition by hydrogen ions adversely influences metal-ligand co-ordination. This fact is exploited in the determination of formation constants by glass electrode potentiometry. It is also a most important factor in simulations such as the one presented in this thesis. The outcome of the metal-hydrogen ion competition depends on a balance between the protonation and the metal ligand formation equilibria. It is important to note that only a simulation, using all the applicable equilibrium constants, will unerringly predict the result of these competitive reactions.

4) The metal ion equilibria in biofluids with which this thesis is concerned are all required to be fairly labile. Inert complexes, being those which are dissociated slowly, must be excluded because the simulation is based on the assumption that the system is at equilibrium.

5) In order to employ concentration formation constants (as in expression 2.3), the activity coefficients of the species in solution must be maintained constant (see 41, 373). As the activity coefficients of charged species, at least, vary with ionic strength, the requirement is not fulfilled in dilute solutions when the concentration

of ionic charges present in the system is altered by the reactions under investigation. In such cases, research workers must resort to attempts to calculate the changes in ionic strength and thus in the activity coefficients; these approaches are often difficult and are subject to increasing inaccuracy as the concentrations are raised. However, the ionic strength of a solution may be maintained materially constant by a relatively high concentration of 'inert', 'background' electrolyte. This swamps the effect of reactions taking place in the solution and justifies the use of equation 2.3. This particular approach is employed in the determination of many formation constants. Thermodynamic equilibrium constants can be obtained by measuring a series of concentration constants at various ionic strengths and extrapolating to infinite dilution. However, the tacit assumption that the supporting electrolyte does not interfere with the reactions being studied is not completely justified. The myth of the non co-ordinating anion has been exposed (392). In fact, the concentration constants one measures in a supporting electrolyte medium reflect the competition of the electrolyte ions with the other components. As the electrolyte is chosen because of the feeble binding abilities of both its cation and anion, the interference is minimal at low background concentrations, especially below 0,25M. (see, for example, 377). Potassium nitrate (with the added advantage that its ions have approximately equal transport numbers(639)) and sodium perchlorate are the salts usually employed. Sodium chloride has also been used but nowadays investigators tend to avoid it because the co-ordination by chloride ion can certainly not be taken as negligible. However, this trend neglects the fact that sodium chloride medium is ideal for the purposes of simulating metal ion distributions in blood and other

biofluids because this eliminates the need to include chloride as a ligand in the model. This is a big advantage as accommodation of chloride binding in this way introduces considerably less error than quantitative considerations of the weak binding by an ion in relatively very high concentration (± 100mM).

2.2 The difficulties with metal protein binding constants.

Whilst the phenomenon of metal protein binding is quite easily demonstrated, it is not a simple matter to establish the quantitative aspects of this. In addition to the difficulties which apply to the measurement of any formation constants the nature of proteins, in particular the multiple equilibria in which they participate, add considerably to the problems associated with both theoretical and practical elucidations. Metal protein binding studies are generally designed to accomplish three objectives (699). The first is to determine the number of binding sites. The second is to estimate the strength of the bonds formed. The last is to establish the identity of the ligand atoms. Techniques which are usually employed include equilibrium dialysis measurements, gel filtration chromatography and potentiometric titrations.

In contrast to low molecular weight systems, proteins exhibit an interesting gradation in binding properties which is based upon a change in the specificity of the interaction with a given type of metal ion. All proteins interact with cations as a consequence of the donor atoms that belong to the amino acid residues making up the protein skeleton. At the other extreme, a number of proteins have binding sites which are very specific for a certain type of ion. Between these two opposite modes of binding, one finds, for various metal ions, differences in (i) selectivity, (ii) strength of the bonds formed and (iii) the number of sites involved. Serum albumin illustrates the situation very well. Ions such as calcium will interact with the carboxylate

groups of the protein. The binding is relatively weak but there are almost one hundred sites available (388). Zinc has been suggested as co-ordinating 16 imidazole side chains at physiological pH (23) although two of the sites appear stronger than the others (32, 33). The specificity on one site for copper at the amino terminal end of serum albumin, is well known (31, 173). The selectivity of this site is due to both the positioning and the variety of ligand atoms. These are a nitrogen from the α-amino terminus (31), two peptide nitrogens and one imidazole donor from HIS(3) (178). These confer such a preference for copper that this metal is not displaced or blocked even in the presence of very high zinc concentrations (366).

The difficulties inherent in any attempted simulation of the metal binding properties of proteins compose the core of objections directed at the concept of computing metal ion distributions in biofluids (Kench, J.E., private communication). This opposition is not without foundation. Indeed, there is as yet no rigorous means of accommodating protein effects in models of metal ion co-ordination in solution. Proteins possess a large number of donor sites which bind to both hydrogen and metal ions. Thus, the macromolecule complexes can exist in numerous forms, differing in their degree of protonation. In practice, it is not possible to determine the concentrations of these individual species (569). However, a selection of experimental methods can provide estimates of the average number of substrate molecules or ions bound to the protein (659, 657, 622, 658). If one makes

some rather sweeping assumptions about the relationships that operate between binding sites, this average number can be used to determine parameters that, under the assumptions, characterize the properties of the macromolecule (657, 662). Unfortunately, these assumptions are rarely good ones. It is usually necessary to consider all sites to be identical. Also, the independence of or co-operativity between the sites is important. If they are considered to be completely independent, the individual binding constants K_i for n equivalent sites are given by

$$K_i = \frac{n-i+1}{i} \cdot K_o \qquad .....(2.4)$$

where K_o is the intrinsic constant applicable to each interaction (657). The factor is a consequence of the statistical effect arising from more than one site. Alternatively, the interaction between binding sites may be taken as highly co-operative. This is exemplified by the oxygen binding of haemoglobin in which attachment at the first site activates the others so that they then become more readily occupied. The potential advantages of such control processes are obvious and thus, some co-operativity is always to be expected. Such features distinguish macromolecules from low molecular weight systems. Interactions between binding sites can arise through (i) steric interference, (ii) electrostatic interactions and (iii) conformational changes induced by binding (657). In fact, most proteins do not fall into either of the extreme categories. Thus, they are not suited to simplified analyses such as equation 2.4, which can be developed from the assumptions discussed above.

To incorporate a protein in a model like Perrin's (369) or that presented in this thesis (see Section 3.2) would strictly require all the equilibrium constants (i) for the proton dissociations and (ii) for the formation of the large variety of possible metal complexes. The full implications of the second requirement are staggering. If the model is to simulate the true competition between protons and metal ions, it needs the metal binding constants for each species that is formed by protonation and deprotonation of the native protein. Whilst it is no doubt possible to leave out many of these equilibrium constants and still obtain a realistic picture, the difficulty is still unsolved: the definition of the important protein species in terms of the number of protons and metal ions that are bound is no less tractable.

As it is not presently possible to adopt the theoretically ideal method of incorporating metal ion binding by proteins in the model, the objectives of this thesis can only be fulfilled if some other satisfactory means of accommodating their effects can be found. Actually there are a number of approaches that can be followed. A brief survey of these reveals that all have limitations. Perrin has chosen to apply equation 2.4 to estimate a stability constant for copper binding to serum albumin in plasma (369). This assumes 16 equivalent and independent sites for copper which, as he acknowledges, is certainly not very realistic. In this author's opinion, the procedure of Branegard and Osterberg is more likely to achieve the desired results. In their model of the calcium reactions of blood plasma, the interaction with serum albumin was represented by formation constants from a single, isolated carboxylate ligand (525).

The protein was regarded as ninety-nine separate carboxylate groups. So, the albumin's concentration rather than the protein's formation constant was multiplied by this figure. The outcome differs from that of equation 2.4 whenever the free concentration of the protein is not taken to be the same as its total concentration. Other workers have determined 'practical' metal protein binding constants (for example, 164, 155). These have an advantage because no assumptions are made about the relationships between binding sites. However, the values are only applicable to the conditions under which they are made. Moreover, they do not often reflect the influence of changes in pH. Apparent pH dependant constants which relate the number of binding sites carrying metal to those which do not can, in fact, be defined (see, for example, 366). However, these require electrostatic interaction factors to allow for charge interactions on the macromolecule. Finally, all the methods mentioned so far tend to focus on one of the more important proteins and thus neglect the influence of all the others.

It is now suggested that the difficulties associated with proteins and the computation of the metal ion distribution among low molecular weight substances can be largely circumvented by a direct method. By supplying the model with either the free concentration of each metal ion or its total concentration *not bound to protein*, the simulation is expected to produce a realistic low molecular weight complex distribution. Provided these values replace the total concentrations of 'exchangeable' metal ion that are usually employed, the effects of metal protein binding are validly excluded. In particular, if the metal ion's free concentration is known, the

low molecular weight complex concentrations depend only on the free concentrations of each ligand. These free ligand concentrations are determined by the model from (i) the total concentration of each ligand not bound to protein, (ii) the pH of the biofluid, (iii) the ligand's acid dissociation constants and (iv) the competition for each ligand by the metal ions in solution.

Unfortunately, the free concentrations of the transition metals in biofluids are all necessarily very low and as yet, remain unmeasured. Furthermore, at least the majority of these concentrations are likely to be below the level of analytical detection for a long time. Thus, the following steps to break the deadlock are proposed.

1) To utilize those free concentrations which have been experimentally measured, for example, free calcium concentrations which have been estimated using ion-specific electrodes (136).

2) To ascertain, where possible, the total concentrations of metal ions not bound to protein. The free concentration of magnesium is not generally as well established as that of calcium. However, the total amount that is bound to protein has been estimated (see Section 3.2). This can be used to calculate the magnesium concentration which constitutes the free ion and low molecular weight fraction.

3) To estimate values for the free metal ion concentrations of each of the transition metal ions. This can be done by using the 'practical constants' which have been measured for the most important binding proteins (see, for example 164, 155, 23). Then, by scanning

in the model a range of concentrations around these values, it is hoped to obtain some idea of the real distribution. If the changes in free concentration do not induce radical alterations in the computed distributions of the metal ions, confidence in the results of the simulation is unlikely to be misplaced.

These suggestions do not pretend to be an ultimate answer to the question concerned with incorporating metal protein binding into a simulation. The approach suffers from a number of limitations which are discussed in detail in Section 3.2.3. However, it is hoped that the proposals will help to free this type of model from the stigma which is attached to studies that ignore the patently important influence of proteins.

2.3 The acquisition of formation constants.

The acquisition of the formation constants for the model presented in this thesis was accomplished by a three phase procedure. To begin with, as many of the applicable values that had been experimentally measured were collected together from the literature. This accumulation did not exclude values which did not correspond to physiological conditions. So, where necessary, the formation constants were then corrected for both temperature and ionic strength. Finally, missing values deemed to be important were estimated by a variety of methods.

An important guideline used throughout the procedure for acquiring formation constants, needs to be emphasised before each of the three phases are described separately. The concept actually has a number of interrelated facets which may be outlined as follows. Every complex species that is to be included in the model requires a corresponding formation constant. The fewer species that are omitted and the more accurate the formation constants used, the smaller will be the error in the computed distribution. Conversely, omitting complex species which really appear in the biofluid in significant concentration is tantamount to using a formation constant equal to zero; it is thus in error and may well distort the simulated result. On such grounds, it is necessary to utilize experimentally measured values, no matter how unreliable the value is adjudged to be whenever there is no alternative. Similarly, it is better to guess formation constants than to leave them out. Omission will always be worse than a guess if the estimate used is lower than the real value. Thus, whilst estimates should be freely made when they are

required, a value lower than that which might otherwise have been used, should be chosen in an attempt to ensure that it is on the low side of its real counterpart.

2.3.1 The literature search.

Preliminary work produced a list of almost one hundred low molecular weight ligands of potential importance to the metal ion distribution in blood plasma. A comprehensive literature search was carried out with the following objectives in mind.

1) To collect as many formation constants pertaining to each of the ligands and the seven types of metal ion named in Section 1.1. This included the protonation constants. In those cases where no value had been measured under physiological conditions particular care was taken to include any reports which could contribute towards an 'educated' assessment of the equilibrium constant operative under the desired conditions.

2) To acquire up-to-date values for the total concentrations of each ligand in blood plasma.

3) To collect evidence in the literature about the state of these ligands in plasma. The fraction of each that is bound to protein, for example, is considered to be excluded from the metal binding pool and must therefore be subtracted from the total amount present in the biofluid.

Two sources of formation constants which deserve specific mention are (i) the special publications of the Chemical Society Nos. 17 and 25 'Stability Constants' edited by Sillén *et al.* and (ii) the values measured experimentally by Perrin *et al.* and listed alongside the accounts of Perrin's blood plasma models (158, 369). The other formation constants used in this work were obtained from the literature appearing subsequent to that covered by the 'Stability Constant' publications. The search extended up to March, 1975. This included a systematic coverage of each ligand of potential importance in plasma and indexed by the Chemical Abstract Service up to and including Volume 80.

In order to catalogue the large number of references that arose from this literature search, a computer program called INDEX was developed. A listing of the program appears in Appendix 5.4. The program was designed to sort and process a data set comprising the suitably coded references which had been accumulated in a random order. The output lists the references chronologically and in order for each author, keyword and journal in turn. These catalogues have also proved most valuable throughout the organisation and writing of this thesis.

Finally, a list of formation constants used in the model, suitably adjusted to the conditions of temperature and ionic strength of blood plasma as discussed in the remaining sections of this chapter, is presented in Appendix 5.6.

2.3.2 Adjustments for temperature and ionic strength.

The theoretical means of calculating an equilibrium constant for a given temperature from one measured at a different temperature has long been established. Assuming that the standard change in heat content ΔH^{o} is independent of temperature, integration of the von't Hoff relationship between the temperature limits T_1 and T_2 yields an expression 2.5 (639).

$$\ln \frac{K_2}{K_1} = \frac{\Delta H^{o}}{R}\left(\frac{T_2 - T_1}{T_1 T_2}\right) \qquad .....(2.5)$$

Actually, the heat of reaction often varies slightly between T_1 and T_2 and in sophisticated treatments this may be accommodated by expressing ΔH^{o} as a function of temperature. This, however, requires a knowledge of the heat capacities of the reactants and products and such data is not generally available. So, in this work, the less exact method has been adopted.

Corrections for temperature were applied whenever necessary throughout the acquisition of the formation constants. This is illustrated in Table 2.1 Data measured by Gergely *et al.* was used to transform their equilibrium constants measured at 25°C (504) into values applicable to 37°C. These were compared with Perrin's formation constants for the same reactions (369,158) which were experimentally determined at 37°C. The comparison revealed that the agreement was reasonable in all cases except one, bearing in mind the slightly different ionic strengths (Δ = 0,05M).

TABLE 2.1

FORMATION CONSTANT TEMPERATURE CORRECTION

Reaction	ΔH^{o} KJ/mole	$\log\left(\frac{K_2}{K_1}\right)$	log K Observed @ 25°C	log K Predicted @ 37°C	log K Observed @ 37°C
Cu+Asn = Cu(Asn)	-26,3	-0,18	7,79	7,61	7,69
Cu(Asn)+Asn = $Cu(Asn)_2$	-27,9	-0,19	6,50	6,31	5,97
Cu+Gln = Cu(Gln)	-22,9	-0,15	7,62	7,47	7,24
Cu(Gln)+Gln = $Cu(Gln)_2$	-25,6	-0,17	6,38	6,21	6,16
Cu+Aba = Cu(Aba)	-22,6	-0,15	8,02	7,81	7,65
Cu(Aba)+Aba = $Cu(Aba)_2$	-27,4	-0,19	6,70	6,51	7,44

Cu = Cu^{2+}

Asn = Asparagine

Gln = Glutamine

Aba = Aminobutyric acid.

Furthermore, the calculated predictions were in better agreement with Perrin's values than the constants measured at 25°C, except the one already mentioned. The exception drew attention to the fact that Perrin's value for the formation constant of copper (aminobutyric acid)$_2$ was extraordinarily high. Further examination, in the light of (i) other independent work cited in 'Stability Constants' Special Publication 25 and (ii) the observation that this ligand tends to exhibit formation constants which are lower than most other amino acids, led to the conclusion that Perrin's value was erroneous. Thus, this was considered to be an exception to the rule that formation constants measured under 'physiological' conditions are used in preference to other values obtained from studies under different temperatures and ionic strengths.

Corrections for ionic strength are not as straightforward as those applied for temperature differences. The reasons for this are twofold. In the first place, the theory involved is not as fundamental or as well established. Secondly, the variations which arise from the non-ideal nature of the system are more pronounced.

In 1923 Debye and Hückel published their theory on the influence of interionic attraction in electrolyte solutions (663, 664). They attributed the departure from ideality of such solutions to the charges carried by the ions and from a mathematical consideration of these electrical interactions derived a limiting expression for activity coefficients of ion pairs in terms of the ionic strength (I). Their relationship has been verified for extremely dilute solutions (70) but attempts to extend it to deal with mixed electrolytes and higher concentrations have been met with limited success. The

extension to concentrated solutions is particularly difficult because many specific properties of the ions need to be taken into account. The application to mixtures is even more complicated, largely because of the presence of more than one kind ion with different sizes (71). One approach to which reference is often made, introduces a term which allows for the finite sizes of ions. This uses equation 2.6 for the activity coefficient, f

$$\log f = \frac{-Az^2 I^{\frac{1}{2}}}{1 + Ba I^{\frac{1}{2}}} \qquad .....(2.6)$$

where A and B are constants, z is the ionic charge and a is the average diameter of the ions in solution. However, it is 'thermodynamically inconsistent' to use equation 2.6 with systems of mixed electrolytes (654). Thus, a number of empirical expressions have been proposed for solutions of mixed electrolytes at moderate concentration. One of these which is in very good agreement with experiment for solutions having an ionic strength up to $I = 0,1M$ is the Davies equation 2.7

$$\log f = -Az^2 \left(\frac{I^{\frac{1}{2}}}{1 + I^{\frac{1}{2}}} \right) - CI \qquad .....(2.7)$$

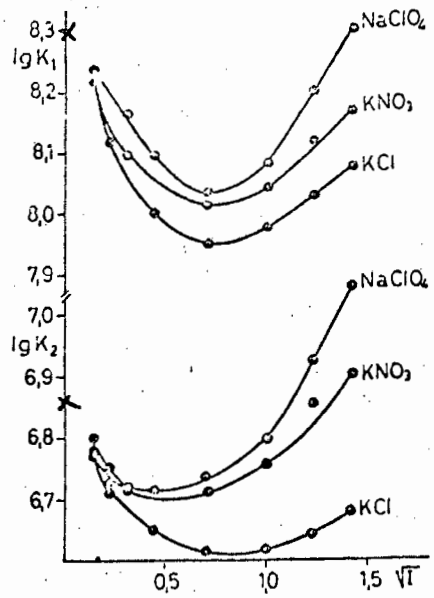
Unfortunately, none of these methods has proved to be much use in the present study. The available data is almost invariably insufficient to determine the parameters which appear in the various equations. Moreover, few of the methods are applicable

to solutions with an ionic strength greater than 0,1M whilst the background electrolyte concentrations used in formation constant measurements are seldom below this figure. The greatest need, in fact, for this type of calculated correction applies to solutions with I = 3,0M. This is for two reasons. A large number of formation constants have been determined under these conditions. Also, the formation constants exhibit minima between I = 0,15M and I = 3,0M so other means which can sometimes be employed (see Section 2.3.4) such as empirical interpolation, become quite unreliable.

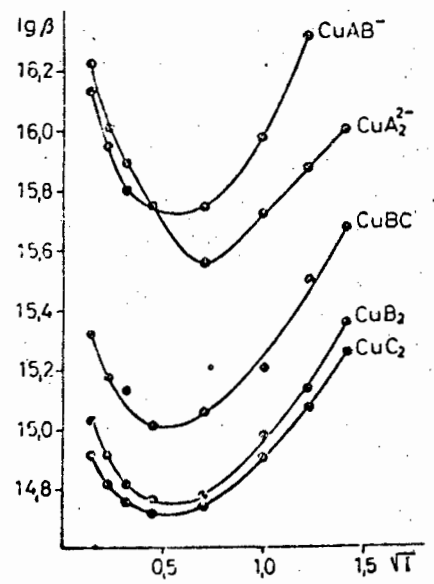
One report by Gergely *et al.* has been of particular value in the attempt to make the model's formation constants representative of the ionic strength of plasma. This study investigated the dependence on ionic strength of the formation constants of parent and mixed complexes of copper (II) with some amino acids (377). The change of each formation constant is depicted not only as a function of ionic strength but also for each of three background electrolytes, potassium chloride, potassium nitrate and sodium perchlorate. These workers have thus established a guideline concerning the approximate variation one can expect for formation constants measured under different conditions. Figure 4 shows their results (377). The following points have all been applied on several occasions on the present work.

1) At the ionic strength of plasma (c.a.0,15M) the nature of the electrolyte used has little influence on the value of the formation constants.

FIGURE 4.



A réz(II)–glicin törzskomplexek stabilitási állandóinak függése a KCl, KNO_3, illetve $NaClO_4$ elektrolitoldatok koncentrációjától. $C_{glicin} = 4 \cdot 10^{-3}$ mól/dm³, $C_{Cu^{2+}} = 2 \cdot 10^{-3}$ mól/dm³



A réz(II) törzs- és vegyes komplexei stabilitási állandóinak függése az ionerősségtől. A: aszparaginsav, B: glicin, C: α-alanin

† from 377

2) The plots all show a minimum. This is in accordance with theoretical considerations, for example, the Davies equation 2.7. Specific applications of this have included the fact that the formation constant applicable to physiological ionic strength is lower than the value reported for infinite dilution and probably greater than that measured at I = 0,2M. Formation constants at infinite dilution are commonly found in the literature.

3) Formation constants measured in 3,0M $NaClO_4$ are very much larger than the corresponding values at 0,15M, often by more than an order of magnitude. On the other hand, formation constants measured at I = 1,0M are very approximately the same value as those applicable to plasma ionic strengths. This is important because many studies use one or other of these high electrolyte concentrations. So, in desperation and only when there is no alternative, corrections can be made by analogy to the behaviour of the systems studied by Gergely *et al.* (see the example in Section 2.3.4).

2.3.3 The computation of ternary complex formation constants.

Many articles dealing with the formation of mixed complexes comment on their biological significance (for example, 336, 502, 221, 334, 97, 183, 453, 279, 186, 307, 308, 309). This is in large measure owing to the realization that ternary protein complexes are the intermediates between the metal ions bound to protein and those belonging to the low molecular weight fraction (see 168, 29, 185, 269). The possibility of ternary complexes arises whenever two or more

ligands are present in solutions of metal ions although this has only become generally recognized during the last two decades (74). All biofluids contain a multitude of potential ligands in high concentraction compared with the transition metal ions so mixed ligand complexes will be the rule rather than the exception. At least the majority of such ternary species in blood plasma will need to be included in the model to ensure its reliability but unfortunately, only a very small fraction of the applicable formation constants have been experimentally measured. Even fewer have been determined under the appropriate conditions of ionic strength and temperature. Thus, the outstanding values need to be calculated before the simulation is attempted. Perrin (369) has set a precedent in this regard by computing estimates of 28 constants each for mixed copper and zinc complexes. However, with n different ligands there are $n(n-1)/2$ possible ternary combinations so that 28 constants seems completely inadequate; the actual number of ternary complexes *for each metal ion* formed by, say, 50 ligands is well over one thousand.

In the presence of equal concentrations of two ligands A and B, the mixed complex MAB is statistically more favoured than MA_2 or MB_2 (74,88). This is simply the outcome of the probability associated with the formation of each species. The binary complexes are only half as likely as the ternary one. Each binary complex is associated with a probability of one in four because the chance that a particular ligand will co-ordinate is exactly 50 percent on each of two occasions. On the other hand, the ternary complex is formed by both of the remaining combinations.

On these grounds, an estimate for the formation constant for the mixed ligand constant is taken to be twice the average of the binary formation constants. Hence,

$$\log \beta^{*}_{MAB} = \tfrac{1}{2}(\log \beta_{MA_2} + \log \beta_{MB_2}) + \log 2 \qquad .....(2.8)$$

and

$$\log \beta_{MAB} = \frac{[MAB]}{[M][A][B]} \doteq \log \beta^{*}_{MAB}$$

Sharma and Schubert have outlined a general approach for calculating statistical factors for the mixed complexes formed when more than two ligands are involved (88). These factors can be quite considerable but owing to the dearth of experimental evidence it is difficult to evaluate their real influence for species more complicated than quaternary complexes.

It would be most surprizing to find that the statistical enhancement of mixed ligand formation constants accounted completely for their observed stability. In fact, this is not the case. The large majority of ternary complexes have formation constants somewhat larger than that predicted by equation 2.8. This reflects a number of factors which are energetically favourable (502). The most common of these are (i) further neutralization of charge, (ii) steric synergism and (iii) the formation of additional bonds, Π-bonds and hydrogen bonds, for example. However, ligand-ligand interactions occasionaly also lead to destabilization. Thus it is conventional to employ a factor, $\Delta.\log \beta$ which expresses the enhanced or diminished stability of the mixed ligand complex after correction for statistical

effects (88, 83).

$$\Delta \log \beta_{MAB} = \log \beta_{MAB} - \log \beta^{*}_{MAB} \qquad .....(2.9)$$

Most studies concerned with the formation constants of ternary complexes provide $\Delta \log \beta$ or analogous values (83, 336, 88, 502, 74, 308, 334, 504, 335, 267). These stabilization factors may be used to compute estimates of the formation constants and indeed, provide one of the easiest means of doing so.

Appendix 5.5 contains a listing of a Fortran program MIX which was developed using the University of Cape Town's UNIVAC 1106 computer. This program was designed to accomplish three tasks; they are dealt with by a single program because they all involve manipulation of the primary formation constant data. The objectives may be detailed as follows.

1) To compute the ternary complex formation constants which have not been experimentally measured, using equation 2.8 and a general stabilization factor $\Delta \log \beta$ provided by the user. The value usually employed is a little greater than zero because except when the two ligands are very similar this is likely to introduce less error than zero itself. The program is required to set up all the possible ternary complexes that can arise from ligands for which MA_2 formation constants are known. As the formation constants it uses are already applicable to the ionic strength and temperature of plasma no further adjustment in this regard is necessary.

2) To correct the ternary formation constants which have been experimentally measured to values more appropriate to plasma conditions. Direct application of the various correction methods used to obtain binary constants for 37°C and I = 0,15M is not favoured because of the widespread dearth of data concerning ternary complexes.

The problem is instead tackled by taking the $\Delta \log \beta$ factor which is observed under the (non-plasma) experimental conditions and after scaling it to take account of the different conditions, substituting it for the theoretical stabilization factor used in (1). The scaling adjustment reflects the difference between the binary constants applicable to plasma and those measured for the experimental solution. In other words, by considering equations 2.8 and 2.9 one obtains $\Delta \log \beta_{MAB}$ (experimental conditions) and scales it to $\Delta \log \beta_{MAB}$ (model conditions) by applying equation 2.10.

$$\Delta \log \beta_{MAB}(\text{model}) = \Delta \log \beta_{MAB}(\text{exp.}) \left(\frac{\beta_{MA_2}(\text{model}).\beta_{MB_2}(\text{model})}{\beta_{MA_2}(\text{exp.}).\beta_{MB_2}(\text{exp.})} \right) \quad \ldots\ldots(2.10)$$

The scaling factor adopted is chosen somewhat arbitrarily. However, its value is seldom far from unity so the error introduced is limited. Moreover, its effect on the relatively small $\Delta \log \beta$ s is much more controlled than it would be if applied directly to the ternary formation constant.

3) To set out the data required by each model. Program MIX assembles the data from three computer files. These contain (i) a list of components desired for the particular occasion, (ii) all the binary formation constants and (iii) the experimentally measured ternary complex formation constants and appropriate stabilization factors. The program only selects the formation constants and corresponding species appearing in the latter two files if they involve components appearing in the first file. This procedure facilitates the numerous manipulations and alterations which are inherent in a study of this kind because it enables one to set up different models without tampering with the primary formation constant data base.

2.3.4 Miscellaneous estimations of formation constants.

For the reasons already stated, the formation constants which are required by a rigorous model but which have not yet been experimentally measured under the appropriate conditions must be estimated by one means or another. These outstanding values fall into two categories. A small fraction have not been measured at all. The remainder have been determined but are not suited to a theoretical adjustment for ionic strength or temperature (as discussed in Section 2.3.2), usually because there is insufficient data available or because the evidence is not mutually compatible. This section outlines the variety of methods used, where possible in concert, to estimate many of the formation constants listed in Appendix 5.6. To illustrate these procedures the estimates for aspartic acid, tabulated with the values upon which they are based,

are presented in Table 2.3 after the following discussion. No formation constants for this ligand under blood plasma conditions of temperature and ionic strength have so far been determined.

1) Numerical interpolation and extrapolation.

This is the device most commonly employed. It is given preference whenever sufficient data is available. It entails estimating the change in the formation constant value produced by changes in temperature or ionic strength. Quite satisfactory values can often be procured by such means. However, a major obstacle encountered was the large disparity often found between values for the same constant reported by different workers. These discrepancies sometimes extend to orders of magnitude (see, for example, 'Stability Constants' Chem. Soc. Spec. Pub. Nos. 17 and 25). Extrapolations by analogy with the ionic strength plots of Gergely *et al.* also proved very valuable (377).

2) Relations between ligand basicity and complex stability.

It is well known that for a series of structurally similar ligands, a linear relationship often exists between the protonation and metal-ligand equilibrium constants (364). Some correlation is to be expected whenever metal ions and protons are in competition for the same ligand site. Sigel has recently extended this approach to include ternary complexes in solution (505). The most important application of this method for estimating formation constants in the present work applies to the ferric and ferrous complexes with amino acids. Few studies have been published about

these equiliabria other than those by Perrin (51, 52, 53). Unfortunately, Perrin's measurements were made under conditions ill-suited to the plasma model. These were in aqueous solution at 20°C and unit ionic strength. In fact, the considerable difference in conditions makes it inadvisable to apply any of the usual methods of correction unless there is absolutely no alternative. However, Perrin has established that there is a linear dependence of the logarithm of stability constants of the iron complexes on the acid ionisation constants of the amino acids. Equation 2.11 is applicable to the combination of a cation with a series of similar ligands.

$$\log K = \alpha pK' + C \qquad .....(2.11)$$

where α and C are constants and K' is the ionisation constant of the ligand. Perrin evaluated the slope of the linear relationship for both ferric (α = 1,8) and ferrous complexes (α = 0,4) so these have been applied to the acid dissociation constants applicable to 37°C and I = 0,15M. to obtain certain of the ferric and ferrous ligand formation constants listed in Appendix 5.6. Provided that the actual form of the linear relationship is not too dependent upon the experimental temperature or ionic strength, the observed change in protonation constants will impose upon the difference between the formation constants of the iron complexes. In fact, the slopes evaluated by Perrin are simply convenient proportionality constants. The application of this procedure to ferrous aspartate is illustrated in Table 2.3 (9). Incidentally, one might note that the magnitude of the correction for both ferric and ferrous complex is small. This is in line with the suggestion made in Section 2.3.2 (3) and applied, for example, in Table 2.3 (17) that formation constants

measured at I = 1,0M often correspond to those applicable to plasma conditions.

3) Chemical analogies.

Whenever no formation constant at all has been determined, the estimation of its value has had to depend upon some kind of chemical analogy. Using the Irving-Williams rule and other observed chemical trends (see Table 2.2 for binding trends of the amino acids, for example), it is often possible to gauge an approximate value. Of course, the more of these analogies which can be brought to bear on any one problem, the better that answer is likely to be. This approach has been used, for example, to obtain most of the magnesium amino acid complex formation constants used in the model. Very few have been measured because of the weak binding and experimental difficulty in monitoring complexation especially by potentiometry. By considering a number of chemical aspects, a satisfactory picture has emerged. At physiological pH magnesium binding is likely to be predominantly to the deprotonated carboxylate function of the amino acid. Thus, formation constants with log $\beta \doteqdot 2,0$ are expected. This figure is a little lower than the majority of reported magnesium-carboxylate interactions (see 'Stability Constants' Chem. Soc. Spec. Pub. Nos. 17 and 25), in line with the general drop in magnitude associated with the transitions $25^{o}C$ - $37^{o}C$ and 0,0M - 0,15M (see Section 2.3.2). Moreover, the magnesium species are likely to be more stable than the analogous calcium ones but less stable than the corresponding manganese compounds. Finally, the general binding trends exhibited by the series of amino acid ligands towards other metals can be superimposed (see Table 2.2). This exercise underlines the principle that even a

poor estimate is usually better than no value at all. Although the magnesium amino acid binding is weak, the complexes formed in plasma are important because of the relatively high concentrations of both components. The considerable influence of the magnesium and calcium amino acid complexes on the computed distribution of the transition metals amongst the low molecular weight ligands in blood plasma is discussed in Section 3.2.3.

TABLE 2.2

TABULATION OF THE BINDING TRENDS* EXHIBITED BY A SERIES OF AMINO ACIDS

Amino acid	Trend	CuL	CuL_2	MnL	MnL_2	ZnL	ZnL_2	ZnL_3
Simple amino acids	0	8,0	14,7	2,5	4,4	4,6	8,5	10,9
Average	0	8,1	14,6	2,6	4,6	4,7	8,4	10,8
Alanine	0	8,0	14,6	2,4	4,3	4,6	8,6	10,7
Aminobutyric acid	-	7,7	14,0			4,4	7,2	
Arginine	-	7,4	13,7			4,0	7,6	
Asparagine	-	7,7	13,7			4,5	7,8	10,0
Glutamic acid	+	8,7	14,9			4,8	8,5	
Glutamine	-	7,2	13,4			4,3	7,9	
Glycine	0	8,0	14,7	2,7	4,8	4,9	9,0	11,3
Histidine	+	9,8	17,5	3,2	6,2	6,3	11,7	
Isoleucine	0	8,0	14,7			4,4	8,1	
Leucine	0	8,0	14,7			4,5	8,6	
Lysine	0	9,3	14,6			3,5	7,0	
Methionine	-	7,7	14,1			4,2	6,9	
Ornithine	+	9,8	14,8			5,9		
Pheynlalanine	-	7,7	14,4			4,5	8,4	
Proline	+	8,7	16,0	2,8	5,5	5,1	9,7	11,2
Serine	-	7,6	14,0	2,5	4,0	4,5	8,3	10,6
Threonine	-	7,6	14,0	2,5	3,9	4,4	8,1	10,1
Tryptophan	+	8,1	15,3			4,5	8,8	11,6
Tyrosine	+	9,1	15,1			6,1		
Valine	0	7,9	14,6	2,3	4,0	4,4	8,2	10,6

Cumulative stability constants measured at 37°C;

I = 0,15M KNO_3 (158, 369, 62)

Simple amino acids = Alanine, Glycine, Leucine, Isoleucine and Valine.

*'TREND' means the strength of binding of the particular amino acid relative to the average for the series of similar ligands.

TABLE 2.3

ESTIMATION OF LOGS OF CUMULATIVE FORMATION CONSTANTS FOR ASPARTIC ACID

AT 37°C ; I = 0,15M.

References with alphabetic characters refer to 'Stability Constants' Chem. Soc. Spec. Pub. Nos 17 and 25 and references therein. Other 'Refs' are those listed for this thesis. See Appendix 5.7 for explanation of symbols.

1) ASP2(+1) H+1(+1) $\log \beta_{101} = 9.20$

Ref	log β	I	t	medium
53L	9.62	0.1	25	KCl
52C	9.46	0.1	30	KCl
52K	9.60	0.1	20	KCl
61B	9.87	0.1	25	KCl
62Ca	9.63	0.1	30	KCl
65R	9.63	0.1	25	KNO_3
Ref 377	9.73	0.1	25	KCl
Ref 377	9.63	0.2	25	KCl

Estimation: log β = 9,7 @ 25;0,10 as average
log β = 9,6 @ 25;0,15 ref 377
Δ ≒ 0,2 from 25 to 30 ref 62Ca more recent
Assume Δ ≒ 0,4 from 25 to 37

2) ASP2(+1) H+1(+2) $\log \beta_{102} = 12.60$

Ref	log K	I	t	medium
61B	3.87	0.1	25	KCl
62Ca	3.79	0.1	30	KCl
63F	3.69	0.2	25	KNO_3
Ref 377	3.75	0.1	25	KCl
Ref 377	3.72	0.2	25	KCl
Ref 267	3.68	0.2	25	KCl
65R	3.71	0.1	25	KNO_3

Estimation: log K = 3,7 @ 25;0,15
Assume log K = 3,4 @ model conditions

continued...

3) ASP2(+1) H+1(+3) $\log \beta_{103} = 14.20$

Ref	log K	I	t	medium
52K	1.88	0.1	20	KCl
53L	1.94	0.1	25	
52A	2.08	0.01	20	
63F	1.92	0.2	25	KNO_3
65R	1.94	0.1	25	KNO_3
Ref 377	1.81	0.1	25	KCl
Ref 377	1.85	0.2	25	KCl

Estimation: log K = 1,9 @ 25;0,15
Assume log K = 1,6 @ 37;0,15 analogy with glutamic acid

4) ASP2(+1) CA+ 2(+1) $\log \beta_{110} = 1.60$

Ref	log K	I	t
53L	1.60	0.1	25
Ref 668	1.53	0.7	37
Ref 668	1.53	0.7	25

Estimation: log K = 1,60 @ 25;0,15
Assume log K = 1.60 @ 37 $\Delta H^o = 0$?Ref 668

5) ASP2(+2) CA+2(+1) $\log \beta_{210} = 2.10$

Guess; $\log K_2$ is unlikely to be less than 0,5

6) ASP2(+1) CU+2(+1) $\log \beta_{110} = 8.50$

Ref	log K	I	t	medium
52C	8.57	0.1	30	KCl
57M	8.40	0.1	25	KNO_3
Ref 377	8.99	0.1	25	KCl
Ref 377	8.86	0.2	25	KCl
Ref 267	8.70	0.2	25	KCl

Estimation: log K = 8,7 @ 25;0,15
Take log K = 8,5 @ 37;0,15 conservative value $\Delta H^o < 0$ for glutamic acid

continued...

7) ASP2(+2) CU+2(+1) $\log \beta_{210}$ 15.20

Ref	log K	I	t	medium
52C	6.78	0.1	30	KCl
Ref 377	6.89	0.1	25	KCl
Ref 377	6.88	0.2	25	KCl
Ref 267	6.96	0.2	25	KCl

Estimation: log K = 6,9 @ 25;0,15
Assume log K = 6,7 @ 37;0,15 : Δ = 0.1 for 5°C

8) ASP2(+1) CU+2(+1) H+1(+1) $\log \beta_{111}$ = 12.20

Ref	log β	I	t	medium
Ref 267	12.38	0.2	25	KCl

9) ASP2(+1) FE+2(+1) $\log \beta_{110}$ = 4.20

Ref	log β	I	t	medium
Ref 53	4.34	1.0	20	KCl

Estimation: log K = 0.4 pK' + C; C = 4.34 - 0.4 x 9.56
(see Section 2.3.4(2)) C = 0.52
log K = 0.4 x 9.2 + 0.52 = 4.2

10) ASP2(+2) FE+2(+1) $\log \beta_{210}$ = 7.75

Ref	log β	I	t
52A	8.5	0.01	20

Estimation: log β = 7.75 conservative value

continued...

11) ASP2(+1) FE+2(+1) $\log \beta_{110} = 11.0$

Ref	log β	I	t
Ref 52	11.4	1.0	20

Estimation: Linear calculation as in (9) with slope = 1.8 (see Section 2.3.4(2))

12) ASP2(+2) FE+3(+1) $\log \beta_{210} = 17.0$

Guess; very conservative value

K_2/K_1 is the approximate minimum of the same ratio with Cu amino acids

13) ASP2(+1) MG+2(+1) $\log \beta_{110} = 2.20$

Ref	log K	I	t	medium
53L	2.43	0.1	25	KCl

Estimation: By analogy with other amino acids (see Section 2.3.4(3))

14) ASP2(+2) MG+2(+1) $\log \beta_{210} = 2.90$

Guess; conservative value c.f. Manganese K_1

15) ASP2(+1) MN+2(+1) $\log \beta_{110} = 3.20$

Ref	log β	I	t
52A	4.0	0.01	20
52K	3.74	0.1	25

Estimation: see Table 2.2 for other amino acid values

continued...

16) ASP2(+2) MN+2(+1) $\log \beta_{210} = 5.20$

Guess; based on K_2/K_1 ratio from Table 2.2

17) ASP2(+1) PB+2(+1) $\log \beta_{110} = 5.80$

Ref	log β	I	t	medium
64R	5.88	1.0	30	KNO_3
ref 311	6.67	3.0	25	$NaClO_4$

See comment in text; Section 2.3.2(3) reference 377

18) ASP2(+2) PB+2(+1) $\log \beta_{210} = 8.20$

Ref	log K	I	t	medium
64R	1.5	1.0	30	KNO_3
Ref 311	2.76	3.0	25	$NaClO_4$

Estimation: log K = 2.4 conservatively
Using K_2/K_1 ratio; c.f. Zn and Mn ratios

19) ASP2(+1) ZN+2(+1) $\log \beta_{110} = 5.80$

Ref	log K	I	t	
52C	5.84	0.1	30	KCl

Estimation: c.f. other Zn amino acid values Table 2.2.
Aspartic acid is invariably a stronger ligand

20) ASP2(+2) ZN+2(+1) $\log \beta_{210} = 9.3$

Ref	log β	I	t	medium
52C	10.15	0.1	30	KCl
52A	10.4	0.01	20	

Estimation: log β unlikely to be greater than 10.0 conservative value
log β = 9.3 is already considerably larger than average 8.4 (Table 2.2)

CHAPTER THREE

THE SIMULATION OF BLOOD PLASMA

3.1 Computer simulation of large equilibrium systems.

A knowledge of the distribution of components and the evaluation of constituent concentrations in chemical equilibrium mixtures is important for many reasons in addition to those associated with the present application to biofluids. The computation of the amounts of specific species present in solutions involved in spectrophotometric, calorimetric and other physical studies is perhaps the most obvious (114, 115). Other examples centre on the determination of optimum conditions for a variety of analytical and chemical separation techniques. Also, the effects produced by the addition of some reagent can be calculated. It has already been implied that one of the primary motivations behind the measurements of formation constants is that they enable evaluations of this sort to be made.

Although the equilibrium concentrations may in principal be evaluated directly from Guldberg and Waage's law of mass action, formulated in 1864, the solution of the appropriate equation is, except in the case of relatively simple systems, by no means straightforward. This is largely the outcome of the non-linear nature of the mathematical relations involved; these complicate any sort of general approach. Under some of the more elementary conditions, specific methods are easier and more economic. So, over the last thirty years this problem has been tackled by many investigators (e.g. 10, 11, 12, 13, 91, 92, 215). With the advent of common high-speed computer availability, the last decade has seen it fall increasingly into the realm of computer application (115, 85). Early work

concentrated on gaseous equilibria and using a variety of optimisation techniques usually approached the question by minimising free energies. With mixtures in solution, a fundamentally analogous means seeks a best fit to the mass action and mass balance expressions. This is more convenient and nowadays more common. The most frequently referenced programs in this regard are HALTAFALL by Ingri *et al.* (112) and COMICS which was developed by Perrin and Sayce (111). There have also appeared a selection of others (49, 219, 110, 214, 119, 245, 227, 327). The trend is hardly surprising because manual methods, especially those with general applicability, are tedious and difficult.

In addition to the problems which are associated with the actual calculation of concentrations, multicomponent mixtures in chemical equilibrium may also exhibit a behaviour which, to those well-versed in the properties of systems with only a few components, may appear at first sight to be a contradiction of Le Chatelier's principle! Broadly speaking, this may arise as follows. When the concentration of any one component in a large, general and well-defined equilibrium system is altered, there is no rule of thumb which will always correctly predict the effect of the change on any specific reaction operating in the solution. The direction of equilibrium shift is determined by the relative magnitudes of all the equilibrium constants acting in concert so the outcome on one particular equilibrium can easily seem anomalous. For example, if the free concentration of a component common to more than one species is raised, the product favoured by a large formation constant can quite possibly increase to a lesser degree than other species whose formation is governed by smaller equilibrium constant values. In the extreme case, the

concentration of the former species may even decrease provided that the equilibrium is disturbed in such a way that the free concentrations of its other components are sufficiently reduced. For this reason, the only method by which the influence of any factor may be properly evaluated is to compute the equilibrium concentrations before and after the change. A comparison of the results which emphasises the differences that have arisen will then reveal the exact effect of the disturbance. This has a bearing on the philosophy which is adopted in this thesis because it denies the validity of short-cuts to the solution which rely on a straightforward chemical intuition.

3.1.1 Program theory and development.

In almost all but the most trivial of computer projects, a balance must be found between the two limiting factors of processing time and core storage requirements. Program development must consequently take place within a framework that not only attempts to achieve an overall coding optimisation but also reflects, in the compromise between the two factors mentioned, the unique demands of the particular problem. The approach adopted towards this question must be formulated before the coding is begun because time and time again it will be found necessary to decide in favour of one or the other.

In the present case, largely because of the considerable number of constituents in living systems, the size of the model will invariably represent one of its most fundamental restrictions.

So, special care needs to be paid to the question of computer core storage. Whilst the value of speed should never be forgotten, whenever its needs would seriously conflict with spatial considerations, the latter must be given priority. This point is pertinent throughout the following discussion.

The problem under consideration here, is to calculate the concentration of all the chemical constituents which exist in a well-defined, single-phase equilibrium mixture. These constituents are classified either as components or as complex species. The components are stoichiometrically independent and are selected as fundamental in the sense that there may be no complex species which comprises a more elementary chemical unit. This definition is arbitrary and chosen merely for convenience - sometimes components have been selected on the grounds of highest concentration at equilibrium. The concentration of each complex species (S) is then fixed by the law of mass action. It is a function of the cumulative stability constant (β) and the free concentrations of each of its components (X). If i is the index for components and j the index for complex species, one has:

$$S_j = \beta_j \prod_i x^{k(i,j)} \qquad (3.1)$$

where k(i,j) is the matrix containing the number of times the ith component appears in the jth complex species. It may be pointed out here that the distinction between metals and ligands in this context is superfluous for mass action is indifferent as to the

nature of the reactant. A list of the symbols used in this section appears in Appendix 5.1.

Generally, the equilibrium system is defined in terms of the total concentrations of each of the components. However, if the free concentration is known instead, that is, of course, just as satisfactory. In most experimental situations, only the free concentrations of hydrogen ion and possibly hydroxyl ion will be available but sometimes free metal ion concentrations and/or free ligand concentrations are also measured. It is therefore important that the program be able to accept the input data in terms of either the total or the free component concentrations.

As there is no direct way to determine the equilibrium concentrations, the approach usually adopted is based on an iterative improvement of some set of initial estimates. By systematic variation of the unknown free component concentrations and calculating via equation 3.1 the corresponding values of all the complex species, it is possible to arrive at the point where the mass balance equations for each component are satisfied. These are:

$$T_m = X_m + \sum_j S \cdot k(m,j) \qquad (3.2)$$

where m is a specific value of i and is the index pertaining to a particular component. T_m is the total concentration of that component. Provided the concentrations of all the constituents are not permitted to be less than zero, once the mass balance relations are all obeyed, the unique solution has been found (see, for example, 216).

It has long been appreciated that the constraints of mass balance and of mass action may be fully described by n nonlinear equations in n unknowns, where n is the number of components whose free concentration must be determined. The question may therefore be solved, in principle, by the application of any nonlinear optimization technique which yields those conditions which produce a set of total calculated concentrations from equation 3.2 that are identical to the given real concentration totals ($^{r}T_{m}$). These calculated values ($^{c}T_{m}$) are obtained by summation over all the species concentrations arising from the current free component estimates. The objective function to be minimised could be, say, the sum of the squares of the differences between the corresponding totals. However, except in the case of smaller systems (where both general and specific techniques are effective), it transpires that an exchange of the particular problem for the general, introduces more disadvantages than it is worth. Equilibrium is dictated by a set of well behaved and easily manipulatable relations and methods of general applicability do not exploit this. These methods also suffer in a number of ways as the nonlinear system expands. General convergence difficulties become more apparent (Zeleznik and Gordon have pointed out that in practice many algorithms fail in this regard (216)). In fact, no general method can be trusted always to converge if the initial estimates are poor ones (621). In large systems, good starting values are likely to be the exception rather than the rule. Gans and Vacca (329, 330) have gone so far as to produce an entirely new program for the calculation of stability constants because both the other programs commonly employed for this (42, 113) exhibit limitations as a result of the iterative procedures

upon which they are based. Above all, most general techniques are unsatisfactory because their computer core storage requirements would seriously limit the magnitude of the models which could be investigated. Large matrix manipulations are inclined to be expensive in terms of both size and time required for computation. Moreover, many methods are affected by round-off errors and thus become incapable of lengthly iteration procedures. Both the Newton-Raphson technique (so often used, with or without modification) (214, 227) and Powell's algorithm for a sum of squared terms (615, 329) have been rejected, for the problem in this thesis, in the light of these arguments. All in all, it appears best to use a simple successive approximation function which varies the current estimates in such a way that the new values can hardly fail to be an improvement on the old ones. Perrin and Sayce have employed such a function in COMICS (111). This is shown as equation 3.3 where the superscripts n, o, r and c denote 'New', 'Old', 'Real' and 'Calculated' quantities respectively.

$$ {}^{n}X_{m} = {}^{o}X_{m} \cdot \left(\frac{{}^{r}T_{m}}{{}^{c}T_{m}} \right)^{\frac{1}{2}} \qquad (3.3) $$

In spite of the fact that at first sight one may anticipate a slow rate of convergence, the function has proved to be remarkably efficient. It is unfortunate that the authors do not indicate exactly why this expression was chosen. Some time after the original publication, Perrin comments that 'for all positive values of x, $x^{\frac{1}{2}}$ is always closer to unity than x^{1} ' (369). Whilst of course, the argument of the square root is indeed required to approach

unity, does this necessarily imply that the new estimation from equation 3.3 will consistently be an improvement on the old one?

Another important aspect of the program development is the means whereby a suitable set of initial approximations are obtained for each component's free concentration. In COMICS, it is assumed that complex formation is negligible and that rough free ligand concentrations can be calculated directly from pK_a values (111). However, when complexation is not minimal, especially in large systems, this approach is redundant and one might just as well put all the free component concentrations equal to the real totals. Moreover, it is this artifical approach which requires COMICS to draw a distinction between metals and ligands and thereby suffer from a lack of optimisation in the program coding. On the other hand, if one substitutes the real total concentrations as the first free estimates, as suggested, one obtains calculated totals ($^{c}T_m$) that are enormous and can hardly be said to represent an astutely chosen set of initial approximations. Perrin's and Sayce's formula (equation 3.3) is not really satisfactory under these extreme conditions, largely because it treats every component independently in a situation where their mutual interplay is most significant. What is actually required is a function which modifies the new value ($^{n}X_m$) on the basis of changes that are about to be imposed on the whole system.

An investigation into the relationships that operate in the equilibrium systems presently under consideration, was undertaken with three broad goals in mind. These may be formulated as attempts (i) to find a more efficient approximation formula, (ii) to discover

why the equation 3.3 used by Perrin and Sayce, in fact, works and (iii) to develop an expression that would cope effectively with very poor initial estimates. The applicable equations were subjected to a parallel consideration in terms of both the real and calculated quantities, as follows.

From equation 3.1, one may write

$$r_{S_j} = \beta_j \prod_i r_{X_i}^{k(i,j)} \qquad .....(3.4),$$

$$c_{S_j} = \beta_j \prod_i c_{X_i}^{k(i,j)} \qquad .....(3.5).$$

Hence, by substitution

$$r_{S_j} = \prod_i \left[\left[\frac{r_{X_i}}{c_{X_i}} \right]^{k(i,j)} \right] . c_{S_j} \qquad .....(3.6)$$

Note that, at this stage, any component whose free concentration is known (so that r_{X_i} / c_{X_i}), cancels.

Moreover, from equation 3.2,

$$r_{T_m} = r_{X_m} + \sum_j \left[r_{S_j} \cdot k(m,j) \right] \qquad .....(3.7),$$

$$c_{T_m} = c_{X_m} + \sum_j \left[c_{S_j} \cdot k(m,j) \right] \qquad .....(3.8).$$

Substituting equation 3.6 into equation 3.7, one obtains

$$r_{T_m} = r_{X_m} + \sum_j \left[\prod_i F_i^{k(i,j)} \cdot c_{S_j} \cdot k(m,j) \right]$$

$$.....(3.9),$$

$$\text{where } F_i = \frac{r_{X_i}}{c_{X_i}} \qquad .....(3.10).$$

Now, extracting F_m by factorization

$$\frac{r_{T_m}}{F_m} = c_{X_m} + \sum_j \left[\left[\prod_i F_i^{k(i,j)} \cdot c_{S_j} \cdot k(m,j) \right] / F_m \right],$$

so that if $$G_j = \frac{\prod_i F_i^{k(i,j)}}{F_m} \qquad \text{.....(3.11)},$$

one obtains :

$$r_{X_m} = \frac{r_{T_m} \cdot c_{X_m}}{c_{X_m} + \sum_j \left[G_j \cdot c_{S_j} \cdot k(m,j) \right]} \qquad \text{.....(3.12)}.$$

Equation 3.12 is an exact expression for the real free component concentrations but it cannot be used to find the solution directly because the set G_j is unknown. These factors G_j include real free component concentrations (compare 3.10 and 3.11) and therefore can only be evaluated once the entire problem has been solved. Nevertheless, the expression 3.12 has considerable value because it may be compared with various approximation formulae in order to discover their properties and to judge their likely performance. To begin with, take G'_j to be some approximation for G_j itself. Then, equation 3.12 transforms into an expression which can possible be employed to improve successively a set of (old) estimates ${}^{o}X_m$ by obtaining (new) values ${}^{n}X_m$ which are closer to ${}^{r}X_m$ than was the case with ${}^{o}X_m$. This expression is :

$$ {}^{n}X_m = \frac{{}^{r}T_m \cdot {}^{o}X_m}{{}^{o}X_m + \sum_j \left[G'_j \cdot {}^{o}S_j \cdot k(m,j) \right]} \qquad \text{.....(3.13)}.$$

For example, if G'_j is obtained by taking ${}^{r}T_i / {}^{c}T_i$ as an approximation for ${}^{r}X_i / {}^{c}X_i$ and applying equations 3.10 and 3.11, it is suggested

(in Appendix 5.2) that an iteration procedure based on 3.13 may be expected to converge on ${}^{r}X_{m}$ provided that all the initial ${}^{c}X_{i} > {}^{r}X_{i}$. This proviso is easily satisfied; for example, by taking the starting values for ${}^{c}X_{m}$ to be equal to the real concentration totals. Equation 3.12 may also be compared with two approximation formulae, 3.14 and 3.15. Under the starting conditions proposed in the last example for equation 3.13, when ${}^{c}X_{i} > {}^{r}X_{i}$, it is demonstrated that the unknown G_j factors are all, effectively, less than unity (see equation 5.5). So, by comparison with equation 3.8, it may be seen that the denominator in equation 3.12 is somewhat less than ${}^{c}T_{m}$. This means that the formula

$$ {}^{n}X_{m} = {}^{o}X_{m} \cdot \frac{{}^{r}T_{m}}{{}^{c}T_{m}} \qquad .....(3.14) $$

will always convert ${}^{o}X_{m}$, which is greater than ${}^{r}X_{m}$, to a value ${}^{n}X_{m}$ which is smaller than the desired real concentration. In other words, it overshoots. The expression has been tried in an iteration procedure but premature application was found to set up an oscillation with very poor or even no convergence power. However, if the equation 3.3 of Perrin and Sayce is re-written as

$$ {}^{n}X_{m} = {}^{o}X_{m} \cdot \frac{{}^{r}T_{m}}{\sqrt{{}^{r}T_{m} \cdot {}^{c}T_{m}}} \qquad .....(3.15) $$

the denominator is seen to be intermediate between ${}^{c}T_{m}$ (which would overshoot) and ${}^{r}T_{m}$ (which yields no improvement). Thus the tendency of 3.14 to overcorrect and to oscillate is curbed and

convergence may be exprected to follow. Another point which merits brief attention at this stage, is the fact that the square root function in equation 3.3 (and equation 3.15), moderates those ratios $({}^{r}T_m/{}^{c}T_m)$ which are far from unity to a greater degree than those which are close to this desired value.

With the preceding points in mind but also by trial and error, the following procedure was adopted. The first few iterations employ a formula 3.13 because it rapidly produces values in the proximity of the solution. Its success is in large measure due to the fact that it does not treat each component individually. The factors G_j' which modify the calculated complex species concentrations include ratios which take into account changes which are about to be imposed on the other free component concentration estimates. These approximation factors G_j' are obtained by assuming that equation 3.15 yields a fraction ${}^{n}X_m/{}^{o}X_m$ that approximates the F_i ratio. That is,

$$F_i' = \left[\frac{r_{T_i}}{c_{T_i}} \right]^{\frac{1}{2}}$$

With the number of iterations as a criterion, this arrangement would be employed exclusively but in fact, the formula requires so much computation that it is only profitable to employ it during the early stages of the iteration procedure. Apart from this economy, the expression is used because it helps to forestall divergence by coping effectively with the large discrepancies that arise when the real total concentrations are used as starting values for the free

concentrations. Once the application of 3.13 is completed, the program moves into an intermediate phase in which the iteration formula of Perrin and Sayce (3.15) is used alone. This expression is all the more satisfactory because it involves almost a minimum of computation (369). Finally, as the solution is approached, formula 3.14 is applied on alternate iteration cycles. Contrasting this formula with equation 3.12 reveals that the former improves progressively as the G_j factors tend to unity during the final stages. This additional facility improves the rate of convergence which would otherwise fall off quite markedly as the estimated free concentrations get closer and closer to the solution. The fall off in convergence rate as the solution is approached is by no means unusual for successive approximation techniques. So, various convergence forcing methods were tried. These efforts were not rewarded by significant improvements in performance and were thus abandoned.

3.1.2 Program coding.

A FORTRAN V program called ECCLES (for Evaluation of Constituent Concentrations in Large Equilibrium Systems) was written and tested on the University of Cape Town's UNIVAC 1106 computer. A program listing and an operation manual which details the program's requirements and capabilities may be found in the appendix. In addition to the mathematics and general principles described in section 3.1.1 special consideration was given to ways which would facilitate the gross manipluations inherent in a simulation of any system as large as the envisaged blood plasma model. To prevent waste when only the components' free concentrations at equilibrium are required, a user option permits the

print-out of the very numerous complex species concentrations to be suppressed. A search-and-sort routine can also be implemented to find and output in order, the forty most predominent species formed at equilibrium from each or just a selected few components. This is particularly helpful with the comparison between two sets of output which reflect some equilibrium displacement, because it focuses attention on any component in question. In addition, it is possible to examine the effects of any systematic alteration in the total or free concentrations of a given component. The program increases the concentration of the component which is being scanned, either by addition of or multiplication by an amount specified. On each occasion, it solves for the new equilibrium values. This procedure is far more efficient than the alternative which would require repetitive execution of the entire program because at the start of each cycle, the previous equilibrium concentrations and not the real totals are used as the new initial estimates. As the displacement is usually fairly small, these previous amounts rarely turn out to be an inferior choice.

The most restricting aspect which applies to any program designed to calculate equilibrium concentrations in the present context is certainly the magnitude of the k(i,j) matrix. The latter defines the number of each component in a complex species. Because of the very limited number of components which can associate to form a chemical unit, the array can be clearly seen to contain a very large proportion of zeros. The saturation of co-ordination is a most common expression of this limit and in the case of many ligands in solution with metal ions, prevents more than, say, ten components

from combining into one complex. So, unless steps are taken to eliminate these superfluous zeros, both core storage and time expended in needless processing will be seriously increased. Moreover, the larger the system, the more acute this detrimental factor will become. Hence, the most important concern of program coding must be to set up and operate within, a bookkeeping routine that minimises this sort of waste.

At present, the bookkeeping in ECCLES centres about a three-dimensional array which replaces the conventional k(i,j) matrix. The new array holds, for each complex species, a pair of numbers which specify not only the number of times the component appears but also the identity of the component itself. Although this increases the number of computer operations which are required to reference the components in a complex species, a considerable saving is achieved, on balance, because only the relevant components are involved. The need to make an arbitrary choice about the maximum number of components in any complex species is inconvenient but an incorrect decision is easily adjusted. It is, in fact, possible to improve further the storage features of the program but by and large, this is undesirable because a more sophisticated bookkeeping technique could easily entail an unwarranted increase in processing time. An improvement that probably will prove to be an exception in this regard, involves storing both of the numbers referred to above in one memory location. This could be achieved by combining the integers, using some reversible arithmetic process such as the muliplication of the first by a factor of ten or one hundred and then adding the second. This would eliminate

the need for the array's third dimension and would result in an overall storage reduction of about 30-40 percent.

3.1.3 Program testing.

The program ECCLES was initially tested on data pertaining to several small equilibrium systems so that the solutions could be easily checked manually. Once it was clear that no elementary coding errors remained, the program was applied to the same data as that used to exemplify COMICS (111, 77). ECCLES converged on the solution in less than twenty seconds compared with the time of somewhat over two minutes needed by COMICS on the same computer, ECCLES has also been tested using the blood plasma model of Hallman *et al*, (158). The results were identical to those published by the above workers and they appear in Table 3.1. To date, ECCLES has successfully tackled an equilibrium model having some 4000 complex species from 55 components (see Section 3.2). This simulation required 70 K of computer core. The equivalent investigation using COMICS would take about 200 K, a figure that exceeds the working capacity of the large majority of computers presently available.

To the best of this author's knowledge, the largest model which has been referred to in the literature contained 'up to a few thousand' species and some thirty components (245). As a listing of the program which was used in this instance cost $50 for printing and handling (private communication), the actual core storage requirements have not been determined. However, taking for comparison, the

TABLE 3.1

COMPUTED DISTRIBUTION OF COPPER AND ZINC IONS AMONG 17 AMINO ACIDS AT PH = 7,4 AS OBTAINED BY 'COMICS' (158) AND 'ECCLES'.

$[Cu^{2+}] = 3,4. 10^{-13}M$; $[Zn^{2+}] = 4,0. 10^{-6}M$.

COMPLEX	Concentration of complex as percentage of total metal.
(Cu. Cystine. Histidine)$^-$	48
(Cu. H. Cystine. Histidine)	37
(Cu. (Histidine)$_2$)	13
(Cu. (Glutamine)$_2$)	0.3
(Cu. OH. Histidine)	0.3
(Cu. H. (Histidine)$_2$)$^+$	0.2
(Cu. Histidine)$^+$	0.2
(Zn. Histidine. Cysteine)$^-$	24
(Zn. Histidine)$^+$	21
(Zn. (Cysteine)$_2$)$^{2-}$	16
(Zn. H. Histidine. Cystine)	5.8
(Zn. (Histidine)$_2$)	5.7
(Zn. Glutamine)$^+$	3.3
(Zn. H. Cysteine. Histidine)	2.2
(Zn. Histidine. Cysteine)	1.7
(Zn. H_2. Histidine. Cystine)	1.5

number of complex species to be 4000 COMICS would need about 120 K of core. This emphasises the fact that the size of a model is very much a function of the number of components as well as the number of complex species.

3.1.4 Discussion.

ECCLES suffers from two chief limitations. Both are of a fundamental nature and arise from the type of simulation for which the program was originally designed, namely the study of metal ion-ligand equilibria in biofluids. The first limitation has already been mentioned; the program is inapplicable to systems other than those confined to a single phase. However, Cumme has noted that, in many instances, a multiphase feature is not necessary for biochemical simulations (119). The second restriction applies to the hydrogen ion mass balance relation. To accommodate hydrolysis, it is conventional to regard the total hydrogen ion concentrations as negative whenever the total hydroxyl ion concentration predominates in aqueous solution (629). This is mathematically quite acceptable even if negative concentrations at first appear somewhat strange! Unfortunately, negative total concentrations are not compatible with the ECCLES iteration procedures, particularly with regard to the square root in Perrin's and Sayce's equation 3.3. By considering the hydrogen ion mass balance separately, it would be possible to apply a different optimization procedure to this component and thereby eliminate the obstacle. However, as the pH and not the total hydrogen ion concentration of most biofluids is experimentally

measurable, this additional facility is not essential and has not been included.

With the exception of size requirements, speed and that, as presently coded, COMICS is unable to accept free concentrations for components other than hydrogen ion, this program and ECCLES are both subject to the same limitations. This is not the case with HALTAFALL, however. The latter Swedish program can simulate titrations because it can process total hydrogen ion concentrations (that can become negative) and it can also deal with multiphase problems. It is acknowledged that the more general nature of HALTAFALL is certainly desirable. In fact, it will become essential for simulations which are required to determine the distribution of reactants between various biofluid compartments in living systems. After all, as Ingri *et al.* have commented 'computer time is getting less and less expensive in comparison with human time' (112). It is obviously bad economics to write programs of a specific nature when the alternative remains open. However, with the computer resources presently available, there is no alternative. A HALTAFALL type of approach would seriously curtail the size of the biosystem which could be investigated. A detailed look at the thousands of metal ion reactions contributing towards the competitive equilibrium in each biofluid is the most useful simulation which can realistically be attempted. Models of the partition between various body compartments still belong to the future. This is not only because the former simulations require all (and more) of the available core storage and would need to be set up simultaneously but also because very little data has so far been accumulated on the distribution coefficients which a partition model would require.

In conclusion, it should be borne in mind that the performance of this type of program, especially considering the non-linear nature of the problem, can really only be judged by trial and error. With limited time available, it is not easy to select, unerringly, either the best iteration procedures or the most revealing test data. Nevertheless, within the context of large systems, the three-tier algorithm which has been developed for ECCLES appears to be robust and quite quick. No doubt, as the computer industry matures and machine capacities increase, the approach outlined or other similar methods will be able to expand until the ultimate goal, the simulation of the entire metal ion picture *in vivo*, is at last achieved.

3.2 The blood plasma models.

It follows from the preceding sections that it is not presently possible to set up and execute a single simulation of blood plasma which will unequivocally reflect the low molecular weight complex distribution of the transition metal ions in the biofluid. The difficulties associated with metal protein binding as discussed in Section 2.2 are not the only obstacle. The concentrations of many components are not completely certain. Even which ligands to include in the model is open to dispute. Some formation constants remain unmeasured. Those that have been determined are subject to experimental error. Proteins are likely to bind ligands as well as metal ions. This binding has not been sufficiently studied to incorporate it in the simulation. Even low molecular weight ligand-ligand interactions occur but as yet remain inadequately understood.

In spite of all these difficulties it is proposed that a satisfactory and informative picture of the low molecular weight complex distribution in plasma can be achieved. This may be accomplished by conducting numerous simulations specifically designed to throw light on the magnitude and nature of the errors introduced by each of the model's various defects.

3.2.1 The composition of the plasma models.

The strategy which is adopted towards the selection of components and complex species is largely dictated by the availability of experimental data. The restrictions thus introduced are seldom

unduly onerous because investigators tend to study the more abundant and more important substances first. The assumptions made and the criteria employed are detailed below. The evaluation of each, however, is reported in Section 3.2.3.

1) The criteria for selection of ligand components.

Initially, just less than one hundred ligands were selected from various tabulations on blood plasma composition (577, 647, 648). This number was reduced as follows. High concentration was chosen as the first criterion. It seems unlikely that a ligand in low abundance will play an important role in metal ion distributions when one considers the relatively high concentrations and high formation constants of substances such as the amino acids and the organic oxyacids (for example, citrate). This eliminated pyridoxine, nitrite, histamine, folic acid, thiamine, serotonin and biotin amongst others. Next, a number of ligands were omitted owing to insufficient formation constant data. The most significant of these were taurine, borate, bilirubin, creatinine, glycocyamine, indican, urea, uric acid, acetone, glucose and glycerol. The defects introduced by these omissions are easily repaired as soon as the necessary data becomes available. Finally, some ligands were eliminated because of weak binding; bromide, fluoride, chloride, iodide and nitrate were considered to contribute to the metal complex distribution only as a result of their background electrolyte effects (see Section 2.1). The list of remaining ligands, used in the plasma models is to be found in Appendix 5.7.

2) Criteria for the selection of metal ion components.

The criterion of high concentration is as important for metal ions as it is in the case of ligands. If the model is to compute a realistic distribution, no metal ion whose concentration is sufficient to compete for ligands and thereby significantly influence the ligand protonation equilibria, can be legitimately neglected. The ions which are thus essential to the model are calcium, magnesium, and zinc. Manganese and lead may also belong to this category. On the other hand, metals such as iron, whose free concentration is very small indeed, do not need to appear because they cannot materially alter the free ligand concentrations. This question is probed at length in Section 3.2.3. Of course, iron and other such metal ions may be included in order to investigate their complex distribution. This work is actually concerned with both iron and copper in addition to the other metals already mentioned because their biological roles have been the most studied and are the best understood. Other metals such as nickel and chromium can easily be incorporated at a later date.

3) Criteria for the selection of complex species.

For the most part, the complex species included in the simulation are those which have been found in experimental studies on individual metal-ligand systems. Also, as indicated in Section 2.3.3, the ternary complexes are considered most important so, where experimentally determined formation constants are not available, these have been estimated instead. The ligands which are taken to participate in mixed ligand complex formation with a particular metal ion are those

for which formation constants for the species ML_2 or $M(LH)_2$ are available. For this reason, a number of ML_2 formation constants have been guessed when no experimental value could be found provided that, at the same time, no reason for the non-existence of the complex could be envisaged. Polynuclear complexes have been widely ignored. This is justified by the very low metal to ligand ratios in blood plasma. In any event, very few formation constants for polynuclear complexes are available. Quaternary complexes have also been excluded from the model. Criteria for selecting those ligands which may participate in the formation of such complexes are not clear-cut. They should certainly encompass a wider field than merely those ligands which form ML_3 complexes but, except in the simplest of circumstances, the factors which influence the stabilization of the complexes are uncertain. Cromer-Morin *et al.* have shown that with ligands that do form ML_3 complexes that statistical factor accounts for the experimentally observed stabilization almost entirely (223). However, the ligands they studied, glycine, alanine and valine, were not very dissimilar so their results are to be expected. The likely effects of omitting quaternary complexes from the simulation are considered in Section 3.2.3. Finally, the redox equilibrium balance needs to be considered. If the data is available, it is possible to include the appropriate redox relationships as constraints upon the simulation. However, owing to the uncertainties which are associated with the free concentrations of transition metal ions it is easier and probably more reliable to regard different oxidation states of the same metal ion as different components. In particular, Cu(I) has been totally ignored. Perrin has commented on the implications of this concerning the redox equilibrium between cystine and cysteine (158, 369). In line with his

conclusions, this study has assumed that both cysteine and cystine are present in plasma (see 666) and that other ligands preferentially stabilize Cu(II) by lowering the oxidation potential of Cu(II)/Cu(I). On the other hand, Fe(II) has been introduced into a few simulations to investigate the possible influence of this ion (see Section 3.2.3).

4) The ligand concentrations.

The ligand concentrations used in the blood plasma simulations are documented in Appendix 5.7. These are usually the average of many reported values; both standard tabulations and more recent literature were consulted (see Appendix 5.7 for references). However, the concentrations of several components were reduced to correspond with the known protein binding of the ligand in question. Although interactions with protein are likely to be a common phenomenon, several substances are very much more tightly bound than the others. The most important of these are salicylic acid, tryptophan, urea, fatty acids, bilirubin, folic acid and fluoride. The last five substances have not been included in the model so protein binding serves to minimise the error which is thus introduced. The total concentration of salicylate in plasma is normally about 1,3mg/100ml which is equivalent to 100μM. The binding to plasma proteins, especially albumin, is well documented (296,375,247). The concentration not bound to protein varies between 0 and 25 percent (247). The level may be raised by doses of aspirin (acetylsalicylic acid) and acute toxic symptoms are produced when about 50 percent is not bound to protein (247). From the study by Lomax (375) it is estimated that a concentration of 5μM is representative of the amount of salicylate available for low molecular weight complexation. The free concentration

of the other component tightly bound by protein, tryptophan, is less certain. The total is about 58μM. Fuller and Roush report that 13 percent is not bound to protein (239) as opposed to the 25 percent cited by Peters (190). A free concentration of 10μM has been adopted in the present work. This is in agreement with a value calculated from the results of McMenamy (556) - particularly if competition for serum albumin binding sites by other ligands is taken into consideration. This author has also provided valuable evidence that the other amino acids are not bound to serum albumin to any significant extent (556, 558).

For reasons of economy, many of the simulations performed have included a single component to represent five very similar amino acids namely alanine, aminobutyric acid, isoleucine, leucine and valine. These ligands are all homologues of glycine and contain no functional groups other than the definitive one. Their metal binding abilities are almost idential as can be seen in Table 2.2. The component has been named the 'average amino acid' (AAA). It has a concentration equal to the total of the concentrations of all the amino acids it represents. Perrin has set a precedent for this procedure (369). Several simulations conducted during the course of this work confirm that it imposes negligible distorition on the models' results (see Section 3.2.3). Perrin, in fact, included glycine in his average component. Whilst, there is no objection to this, glycine has been included separately in the simulations of this thesis simply for purposes of comparison with the average amino acid component. This provides a further check on the validity of the simplification.

5) The metal concentrations.

Owing to metal protein binding and the factors detailed in Section 2.2, it is not possible to arrive at an unequivocal concentration which can be used for each of the metal ions. Thus, it is intended to employ a range of values for the uncertain concentrations. This is reported in Section 3.2.3. Meanwhile, a set of approximate values needs to be determined in order that they may be used for purposes of comparison (see Table 3.2). Also they will help to establish a reasonable concentration range that should be scanned. The means whereby these estimates can be obtained is now described for each metal ion in turn.

(a) Calcium (194, 525, 171, 202, 198, 136, 142).

Experimental studies on calcium in blood plasma are more advanced than is the case of any of the other metal ions. The free concentration has been measured frequently; the best determinations have employed specific ion sensitive electrodes to arrive at a value of $[Ca^{++}]$=1,12mM (202). In fact most estimates do not fall far from this. Greater precision cannot be expected because of considerable physiological variation. Changes of up to 20 percent of the free ion concentration are regarded as normal (577). Taking the mean total calcium concentration in serum to be 2,5mM, about 1,0mM is bound to protein (136). This leaves about 0,3mM of the calcium to appear as low molecular weight complexes.

(b) Copper.

The total concentration of copper in blood plasma is about 18μM but

almost all of this is very tightly bound in ceruloplasmin. The 'exchangeable' non-ceruloplasmin copper concentration is only about 1μM (133, 144, 135, 491, 369). Futhermore, the larger percentage of this exchangeable metal ion fraction is bound to serum albumin (190). The blood plasma model requires either the total copper bound to low molecular weight ligands (T_{LMWF}) or the free concentration. There are several approaches which help to estimate these quantities. Lau and Sarkar have determined a practical dissociation constant for the copper-albumin binary complex (185). By assuming that (i) the vast proportion of exchangeable copper in plasma is bound to albumin and (ii) this quantity is negligible compared with the total albumin concentration of 500μM (190) it is possible to solve for a free copper concentration.

$$K_D = \frac{[\text{Alb}][\text{Cu}^{2+}]}{[\text{Cu Alb}]} = 6{,}6 \times 10^{-17}$$

By substitution of $[\text{Alb}] = 500\mu M$ and $[\text{Cu Alb}] = 1\mu M$, this yields:

$$[\text{Cu}^{2+}] \doteq 1 \times 10^{-19} M$$

In plasma, other cations will compete for the copper binding site so this figure may be regarded as a minimum value. It should be noted here that in any event, the free copper concentration is below the level which could be measured by ion selective electrodes. This is approximately $10^{-9}M$ (667). On the other hand, the total concentration of copper bound to the low molecular weight fraction is

indicated by several reports in the literature. Neumann and Sass-Kortsak show that at physiological ratios of copper to serum albumin about 0,4 percent of the metal ion is ultrafiltratable. If this is taken to represent the low molecular weight complex fraction, one obtains $T_{LMWF} \doteqdot 5 \times 10^{-9}M$. This is not far from a value cited by Peters (190). Furthermore it is in reasonable agreement with calculations made by Osterberg (319). Osterberg estimates that the copper albumin to copper mixed amino acid complex ratio will be about 10^6. This puts $T_{LMWF} = 10^{-12}M$ as a lower limit.

(c) Ferric iron.

The total concentration of plasma iron is about 22μM and at least 99,99999 percent of it is bound to transferrin (see Section 3.2.2). The free concentration of ferric ion is limited by the solubility product to a maximum of about $10^{-18}M$ under physiological conditions of pH (138). In fact, the free concentration is likely to be considerably lower than this. A number of iron transferrin binding constants have been measured (see Section 1.3.1). The most appropriate and easy to apply is the apparent constant for physiological conditions measured by Aasa *et al.* (162, 611). They have proposed

$$\frac{[FeTr]}{[Fe^{3+}][Tr]} = 5 \times 10^{23} M^{-1}$$

so that

$$[Fe^{3+}] = 2,2 \cdot 10^{-5}/3,3.10^{-5} \cdot 5,10^{23}$$
$$\doteqdot 10^{-24}M$$

(d) Lead.

The estimation of even the most approximate free concentration of plumbous ions in blood plasma has proved most difficult. The reasons for this are twofold. Lead is not specifically associated with any particular plasma protein (225). Moreover, the studies on the binding of this metal to serum albumin are in disagreement concerning the nature of the interaction and the sites at which it takes place (22, 28). The total concentration of lead in plasma varies quite considerably; average levels are about 0,5μM (577, 647, 648, 534) but increase to 3,0μM before it is possible to diagnose plumbism (311). It is estimated from the data provided by Gurd and Murray (28) that the maximum free concentration is below 10^{-9}M. However, as many proteins other than albumin compete for the metal the normal value is likely to be considerably lower than this.

(e) Magnesium (194, 143, 272, 531, 532, 148).

Few investigators have attempted to estimate free magnesium ion concentrations in plasma because measurements using selective ion electrodes are not presently reliable, especially in the presence of other metal ions. This is in contrast to the position with calcium. However, Heaton has obtained as approximate value from measurements of adsorption by a cation exchange membrane (532). He found that ionized magnesium averaged 79 percent of the ultrafiltratable magnesium in serum. This yields a free concentration of about 530μM. Many experiments have determined the fraction of magnesium not bound to protein. The average is close to 650μM of

of the cation. Unfortunately, a number of these studies appear to neglect the low molecular weight complex fraction (see, for example, 143 and 148). In any event, it seems unlikely that the free magnesium fraction will constitute less than 530/650, that is about 80 percent of the non-protein bound amount.

(f) Manganese (262, 149, 257, 332, 155, 612, 554).

There has been a great deal of controversy about manganese in blood plasma. Conflicting assertions concerning the protein to which the metal is selectively bound have appeared even quite recently (462,257). Furthermore, the total concentration in plasma which is generally reported to be approximately 1,0μM (577, 534, 647, 648) has been attributed to contamination by workers using the very sensitive neutron activation procedure (612). It seems best, therefore, to determine a maximum free concentration from binding constants measured for serum albumin and scan downwards from there. Neglecting all the weak binding sites in view of the low metal to protein ratio, a free concentration of approximately 5.10^{-8}M is obtained (from 155, 149, 257, 332). Making the assumptions analogous to those for copper, iron and zinc (see (b), (c), and (g)) the concentration is obtained from

$$K = \frac{[\text{Mn Alb}]}{[\text{Mn}]\,[\text{Alb}]} \doteq 3,10^{4}$$

(g) Zinc.

The total concentration of zinc in plasma is about 16μM (577, 606, 196, 669, 164) and not 46μM as is so often claimed (286, 158, 369, 647, 648, 534 etc.). The erroneous value appears to stem from a 1948 report by Vallee and Gibson (670). Of the total, some 35 percent is firmly bound in a metallo protein identified as an α_2-macroglobulin (345, 577). This means that the concentration of exchangeable zinc in plasma is about 10μM. Most of this is loosely bound to serum albumin (see Section 1.3.1). From the study of zinc binding to serum proteins by Prasad and Oberleas it seems that less than two percent of the 10μM will be available to the low molecular weight fraction (140). Furthermore, an approximate value of the free zinc concentration in plasma can be obtained from a practical constant evaluated by Giroux and Henkin (164). The constant is derived from measurements on the competition set up in serum between the albumin protein and the low molecular weight amino acids. The workers found that

$$K = \frac{[\text{Zn Alb}]}{[Zn^{2+}][\text{Alb}]} \doteqdot 10^7$$

Making the same assumptions as in the case of copper, iron and manganese namely that (i) almost all the metal is protein bound and (ii) that as the albumin/zinc ratio is so large the free albumin concentration is equal to the total value, one finds

$$[Zn^{2+}] \doteqdot 10^{-9}M$$

TABLE 3.2

METAL ION CONCENTRATIONS USED IN THE SIMULATIONS

Metal Ion	Free concentration estimates used as average	Free concentration range scanned	Estimated total low molecular weight metal complex concentration	Approximate total 'exchangeable' metal ion concentration
Ca^{2+}	1,14mM	Fixed	300µM	2,45mM
Cu^{2+}	10^{-18}M	10^{-19}M-10^{-7}M	10^{-12}M-10^{-9}M	1µM
Fe^{3+}	10^{-23}M	10^{-24}M-10^{-18}M		
Pb^{2+}	10^{-14}M	10^{-16}M-10^{-9}M		500nM
Mg^{2+}	520µM	510µM-550µM	120µM	900µM
Mn^{2+}	10^{-12}M	10^{-15}M-10^{-8}M		10^{-8}M-10^{-6}M
Zn^{2+}	10^{-9}M	10^{-11}M-10^{-6}M	10^{-7}M-10^{-6}M	10µM

The model can be used to determine whether this free concentration yields a total low molecular weight zinc complex concentration in agreement with the 200nM maximum which is estimated from the experiments of Prasad and Oberleas mentioned above. This check does away with the need to employ other methods of estimating the free zinc concentration. For example, an approach using the imidazole binding constant, at sixteen identical and independent sites, as evaluated by Gurd and Goodman(23) is not necessary.

3.2.2 The results

'Without generalisation there is no meaning and
without concreteness there is no significance.'

A.N. Whitehead
(cited in ref. 647)

Throughout the following presentation of the results of the blood plasma simulations, a deliberate attempt has been made to avoid tabulating the absolute values which the models have produced. This is in recognition of the fact that such values reflect most accurately the error in the parameters from which they are generated. Instead, it seems more propitious to record the results in a manner which is as independent of the variation in the parameter values as possible. With this in mind, the formation of each complex is expressed as a percentage of the total concentration of the relevant metal ion contained in the low molecular weight complex fraction. The constancy of these percentages is discussed at greater length below. On the other hand, such generalisation is not always the most suitable means of comparing the models' results with experimentally measured concentrations. They also cannot be used to calculate approximate concentrations of complex species

which have not been included in the model, to judge whether they may be significant. For this reason, absolute concentrations have sometimes also been presented. However, the uncertainty associated with these values should not be discounted (see Section 3.2.1). If a realistic picture is to emerge, it is essential to maintain a balanced perspective between the general and the specific aspects of the results.

Before the computation of the metal ion distributions was initiated, it was necessary to choose a representative pH for the plasma solution. An average value of $-\log [H] = 7,4$ was adopted. The activity coefficient is unlikely to alter the concentration obtained from hydrogen ion activity by more than 0,1 log units (see 394) so, as this is well within the normal physiological variation, the coefficient has been neglected. Two limits of plasma pH corresponding to $-\log [H] =$ 7,6 and 7,2 respectively were also selected. This was in order to evaluate the influence of hydrogen ion concentration on the metal complexes formed in plasma. It is in line with the idea that as many parameters as possible should be varied to gauge their effect on the computed distributions. Although very few of the formation constants listed in Appendix 5.6 apply to complex species defined in terms of hydroxyl ion instead of negative hydrogen ion indices, a few exceptions do occur. These were not converted as a general rule due to uncertainty in the respective values of the *experimental* dissociation constant of water, pK'_w. So, the hydroxyl ion concentration in the plasma medium needs to be calculated instead. A value of $pK'_w =$ 13,62 was used for this purpose (see 321 and the reference therein).

Using the free metal ion concentrations listed in Table 3.2 as those which, in this author's opinion are representative of the true

values in plasma (see Section 3.2.1) distributions of the metal ions Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Pb^{2+}, Mn^{2+} and Zn^{2+} amonst 35 ligands were computed using program ECCLES. The formation of 3967 complex species was simulated. The program was instructed to print out the 40 most predominant complexes formed with each component in turn. The output also included the free ligand concentrations and the total concentration of each metal contained in the low molecular weight fraction. The results are to be found in Table 3.3 and 3.4 respectively. The percentage of the total low molecular weight metal ion concentration bound by the most predominant complexes for each metal ion is listed in Table 3.5 for each complex under three different conditions of pH. These results all apply to the 'primary' model, the parameters and data for which are provided in Appendices 5.6 and 5.7.

The results of the 'primary' model exhibit a number of aspects which merit specific attention. Possibly the most striking is the effect of the relatively limited changes in hydrogen ion concentration. It is not surprizing to find that the total concentration of each metal complexed to low molecular weight ligands increases with pH. However, the marked alteration in the computed distribution as shown in Table 3.5 is possibly less expected. Moreover, it is no simple matter to foresee the direction of the change undergone by several of the complexes individually. Whilst there is often no discernable alteration, both increases and decreases in the degree of complexation can be observed for increasing pH. The changes are really only predictable when two complexes which differ only in their state of protonation are in obvious competition (for example, the two most abundant copper complexes). The metal ions in plasma appear to form complexes in a manner that is reminiscent of the categories suggested

TABLE 3.3

TOTAL CONCENTRATIONS OF METAL IONS IN THE LOW MOLECULAR WEIGHT COMPLEX FRACTION IN PLASMA

METAL ION	FREE ION CONC(M)	TOTAL CONCENTRATIONS -log [H]=7,2	-log [H]=7,4	-log [H]=7,6
Ca^{2+}	$1,14x10^{-3}$	$1,41x10^{-3}$M	$1,43x10^{-3}$M	$1,47x10^{-3}$M
Cu^{2+}	10^{-18}	$3,35x10^{-12}$M	$1,12x10^{-11}$M	$4,91x10^{-11}$M
Fe^{3+}	10^{-23}	$3,84x10^{-13}$M	$6,71x10^{-13}$M	$1,34x10^{-12}$M
Pb^{2+}	10^{-14}	$1,43x10^{-11}$M	$3,82x10^{-11}$M	$1,20x10^{-10}$M
Mg^{2+}	$5,20x10^{-4}$	$6,42x10^{-4}$M	$6,53x10^{-4}$M	$6,72x10^{-14}$M
Mn^{2+}	10^{-12}	$1,57x10^{-12}$M	$1,63x10^{-12}$M	$1,71x10^{-12}$M
Zn^{2+}	10^{-9}	$4,67x10^{-8}$M	$1,58x10^{-7}$M	$8,52x10^{-7}$M

TABLE 3.4

FREE LIGAND CONCENTRATIONS IN PLASMA.

(Simulation for $-\log [H] = 7,4$)

LIGAND	CONC(M)
AAA*	$7,74 \times 10^{-6}$
Arginate	$3,46 \times 10^{-6}$
Asparaginate	$2,72 \times 10^{-6}$
Aspartate	$7,75 \times 10^{-8}$
Citrullinate	$1,28 \times 10^{-6}$
Cysteinate	$5,89 \times 10^{-9}$
Cystinate	$4,35 \times 10^{-7}$
Glutamate	$4,83 \times 10^{-7}$
Glutaminate	$1,86 \times 10^{-5}$
Glycinate	$2,51 \times 10^{-6}$
Histidinate	$2,41 \times 10^{-6}$
Hydroxyprolinate	$8,66 \times 10^{-8}$
Lysinate	$4,83 \times 10^{-9}$
Methionate	$8,64 \times 10^{-7}$
Ornithinate	$5,73 \times 10^{-9}$
Phenylalanate	$1,99 \times 10^{-6}$
Prolinate	$2,41 \times 10^{-7}$
Serinate	$4,25 \times 10^{-6}$
Threoninate	$6,96 \times 10^{-6}$
Tryptophanate	$1,99 \times 10^{-7}$
Tyrosinate	$3,52 \times 10^{-9}$

LIGAND	CONC(M)
Carbonate	$3,23 \times 10^{-5}$
Phosphate	$3,79 \times 10^{-8}$
Silicate	$2,67 \times 10^{-10}$
Sulphate	$2,04 \times 10^{-4}$
Thiocynate	$1,40 \times 10^{-5}$
Ammonia	$5,73 \times 10^{-7}$
Citrate	$2,67 \times 10^{-5}$
Lactate	$1,72 \times 10^{-3}$
Malate	$3,11 \times 10^{-5}$
Oxalate	$7,70 \times 10^{-6}$
Pyruvate	$9,41 \times 10^{-5}$
Salicylate	$1,25 \times 10^{-11}$
Succinate	$4,10 \times 10^{-5}$
Ascorbate	$4,78 \times 10^{-8}$

*AAA = Alanate, Aminobutrate, Isoleucinate, Leucinate and Valinate combined.

TABLE 3.5

PREDOMINANT COMPLEXES OF EACH METAL ION FOUND BY SIMULATION OF BLOOD PLASMA

(Symbols defined in Appendix 5.7)

COMPLEX	PERCENTAGE OF THE TOTAL METAL BOUND IN THE LOW MOLECULAR WEIGHT COMPLEX FRACTION		
	-log [H]=7,2	-log [H]=7,4	-log [H]=7,6
CA+2(1) CO32(1) H+1(1)	8	8	8
CA+2(1) LTA1(1)	5	5	5
CA+2(1) CTA3(1)	4	4	4
CA+2(1) CO32(1)	1	2	4
CU+2(1) CIS2(1) HIS1(1)	21	30	43
CU+2(1) CIS2(1) HIS1(1) H+1(1)	28	24	17
CU+2(1) HIS1(2)	17	16	14
CU+2(1) HIS1(1) THR1(1)	9	8	7
CU+2(1) HIS1(1) SER1(1)	5	5	4
CU+2(1) HIS1(1) AAA1(1)	4	4	4
CU+2(1) HIS1(1) GLN1(1)	2	2	2
CU+2(1) HIS1(1) GLU2(1)	2	2	2
CU+2(1) HIS1(1) GLY1(1)	1	1	1
FE+3(1) CTA3(1) OH-1(1)	99	99	99
FE+3(1) CTA3(1) SLA2(1)	-	-	-
FE+3(1) CTA3(1) GLU2(1)	-	-	-
FE+3(1) CTA3(1) OXA2(1)	-	-	-
FE+3(1) CTA3(1) GLN1(1)	-	-	-
FE+3(1) CTA3(1) GLY1(1)	-	-	-
FE+3(1) CTA3(1) SCA2(1)	-	-	-
FE+3(1) CTA3(1) SER1(1)	-	-	-
PB+2(1) CYS2(1)	73	77	80
PB+2(1) CYS2(1) CTA3(1)	10	10	11
PB+2(1) CIS2(1) H+1(1)	12	7	4
PB+2(1) CYS2(1) P043(1) H+1(1)	4	5	5
PB+2(1) CYS2(2)	-	-	3
MG+2(1) CO32(1) H+1(1)	8	8	8
MG+2(1) CTA3(1)	5	5	5
MG+2(1) LTA1(1)	3	3	3
MG+2(1) CO32(1)	1	2	4
MG+2(1) P043(1) H+1(1)	1	2	2
MN+2(1) CO32(1) H+1(1)	25	25	25

(continued)

TABLE 3.5 (continued)

COMPLEX	PERCENTAGE OF THE TOTAL METAL BOUND IN THE LOW MOLECULAR WEIGHT COMPLEX FRACTION		
	-log [H]=7,2	-log [H]=7,4	-log [H]=7,6
MN+2(1) CTA3(1)	3	3	2
MN+2(1) C032(1)	1	3	5
MN+2(1) P043(1) H+1(1)	2	2	2
MN+2(1) OXA2(1)	2	2	1
MN+2(1) S042(1)	1	1	1
MN+2(1) LTA1(1)	1	1	1
ZN+2(1) CYS2(1) CTA3(1)	43	36	22
ZN+2(1) CYS2(2)	9	21	41
ZN+2(1) CYS2(1) HIS1(1)	10	15	18
ZN+2(1) CYS2(1)	4	4	2
ZN+2(1) HIS1(1)	6	3	1
ZN+2(1) CYS2(2) H+1(1)	1	2	2
ZN+2(1) HIS1(2)	2	2	1
ZN+2(1) HIS1(1) CYS2(1) H+1(1)	2	1	-
ZN+2(1) CYS2(1) CIS2(1) H+1(1)	1	1	1
ZN+2(1) CYS2(1) GLN1(1)	-	1	1
ZN+2(1) AAA1(1) CYS2(1)	-	1	1
ZN+2(1) C032(1) H+1(1)	1	-	-
ZN+2(1) CTA3(1)	1	-	-

by R.J. Williams (see Table 1.1). Ternary complexes account for the large percentage of those formed by copper and ferric iron. Binary complexes are favoured by calcium, magnesium and manganese. Zinc and lead are intermediate in this respect. The concentrations of copper and iron complexes are respectively, some seven and ten orders of magnitude larger than the free metal ion concentrations. This fact lends credibility to proposals that low molecular weight complexes can have important biological roles in spite of the extremely low free ion levels of certain transition metals. It is interesting to note that all the more important copper complexes contain histidinate. Citrate plays an analogous role in the formation of ternary ferric complexes. It is to illustrate this aspect that the most predominant complexes of iron other than ferric hydroxy citrate are included in Table 3.5 even though they account for less than one percent of the total metal ion *in toto*. Another reason is that the formation constants used for these ternary species are all most probably too low. This is due to the deliberate policy of conservatism adopted during the estimation of formation constants (see Section 2.3). With very few exceptions, no measurements of the formation constants of ML_2 complexes have been made where M is ferric ion and L is an amino acid. Thus, it is to be expected that a more even distribution of iron amongst low molecular weight ligands will emerge when the necessary formation constant values have been determined. The predominance of the ferric hydroxy citrate species, on the other hand, is not surprizing as it reflects the tendency of ferric ion to hydrolyse in aqueous solution (see 619). Lead appears to be mainly bound by the sulphur donors, cysteinate and cystinate. The other amino acid in the model which contains sulphur is methionine. It does not appear

to compete effectively for lead. This is due to (i) the noticeably small formation constant of the lead methionine complex compared, say, with the cysteine equivalent and (ii) the fact that methionine tends to complex calcium and magnesium more than the other two sulphur amino acids. The similarities of the predominant complexes formed by lead and zinc are of interest. The ternary cysteine - citrate species is important in both cases. One of the poisonous aspects of lead overload may be the displacement of zinc by lead from cysteine moeities of certain enzymes. Simulating the effect of high lead concentrations on the distribution of these two metals amongst low molecular weight ligands (*vide infra*) actually shows that lead complexation occurs at the expense of zinc cysteinate complexes. Finally, it may be noted that the predominant species shown in Table 3.5 cannot be selected in a simplistic way looking only at the magnitude of their formation constants. This is true of all multicomponent systems.

It should be recorded that a simulation of the 'primary' model which included ferrous complexes revealed that their distribution pattern is akin to those of manganese, calcium and magnesium. Predominant complex species turn out to be ferrous-bicarbonate, ferrous-carbonate, ferrous-ascorbate, ferrous-histidinate and ferrous citrate. These results are not shown in Table 3.5 because the formal concentrations of ferrous ion and the very existence of the complexes in plasma is uncertain.

Using the 'primary' model as a reference, the free concentration of each metal ion in turn was varied through the range depicted in Table 3.2. The outcome of each concentration scan was then evaluated by comparison with the results of the 'primary' model. The concentrations of the complex species obviously increased in a manner corresponding to the rise of each component. However, as one would expect, the increase was also reflected in the total concentration of the metal contained in the low molecular weight complex fraction. In fact, the change in individual concentrations is paralleled by the changed in total concentration to the extent that the percentages recorded in Table 3.5 remain constant. In other words, these percentages are, to a precision of one percent, independent of the free metal ion concentrations which one chooses for a given model, within the ranges shown in Table 3.2. This may be at first sight rather surprizing because in general the complex distribution of a given system does change when the component concentrations are varied. Thus, the behaviour of the plasma system which is presently being simulated is exceptional. The reason for this is to be found in the fact that the formation of low molecular weight complexes in the biofluid is minimal. The very low total concentrations of the transition metal ions, the weak binding of the calcium and magnesium cations and the lowering of free metal ion concentrations by protein binding are all contributory factors. In consequence, the concentration of each complex species is altered in direct proportion to the change in free concentration of its metal ion component. This is evident from the general equation 3.1, provided that the free concentrations of the other components are unaffected by equilibrium displacements. The condition is not as a general rule fulfilled because the complex

formation tends to reduce the availability of the ligands (see Section 3.1). However, in the plasma simulations, the free concentrations of the ligands are usually several orders of magnitude larger than the complex concentrations. This is an outcome of the extremely low free metal ion concentrations. Thus, the free ligand concentrations are not significantly altered by changes in the complex concentration. So, the percentage of metal ion appearing in a given complex species is independent of the free metal ion concentration.

Of course, in systems where the complex formation is not negligible by comparison with the free ligand concentrations, the constancy of the percentages in Table 3.5 as described above, will no longer occur. In the case of the present models, deviation is in fact observed towards the upper limits of the free concentration range set out in Table 3.2 for magnesium, lead and zinc. Even in these extreme instances, the displacement observed is not large. The variation is usually confined to one percent and in no case exceeds five percent. This is especially gratifying in view of the unlikelihood of these higher concentrations *in vivo* (see Section 3.2.1). As is to be expected, the complexes of the metal whose concentration is scanned are influenced the most but the effect may be seen with the complexes of other metals as well. These results were extended and substantiated in two ways. A simulation using concentrations at the upper limit of the scanning range for all the metals produced a computed distribution the same, within a tolerance of two percent, as that shown in Table. 3.5. Then, all the simulations were repeated using the hydrogen ion concentration limits of $-\log [H] = 7,2$ and $7,6$. Although the computed distributions are quite different in each case (see Table 3.5), the percentages obtained were again independent of the

free metal ion concentrations that were adopted. This confirms conclusion that the overall picture of the metal ion distribution amongst low molecular weight ligands in plasma is not going to be upset by an improved knowledge concerning metal-protein binding in plasma even if this alters the estimates for the free metal ion concentrations.

3.2.3 Model evaluation.

One of the most powerful motivating factors leading to the development of the models presented in this thesis is the fact that the low molecular weight complexes of the transition metals exist in plasma in amounts which are not measurable by experiment. This is the outcome of their very low concentrations, their labile nature and the complexity of the system. There is therefore no direct means of verifying either the qualitative or quantitative results of this kind of simulation. Moreover, one is confronted with the problem of substantiating the theoretical construction by some other, less direct method: to neglect to test experimentally both the model and the hypotheses upon which it is founded is to violate the principles of the scientific method.

The inability to make direct measurements does not preclude the application of the scientific method. The detection and measurement of postulated low molecular weight species in plasma is certainly not the only acceptable test. Many theories in science are established entirely upon circumstantial evidence - a fact which in no way implies discredit. The paramount criterion, by which all hypotheses must ultimately stand or fall is the ability of models

which embody the hypotheses to produce verifiable predictions. This aspect is treated in Section 3.3. Before such considerations are made, however, it is proper to impose a number of internal checks. For example, it is obviously necessary to ensure that different aspects of the simulation are at least self-consistent. Furthermore, it is important to evaluate the error which is imposed by (i) the simplification inherent in any model and (ii) inaccuracies in the formation constants. It is only when this preliminary evaluation has been accomplished that the model is ready to be compared and contrasted with reality.

The 'internal' evaluation of the model which is now to be discussed, falls into two categories. The first is concerned with the effects due to numerical uncertainty in the data for the model. The second category deals with the error which results from the omission of complex species from the simulated system. The evaluation of the influence of both these factors on the computed distributions will, hopefully, yield some idea of the confidence which can be placed in the model's results as far as reproducibility is concerned. One would be interested in the effects of changes in (i) the formation constants supplied, (ii) the estimates of component concentrations and (iii) the complexes appearing in the simulated system. Thus, it is the model's precision rather than its accuracy which is investigated.

The majority of the investigations which are described below are accomplished by comparison of the results of simulations in whch the parameter in question has been varied. Although a less

empirical approach may be thought desirable, this is generally not possible. The complexity of multicomponent systems means that comprehensive theoretical analyses are quite out of reach. Moreover, most simple treatments are open to criticism for one reason or another. So, it seems best to credit empirical results whenever they can be successfully merged into a broad pattern.

The choice of ligand concentrations as described in Section 3.2.1 (4) is not an easy one. This is due (i) to the large number of low molecular weight substances in blood plasma and (ii) to the unknown extent of ligand-protein and ligand-ligand interaction. Only the most pronounced protein binding has been able to be accommodated. However, these major adjustments make it unlikely that the effective concentrations of the majority of ligands used will be far from the values adopted. In any event, there is no good purpose in reducing all the ligand concentrations by some constant proportion because the significant errors are going to arise when the ligand concentration ratios rather than the absolute concentrations are incorrect. The effect of employing radically different ratios is well illustrated by the ligands salicylate and tryptophan. These have been investigated because they are both predominantly bound to protein in plasma (see Section 3.2.1 (4)). The exact extent of this protein binding is uncertain. It transpires that this factor is most important in the case of salicylate. If protein binding is ignored, the ligand concentration is increased by a factor of twenty causing a redistribution of iron in favour of the ferric salicylate citrate complex (see Section 3.2.1 and Table 3.5). On the other hand, tryptophan is not involved in any of the major complexes found in plasma and thus, it may be concluded that the extent of protein binding is immaterial.

As the question concerning the free metal ion concentrations has been dealt with at length in Sections 3.2.1 (5) and 3.2.2, there is no need to reconsider the details here. Suffice it to say that the computed distribution is insensitive to errors in this regard unless unrealistically high concentrations are utilized.

The effect of errors in the formation constants of complex species in the simulated system is similar to the effect of most of the possible defects presently under consideration. The picture which has been discerned is simply that the error is only significant if it applies directly to predominant species. In other words, even moderately large errors in the formation constants of species which are found to exist in concentrations some orders of magnitude below the concentrations of the complexes shown in Table 3.5, can have little influence on the computed distribution. Moreover, there appears to be no cumulative effect of the errors pertaining to minor complexes. This is in no way surprizing but needs to be stated specifically because to assume as much without confirmation would be unwise. As a result of this observation, it is possible to focus on the relevant factors which may produce an erroneous computed distribution. To begin with, the protonated forms of each ligand are invariably important. Only in the case of sulphate and some of the organic oxy-acids such as citrate, malate, lactate and oxalate do the calcium and magnesium complex concentrations exceed the concentration of the singly protonated ligand. Fortunately the acid dissociation constants are likely to be amongst the most accurately known. Many have been measured under the model conditions. The rest have been determined under a wide range of temperature and ionic strength so adjustment procedures (see Section 2.3) have been facilitated. The same cannot be said for the calcium and magnesium complexes. The accurate determination of

the applicable formation constants will enhance the quantitative results of the simulations considerably. This is particularly true for the series of amino acids as ligands. Although calcium and magnesium are recognised to be weakly co-ordinating, the relatively high concentrations of these cations means that they form the most predominant (metal ion) complexes with all the important ligands except cysteine. Apart from these circumstances, the present model actually performs a valuable service towards the computation of accurate metal ion distributions in plasma by isolating the set of major species for each type of metal ion. By concentrating efforts to obtain very accurate formation constants for these important complexes, the goal may be achieved surprizingly quickly.

It is evident that regardless of the size of the model which may be constructed, some complex species existing in a mixture as complicated as blood plasma will inevitably be omitted. In view of this, it is important to be able to judge the extent of the effects which may arise because the model is incomplete. This has been attempted by undertaking a fairly intensive program during which many simulations were conducted using 'segments' of the primary model. On the one hand, individual species were excluded. Alternatively, all those complexes formed from one or more components were removed. This procedure showed that the segmentation of the model has an outcome similar to that described in context of numerical uncertainty in the model's parameters. Only the omission of the predominant species seriously disrupted the computed distribution (*vide infra*). Indeed, omission of a complex is tantamount to using a formation constant of zero and as such is an extreme case of error in this parameter. Nevertheless, the picture which emerges is somewhat surprizing. The very large errors involved are alone sufficient to

suggest that they might introduce distortion into the results. In addition to this, it is rare to find a simulation whch neglects some important relationships without upsetting the manifestation of the others. As in the case of the uncertainty associated with the free metal ion concentrations where a variation of several orders of magnitude did not alter the percentage of metal ion bound in a particular complex, the reason for this exceptional behaviour is to be found in the very small degree of metal complex formation in plasma. Once again, provided that the ligands in question are represented, their free concentrations are for the most part independent of factors other than the hydrogen ion concentration and the relevant protonation constants.

The generalisations asserted in the above paragraphs pertain only to the overall picture of the distribution of metal ion complexes in plasma. They are not intended to convey the impression that the simulation is quantitatively unaffected by the omission of various equilibrium constraints. In fact, quite the reverse is true. Whilst the order of the complexes, sorted according to highest concentration, is often difficult to upset, even this happens when the computed concentrations of two or more of them are of equal orders of magnitude. However, as evident from Table 3.5, the percentage of metal ion bound in the most predominant complexes usually falls off rapidly so small fluctuations do not disturb the broad picture. The situation is well illustrated by a comparison of the results of the 'primary ' model of this thesis with those of Perrin (158) shown in Table 3.1. Perrin's is a subset of the former model. To begin with, the percentage of each metal ion bound in a given form differs

noticeably. This is in spite of the fact that the major copper species are found to be the same in both cases. The very marked decrease in the percentage of copper distributed amongst the low molecular weight ligands as observed by Perrin is now suggested to be less pronounced. This is due to Perrin's failure to include important equilibrium reactions. This was partially rectified in a subsequent simulation by Perrin himself; the copper was then shown to be spread slightly more evenly than had been predicted earlier. However, the major difference between Perrin's computed distributions for copper and those which are presented in this thesis stem from the calcium and magnesium amino acid complex reactions which the earlier work omitted. These cations do react with amino acids to a limited extent. The dominant interaction is most probably with the 'hard' carboxylate function of these ligands. So, whilst the degree of complexation is not sufficient to seriously disrupt the computed distribution, it does alter the availability of the ligands (as manifest by their free concentrations) to a certain degree. On the other hand, both Perrin (158, 369) and Giroux and Henkin (164) failed to find a most important zinc species because they did not include citrate in their simulations. Thus, the ternary zinc citrate cysteine complex has been previously overlooked. It is obvious that omission of a major species or important component is always going to produce a defect in the model's results. Even such a gross error, however, does not appear to radically disturb the relative distribution of the other complexes which have been considered. For example, the omission of either of the two most predominant ferric complexes merely reflects as a redistribution of iron amongst the other major contenders. The quantitative effects are disastrous. The total concentration of iron bound to low molecular weight ligands, for one thing, can drop a

hundredfold. But the order of the other complexes is quite unaltered (see Table 3.6). Furthermore, Perrin's results for zinc (158, 369) show all the important complexes that had not been omitted. More important, they did not foster belief in a completely false 'pretender'. One might also note that the order of Perrin's zinc complexes in terms of concentration differs from that shown in Table 3.5. This is partially due to the similarity between the concentrations of the predominant zinc complexes. It has been noted previously that the order of the complexes in Table 3.5 can become altered if their concentrations are close together.

In view of the fact that the omission of important complex species, rather than some other fault, is the factor most likely to cause the models' results to be in serious error, it seems sensible to examine the possibility of the models, in this thesis, suffering from this kind of defect. The most obvious starting point in this regard is a consideration of the ligands which have been neglected. These include bilirubin, sugars such as glucose and fructose, histamine and the low molecular weight peptides. Bilirubin does form complexes with Ca^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+} (730) but these are unstable *in vitro* and this substance does not appear to be a strong ligand. Carbohydrates, on the other hand, are likely to be important iron binders at least (161, 374). Glucose, in particular, has a high plasma concentration (577). Histamine, in spite of a low plasma concentration, is a powerful ligand and thus may well prove to be important. This ligand is prone to form ternary complexes (58, 60, 334). Perrin has commented that peptide chains made up of simple amino acids are unlikely to prove effective binding agents for copper and zinc in plasma (369). This is probably the case due to the low concentrations of these compounds normally

TABLE 3.6

PREDOMINANT COMPLEXES OF FERRIC ION FOUND BY SIMULATION OF BLOOD PLASMA WHEN THE FERRIC HYDROXY CITRATE COMPLEX IS OMITTED FROM THE MODEL.

(Symbols defined in Appendix 5.7)

COMPLEX	PERCENTAGE OF THE TOTAL FERRIC IRON BOUND IN THE LOW MOLECULAR WEIGHT COMPLEX FRACTION		
	-log [H]=7,2	-log [H]=7,4	-log [H]=7,6
FE+3(1) CTA3(1) SLA2(1)	30	34	36
FE+3(1) CTA3(1) GLU2(1)	11	12	13
FE+3(1) CTA3(1) OXA2(1)	18	12	6
FE+3(1) CTA3(1) GLN1(1)	5	5	5
FE+3(1) CTA3(1) GLY1(1)	5	5	5
FE+3(1) CTA3(1) SCA2(1)	8	5	3
FE+3(1) CTA3(1) SER1(1)	4	4	4
FE+3(1) CTA3(1) AAA1(1)	4	4	4
FE+3(1) CTA3(1) THR1(1)	4	4	4
FE+3(1) CTA3(1) PHE1(1)	2	2	2
FE+3(1) CTA3(2)	2	2	-
FE+3(1) CTA3(1) ARG1(1)	1	1	1
FE+3(1) CTA3(1) SLA2(1)	1	1	1

Total concentration of ferric ion in the low molecular weight complex fraction in plasma.

$-\log [H] = 7,2 \quad : - \quad 3,15 \times 10^{-15}$ M

$-\log [H] = 7,4 \quad : - \quad 4,90 \times 10^{-15}$ M

$-\log [H] = 7,6 \quad : - \quad 9,18 \times 10^{-15}$ M

(c.f. Table 3.3)

present in the biofluid (577). This is in spite of a recent publication by Agarwal and Perrin which implies otherwise (499). In any event, opinion about all these ligands must remain neutral until formation constants for each with a range of metal ions become available. The same applies to the question of quaternary complexes although it presently appears that these will not be very important.

Finally, it is instructive to note that the independence which the complexes have been found to exhibit with regard to each other is a direct outcome of the fact that the model is designed to accommodate protein binding. Metal-protein species are actually the most predominant transition metal complexes in plasma. The way that the model is set up ensures that the distribution of the complexes in relatively low abundance is not upset by an absence of knowledge about the major species.

3.3 General discussion.

As it is the ability of models to yield verifiable predictions that serves as the yardstick of their value and validity, it is surely on this aspect that the final analysis of any model must focus. It is not necessary to formulate in advance the sort of prediction by which the model will ultimately be judged; the results themselves must suggest the direction of subsequent experiments. However, it is important to consider the possible applications of the model. In particular, it is desirable to outline those which may provide the framework within which predictions can be made and experiments then designed to test them. Thus, whilst it is not the responsibility of the model-builder himself to establish the absolute value of his results (by their very nature, models often predate the experimental means of substantiation), the onus is on him to clarify the relationship between reality and the simulation.

In the present context there are considerable grounds which provide assurance concerning the reliability of the model's mathematical construction. There can be little concern with the thermodynamic theory and relationships that have been employed. In this type of situation, chemists universally depend upon the conservation of matter; the equilibrium functions are also exceedingly well established. Similar calculations, for example, are employed whenever formation constants are determined. The only uncertainties are those associated with (i) the applicability of the thermodynamic theory and (ii) the experimental error in the parameters of the model. These have been discussed in Sections 2.1 and 3.2 respectively. Neither factor seems likely to invalidate the results of this work.

The experimental error, which presently merits the most concern, is constantly being diminished as research in this field progresses. Moreover, whilst the difficulty in verifying the predictions of analogous computations becomes insurmountable as the system gets more complicated or the concentrations involved are decreased, there is no reason to believe that a discontinuity separates these extreme cases from the simple and tractable ones. In this regard, it is of special interest to consider the astonishingly low free ion concentrations which have been utilized in the simulations. This applies only to the transition metals. In the case of iron, for example, concentrations as low as 10^{-24}M have been used. These concentrations refer to solutions in which there is less than a single free ferric ion in one litre! This, in fact, reflects not so much on the actual concentration of the ion species (which, indeed, need not be present at all) but rather, expresses the chemical potential of such an ion with respect to the other ferric species present in the solution. Concern that a thermodynamic treatment might fail because it no longer deals with a number of particles sufficient to ensure statistical effects, is therefore unfounded. The situation is analogous to that of the aqueous solution in calomel electrodes (Schwarzenbach, G. - private communication). The insolubility of mercurous chloride ensures that the concentration of the metal ion is so low that it is certainly non-existent in aqueous chloride solutions. Nevertheless, a non-zero concentration, equivalent to one ion in a volume of water larger than that of the earth, may be utilized in a consideration of the reactions and thermodynamics of the electrode system.

Turning to the model in its physiological context, it appears that most of the predictions will arise from the application

of results towards an interpretation of the mechanisms of various processes. For example, major complex species postulated by the model are suggested to participate in the transport of metal ions into and out of protein structures in plasma. This, of course, does not apply to situations where the metal may be transferred directly from one protein to another. The exchange of ferric ions between transferrin molecules is therefore according to the results in Table 3.5 predicted to take place via a mixed complex involving only one citrate ligand rather than as the dicitrato complex as previously implied (611, 404). This applies to the exchange in plasma and is not necessarily applicable to *in vitro* experiments. However, it is encouraging to note that Bates *et al.* have studied the kinetics of iron (III) exchange between chelates and transferrin and their work confirms the prediction in so far as they find that the iron-dicitrate is not the most reactive species (174). Similarly, it may be concluded from Table 3.5 that the exchange of copper and possibly lead between proteins in plasma involves mixed ligand complexes. The opposite conclusion is drawn for manganese.

A most direct assurance that the model is not grossly in error is provided by a comparison of Tables 3.2 and 3.3. It is evident that the simulation predicts total low molecular weight metal complex concentrations (see equation 3.2) in very satisfactory agreement with those estimated from experimental considerations (see Section 3.2.1) for calcium, copper, magnesium and zinc. Moreover, this establishes that the free metal ion concentrations chosen for the 'primary' model are reasonably accurate. The negative aspect of this has even greater absolute significance. The possibility of free concentrations as large or larger than the upper limits which have been scanned (see

Section 3.2.2) is denied. Such high values produce complex concentractions incompatible with a number of experimental measurements (see, for example 155, 135, 194, 140 and other references cited in Section 3.2.1 (a), (b), (e) and (g)).

Interesting implications of the model concern the effect of administered therapeutics on the plasma metal ion distribution. It is clear from the model's results that if a ligand is to compete effectively against those normally present, it will need to be both powerful and in relatively high concentration. Indeed, very few therapeutics will be able to exert a noticeable influence on the physiological distribution of the transition metal ions. However, those which are potentially capable of disrupting the equilibria are of great interest. Doses of aspirin, for example, are capable of increasing the salicylic acid concentrations in plasma tenfold (533). The effect of even such mild treatment is to produce a surge in the salicylic acid not bound to protein (375, 247). The model shows clearly that this will, in turn, cause a marked alteration in the ferric ion distribution amongst the low molecular weight ligands with the emergence of the mixed citrate-salicylic acid complex (see Table 3.7). Thus, the widespread use of aspirin as a multipurpose therapeutic may have a pronounced influence on iron metabolism, depending on the role of low molecular weight complexes in this context.

Possibly the most important application of models such as the one presented in this thesis concerns the transport of transition metal ions through biological membranes. Very little is known about this subject so it provides a fertile area for making predictions without the prior knowledge of experiment. This is not meant to imply

TABLE 3.7

PREDOMINANT LOW MOLECULAR WEIGHT FERRIC COMPLEXES* FOUND BY SIMULATION OF BLOOD PLASMA FOR INCREASING CONCENTRATIONS OF SALICYLATE THAT MAY BE PRODUCED BY DOSES OF ASPIRIN (ACETYLSALICYLIC ACID).

(Symbols defined in Appendix 5.7)

COMPLEX	Concentration of salicylate.			
	5μM	50μM	100μM	500μM
FE+3(1) CTA3(1) OH-1	99(1)	97(1)	94(1)	77(1)
FE+3(1) CTA3(1) SLA2(1)	(2)	2(2)	5(2)	19(2)
FE+3(1) CTA3(1) GLU2(1)	(3)	(3)	(5)	(6)
FE+3(1) CTA3(1) OXA2(1)	(4)	(5)	(6)	(7)
FE+3(1) SLA2(1) OXA2(1)	(14)	(4)	(3)	(4)
FE+3(1) SLA2(1) GLU2(1)	(26)	(14)	(13)	(5)
FE+3(1) SLA2(2)	(-)	(12)	(4)	2(3)

* Given as the percentage of the total low molecular weight ferric ion fraction. Figures in parentheses are the positions of each complex after they had been sorted according to highest concentration @ $-\log[H] = 7,4$.

that this field of research has been neglected. On the contrary, much effort has been directed at elucidating the nature of biological membranes. Their physiological significance is obvious and so their transport properties, in particular, have been intensively studied. However, the understanding of life processes is rarely easy and membrane phenomena have proved no exception to the general rule. Most work has been confined to organic molecules (370) and the more abundant inorganic ions such as Na^+, K^+, Cl^-, HCO_3^-, H^+ (564). Very little has dealt with the transition metals. Thus, it is necessary to formulate predictions pertaining to the transition metal ions from a knowledge of these which are better understood. Paradoxically, small organic molecules are probably more analogous than the alkali metal ions. This is because the latter are known to participate in active transport processes which require metabolic energy and involve astonishingly selective reactions. On the other hand, at least part of the transition metal movement through membranes is believed to occur by passive diffusion. Their penetration is invariably slow, suggesting passive flux (677). Active processes certainly do not account for the total intestinal absorption of iron, copper and zinc (*vide infra* and also Section 1.2.3).

Little can be concluded about the model's results in terms of the active transport of transition metal ions through membranes. This will have to wait until considerably more has been learned about such processes. One can only opine that it is not improbable that low molecular weight conplexes participate in one way or another. After all, low molecular weight complexing agents have been implicated in the transport of alkaline metal ions through membranes (see, for

example, 675). Furthermore, the very low free metal ion concentrations of the transition elements require that complexes rather than the hydrated ion species participate in many physiological processes. These facts should all be evaluated in the knowledge that the movement of macromolecules through membranes is generally restricted. This limitation on the nature of carrier ligands applies equally to active and passive transport. It should thus be borne in mind throughout the present discussion.

Far more is known about passive transport than about the active process. The fact which is possibly of greatest applicability is that small, uncomplexed inorganic ions cannot cross thin lipid membranes at a measurable rate (676). Similar restrictions apply to small organic ions (370). The amount of energy required to transfer a charge of small dimensions from water into hydrocarbon is totally prohibitive (676). This is well illustrated by organic ligands whose rate of transport is observed to be very dependant on the protonation constant of the substance and the pH on either side of the membrane. The molecule can only migrate through the membrane in the non-ionised form of the weak acid or base (370). The magnitude of the flux is a function of the difference in concentration of the neutral species in the solutions on either side of the membrane barrier. A similar situation applies to the low molecular weight complexes of the transition metal ions. Only the neutral complexes will be lipid soluble. This criterion underlies an important application of the model The concentration of the neutral species in two biofluids may be calculated and compared. The simulations can also indicate how the distribution changes with pH. So, the results can be used to predict the direction of passive diffusion through a membrane separating the two biofluids.

Moreover, the search for conditions which will either increase or decrease the flux should be facilitated.

Consider a person suffering from transition metal overload. It is obviously desirable to be able to administer a drug that will bind the offensive cation, thereby enabling it to be excreted. This applies equally to patients who have been poisoned by excessive intake (e.g. those suffering from plumbism) and to those afflicted by a metabolic disorder such as Wilson's disease. The difficulties experienced by those seeking more efficient medical chelating agents are discussed in Section 1.2.4. In this regard, the value of the model lies in its ability to test that potential of large numbers of drugs quickly and cheaply. The treatment of Wilson's disease by penicillamine provides a good illustration of the sort of computer investigation which can eliminate much of the randomness which has often characterized the search for better therapeutics. It has been shown that the mode of action of penicillamine depends upon the fact that it renders plasma copper more available for diffusion across a membrane (341). The experiments have been conducted both *in vitro* and *in vivo*. There is a very marked rise in urine copper within hours of the administration of the drug. Table 3.8 shows a number of important aspects which are disclosed by simulating the low molecular weight metal ion distributions of plasma in which the concentration of the tetramethylcystine (the oxidation product of penicillamine) is systematically increased. A similar model has been produced by Perrin (369). Whilst the overall picture is unchanged, differences have arisen because the present model contains estimates of the formation constants for a substantially larger number of tetramethyl cystine complexes. It can be seen from Table 3.8 that penicillamine therapy can raise the concentration of the low

TABLE 3.8

MODEL RESULTS FOR THE TREATMENT OF WILSON'S DISEASE WITH PENICILLAMINE*

a) Changes in the total concentrations of metal ions bound to low molecular weight ligands expressed as a percentage of the normal value.

Metal ion	Total concentrations of tetramethyl cystine in plasma					
	0μM	50μM	100μM	150μM	200μM	300μM
Ca^{2+}	1001	100	100	100	100	100
Cu^{2+}	100	110	126	175	224	286
Fe^{3+}	100	100	100	100	100	101
Pb^{2+}	100	103	107	110	115	122
Mg^{2+}	100	100	100	100	100	100
Mn^{2+}	100	100	100	100	100	100
Zn^{2+}	100	103	107	111	117	127

b) Changes in the percentage of neutral species in plasma

Metal ion	Total concentration of tetramethyl cystine in plasma			
	0μM	100μM	200μM	300μM
Cu^{2+}	62	66	66	68
Zu^{2+}	8	8	8	11

*Penicillamine is taken to be totally oxidized in plasma to tetramethyl cystine.

molecular weight copper fraction quite considerably. Small increases in the corresponding fractions of zinc and lead are also evident. The total concentration of low molecular weight complexes for the remaining metal ions are substantially unaltered. This is an important property of a good medical chelating agent; the distributions of the ions other than the metal which is in excess should be distrubed as little as possible. Further, the model reveals that whilst the percentage of neutral copper species is increased, the same change only affects zinc at high therapeutic concentrations. So, it would seem that the beneficial removal of copper would continue as the size of the penicillamine dose was stepped-up but that at higher levels one might find that this is accompanied by an undesirable loss of zinc. Uncertainty is warranted here because nothing is as yet known about the corresponding complex distributions in urine. Only when the two biofluids can be compared will it become possible to properly investigate the phenomena in question.

Another aspect of the application of the model to the question of transition metal ion transport through membranes concerns the absorption of iron. Iron deficiency anaemia is a very widespread condition which provides scope for further investigation. Persons who have been diagnosed as iron deficient can only absorb a small fraction of the iron therapeutics presently available due to the body's protective mechanism described in Section 1.2.4. The tolerance of an individual towards most iron compounds is limited. In addition to these drawbacks, alternatives to oral therapy are expensive and thus beyond the reach of the majority which require this kind of treatment. It is therefore evident that there is a pronounced need for a simple, cheaply manufactured iron complex which can significantly enhance

considerable stability of the computed distribution of the metal ions amongst low molecular weight ligands has some profound implications. To begin with it indicates that neither metal ion deficiency or overload is likely to alter the gross distribution. The negligible influence of the majority of therapeutics on the low molecular weight complex distribution of the transition metal ions has already been mentioned. It may well transpire that, based on the evidence of the model, a given complex or even the entire low molecular weight fraction may be eliminated as possible participants in various physiological processes under investigation.

Finally, it is necessary to consider the future of the work that has been presented in this thesis. Much remains which can be done to improve the detail of the blood plasma model itself. The present project has helped to delineate the most pressing tasks. These are (i) the accurate determination of all the as yet unmeasured formation constnts for the important complex species (bicarbonate complexes and calcium and magnesium amino acid species, in particular), (ii) the experimental measurement of many more mixed ligand complex formation constants and (iii) the improvement of estimates of the free metal ion concentrations in plasma, especially that of magnesium. As far as the simulation of other solutions is concerned, numerous possibilities exist. Intestinal fluid, cerebrospinal fluid, gastric juice, urine and cytoplasm are obvious examples. Simulations of these body fluids are pre-requisites of predictions about the passive diffusion of transition metal ions through membranes *in vivo*. These models only await the necessary experimental data. Sea, river and lake waters can also be simulated to great advantage. The same can be said of many industrial solutions. There must be considerable potential in the

absorption of the metal. A consideration of the iron overload disease, siderosis, suggests that the normal iron absorption pathways can be by-passed by low molecular weight complexes. This, in fact, has been incorporated in the description of iron absorption by Forth and Rummel (400). So, the computer models are clearly applicable. Indeed, a less sophisticated but analogous approach has been taken by Schubert when he calculates (using calcium as the only competitive metal ion) the 'effective stability' of iron complexes under physiological conditions (726). An elementary model calculation of the concentrations of iron complexes over a range of physiological pH values has, in fact, already been published (316). However, once again it is necessary to refrain from making definite predictions until the complex distribution in both plasma and intestinal fluid have been simulated. Even this advance may prove insufficient because the mucosal cell may well behave as an additional compartment and not simply as a single phase partition.

Still mindful of the limiting factors which have been stressed above, it is tempting to speculate about the results of the model with regard to the body's inability to excrete iron (see Section 1.2.3). It is shown in Table 3.5 that all the predominant low molecular weight ferric complexes are negatively charged. This could well be biologically significant because it would tend to prevent loss of the metal through biological membranes. It is certainly true that the homeostatic control mechanism for iron is unlikely to have evolved as it did had the metal been difficult to retain *in vivo*.

In spite of all the applications that one might envisage, it is important ot recognise that the negative evidence produced by the model may ultimately prove to be that of greatest value. The

simulation of systems concerned with metal extraction, for example. In all these cases, the investigation of the metal complex distribution is motivated by the fact that only in the light of such knowledge can one truly understand the chemical processes which are taking place in the solution. However, no model can ever be complete. No model can behave exactly as the real system. 'It does require maturity to realize that models are to be used but not to be believed.' (Henri Theil in 'Principles of Econometrics' , 1971). The best that can be done is to strive to simulate the major relationships which regulate the real behaviour by over-riding the effect of the less important aspects. Eventually, this can establish the validity of the hypotheses which the model manifests. It is in this spirit that the blood plasma model is presented. One should not overemphasise the shortcomings of the simulation, especially during the early stages: it is always a pity not to advance for fear of taking the first few steps. Furthermore, it is important to remember that accuracy is of greater value than precision. As Perrin has suggested (369), it is well to recall Tukey's Principle: 'Far better an approximate answer to the right question, which is often vague, than an exact answer to the wrong question, which can always be made precise.'

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APPENDICES

APPENDIX 5.1

List of Symbols

a) Concentrations

S - the concentration of a complex species

X - the free concentration of a component

T - the total concentration of a component

b) Indices

i - the general indix for components

j - the general index for complex species

m - the specific component index

p - the index for a metal ion component

q - the index for a ligand component

r - the index for a hydrogen ion component

c) Superscripts

c - denotes a 'calculated' quantity

r - denotes a 'real' quantity

o - denotes an 'old' value in an iteration

n - denotes a 'new' value in an iteration

t - denotes a 'thermodynamic' formation constant

d) Parameters

β - the cumulative stability constant of a complex species as defined by equations 2.1, 2.3 and 3.1

k - the matrix which defines the components and their multiplicity in a complex species

e) Factors

F - a factor defined by equation 3.10

G - a factor defined by equation 3.11

F' & G' - approximations for F and G respectively

f) Miscellaneous symbols

M - metal ion

L - ligand

A - ligand (as opposed to ligand B)

B - ligand (as opposed to ligand A)

H - hydrogen ion

f - activity coefficient

I - ionic strength

T - thermodynamic temperature

t - temperature in Celsius

APPENDIX 5.2

A consideration of the convergence properties of expression 3.13.

It is required to investigate expression 3.13 and to establish whether it may be a suitable iteration formula that, by successive approximation, may converge on the real free component concentrations, ${}^{r}X_{m}$, when the approximation $({}^{r}T_{i}/{}^{c}T_{i}) \doteq ({}^{r}X_{i}/{}^{c}X_{i})$ is used to obtain G'_{j}. One has,

$$n_{X_m} = \frac{{}^{r}T_{m} \cdot {}^{o}X_{m}}{{}^{c}X_{m} + \sum_{j}\left[G'_{j} \cdot {}^{c}S_{j} \cdot k(m,j)\right]} \qquad \ldots\ldots(3.13).$$

Equation 3.12 provides an exact expression for ${}^{r}X_{m}$ in terms of G_{j} factors. If one begins by setting all ${}^{c}X_{i} = {}^{r}T_{i}$, then

$${}^{r}X_{i} < {}^{c}X_{i} \qquad \ldots\ldots(5.1)$$

and

$${}^{r}T_{i} < {}^{c}T_{i} \qquad \ldots\ldots(5.2)$$

(see equations 3.7 and 3.8 with 3.4 and 3.5).

We have

$$\frac{{}^{r}X_{i\neq m}\ k(i,j)}{{}^{c}X_{i\neq m}\ k(i,j)} < 1 \qquad \ldots\ldots(5.3)$$

and

$$\frac{{}^{r}X_{m}\ k(m,j) - 1}{{}^{c}X_{m}\ k(m,j) - 1} \leqslant 1 \qquad \ldots\ldots(5.4)$$

except when $k(m,j) = 0$. Hence, from equations 3.10 and 3.11, because the product of 5.3 and 5.4 is equal to G_j,

$$G_j < 1 \qquad \text{.....(5.5)}$$

except when $k(m,j) = 0$. From equations 3.8 and 3.12, it follows that

$$\frac{r_{X_m}}{c_{X_m}} = \frac{r_{T_m}}{c_{X_m} + \sum_j \left[G_j \cdot {}^{c}S_j \cdot k(m,j) \right]} > \frac{r_{T_m}}{c_{T_m}} \qquad \text{.....(5.6)}$$

Therefore, in general

$$\frac{r_{X_i}}{c_{X_i}} > \frac{r_{T_i}}{c_{T_i}}$$

Note that when $k(m,j) = 0$, the term in the summation becomes zero and the magnitude of G_j is then irrelevant. Hence, if $({}^{r}T_i/{}^{c}T_i)$ is substituted as an approximation for the factor $({}^{r}X_m/{}^{c}X_m)$ in equation 3.13 (see equations 3.10 and 3.11), it follows from equation 5.6 that

$$G_j' < G_j \qquad \text{.....(5.7)}$$

In such a case, a comparison of equations 3.12 and 3.13 where ${}^{o}X_m \equiv {}^{c}X_m$, reveals that

$$ {}^{n}X_m > {}^{r}X_m \tag{5.8} $$

for convergence, then, all that is necessary is

$$ {}^{n}X_m < {}^{o}X_m \tag{5.9} $$

Although this is not an unreasonable expectation, it is difficult to prove in the general case. This is not very important, however, because as discussed in Section 3.1.3, formula 3.13 is only utilized profitably during the initial stages of the iteration procedure. Its chief task is to improve the poor initial estimates obtained by setting ${}^{o}X_i = {}^{r}T_i$. Under these circumstances, for the first iteration, the inequality 5.9 is certainly true. This follows from equation 3.13 where the suggested substitution for starting values ensures that the denominator is greater than ${}^{r}T_m$ because a number of terms in the summation are non-zero and are added to ${}^{o}X_m = {}^{r}T_m$.

appendix 5.3

LISTING AND INSTRUCTIONS FOR PROGRAM ECCLES.

THIS PROGRAM COMPUTES THE EQUILIBRIUM CONCENTRATIONS OF METAL ION LIGAND COMPLEXES IN LARGE AQUEOUS EQUILIBRIUM SYSTEMS. IT REQUIRES THE FORMATION CONSTANTS FOR ALL THE COMPLEXING REACTIONS AS WELL AS THE TOTAL OR FREE CONCENTRATIONS OF THE COMPONENTS PRESENT IN THE MIXTURE. IT ALSO NEEDS A FREE HYDROGEN ION CONCENTRATION.

EACH COMPONENT MUST BE ASSIGNED A SYMBOL WHICH IS FOUR CHARACTERS IN LENGTH. THE SYMBOL FOR HYDROGEN ION IS 'H +1' (NOTE THE BLANK SPACE). EACH COMPLEX SPECIES IS THEN DEFINED BY A STRING OF COMPONENT SYMBOLS FOLLOWED IMMEDIATELY BY THE NUMBER OF TIMES THAT COMPONENT APPEARS IN THE COMPLEX - THE NUMBER IS PLACED WITHIN PARENTHESES. THE COMPONENT SYMBOLS IN THE STRING DEFINING THE COMPLEX SPECIES ARE DELIMITED BY ONE BLANK SPACE.

THE FIRST THREE INPUT CARDS SHOULD CONTAIN ALPHANUMERIC INFORMATION FOR IDENTIFICATION PURPOSES. THE EXPERIMENT TITLE, THE INVESTIGATORS NAME AND THE DATE ARE SUGGESTED.

ECCLES CAN 'MONITOR' EITHER ALL OR UP TO TEN SELECTED COMPONENTS. THIS MEANS THAT THE COMPLEX SPECIES WHICH CONTAIN THE COMPONENT IN QUESTION ARE SORTED INTO ORDER OF HIGHEST CONCENTRATION. THE FORTY MOST PREDOMINANT COMPLEX SPECIES FOR EACH 'MONITORED' COMPONENT ARE PRINTED OUT IN ORDER. THE INSTRUCTION FOR THIS IS SUPPLIED ON THE FOURTH INPUT CARD. IF THE COMPONENTS ARE ALL TO BE 'MONITORED', THE CARD SHOULD READ

TRUE ALL

IF ONLY A FEW SELECTED COMPONENTS ARE OF INTEREST, THE CARD SHOULD READ

TRUE XXXX YYYY ZZZZ

WHERE XXXX, YYYY AND ZZZZ ARE THE SYMBOLS OF THE COMPONENTS TO BE 'MONITORED'. OTHERWISE, THE CARD SHOULD READ

FALSE

ECCLES CAN SCAN THE CONCENTRATION OF ANY GIVEN COMPONENT. THE EQUILIBRIUM

CONCENTRATIONS ARE COMPUTED AT EACH STAGE. THE STARTING CONCENTRATION IS TAKEN TO BE THE VALUE SUPPLIED AS THE COMPONENT CONCENTRATION IN THE MAIN BODY OF THE DATA. THE SYMBOL OF THE COMPONENT WHOSE CONCENTRATION IS TO BE SCANNED, THE INCREMENT FACTOR BY WHICH THE STARTING VALUE MUST BE INCREASED AND THE MAXIMUM VALUE OF THE SCANNED CONCENTRATION AT WHICH THE SCAN MUST TERMINATE, ARE INDICATED ON THE FIFTH INPUT CARD. IF THERE IS TO BE NO SCAN, THE CARD SHOULD SIMPLY READ

FALSE

IF THE CONCENTRATION IS TO BE INCREASED MULTIPLICATIVELY, INSTEAD OF ADDITIVELY, A CARD READING 'MULTIPLY' MUST IMMEDIATLY FOLLOW THE CARD CONTAINING THE SCAN PARAMETERS.

THE OUTPUT OF THE CONCENTRATIONS OF EACH COMPLEX SPECIES CAN BE SUPPRESSED, THIS IS INDICATED BY A CARD READING

SUPPRESS OUTPUT

IN THE CASE OF VERY LARGE SYSTEMS WHICH ARE BEING SUBJECTED TO REPEATED ANALYSIS THE INPUT CHECKING PROCEDURES WITHIN THE PROGRAM CAN BE SWITCHED OFF BY A CARD READING

OMIT INPUT CHECKS

THIS IS NOT ADVISABLE UNLESS THE USER IS ABSOLUTELY CERTAIN THE INPUT IS ERROR-FREE.

THE CARDS BEARING THE TOTAL CONCENTRATIONS OF THE COMPONENTS ARE ENTERED NEXT. THESE ARE ANNOUNCED BY A CARD READING

TOTAL CONCENTRATIONS

THEN, THE CARDS WITH THE KNOWN FREE CONCENTRATIONS FOLLOW. THESE ARE HEADED BY A CARD READING

FREE CONCENTRATIONS

FINALLY, THE FORMATION CONSTANT DATA FOLLOWS A CARD READING

SPECIES FORMATION CONSTANTS

THE DATA IS SET UP AS IN APPENDICES 5.7 & 5.6, FOR EXAMPLE. AS PRESENTLY DIMEN ECCLES WILL ACCEPT UP TO 45 COMPONENTS WITH TOTAL CONCENTRATION SUPPLIED, 10 COMPONENTS WITH GIVEN FREE CONCENTRATIONS AND 4000 COMPLEX SPECIES.

```
PROGRAM  ECCLES.
---------------


C      DEVELOPED AT THE UNIVERSITY OF CAPE TOWN DURING 1974.
C
C      THIS PROGRAM EVALUATES THE COMPONENT CONCENTRATIONS IN LARGE
C      EQUILIBRIUM SYSTEMS.   IT IS DESIGNED TO REFLECT THE MOST
C      PRONOUNCED EFFECTS OF SYSTEMATIC CONCENTRATION CHANGES IN
C      THE EQUILIBRIUM MIXTURE.
C
C      THE PROGRAM IS WRITTEN IN FORTRAN V.



C      SECTION ONE.      STORAGE ALLOCATION AND FORMAT STATEMENTS.


       COMPILER(XM=2)
       PARAMETER  NS=4000, NX=45, NXX=10, NKEY=4
       PARAMETER  NLINES = 55, NSTOP = 500
       PARAMETER  NND = NKEY * 2, NXP1 =NX + 1, NSP1 = NS + 1
       PARAMETER  NXXX = NX + NXX
       INTEGER  OUT, TITLE(12), DATE(6), CHECK(NXXX)
       DOUBLE PRECISION  HOLD(NS), SPECIE(NS)
       LOGICAL SCAN, MULTIP, SELECT, NONALL, CMPRES, UCHEK,
      1 NOWIT, SUPRES, TRIAL
       DIMENSION  KEY(NKEY,NS,2), CONST(NS), NUM1(NS), NUM2(NS), X(NX),
      1 TREAL(NX), TCALC(NXXX), CRIT(NX), XX(NXX), NAME(6), NDUM(NND),
      2 DUM(NX), NCOMP(NXXX), IQUE(NS), NKY(NKEY,2),
      3 COMMIN(NXXX), IMON(10), CONC(NS)
       EQUIVALENCE (HOLD(1), IQUE(1)), (IQUE(NSP1), CONC(1))
       COMMON KEY




10401 FORMAT(12A6)
10402 FORMAT(L5,1X,10(A4,1X))
10403 FORMAT(L5,1X,A4,2X,3G10.4)
10404 FORMAT(A4,A1,2G10.4)
10405 FORMAT(G9.5,1X,8(A4,1X,I2,2X))
20400 FORMAT(//'0',12A6,///)
20401 FORMAT('0','THE MAXIMUM NUMBER OF VARIABLE COMPONENTS IS',I3)
20402 FORMAT('0','THE MAXIMUM NUMBER OF FIXED COMPONENTS IS',I3)
20403 FORMAT('0','THE MAXIMUM NUMBER OF SPECIES IS',I5)
20404 FORMAT('0','SPECIE NUMBER',I5,' HAS TOO MANY COMPONENTS')
20405 FORMAT('0','THE PROGRAM LIMITS HAVE BEEN EXCEEDED,')
20406 FORMAT('0','EXECUTION TERMINATED,   ERROR ON CARD',I5,/////)
20407 FORMAT('0','ALL SPECIES MUST HAVE MORE THAN ONE COMPONENT,')
```

```
20408 FORMAT('0','COMPONENT ERROR IN SPECIE NUMBER',I5)
20409 FORMAT('0','A SPECIE WITH NO VARIABLE COMPONENTS IS SUPERFLUOUS')
20410 FORMAT(//'0','EXECUTION TERMINATED,'/' ','COMPONENT NUMBER',I3,
     1 ' IS NEVER REFERENCED,'//////)
20411 FORMAT('0','SCAN COMPONENT UNIDENTIFIABLE.')
20412 FORMAT(//'0','SPECIE DUPLICATION,'/' ','SPECIE NUMBER',
     1 I5,' HAS ALREADY BEEN ENTERED,'//)
20413 FORMAT(///'0','******* WARNING ********'/' ',
     1 'IF THE SOLUTION IS AQUEOUS, A FREE HYDROGEN CONCENTRATION IS REQ
     2UIRED,',///)
20414 FORMAT(//'0','COMPONENT DUPLICATION,'/' ','COMPONENT',I4,
     1 ' HAS ALREADY BEEN ENTERED.')
20415 FORMAT('0','MONITOR COMPONENT UNIDENTIFIABLE,')
20416 FORMAT(//'0','A MONITORED COMPONENT MUST APPEAR IN AT LEAST THREE
     1 SPECIES' :/' ',A4,' HAS BEEN REMOVED FROM THE LIST,',///)
20417 FORMAT(//'0','THE SCAN INCREMENT FACTOR IS TOO SMALL,')
20418 FORMAT(//'0','THE MAXIMUM SCAN CONCENTRATION IS LOWER THAN THE STA
     1RTING VALUE,')
20419 FORMAT(' ','THE CONC, OF THE SCANNED COMPONENT MUST INCREASE,')
20420 FORMAT(//'0','MODEL SELECTION COMPONENTS MUST BE SPECIFIED ON THE
     1MONITOR CARD,',//)
20801 FORMAT(//'0','EXECUTION TERMINATED AS A RESULT OF NON-CONVERGENCE,
     1',///)
21000 FORMAT('1'//'0',5(1H*),2(6X,4(1H*)),5X,1H*,4X,2(5X,5(1H*)),/' ',
     1 6(1H*,9X),/' ',4(1H*),6X,3(1H*,9X),4(1H*),6X,5(1H*),/' ',
     2 5(1H*,9X),4X,1H*,/' ',5(1H*),2(6X,4(1H*)),3(5X,5(1H*)),/' ',
     3 55(1H-),/////////'0',T25,40(2H<>),2(/' ',T25,2H<>,T103,2H<>,
     4 /' ',T25,4H<>  ,12A6,4H  <>,/' ',T25,2H<>,T103,2H<>),/' ',T25,
     5 40(2H<>),//////'0',T25,'TOTAL NUMBER OF COMPONENTS =',I4,
     6 /' ',T25,'NUMBER OF COMPONENTS WITH FIXED FREE CONCS, =',
     7 I3,/' ',T25,'TOTAL NUMBER OF SPECIES =',I5,//)
21001 FORMAT('1',T50,'ECCLES: SCAN CYCLE NUMBER',I3,/' ',T50,30(1H*)///)
21002 FORMAT(//'0',' CONCENTRATIONS OF THE FREE COMPONENTS,',/' '
     1 37(1H-)///)
21003 FORMAT(' COMP,',T14,'COMP,',T34,'REAL',T54,'CALC,',T92,'COMP,',/
     1 3X,'NO,',T14,'CONC,',T34,'TOTAL',T54,'TOTAL',T92,'NAME',//)
21004 FORMAT(' ',I4,2X,4(1PG15,6,5X),T92,A4,I5)
21005 FORMAT(' ',I4,2X,1PG15,6,6X,'FIXED FREE CONC,',3X,2(1PG15,6,5X),
     1  T92,A4,I5)
21006 FORMAT('1',/'0','CONCENTRATIONS OF THE SPECIES,',/' ',29(1H-),//)
21007 FORMAT('0',1X,'SPECIE',T14,'SPECIE',T32,'LOG STAB,',T50,
     1 'COMPOSITION',/' ',2X,'NO,',T15,'CONC,',T34,'CONST,',//)
21008 FORMAT(' ',I4,2X,2(1PG15,6,5X),T50,10(A4,'(',I2,') '))
21009 FORMAT(1H1)
21010 FORMAT(/'0','THE SOLUTION WAS REACHED AFTER',I4,' ITERATIONS,')
21011 FORMAT('0',T25,'OUTPUT',2A6)
21012 FORMAT('0',T25,'NO SCAN REQUIRED')
21013 FORMAT('0',T25,'SCAN COMPONENT ',T45,A4)
21014 FORMAT('0',T25,'COMPONENT MONITOR ',A3)
21015 FORMAT('0',T25,'INPUT ERROR DETECTORS ',A3)
21016 FORMAT('1',/'0','MONITORED COMPONENT  ',A4,/' ',25(1H-),///)
21017 FORMAT('1','THE FOLLOWING COMPONENTS DO NOT APPEAR IN THE SELECTED
     1 MODEL,',///)
21018 FORMAT(' ',A4)
```

```
21019 FORMAT('0',T25,'MULTIPLICATIVE INCREMENT,')
21020 FORMAT('0',T25,'ADDITIVE INCREMENT,')
21021 FORMAT('0',T25,'MODEL SELECTOR ',A3)
31001 FORMAT(G9.3,1X,8(A4,'(',I2,') '))
99999 FORMAT('1',34H'WELL DONE, ECCLES' SAID MORIARTY.,//////)


C     SECTION TWO.      INITIALISATION.


  200 NUL = 0
      IN = 8
      OUT = 5
      NN = 4
      N1 = NXP1
      N2 = NXX + 1
      N3 = NSP1
      MULTIP = .FALSE.
      SUPRES = .FALSE.
      TRIAL = .FALSE.
      NOVALL = .FALSE.
      SELECT = .FALSE.
      UCHEK = .FALSE.
      DO 201 I=1,NXXX
  201 CHECK(I) = 0
      DO 202 J=1,NS
      DO 202 L=1,NKEY
  202 KEY(L,J,2) = 0
      KOUNT = 0
      GO TO 400


C     SECTION THREE.      INPUT (WITHOUT ERROR DETECTION).


  300 NN = 0
      DO 301 NI=1,N1
      READ(IN,10401)  IXA
      IF(IXA.EQ.'FREE C'.OR.IXA.EQ.'SPECIE')  GO TO 302
      READ(NUL,10404) NCOMP(NI), IXA, TREAL(NI), COMMIN(NI)
  301 CONTINUE
  302 NI = NI - 1
      NII = 0
      IF(IXA.EQ.'SPECIE') GO TO 305
      N = NI
      DO 303 NII = 1,N2
      N = N + 1
      READ(IN,10401)  IXA
      IF(IXA.EQ.'SPECIE')  GO TO 304
      READ(NUL,10404) NCOMP(N), IXA, XX(NII), COMMIN(N)
  303 CONTINUE
  304 NII = NII - 1
```

```
  305 NIII = NI + NII
      DO 311 NJ=1,N3
      READ(IN,10405,END=312) CONST(NJ), NDUM
      NUM1(NJ) = 0
      NUM2(NJ) = 0
      NPM = 1
      DO 310 N=1,NND,2
      IF(NDUM(N).EQ.'    ')  GO TO 311
  306 DO 307 M=NPM,NIII
      IF(NDUM(N).EQ.NCOMP(M)) GO TO 308
  307 CONTINUE
      IF(NPM.EQ.1) GO TO 432
      NPM = 1
      GO TO 306
  308 CHECK(M) = CHECK(M) + 1
      NPM = M
      IF(M.GT.NI)  GO TO 309
      L = NUM1(NJ) + 1
      NUM1(NJ) = L
      KEY(L,NJ,1) = M
      GO TO 310
  309 L = 'KEY - NUM2(NJ)
      NUM2(NJ) = NUM2(NJ) + 1
      KEY(L,NJ,1) = M - NI
  310 KEY(L,NJ,2) = NDUM(N+1)
  311 CONTINUE
  312 NJ = NJ - 1
      GO TO 450


C     SECTION FOUR.     INPUT (WITH ERROR DETECTION).


  400 READ(IN,10401)  TITLE
      IF(TITLE(1).EQ.'ABORT ')  WRITE(OUT,20400)  TITLE
      IF(TITLE(1).EQ.'ABORT ')  GO TO 9999
      READ(IN,10401)  NAME
      READ(IN,10401)  DATE
      READ(IN,10402)  NONIT, IMON
      IF(IMON(1).EQ.'ALL ')  MONALL = .TRUE.
      NN = 5
      READ(IN,10403,ERR=432)  SCAN, ISCN, SCNINC, SCNMAX
      NN = 6
      READ(IN,10401)  IXA
      IF(IXA.EQ.'MULTIP')  MULTIP = .TRUE.
      IF(MULTIP)  READ(IN,10401)  IXA
      IF(MULTIP)  NN = 7
      IF(IXA.EQ.'SELECT')  SELECT = .TRUE.
      IF(SELECT)  READ(IN,10401)  IXA
      IF(SELECT)  NN = NN + 1
      IF(.NOT.NONIT.AND.SELECT)  GO TO 443
      IF(IXA.EQ.'COMPRE') CMPRES = .TRUE.
      IF(CMPRES) READ(IN,10401) IXA
```

```
      IF(CHPRES) NN = NN + 1
      IF(IXA.EQ.'SUPRES')  SUPRES = .TRUE.
      IF(SUPRES)  READ(IN,10401)  IXA
      IF(SUPRES)  NN = NN + 1
      IF(IXA.EQ.'TRIAL ')  TRIAL = .TRUE.
      IF(TRIAL)  READ(IN,10401)  IXA
      IF(TRIAL)  NN = NN + 1
      IF(IXA.EQ.'OMIT I')  UCHEK = .TRUE.
      IF(UCHEK)  READ(IN,10401)  IXA
      IF(UCHEK)  GO TO 300
      IF(IXA.NE.'TOTAL ')  GO TO 431



      NN = NN + 1
      READ(IN,10404,ERR=432) NCOMP(I), IXA, TREAL(I), COMMIN(I)
      IF(IXA.NE.' ')  GO TO 432
      DO 401 NI=2,N1
      NN = NN + 1
      READ(IN,10401)  IXA
      IF(IXA.EQ.'FREE C'.OR.IXA.EQ.'SPECIE') GO TO 402
      READ(NUL,10404,ERR=432) NCOMP(NI), IXA, TREAL(NI), COMMIN(NI)
      IF(IXA.NE.' ')  GO TO 432
      N = NI - 1
      DO 401 I=1,N
      IF(NCOMP(NI).EQ.NCOMP(I))  GO TO 439
401   CONTINUE
      N = NX
      WRITE(OUT,20401)  N
      GO TO 431
402   NI = NI - 1
      NII = 0
      IF(IXA.EQ.'SPECIE') WRITE(OUT,20413)
      IF(IXA.EQ.'SPECIE') GO TO 405
      N = NI
      DO 403 NII=1,N2
      N = N + 1
      NN = NN + 1
      READ(IN,10401)  IXA
      IF(IXA.EQ.'SPECIE') GO TO 404
      READ(NUL,10404,ERR=432)  NCOMP(N), IXA, XX(NII), COMMIN(N)
      IF(IXA.NE.' ')  GO TO 432
      N = N - 1
      DO 403 I=1,N
      IF(NCOMP(N).EQ.NCOMP(I))  GO TO 439
403   CONTINUE
      N = NXX
      WRITE(OUT,20402) N
      GO TO 431
404   NII = NII - 1
405   NIII = NI + NII
      DO 412 NJ=1,N3
      NN = NN + 1
      READ(IN,10405,ERR=432,END=436)  CONST(NJ), NDUM
```

```
      NUM1(NJ) = 0
      NUM2(NJ) = 0
      NPM = 1
      DO 411 N=1,NMD,2
      IF(NDUM(N).EQ.'     ')  GO TO 411
  406 DO 407 M=NPM,NIII
      IF(NDUM(M).EQ.NCOMP(M))  GO TO 408
  407 CONTINUE
      IF(NPM.EQ.1)  GO TO 434
      NPM = 1
      GO TO 406
  408 CHECK(M) = CHECK(M) + 1
      NPM = M
      IF(NUM1(NJ)+NUM2(NJ).GE.NKEY)  GO TO 430
      IF(M.GT.NII)  GO TO 409
      L = NUM1(NJ) + 1
      NUM1(NJ) = L
      KEY(L,NJ,1) = M
      GO TO 410
  409 L = NKEY - NUM2(NJ)
      NUM2(NJ) = NUM2(NJ) + 1
      KEY(L,NJ,1) = M - NI
  410 IF(NDUM(N+1).EQ.0)  GO TO 434
      KEY(L,NJ,2) = NDUM(N+1)
  411 CONTINUE
      IF(NUM1(NJ)+NUM2(NJ).LE.1)  GO TO 433
      IF(NUM1(NJ).LE.0)  GO TO 435
  412 CONTINUE
      NJ = NJ - 1


C     OUTPUT ERROR MESSAGES.

      WRITE(OUT,20403)  NJ
      GO TO 431
  430 WRITE(OUT,20404)  NJ
  431 WRITE(OUT,20405)
  432 WRITE(OUT,20406)  NN
      GO TO 9999
  433 WRITE(OUT,20407)
  434 WRITE(OUT,20408)  NJ
      GO TO 432
  435 WRITE(OUT,20409)
      GO TO 434
  436 NJ = NJ - 1
      DO 437 I=1,NIII
      IF(CHECK(I).LE.0)  GO TO 438
  437 CONTINUE
      GO TO 450
  438 WRITE(OUT,20410)  I
      GO TO 9999
  439 WRITE(OUT,20414)  I
```

```
      GO TO 432
  440 WRITE(OUT,20417)
      GO TO 442
  441 WRITE(OUT,20418)
  442 WRITE(OUT,20419)
      NN = 5
      GO TO 432
  443 WRITE(OUT,20420)
      GO TO 432


C     POSITION THE TITLE AND DATE ALPHANUMERIC STRINGS.

  450 N1 = 1
  451 IF(DATE(6).NE.'      ') GO TO 453
      N1 = N1 + 1
      DO 452 N=6,N1,-1
  452 DATE(N) = DATE(N-1)
      DATE(N1-1) = '      '
      IF(N1.LT.6)  GO TO 451
  453 IF(TITLE(12).NE.'      '.OR.TITLE(11).NE.'      ')  GO TO 460
      DO 454 N=10,1,-1
      IF(TITLE(N).NE.'      ')  GO TO 455
  454 CONTINUE
      GO TO 460
  455 N1 = (12-N) / 2
      DO 457 N=1,N1
      DO 456 NN=11+1,-1
      N2 = NN + 1
  456 TITLE(N2) = TITLE(NN)
  457 TITLE(1) = '      '


C     SWITCH THE ORDER OF THE COMPONENTS WITH FIXED FREE CONCENTRATIONS.

  460 DO 463 J=1,NJ
      IF(NUM2(J).LE.1) GO TO 463
      N1 = NKEY - NUM2(J) + 1
      N = NUM2(J) + 1
      DO 461 L=N1,NKEY
      N = N - 1
      NKY(N,1) = KEY(L,J,1)
  461 NKY(N,2) = KEY(L,J,2)
      N = 0
      DO 462 L=N1,NKEY
      N = N + 1
      KEY(L,J,1) = NKY(N,1)
  462 KEY(L,J,2) = NKY(N,2)
  463 CONTINUE
```

```
C     NUMBER THE SCAN AND MONITORED COMPONENTS.

      IF(.NOT.SCAN)  GO TO 472
      DO 470 I=1,NIII
      IF(NCOMP(I).EQ.ISCN)  GO TO 471
  470 CONTINUE
      NN = 5
      WRITE(OUT,20411)
      GO TO 432
  471 ISCN = I
      IF(.NOT.MULTIP.AND.SCNINC.LE.0.000)  GO TO 440
      IF(MULTIP.AND.SCNINC.LE.1.000)  GO TO 440
      IF(I.LE.NI.AND.TREAL(I).GT.SCNMAX)  GO TO 441
      IF(I.GT.NI.AND.XX(I-NI).GT.SCNMAX)  GO TO 441
  472 IF(.NOT.MONIT)  GO TO 480
      IF(MONALL)  GO TO 480
      K = 1
  473 N = 1
  474 DO 475 I=N,NIII
      IF(IMON(K).EQ.NCOMP(I))  GO TO 476
  475 CONTINUE
      IF(N.NE.1)  GO TO 473
      NN = 4
      WRITE(OUT,20415)
      GO TO 432
  476 IF(CHECK(I).GE.3)  GO TO 478
      WRITE(OUT,20416) NCOMP(I)
      IF(IMON(K+1).EQ.'    '.OR.K.EQ.10)  GO TO 479
      DO 477 M=K,9
  477 IMON(K) = IMON(K+1)
      IMON(10) = '      '
      GO TO 474
  478 IMON(K) = I
      K = K + 1
      IF(K.GT.10)  GO TO 479
      N = I
      IF(IMON(K).NE.'    ')  GO TO 474
  479 NMON = K - 1


C     TEST FOR SPECIE UNIQUENESS.

  480 IF(UCHEK)  GO TO 500
      DO 483 J=1,NJ
      IQUE(J) = 0
      NL = NUM1(J)
      DO 481 L=1,NL
  481 IQUE(J) = IQUE(J) + (KEY(L,J,1) * KEY(L,J,2))
```

```
      IF(NUM2(J).EQ.0) GO TO 483
      NL = NKEY - NUM2(J) + 1
      DO 482 L=NL,NKEY
  482 IQUE(J) = IQUE(J) + (KEY(L,J,1) * KEY(L,J,2))
  483 CONTINUE



      N1 = 2
  484 NN = N1 - 1
      N = N1
  485 DO 486 J=N,NJ
      IF(IQUE(NN).EQ.IQUE(J)) GO TO 488
  486 CONTINUE
  487 N1 = N1 + 1
      IF(N1.GT.NJ) GO TO 500
      GO TO 484
  488 IF(NUM1(J).NE.NUM1(NN).OR.NUM2(J).NE.NUM2(NN)) GO TO 496
      IF(KEY(1,J,1).NE.KEY(1,NN,1)) GO TO 492
      DO 490 L=1,NKEY
      DO 490 N2=1,2
      IF(KEY(L,J,N2).NE.KEY(L,NN,N2)) GO TO 492
  490 CONTINUE
  491 WRITE(OUT,20412) NN
      NN = J + NI + NII + 8
      IF(MULTID) NN = NN + 1
      IF(SUPRES) NN = NN + 1
      IF(SELECT) NN = NN + 1
      IF(CMPRES) NN = NN + 1
      GO TO 432
  492 N3 = 1
      NL = NUM1(J)
  493 DO 495 L=N3,NL
      DO 494 I=N3,NL
      IF(KEY(L,J,1).EQ.KEY(I,NN,1).AND.KEY(L,J,2).EQ.KEY(I,NN,2))
     1  GO TO 495
  494 CONTINUE
      GO TO 496
  495 CONTINUE
      IF(NL.EQ.NKEY.OR.NUM2(J).EQ.0) GO TO 491
      N3 = NKEY - NUM2(J) + 1
      NL = NKEY
      GO TO 493
  496 IF(J.EQ.NJ)  GO TO 487
      N = J + 1
      GO TO 485


C     SECTION FIVE.     CONSTANT EVALUATION.


  500 IF(TRIAL)  GO TO 1000
      DO 501 I=1,NI
```

```
      X(I) = TREAL(I)
  501 CRIT(I) = TREAL(I) * 0.000001
  502 NNN = 0
      DO 504 J=1,NJ
      HOLD(J) = 10.000 ** CONST(J)
      IF(NUM2(J).EQ.0)  GO TO 504
      N1 = NKEY - NUM2(J) + 1
      DO 503 L=N1,NKEY
      II = KEY(L,J,1)
  503 HOLD(J) = HOLD(J) * (XX(II) ** KEY(L,J,2))
  504 CONTINUE


C     SECTION SIX.     EVALUATION OF THE CALCULATED SPECIE CONCENTRATIONS.


  600 DO 601 J=1,NJ
      SPECIE(J) = HOLD(J)
      NL = NUM1(J)
      DO 601 L=1,NL
      I = KEY(L,J,1)
  601 SPECIE(J) = SPECIE(J) * (X(I) ** KEY(L,J,2))


C     SECTION SEVEN.     EVALUATION OF THE CALCULATED TOTAL CONCENTRATIONS.


  700 DO 701 I=1,NI
  701 TCALC(I) = X(I)
      DO 702 J=1,NJ
      NL = NUM1(J)
      DO 702 L=1,NL
      I = KEY(L,J,1)
  702 TCALC(I) = TCALC(I) + (SPECIE(J) * KEY(L,J,2))


C     SECTION EIGHT.     TEST FOR CONVERGENCE.


  800 N1 = 0
      NNN = NNN + 1
      DO 801 I=1,NI
      IF(ABS(TREAL(I)-TCALC(I)).LE.CRIT(I))  N1 = N1 + 1
  801 CONTINUE
      IF(NNN.LT.NSTOP.AND.N1.LT.NI)  GO TO 900
      IF(NNN.LT.NSTOP)  GO TO 1000
      WRITE(OUT,20801)
      SCAN = .FALSE.
      GO TO 1000
```

```
C     SECTION NINE.     EVALUATION OF THE COMPONENTS NEW FREE CONCENTRATIONS.

  900 IF(NNN.GT.25.AND.MOD(NNN,2).EQ.1)  GO TO 908
      IF(NNN.GE.4)  GO TO 910
      DO 901 I=1,NI
  901 DUM(I) = SQRT(TREAL(I)/TCALC(I))
  902 DO 907 M=1,NI
      DEN = X(M)
      DO 906 J=1,NJ
      NL = NUMI(J)
      DO 903 L=1,NL
      IF(KEY(L,J,1).EQ.M)  GO TO 904
  903 CONTINUE
      GO TO 906
  904 FACTOR = 1.000
      DO 905 LL=1,NL
      I = KEY(LL,J,1)
  905 FACTOR = FACTOR * (DUM(I) ** KEY(LL,J,2))
      DEN = DEN + (FACTOR*SPECIE(J)*KEY(L,J,2)/DUM(M))
  906 CONTINUE
      DUM(M) = X(M)
      X(M) = X(M) * TREAL(M) / DEN
  907 DUM(M) = X(M) / DUM(M)
      GO TO 600
  908 DO 909 I=1,NI
  909 X(I) = X(I) * TREAL(I)/TCALC(I)
      GO TO 600
  910 DO 911 I=1,NI
  911 X(I) = X(I) * SQRT(TREAL(I)/TCALC(I))
      GO TO 600


C     SECTION TEN.      OUTPUT.

 1000 KOUNT = KOUNT + 1
      IF(KOUNT.NE.1) GO TO 1002
      WRITE(OUT,21000) TITLE, NAME, DATE, NIII, NII, NJ
      N1 = '  COMP'
      N2 = 'LETE  '
      IF(CMPRES) N1 = ' COMPR'
      IF(CMPRES) N2 = 'ESSED '
      IF(SUPRES)  N1 = ' SUPRE'
      IF(SUPRES)  N2 = 'SSED  '
      WRITE(OUT,21011) N1, N2
      N1 = 'OFF'
      IF(MONIT)  N1 = 'ON '
      IF(MONALL)  N1 = 'ALL'
      WRITE(OUT,21014)  N1
      N1 = 'OFF'
      IF(SELECT)  N1 = 'ON '
      WRITE(OUT,21021)
```

```
      N1 = 'ON '
      IF(NCHEK) N1 = 'OFF'
      WRITE(OUT,21015) N1
      IF(SCAN) GO TO 1001
      WRITE(OUT,21012)
      GO TO 1002
 1001 WRITE(OUT,21013) NCOMP(ISCN)
      IF(MULTIP)  WRITE(OUT,21019)
      IF(.NOT.MULTIP)  WRITE(OUT,21020)
 1002 IF(SCAN) WRITE(OUT,21001) KOUNT
      IF(.NOT.SCAN) WRITE(OUT,21009)
      WRITE(OUT,21002)
      WRITE(OUT,21003)
      IF(TRIAL)  GO TO 1050


C     SUM TOTAL CONCENTRATIONS FOR SPECIES WITH FREE CONCS. FIXED.

      DO 1011 II=1,NII
      M = MI + II
 1011 TCALC(M) = XX(II)
      DO 1013 J=1,NJ
      IF(NUM2(J).EQ.0) GO TO 1013
      NL = NKEY - NUM2(J) + 1
      DO 1012 L=NL,NKEY
      II = KEY(L,J,1)
      M = MI + II
 1012 TCALC(M) = TCALC(M) + (SPECIE(J) * KEY(L,J,2))
 1013 CONTINUE


C     OUTPUT COMPONENT CONCENTRATIONS.

 1050 N = NLINES - 15
      IF(SCAN) N = N - 10
      NPM = ((NIII - N) / (NLINES - 9)) + 2
      IF(NIII.LE.N) NPM = 1
      N2 = (NIII / NPM) + 1
      IF(N.GE.N2) N = N2
      IF(N.GE.N2) GO TO 1051
      N2 = ((NIII - N) / (NPM - 1)) + 1
 1051 NPM = N
      DO 1052 I=1,NI
      IF(I.LT.NPM.OR.I.EQ.NI) GO TO 1052
      NPM = NPM + N2
      WRITE(OUT,21009)
      WRITE(OUT,21003)
 1052 WRITE(OUT,21004) I, X(I), TREAL(I), TCALC(I), COMMIN(I),
     1 NCOMP(I), CHECK(I)
```

```
      N1 = NI
      DO 1061 II=1,NII
      N1 = N1 + 1
      IF(N1.LT.NPM.OR.II.EQ.NII) GO TO 1061
      NPM = NPM + N2
      WRITE(OUT,21009)
      WRITE(OUT,21003)
 1061 WRITE(OUT,21005) N1, XX(II), TCALC(N1), COMMIN(N1),
     1 NCOMP(N1), CHECK(N1)


C     OUTPUT SPECIE CONCENTRATIONS.

      IF(SUPRES)  GO TO 1077
      N = NLINES - 15
      NPM = ((NJ - N) / (NLINES - 9)) + 2
      IF(NJ.LE.N) NPM = 1
      N2 = (NJ / NPM) + 1
      IF(N.GE.N2) N = N2
      IF(N.GE.N2) GO TO 1071
      N2 = ((NJ - N) / (NPM - 1)) + 1
 1071 N1 = 1
      NPM = N
      WRITE(OUT,21006)
      WRITE(OUT,21007)
      DO 1076 J=1,NJ
      IF(N1.LT.NPM.OR.J.EQ.NJ) GO TO 1072
      NPM = NPM + N2
      WRITE(OUT,21009)
      WRITE(OUT,21007)
 1072 N =1
      N3 = 1
      NL = NUM1(J)
 1073 DO 1074 L=N3,NL
      M = KEY(L,J,1)
      IF(NL.EQ.NKEY.AND.NUM2(J).NE.0) M = M + NI
      IF(CHPRES.AND.SPECIE(J).LT.COMMIN(M)) GO TO 1076
      NDUM(N) = NCOMP(M)
      NDUM(N+1) = KEY(L,J,2)
 1074 N = N + 2
      IF(NUM2(J).EQ.0.OR.NL.EQ.NKEY) GO TO 1075
      NL = NKEY
      N3 = NKEY - NUM2(J) + 1
      GO TO 1073
 1075 NN = N  - 1
      WRITE(OUT,21008) J, SPECIE(J), CONST(J), (NDUM(N), N=1,NN)
      N1 = N1 + 1
 1076 CONTINUE
      IF(TRIAL)  GO TO 9999
      WRITE(OUT,21010)  NNN
```

```
 1077 IF(NPN.GE.NSTOP)  WRITE(OUT,20801)
      IF(.NOT.MONIT)  GO TO 1095
      DO 1078 I=1,NIII
 1078 CHECK(I) = 0


C       OUTPUT THE MONITORED COMPONENT RESULTS


      IF(MONALL)  NMON = NIII
      DO 1090 K=1,NMON
      NPN = NLINES - 15
      M = K
      IF(.NOT.MONALL)  M = IMON(K)
      NI = 0
      WRITE(OUT,21016)  NCOMP(M)
      IF(M.GT.NI)  GO TO 1087
      DO 1081 J=1,NJ
      NL = NUM1(J)
      DO 1080 L=1,NL
      IF(KEY(L,J,1).NE.M)  GO TO 1080
      NI = NI + 1
      CONC(NI) = SPECIE(J)
      IQUE(NI) = J
      GO TO 1081
 1080 CONTINUE
 1081 CONTINUE
 1082 CALL SORT(CONC,IQUE,NI)
      WRITE(OUT,21007)
      DO 1086 I=1,100
      J = IQUE(I)
      N = 1
      N3 = 1
      NL = NUM1(J)
 1083 DO 1084 L=N3,NL
      M = KEY(L,J,1)
      IF(NL.EQ.NKEY.AND.NUM2(J).NE.0)  M = M + NI
      NDUM(N) = NCOMP(M)
      IF(SELECT) CHECK(M) = CHECK(M) + 1
      NDUM(N+1) = KEY(L,J,2)
 1084 N = N + 2
      IF(NUM2(J).EQ.0.OR.NL.EQ.NKEY)  GO TO 1085
      NL = NKEY
      N3 = NKEY - NUM2(J) + 1
      GO TO 1083
 1085 NN = N - 1
      IF(SELECT)  WRITE(28,31001)  CONST(J), (NDUM(N), N=1,NN)
      IF(I.LE.NPN) WRITE(OUT,21008)J,SPECIE(J),CONST(J),(NDUM(N),N=1,NN)
      IF(I.GE.NI)  GO TO 1090
 1086 CONTINUE
      GO TO 1090
 1087 M = M - NI
      DO 1089 J=1,NJ
```

```
      IF(NUM2(J).EQ.0)  GO TO 1089
      NL = NKEY - NUM2(J) + 1
      DO 1088 L=NL,NKEY
      IF(KEY(L,J,1).NE.N)  GO TO 1088
      N1 = N1 + 1
      CONC(N1) = SPECIE(J)
      IGUE(N1) = J
      GO TO 1089
 1088 CONTINUE
 1089 CONTINUE
      GO TO 1082
 1090 CONTINUE


 1091 IF(.NOT.SELECT)  GO TO 1095
      NPM = 1
 1092 DO 1093 I=NPM,NIII
      IF(CHECK(I).EQ.0)  GO TO 1094
 1093 CONTINUE
      GO TO 1095
 1094 IF(NPM.EQ.1)  WRITE(OUT,21017)
      NPM = I + 1
      WRITE(OUT,21018)  NCOMP(I)
      IF(NPM.LE.NIII)  GO TO 1092
 1095 IF(.NOT.SCAN)  GO TO 9999


C     SECTION ELEVEN.     SCAN MECHANISM.


 1100 IF(ISCN.GT.NI)  GO TO 1101
      IF(TREAL(ISCN).LE..1E-24)  X(I) = SCNINC
      IF(.NOT.MULTIP)  TREAL(ISCN) = TREAL(ISCN) + SCNINC
      IF(MULTIP)  TREAL(ISCN) = TREAL(ISCN) * SCNINC
      IF(TREAL(ISCN).LE..1E-24)  X(I) = SCNINC
      IF(TREAL(ISCN).GT.SCNMAX)  GO TO 9999
      CRIT(ISCN) = TREAL(ISCN) * 0.000001
      GO TO 502
 1101 II = ISCN - NI
      IF(KOUNT.EQ.1)  SCNMIN = XX(II)
      IF(.NOT.MULTIP)  XX(II) = XX(II) + SCNINC
      IF(MULTIP)  XX(II) = XX(II) * SCNINC
      IF(XX(II).LE.SCNMAX)  GO TO 502



 9999 WRITE(OUT,99999)
      IF(SELECT)  ENDFILE 28


      SUBROUTINE SORT(ARRAY,NARRAY,N)
```

```
      DIMENSION ARRAY(N), HARRAY(N)
      REAL LAYBY
      INTEGER OLD
      M = 2
      DO 1 J=1,N
      M = M + M
      IF(N.GT.M)  GO TO 2
    1 CONTINUE
    2 M = M - 1
    3 M = (M-1) / 2
    4 NN = N - M
      DO 7 I=1,NN
      OLD = I + M
      LAYBY = ARRAY(OLD)
      HLAYBY = HARRAY(OLD)
      DO 5 J=1,J,M
      NEW = OLD + M
      IF(LAYBY.LE.ARRAY(NEW))  GO TO 6
      ARRAY(OLD) = ARRAY(NEW)
      HARRAY(OLD) = HARRAY(NEW)
      OLD = NEW
    5 CONTINUE
    6 ARRAY(OLD) = LAYBY
      HARRAY(OLD) = HLAYBY
    7 CONTINUE
      IF(M.GT.1)  GO TO 3
      RETURN
      END
```

appendix 54

LISTING AND INSTRUCTIONS FOR PROGRAM INDEX.

PROGRAM INDEX IS USED TO PRODUCE LISTS OF LITERATURE REFERENCES SORTED IN ORDER OF (1) JOURNAL NAMES, (2) AUTHOR NAMES, (3) KEYWORDS, (4) DATE AND (5) CHEMICAL ABSTRACT NUMBER. THE INPUT CONSISTS OF THE ALPHANUMERIC STRINGS WHICH SPECIFY EACH AUTHOR, JOURNAL, BOOK AND KEYWORD AS WELL AS THE SET OF REFERENCES IN CODED FORM. THIS CODING ELIMINATES STORAGE WASTE BY MINIMIZING ALPHANUMERIC DUPLICATION OF AUTHOR AND JOURNAL NAMES WHEN THEY APPLY TO MORE THAN ONE REFERENCE. EACH AUTHOR, JOURNAL, BOOK, TITLE AND KEYWORD IS ASSIGNED A SEQUENCE NUMBER. THESE NUMBERS ARE PLACED IN FRONT OF EACH ENTRY . THE LISTS THUS ASSEMBLED FOR (1) AUTHOR, (2) JOURNAL, (3) BOOK TITLE AND (4) KEYWORDS COMPRISE THE FIRST SECTION OF THE DATA. EACH LIST MUST BE HEADED BY A CARD SPECIFYING WHICH LIST IS TO FOLLOW. THE LISTS ARE DELIMITED BY A BLANK CARD. THE SEQUENCE NUMBERS ARE STARTED AFRESH FOR EACH LIST FOR EXAMPLE,

```
AUTHOR LIST
001 SMITH H.K.
002 CHARLES M.
003 DALLY P.L.O.M.
...
...
...
122 MASON N.H.

JOURNAL LIST
001 BOOKS
002 J. CHEM SOC.
003 J. CHEM SOC. DALTON
004 J. CHEM SOC. A
005 J. CHEM SOC. B
006 J. CHEM SOC. PERKIN
007 J. CHEM SOC. FARADAY
008 J. CHEM EDUCATION
...
...
...
145 TALANTA

BOOK LIST
001 THE CHEMISTRY OF THE TRACE METALS IN VIVO.
002 THE CHEMISTRY OF COMPLEX EQUILIBRIA.
```

...
...
...
024 THE METALS OF LIFE.

KEYWORD LIST
001 COPPER
002 CALCIUM
...
...
...
104 ZINC

REFERENCES

THE SECOND PART OF THE DATA CONSISTS OF THE CODED REFERENCES. THESE ARE A COLLECTION OF NUMBERS FOR (1) THE SEQUENCE NUMBER OF THE REFERENCE, (2) THE CHEMICAL ABSTRACT NUMBER, (3) THE SEQUENCE NUMBER OF THE JOURNAL, (4) THE YEAR, (5) THE VOLUME, (6) THE PAGE, (7) AN ALPHANUMERIC SET OF FOUR CHARACTERS AS A PERSONAL FILING LABEL AND (8) SUCCESIVE AUTHOR NUMBERS. THE NUMBERS ARE ENTERED IN THE ORDER NAMED AND ARE FORMATTED AS SHOWN BELOW. THE SECOND CARD BEARS THE STRING OF KEYWORD NUMBERS. THE THIRD AND SUBSEQUENT CARDS CARRY THE TITLE OF THE REFERENCE AS ALPHANUMERIC STRINGS OF 80 CHARACTERS EACH. EACH REFERENCE IS DELIMITED BY A BLANK CARD.

PLEASE NOTE:

(1) THE FIRST JOURNAL MUST BE ENTITLED 'BOOKS'. BOOKS ARE REFERENCED BY INDICATING JOURNAL 0001. THE VOLUME NUMBER IS THEN TAKEN TO BE THE SEQUENCE NUMBER OF THE BOOK.

(2) THE SIX JOURNALS FOLLOWING 'BOOKS' ARE ASSUMED, BY THE PROGRAM, TO BE WITHOUT VOLUME NUMBERS (E.G. J.C.S.)

(3) IF THE YEAR IS CODED AS 9999 THEN THIS IS OMITTED WHENEVER THE REFERENCE IS PRINTED OUT.

(4) THE SPACE FOR THE CHEMICAL ABSTRACT NUMBER MAY BE LEFT BLANK.

(5) IF THE PAGE NUMBER OF A REFERENCE TO A BOOK IS ZERO, THE TITLE CARD OF THE REFERENCE IS NOT PRINTED OUT. A DUMMY CARD IS NEVERTHELESS REQUIRED.

(6) LISTS OF REFERENCES, AUTHORS, JOURNALS, BOOKS AND KEYWORDS CAN ALL BE ACCUMULATED IN A RANDOM ORDER.

EXAMPLES OF REFERENCES.

0001 12345678 0123 1975 0001 1234 FILE 0987 0678

```
 099 122 005
THE TITLE GOES HERE.

0002          0001 1967 0003 0000 BOOK 0476 0244 0123 0067
 007 099
THIS REFERS TO A BOOK.  THE CARD WOULD NOT BE PRINTED OUT.

0003 45000564 0002 1975 0000 1652 JCS. 0065
 005
THIS REFERS TO AN ARTICLE IN J.C.S.



PROGRAM INDEX REQUIRES TWO TEMPORARY WORKING FILES.   THESE MUST BE
ASSIGNED LOGICAL UNIT NUMBERS 11 & 12.



PROGRAM  INDEX.
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C     THIS PROGRAM SETS UP AND OPERATES A LITRATURE INDEX AND CATALOGUE.
C
C     IT WAS DEVELOPED AT THE UNIVERSITY OF CAPE TOWN DURING 1975.


      IMPLICIT INTEGER(A-Z)
      PARAMETER MR=800, MA=1000, MJ=200, MK=200, MB=100, MRST=MAX(MA,MK)
      PARAMETER MRA = 1800,MRK = 3000,MRT = MR*2
      PARAMETER  MAZ=7*MA, MBZ=21*MB, MJZ=12*MJ, MKZ=12*MK
      COMMON /ONE/   REF(9,MR), REFA(2,MRA), REFK(2,MRK)
      COMMON /TWO/   BOOK(21,MB), JURNAL(12,MJ)
      COMMON /THREE/ AUTHOR(7,MA), KEY(12,MK)
      COMMON /FOUR/  R(MRST), S(MRST), T(MRST)
      COMMON /FIVE/  LINE30(30), NUMBER(20), LINES, NLINES
      COMMON /SIX/   LABS, LKEY, LTIT
      COMMON /SEVEN/ MR, MA, MJ, MK, MB, MRA, MRK, MRT
      COMMON /EIGHT/ IN, OUT, REFT, SEQF, OUTF, SWIFT
      DATA  AUTHOR / MAZ*'     '/ BOOK / MBZ*'     '/ JURNAL / MJZ*'     '/
      DATA  KEY / MKZ*'     '/
      DATA  NUMBER/'1','2','3','4','5','6','7','8','9','10',
     1  '11','12','13','14','15','16','17','18','19','20'/
      LOGICAL  LABS, LKEY, LTIT, SWIFT


      IN = 8
      OUT = 5
```

```
      REFT = 12
      SCRF = 11
      OUTF = 13
      LINES = 7
      LABS = .FALSE.
      LKEY = .FALSE.
      LTIT = .TRUE.
      SHIFT = .FALSE.
      DEFINE FILE REFT(NRT,80,E,V)
      CALL CREATE
      WRITE(OUT,9999)
      CALL LIST(AUTHOR,7,NA,OUT)
      WRITE(OUT,9999)
      CALL LIST(JURNAL,12,NJ,OUT)
      WRITE(OUT,9999)
      CALL LIST(BOOK,21,NB,OUT)
      WRITE(OUT,9999)
      CALL LIST(KEY,12,NK,OUT)
      WRITE(OUT,9999)
      IF(SHIFT)  GO TO 100
      CALL SELECT(3,LINES)
      WRITE(OUT,9999)
      CALL SELECT(2,LINES)
      WRITE(OUT,9999)
  100 LINES = 7
      LTIT = .TRUE.
      IF(SHIFT)  LTIT = .FALSE.
      CALL SELECT(1,LINES)
      WRITE(OUT,9999)
      IF(SHIFT)  STOP OK
      CALL SELECT(4,LINES)
      WRITE(OUT,9999)
      CALL SELECT(5,LINES)
      WRITE(OUT,9999)
 9999 FORMAT('1')
      END




      SUBROUTINE CREATE

C     THIS SUBROUTINE SETS UP THE INDEX SYSTEM FROM THE DATA ON CARDS.

      IMPLICIT INTEGER(A-Z)
      PARAMETER NR=800,MA=1000, MJ=200, MK=200, MB=100, MRST=MAX(MA,MK)
      PARAMETER  MRA = 1800, MRK = 3000
      COMMON /ONE/    REF(9,NR), REFA(2,MRA), REFK(2,MRK)
      COMMON /TWO/    BOOK(21,MB), JURNAL(12,MJ)
      COMMON /THREE/ AUTHOR(7,MA), KEY(12,MK)
      COMMON /FOUR/  R(MRST), S(MRST), T(MRST)
```

```
      COMMON /FIVE/  LINE30(30), NUMBER(20), LINES, NLINES
      COMMON /SEVEN/ NR, NA, NJ, NK, NC, NRA, NRK, NRT
      COMMON /EIGHT/ IN, OUT, REFT, SEQF, OUTF, SWIFT
      LOGICAL SWIFT
      DIMENSION  LINE20(20), LINE14(14)
      EQUIVALENCE  (LINE30(1), LINE20(1), LINE14(1))



1101  FORMAT(A4)
1201  FORMAT(I4,I9,4I5,1X,A4,12I5)
1221  FORMAT(20I4)
1241  FORMAT(20A4)
3251  FORMAT(///'0','A TITLE MAY NOT BE LONGER THAN FIVE LINES.')
3271  FORMAT(///'0','TOO MANY REFERENCES.')
3290  FORMAT('1')
2310  FORMAT(I0,2I5,4I9,1X,A4,3(1X,I4))
2311  FORMAT(20I5)
3531  FORMAT('1','THE FOLLOWING REFERENCE(S) HAVE BEEN DUPLICATED.',///)
3532  FORMAT('0',10A4)
3533  FORMAT(' ',2X,3(I5,',',),I5,/'0','SORTED POSITIONS',2X,2I5,/' ',
     1 'UNSORTED POSITIONS',2I5,////)
3541  FORMAT(///'0','THE ORIGINAL REFERENCE LIST HAS BEEN SORTED AS FOLL
     10WS:',///)
3542  FORMAT('0',10I5)
3564  FORMAT('0','JOURNAL',I4,' IS NEVER REFERENCED.',////)
3574  FORMAT('0','AUTHOR',I4,' IS NEVER REFERENCED.',////)
3584  FORMAT('0','KEYWORD',I4,' IS NEVER REFERENCED.',////)
3600  FORMAT('0','THE PROGRAM LIMITS HAVE BEEN EXCEEDED IN SUBROUTINE CR
     1EATE')
3610  FORMAT('0','ERROR ON CARD NUMBER',I5,////)
3620  FORMAT('0','DELINITER ERROR. STRINGS OF BLANKS IN TITLES ARE UNACC
     1EPTABLE.')
3660  FORMAT('0','PROBLEM ',3A4,/' ','EXECUTION TERMINATED.')
3661  FORMAT('0','NUMBER',I4,' IS NEVER REFERENCED.',////)
3701  FORMAT('1','INDEX SYSTEM CREATED.',/' ',20(1H*),///'0',T25,'NUMBER
     1 OF DATA CARDS =',I5,/' ',T25,'NUMBER OF REFERENCES =',I5,/' ',
     2 T25,'NUMBER OF AUTHORS =',I5,/' ',T25,'NUMBER OF KEYWORDS =',
     3 I5,/' ',T25,'NUMBER OF JOURNALS =',I4,/' ',T25,'NUMBER OF BOOKS =
     4 ',I4,/' ',T25,'NUMBER OF AUTHOR ENTRIES =',I5,/' ',T25,
     5 'NUMBER OF KEYWORD ENTRIES =',I5,/' ',T25,
     6 'NUMBER OF TITLE LINES =',I5,/////)



      NN = 1
      READ(IN,1101) IXA
      IF(IXA.EQ.'SWIF')  SWIFT = .TRUE.
      IF(SWIFT)  READ(IN,1101)  IXA
      IF(IXA.NE.'AUTH')  GO TO 610
      I = MA
      CALL NULIST(AUTHOR,I,7,NA,NN,IN,OUT,LINE20,R,S,T)
      IF(NA.EQ.0)  GO TO 610
```

```
READ(IN,1101) IXA
NN = NN + 1
IF(IXA.NE.'JOUR')  GO TO 610
I = NJ
CALL NULIST(JURNAL,I,12,NJ,NN,IN,OUT,LINE20,R,S,T)
IF(NJ.EQ.0)  GO TO 610



READ(IN,1101) IXA
NN = NN + 1
IF(IXA.NE.'BOOK')  GO TO 610
I = NB
CALL NULIST(BOOK,I,21,NB,NN,IN,OUT,LINE20,R,S,T)
IF(NB.EQ.0)  GO TO 610



READ(IN,1101) IXA
NN = NN + 1
IF(IXA.NE.'KEY')  GO TO 610
I = NK
CALL NULIST(KEY,I,12,NK,NN,IN,OUT,LINE20,R,S,T)
IF(NK.EQ.0)  GO TO 610

|
|
IRA = 0
IRK = 0
IRT = 1
FIND(REFT,IRT)
READ(IN,1101) IXA
NN = NN + 1
IF(IXA,NE.'REFE')  GO TO 610
DO 270 NR=1,NR
READ(IN,1201)  REF(9,NR), LINE14
NN = NN + 1
IF(LINE14(2).EQ.0)  GO TO 280
IF(REF(9,NR).NE.NR)  GO TO 680
REF(1,NR) = LINE14(1)
REF(2,NR) = LINE14(2)
IF(REF(2,NR).GT.NJ)  GO TO 630
REF(3,NR)= LINE14(3)
IF(LINE14(4).EQ.0.AND.LINE14(2).GT.7)  LINE14(4) = LINE14(3)
REF(4,NR) = (LINE14(4) * 10000) + LINE14(5)
REF(8,NR) = LINE14(6)
IF(SWIFT)  REF(8,NR) = '     '
REF(6,NR) = IRA
DO 210 I = 7,14
IF(LINE14(I).EQ.0)  GO TO 220
IRA = IRA + 1
REFA(I,IRA) = LINE14(I)
```

```
      IF(REFA(1,IRA).GT.NA)  GO TO 640
  210 CONTINUE
  220 IF(IRA.EQ.REF(6,NR))  GO TO 640
      REF(6,NR) = ((IRA - REF(6,NR)) * 10000) + REF(6,NR) + 1



      READ(IN,1221)  LINE20
      NN = NN + 1
      REF(7,NR) = IRK
      DO 230 I = 1,20
      IF(LINE20(I).EQ.0)  GO TO 240
      IRK = IRK + 1
      REFK(1,IRK) = LINE20(I)
      IF(REFK(1,IRK).GT.NK)  GO TO 650
  230 CONTINUE
  240 IF(IRK.EQ.REF(7,NR))  GO TO 650
      REF(7,NR) =((IRK - REF(7,NR)) * 10000) + REF(7,NR) + 1



      REF(5,NR) = IRT
      DO 250 I=1,6
      READ(IN,1241)  LINE20
      IF(LINE20(1).EQ.'    ')  GO TO 260
      IF(IXA.EQ.'    ') GO TO 620
      WRITE(REFT'IRT,1241)  LINE20
      IRT = IRT + 1
      FIND(REFT'IRT)
      IXA = LINE20(11)
  250 NN = NN + 1
      WRITE(OUT,3251)
      GO TO 600
  260 NN = NN + 1
      IXA = 1
  270 REF(5,NR) = ((IRT - REF(5,NR)) * 10000) + REF(5,NR)
      WRITE(OUT,3271)
      GO TO 600
  280 NR = NR - 1
      NRA = IRA
      NRK = IRK
      NRT = IRT


      WRITE(OUT,3290)
      DO 291 IR=1,NR
  291 CALL CATOUT(IR,6,,FALSE,,'    ')



      DO 310 IR=1,NR
      J = REF(2,IR)
      R(IR) = (REF(3,IR) * 10000) + JURNAL(1,J)
      S(IR) = REF(4,IR)
```

```
310 T(IR) = IR
    CALL ORDER(R,S,T,NR)
    DO 320 IR=1,NR
    J = T(IR)
    CALL SPLIT(REF(6,J),IRA,IXA)
    IA = IXA - IRA
    CALL SPLIT(REF(7,J),IRK,IRT)
    IK = IRT -IRK
    WRITE(SEQF,2310) (REF(I,J), I=1,9), IA, IK
    WRITE(SEQF,2311) (REFA(1,I), I=IRA,IXA)
320 WRITE(SEQF,2311) (REFK(1,I), I=IRK,IRT)
    ENDFILE SEQF
    REWIND  SEQF
    IRA = 1
    IRK = 1
    DO 350 IR=1,NR
    READ(SEQF,2310) (REF(I,IR), I=1,9), IA, IK
    IXA = IRA + IA
    READ(SEQF,2311) (REFA(1,I), I=IRA,IXA)
    DO 330 I=IRA,IXA
330 REFA(2,I) = IR
    REF(6,IR) = ((IA + 1) * 10000) + IRA
    IRA = IXA + 1
    IXA = IRK + IK
    READ(SEQF,2311) (REFK(1,I), I=IRK,IXA)
    DO 340 I=IRK,IXA
340 REFK(2,I) = IR
    REF(7,IR) = ((IK + 1) * 10000) + IRK
350 IRK = IXA + 1
    REWIND  SEQF



500 I = 2
510 DO 520 IR=I,NR
    J = IR - 1
    IF(REF(4,IR).NE.REF(4,J))  GO TO 520
    IF(REF(2,IR).EQ.REF(2,J))  GO TO 530
520 CONTINUE
    GO TO 540
530 IF(NN.GT.0)  WRITE(OUT,3531)
    NN = J
    IJ = REF(2,IR)
    J = JOURNAL(2,IJ) + 2
    WRITE(OUT,3532)  (JOURNAL(I,IJ), I=3,J)
    I = REF(4,IR) / 10000
    J = REF(4,IR) - (I * 10000)
    WRITE(OUT,3533)  IJ, REF(3,IR), I, J, NN, IR, T(NN), T(IR)
    NN = - 1
    I = IR + 1
    GO TO 510
540 IF(NN.GT.0)  GO TO 560
    WRITE(OUT,3541)
    DO 550 I=1,NR,10
```

```
      J = I + 9
      IF(J.GT.NR)  J = NR
  550 WRITE(OUT,3542)  (T(IJ), IJ=I,J)
      STOP CRE3



  560 DO 566 IJ=1,NJ
      DO 563 IR=1,NR
      IF(REF(2,IR).EQ.IJ)  GO TO 566
  563 CONTINUE
      NN = -1
      WRITE(OUT,3564)  IJ
  566 CONTINUE
      DO 576 IA=1,NA
      DO 573 IRA=1,NRA
      IF(REFA(1,IRA).EQ.IA)  GO TO 576
  573 CONTINUE
      IF(NN.GT.0)  NN = -2
      IF(NN.EQ.-1)  NN = -10
      WRITE(OUT,3574)  IA
  576 CONTINUE
      DO 586 IK=1,NK
      DO 583 IRK=1,NRK
      IF(REFK(1,IRK).EQ.IK)  GO TO 586
  583 CONTINUE
      IF(NN.GT.0)  NN = -3
      IF(NN.EQ.-1.OR.NN.EQ.-2)  NN = -10
      WRITE(OUT,3584)  IK
  586 CONTINUE
      IF(NN.GT.0)  GO TO 700
      I = 'ONLY'
      IF(NN+2) 590, 640, 630
  590 IF(NN.EQ.-3)  GO TO 650
      IXA = 'GENE'
      J = 'RAL '
      I = '    '
      GO TO 670



  600 WRITE(OUT,3600)
  610 WRITE(OUT,3610)  NN
      STOP CRE4
  620 WRITE(OUT,3620)
      GO TO 610
  630 IXA = 'JOUR'
      J = 'NAL '
      GO TO 660
  640 IXA = 'AUTH'
      J = 'OR  '
      GO TO 660
  650 IXA = 'KEYW'
      J = 'ORD '
```

```
  660 IF(NN.GT.0)  I = '    '
  670 WRITE(OUT,3660)  IXA, J, I
      IF(NN.GT.0)  GO TO 610
      STOP CRES
  680 IXA = 'REFE'
      J = 'RENC'
      I = 'E   '
      GO TO 670
  700 WRITE(OUT,3701)  NN, NR, NA, NK, NJ, NB, NRA, NRK, NRT
      RETURN
      END



      SUBROUTINE SELECT(NCAT,LINES)

C     THIS SUBROUTINE SELECTS AND SETS UP THE APPROPRIATE CATALOGUE
C     OUTPUT PROCEDURES.

      IMPLICIT INTEGER(A-Z)
      PARAMETER  NR=800, NA=1000, NJ=200, NK=200, NB=100
      PARAMETER  NRA = 1800, NRK = 3000
      COMMON /ONE/    REF(9,NR), REFA(2,NRA), REFK(2,NRK)
      COMMON /TWO/    BOOK(21,NB), JURNAL(12,NJ)
      COMMON /THREE/ AUTHOR(7,NA), KEY(12,NK)
      COMMON /SIX/    LABS, LKEY, LTIT
      COMMON /SEVEN/ NR, NA, NJ, NK, NB, NRA, NRK, NRT
      COMMON /EIGHT/ IN, OUT, REFT, SEQF, OUTF
      LOGICAL  PAGE, LABS, LKEY, LTIT


 3501 FORMAT('1')
 3700 FORMAT(//'0',,'EXECUTION TERMINATED.   ERROR IN DATA ARRAYS,',////)



      GO TO(100,200,300,400,500,100), NCAT


  100 LKEY = .FALSE.
      LABS = .FALSE.
      PAGE = .TRUE.
      DO 110 IR=1,NR
      IF(REF(3,IR).GT.0)  GO TO 120
  110 CONTINUE
      GO TO 700
  120 CALL CATOUT(IR,1,PAGE,REF(3,IR))
      N = IR + 1
```

```
      DO 130 IR=N,NR
      IF(REF(3,IR).GT.0)  GO TO 140
  130 CONTINUE
      GO TO 700
  140 N = IR + 1
      DO 150 J=N,NR
      NEXT = REF(3,J)
      IF(NEXT.LT.0)  GO TO 150
      CALL CATOUT(IR,1,PAGE,NEXT)
      IR = J
  150 CONTINUE
      CALL CATOUT(IR,6,PAGE,NEXT)
      IF(NCAT.EQ.6)  GO TO 600
      RETURN


  200 LTIT = .TRUE.
      LABS = .FALSE.
      LKEY = .FALSE.
      LINES = 7
      CALL SCAN(AUTHOR,7,NA,REFA,NRA,2,NCAT,OUT)
      RETURN


  300 LTIT = .TRUE.
      LKEY = .TRUE.
      LABS = .TRUE.
      LINES = 11
      CALL SCAN(JURNAL,12,NJ,REF,NR,9,NCAT,OUT)
      RETURN


  400 LTIT = .TRUE.
      LKEY = .FALSE.
      LABS = .FALSE.
      LINES = 7
      CALL SCAN(KEY,12,NK,REFK,NRK,2,NCAT,OUT)
      RETURN


  500 LAST = 0
      PAGE = .FALSE.
      WRITE(OUT,3501)
      NEXT =  99999999
      LTIT = .FALSE.
      LABS = .TRUE.
      LKEY = .FALSE.
      LINES = 5
      DO 520 IR=1,NR
      IF(REF(1,IR).NE.0)  GO TO 510
```

```
      LAST = LAST + 1
      GO TO 520
  510 IF(REF(1,IR).GE.NEXT) GO TO 520
      NEXT = REF(1,IR)
      KEEP = IR
  520 CONTINUE
      DO 530 IR=1,NR
      IF(REF(1,IR).NE.0)  GO TO 530
      LAST = LAST - 1
      IF(LAST.LE.0)  GO TO 540
      CALL CATOUT(IR,6,PAGE,NEXT)
  530 CONTINUE
      GO TO 550
  540 CALL CATOUT(IR,NCAT,PAGE,NEXT)
  550 LAST = NEXT
      IR = KEEP
      NEXT = 99999999
      DO 560 N=1,NR
      IF(REF(1,N).GE.NEXT.OR.REF(1,N).LE.LAST)  GO TO 560
      NEXT = REF(1,N)
      KEEP = N
  560 CONTINUE
      IF(NEXT.GT.LAST)  GO TO 540
      CALL CATOUT(IR,6,PAGE,NEXT)
      RETURN



  600 DO 610 IR=1,NR
      IF(REF(3,IR).LT.0)  REF(3,IR) = -REF(3,IR)
  610 CONTINUE
      RETURN



  700 WRITE(OUT,3700)
      STOP
      END




      SUBROUTINE SCAN(AJK,WAJK,NAJK,R,NR,WR,NCAT,OUT)


C     THIS SUBROUTINE SCANS THE REFFRENCES FOR EACH JOURNAL, AUTHOR OR
C     KEYWORD AND THEN HAS THEM PRINTED BY CALLING THE OUTPUT ROUTINE.


      IMPLICIT INTEGER(A-Z)
      PARAMETER  MA = 1000, MK = 200, MJ = 200, MB = 100
      COMMON /TWO/   BOOK(21,MB), JURNAL(12,MJ)
      COMMON /THREE/ AUTHOR(7,MA), KEY(12,MK)
```

```
      COMMON /FIVE/  LINE30(30),  NUMBER(20), LINES, NLINES
      DIMENSION  AJK(MAJK,NAJK), R(NR,NR), LINE10(10)
      EQUIVALENCE  (LINE30(1), LINE10(1))
      LOGICAL PAGE


 3571 FORMAT(' ',88(1H*),10A4,/)



      CR = 1
      IF(MR.EQ.2)  CR = 2
      PAGE = .TRUE.
      DO 110 X=1,NAJK
      IF(AJK(1,X).EQ.1) GO TO 200
  110 CONTINUE
      STOP SCAN1

C     X IS THE SUBSCRIPT OF THE CURRENT AUTHOR, JOURNAL OR KEYWORD.
C     NEXT IS THE SUBSCRPIT OF THE FOLLOWING AUTHOR, JOURNAL OR KEYWORD.

  200 N = NAJK + 1
      DO 600 INCR=2,N
      DO 210 NEXT=1,NAJK
      IF(AJK(1,NEXT).EQ.INCR)  GO TO 300
  210 CONTINUE
      NEXT = NCAT
      NCAT = 6

C     IF NCAT = 6, THIS SHOULD BE THE LAST REFERENCE,
C     THE ROUTINE CATOUT WILL PAGE BUT NOT OUTPUT A HEADING.

  300 JR = 1
      DO 310 IR=NR,1,-1
      IF(R(CR,IR).EQ.X)  GO TO 320
  310 CONTINUE
      STOP SCAN2
  320 LAST = IR



  400 DO 410 IR=JR,NR
      IF(R(CR,IR).EQ.X)  GO TO 500
  410 CONTINUE
      STOP SCAN3



  500 JR = IR + 1
      IF(.NOT.PAGE.AND.IR.EQ.LAST)  X = NEXT
      IF(NCAT.EQ.3)  GO TO 520
      IF(NCAT.EQ.2.OR.NCAT.EQ.4)  GO TO 510
      IF(NEXT.EQ.3)  GO TO 520
  510 IR = R(2,IR)
```

```
  520 CALL CATOUT(IR,NCAT,PAGE,X)
      IF(JR.LE.LAST)  GO TO 400
      X = NEXT
      IF(NCAT.EQ.6)  GO TO 700
      IF(NLINES.LE.2)  GO TO 600
      DO 530 I=1,10
  530 LINE10(I) = '****'
      N = 2
      IF(NCAT-3)  , 570, 550
      N1 = AUTHOR(2,X) + 2
      LINE10(2) = '    '
      DO 540 I=3,N1
      N = N + 1
  540 LINE30(N) = AUTHOR(I,X)
      GO TO 590
  550 N1 = KEY(2,X) + 2
      IF(N1.GT.10)  N = 12 - N1
      IF(N.GE.1)  LINE10(N) = '    '
      DO 560 I=3,N1
      N = N + 1
  560 LINE10(N) = KEY(I,X)
      GO TO 590
  570 N1 = JURNAL(2,X) + 2
      IF(N1.GT.10)  N = 12 - N1
      IF(N.GE.1)  LINE10(N) = '    '
      DO 580 I=3,N1
      N = N + 1
  580 LINE10(N) = JURNAL(I,X)
  590 N = N + 1
      LINE10(N) = '    '
      WRITE(OUT,3571)  LINE10
      NLINES = NLINES + 2
  600 CONTINUE
      STOP SCAN4



  700 NCAT = NEXT
      IF(INCR.EQ.NAJK+1)  RETURN
      STOP SCAN5
      END




      SUBROUTINE LIST(ARRAY,NW,NN,OUT)

C     THIS SUBROUTINE PRINTS OUT THE AUTHOR, JOURNAL, BOOK AND KEYWORD
C     LISTS IN BOTH THEIR ENTERED AND ALPHABETICALLY SORTED ORDER.

      IMPLICIT INTEGER(A-Z)
```

```
      DIMENSION  ARRAY(NW,NN)


 3130 FORMAT(' ',I5,5X,5A4,24X,I5,5X,5A4)
 3140 FORMAT(' ',I5,5X,10A4,4X,I5,5X,10A4)
 3150 FORMAT(' ',2I5,5X,20A4)


      DO 160 POS=1,NN
      DO 110 J=1,NN
      IF(ARRAY(1,J).EQ.POS) GO TO 120
  110 CONTINUE
      STOP LIST
  120 K = ARRAY(2,J) + 2
      IF(NN-12) 130,140,150
  130 WRITE(OUT,3130)  POS,(ARRAY(I,POS), I=3,7), J,(ARRAY(I,J), I=3,K)
      GO TO 160
  140 WRITE(OUT,3140)  POS,(ARRAY(I,POS),I=3,12), J,(ARRAY(I,J),I=3,K)
      GO TO 160
  150 WRITE(OUT,3150)  POS, J, (ARRAY(I,J), I=3,K)
  160 CONTINUE
      RETURN
      END



      SUBROUTINE NULIST(ARRAY,MARR,MARRW,NARR,NN,IN,OUT,LINE20,R,S,T)

C     THIS SUBROUTINE INPUTS THE AUTHOR, JOURNAL, BOOK AND KEYWORD LISTS
C     FROM DATA CARDS.

      IMPLICIT INTEGER(A-Z)
      DIMENSION ARRAY(MARRW,MARR), LINE20(20), R(MARR), S(MARR), T(MARR)
      DIMENSION  V(10), U(10), W(10)


 1011 FORMAT(I3,1X,18A4,A3)
 1121 FORMAT(A4)
 3122 FORMAT(//'0','THE PROGRAM LIMITS HAVE BEEN EXCEEDED.')


      DO 120 NARR=1,MARR
      READ(IN,1011)  LINE20
      NN = NN + 1
      IF(LINE20(1).EQ.0)  GO TO 140
      IF(LINE20(1).NE.NARR)  GO TO 130
```

```
      DO 110 IARR=3,NARRY
      J = IARR - 1
      M = J
      IF(J.LE.19)  M = M + 1
      IF(LINE20(J).EQ.'    '.AND.LINE20(M).EQ.'    ')  GO TO 120
  110 ARRAY(IARR,NARR) = LINE20(J)
      J = J + 1
  120 ARRAY(2,NARR) = J - 2
      READ(IN,1121)  J
      IF(J.EQ.'    ')  GO TO 200
      WRITE(OUT,3122)
  130 NARR = 0
      RETURN
  140 NARR = NARR - 1

C     BEGIN SORT ON THE FIRST EIGHT CHARACTERS.

  200 DO 210 IARR=1,NARR
      R(IARR) = ARRAY(3,IARR)
      S(IARR) = ARRAY(4,IARR)
  210 T(IARR) = IARR
      CALL ORDER(R,S,T,NARR)
      DO 220 IARR=1,NARR
      J = T(IARR)
  220 ARRAY(1,J) = IARR

C     THE SORT IS NOW CONTINUED WITH THOSE ENTRIES WHOSE FIRST EIGHT
C     CHARACTERS ARE IDENTICAL.

      M = 0
      DO 370 IARR=2,NARR
      IF(IARR.LE.M)  GO TO 370
      M = IARR
      J = IARR - 1
      IF(S(IARR).NE.S(J))  GO TO 370
      IF(R(IARR).NE.R(J))  GO TO 370

      DO 310 K=IARR,NARR
      IF(S(IARR).NE.S(K))  GO TO 320
  310 CONTINUE
      K = K + 1

C     POSITIONS J TO K ARE UNSORTED.

  320 K = K - 1
      IF(K.GT.IARR)  GO TO 340

C     J AND IARR ARE ADJACENT ....

      I = T(J)
      K = T(IARR)
      IF(ARRAY(5,I).LT.ARRAY(5,K))  GO TO 370
      IF(ARRAY(5,I).GT.ARRAY(5,K))  GO TO 330
      IF(ARRAY(6,I).LE.ARRAY(6,K))  GO TO 370
```

```
C      ..... AND INCORRECTLY POSITIONED.   SWOP THEM.

  330 ARRAY(1,I)  = IARR
      ARRAY(J,K)  = J
      GO TO 370

C       THERE ARE THREE OR MORE UNSORTED SEQUENTIAL VALUES.

  340 L = 0
      DO 350 I=J,K
      L = L + 1
      M = T(I)
      U(L) = ARRAY(5,M)
      V(L) = ARRAY(6,M)
  350 W(L) = M
      CALL ORDER(U,V,W,L)
      J = J - 1
      DO 360 I=1,L
      M = W(I)
  360 ARRAY(1,M) = I + J
      M = K
  370 CONTINUE
      RETURN
      END



      SUBROUTINE ORDER(R,S,T,N)

C     THIS ROUTINE SORTS THE VALUES IN THE THREE ARGUMENT ARRAYS.  THE
C     PRIMARY SORT IS BASED ON THE FIRST ARRAY VALUE BUT WHENEVER TWO OR
C     MORE OF THESE ARE IDENTICAL THE ORDER IS BASED ON THE VALUE IN THE
C     SECOND ARRAY.   THE THIRD ARRAY SIMPLY TAGS ALONG AND IS USED TO
C     HOLD THE ORIGINAL POSITION OF THE SORTED NUMBER PAIRS.


      IMPLICIT INTEGER(A-Z)
      DIMENSION R(N), S(N), T(N)



      M = 2
      DO 1 J=1,N
      M = M + M
      IF(M.GT.N)  GO TO 2
    1 CONTINUE
    2 M = M - 1
    3 M = (M - 1) / 2
      MM = N - M
      DO 8 I=1,MM
```

```
      OLD = I + N
      LR = R(OLD)
      LS = S(OLD)
      LT = T(OLD)
      DO 6 J=I,I+N
      NEW = OLD - N
      IF(LR-R(NEW)) 5,4,7
    4 IF(LS-S(NEW)) 5,7,7
    5 R(OLD) = R(NEW)
      S(OLD) = S(NEW)
      T(OLD) = T(NEW)
      OLD = NEW
    6 CONTINUE
    7 R(OLD) = LR
      S(OLD) = LS
      T(OLD) = LT
    8 CONTINUE
      IF(N.GT.1)  GO TO 3
      RETURN
      END



      FUNCTION LENGTH(ARGN)

C     THIS FUNCTION RETURNS THE NUMBER OF DIGITS REQUIRED BY THE VARIABLE
C     FORMAT STATEMENT TO OUTPUT THE ARGUMENT IN QUESTION.

      IMPLICIT INTEGER(A-Z)

      NUMBER = ARGN
      DO 100 I=1,11
      IF(NUMBER.LE.0)  GO TO 200
  100 NUMBER = NUMBER / 10
      STOP LENGTH
  200 LENGTH = I - 1
      IF(LENGTH.LE.0)  LENGTH = 1
      RETURN
      END



      SUBROUTINE SPLIT(I,J,K)


      K = I / 10000
      J = I - (K * 10000)
      K = J + K - 1
      RETURN
      END
```

```
      SUBROUTINE CATOUT(IR,NCAT,PAGE,NEXT)

C     THIS SUBROUTINE OUTPUTS REFERENCES FOR THE YEAR, AUTHOR, JOURNAL,
C     KEYWORD AND CHEMICAL ABSTRACT NUMBER CATALOGUES.

      IMPLICIT INTEGER(A-Z)
      PARAMETER  MR=800, MA=1000, MJ=200, MK=200, MB=100
      PARAMETER  MRA = 1800, MRK = 3000
      PARAMETER  OUT = 5, REPT = 12, NLINES = 57
      COMMON /ONE/   REF(9,MR), REFA(2,MRA), REFK(2,MRK)
      COMMON /TWO/   BOOK(21,MB), JOURNAL(12,MJ)
      COMMON /THREE/ AUTHOR(7,MA), KEY(12,MK)
      COMMON /FIVE/  LINE30(30), NUMBER(20), LINES, NLINES
      COMMON /SIX/   LABS, LKEY, LTIT
      DIMENSION  LINE20(20), LINE10(10), VORMAT(14)
      EQUIVALENCE  (LINE30(1), LINE20(1)), (LINE10(1), LINE30(21))
      LOGICAL  PAGE, LABS, LTIT, LKEY
      DATA VORMAT /'(1H ',',8X,',' ',',A4,',',2X,',','I4,',','1H,',',2X,I',
     1   ' ',',1H,',',2X,',','I',' ',')'/


 3240 FORMAT(' ',I4,4X,30A4)
 2300 FORMAT(20A4)
 3310 FORMAT(' ',A4,4X,20A4)
 3600 FORMAT(9X,'CHEMICAL ABSTRACT',I4,':',I6,10X,'FILING CODE  ',A4)
 3610 FORMAT(' ')
 3720 FORMAT('1',T110,I6)
 3801 FORMAT('1',T110,I3,':',I6)
 3830 FORMAT('1',T90,10A4)


      IF(PAGE)  GO TO 710
  110 IF(REF(3,IR).LT.0)  RETURN
      IF(.NOT.LTIT)  GO TO 200
      CALL SPLIT(REF(5,IR),IRT,NRT)
      FIND(REPT,IRT)

C     OUTPUT AUTHORS' NAMES

  200 CALL SPLIT(REF(6,IR),IRA,NRA)
      N = 1
      DO 220 J=IRA,NRA
      IA = REFA(1,J)
      L = AUTHOR(2,IA) + 2
      DO 210 M=3,L
```

```
      LINE30(N) = AUTHOR(M,IA)
  210 N = N + 1
      IF(N.EQ.30)  GO TO 240
      LINE30(N) = '    '
  220 N = N + 1
      IF(N.EQ.30)  GO TO 240
      DO 230 M=N,30
  230 LINE30(M) = '    '
  240 WRITE(OUT,3240)  REF(9,IR), LINE30
      NLINES = NLINES + 1
      IJ = REF(2,IR)
      IB = REF(4,IR) / 10000
      J = REF(4,IR) - (IB * 10000)
      IF(IJ.EQ.1.AND.J.EQ.0)  GO TO 420
      IF(.NOT.LTIT)  GO TO 400

C     OUTPUT THE TITLE

      IFR = '    '
      IF(.NOT.LABS)  IFR = REF(8,IR)
  300 READ(REFT'IRT,2300)  LINE20
      IF(IRT.GE.URT)  GO TO 310
      IRT = IRT + 1
      FIND(REFT'IRT)
      WRITE(OUT,3310)  IFR, LINE20
      NLINES = NLINES + 1
      IFR = '    '
      GO TO 300
  310 WRITE(OUT,3310)  IFR, LINE20
      IFR = '    '
      NLINES = NLINES + 1

C     OUTPUT JOURNAL REFERENCE

  400 IF(IJ.EQ.1)  GO TO 420
      K = JURNAL(2,IJ)
      VORMAT(3) = NUMBER(K)
      IF(REF(3,IR).GE.3000)  GO TO 450
      IF(IB.EQ.0)  GO TO 430
      M = LENGTH(IB)
      VORMAT(9) = NUMBER(M)
      VORMAT(10) = '1H,'
      N = LENGTH(J)
      VORMAT(13) = NUMBER(N)
      K = K + 2
  410 WRITE(OUT,VORMAT)  (JURNAL(L,IJ), L=3,K), REF(3,IR), IB, J
      GO TO 460
  420 K = BOOK(2,IB)
      VORMAT(3) = NUMBER(K)
      IF(REF(3,IR).GE.3000)  GO TO 450
  430 K = K + 2
      M = LENGTH(J)
      VORMAT(9) = NUMBER(M)
      VORMAT(10) = '    '
```

```
      VORMAT(13) = NUMBER(10)
      IF(IB.EQ.0)  GO TO 440
      IF(J.EQ.0)  GO TO 435
      WRITE(OUT,VORMAT)  (BOOK(L,IB), L=3,K), REF(3,IR), J
      GO TO 460
  435 VORMAT(7) = ' ,  '
      WRITE(OUT,VORMAT) (BOOK(L,IB), L=3,K), REF(3,IR)
      VORMAT(7) = '1H,,'
      GO TO 460
  440 WRITE(OUT,VORMAT) (JURNAL(L,IJ), L=3,K), REF(3,IR), J
      GO TO 460
  450 K = K + 2
      IF(IJ.EQ.1)  WRITE(OUT,VORMAT)  (BOOK(L,IB), L=3,K)
      IF(IJ.NE.1)  WRITE(OUT,VORMAT)  (JURNAL(L,IJ), L=3,K)
  460 NLINES = NLINES + 1
      IF(.NOT.LKEY)  GO TO 580

C     OUTPUT KEYWORD STRINGS

      N = 1
      CALL SPLIT(REF(7,IR),J,K)
      DO 510 N2=1,20
  510 LINE20(N2) = '    '
      DO 570 L=J,K
      IK = REFK(1,L)
      N1 = KEY(2,IK)
      IF(N+N1.LE.20)  GO TO 550
      N = 100
  530 WRITE(OUT,3310)  IFR, LINE20
      NLINES = NLINES + 1
      IF(L.EQ.K.AND.N.NE.100)  GO TO 580
      DO 540 N2=1,20
  540 LINE20(N2) = '    '
      N = 1
  550 N1 = N1 + 2
      DO 560 M=3,N1
      LINE20(N) = KEY(M,IK)
  560 N = N + 1
      IF(L.EQ.K)  GO TO 530
      IF(N.GE.20) GO TO 570
      LINE20(N) = '    '
      N = N + 1
  570 CONTINUE
  580 IF(.NOT.LABS)  GO TO 610

C     OUTPUT CHEMICAL ABSTRACT NUMBER

      J = REF(1,IR) / 1000000
      K = REF(1,IR) - (J * 1000000)
      WRITE(OUT,3600)  J, K, REF(8,IR)
      NLINES = NLINES + 1
  610 NLINES = NLINES + 1
      J = NLINES - NLINES
      IF(J.LE.LINES)  GO TO 710
```

```
      WRITE(OUT,3610)
      RETURN

C     OUTPUT PAGE HEADING

  700 PAGE = .FALSE.
      GO TO 110
  710 NLINES = 0
      N = 2
      LINE10(1) = '     '
      LINE10(2) = '     '
      GO TO(720,730,750,780,800,830), NCAT
  720 WRITE(OUT,3720)  NEXT
      GO TO 840
  730 N1 = AUTHOR(2,NEXT) + 2
      DO 740 J=3,N1
      N = N + 1
  740 LINE10(N) = AUTHOR(J,NEXT)
      GO TO 810
  750 IF(NEXT.EQ.1)  GO TO 770
      N1 = JURNAL(2,NEXT) + 2
      IF(N1.GT.10)  N = 12 - N1
      DO 760 J=3,N1
      N = N + 1
  760 LINE10(N) = JURNAL(J,NEXT)
      GO TO 810
  770 N = 4
      LINE10(3) = '  BO'
      LINE10(4) = 'OKS '
      GO TO 810
  780 N1 = KEY(2,NEXT) + 2
      IF(N1.GT.10)  N = 12 - N1
      DO 790 J=3,N1
      N = N + 1
  790 LINE10(N) = KEY(J,NEXT)
      GO TO 810
  800 J = NEXT / 1000000
      K = NEXT - (J * 1000000)
      WRITE(OUT,3801)  J, K
      GO TO 840
  810 N = N + 1
      IF(N.GT.10)  GO TO 830
      DO 820 J=N,10
  820 LINE10(J) = '     '
  830 WRITE(OUT,3830)  LINE10
  840 IF(PAGE) GO TO 700
      RETURN
      END
```

appendix 5-5

LISTING AND INSTRUCTIONS FOR PROGRAM MIX.

THIS PROGRAM SETS UP THE INPUT FOR PROGRAM ECCLES. IT READS IN DATA FROM THREE FILES. THE FIRST CONTAINS THE TITLE CARDS, OTHER CONTROL CARDS AND THE LIST OF COMPONENTS THAT ARE TO BE INCORPORATED IN THE MODEL, WITH THEIR CONCENTRATIONS. THIS IS THE ONLY FILE WHICH IS SPECIFICALLY PREPARED FOR INDIVIDUAL SIMULATIONS. THE OTHER TWO CONTAIN GENERAL IMFORMATION PERTAINING TO COMPLEX SPECIES FORMATION CONSTANTS. THE VALUES WHICH ARE REQUIRED FROM BOTH THESE FILES ARE SELECTED BY THE PROGRAM. INAPPLICABLE DATA ARE IGNORED. THE FIRST FORMATION CONSTANT FILE CONTAINS ALL THE CONSTANTS OTHER THAN THOSE CONCERNED WITH TERNARY COMPLEX FORMATION. THUS, THIS FILE HOLDS THE FORMATION CONSTANTS OF THE BINARY COMPLEXES, THE PROTONATED SPECIES, HYDROXY SPECIES ETC. THE OTHER FILE CONTAINS DATA ON TERNARY COMPLEX FORMATION.

THE BINARY FORMATION CONSTANT FILE IS FORMATTED AS DESCRIBED FOR PROGRAM ECCLES. THE TERNARY FORMATION CONSTANT DATA CAN BE FORMATTED IN A NUMBER OF WAYS, AS FOLLOWS. EACH ENTRY IS DELINEATED BY A BLANK CARD.

1) DIRECT VALUES CAN BE INSERTED, AS DESCRIBED FOR PROGRAM ECCLES.

2) IF THE TERNARY FORMATION CONSTANTS ARE PRECEDED BY A SET OF APPLICABLE BINARY CONSTANTS FOR THE ML2 OR M(LH)2 COMPLEXES, PROGRAM MIX SCALES THE VALUES BY COMPARING THE BINARY CONSTANTS SUPPLIED WITH THOSE FOUND IN THE BINARY FORMATION CONSTANT FILE.

3) STABILISATION FACTORS FOR A SET OF SIMILAR LIGANDS WHICH FORM A MIXED COMPLEX WITH ONE METAL AND WITH A COMMON PRIMARY LIGAND CAN BE INSERTED. THE LIST OF SYMBOLS FOR THE SECONDARY LIGANDS IN QUESTION AND THE INDIVIDUAL STABILIZATION FACTORS FOLLOW THE BINARY ML2 FORMATION CONSTANT FOR THE PRIMARY LIGAND.

PROGRAM MIX MUST ALSO BE PROVIDED WITH A LIST OF THE METAL ION'S SYMBOLS AND THE CORRESPONDING GENERAL STABILISATAION FACTORS. THESE ARE INPUT AS CARD DATA, RATHER THAN FROM A FILE. THE COMPONENT DATA FILE IS ASSIGNED LOGICAL UNIT NUMBER 24, THE BINARY FORMATION CONSTANT FILE NUMBER 25 AND THE MIXED LIGAND COMPLEX FILE NUMBER 26.

```
      PROGRAM  MIX.
      -------------


C     DEVELOPED AT THE UNIVERSITY OF CAPE TOWN DURING 1975.
C
C     THIS PROGRAM PRODUCES, ON FILE, THE EQUILIBRIUM DATA REQUIRED AS INPUT
C     FOR PROGRAM ECCLES.
C
C     FOR A GIVEN SET OF MODEL COMPONENTS, IT SELECTS THE APPROPRIATE COMPLEX
C     SPECIES FROM A STANDARD FORMATION CONSTANT DATA FILE AND OUTPUTS THESE
C     AS WELL AS PROJECTED VALUES FOR ALL THE 1:1:1 MIXED LIGAND CONSTITUENTS
C     OF THE MIXTURE.  A USER SPECIFIED DEFAULT VALUE IS USED AS A
C     STABILISATION FACTOR UNLESS A MORE SPECIFIC VALUE CAN BE TAKEN DIRECTLY
C     OR CALCULATED FROM DATA SUPPLIED IN A FILE ON MIXED LIGAND COMPLEXES.
C     (THIS FILE MAY ALSO CONTAIN SPECIES TO BE OUTPUT WITHOUT ALTERATION).
C     SPECIES WHICH HAVE COMPONENTS THAT DO NOT APPEAR IN THE COMPONENT LIST
C     ARE IGNORED.   THIS FACILITATES MANIPULATION OF THE INPUT TO ECCLES.



C     SECTION ONE       STORAGE ALLOCATION AND FORMAT STATEMENTS.



      PARAMETER  NS=900, NX=50, NXX=10, NKEY=5
      PARAMETER  NND = NKEY * 2, NXP1 = NX + 1, NSP1 = NS + 1
      PARAMETER  NXXX = NX + NXX, NMIX = (NX**2-NX)/2 + 25
      INTEGER    OUT, OUTF, HCOMP, DUP, OMIT
      DIMENSION  CONST(NS), NCOMP(NXXX), NDUM(NND), KEY(NKEY,NS,2)
      DIMENSION  NUM1(NS), NUM2(NS)
      DIMENSION  CONSTH(NXXX), NCOMP(NXXX), CONSTH(NX), HCOMP(NX)
      DIMENSION  CMIX(NMIX), STAB(NMIX), CBUF(NXXX)
      DIMENSION  DUP(NX,2), OMIT(NX,2)



10301 FORMAT(2A4,12A6)
10401 FORMAT(G9.4,1X,8(A4,1X,I2,2X))
10501 FORMAT(A4,1X,G10.4)
20401 FORMAT(G9.4,1X,8(A4,'(',I2,')',1X))
20999 FORMAT('ABORT THIS RUN.   ERROR IN THE MIXED LIGAND CONSTANT CALCU
     1LATIONS.')
30301 FORMAT(' ',2A4,12A6)
30302 FORMAT(//'0','END OF COMPONENT DATA.   CARD IMAGES READ =',I3,///)
30401 FORMAT(' ','OMIT....  ',1PG10.4,1X,8(A4,'(',I2,')',1X))
30402 FORMAT(//'0','THE SPECIE ON CARD NUMBER ',I4,' IS IN ERROR',////)
30403 FORMAT(//'0','END OF BASIC STABILITY CONSTANT DATA.   CARD IMAGES
     1READ =',I4,5X,'NUMBER OF SPECIES = ',I4,////)
30501 FORMAT('0',A4,' IS NOT IN THE COMPONENT LIST.',/' PLEASE RETYPE.
```

```
     *    TYPE END TO EXIT.*)
30502 FORMAT('0',*****   CHECK SPECIE NUMBER',I5,//)
30601 FORMAT('0',*EXECUTION TERMINATED.  MIXED SPECIE ARRAYS TOO SMALL*)
30602 FORMAT('0',*OMIT THE PREDICTION FOR*,5X,1PG10.4,5X,A4,*( 1) *,
     1 A4,*( 2) H +I( 1)*,4X,I6)
30603 FORMAT('0',*COMBINE PREDICTIONS FOR IDENTICAL MIXED SPECIES.*,
     1 /* *,1PG10.4,5X,3(A4,*( 1) *),*H +1( 1)*,5X,2(1PG10.4),3I6)
30604 FORMAT(* *,A4,5X,1PG10.4)
30605 FORMAT(//'0',*THE FOLLOWING COMPONENTS ARE USED:*,///)
30606 FORMAT(//'0',*TOTAL NUMBER =*,I4,/* *,*NUMBER OF BARE LIGANDS =*,
     1  I4,/* *,*NUMBER WITH ONE PROTON =*,I4,/////)
30731 FORMAT(* OUTPUT TO FILE *,10X,1PG10.4,1X,8(A4,*(*,I2,*)*,1X))
30741 FORMAT(* COMPONENT NOT IN LIST,*)
30742 FORMAT('0',*OMIT THE SPECIE ON CARD NUMBER*,I4,//)
30745 FORMAT(///'0',*ERROR ON CARD NUMBER*,I4,//)
30746 FORMAT(* MODE CODE =*,I4,//)
30747 FORMAT(///'0',*ERROR TERMINATION.  FORMAT ERROR ON CARD*,I5,///)
30762 FORMAT('0',*PREDICTIONS BASED ON SPECIES NOT APPEARING IN THE BASI
     1C DATA ARE ILLEGAL.*)
30763 FORMAT('0',*PREDICTION FOR *,8(A4,*(*,I2,*)*,1X))
30764 FORMAT(* *,T40,4(1PG10.4))
30761 FORMAT(//'0',*A BINARY CONSTANT FOR MIXED LIGAND STABILITY CALCULA
     1TION IS MISSING.*)
30771 FORMAT(* *,*ADJUST THE STAR. FACTOR *,10X,3(A4,*( 1)*,1X),*H +1(*,
     1  I2,*)*,4X,2(1PG10.4))
30781 FORMAT(*REPLACED SPECIE*,10X,1PG10.4,1X,8(A4,*(*,I2,*)*,1X))
30801 FORMAT('0',I5,* CONSTANTS FOR THE MIXED LIGAND COMPLEXES OF *,A4,
     1* HAVE BEEN PRODUCED.*)
30802 FORMAT('0',*NUMBER OF APPLICABLE CARDS IN THE MIXED-DATA FILE =*,
     1  I5,////)
30901 FORMAT(//'0',I5,* CONSTITUENTS FOR ECCLES!*)
30902 FORMAT(//'0',*END MIX.*,/*1*)
30999 FORMAT(///'0',*ABORT ECCLES RUN.*)


C     SECTION TWO       INITIALISATION.


  200 IN = 8
      IN1 = 24
      IN2 = 25
      IN3 = 26
      OUT = 5
      OUTF = 27
      N1 = NXP1
      N2 = NXX + 1
      N3 = NSP1
      NNN = 0


C     SECTION THREE       COMPONENT DATA INPUT AND OUTPUT.
```

```
300 READ(IN1,10301) IXA, IXB, NDUM, I, J
    NNN = NNN + 1
    WRITE(OUTF,10301) IXA, IXB, NDUM, I, J
    WRITE(OUT,30301) IXA, IXB, NDUM, I, J
    IF(IXA.NE.'TOTA') GO TO 300


    DO 301 NI=1,N1
    READ(IN1,10301) IXA, IXB, I, J, K
    NNN = NNN + 1
    WRITE(OUTF,10301) IXA, IXB, I, J, K
    WRITE(OUT,30301) IXA, IXB, I, J, K
    IF(IXA.EQ.'FREE') GO TO 302
301 NCOMP(NI) = IXA
    STOP
302 NI = NI - 1


    N = NI
    DO 303 NII=1,N2
    READ(IN1,10301) IXA, IXB, I, J, K
    NNN = NNN + 1
    WRITE(OUTF,10301) IXA, IXB, I, J, K
    WRITE(OUT,30301) IXA, IXB, I, J, K
    IF(IXA.EQ.'SPEC') GO TO 304
    N = N + 1
303 NCOMP(N) = IXA
    STOP
304 NII = NII - 1
    NIII = NI + NII
    WRITE(OUT,30302) NNN
    NNN = 0


C     SECTION FOUR      BASIC STABILITY CONSTANT DATA INPUT AND OUTPUT.


400 DO 412 NJ=1,N3
401 READ(IN2,10401,END=414) CONST(NJ), NDUM
    NNN = NNN + 1
    NUM1(NJ) = 0
    NUM2(NJ) = 0
402 NPM = 1
    DO 407 N=1,NND,2
    IF(NDUM(N).EQ.'    ') GO TO 408
403 DO 404 M=NPM,NIII
    IF(NDUM(N).EQ.NCOMP(M)) GO TO 405
404 CONTINUE
    IF(NPM.EQ.1)  NUM1(NJ) = 0
    IF(NPM.EQ.1) GO TO 409
```

```
      NPM = 1
      GO TO 403
  405 NPM = M
      IF(M.GT.NI) GO TO 406
      L = NUM1(NJ) + 1
      NUM1(NJ) = L
      KEY(L,NJ,1) = M
      GO TO 407
  406 L = NKEY - NUM2(NJ)
      NUM2(NJ) = NUM2(NJ) + 1
      KEY(L,NJ,1) = M - NI
  407 KEY(L,NJ,2) = NDUM(N+1)
  408 IF(NUM1(NJ)+NUM2(NJ).LE.1) GO TO 413
  409 DO 410 N=2,NND,2
      IF(NDUM(N).EQ.0) GO TO 411
  410 CONTINUE
      N = N + 2
  411 NN = N - 2
      IF(NUM1(NJ).GT.0) GO TO 412
      WRITE(OUT,30401) CONST(NJ), (NDUM(N), N=1,NN)
      GO TO 401
  412 WRITE(OUT,20401) CONST(NJ), (NDUM(N), N=1,NN)
      STOP
  413 WRITE(OUT,30402) NNN
      GO TO 9999
  414 NJ = NJ - 1
      WRITE(OUT,30403) NNN, NJ
      NTOT = NJ
      N = NI
      DO 415 II=1,NII
      N = N +1
      IF(NCOMP(N).EQ.'H +1') GO TO 500
  415 CONTINUE
      STOP


C     SECTION FIVE      SELECTION OF THE MIXED COMPLEX COMPONENTS.


  500 READ(IN,10501,END=900) METAL, STARF
      IF(METAL.EQ.'END ',OR.METAL.EQ.'    ') GO TO 900
      DO 501 III=1,NIII
      IF(NCOMP(III).EQ.METAL) GO TO 502
  501 CONTINUE
      WRITE(OUT,30501) METAL
      GO TO 500
  502 METAL = III
      NM = 0
      NH = 0
      NT = 0
      NNN = 0
      METALF = 0
      IF(METAL.GT.NI) METALF = METAL - NI
```

```
      DO 516 J=1,NJ
      IF(METALF.GT.0) GO TO 504
      NL = NUM1(J)
      DO 503 L=1,NL
      IF(KEY(L,J,1).EQ.METAL) GO TO 506
  503 CONTINUE
      GO TO 516
  504 IF(NUM2(J).EQ.0) GO TO 516
      NL = NKEY - NUM2(J) + 1
      DO 505 L=NL,NKEY
      IF(KEY(L,J,1).EQ.METALF) GO TO 506
  505 CONTINUE
      GO TO 516
  506 IF(KEY(L,J,2).NE.1) GO TO 516
      KEY(L,J,2) = 2
      NN = NUM1(J)
      NL = NKEY - NUM2(J) + 1
      DO 507 M=1,NKEY
      IF(M.GT.NN.AND.M.LT.NL) GO TO 507
      IF(KEY(M,J,2).NE.2) GO TO 515
  507 CONTINUE
      NN = NN + NUM2(J)
      IF(NN.GT.2) GO TO 508
      NN = NN + 1
      CONSTH(NN) = CONST(J) / 2.0
      HCOMP(NN) = KEY(1,J,1)
      IF(L.EQ.1) HCOMP(NN) = KEY(NKEY,J,1) + NI
      GO TO 515
  508 IF(NN.GT.3) GO TO 515
      NL = NKEY - 1
      IF(KEY(NL,J,1).NE.II.AND.KEY(NKEY,J,1).NE.II) GO TO 514
      NH = NH + 1
      IF(NUM2(J).EQ.1) GO TO 513
      IF(L.GT.NUM1(J)) GO TO 512
      HCOMP(NH) = KEY(NL,J,1) + NI
      IF(KEY(NL,J,1).EQ.II) HCOMP(NH) = KEY(NKEY,J,1) + NI
  511 CONSTH(NH) = CONST(J) / 2.0
      GO TO 515
  512 HCOMP(NH) = KEY(1,J,1)
      GO TO 511
  513 HCOMP(NH) = KEY(1,J,1)
      IF(L.EQ.1) HCOMP(NH) = KEY(2,J,1)
      GO TO 511
  514 WRITE(OUT,30502) J
  515 KEY(L,J,2) = 1
  516 CONTINUE


C     SECTION SIX       CALCULATION OF THE UNSTABILISED MIXED LIGAND CONSTANTS.


  600 NNH = NM + NH
      NNX = ((NNH ** 2) - NNH) / 2
      IF(NNX.GT.NMIX) GO TO 605
```

```
      DO 601 M=1,MMX
  601 STAB(M) = STABF
      DO 602 I=1,NH
      M = MM + I
      MCOMP(M) = MCOMP(I)
  602 CONSTM(I) = CONSTH(I)


      J = 0
      DO 604 M=2,MMH
      N = M - 1
      DO 604 I=1,N
      J = J + 1
  604 CMIX(J) = CONSTM(M) + CONSTH(I)
      GO TO 610
  605 WRITE(OUT,30601)
      GO TO 9999



  610 WRITE(OUT,30605)
      DO 611 M=1,MMH
      MPM = MCOMP(M)
      MCOMP(M) = MCOMP(MPM)
  611 WRITE(OUT,30604)  MCOMP(M), CONSTM(M)
      WRITE(OUT,30606)  MMH, MM, NH
      DO 612 M=1,MM
      MPM = MCOMP(M)
  612 MCOMP(M) = MCOMP(MPM)



  620 ND = 0
      DO 623 I=1,NH
      DO 621 M=1,MM
      IF(MCOMP(I).EQ.MCOMP(M))  GO TO 622
  621 CONTINUE
      GO TO 623
  622 ND = ND + 1
      DUP(ND,1) = I + MM
      DUP(ND,2) = M
  623 CONTINUE
      OMIT(1,1) = 0
      NZ = 0
      IF(ND.EQ.0)  GO TO 700
      DO 626 I=1,ND
      DO 626 L=I,ND
      M = DUP(I,1) - 1
      IF(I.NE.L)  GO TO 624
      M = (((M ** 2) - M) / 2) + DUP(I,2)
      J = 0
      N3 = DUP(L,2)
      XA = CMIX(M) + STAB(M)
      WRITE(OUT,30602)  XA, MCOMP(METAL), MCOMP(N3), M
```

```
      GO TO 625
  624 J = (((M ** 2) - M) / 2) + DUP(L,2)
      M = DUP(L,1) - 1
      M = (((M ** 2) - M) / 2) + DUP(I,2)
      XA = ((CMIX(J) + CMIX(M)) / 2.0) + STABF
      N1 = J - I
      N3 = DUP(I,1)
      N2 = DUP(L,2)
      WRITE(OUT,30603)  XA, NCOMP(METAL), NCOMP(N3), NCOMP(N2), CMIX(J),
     1  CMIX(M), J, M, N1
      CMIX(J) = XA - STABF
  625 NZ = NZ + 1
      NMX = NMX - 1
      OMIT(NZ,2) = J
  626 OMIT(NZ,1) = M



C     SECTION SEVEN       INPUT, PROCESS AND OUTPUT MIXED LIGAND DATA.


  700 KSW = 0
  701 READ(IN3,10401,ERR=746,END=800)  XA, NDUM
      NNM1 = NMN + 1
      IF(NDUM(1).EQ.'     ')  GO TO 770
      IF(NDUM(1).NE.NCOMP(METAL))  GO TO 700
      IF(KSW.EQ.100)  KSW = 0
      NT = NT + 1
      I = 1
      DO 705 N=4,NND,2
      IF(NDUM(N).EQ.0)  GO TO 706
      N1 = N - 1
  702 N2 = I
  703 DO 704 I=N2,NIII
      IF(NCOMP(I).EQ.NDUM(N1))  GO TO 705
  704 CONTINUE
      IF(N2.EQ.1)  GO TO 740
      N2 = 1
      GO TO 703
  705 CONTINUE
      N = N + 2
  706 NN = N - 2



  710 IF(KSW.NE.0)  GO TO 720
      IF(NDUM(2).NE.1)  GO TO 730
      IF(NDUM(4).EQ.2)  GO TO 711
      KSW = 87
      IF(NDUM(4).NE.1.OR.NDUM(6).NE.1)  GO TO 730
      IF(NDUM(8).EQ.0.AND.NDUM(7).EQ.'     ')  GO TO 780
      IF(NDUM(7).NE.'     '.AND.NDUM(7).NE.'H +1')  GO TO 730
      IF(NDUM(8).LE.2.AND.NDUM(7).EQ.'H +1')  GO TO 780
```

```
      GO TO 730
  711 IF(NDUM(6).EQ.0.AND.NDUM(8).EQ.0)  GO TO 750
      IF(NDUM(6).EQ.2.AND.NDUM(5).EQ.'H +1')  GO TO 750
      GO TO 730



  720 IF(KSW.EQ.31)  GO TO 723
      IF(NDUM(2).NE.1)  GO TO 730
      IF(KSW.NE.52)  GO TO 722
      IF(NDUM(4).NE.2)  GO TO 721
      IF(NDUM(8).NE.0)  GO TO 745
      IF(NDUM(6).EQ.0)  GO TO 752
      IF(NDUM(6).EQ.2.AND.NDUM(5).EQ.'H +1')  GO TO 752
      GO TO 745
  721 IF(NDUM(4).NE.1.AND.NDUM(6).NE.1)  GO TO 745
      IF(NDUM(8).EQ.0)  GO TO 760
      IF(NDUM(8).LE.2.AND.NDUM(7).EQ.'H +1')  GO TO 760
      GO TO 745
  722 IF(KSW.NE.87)  GO TO 725
  723 IF(NDUM(4).NE.1.OR.NDUM(6).NE.1)  GO TO 730
      KSW = 87
  724 IF(NDUM(8).EQ.0)  GO TO 730
      IF(NDUM(8).LE.2.AND.NDUM(7).EQ.'H +1')  GO TO 780
      GO TO 730
  725 IF(KSW.NE.61)  GO TO 745
      IF(NDUM(4).NE.1.AND.NDUM(6).NE.1)  GO TO 745
      GO TO 724



  730 KSW = 31
  731 IF(KSW.GE.50.AND.KSW.LT.80)  GO TO 741
      WRITE(OUT,30731)  XA, (NDUM(N), N=1,NN)
      WRITE(OUTF,20401) XA, (NDUM(N), N=1,NN)
      NTOT = NTOT + 1
      GO TO 701



  740 WRITE(OUT,30741)
  741 WRITE(OUT,30742)  NNN
      IF(KSW.NE.0)  GO TO 701
      IF(NDUM(4).NE.2)  GO TO 701
      IF(NDUM(6).EQ.0.OR.(NDUM(5).EQ.'H +1'.AND.NDUM(6).EQ.2)) KSW = 100
      GO TO 701



  745 WRITE(OUT,30745)  NNN
      WRITE(OUT,30746)  KSW
      GO TO 9999
  746 NNN = NNN + 1
      WRITE(OUT,30747) NNN
```

```
      GO TO 9999


  750 DO 751 I=1,NNN
  751 CBUF(I) = -1001.0
      KSW = 52


  752 IF(NDUM(5).EQ.'H +I') GO TO 753
      N1 = 1
      N2 = NN
      GO TO 754
  753 N1 = NN + 1
      N2 = NNN
  754 DO 755 I=N1,N2
      IF(NDUM(3).EQ.NCOMP(I)) GO TO 756
  755 CONTINUE
      IF(KSW.EQ.71) GO TO 775
      GO TO 741
  756 IF(CBUF(I).GT.-100.0) GO TO 745
      IF(KSW.EQ.71) GO TO 771
      NB = I
      CBUF(I) = XA / 2.0
      GO TO 701



  760 IF(KSW.NE.52) GO TO 745
      KSW = 61
      GO TO 780


  761 IF(CBUF(LX).GT.-100.0.AND.CBUF(LY).GT.-100.0) GO TO 766
      IF(ND.NE.0) GO TO 763
  762 WRITE(OUT,30761)
      GO TO 745
  763 N1 = 0
      N2 = 0
      DO 764 I=1,ND
      IF(DUP(I,1).EQ.LX) N1 = DUP(I,2)
      IF(DUP(I,1).EQ.LY) N2 = DUP(I,2)
  764 CONTINUE
      IF(N1.EQ.0.OR.N2.EQ.0) GO TO 765
      LX = N1
      LY = N2
      IF(CBUF(LX).GT.-100.0.AND.CBUF(LY).GT.-100.0) GO TO 766
  765 WRITE(OUT,30762)
      GO TO 762
  766 XB = XA - (CBUF(LX) + CBUF(LY))
      WRITE(OUT,30763) (NDUM(N), N=1,NN)
      WRITE(OUT,30764) CBUF(LX), CBUF(LY), XB, XA
      STAB(J) = XB * ((CONSTM(LX) + CONSTM(LY)) / (CBUF(LX) + CBUF(LY)))
      XB = CMIX(J) + STAB(J)
```

```
      WRITE(OUT,30764)  CONSTM(LX), CONSTM(LY), STAB(J), XB
      GO TO 701


  770 IF(NDUM(3).EQ.'     ')  GO TO 700
      IF(KSW.EQ.100)  NT = NT + 1
      IF(KSW.EQ.0.OR.KSW.EQ.100)  GO TO 701
      IF(KSW.NE.52.AND.KSW.NE.71.AND.KSW.NE.72)  GO TO 745
      NT = NT + 1
      KSW = 71
      LX = NB
      XB = CONSTM(LX) / CBUF(LX)
      GO TO 752

  771 LY = I
      CBUF(I) = XA
      KSW = 72
      GO TO 785
  772 STAB(J) = XA * XB
      N1 = 0
      IF(LX.GT.NM)  N1 = N1 + 1
      IF(LY.GT.NM)  N1 = N1 + 1
      WRITE(OUT,30771)  NCOMP(METAL),NCOMP(LX),NCOMP(LY),N1,XA,STAB(J)
      GO TO 701


  775 DO 776 I=1,NIII
      IF(NDUM(3).EQ.NCOMP(I))  GO TO 741
  776 CONTINUE
      GO TO 740


  780 IF(NDUM(3).EQ.NDUM(5))  GO TO 745
      LX = 0
      LY = 0
      IF(NDUM(8).EQ.0)  GO TO 782
      N1 = NM + 1
      DO 781 I=N1,NMH
      IF(NDUM(3).EQ.NCOMP(I))  LX = I
      IF(NDUM(5).EQ.NCOMP(I))  LY = I
  781 CONTINUE
      IF(NDUM(8).EQ.2)  GO TO 735
      IF(LX.EQ.0.AND.LY.EQ.0)  GO TO 731
      N2 = 0
      N3 = 0
      IF(LX.EQ.0.OR.LY.EQ.0)  GO TO 782
      N1 = MIN(LX,LY)
      N2 = LX
      N3 = LY
      LX = 0
```

```
      LY = 0
  782 DO 783 I=1,NN
      IF(LX.EQ.0.AND.NDUM(3).EQ.NCOMP(I))  LX = I
      IF(LY.EQ.0.AND.NDUM(5).EQ.NCOMP(I))  LY = I
  783 CONTINUE
      IF(NDUM(3).EQ.0)  GO TO 785
      IF(LX.EQ.0.OR.LY.EQ.0)  GO TO 784
      IF(N1.EQ.N2)  LX = N2
      IF(N1.EQ.N3)  LY = N3
      GO TO 785
  784 IF(LX.EQ.0.AND.LY.EQ.0)  GO TO 731
      IF(LX.EQ.0)  LX = N2
      IF(LY.EQ.0)  LY = N3



  785 IF(LX.EQ.0.OR.LY.EQ.0)  GO TO 731
      L = MAX(LX,LY)
      M = L - 1
      I = LX
      IF(I.EQ.L)  I = LY
      L = M - 1
      J = ((M * L) / 2) + I
      IF(NZ.EQ.0)  GO TO 787
      DO 786 I=1,NZ
      IF(J.EQ.OMIT(I,1))  J = OMIT(I,2)
  786 CONTINUE
  787 IF(J.LE.0)  GO TO 745
      IF(KSW.EQ.72)  GO TO 772
      IF(KSW.EQ.61)  GO TO 761
      IF(KSW.NE.87)  GO TO 745
      CHIX(J) = XA - STAB(J)
      WRITE(OUT,30781)  XA, (NDUM(N), N=1,NN)
      GO TO 731


C     SECTION EIGHT      OUTPUT THE CALCULATED MIXED LIGAND FORMATION CONSTANTS.


  800 REWIND IN3
      DO 801 I=1,NMX
  801 CHIX(I) = CHIX(I) + STAB(I)
      NDUM(1) = NCOMP(METAL)
      NDUM(2) = 1
      NDUM(3) = NCOMP(1)
      NDUM(4) = 1
      NDUM(5) = NCOMP(2)
      NDUM(6) = 1
      NDUM(7) = 'H +1'
      J = 0
      DO 804 M=2,NMH
      N = M - 1
      DO 804 I=1,N
```

```
      J = J + 1
      NDUM(3) = NCOMP(I)
      NDUM(5) = NCOMP(N)
      NN = 6
      IF(N.LE.NN)  GO TO 803
      NN = 8
      NDUM(8) = 1
      IF(I.GT.NN) NDUM(8) = 2
      IF(OMIT(I,I).EQ.0)  GO TO 803
      DO 802 K=1,NZ
      IF(OMIT(K,I).EQ.J)  GO TO 804
  802 CONTINUE
  803 NTOT = NTOT + 1
      WRITE(OUTF,20401) CMIX(J), (NDUM(N), N=1,NN)
  804 CONTINUE
      WRITE(OUT,30801)  NMX, NCOMP(METAL)
      WRITE(OUT,30802)  NT
      GO TO 500


C     SECTION NINE       FINISH!


  900 NTOT = NTOT + NIII
      WRITE(OUT,30901) NTOT
      GO TO 901
 9999 WRITE(OUT,30999)
      ENDFILE OUTF
      REWIND OUTF
      WRITE(OUTF,20999)
  901 WRITE(OUT,30902)
      ENDFILE OUTF
      END
```

appendix 5·6

FORMATION CONSTANT DATA USED FOR THE BLOOD PLASMA MODEL.
(TABULATED AS INPUT DATA FOR PROGRAM ECCLES)

```
 9.40      AAA1(+1) H +1(+1)                          E
11.76      AAA1(+1) H +1(+2)                          E
 1.20      AAA1(+1) CA+2(+1)                          E
 1.58      AAA1(+2) CA+2(+1)                          E
 7.98      AAA1(+1) CU+2(+1)                          E
14.66      AAA1(+2) CU+2(+1)                          E
10.89      AAA1(+1) CU+2(+1) H +1(+1)                 E
 3.28      AAA1(+1) FE+2(+1)                          E
 6.20      AAA1(+2) FE+2(+1)                          E
 9.53      AAA1(+1) FE+3(+1)                          E
15.34      AAA1(+2) FE+3(+1)                          E
 4.17      AAA1(+1) PB+2(+1)                          E
 6.17      AAA1(+2) PB+2(+1)                          E
 8.80      AAA1(+2) PB+2(+1) OH-1(+1)                 E
 1.80      AAA1(+1) MG+2(+1)                          E
 2.30      AAA1(+2) MG+2(+1)                          E
 2.59      AAA1(+1) MN+2(+1)                          E
 4.60      AAA1(+2) MN+2(+1)                          E
 5.47      AAA1(+3) MN+2(+1)                          E
10.00      AAA1(+1) MN+2(+1) H +1(+1)                 E
12.80      AAA1(+2) MN+2(+1) H +1(+1)                 E
 4.56      AAA1(+1) ZN+2(+1)                          E
 8.50      AAA1(+2) ZN+2(+1)                          E
10.87      AAA1(+3) ZN+2(+1)                          E
 9.502     ALA1(+1) H +1(+1)                          D   158 98 83 62
11.879     ALA1(+1) H +1(+2)                          D   158 98 83 62
 1.20      ALA1(+1) CA+2(+1)                          E   668
 1.80      ALA1(+2) CA+2(+1)                          F
 8.01      ALA1(+1) CU+2(+1)                          D   158 83 279
14.64      ALA1(+2) CU+2(+1)                          D   158 279 83
10.57      ALA1(+1) CU+2(+1) H +1(+1)                 D   158
 3.50      ALA1(+1) FE+2(+1)                          E   53
 6.90      ALA1(+2) FE+2(+1)                          E   STAB
10.00      ALA1(+1) FE+3(+1)                          E   STAB   52
 4.20      ALA1(+1) PB+2(+1)                          E   STAB
 6.90      ALA1(+2) PB+2(+1)                          E   STAB
 9.10      ALA1(+2) PB+2(+1) OH-1(+1)                 E   STAB
 1.70      ALA1(+1) MG+2(+1)                          E   STAB
 2.20      ALA1(+2) MG+2(+1)                          F
 2.40      ALA1(+1) MN+2(+1)                          D   62
```

4.28	ALA1(+2) MN+2(+1)		D	62
5.70	ALA1(+3) MN+2(+1)		D	62
10.00	ALA1(+1) MN+2(+1)	H +1(+1)	F	
12.85	ALA1(+2) MN+2(+1)	H +1(+1)	D	62
4.57	ALA1(+1) ZN+2(+1)		D	158 62 98
8.56	ALA1(+2) ZN+2(+1)		D	158 62 98
10.65	ALA1(+3) ZN+2(+1)		D	158 98
3.96	ALA1(+1) ZN+2(+1)	H +1(-1)	D	158
16.40	ALA1(+2) FE+3(+1)		F	
9.24	ARA1(+1) H +1(+1)		D	369 83 53 518
11.69	ARA1(+1) H +1(+2)		E	STAB 518 83
1.10	ARA1(+1) CA+2(+1)		F	
1.40	ARA1(+2) CA+2(+1)		F	
7.65	ARA1(+1) CU+2(+1)		D	369 518 83
14.10	ARA1(+2) CU+2(+1)		E	STAB 369 518 83
3.40	ARA1(+1) FE+2(+1)		E	53
5.00	ARA1(+2) FE+2(+1)		F	
9.00	ARA1(+1) FE+3(+1)		E	52
4.00	ARA1(+1) PB+2(+1)		F	
6.00	ARA1(+2) PB+2(+1)		F	
8.00	ARA1(+1) PB+2(+1)	OH-1(+1)	F	
1.60	ARA1(+1) MG+2(+1)		F	
2.20	ARA1(+2) MG+2(+1)		F	
2.25	ARA1(+1) MN+2(+1)		F	
4.00	ARA1(+2) MN+2(+1)		F	
4.42	ARA1(+1) ZN+2(+1)		D	369 518
7.15	ARA1(+2) ZN+2(+1)		D	369 518
16.00	ARA1(+2) FE+3(+1)		F	
8.82	ARG1(+1) H +1(+1)		D	158 51 333
10.64	ARG1(+1) H +1(+2)		D	158 51
1.40	ARG1(+1) CA+2(+1)		E	STAB 668 333
1.80	ARG1(+2) CA+2(+1)		F	
7.38	ARG1(+1) CU+2(+1)		D	158 333
13.66	ARG1(+2) CU+2(+1)		D	158 333
3.17	ARG1(+2) CU+2(+2)	H +1(-2)	D	158
3.00	ARG1(+1) FE+2(+1)		E	STAB 53
4.40	ARG1(+2) FE+2(+1)		F	
8.00	ARG1(+1) FE+3(+1)		E	STAB 51
15.00	ARG1(+2) FE+3(+1)		F	
3.50	ARG1(+1) PB+2(+1)		E	STAB
5.00	ARG1(+2) PB+2(+1)		F	
9.00	ARG1(+1) PB+2(+1)	OH-1(+1)	F	
1.80	ARG1(+1) MG+2(+1)		E	STAB 333
2.30	ARG1(+2) MG+2(+1)		F	
2.35	ARG1(+1) MN+2(+1)		E	STAB 333
3.90	ARG1(+2) MN+2(+1)		E	STAB
4.03	ARG1(+1) ZN+2(+1)		D	158 333
7.56	ARG1(+2) ZN+2(+1)		D	158 333
4.25	ARG1(+1) ZN+2(+1)	H +1(-1)	D	158
8.68	ASN1(+1) H +1(+1)		D	369 68 51 52 504
10.91	ASN1(+1) H +1(+2)		D	369 68 51 52 504
1.10	ASN1(+1) CA+2(+1)		F	
1.40	ASN1(+2) CA+2(+1)		F	
7.69	ASN1(+1) CU+2(+1)		D	369 312 504

13.66	ASN1(+2) CU+2(+1)		D	369 312 504
3.50	ASN1(+2) CU+2(+1) H +1(-1)		E	504
3.40	ASN1(+1) FE+2(+1)		E	53 312
6.00	ASN1(+2) FE+2(+1)		E	STAB 312
8.00	ASN1(+3) FE+2(+1)		E	312
8.40	ASN1(+1) FE+3(+1)		E	52
4.00	ASN1(+1) PB+2(+1)		E	STAB 311
6.00	ASN1(+2) PB+2(+1)		E	STAB 311
7.00	ASN1(+3) PB+2(+1)		E	311
9.20	ASN1(+2) PB+2(+1) OH-1(+1)		E	STAB
1.80	ASN1(+1) MG+2(+1)		F	
2.30	ASN1(+2) MG+2(+1)		E	STAB
2.40	ASN1(+1) MN+2(+1)		E	312
4.00	ASN1(+2) MN+2(+1)		E	STAB 312
4.45	ASN1(+1) ZN+2(+1)		D	369 312
7.95	ASN1(+2) ZN+2(+1)		D	369 312
10.00	ASN1(+3) ZN+2(+1)		E	312
14.75	ASN1(+2) FE+3(+1)		F	
9.20	ASP2(+1) H +1(+1)		E	STAB 68 377 53
12.60	ASP2(+1) H +1(+2)		E	STAB 377 267
14.20	ASP2(+1) H +1(+3)		E	STAB 377
1.60	ASP2(+1) CA+2(+1)		F	STAB 668
2.10	ASP2(+2) CA+2(+1)		F	
2.50	ASP2(+1) CU+2(+1)		E	STAB 377 267
15.20	ASP2(+2) CU+2(+1)		E	STAB 377 267
12.20	ASP2(+1) CU+2(+1) H +1(+1)		E	267
4.20	ASP2(+1) FE+2(+1)		E	53
7.75	ASP2(+2) FE+2(+1)		E	STAB
11.00	ASP2(+1) FE+3(+1)		E	53
5.80	ASP2(+1) PB+2(+1)		E	STAB 311
8.20	ASP2(+2) PB+2(+1)		E	STAB 311
8.00	ASP2(+1) PB+2(+1) OH-1(+1)		F	
2.20	ASP2(+1) MG+2(+1)		E	STAB
2.90	ASP2(+2) MG+2(+1)		F	
3.20	ASP2(+1) MN+2(+1)		E	STAB
5.20	ASP2(+2) MN+2(+1)		F	
5.80	ASP2(+1) ZN+2(+1)		E	STAB 311
9.30	ASP2(+2) ZN+2(+1)		E	STAB
17.10	ASP2(+2) FE+3(+1)		F	
8.70	CIT1(+1) H +1(+1)		E	STAB 333 249
10.40	CIT1(+1) H +1(+2)		E	STAB 249
1.20	CIT1(+1) CA+2(+1)		E	333
1.50	CIT1(+2) CA+2(+1)		E	333
7.00	CIT1(+1) CU+2(+1)		E	333 249
13.00	CIT1(+2) CU+2(+1)		E	333 249
3.00	CIT1(+1) FE+2(+1)		F	
4.80	CIT1(+2) FE+2(+1)		F	
8.00	CIT1(+1) FE+3(+1)		F	
14.42	CIT1(+2) FE+3(+1)		F	
4.00	CIT1(+1) PB+2(+1)		E	249
6.00	CIT1(+2) PB+2(+1)		F	
8.00	CIT1(+1) PB+2(+1) OH-1(+1)		F	
1.60	CIT1(+1) MG+2(+1)		E	333
2.10	CIT1(+2) MG+2(+1)		F	

1.70	CIT1(+1) MN+2(+1)		E	333
2.60	CIT1(+2) MN+2(+1)		F	
4.00	CIT1(+1) ZN+2(+1)		E	333 249 164
7.00	CIT1(+2) ZN+2(+1)		E	249 164
10.23	CYS2(+1) H +1(+1)		D	158 68 337 678
18.30	CYS2(+1) H +1(+2)		D	158 68 337 678
20.20	CYS2(+1) H +1(+3)		D	158 68 678
2.30	CYS2(+1) CA+2(+1)		E	273
2.70	CYS2(+2) CA+2(+1)		F	
6.00	CYS2(+1) FE+2(+1)		E	STAB 273
11.00	CYS2(+2) FE+2(+1)		E	STAB 273
11.70	CYS2(+1) PB+2(+1)		E	STAB 273 311
16.00	CYS2(+2) PB+2(+1)		E	STAB 273 311
18.40	CYS2(+3) PB+2(+1)		E	273 311
2.60	CYS2(+1) MG+2(+1)		E	273
3.40	CYS2(+2) MG+2(+1)		F	
4.10	CYS2(+1) MN+2(+1)		E	STAB 273
7.10	CYS2(+2) MN+2(+1)		E	273
8.99	CYS2(+1) ZN+2(+1)		E	STAB 273 678
17.98	CYS2(+2) ZN+2(+1)		D	STAB 158 273 678
20.50	CYS2(+3) ZN+2(+1)		E	273
24.33	CYS2(+2) ZN+2(+1)	H +1(+1)	D	158
29.86	CYS2(+2) ZN+2(+1)	H +1(+2)	D	158
42.50	CYS2(+4) ZN+2(+3)		F	
48.60	CYS2(+4) ZN+2(+3)	H +1(+1)	D	158
8.69	CIS2(+1) H +1(+1)		D	158
16.64	CIS2(+1) H +1(+2)		D	158
19.34	CIS2(+1) H +1(+3)		D	158
10.00	CIS2(+1) CA+2(+1)	H +1(+1)	F	
19.50	CIS2(+2) CA+2(+1)	H +1(+2)	F	
6.80	CIS2(+1) CU+2(+1)		D	158
16.20	CIS2(+1) CU+2(+1)	H +1(+1)	D	158
28.07	CIS2(+2) CU+2(+2)		D	158
27.50	CIS2(+2) CU+2(+1)	H +1(+2)	D	158
17.70	CIS2(+1) FE+3(+1)	H +1(+1)	F	
25.80	CIS2(+2) FE+3(+1)	H +1(+1)	F	
31.28	CIS2(+2) FE+3(+1)	H +1(+2)	F	
16.20	CIS2(+1) PB+2(+1)	H +1(+1)	F	
22.00	CIS2(+2) PB+2(+1)	H +1(+2)	F	
10.70	CIS2(+1) MG+2(+1)	H +1(+1)	F	
20.00	CIS2(+2) MG+2(+1)	H +1(+2)	F	
11.00	CIS2(+1) MN+2(+1)	H +1(+1)	F	
23.00	CIS2(+2) MN+2(+1)	H +1(+2)	F	
13.33	CIS2(+1) ZN+2(+1)	H +1(+1)	D	369
26.00	CIS2(+2) ZN+2(+1)	H +1(+2)	D	369
9.39	GLU2(+1) H +1(+1)		D	158 267 51
13.54	GLU2(+1) H +1(+2)		D	158 267
15.67	GLU2(+1) H +1(+3)		D	158 267 51
1.50	GLU2(+1) CA+2(+1)		E	STAB 668
1.90	GLU2(+2) CA+2(+1)		F	
8.74	GLU2(+1) CU+2(+1)		D	158 267
14.91	GLU2(+2) CU+2(+1)		D	158 267
12.79	GLU2(+1) CU+2(+1)	H +1(+1)	D	158 267
3.40	GLU2(+1) FE+2(+1)		E	53

6.00	GLU2(+2) FE+2(+1)		F
11.20	GLU2(+1) FE+3(+1)		E 53
18.70	GLU2(+2) FE+3(+1)		F
4.50	GLU2(+1) PB+2(+1)		E STAB
6.00	GLU2(+2) PB+2(+1)		E STAB
8.30	GLU2(+1) PB+2(+1)	OH-1(+1)	F
2.00	GLU2(+1) MG+2(+1)		E STAB
2.70	GLU2(+2) MG+2(+1)		F
3.05	GLU2(+1) MN+2(+1)		E STAB
4.90	GLU2(+2) MN+2(+1)		F
4.76	GLU2(+1) ZN+2(+1)		D 158
8.54	GLU2(+2) ZN+2(+1)		D 158
8.83	GLN1(+1) H +1(+1)		D 158 69 379 515 504
10.98	GLN1(+1) H +1(+2)		D 158 69 379 515 504
1.00	GLN1(+1) CA+2(+1)		F
1.40	GLN1(+2) CA+2(+1)		F
7.24	GLN1(+1) CU+2(+1)		D 158 69 379 515 504
13.40	GLN1(+2) CU+2(+1)		D 158 69 379 515 504
3.50	GLN1(+1) FE+2(+1)		E 69
6.00	GLN1(+2) FE+2(+1)		E 69
8.00	GLN1(+3) FE+2(+1)		E 69
8.00	GLN1(+1) FE+3(+1)		F
14.79	GLN1(+2) FE+3(+1)		F
4.00	GLN1(+1) PB+2(+1)		E 311
7.00	GLN1(+2) PB+2(+1)		E 311
8.00	GLN1(+3) PB+2(+1)		E 311
8.00	GLN1(+1) PB+2(+1)	OH-1(+1)	F
1.70	GLN1(+1) MG+2(+1)		F
2.20	GLN1(+2) MG+2(+1)		F
2.60	GLN1(+1) MN+2(+1)		E 69 515
4.00	GLN1(+2) MN+2(+1)		E 69
4.27	GLN1(+1) ZN+2(+1)		D 158 69 515
7.94	GLN1(+2) ZN+2(+1)		D 158 69 515
10.00	GLN1(+3) ZN+2(+1)		E 69
9.38	GLY1(+1) H +1(+1)		D 158 83 267
11.76	GLY1(+1) H +1(+2)		D 158 83 267
1.30	GLY1(+1) CA+2(+1)		E STAB 668
1.70	GLY1(+2) CA+2(+1)		F
8.02	GLY1(+1) CU+2(+1)		D 158 83
14.67	GLY1(+2) CU+2(+1)		D 158 83
10.11	GLY1(+1) CU+2(+1)	H +1(+1)	D 158
3.01	GLY1(+1) FE+2(+1)		E STAB 53
5.50	GLY1(+2) FE+2(+1)		F
9.40	GLY1(+1) FE+3(+1)		E 51
16.50	GLY1(+2) FE+3(+1)		F
4.50	GLY1(+1) PB+2(+1)		E STAB
6.00	GLY1(+2) PB+2(+1)		E STAB
2.10	GLY1(+1) MG+2(+1)		F
2.70	GLY1(+2) MG+2(+1)		D 62
2.71	GLY1(+1) MN+2(+1)		D 62
4.76	GLY1(+2) MN+2(+1)		D 162
5.52	GLY1(+3) MN+2(+1)		D 62
10.02	GLY1(+1) MN+2(+1)	H +1(+1)	D 62
12.89	GLY1(+2) MN+2(+1)	H +1(+1)	D 62

4.205	GLY1(+1)	ZN+2(+1)		D	62
9.00	GLY1(+2)	ZN+2(+1)		D	158 668 317
11.31	GLY1(+3)	ZN+2(+1)		D	158 668 317
9.40	GLY1(+1)	ZN+2(+1)	H +1(+1)	D	158 668 317
3.70	GLY1(+1)	ZN+2(+1)	H +1(-1)	D	158
10.00	GLY1(+1)	PB+2(+1)	H +1(+1)	E	317
3.00	GLY1(+1)	PB+2(+1)	H +1(-1)	E	317
8.916	HIS1(+1)	H +1(+1)		D	158 311 679
14.872	HIS1(+1)	H +1(+2)		D	158 311 679
17.113	HIS1(+1)	H +1(+3)		D	158 311 679
1.40	HIS1(+1)	CA+2(+1)		F	
1.80	HIS1(+2)	CA+2(+1)		F	
9.80	HIS1(+1)	CU+2(+1)		D	158 66 306 679
17.50	HIS1(+2)	CU+2(+1)		D	158 66 306 679
13.92	HIS1(+1)	CU+2(+1)	H +1(+1)	D	158 66 306 679
2.46	HIS1(+1)	CU+2(+1)	H +1(-1)	D	158 66 306
23.15	HIS1(+2)	CU+2(+1)	H +1(+1)	D	158 66 306 679
7.38	HIS1(+2)	CU+2(+2)	H +1(-2)	D	158 66 306
26.03	HIS1(+2)	CU+2(+1)	H +1(+2)	E	66 306
5.20	HIS1(+1)	FE+2(+1)		E	STAB 63
9.50	HIS1(+2)	FE+2(+1)		E	STAB 63
4.20	HIS1(+1)	FE+3(+1)		E	STAB 52
7.65	HIS1(+2)	FE+3(+1)		F	
5.96	HIS1(+1)	PB+2(+1)		D	311 57
9.00	HIS1(+2)	PB+2(+1)		D	311 57
8.00	HIS1(+1)	PB+2(+1)	OH-1(+1)	F	
2.20	HIS1(+1)	MG+2(+1)		F	
2.90	HIS1(+2)	MG+2(+1)		F	
3.24	HIS1(+1)	MN+2(+1)		D	57 63
6.16	HIS1(+2)	MN+2(+1)		D	63 57
6.32	HIS1(+1)	ZN+2(+1)		D	57 63
11.68	HIS1(+2)	ZN+2(+1)		D	63 57
11.12	HIS1(+1)	ZN+2(+1)	H +1(+1)	D	158
9.30	HYP1(+1)	H +1(+1)		E	680 288 51
11.20	HYP1(+1)	H +1(+2)		E	53
1.00	HYP1(+1)	CA+2(+1)		E	STAB
1.30	HYP1(+2)	CA+2(+1)		F	
8.10	HYP1(+1)	CU+2(+1)		E	STAB 680
14.50	HYP1(+2)	CU+2(+1)		E	STAB 680
3.80	HYP1(+1)	FE+2(+1)		E	53
6.00	HYP1(+2)	FE+2(+1)		F	
8.50	HYP1(+1)	FE+3(+1)		F	
15.00	HYP1(+2)	FE+3(+1)		E	51
3.50	HYP1(+1)	PB+2(+1)		F	
4.80	HYP1(+2)	PB+2(+1)		F	
8.00	HYP1(+1)	PB+2(+1)	OH-1(+1)	F	
1.70	HYP1(+1)	MG+2(+1)		F	
2.30	HYP1(+2)	MG+2(+1)		F	
2.70	HYP1(+1)	MN+2(+1)		F	
4.90	HYP1(+2)	MN+2(+1)		F	
4.50	HYP1(+1)	ZN+2(+1)		F	
8.50	HYP1(+2)	ZN+2(+1)		E	STAB
9.36	ILE1(+1)	H +1(+1)		D	158
11.72	ILE1(+1)	H +1(+2)		D	158

1.10	ILE1(+1) CA+2(+1)		E	668
1.40	ILE1(+2) CA+2(+1)		F	
7.95	ILE1(+1) CU+2(+1)		D	158
14.68	ILE1(+2) CU+2(+1)		D	158
0.32	ILE1(+1) CU+2(+1)	H +1(-1)	D	158
3.00	ILE1(+1) FE+2(+1)		F	
5.00	ILE1(+2) FE+2(+1)		F	
9.00	ILE1(+1) FE+3(+1)		F	
15.40	ILE1(+2) FE+3(+1)		F	
3.80	ILE1(+1) PB+2(+1)		F	
5.30	ILE1(+2) PB+2(+1)		F	
9.00	ILE1(+1) PB+2(+1)	OH-1(+1)	F	
1.80	ILE1(+1) MG+2(+1)		F	
2.40	ILE1(+2) MG+2(+1)		F	
2.60	ILE1(+1) MN+2(+1)		F	
4.70	ILE1(+2) MN+2(+1)		F	
4.40	ILE1(+1) ZN+2(+1)		D	158
8.08	ILE1(+2) ZN+2(+1)		D	158
3.62	ILE1(+1) ZN+2(+1)	H +1(-1)	D	158
15.25	ILE1(+2) ZN+2(+1)	H +1(+1)	D	158
9.36	LEU1(+1) H +1(+1)		D	158 251
11.72	LEU1(+1) H +1(+2)		D	158 251
1.10	LEU1(+1) CA+2(+1)		E	668
1.40	LEU1(+2) CA+2(+1)		F	
8.04	LEU1(+1) CU+2(+1)		D	158
14.69	LEU1(+2) CU+2(+1)		D	158
11.49	LEU1(+1) CU+2(+1)	H +1(+1)	D	158
19.43	LEU1(+2) CU+2(+1)	H +1(+1)	D	158
3.32	LEU1(+1) FE+2(+1)		E	53
5.00	LEU1(+2) FE+2(+1)		F	
9.49	LEU1(+1) FE+3(+1)		E	51
15.50	LEU1(+2) FE+3(+1)		F	
4.00	LEU1(+1) PB+2(+1)		F	
5.50	LEU1(+2) PB+2(+1)		F	
9.00	LEU1(+1) PB+2(+1)	OH-1(+1)	F	
1.80	LEU1(+1) MG+2(+1)		F	
2.30	LEU1(+2) MG+2(+1)		F	
2.60	LEU1(+1) MN+2(+1)		E	STAB 253
4.80	LEU1(+2) MN+2(+1)		E	STAB
4.51	LEU1(+1) ZN+2(+1)		D	158
8.56	LEU1(+2) ZN+2(+1)		D	158
4.25	LEU1(+1) ZN+2(+1)	H +1(-1)	D	158
15.17	LEU1(+2) ZN+2(+1)	H +1(+1)	D	158
10.39	LYS1(+1) H +1(+1)		D	369 681
19.35	LYS1(+1) H +1(+2)		D	369 681 682
21.55	LYS1(+1) H +1(+3)		D	369 682
11.60	LYS1(+1) CA+2(+1)	H +1(+1)	E	668
21.30	LYS1(+2) CA+2(+1)	H +1(+2)	F	
7.30	LYS1(+1) CU+2(+1)		E	682
14.60	LYS1(+2) CU+2(+1)		D	369
17.91	LYS1(+1) CU+2(+1)	H +1(+1)	D	369
25.87	LYS1(+2) CU+2(+1)	H +1(+1)	D	369
34.77	LYS1(+2) CU+2(+1)	H +1(+2)	D	369
13.80	LYS1(+1) FE+2(+1)	H +1(+1)	E	STAB

25.00	LYS1(+2)	FE+2(+1) H +1(+2)	F	
18.40	LYS1(+1)	FE+3(+1) H +1(+1)	F	
35.87	LYS1(+2)	FE+3(+1) H +1(+2)	F	
14.10	LYS1(+1)	PB+2(+1) H +1(+1)	F	
25.86	LYS1(+2)	PB+2(+1) H +1(+2)	F	
12.10	LYS1(+1)	MG+2(+1) H +1(+1)	F	
22.70	LYS1(+2)	MG+2(+1) H +1(+2)	F	
12.20	LYS1(+1)	MN+2(+1) H +1(+1)	E	STAB
24.35	LYS1(+2)	MN+2(+1) H +1(+2)	F	
3.50	LYS1(+1)	ZN+2(+1)	D	369
7.00	LYS1(+2)	ZN+2(+1)	D	369
14.56	LYS1(+1)	ZN+2(+1) H +1(+1)	D	369
20.01	LYS1(+2)	ZN+2(+1) H +1(+1)	D	369
28.80	LYS1(+2)	ZN+2(+1) H +1(+2)	D	369
18.30	LYS1(+2)	FE+2(+1) H +1(+1)	F	
28.88	LYS1(+2)	FE+3(+1) H +1(+1)	F	
18.90	LYS1(+2)	PB+2(+1) H +1(+1)	F	
19.50	LYS1(+2)	MN+2(+1) H +1(+1)	F	
8.91	MET1(+1)	H +1(+1)	D	158 253
11.17	MET1(+1)	H +1(+2)	D	158 253
1.20	MET1(+1)	CA+2(+1)	E	668
1.60	MET1(+2)	CA+2(+1)	F	
7.67	MET1(+1)	CU+2(+1)	D	158
14.93	MET1(+2)	CU+2(+1)	D	158
3.15	MET1(+1)	FE+2(+1)	E	53
4.90	MET1(+2)	FE+2(+1)	F	
8.60	MET1(+1)	FE+3(+1)	E	51
14.94	MET1(+2)	FE+3(+1)	F	
4.30	MET1(+1)	PB+2(+1)	E	STAB
7.50	MET1(+2)	PB+2(+1)	E	STAB
8.20	MET1(+1)	PB+2(+1) OH-1(+1)	F	
1.80	MET1(+1)	MG+2(+1)	F	
2.40	MET1(+2)	MG+2(+1)	F	
2.70	MET1(+1)	MN+2(+1)	E	STAB 253
4.25	MET1(+2)	MN+2(+1)	E	STAB
4.22	MET1(+1)	ZN+2(+1)	D	158
6.93	MET1(+2)	ZN+2(+1)	D	158
10.22	ORN1(+1)	H +1(+1)	D	158 333
18.77	ORN1(+1)	H +1(+2)	D	158 333
20.69	ORN1(+1)	H +1(+3)	D	158
11.40	ORN1(+1)	CA+2(+1) H +1(+1)	E	333
21.30	ORN1(+2)	CA+2(+1) H +1(+2)	F	
9.78	ORN1(+1)	CU+2(+1)	D	158
14.77	ORN1(+2)	CU+2(+1)	D	158
17.42	ORN1(+1)	CU+2(+1) H +1(+1)	D	158 333
24.73	ORN1(+2)	CU+2(+1) H +1(+1)	D	158
33.64	ORN1(+2)	CU+2(+1) H +1(+2)	D	158 333
0.89	ORN1(+1)	CU+2(+1) H +1(-1)	D	158
12.76	ORN1(+1)	FE+2(+1) H +1(+1)	E	53
24.25	ORN1(+2)	FE+2(+1) H +1(+2)	F	
15.47	ORN1(+2)	FE+2(+1) H +1(+1)	F	
18.28	ORN1(+1)	FE+3(+1) H +1(+1)	E	51
35.34	ORN1(+2)	FE+3(+1) H +1(+2)	F	
28.52	ORN1(+2)	FE+3(+1) H +1(+1)	F	

13.90	ORN1(+1) PB+2(+1) H +1(+1)	F	
18.50	ORN1(+2) PB+2(+1) H +1(+1)	F	
25.50	ORN1(+2) PB+2(+1) H +1(+2)	F	
9.00	ORN1(+1) PB+2(+1) OH-1(+1)	F	
11.90	ORN1(+1) MG+2(+1) H +1(+1)	E	333
22.20	ORN1(+2) MG+2(+1) H +1(+2)	F	
12.02	ORN1(+1) MN+2(+1) H +1(+1)	E	333
19.00	ORN1(+2) MN+2(+1) H +1(+1)	F	
23.70	ORN1(+2) MN+2(+1) H +1(+2)	F	
5.96	ORN1(+1) ZN+2(+1)	D	158
14.08	ORN1(+1) ZN+2(+1) H +1(+1)	D	158 333
19.31	ORN1(+2) ZN+2(+1) H +1(+1)	D	158
27.62	ORN1(+2) ZN+2(+1) H +1(+2)	D	158
2.59	ORN1(+1) ZN+2(+1) H +1(-1)	F	
8.89	PHE1(+1) H +1(+1)	D	158 253 83
10.49	PHE1(+1) H +1(+2)	E	253 68 83
1.00	PHE1(+1) CA+2(+1)	F	
1.60	PHE1(+2) CA+2(+1)	F	
7.65	PHE1(+1) CU+2(+1)	E	STAB 67
14.41	PHE1(+2) CU+2(+1)	E	STAB 67
3.15	PHE1(+1) FE+2(+1)	E	67 53
5.36	PHE1(+2) FE+2(+1)	E	STAB 67
8.85	PHE1(+1) FE+3(+1)	E	67 51
16.00	PHE1(+2) FE+3(+1)	E	67
4.00	PHE1(+1) PB+2(+1)	E	311
7.20	PHE1(+2) PB+2(+1)	E	311
8.00	PHE1(+1) PB+2(+1) OH-1(+1)	F	
1.70	PHE1(+1) MG+2(+1)	F	
2.30	PHE1(+2) MG+2(+1)	F	
2.65	PHE1(+1) MN+2(+1)	E	253
4.30	PHE1(+2) MN+2(+1)	F	
4.50	PHE1(+1) ZN+2(+1)	D	369
8.36	PHE1(+2) ZN+2(+1)	D	369 164
10.336	PRO1(+1) H +1(+1)	D	158 288 680
12.35	PRO1(+1) H +1(+2)	D	158 288 680
1.30	PRO1(+1) CA+2(+1)	F	
1.70	PRO1(+2) CA+2(+1)	F	
8.68	PRO1(+1) CU+2(+1)	D	158 288 680
16.00	PRO1(+2) CU+2(+1)	D	158 288 680
10.64	PRO1(+1) CU+2(+1) H +1(+1)	D	158
4.00	PRO1(+1) FE+2(+1)	E	53
7.00	PRO1(+2) FE+2(+1)	E	STAB
9.69	PRO1(+1) FE+3(+1)	E	51
17.90	PRO1(+2) FE+3(+1)	F	
4.00	PRO1(+1) PB+2(+1)	F	
6.00	PRO1(+2) PB+2(+1)	F	
8.00	PRO1(+1) PB+2(+1) OH-1(+1)	F	
1.90	PRO1(+1) MG+2(+1)	F	
2.40	PRO1(+2) MG+2(+1)	E	STAB
2.84	PRO1(+1) MN+2(+1)	D	62
5.53	PRO1(+2) MN+2(+1)	D	62
6.74	PRO1(+3) MN+2(+1)	D	62
11.84	PRO1(+1) MN+2(+1) H +1(+1)	D	62
14.92	PRO1(+2) MN+2(+1) H +1(+1)	D	62

5.13	PRO1(+1)	ZN+2(+1)	D	158
9.69	PRO1(+2)	ZN+2(+1)	D	158
11.16	PRO1(+3)	ZN+2(+1)	D	158
3.22	PRO1(+1)	ZN+2(+1) H +1(-1)	D	158
0.06	PRO1(+2)	ZN+2(+1) H +1(-1)	D	158
8.84	SER1(+1)	H +1(+1)	D	156
11.02	SER1(+1)	H +1(+2)	D	158
1.30	SER1(+1)	CA+2(+1)	E	STAB 668
1.70	SER1(+2)	CA+2(+1)	F	
7.565	SER1(+1)	CU+2(+1)	D	158 69
14.01	SER1(+2)	CU+2(+1)	D	158 69
3.35	SER1(+1)	FE+2(+1)	E	STAB 51 69
6.00	SER1(+2)	FE+2(+1)	E	STAB
8.00	SER1(+3)	FE+2(+1)	F	
8.70	SER1(+1)	FE+3(+1)	E	53
15.90	SER1(+2)	FE+3(+1)	F	
4.30	SER1(+1)	PB+2(+1)	E	311
7.20	SER1(+2)	PB+2(+1)	F	
8.30	SER1(+3)	PB+2(+1)	F	
8.00	SER1(+1)	PB+2(+1) OH-1(+1)	F	
1.80	SER1(+1)	MG+2(+1)	F	
2.40	SER1(+2)	MG+2(+1)	F	
2.48	SER1(+1)	MN+2(+1)	E	STAB 69
4.00	SER1(+2)	MN+2(+1)	E	STAB 69
4.47	SER1(+1)	ZN+2(+1)	D	158
8.31	SER1(+2)	ZN+2(+1)	D	158
10.56	SER1(+3)	ZN+2(+1)	D	158
8.71	THR1(+1)	H +1(+1)	D	158
10.91	THR1(+1)	H +1(+2)	D	158
1.10	THR1(+1)	CA+2(+1)	E	668
1.40	THR1(+2)	CA+2(+1)	F	
7.55	THR1(+1)	CU+2(+1)	D	158
14.91	THR1(+2)	CU+2(+1)	D	158
3.31	THR1(+1)	FE+2(+1)	E	STAB 53
6.00	THR1(+2)	FE+2(+1)	E	STAB
8.40	THR1(+1)	FE+3(+1)	E	51
15.40	THR1(+2)	FE+3(+1)	F	
4.40	THR1(+1)	PB+2(+1)	F	
7.40	THR1(+2)	PB+2(+1)	F	
8.00	THR1(+1)	PB+2(+1) OH-1(+1)	F	
1.70	THR1(+1)	MG+2(+1)	F	
2.30	THR1(+2)	MG+2(+1)	F	
2.56	THR1(+1)	MN+2(+1)	E	STAB
3.90	THR1(+2)	MN+2(+1)	E	STAB
4.43	THR1(+1)	ZN+2(+1)	D	158
8.14	THR1(+2)	ZN+2(+1)	D	158
10.09	THR1(+3)	ZN+2(+1)	D	158
9.09	TRP1(+1)	H +1(+1)	D	158 63
11.55	TRP1(+1)	H +1(+2)	D	158 63
1.20	TRP1(+1)	CA+2(+1)	F	
1.60	TRP1(+2)	CA+2(+1)	F	
8.05	TRP1(+1)	CU+2(+1)	D	158 63
15.32	TRP1(+2)	CU+2(+1)	D	158 63
3.45	TRP1(+1)	FE+2(+1)	E	63 53

6.20	TRP1(+2) FE+2(+1)		E STAB 63
8.40	TRP1(+1) FE+3(+1)		E 51
16.15	TRP1(+2) FE+3(+1)		F
4.40	TRP1(+1) PB+2(+1)		E 311
8.50	TRP1(+2) PB+2(+1)		E 311
8.00	TRP1(+1) PB+2(+1)	OH-1(+1)	F
1.80	TRP1(+1) MG+2(+1)		F
2.30	TRP1(+2) MG+2(+1)		F
2.60	TRP1(+1) MN+2(+1)		E 63
4.30	TRP1(+2) MN+2(+1)		E 63
4.50	TRP1(+1) ZN+2(+1)		E 158 63
8.76	TRP1(+2) ZN+2(+1)		D 158 63
11.61	TRP1(+3) ZN+2(+1)		D 158
10.06	TYR2(+1) H +1(+1)		D 369
19.00	TYR2(+1) H +1(+2)		D 369
21.35	TYR2(+1) H +1(+3)		D 369
11.20	TYR2(+1) CA+2(+1)	H +1(+1)	E STAB 668
20.60	TYR2(+2) CA+2(+1)	H +1(+2)	D 369
9.32	TYR2(+1) CU+2(+1)		D 369
15.09	TYR2(+2) CU+2(+1)		D 369
17.63	TYR2(+1) CU+2(+1)	H +1(+1)	D 369
25.08	TYR2(+2) CU+2(+1)	H +1(+1)	D 369
34.48	TYR2(+2) CU+2(+1)	H +1(+2)	D 369
3.00	TYR2(+1) FE+2(+1)		F
13.50	TYR2(+1) FE+2(+1)	H +1(+1)	F
19.00	TYR2(+2) FE+2(+1)	H +1(+1)	F
27.00	TYR2(+2) FE+2(+1)	H +1(+2)	E STAB
7.00	TYR2(+1) FE+3(+1)		F
18.00	TYR2(+1) FE+3(+1)	H +1(+1)	F
28.24	TYR2(+2) FE+3(+1)	H +1(+1)	F
35.07	TYR2(+2) FE+3(+1)	H +1(+2)	F
3.50	TYR2(+1) PB+2(+1)		F
14.16	TYR2(+1) PB+2(+1)	H +1(+1)	F
23.17	TYR2(+2) PB+2(+1)	H +1(+2)	F
21.00	TYR2(+2) PB+2(+1)	H +1(+1)	F
11.60	TYR2(+1) MG+2(+1)	H +1(+1)	F
22.10	TYR2(+2) MG+2(+1)	H +1(+2)	F
1.20	TYR2(+1) MN+2(+1)		F
12.36	TYR2(+1) MN+2(+1)	H +1(+1)	F
19.28	TYR2(+2) MN+2(+1)	H +1(+1)	F
23.90	TYR2(+2) MN+2(+1)	H +1(+2)	E STAB
6.08	TYR2(+1) ZN+2(+1)		F
14.27	TYR2(+1) ZN+2(+1)	H +1(+1)	F
27.90	TYR2(+2) ZN+2(+1)	H +1(+2)	F
21.00	TYR2(+2) ZN+2(+1)	H +1(+1)	F
9.32	VAL1(+1) H +1(+1)		D 158
11.66	VAL1(+1) H +1(+2)		D 158
1.10	VAL1(+1) CA+2(+1)		E 668
1.40	VAL1(+2) CA+2(+1)		F
7.90	VAL1(+1) CU+2(+1)		D 158
14.55	VAL1(+2) CU+2(+1)		D 158
3.25	VAL1(+1) FE+2(+1)		E 53
5.20	VAL1(+2) FE+2(+1)		F
9.20	VAL1(+1) FE+3(+1)		E 51

16.20	VAL1(+2)	FE+3(+1)	
3.60	VAL1(+1)	PB+2(+1)	
5.60	VAL1(+2)	PB+2(+1)	
8.80	VAL1(+2)	PB+2(+1)	OH-1(+1)
1.70	VAL1(+1)	MG+2(+1)	
2.20	VAL1(+2)	MG+2(+1)	
2.337	VAL1(+1)	MN+2(+1)	
3.97	VAL1(+2)	MN+2(+1)	
5.19	VAL1(+3)	MN+2(+1)	
12.73	VAL1(+1)	MN+2(+1)	H +1(+1)
13.40	VAL1(+2)	MN+2(+1)	H +1(+1)
4.44	VAL1(+1)	ZN+2(+1)	
8.24	VAL1(+2)	ZN+2(+1)	
10.62	VAL1(+3)	ZN+2(+1)	
4.18	VAL1(+1)	ZN+2(+1)	H +1(-1)
10.24	C032(+1)	H +1(+1)	
16.54	C032(+1)	H +1(+2)	
2.90	C032(+1)	CA+2(+1)	
10.90	C032(+1)	CA+2(+1)	H +1(+1)
5.50	C032(+1)	CU+2(+1)	
8.30	C032(+2)	CU+2(+1)	
13.00	C032(+1)	CU+2(+1)	H +1(+1)
14.20	C032(+2)	CU+2(+1)	H +1(+2)
4.50	C032(+1)	FE+2(+1)	
6.00	C032(+2)	FE+2(+1)	
12.00	C032(+1)	FE+2(+1)	H +1(+1)
13.00	C032(+2)	FE+2(+1)	H +1(+2)
6.00	C032(+1)	FE+3(+1)	
9.00	C032(+2)	FE+3(+1)	
13.00	C032(+1)	FE+3(+1)	H +1(+1)
14.50	C032(+2)	FE+3(+1)	H +1(+2)
3.30	C032(+1)	PB+2(+1)	
6.40	C032(+2)	PB+2(+1)	
11.80	C032(+1)	PB+2(+1)	H +1(+1)
13.50	C032(+2)	PB+2(+1)	H +1(+2)
14.20	C032(+3)	PB+2(+1)	H +1(+3)
2.90	C032(+1)	MG+2(+1)	
10.90	C032(+1)	MG+2(+1)	H +1(+1)
3.10	C032(+1)	MN+2(+1)	
11.50	C032(+1)	MN+2(+1)	H +1(+1)
3.30	C032(+1)	ZN+2(+1)	
11.80	C032(+1)	ZN+2(+1)	H +1(+1)
11.7	H +1(-1)	CA+2(+1)	
7.6	H +1(-1)	CU+2(+1)	
10.49	H +1(-2)	CU+2(+2)	
8.7	H +1(-1)	FE+2(+1)	
2.9	H +1(-1)	FE+3(+1)	
7.7	H +1(-1)	PB+2(+1)	
11.5	H +1(-1)	MG+2(+1)	
10.1	H +1(-1)	MN+2(+1)	
9.03	H +1(-1)	ZN+2(+1)	
5.9	H +1(-2)	FE+3(+1)	
11.30	P043(+1)	H +1(+1)	
18.00	P043(+1)	H +1(+2)	

F				
E	STAB			
E	STAB			
E	STAB			
F				
F				
D	62			
D	62			
D	62			
D	62			
D	62			
D	158			
D	158			
D	158			
D	158			
E	STAB	684	270	
E	STAB	684	270	
E	STAB	246	434	
E	STAB	246	434	
E	STAB	434		
E	STAB			
F	434			
F	424			
F	434			
F	434			
F	434			
F	434			
F	434			
F	434			
F	434			
F	434			
F				
E	STAB			
F				
E	STAB			
E	STAB			
E - LOWENTHAL R., PRIVATE COMM.				
E	STAB - LOWENTHAL R.			
F				
E	STAB			
F				
F				
D	62			
D	62			
D	62			
E	STAB	434		
E	STAB	434		
E	STAB	434		
D				
D	62			
D	62			
F	434			
D	394			
D	394			

19.92	PO43(+1) H +1(+3)	D	394	
39.50	PO43(+2) H +1(+5)	D	394	
36.72	PO43(+2) H +1(+4)	D	394	
29.68	PO43(+2) H +1(+3)	D	394	
12.60	PO43(+1) CA+2(+1) H +1(+1)	D	321	
18.60	PO43(+1) CA+2(+1) H +1(+2)	D	321	
16.30	PO43(+2) CA+2(+2) H +1(+2)	D	321	
32.30	PO43(+2) CA+2(+1) H +1(+3)	D	321	
25.30	PO43(+2) CA+2(+1) H +1(+2)	F		
14.60	PO43(+1) CU+2(+1) H +1(+1)	D	321	
19.30	PO43(+1) CU+2(+1) H +1(+2)	D	321	
34.00	PO43(+2) CU+2(+1) H +1(+3)	D	321	
31.80	PO43(+2) CU+2(+2) H +1(+2)	D	321	
27.00	PO43(+2) CU+2(+1) H +1(+2)	F		
13.50	PO43(+1) FE+2(+1) H +1(+1)	F		
26.00	PO43(+2) FE+2(+1) H +1(+2)	F		
17.00	PO43(+1) FE+3(+1) H +1(+1)	F		
21.00	PO43(+1) FE+3(+1) H +1(+2)	E	STAB	
28.00	PO43(+2) FE+3(+1) H +1(+2)	F		
14.00	PO43(+1) PB+2(+1) H +1(+1)	E	337	
26.30	PO43(+2) PB+2(+1) H +1(+2)	F		
3.40	PO43(+1) MG+2(+1)	D	321	
13.10	PO43(+1) MG+2(+1) H +1(+1)	D	321	
18.70	PO43(+1) MG+2(+1) H +1(+2)	D	321	
32.30	PO43(+2) MG+2(+1) H +1(+3)	D	321	
27.60	PO43(+2) MG+2(+2) H +1(+2)	D	321	
25.30	PO43(+2) MG+2(+1) H +1(+2)	F		
13.30	PO43(+1) MN+2(+1) H +1(+1)	F		
25.60	PO43(+2) MN+2(+1) H +1(+2)	F		
13.70	PO43(+1) ZN+2(+1) H +1(+1)	D	321	
19.20	PO43(+1) ZN+2(+1) H +1(+2)	D	321	
33.30	PO43(+2) ZN+2(+1) H +1(+3)	D	321	
31.00	PO43(+2) ZN+2(+2) H +1(+2)	D	321	
26.30	PO43(+2) ZN+2(+1) H +1(+2)	F		
11.40	SIL2(+1) H +1(+1)	E	STAB	
20.50	SIL2(+1) H +1(+2)	E	STAB	
3.00	SIL2(+1) CA+2(+1)	F		
11.79	SIL2(+1) CA+2(+1) H +1(+1)	F		
25.69	SIL2(+2) CA+2(+1) H +1(+2)	F		
20.40	SIL2(+1) FE+3(+1) H +1(+1)	F		
4.17	SIL2(+1) MG+2(+1)	F		
12.04	SIL2(+1) MG+2(+1) H +1(+1)	F		
26.62	SIL2(+2) MG+2(+1) H +1(+2)	F		
1.60	SO42(+1) H +1(+1)	E	STAB	
1.20	SO42(+1) CA+2(+1)	E	STAB	434 246 525
2.20	SO42(+1) CU+2(+1)	E	524	
1.00	SO42(+1) FE+2(+1)	E	STAB	
2.50	SO42(+1) FE+3(+1)	E	STAB	
2.50	SO42(+1) FE+3(+1) H +1(+1)	E	STAB	
2.60	SO42(+2) FE+3(+1)	E	STAB	
2.20	SO42(+1) PB+2(+1)	E	STAB	
1.50	SO42(+1) MG+2(+1)	E	STAB	434 246
2.00	SO42(+1) MN+2(+1)	E	STAB	719
2.20	SO42(+1) ZN+2(+1)	E	STAB	

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 0.50    SCN1(+1) H +1(+1)                                E   STAB
 2.00    SCN1(+1) CU+2(+1)                                E   STAB
 2.40    SCN1(+2) CU+2(+1)                                E   STAB
 0.40    SCN1(+1) FE+2(+1)                                E   STAB
 2.30    SCN1(+1) FE+3(+1)                                E   STAB
 3.30    SCN1(+2) FE+3(+1)                                F
 1.00    SCN1(+1) PB+2(+1)                                E   STAB
 1.50    SCN1(+2) PB+2(+1)                                E   STAB
 1.05    SCN1(+1) MN+2(+1)                                E   STAB  287
 1.20    SCN1(+1) ZN+2(+1)                                E   STAB
 1.60    SCN1(+2) ZN+2(+1)                                E   STAB
 2.00    SCN1(+3) ZN+2(+1)                                E   STAB
 2.40    SCN1(+4) ZN+2(+1)                                E   STAB
 8.95    NH3O(+1) H +1(+1)                                E   STAB  98
 3.90    NH3O(+1) CU+2(+1)                                E   STAB
 7.13    NH3O(+2) CU+2(+1)                                E   STAB
 9.90    NH3O(+3) CU+2(+1)                                E   STAB
11.90    NH3O(+4) CU+2(+1)                                E   STAB
 1.30    NH3O(+1) FE+2(+1)                                E   STAB
 1.90    NH3O(+2) FE+2(+1)                                E   STAB
 3.00    NH3O(+4) FE+2(+1)                                E   STAB
 0.50    NH3O(+1) MN+2(+1)                                E   STAB
 2.25    NH3O(+1) ZN+2(+1)                                E   STAB  98
 4.55    NH3O(+2) ZN+2(+1)                                E   STAB  98
 6.85    NH3O(+3) ZN+2(+1)                                F   STAB  98
 9.01    NH3O(+4) ZN+2(+1)                                E   STAB  98
15.50    NH3O(+1) CU+2(+1) OH-1(+3)                       E   STAB
15.00    NH3O(+2) CU+2(+1) OH-1(+2)                       E   STAB
14.50    NH3O(+3) CU+2(+1) OH-1(+1)                       E   STAB
14.00    NH3O(+1) ZN+2(+1) OH-1(+3)                       E   STAB
13.10    NH3O(+2) ZN+2(+1) OH-1(+2)                       E   STAB
11.50    NH3O(+3) ZN+2(+1) OH-1(+1)                       E   STAB
10.30    NH3O(+2) ZN+2(+1) OH-1(+1)                       E   STAB
 8.80    NH3O(+1) ZN+2(+1) OH-1(+1)                       E   STAB
 5.53    CTA3(+1) H +1(+1)                                D   460 26 516 691 457
 9.74    CTA3(+1) H +1(+2)                                D   460 26 516 691 457
12.49    CTA3(+1) H +1(+3)                                D   460 26 619 512 457
 3.26    CTA3(+1) CA+2(+1)                                D   460 254 255 256 244 692 425
 4.00    CTA3(+2) CA+2(+1)                                E   STAB  692
 7.53    CTA3(+1) CA+2(+1) H +1(+1)                       D   STAB  460 547
10.95    CTA3(+1) CA+2(+1) H +1(+2)                       E   STAB
16.00    CTA3(+2) CA+2(+1) H +1(+2)                       E   STAB
 4.75    CTA3(+1) CU+2(+1)                                E   STAB  524 691
 7.80    CTA3(+2) CU+2(+1)                                E   STAB  524 691
 8.00    CTA3(+1) CU+2(+1) H +1(+1)                       E   STAB  691
 9.30    CTA3(+2) CU+2(+1) H +1(+1)                       E   691
 4.90    CTA3(+1) FE+2(+1)                                E   STAB  440
 8.30    CTA3(+1) FE+2(+1) H +1(+1)                       E   STAB  440
 6.00    CTA3(+2) FE+2(+1)                                F
10.90    CTA3(+1) FE+3(+1)                                E   STAB  440 512
11.80    CTA3(+1) FE+3(+1) H +1(+1)                       E   STAB  440
16.00    CTA3(+2) FE+3(+1)                                E   512
17.00    CTA3(+2) FE+3(+1) H +1(+1)                       F
19.00    CTA3(+2) FE+3(+1) H +1(+2)                       F
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8.00	CTA3(+1) FE+3(+1) H +1(-1)	E	440
4.00	CTA3(+1) PB+2(+1)	E	STAB 516
8.00	CTA3(+1) PB+2(+1) H +1(+1)	E	STAB 516
5.50	CTA3(+2) PB+2(+1)	E	STAB 516
6.00	CTA3(+3) PB+2(+1)	E	STAB
10.00	CTA3(+1) PB+2(+1) H +1(+2)	E	516
3.34	CTA3(+1) MG+2(+1)	D	460 457
4.10	CTA3(+2) MG+2(+1)	F	STAB
7.32	CTA3(+1) MG+2(+1) H +1(+1)	D	460 457
3.20	CTA3(+1) MN+2(+1)	E	STAB 720
5.00	CTA3(+1) MN+2(+1) H +1(+1)	E	STAB 720
4.30	CTA3(+2) MN+2(+1)	F	
4.60	CTA3(+1) ZN+2(+1)	E	STAB 457
8.50	CTA3(+1) ZN+2(+1) H +1(+1)	E	STAB 457
10.95	CTA3(+1) ZN+2(+1) H +1(+2)	E	STAB
6.60	CTA3(+2) ZN+2(+1)	F	
3.70	LTA1(+1) H +1(+1)	E	STAB
1.55	LTA1(+1) CA+2(+1)	E	STAB 695
2.00	LTA1(+2) CA+2(+1)	E	STAB
2.20	LTA1(+1) CU+2(+1)	E	STAB 694 695 696 697
3.00	LTA1(+2) CU+2(+1)	E	STAB 694 695 696
3.03	LTA1(+3) CU+2(+1)	E	STAB 694
1.55	LTA1(+1) FE+2(+1)	F	
4.00	LTA1(+1) FE+3(+1)	E	STAB
6.00	LTA1(+2) FE+3(+1)	F	
1.75	LTA1(+1) PB+2(+1)	F	
2.50	LTA1(+2) PB+2(+1)	E	STAB 727
1.30	LTA1(+1) MG+2(+1)	E	STAB
1.60	LTA1(+2) MG+2(+1)	E	STAB
1.00	LTA1(+1) MN+2(+1)	E	STAB
1.40	LTA1(+2) MN+2(+1)	E	STAB
1.75	LTA1(+1) ZN+2(+1)	E	STAB
2.75	LTA1(+2) ZN+2(+1)	E	STAB
4.81	MLA2(+1) H +1(+1)	E	STAB
8.05	MLA2(+1) H +1(+2)	E	STAB
2.00	MLA2(+1) CA+2(+1)	E	STAB 698
5.80	MLA2(+1) CA+2(+1) H +1(+1)	E	STAB
8.80	MLA2(+1) CA+2(+1) H +1(+2)	E	STAB
4.00	MLA2(+1) CU+2(+1)	E	STAB 698
5.50	MLA2(+2) CU+2(+1)	E	STAB
8.00	MLA2(+1) CU+2(+1) H +1(+1)	E	STAB 698
9.80	MLA2(+1) CU+2(+1) H +1(+2)	E	STAB
10.60	MLA2(+2) CU+2(+1) H +1(+2)	E	STAB
2.40	MLA2(+1) FE+2(+1)	E	STAB
6.70	MLA2(+1) FE+3(+1)	E	STAB
2.10	MLA2(+1) PB+2(+1)	E	728
2.60	MLA2(+2) PB+2(+1)	E	728
2.90	MLA2(+3) PB+2(+1)	E	728
1.20	MLA2(+1) MG+2(+1)	E	STAB
5.90	MLA2(+1) MG+2(+1) H +1(+1)	E	STAB
8.40	MLA2(+1) MG+2(+1) H +1(+2)	E	STAB
2.00	MLA2(+1) MN+2(+1)	E	STAB
2.60	MLA2(+1) ZN+2(+1)	E	STAB
7.40	MLA2(+1) ZN+2(+1) H +1(+1)	E	STAB

3.85	OXA2(+1) H +1(+1)	E	STAB	700
5.10	OXA2(+1) H +1(+2)	E	STAB	699
1.50	OXA2(+1) CA+2(+1)	E	STAB	703
2.40	OXA2(+2) CA+2(+1)	E	STAB	
4.60	OXA2(+1) CU+2(+1)	E	STAB	
8.20	OXA2(+2) CU+2(+1)	E	STAB	
5.85	OXA2(+1) CU+2(+1) H +1(+1)	E	STAB	
3.10	OXA2(+1) FE+2(+1)	E	STAB	
6.00	OXA2(+2) FE+2(+1)	E	STAB	
8.00	OXA2(+1) FE+3(+1)	E	STAB	701
14.20	OXA2(+2) FE+3(+1)	E	STAB	
19.00	OXA2(+3) FE+3(+1)	E	STAB	
8.00	OXA2(+1) FE+3(+1) H +1(+1)	E	STAB	
3.40	OXA2(+1) PB+2(+1)	E	STAB	707
5.70	OXA2(+2) PB+2(+1)	E	STAB	707
2.40	OXA2(+1) MG+2(+1)	E	STAB	
3.50	OXA2(+2) MG+2(+1)	E	STAB	
3.50	OXA2(+1) MN+2(+1)	E	STAB	708
4.80	OXA2(+2) MN+2(+1)	E	STAB	
4.40	OXA2(+1) ZN+2(+1)	E	STAB	
7.00	OXA2(+2) ZN+2(+1)	E	STAB	
7.50	OXA2(+3) ZN+2(+1)	E	STAB	
5.15	OXA2(+1) ZN+2(+1) H +1(+1)	E	STAB	
7.55	OXA2(+2) ZN+2(+1) H +1(+2)	E	STAB	
2.30	PVA1(+1) H +1(+1)	E	STAB	710
0.75	PVA1(+1) CA+2(+1)	E	STAB	
2.20	PVA1(+1) CU+2(+1)	E	STAB	711 250
4.20	PVA1(+2) CU+2(+1)	E	STAB	250
1.20	PVA1(+1) FE+2(+1)	F		
1.90	PVA1(+2) FE+2(+1)	F		
4.00	PVA1(+1) FE+3(+1)	F		
6.00	PVA1(+2) FE+3(+1)	F		
1.50	PVA1(+1) PB+2(+1)	E	250	
2.80	PVA1(+2) PB+2(+1)	E	250	
0.75	PVA1(+1) MG+2(+1)	F		
1.00	PVA1(+1) MN+2(+1)	E	STAB	
1.50	PVA1(+1) ZN+2(+1)	E	710	
2.20	PVA1(+2) ZN+2(+1)	E	STAB	710
13.000	SLA2(+1) H +1(+1)	D	STAB	
15.814	SLA2(+1) H +1(+2)	D	STAB	
0.20	SLA2(+1) CA+2(+1)	E	STAB	
0.30	SLA2(+2) CA+2(+1)	F		
13.10	SLA2(+1) CA+2(+1) H +1(+1)	F		
10.133	SLA2(+1) CU+2(+1)	D	STAB	
18.20	SLA2(+2) CU+2(+1)	D	STAB	
6.00	SLA2(+1) FE+2(+1)	E	STAB	
10.00	SLA2(+2) FE+2(+1)	E	STAB	
16.00	SLA2(+1) FE+3(+1)	E	STAB	512
27.00	SLA2(+2) FE+3(+1)	E	STAB	512
34.00	SLA2(+3) FE+3(+1)	E	STAB	
17.00	SLA2(+1) FE+3(+1) H +1(+1)	E	STAB	
5.50	SLA2(+1) PB+2(+1)	F		
9.00	SLA2(+2) PB+2(+1)	F		
0.25	SLA2(+1) MG+2(+1)	F		

0.35	SLA2(+2) MG+2(+1)		F	
5.50	SLA2(+1) MN+2(+1)		E	STAB
9.00	SLA2(+2) MN+2(+1)		E	STAB
6.50	SLA2(+1) ZN+2(+1)		E	STAB
10.50	SLA2(+2) ZN+2(+1)		F	
5.19	SCA2(+1) H +1(+1)		E	512
9.13	SCA2(+1) H +1(+2)		E	512 316
1.00	SCA2(+1) CA+2(+1)		E	STAB
1.50	SCA2(+2) CA+2(+1)		F	
5.60	SCA2(+1) CA+2(+1)	H +1(+1)	E	STAB
2.40	SCA2(+1) CU+2(+1)		E	STAB
3.40	SCA2(+2) CU+2(+1)		F	
6.60	SCA2(+1) CU+2(+1)	H +1(+1)	E	STAB
1.42	SCA2(+1) FE+2(+1)		E	316
2.92	SCA2(+2) FE+2(+1)		E	316
7.30	SCA2(+1) FE+3(+1)		E	STAB 512
12.30	SCA2(+2) FE+3(+1)		E	512
1.40	SCA2(+1) PB+2(+1)		E	STAB
2.50	SCA2(+2) PB+2(+1)		F	
1.00	SCA2(+1) MG+2(+1)		E	STAB
1.50	SCA2(+2) MG+2(+1)		F	
5.50	SCA2(+1) MG+2(+1)	H +1(+1)	E	STAB
1.30	SCA2(+1) MN+2(+1)		E	STAB
1.50	SCA2(+1) ZN+2(+1)		E	STAB
2.30	SCA2(+2) ZN+2(+1)		F	
5.90	SCA2(+1) ZN+2(+1)	H +1(+1)	E	STAB
10.35	ACA2(+1) H +1(+1)		E	316
14.31	ACA2(+1) H +1(+2)		E	316
10.45	ACA2(+1) CA+2(+1)	H +1(+1)	E	STAB 233 234 235 713 714
11.35	ACA2(+1) CU+2(+1)	H +1(+1)	E	STAB
7.10	ACA2(+1) FE+2(+1)		F	
11.00	ACA2(+1) PB+2(+1)	H +1(+1)	E	713 714
10.45	ACA2(+1) MG+2(+1)	H +1(+1)	F	
10.80	ACA2(+1) MN+2(+1)	H +1(+1)	F	
11.00	ACA2(+1) ZN+2(+1)	H +1(+1)	F	

THE FORMATION CONSTANTS ARE GIVEN AS LOGS. THE SYMBOLS USED ARE DEFINED IN APPENDIX 5.7. THE COLUMN OF ALPHABETIC CHARACTERS, THE WORD 'STAB,' AND THE NUMBERS ON THE RIGHT HAND SIDE SERVE ONLY TO INDICATE THE SOURCE OF THE FORMATION CONSTANT. THEY DO NOT APPEAR IN THE ECCLES DATA FILE. 'STAB,' MEANS THAT A VALUE FOR THE FORMATION CONSTANT OF THE COMPLEX SPECIES IN QUESTION APPEARS IN THE CHEN, SOC. SPECIAL PUBLICATIONS NOS. 17 OR 25 ON 'STABILITY CONSTANTS', THE REFERENCE NUMBERS ARE THOSE LISTED FOR THIS THESIS.

THE ALPHABETIC CHARACTERS INDICATE THE FOLLOWING:

D - DIRECT MEASUREMENT: THE FORMATION CONSTANT HAS BEEN DETERMINED UNDER PHYSIOLOGICAL CONDITIONS OF TEMPERATURE AND IONIC STRENGTH.

E - ESTIMATED: THE FORMATION CONSTANT HAS BEEN MEASURED EXPERIMENTALLY BUT HAS BEEN ADJUSTED TO SUIT THE MODEL.

G - GUESSED: NO FORMATION CONSTANT HAS BEEN FOUNDIN THE LITERATURE. THE VALUE HAS BEEN ESTIMATED USING VARIOUS TYPES OF CHEMICAL TREND.

DATA USED FOR THE TERNARY FORMATION CONSTANTS.

9.06	ZN+2(+1)	AAA1(+2)		FROM REF 98
8.54	ZN+2(+1)	ALA1(+2)		
9.06	ZN+2(+1)	GLY1(+2)		
4.80	ZN+2(+1)	NH30(+2)		
7.17	ZN+2(+1)	ALA1(+1)	NH30(+1)	
7.52	ZN+2(+1)	AAA1(+1)	NH30(+1)	
7.52	ZN+2(+1)	GLY1(+1)	NH30(+1)	
8.92	ZN+2(+1)	ALA1(+1)	NH30(+2)	FROM REF 98
10.28	ZN+2(+1)	ALA1(+2)	NH30(+1)	
9.14	ZN+2(+1)	GLY1(+1)	NH30(+2)	
10.52	ZN+2(+1)	GLY1(+2)	NH30(+1)	
9.14	ZN+2(+1)	AAA1(+1)	NH30(+2)	
10.52	ZN+2(+1)	AAA1(+2)	NH30(+1)	
4.80	ZN+2(+1)	NH30(+2)		
0.40		AAA1		
0.40		ABA1		
0.40		APG1		
0.40		ASN1		
0.30		ASP2		

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 0.40                CIT1
 0.40                CYS2
 0.40                CIS2
 0.40                GLU2
 0.40                GLN1
 0.40                HIS1
 0.40                HYP1
 0.40                ILE1
 0.40                LEU1
 0.40                LYS1
 0.40                MET1
 0.40                ORN1
 0.40                PHE1
 0.40                PRO1
 0.30                SER1
 0.40                THR1
 0.40                TRP1
 0.40                TYR2
 0.40                VAL1

 8.09      CU+2(+1)  CTA3(+2)                          FROM REF 486
16.30      CU+2(+1)  PRO1(+2)
14.20      CU+2(+1)  CTA3(+1)  PRO1(+1)

 3.20      CU+2(+1)  LTA1(+2)
 0.45                AAA1
 0.45                ALA1
 0.45                ABA1
 0.45                ARG1
 0.45                ASN1
 0.35                ASP2
 0.45                CIT1
 0.45                CYS2
 0.45                CIS2
 0.45                GLU2
 0.45                GLN1
 0.45                GLY1
 0.45                HIS1
 0.45                HYP1
 0.45                ILE1
 0.45                LEU1
 0.45                LYS1
 0.45                MET1
 0.45                ORN1
 0.45                PHE1
 0.45                PRO1
 0.35                SER1
 0.45                THR1
 0.45                TRP1
 0.45                TYR2
 0.45                VAL1

11.80      ZN+2(+1)  PVA1(+1)  GLY1(+2)          IN 'STABILITY CONSTANTS'
14.20      ZN+2(+1)  PVA1(+2)  GLY1(+2)
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 9.00     ZN+2(+1) PVA1(+1) ILE1(+2)
11.70     ZN+2(+1) PVA1(+2) ILE1(+2)
 9.90     ZN+2(+1) PVA1(+1) ALA1(+2)
10.00     ZN+2(+1) PVA1(+2) ALA1(+2)

 1.95     ZN+2(+1) PVA1(+2)                                IN 'STABILITY CONSTANTS'
 9.01     ZN+2(+1) GLY1(+2)
 8.52     ZN+2(+1) ALA1(+2)
 8.49     ZN+2(+1) ILE1(+2)
 7.58     ZN+2(+1) PVA1(+1) GLY1(+1)
 6.79     ZN+2(+1) PVA1(+1) ALA1(+1)
 6.76     ZN+2(+1) PVA1(+1) ILE1(+1)

 1.98     ZN+2(+1) PVA1(+2)
 0.70              AAA1
 0.90              ABA1
 0.70              ARG1
 0.70              ASN1
 0.60              ASP2
 0.70              CIT1
 0.70              CYS2
 0.70              CIS2
 0.70              GLU2
 0.70              GLN1
 0.70              HIS1
 0.90              LEU1
 0.70              LYS1
 0.70              MET1
 0.70              ORN1
 0.70              PHE1
 0.70              PRO1
 0.60              SER1
 0.70              THR1
 0.70              TRP1
 0.70              TYR2
 0.90              VAL1

 8.09     CU+2(+1) CTA3(+2)
 1.00              AAA1
 1.00              ALA1
 1.00              ABA1
 1.00              ARG1
 1.00              ASN1
 0.90              ASP2
 1.00              CIT1
 1.00              CYS2
 1.00              CIS2
 1.00              GLU2
 1.00              GLN1
 1.00              GLY1
 1.00              HIS1
 1.00              HYP1
 1.00              ILE1
 1.00              LEU1
```

1.00		LYS1	
1.00		MET1	
1.00		ORN1	
1.00		PHE1	
0.90		SER1	
1.00		THR1	
1.00		TRP1	
1.00		TYR2	
1.00		VAL1	
15.76	CU+2(+1)	ASP2(+2)	
0.45		AAA1	
0.45		ABA1	
0.45		ARG1	
0.45		ASN1	
0.45		CIT1	
0.45		CYS2	
0.45		CYS2	
0.45		GLN1	
0.45		HIS1	
0.45		HYP1	
0.45		ILE1	
0.45		LEU1	
0.45		LYS1	
0.45		MET1	
0.45		ORN1	
0.45		PHE1	
0.45		PRO1	
0.25		SER1	
0.45		THR1	
0.45		TRP1	
0.45		TYR2	
0.45		VAL1	
14.61	CU+2(+1)	GLU2(+2)	FROM REF 379
18.45	CU+2(+1)	HIS1(+2)	
26.85	CU+2(+1)	HIS1(+2) H +1(+2)	
14.59	CU+2(+1)	SER1(+2)	
14.69	CU+2(+1)	THR1(+2)	
17.62	CU+2(+1)	HIS1(+1) GLU2(+1)	
21.65	CU+2(+1)	HIS1(+1) GLU2(+1) H +1(+1)	
17.54	CU+2(+1)	HIS1(+1) SER1(+1)	
21.70	CU+2(+1)	HIS1(+1) SER1(+1) H +1(+1)	
17.58	CU+2(+1)	HIS1(+1) THR1(+1)	
21.80	CU+2(+1)	HIS1(+1) THR1(+1) H +1(+1)	
15.01	CU+2(+1)	AAA1(+2)	FROM REF 83
14.99	CU+2(+1)	ALA1(+2)	
14.93	CU+2(+1)	ABA1(+2)	
15.11	CU+2(+1)	GLY1(+2)	
14.77	CU+2(+1)	PHE1(+2)	
14.57	CU+2(+1)	SER1(+2)	
14.77	CU+2(+1)	THR1(+2)	
34.84	CU+2(+1)	TYR2(+2) H +1(+2)	

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15.32     CU+2(+1) AAA1(+1) GLY1(+1)
15.25     CU+2(+1) AAA1(+1) PHE1(+1)
15.09     CU+2(+1) AAA1(+1) SER1(+1)
15.18     CU+2(+1) AAA1(+1) THR1(+1)
25.21     CU+2(+1) AAA1(+1) TYR2(+1) H +1(+1)
15.27     CU+2(+1) ALA1(+1) ABA1(+1)
15.24     CU+2(+1) ALA1(+1) PHE1(+1)
15.23     CU+2(+1) ALA1(+1) THR1(+1)
25.23     CU+2(+1) ALA1(+1) TYR2(+1) H +1(+1)
15.28     CU+2(+1) ABA1(+1) GLY1(+1)
15.21     CU+2(+1) ABA1(+1) PHE1(+1)
15.06     CU+2(+1) ABA1(+1) SER1(+1)
15.16     CU+2(+1) ABA1(+1) THR1(+1)
25.18     CU+2(+1) ABA1(+1) TYR2(+1) H +1(+1)
15.30     CU+2(+1) GLY1(+1) PHE1(+1)
15.24     CU+2(+1) GLY1(+1) THR1(+1)
25.24     CU+2(+1) GLY1(+1) TYR2(+1) H +1(+1)
15.00     CU+2(+1) PHE1(+1) SER1(+1)
15.09     CU+2(+1) PHE1(+1) THR1(+1)
25.09     CU+2(+1) PHE1(+1) TYR2(+1) H +1(+1)
14.95     CU+2(+1) SER1(+1) THR1(+1)
24.96     CU+2(+1) SER1(+1) TYR2(+1) H +1(+1)
25.06     CU+2(+1) THR1(+1) TYR2(+1) H +1(+1)

15.76     CU+2(+1) ASP2(+2)                          FROM REF 335
14.74     CU+2(+1) GLU2(+2)
14.84     CU+2(+1) GLY1(+2)
15.78     CU+2(+1) ASP2(+1) GLY1(+1)
15.63     CU+2(+1) ASP2(+1) GLU2(+1)
15.10     CU+2(+1) GLU2(+1) GLY1(+1)

14.82     CU+2(+1) ALA1(+2)                          FROM REF 81
15.02     CU+2(+1) GLY1(+2)
14.57     CU+2(+1) SER1(+2)
14.79     CU+2(+1) VAL1(+2)
15.05     CU+2(+1) ALA1(+1) GLY1(+1)
14.91     CU+2(+1) ALA1(+1) SER1(+1)
15.20     CU+2(+1) ALA1(+1) VAL1(+1)
14.66     CU+2(+1) GLY1(+1) SER1(+1)
15.06     CU+2(+1) GLY1(+1) VAL1(+1)
14.84     CU+2(+1) SER1(+1) VAL1(+1)

17.00     PB+2(+1) CYS2(+2)                          FROM REF 337
29.00     PB+2(+1) PO43(+2) H +1(+2)
 8.50     PB+2(+1) CTA3(+2)
27.53     PB+2(+1) CYS2(+1) PO43(+1) H +1(+1)
18.27     PB+2(+1) CYS2(+1) CTA3(+1)
20.95     PB+2(+1) PO43(+1) CTA3(+1) H +1(+1)

19.30     ZN+2(+1) CYS2(+2)                          FROM REF 337
29.00     ZN+2(+1) PO43(+2) H +1(+2)
 8.70     ZN+2(+1) CTA3(+2)
25.14     ZN+2(+1) CYS2(+1) PO43(+1) H +1(+1)
16.59     ZN+2(+1) CYS2(+1) CTA3(+1)
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21.72	ZN+2(+1)	PO43(+1)	CTA3(+1)	H +1(+1)	
18.51	CU+2(+1)	CIS2(+1)	HIS1(+1)		FROM REF 158
25.80	CU+2(+1)	CIS2(+1)	HIS1(+1)	H +1(+1)	
30.69	CU+2(+1)	CIS2(+1)	HIS1(+1)	H +1(+2)	
39.15	CU+2(+2)	CIS2(+1)	HIS1(+2)	H +1(+1)	
11.62	ZN+2(+1)	CIS2(+1)	HIS1(+1)		FROM REF 158
19.55	ZN+2(+1)	CIS2(+1)	HIS1(+1)	H +1(+1)	
26.35	ZN+2(+1)	CIS2(+1)	HIS1(+1)	H +1(+2)	
15.23	ZN+2(+1)	CYS2(+1)	HIS1(+1)		
21.60	ZN+2(+1)	CYS2(+1)	HIS1(+1)	H +1(+1)	
26.50	ZN+2(+1)	CYS2(+1)	HIS1(+2)	H +1(+1)	
10.75	ZN+2(+1)	HIS1(+1)	GLU2(+1)		
17.72	ZN+2(+1)	HIS1(+1)	GLY1(+1)	H +1(+1)	
23.10	ZN+2(+1)	HIS1(+1)	GLY1(+2)	H +1(+1)	
22.79	ZN+2(+1)	HIS1(+2)	GLY1(+1)	H +1(+1)	
14.29	CU+2(+1)	ASN1(+2)			FROM REF 504
14.00	CU+2(+1)	GLN1(+2)			
14.83	CU+2(+1)	GLY1(+2)			
14.35	CU+2(+1)	SER1(+2)			
14.91	CU+2(+1)	ASN1(+1)	GLY1(+1)		
14.62	CU+2(+1)	ASN1(+1)	SER1(+1)		
14.78	CU+2(+1)	GLN1(+1)	GLY1(+1)		
14.53	CU+2(+1)	GLN1(+1)	SER1(+1)		
10.90	FE+3(+1)	CTA3(+2)			
1.40		LTA1			
1.50		MLA2			
1.60		OXA2			
1.30		PVA1			
1.50		SLA2			
1.50		SCA2			
0.90		AAA1			
0.90		ALA1			
0.90		ABA1			
0.90		ARG1			
0.90		ASN1			
0.90		ASP2			
0.90		CIT1			
0.90		CYS2			
0.90		CIS2			
0.90		GLU2			
0.90		GLN1			
0.90		GLY1			
0.90		HIS1			
0.90		HYP1			
0.90		ILE1			
0.90		LEU1			
0.90		LYS1			
0.90		MET1			
0.90		ORN1			
0.90		PHE1			

```
0.90                SER1
0.90                THR1
0.90                TRP1
0.90                TYR2
0.90                VAL1

6.50      FE+3(+1)  OXA2(+2)
1.00                LTA1
1.00                MLA2
0.90                PVA1
1.00                SCA2
1.00                SLA2

9.98      CU+2(+1)  OXA2(+2)                                  FROM REF 630
4.00      CU+2(+1)  SCA2(+2)
7.80      CU+2(+1)  OXA2(+1)  SCA2(+1)

9.98      CU+2(+1)  OXA2(+2)
1.20                CTA3
0.80                LTA1
1.00                MLA2
0.80                PVA1
1.00                SLA2
```

appendix 5·7

EXAMPLE OF BLOOD PLASMA MODEL COMPONENT CONCENTRATIONS
AND OF THE CORRESPONDING INPUT DATA FOR PROGRAM ECCLES.

```
SIMULATION OF THE METAL ION / LIGAND DISTRIBUTION IN BLOOD PLASMA.
P.M. MAY
DECEMBER 1975
TRUE  CU+2 CA+2 MG+2 MN+2 FE+3 ZN+2
TRUE  CU+2     10000.0     0.001
MULTIPLY
SUPRESS OUTPUT    :
TOTAL CONCENTRATIONS
AAA1 0.000786          (AVERAGE AMINO ACID COMPONENT)
ARG1 0.000185          (ARGININE)
ASN1 0.000055          (ASPARAGINE)
ASP2 0.000005          (ASPARTIC ACID)
CIT1 0.000027          (CITRULLINE)
CYS2 0.000023          (CYSTEINE)
CIS2 0.000040          (CYSTINE)
GLU2 0.000048          (GLUTAMIC ACID)
GLN1 0.000521          (GLUTAMINE)
GLY1 0.000243          (GLYCINE)
HIS1 0.000085          (HISTIDINE)
HYP1 0.000007          (HYDROXYPROLINE)
LYS1 0.000178          (LYSINE)
MET1 0.000029          (METHIONINE)
ORN1 0.000058          (ORNITHINE)
PHE1 0.000064          (PHENYLALANINE)
PRO1 0.000211          (PROLINE)
SER1 0.000122          (SERINE)
THR1 0.000150          (THREONINE)
TRP1 0.000010          (TRYPTOPHAN)
TYR2 0.000058          (TYROSINE)
C032 0.024500          (CARBONATE)
P043 0.000381          (PHOSPHATE)
SIL2 0.000138          (SILICATE)
S042 0.000211          (SULPHATE)
SCN1 0.000014          (THIOCYNATE)
NH30 0.000021          (AMMONIA)
CTA3 0.000113          (CITRATE)
LTA1 0.001818          (LACTATE)
MLA2 0.000035          (MALATE)
OXA2 0.000009          (OXALATE)
PVA1 0.000095          (PYRUVATE)
```

```
SLA2 0.000005          (SALICYLATE)
SCA2 0.000042          (SUCCINATE)
ACA2 0.000043          (ASCORBATE)
FREE CONCENTRATIONS
H +1 0.00000004        (HYDROGEN IONS)
OH-1 0.00000060        (HYDROXYL IONS)
CA+2 0.00114           (CALCIUM IONS)
CU+2   1.0E-18         (CUPROUS IONS)
FE+3   1.0E-23         (FERRIC IONS)
PB+2   1.0E-14         (PLUMBOUS IONS)
MG+2 0.00052           (MAGNESIUM IONS)
MN+2   1.0E-12         (MANGANOUS IONS)
ZN+2   1.00E-9         (ZINC IONS)
SPECIE CONSTANTS.
```

THE NAMES IN PARENTHESES ONLY SERVE TO IDENTIFY THE SYMBOLS. THEY DO NOT APPEAR IN THE ECCLES DATA FILE. ALL CONCENTRATIONS ARE IN MOLES/LITRE.

MOST OF THE CONCENTRATION DATA IS TAKEN FROM STANDARD BIOLOGICAL DATA HANDBOOKS (FOR EXAMPLE, 577, 647, 648). OTHER REFERENCES USED INCLUDE 158, 369, 666, 145, 416, 722, 723, 724, 725, 731, 732, 733, 734, 735, 193, 247.