THE STRUCTURE AND FUNCTION OF APOFERRITIN

by

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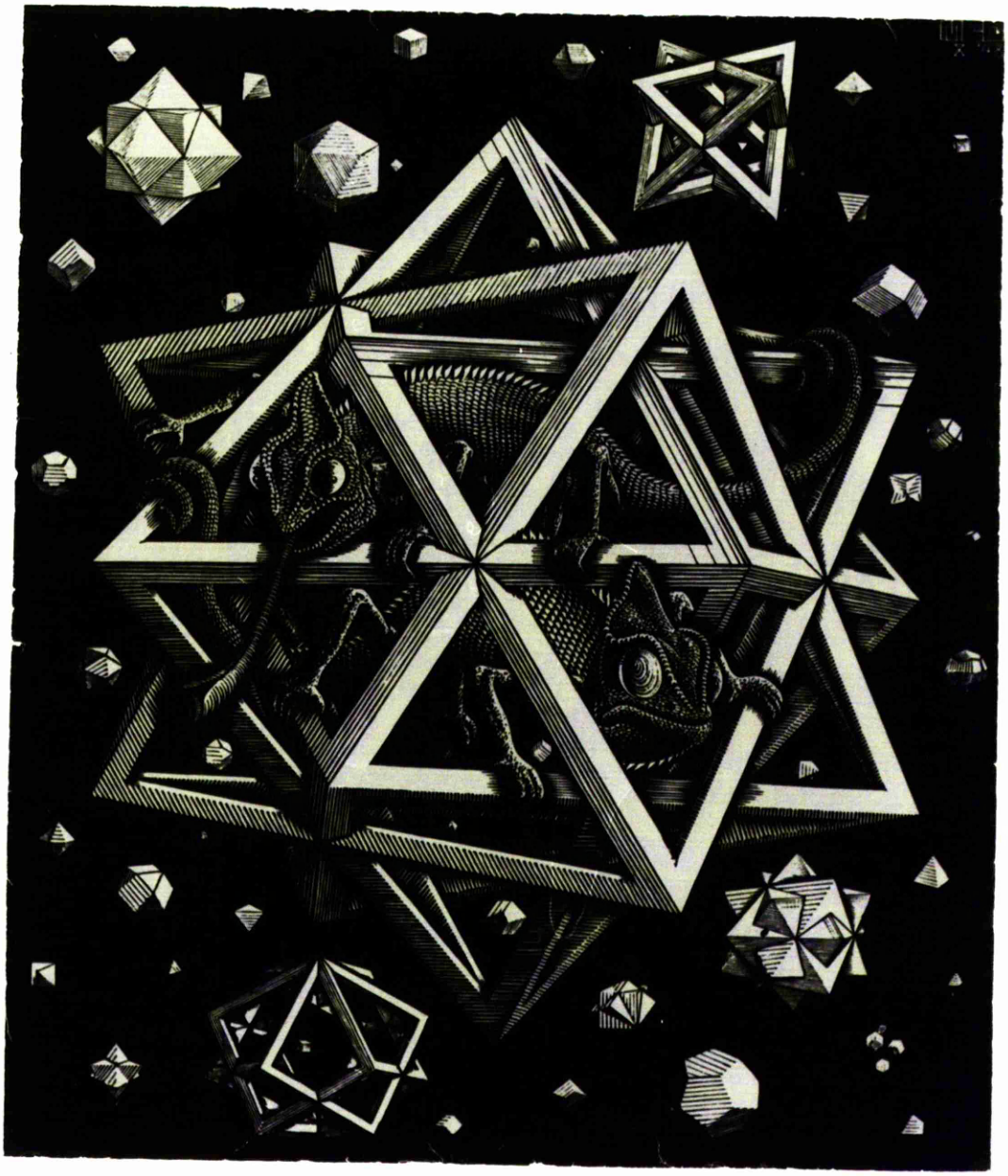


INTRODUCTION

"The reasonings about the wonderful and intricate operations of Nature are so full of uncertainty, that, as the Wise-man truly observes, hardly do we guess aright at the things that are upon earth, and with labour do we find the things that are before us."

(Stephen Hales, 1727)

Despite the fact that in ancient times, Egyptian, Greek and Roman physicians were aware of the importance of iron as a therapeutic agent it was not until some three hundred years ago that important contributions to our knowledge of the biological role of iron were made, and since that time our understanding of the subject has developed enormously.

It was at the end of the seventeenth century that Sydenham (1850) first described the mode of action of iron in the affliction known, both to the veterinarian and the botanist alike, as chlorosis. Some years later, in 1713, Lemmery and Geoffroy (1903) observed the presence of iron in both animal and vegetable tissues. Following this work several tissues were examined and shown to contain various types of "haemin" iron, however, as a result of the difficulty in analysing such small quantities of this trace element, some of these findings were misinterpreted and were the basis of controversies. One such controversy was between Levy (1889) and McMunn (1886) and concerned the distribution of iron in tissues. Levy was convinced that haem was a chemical unique to red blood cells while the studies of McMunn demonstrated the ubiquity of this substance. By 1867 Hoppe-Seyler (1890) had managed to isolate and crystallise haemoglobin from red blood corpuscles.

Up to about this time iron had always been found as haem (i.e. complexed with porphyrin) and non-haem iron was not in fact

described until the present century. It was in 1925 that the two French scientists, Fontès and Thivolle (1925), succeeded in demonstrating the presence of such non-haem iron in the circulating plasma and Barkam (1927) later showed that the iron was protein-bound and also described a method for the determination of such iron.

At about this time histologists had observed small, dark, water-insoluble granules which they called haemosiderin and they assumed these to be the result of rapid destruction of red blood cells (Cook, 1929). In 1934 Laufberger (1934) isolated from horse spleen a brown-coloured material which he called ferritin and by 1937 he had managed to purify and crystallise some ferritin with cadmium sulphate (Laufberger, 1937). In 1949, Surgenor, Koechlin and Strong (1949) isolated siderophilin (transferrin), a serum protein with the specific properties of binding and transporting iron, which Koechlin (1952) managed to crystallise some three years later.

Historically speaking, we are to all intents and purposes now at the point in time in which we started gaining, as is witnessed by the ever increasing bibliography, a more profound understanding of the actual function fulfilled by iron as a trace element in the physiology, biochemistry and pathology of the cell at the molecular level.

When we speak of iron in the context of biochemistry we often think only of haemoglobin and possibly of some of the problems concerning anaemias. However, if we ever had recourse to the extensive bibliography we would soon see that the importance of iron in cell function is far from limited to haematology. Instead it is found that iron participates in transport and storage and is also involved in numerous enzymatic systems owing to its

ability to catalyse oxido-reduction mechanisms by a simple change of valency. The main classes of iron-containing proteins are listed in Table 1.

Such proteins are intimately associated with both the physical and the chemical activities that constitute the normal functioning of the cell. It is now a well known fact that while some proteins serve as important structural elements of the body (hair, collagen), others may be enzymes, hormones, proteins associated with genes (histones), oxygen carriers, proteins that are concerned with immunological defence (antibodies) and a variety of other functions. In essence, it has become the object of the protein chemist to attempt to explain the particular physiological and biochemical functions of these macromolecules in terms of their molecular structure. The nature of the present problem was to consider the iron-storage protein, ferritin, in the light of such a structure-function relationship.

General Properties of Ferritin and Apoferritin.

Haemoglobin and myoglobin together account for about three-quarters of the total body iron while about 90% of the remainder is in the form of the two principal iron-storage proteins, ferritin and haemosiderin.

For haemosiderin it will suffice at this stage to merely indicate the probable relationship that this molecular species has with ferritin and to briefly outline some of the similar and some of the dissimilar properties that it has with ferritin. Haemosiderin is a dark, water-insoluble, granular substance which can easily be seen microscopically and, electron micrographs of this material show aggregates of electron-dense particles. Principally because of this ease of detection and because its

Table 1. The table lists the main classes of iron-containing proteins and was taken from 'Biochemical Functions of Iron', by B-G. Malmstrom, in "Iron Deficiency, Pathogenesis, Clinical Aspects, Therapy", edited by L. Hallberg, H-G. Harweth and A. Vannoti, Academic Press, London and New York, (1969), p. 10.

Protein	Haem (H) or non-Haem (N)	Function
Haemoglobin, myoglobin	H	O_2 Carrier
Hydroperoxidases (catalase, peroxidase)	H	Enzymes with H_2O_2 as substrate
Cytochromes	H	Electron carriers
Cytochrome c oxidase[a]	H	Terminal oxidase
Flavoprotein dehydrogenases and oxidases	N	Oxidising enzymes
Oxygenases[b]	N[c]	Enzymes catalysing incorporation of O_2 into substrates
Ferredoxin	N	Electron carrier
Haemerythrin	N	O_2 Carrier
Transferrins (blood, egg-white, milk)	N	Transport, storage
Ferritin	N	Transport, storage

[a]This enzyme contains copper as well

[b]Not all members of this class are iron-proteins

[c]One oxygenase (tryptophan 2,3-dioxygenase) is a haem-protein

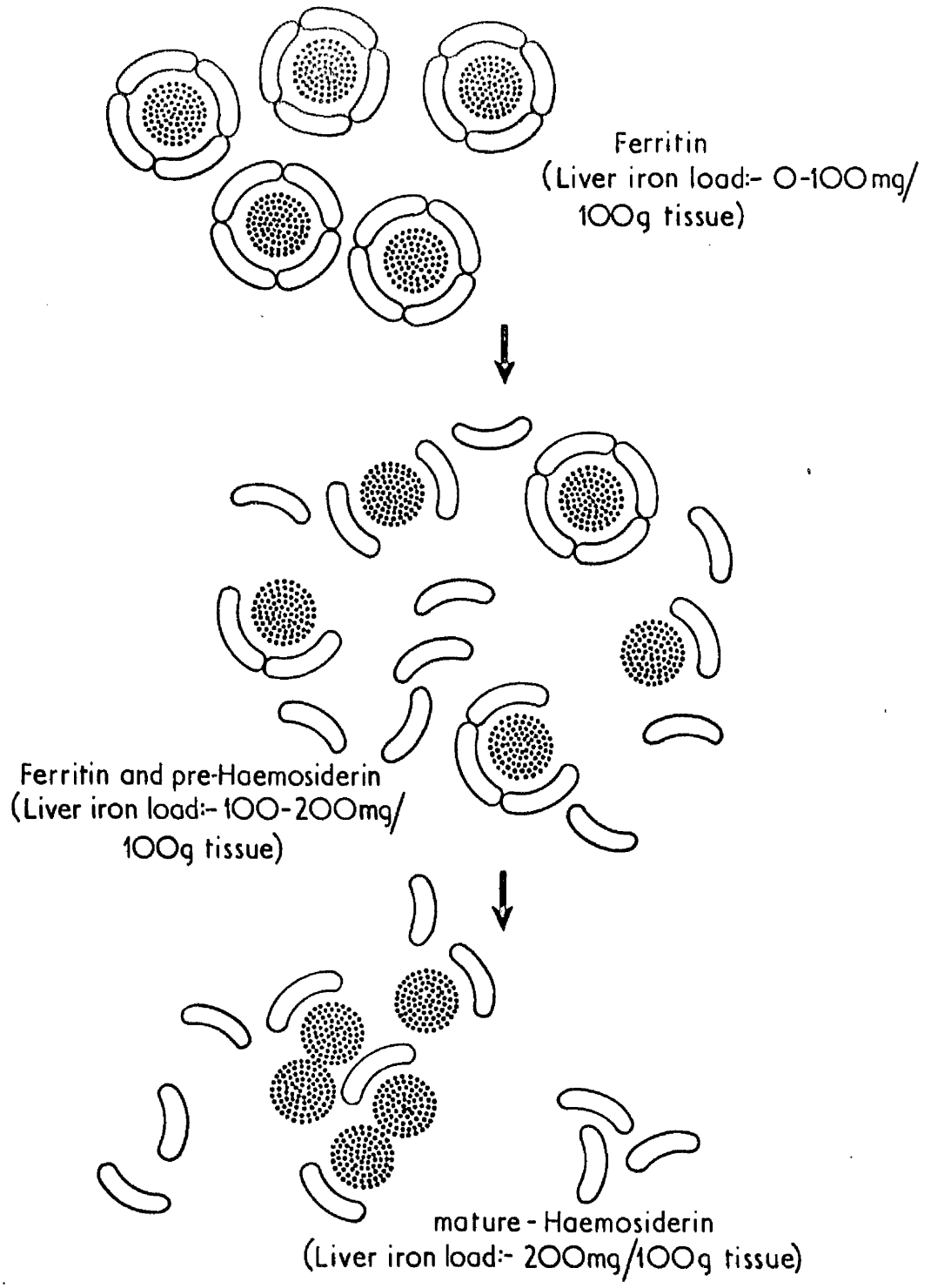
solubility properties tended to defy conventional systematic biochemical analysis it received considerably more attention from histologists and anatomists than from the biochemists. On the other hand, ferritin, despite its empirical interest to electron microscopists, was a much more suitable target for the interest of the protein chemist principally because of its water solubility and relative ease of extraction. The number of individuals who had actually carried out experimental work on both these materials remained extremely small and this dichotomy persisted until just about a decade ago. This gap between the various disciplines was finally bridged as a result of the work carried out principally by the two American scientists, Shoden and Sturgeon (1958, 1959, 1960, 1961, 1962a,b, 1963). The results of their intensive investigation revealed that haemosiderin was a rather heterogeneous entity, the composition of which was very variable. It accounted for about 20% of the total storage form of iron in the normal liver and was composed of dense granules which varied in size from less than 1 micron in diameter to about 5 microns. On quantitative analysis these granules had a substantially greater iron content than ferritin, the nitrogen content was much less and the phosphorus was again much greater. On dissolving in mercapto-acetic acid it showed, unlike ferritin, a peak at 412 nm which was assumed to be associated with the presence of porphyrin. Immediately prior to these studies certain workers (McKay and Fineberg, 1958; Richter, 1959; Wohler, 1960) had observed that the protein component of haemosiderin showed certain similarities to that of ferritin although McKay and Fineberg (1958) had also demonstrated the presence of other proteins. Michaelis, Coryell and Granick (1943) had also shown that the iron of haemosiderin was, for the main part, in the same magnetic

state as that in ferritin. The question that Shorden and Sturgeon asked themselves was, "What was the relationship between these two species ?" They had many similarities but just as many, if not more, dissimilar properties. They tackled this problem by considering what influenced the proportion of haemosiderin and ferritin in liver and they studied the kinetics of iron-storage by a series of double-label radio-isotopic experiments. Analysis of their data suggested that in the parenchymal cell, ferritin iron was the precursor of haemosiderin iron. Figure 1 shows schematically the proposed relationship that these workers envisaged and certainly at present it seems to take into account all of the available experimental data.

This now brings us to consider ferritin, that protein which accounts for the other 80% of the total body iron-storage capacity. The iron in ferritin is stored until the need for its function arises and is then readily available for utilisation elsewhere in the body, be it for haemoglobin or myoglobin synthesis or for cellular enzymes. It acts as a reserve depot against the possibility of an increased iron loss due to bleeding. Iron is also required during periods of rapid growth or for its transport to the foetus. Recently Arora, Lynch, Whitley and Alfrey (1970) published a paper entitled "The Ubiquity and Significance of Human Ferritin". Although probably the only criticism of this publication is the grammatical error in the title, a paper entitled "The Ubiquity of Ferritin" would certainly not be too far from the truth.

Ferritin has now been found in species as diverse as the marine Elasmobranch, Scyllium canicular and invertebrates (Teece, 1952; Roche, Bessis, Brenton-Gorius and Stralin, 1961; Towe, Lowenstam and Nesson, 1963), in plants as phytoferritin

Figure 1. This figure illustrates schematically the proposed relationship between ferritin and haemosiderin as a result of the investigations carried out by Shoden and Sturgeon (see text for references).


Ferritin
(Liver iron load:- 0-100mg/
100g tissue)
Ferritin and pre-Haemosiderin
(Liver iron load:- 100-200mg/
100g tissue)
mature - Haemosiderin
(Liver iron load:- 200mg/100g tissue)

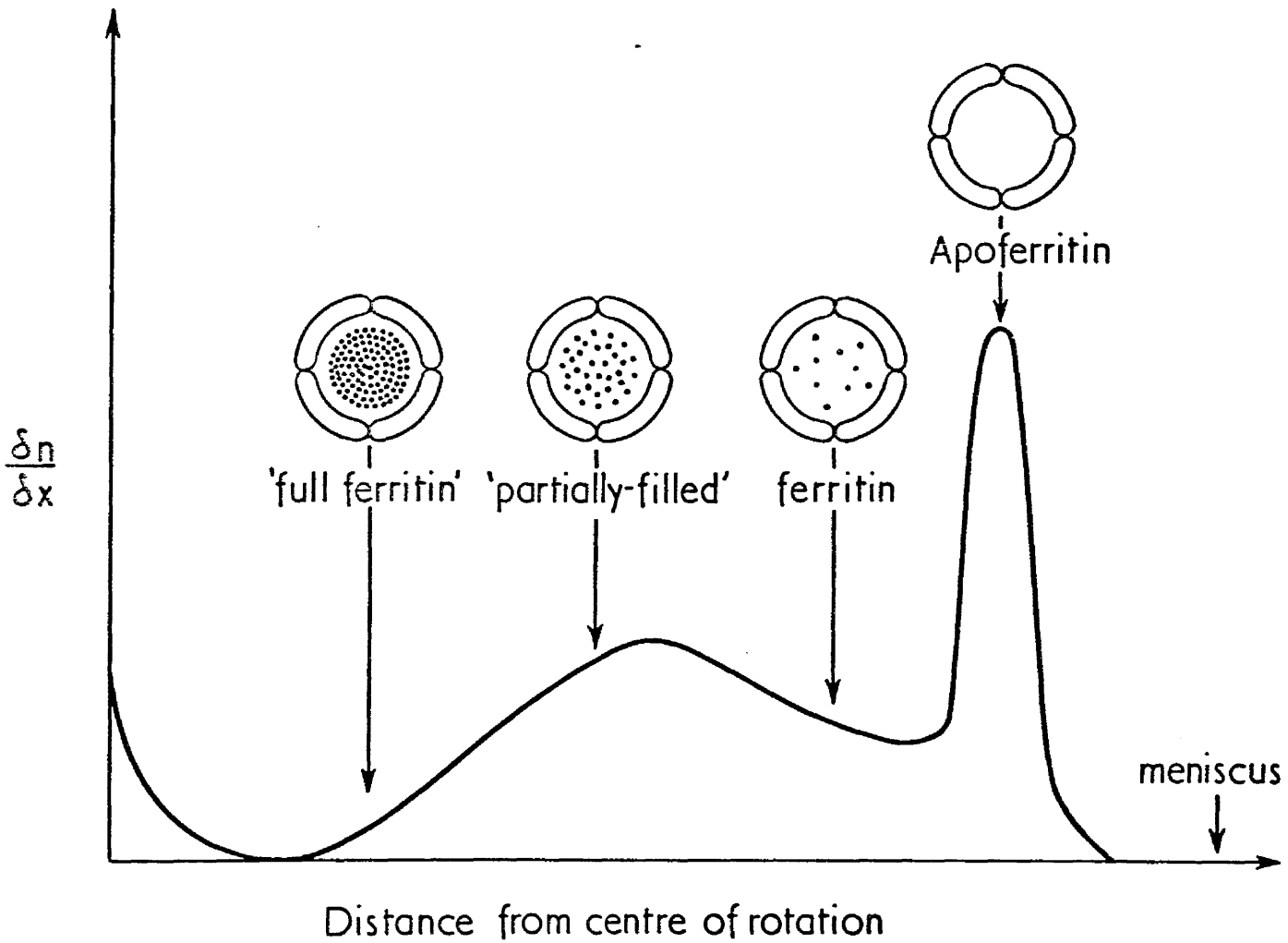
(Hyde, Hodge, Kahn and Birnstein, 1963), in the mycelium, sporangiospores and spores of the fungus Phycomyces blakesleeanus (David, 1969), in the hepatopancreas of Octopus vulgaris Lam (Nandi, Muzii and Puca, 1971), the dolphin (Delphinus cetacea) spleen (Kato and Shimada, 1970), in the eggs and early embryos of Rana piquiens (Brown and Caston, 1962), in tuna fish (Thunnus obesis) spleen (Kato, Shinjo and Shimada, 1968), and in a great number of mammals.

In mammals it is principally found in those organs which are intimately concerned with the reticuloendothelial system, namely the liver, spleen and bone marrow although ferritin has also been found to a lesser extent in extracts of the adrenal, brain, circulating plasma (in certain pathological conditions), heart, intestine, kidney, lung, neoplasms, pancreas, placenta, skeletal muscle, skin, testes and in the thyroid (Arora, Lynch, Whitley and Alfrey, 1970).

It has been shown by a number of workers (later section) that the iron contained in ferritin could be removed by reduction of the ferric iron to the ferrous state to give a colourless, iron-free protein called apoferritin. Rothen (1944) demonstrated that, when a fraction of ferritin was fractionated on a molecular weight basis, a distribution profile was obtained as shown in Figure 2 and that such a profile is clearly indicative of a heterogeneous species. He showed that the more slowly moving substance was in fact the iron-free protein, apoferritin, and that this constituted 25% of a ferritin preparation. Unlike the heterogeneity exhibited by the iron-containing ferritin particles, apoferritin gave on sedimentation a very sharp boundary and was considered to be a very homogeneous protein with a sedimentation coefficient of about 17.6 Svedberg units. As will be considered

Figure 2. Typical distribution profile obtained by sedimenting a sample of horse spleen ferritin. Such a sedimentation pattern was interpreted as shown in the figure, with the sharp uniform peak representing iron-free apoferritin while the more diffuse peak is composed of protein molecules differing only in their iron content.

Sedimentation Pattern of Horse Spleen Ferritin



in greater detail at a later stage, ferritin is thought to be a hollow protein shell surrounding an insoluble micelle of ferric hydroxyphosphate. Thus the heterogeneity exhibited by ferritin was assumed to result from varying amounts of iron contained within the protein shell and Van Bruggen (1962) claims to have demonstrated the presence of such full, empty and partially full molecules by electron microscopy and he has also estimated the number of each of these in various samples. This qualitative interpretation of Rothen's experimental data is also shown in Figure 2.

Isolation and Purification of Ferritin and Apoferritin.

Despite the fact that many protein and enzyme extractions and purifications are laborious and time-consuming they are an essential preliminary to any systematic investigation. By many standards, the extraction and purification of ferritin is a relatively simple procedure and the reasons that make it so are essentially three:

(a) the source of the material is both rich and reliable (at least for most of the ferritins so far studied).

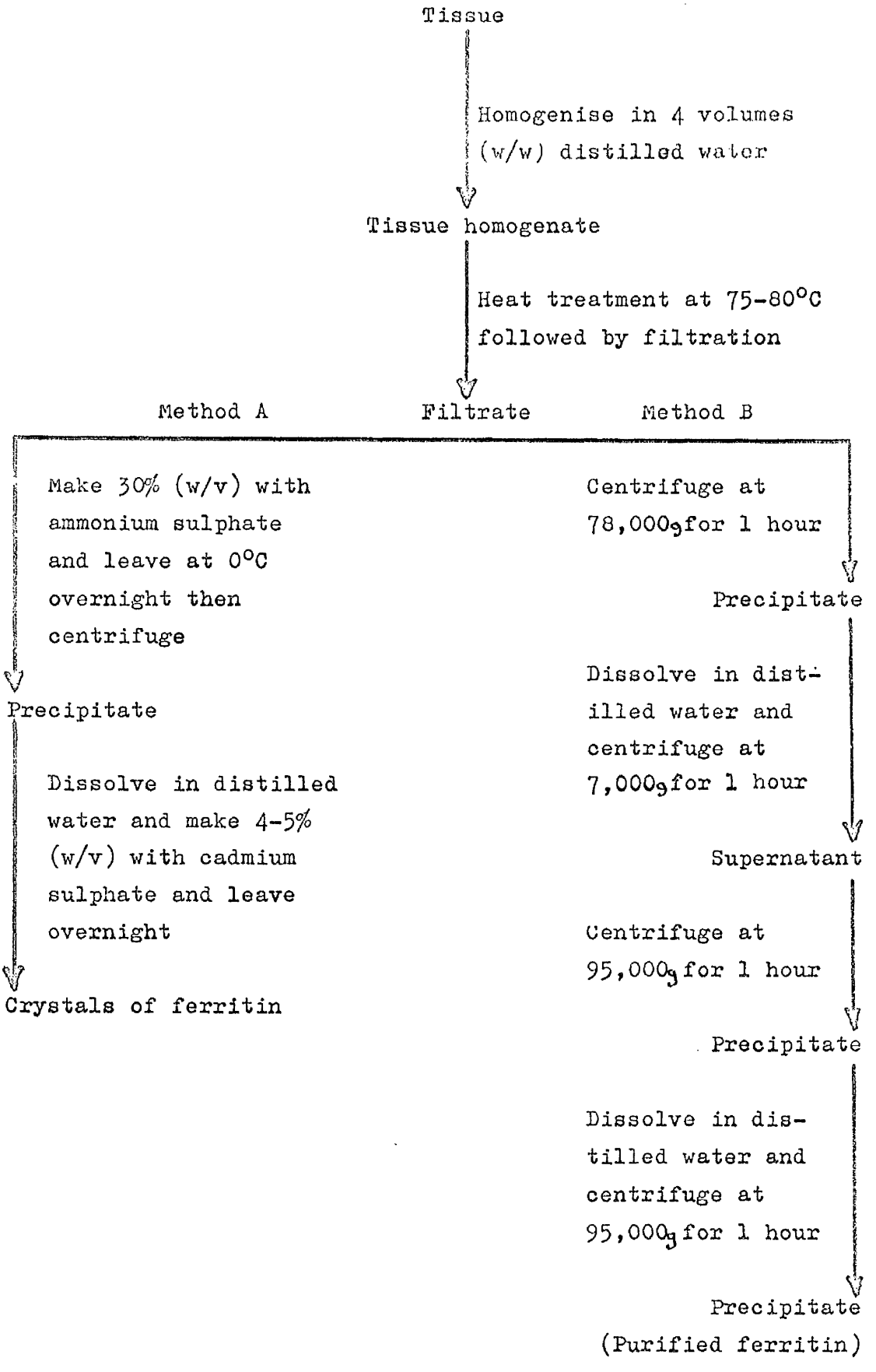
(b) the protein is heat-stable at 75-80^{o}C and,

(c) the large size of the protein-iron complex.

There are principally two routine methods for the isolation and purification of ferritin and these are shown in Figure 3. Method A makes use of the fact that ferritin can be crystallised from a 4-5% cadmium sulphate solution (Granick, 1942), while Method B utilises the large size and weight of ferritin to isolate this protein by ultracentrifugation (Penders, De Rooij-Dijk and Leijnse, 1968).

The type of ferritin crystals obtained by Method A are optically isotropic and the crystal form they adopt is that of a

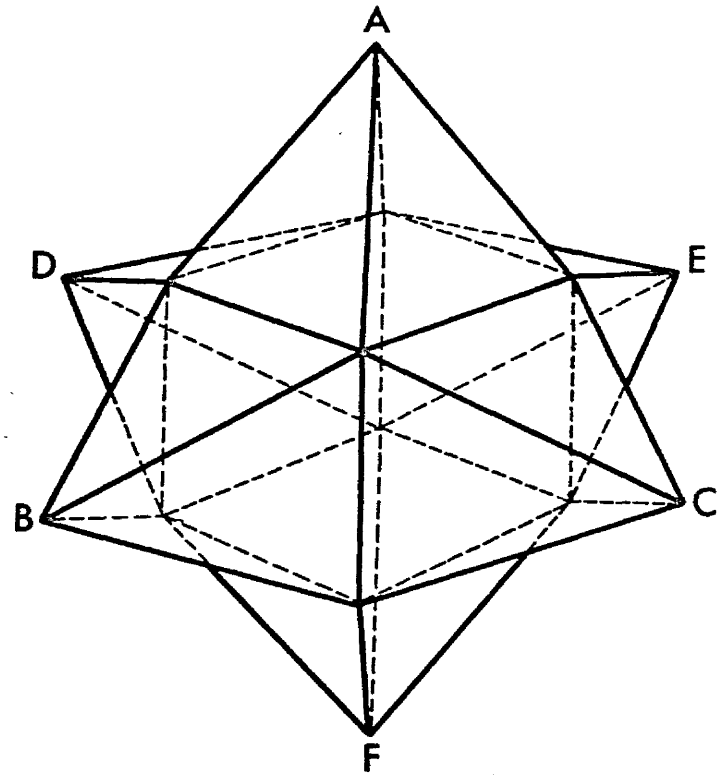
Figure 3. Principal routine methods for the isolation and purification of ferritin.


Tissue
Homogenise in 4 volumes (w/w) distilled water
Tissue homogenate
Heat treatment at 75-80°C followed by filtration
Filtrate
Method A
Make 30% (w/v) with ammonium sulphate and leave at 0°C overnight then centrifuge
Precipitate
Dissolve in distilled water and make 4-5% (w/v) with cadmium sulphate and leave overnight
Crystals of ferritin
Method B
Centrifuge at 78,000g for 1 hour
Precipitate
Dissolve in distilled water and centrifuge at 7,000g for 1 hour
Supernatant
Centrifuge at 95,000g for 1 hour
Precipitate
Dissolve in distilled water and centrifuge at 95,000g for 1 hour
Precipitate
(Purified ferritin)

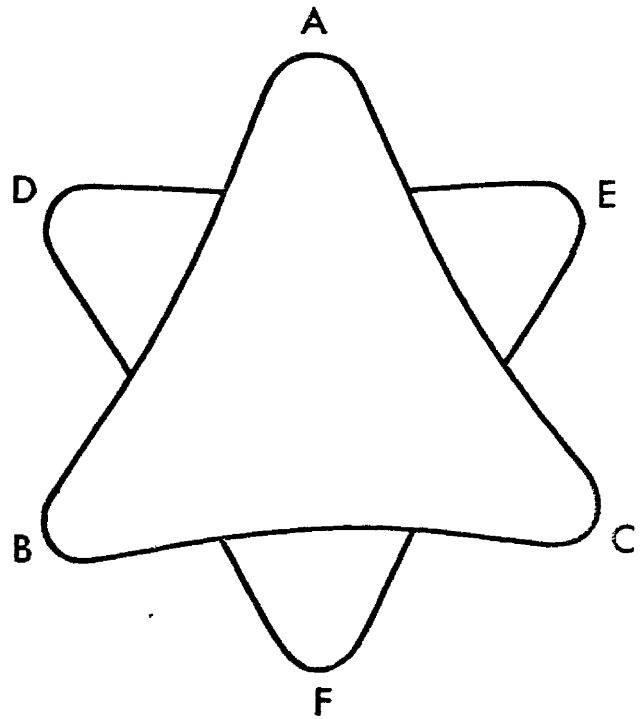
twinned octahedron (tetrahedral pyramids on each face of a cube) although the edges are found to be slightly curved as shown schematically in Figure 4.

It is generally accepted that the storage and mobilisation of iron in the body is controlled by its oxidation and reduction. In ferritin the iron exists in the ferric state and several workers have shown that it is possible to remove this by certain reducing agents. These can be roughly classified as two types, (a) the chemical reducing agents and (b) the biological reducing agents. Class (a), typified by reagents like sodium dithionite ($Na_2S_2O_4$), provide a useful laboratory method for the removal of the iron in the preparation of apoferritin (Granick and Michaelis, 1943; Behrens and Taubert, 1952), and class (b) consists of reagents like cysteine, glutathione, ascorbic acid, glucose and ATP (Mazur, Baez and Shorr, 1955b; Bielig and Bayer, 1955b; Miller and Perkins, 1969) which have been shown to release the ferric iron from ferritin, although at a much slower rate, the implication being that the latter may be concerned with such transfer *in vivo*. In this context, xanthine oxidase has been accredited with the physiological role of ferritin iron reduction and mobilisation (Mazur and Green, 1959). However, Davis and Deller (1946) have shown that several agents which inhibit xanthine oxidase have negligible effect on *in vivo* iron absorption. Release of iron from ferritin in tissue slices under anaerobic conditions has also been demonstrated (Mazur, Baez and Shorr, 1955). It was, in effect, in the search for such physiological agents involved in iron transfer from ferritin that some workers demonstrated that the transfer was affected by low molecular weight chelating agents (Miller and Perkins, 1969; Pape, Multani, Stitt and Saltman, 1968b). Although it is generally accepted

Figure 4. This figure shows schematically the crystalline form adopted by both ferritin and apoferritin. This is essentially that of a twinned octahedron as shown, the only difference being that in the crystal the edges are slightly curved. This schematic representation can be compared with actual crystals as shown in Figure 14.



Twinned Octahedron
(tetrahedra set on each face of a cube)



Ferritin Crystal Form
(optically isotropic with slight curved edges)

that these have an effect on transfer, there is no complete agreement as to their mode of action since some workers believe that these chelators work in the absence of redox systems (Pape, Multani, Stitt and Saltman, 1968b) while others believe that reducing agents are necessary and that these chelators merely enhance transfer (Miller and Perkins, 1969).

The apoferritin obtained by any of these techniques is colourless and crystallises from cadmium sulphate as octahedral crystals which are isomorphous with those of ferritin.

Structural Aspects of Ferritin and Apoferritin.

Gross Structure of Ferritin and Apoferritin.

Until about twenty years ago conflicting interpretations of the data existed as to the nature of the gross structure of these proteins. There existed at this time two schools of thought, the first believing that the iron micelles were situated outside the apoferritin shell while the second believed the converse to be true. Almost certainly the reason for the existence of such controversies over the then existing data was because such experimental data was obtained from rather indirect approaches to the solution of the problem. Thus Granick (1951) believed the micelles to exist on the surface of the protein because of the differences in the heat-coagulation profiles between solutions of ferritin and apoferritin. A ferritin solution coagulates reversibly at 60^{o}C whereas apoferritin does not and Granick believed that this was due to the presence of iron micelles on the surface of the protein (Granick, 1942). Also as it was shown that cysteine could effect the reduction of ferritin iron (Mazur, Litt and Shorr, 1950b) and since Granick considered that cysteine was too large

to penetrate between the surface polypeptide chains of apoferritin, he concluded that the micelles must be on the surface. The evidence for the existence of an iron-micelle surrounded by a protein shell seemed to be somewhat more weighty. Both Rothen (1944) and Mazur and Shorr (1950) demonstrated that ferritin and apoferritin have identical electrophoretic mobilities and since such mobilities depend upon surface charge densities, it was argued by these workers that the micelle could not be situated on the outside of the protein. Mazur and Shorr (1950) also showed that a precipitin-antibody to horse spleen ferritin would precipitate an identical amount of horse spleen apoferritin ~~protein~~ (based on its nitrogen content) and Mazur, Litt and Shorr (1950a) further demonstrated that solutions of ferritin and apoferritin have identical viscosities.

Such was the state of opinion when, in 1954, Farrant (1954) first demonstrated directly by electron microscopy that the iron micelles were situated on the inside of a hollow protein shell. This study by Farrant (1954) has since been confirmed by a variety of physical techniques including low resolution X-ray diffraction measurements on wet crystals (Harrison, 1963) and small angle X-ray scattering (Bielig, Kratky, Steiner and Wawra, 1963; Bielig, Kratky, Rohns and Wawra, 1964; Fischbach and Anderegg, 1965) as well as a more detailed investigation by many electron microscopists (Kuff and Dalton, 1957; Labaw, Wyckoff, 1957; Richter, 1959b; Bessis and Breton-Gorius, 1960; Van Bruggen, Wiebenga, and Gruber, 1960). As a result of these studies it appears at present that ferritin consists of a hollow, roughly spherical protein shell, apoferritin, with internal and external diameters of about 60 - 70Å and 110 - 120Å respectively, surrounding a micelle of ferric hydroxyphosphate which can vary in size within

the interior. The molecular weight for the apoferritin shell has been determined by a number of physical techniques and shown to be in the range 430,000 - 480,000 (Rothen, 1944; Harrison, 1963; Fischbach and Anderegg, 1965; Harrison, 1959; Richter and Walker, 1967) although De Bornier (1957) obtained a much smaller value, 340,000 daltons, by osmotic pressure measurements.

Because of the heterogeneity exhibited by ferritin, a molecular weight has no real significance. However, it is useful to know the highest molecular weight for ferritin since this can be used to evaluate the maximum possible amount of iron contained within the protein shell. Based on the calculations made by Harrison (1964), except to extend over the range of molecular weight for apoferritin, we can calculate a molecular weight for full ferritin and hence calculate the number of atoms of iron that can exist within the protein shell. The highest Fe:N ratios that have been reported lie within the range 2.5 - 2.9 (Van Bruggen, 1962; Mazur, Litt and Shorr, 1950a; Philippot and De Bornier, 1959). Using this fact and assuming that the nitrogen of apoferritin constitutes 16.1% of the protein and that the iron accounts for 59% of the weight of the core (Granick and Michaelis, 1943), the molecular weight computed for full ferritin is 720,000 - 900,000 which is in good agreement with experimentally determined values (Fischbach and Anderegg, 1965; Harrison, 1964). From such treatment it can be calculated that the hollow centre of the protein shell can accomodate about 4,000 atoms of iron.

The arrangement of these iron atoms within the micelle is still not known with any degree of certainty. Treatment of ferritin with 1N sodium hydroxide for ten minutes at room temperature caused the protein shell to be stripped from the iron core and micelles so obtained were found to have a decreased phospho-

rus: iron ratio which tended to favour the view that phosphorus was at the interface of the micelle and the protein. However, this ratio never reached zero which seems reasonably interpreted as indicating that phosphate is present both on the surface and also within the micelle (Granick and Hahn, 1944; Harrison, Fischbach, Hoy and Haggis, 1967). The role of phosphorus, if any, is still uncertain and has yet to be determined. However, the indications are from recent reconstitution studies that the requirement for phosphorus is not fundamental to the synthesis of ferritin (see later section). Iron core analogues have also recently been prepared and the nature of these and their interaction with protein will be considered at a later stage.

Granick and Hahn (1944) have suggested that a possible composition of the micelle is $\left[(FeOOH)_8.(FeO.PO_3H_2)\right]_n$ and Michaelis, Coryell and Granick (1943) have studied the magnetic properties of the iron in ferritin. The iron in the micelles is paramagnetic and has a magnetic susceptibility of 3.8 Bohr magnetons. Several workers have noticed a certain degree of order in the micellar structure and have proposed several substructures for these, be it for four subunits at the corner of a square (Farrant, 1954) or at the vertices of a tetrahedron (Richter, 1959 b) or of six subunits at the vertices of an octahedron (Bessis and Breton-Gorius; Muir, 1960) or at the vertices of a trilateral prism (Van Bruggen, Wiebenga and Gruber, 1960). However, as has been the case so many times before, the experimental data concerning such substructures is conflicting. Harrison (1963), Fischbach and Anderegg (1965), Haggis (1965) and Harrison, Fischbach, Hoy and Haggis (1967) have demonstrated by a variety of physical and optical techniques that the micelle appears to be a uniform species and suggested that perhaps the observations of substruct-

ure within the micelles were artifacts of the mode of preparation of the samples for electron microscopy.

It was seen on page 10 that as a result of its variable iron content, ferritin was a heterogeneous species while the iron-free apoferritin was homogeneous, at least with respect to molecular weight. However, further studies on apoferritin demonstrated a polymorphism resulting from self-association phenomena.

Polymorphism of Ferritin and Apoferritin.

By ultracentrifugation, Rothen (1944) had observed for human and horse apoferritin a faster moving substance (S^{0}_{20} = 25 Svedberg units) which accounted for approximately 1 - 10% of the total protein and considered this to be an impurity. More recently, however, this has been shown not to be the case. This minor component is present even after ten recrystallisations (Harrison and Gregory, 1965) and was unambiguously observed, together with other minor components, by starch gel electrophoresis and by polyacrylamide gel electrophoresis by a number of workers (Saadi, 1962; Richter, 1963a,b, 1964; Fine and Harris, 1963; Kopp, Vogt and Maass, 1963, 1964; Theron, Hawtrey and Schirren, 1963; Carnevali and Teece, 1964; Suran and Tarver, 1965). Although certain investigators originally believed that these bands were in no way attributable to either stable polymers of apoferritin or to apoferritin molecules in reversible association-dissociation equilibrium (Kopp, Vogt and Maass, 1963; Richter, 1964), it has now become currently accepted that this is, in fact, the explanation for such heterogeneity. The reasons for such general acceptance stemmed from an ultracentrifugal study of the problem. It was demonstrated that such fractions could be observed by sedimentation in the ultracentrifuge and that the various fractions corres-

ponded to those visualised by electrophoretic studies. The various components of ferritin and apoferritin have been termed $\alpha, \beta, \gamma, \delta$, and ϵ and it has been shown by a number of workers that these species correspond to monomers, dimers, trimers, tetramers and pentamers respectively (Harrison and Gregory, 1965; Suran and Tarver, 1965; Kopp, Vogt and Sund, 1966).

Kopp et al (1964) suggested that the α, β and γ fractions of ferritin might have different iron binding capacities and this was what was found in a recent study with model experiments for the study of iron transfer by Miller and Perkins (1969). They found that the order of iron uptake of the various fractions of ferritin from transferrin was $\alpha > \beta > \gamma$. However, these fractions were shown to differ in neither their iron content nor in their amino acid composition and this then led Miller and Perkins (1969) to interpret their data as resulting from some form of steric factor and that, in an undefined way, some iron binding sites were masked by polymerisation (see later section). The ultracentrifugal studies gave the values for the sedimentation coefficients of the α, β, and γ bands as 17-18 S, 24-25 S, and 33S respectively. As Harrison and Gregory (1965) pointed out, the ratio of the sedimentation coefficients, 1.4 (β/α) and 1.9 (γ/α) agree reasonably well with the ratios calculated from Perrin's formulae (Svedberg and Pedersen, 1940) which are 1.52 for dimer/monomer and 1.87 - 1.98 for trimer/monomer (limits are for linear or triangular arrangements respectively).

It was stated earlier that in view of all these data there now seems to be general agreement at present that such polydispersity arises as a result of association phenomena, however, there is no general agreement as to whether such association is reversible or irreversible. Harrison and Gregory (1965) and

Williams and Harrison (1960) proposed that in the oligomeric state, the monomer units comprising it were covalently bound. This assumption was based on the resistance of the oligomers to various chemical denaturants and that the existence of such oligomers exhibited no concentration dependence and they were stable even on storage. They showed that no dissociation of oligomers occurred in 8M urea, 2 - 6 M guanidine hydrochloride, 1% sodium dodecyl sulphate (for hydrophobic interactions); 2-mercaptoethanol- 8M urea, dithiothreitol (for disulphide bonds); pronase (for peptide bonds); EDTA (for bivalent cations) and desferrioxamine (for ferric ions). On the other hand, Richter and Walker (1967) showed by a combination of light-scattering, gel electrophoresis, ultracentrifugation and electron microscopy that in fact the polymerisation was reversible and they demonstrated a marked concentration dependence. The free energy changes for the association monomer to dimer, ΔG^{o} = -7.72 kcal/mole, and for dimer to trimer, ΔG^{o} = -7.59 kcal/mole, were shown by these workers to be incompatible with covalent binding.

As an understanding of the quaternary structure of a protein plays an important part in the understanding of its biological function it was of interest to see if additional levels of structural organisation existed for the apoferritin molecule, other than association products of the protein molecule.

Subunit Structure of Ferritin and Apoferritin.

Physical and Chemical Evidence for Subunits.

The existence of subunits for ferritin and apoferritin was first categorically demonstrated by Harrison in 1959 (Harrison, 1959). This was done by X-ray diffraction studies which revealed that the space group of apoferritin was probably F432 with four

molecules per unit cell and each molecule having a point group symmetry 432. Harrison interpreted this as suggesting 24n identical subunits and a possible molecular structure would be one with the subunits at each of the vertices of a snub cube. Saadi, Shapira and Dreyfus (1961) concluded from their study on the number and nature of the tryptic peptides of apoferritin and from the amino acid composition that there were about 30 subunits of molecular weight approximately 15,600. This work was repeated by Harrison and Hofmann (1962) and they found only one trytophan-containing peptide, as did Saadi et al (1961), but rather more major peptides. From this data it was suggested that the number of subunits was not more than 24 and not less than 12 and that the most probable number was 20 with a molecular weight of 24,000. This was further confirmed on calculating a minimum molecular weight based on the amino acid composition. By this method, a value of 23,000 for the subunit molecular weight was obtained on the basis of the tryptophan content (Harrison, Hofmann and Mainwaring, 1962; Hofmann and Harrison, 1962) for a molecular weight of 480,000 for the apoferritin molecule (Harrison, 1960). This was also a value suggested by Harrison (1963) from a more detailed examination of the diffraction patterns. This latter study revealed that the crystals exhibited pseudo 5:3:2 or 5:2 symmetry and Harrison suggested a structure having pseudo-icosahedral symmetry, twenty subunits at the vertices of a pentagonal dodecahedron. Hofmann and Harrison (1963) managed to degrade the apoferritin into subunits by treating with sodium dodecyl sulphate and were able to determine the molecular weight of the protein-detergent complex which, after correction for the amount of bound detergent, yielded a molecular weight for the subunit of 25,000 - 27,000. The concept of a twenty-subunit model for the quaternary

structure of apoferritin was also confirmed by quantitative N-terminal determinations. Suran (1966) demonstrated that the N-terminus is N-acetylated and Mainwaring and Hofmann (1968) determined the bound acetyl groups and found a value of 19.6 ± 1 mole per mole of apoferritin (molecular weight 480,000). They also demonstrated that carboxypeptidase B released 18-19 moles of arginine per mole of apoferritin.

Because of its obvious architectural similarity with small spherical viruses, Harrison and Gregory (1968) studied some of those methods which had been used to degrade viral particles. By this approach it was found that treatment of protein with ice-cold 67% (v/v) solution of acetic acid for one hour was sufficient to disaggregate apoferritin just as it was for tobacco mosaic virus (Fraenkel-Conrat, 1957). Also it had been shown that dilute alkali slowly disaggregates the protein, but only to about 50% and this process is complicated by alkaline hydrolysis of peptide bonds (Hofmann and Harrison, 1963).

More recently Smith-Johannsen and Drysdale (1969) determined the subunit molecular weight by Sephadex gel filtration on Sephadex G-200 with sodium dodecyl sulphate contained in the eluting buffer. The value that these workers obtained for the apparent molecular weight was of the order of 12,000.

David and Easterbrook (1971) determined the molecular weight of the apoferritin subunit obtained from the ferritin of the fungus Phycomyces by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and showed this to be identical to that of horse spleen apoferritin. Vaněček and Keil (1969), using the fact that apoferritin can be dissociated by lyophilisation (Hofmann and Harrison, 1963), fractionated horse spleen apoferritin that had been lyophilised and resolved this into at least three components,

one which was probably undissociated material and two others, one of which eluted ahead of bovine pancreatic ribonuclease (molecular weight 13,000). They also showed that the amino acid composition of these components were identical and that the smallest component may represent subunits.

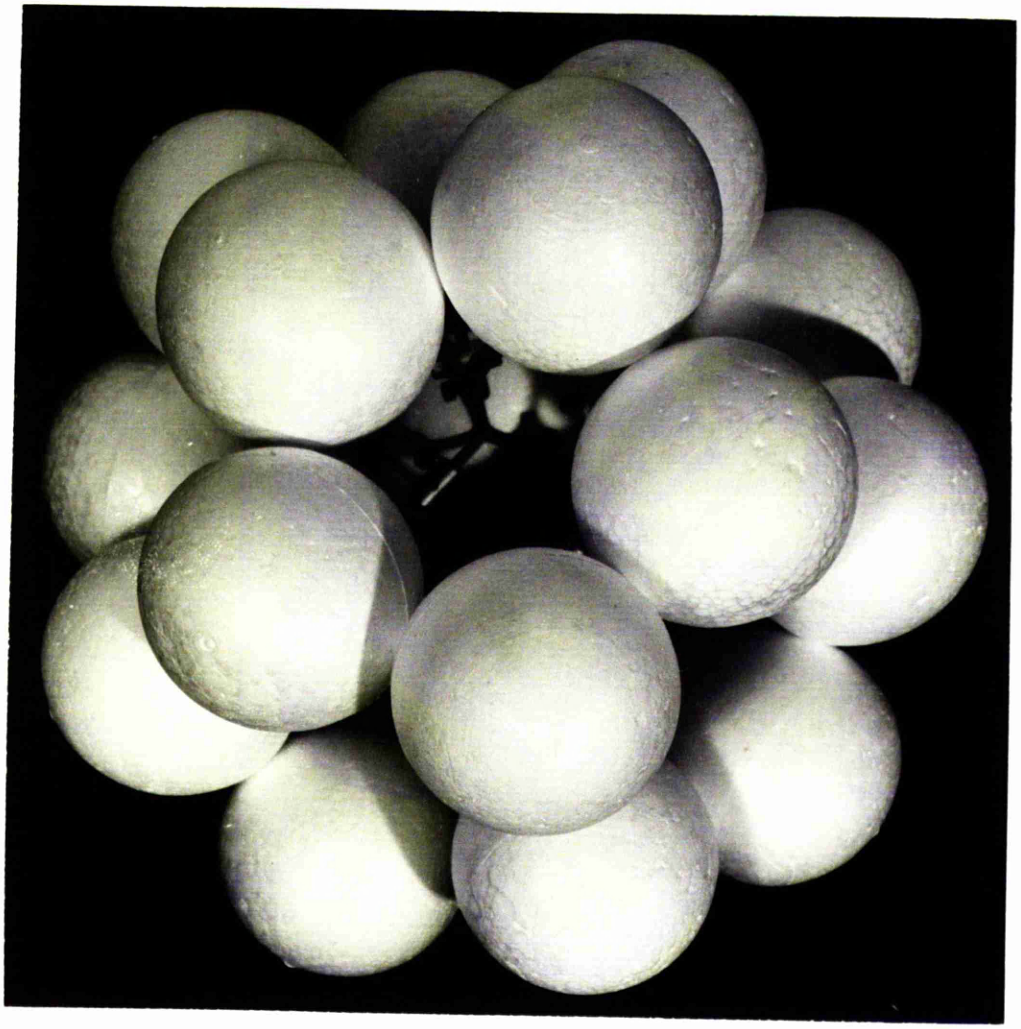
Spatial Arrangement of Subunits in Apoferritin Molecule.

The results obtained from the various physical and chemical studies on the apoferritin subunit strongly suggested that apoferritin consisted of twenty identical subunits.

Figure 5 shows a possible spatial arrangement for a twenty-subunit model. This is an arrangement in which the subunits are situated at the apices of a pentagonal dodecahedron. For such a model to be sterically possible it has been shown that the twenty subunits, being themselves asymmetric, cannot occupy identical environments (Casper and Klug, 1963). This, as Hanson (1968) has shown, necessitates recourse to one of two possibilities. (a) there must be two types of subunit which possess the same primary structure but adopt one of two alternative conformations or (b) there must be present two different types of subunit which can either be completely different or resemble each other closely.

Such subunit heterogeneity has recently received the attention of Drysdale (1970) who, by isoelectric focusing on gels, observed such polydispersity and suggested that it may in fact be due to different subunits. Urushizaki, Fukuda, Matsuda, Niitsu, Yokota and Kitango (1970) and Urushizaki, Niitsu, Ishitami, Matsuda and Fukuda (1971), using free-flow isoelectric focusing also demonstrated heterogeneity in samples of rat liver and horse spleen and concluded that this probably represented a polymorphism at the level of the subunits. However, in a study using Sephadex

Figure 5. Model of a proposed quaternary structure for apoferritin. This is an arrangement of twenty subunits in which each subunit is situated at an apex of a pentagonal dodecahedron and is viewed perpendicular to a pentagonal face.



G-75 superfine as the supporting media for thin layer isoelectric focusing, Radola and Delincee (1971) obtained only a single band for ferritin.

High resolution electron microscopy has been used as a method of indicating molecular shapes and the investigation of Easterbrook (1970) has tended to support the concept of a twenty subunit structure for apoferritin. Figure 6 shows ferritin molecules negatively stained with uranyl acetate (mag. 500,000). Figure 7 shows photographic enlargement (x10) of various selected areas and contain sufficient surface detail to allow interpretation in terms of the quaternary structure of a possible model.

Amino Acid Sequence Studies on the Apoferritin Subunit.

In the last five years or so sequence data on apoferritin has been slowly accumulating.

The N and C-terminal peptides have been determined by Suran (1966) and Mainwaring and Hofmann (1968) and these are shown schematically in Figure 8.

The fact that these workers find unique sequences for both of these regions of the polypeptide chain tends to support the view that the subunits are identical.

Another interesting aspect in protein structure-function studies is to examine the protein from a variety of organs and species and see if these differ from each other in any way. From such studies it may be possible to determine those features of the structure of the protein that might be important for biological function and also to determine those features of the structure that are unimportant for the protein's catalytic activity.

Figure 6. High resolution electron micrograph of ferritin molecules negatively stained with uranyl acetate. (Easterbrook, 1970).

Magnification x 500,000

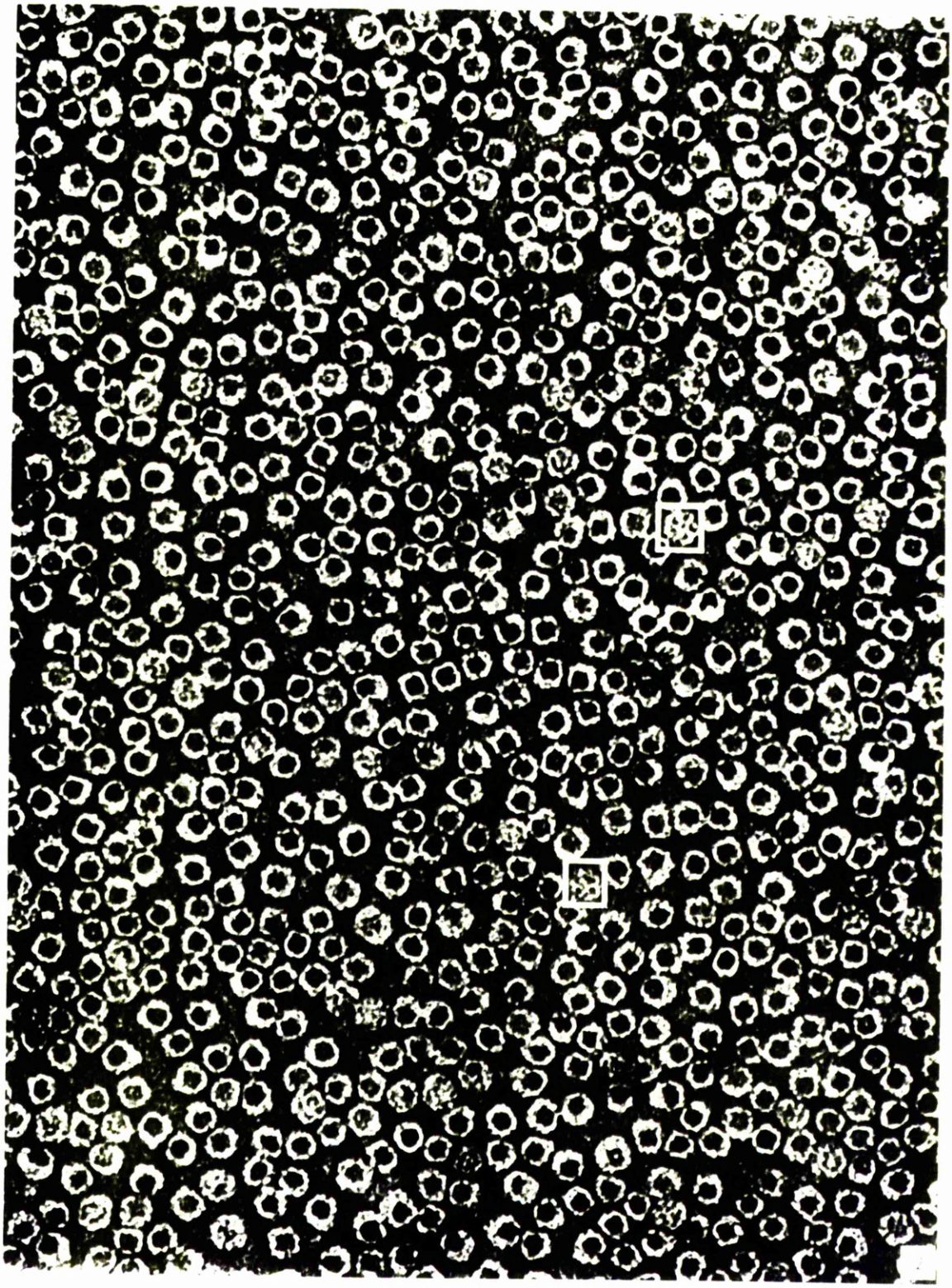
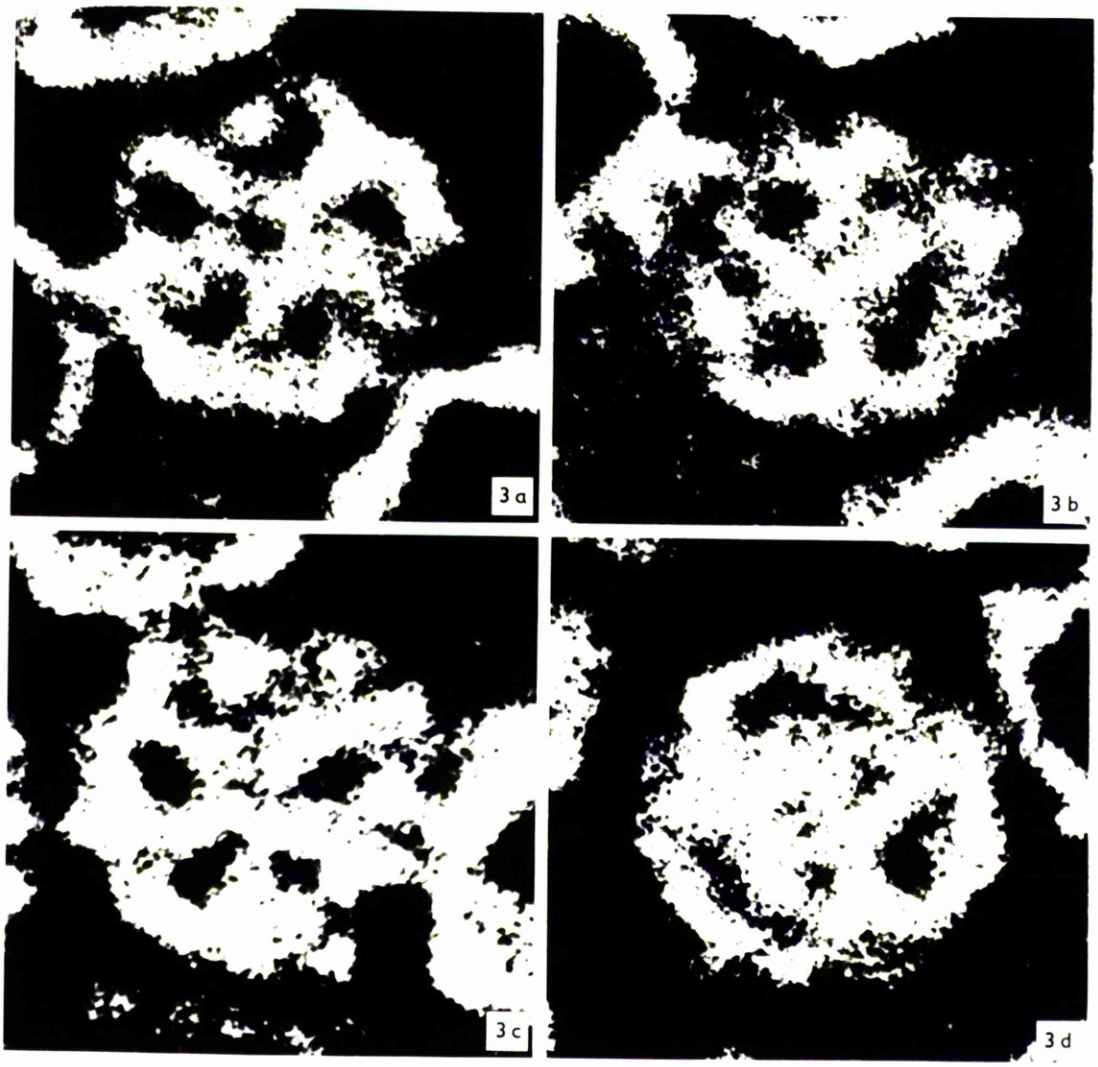
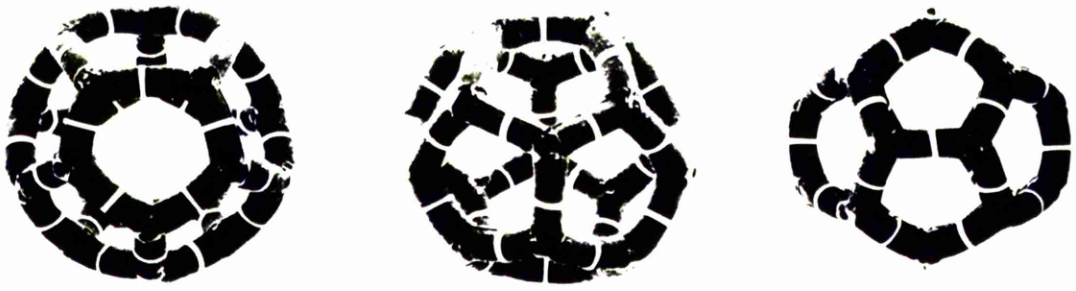


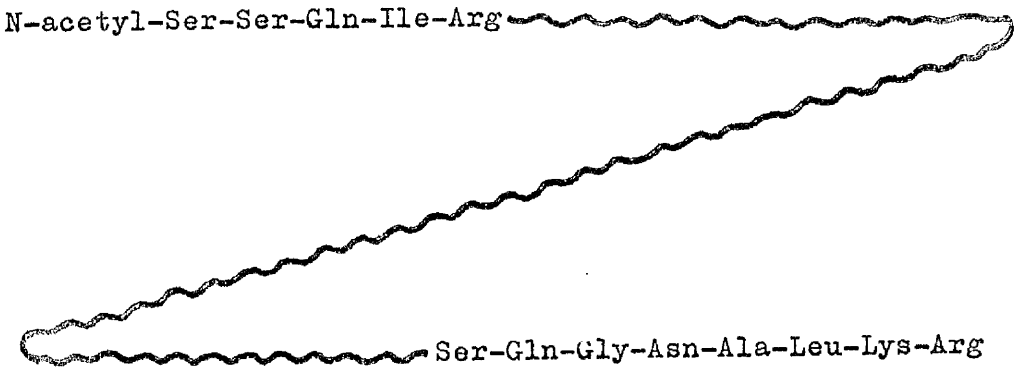
Figure 7. High resolution electron micrograph of ferritin molecules. These micrographs were obtained by photographic enlargement of individual molecules from the previous Figure. The fine detail present is interpreted in terms of a model of a pentagonal dodecahedron as shown. (Easterbrook, 1970). Magnification x 4.4 million





4a 4b 4c

Figure 8. Schematic representation of the known sequences of horse spleen apoferritin. The sequence starting N-acetyl-Ser- represents the N-terminus of the protein while the sequence ending in -Lys-Arg constitutes the C-terminal peptide.


N-acetyl-Ser-Ser-Gln-Ile-Arg
Ser-Gln-Gly-Asn-Ala-Leu-Lys-Arg

Organ and Species Specificity of Ferritin and Apoferritin.

Gone are the days when it was imagined that each protein was a unique and indivisible entity. The one cistron-one polypeptide chain postulate does not, of course, imply that there cannot be more than one form of an enzyme or protein and in fact isoenzymes - the products of different genomes - are now known to be common. Proteins vary, moreover, according to the organ and organism from which they are derived, so that if such polymorphic diversity is encountered, the number of distinct proteins exhibiting a given function becomes legion. The realization that humans do not all share the same stock of proteins came with Landsteiner's demonstration of the ABO blood group system (Landsteiner, 1901) and more recently with the observations of Pauling, Itano, Singer and Wells (1950) who found that the haemoglobin of patients with sickle cell anaemia was electrophoretically different and therefore chemically different from normal haemoglobin.

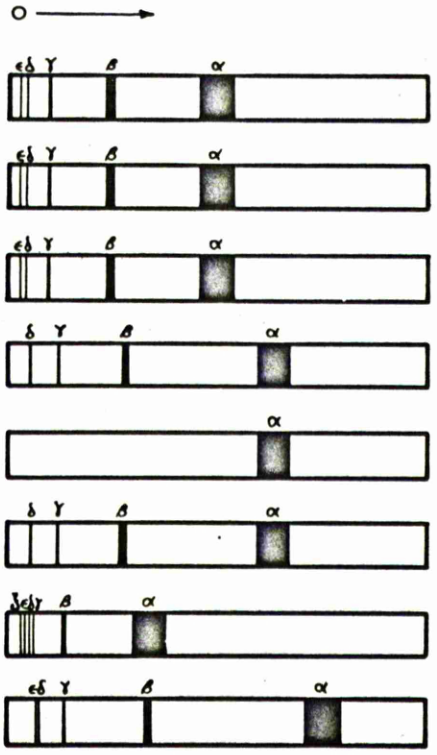
Since then, of course, many protein polymorphisms have been detected in man and other species. More than one hundred variant forms of haemoglobin alone are known, most of them differing by a single amino acid substitution from the normal molecule, and presumably arising by a single base change in its gene. Harris (1969) has shown that about one third of all the amino acid substitutions provide a change in electric charge, and it is probably only these substitutions that are detected by starch gel electrophoresis. The proteins studied so far, mainly enzymes, appear to have a heterozygosity of five per cent per locus. Correcting this to allow for undetected variants, the conclusion is that each individual is heterozygous in about 15% of the genetic loci and Lewis (1971) believes that it is "probable that almost every protein, structural or enzymatic, can be made to reveal some

degree of variability".

That such a degree of variability existed for ferritins from different species was demonstrated by several workers (Theron, Hawtrey and Schirren, 1963; Richter, 1964; Kopp, Vogt and Maass, 1964; Zimiri and Mason, 1968) but it remains controversial as to whether or not different normal tissues in individual animals produce distinct ferritins. These workers showed that the horse ferritins, human ferritins and rat ferritins were different for the different species and also that the ferritin extracted from either human or rat neoplasms differed from those of the normal tissues (Richter, 1963b, 1964, 1965; Richter and Lee, 1970; Lee and Richter, 1971b). However, within a species Richter (1964, 1970) claimed to demonstrate that no such differences existed between ferritins from different organs. The results that Richter obtained with various types of ferritin by means of polyacrylamide gel electrophoresis are summarized in Figure 9.

On the other hand, Alfrey, Lynch and Whitley (1967) reported that they observed electrophoretic differences between ferritins isolated from human marrow, spleen, liver and reticulocytes and this was confirmed by Gabuzda and Gardner (1967) for rabbit ferritins although they failed to resolve the human liver and human spleen proteins electrophoretically. Alfrey et al (1967) also observed two ferritins derived from human bone marrow and Gabuzda and Pearson (1968, 1969) reported the presence of a similar polymorphism within one organ when they observed two ferritins in rabbit bone marrow and they called these 'catabolic' (of reticuloendothelial origin) and 'anabolic' (of erythroblastic origin). However, Yamada and Gabuzda (1970) have since repeated this work and found only one type of ferritin in normal human bone marrow and showed this to be of the 'catabolic' ver-

Figure 9. Results of polyacrylamide gel electrophoresis of a variety of ferritins from different organs and species.



Human Ferritin
(Liver, Spleen, Kidney)

SK - 1 cells
SK - 2 cells
} From embryonic human skin

HeLa cells
KB cells
HEP - 2 cells
} From human carcinomas

Rats (Wistar, Sprague-Dawley, Long- Evans)
(Liver, Spleen, Kidney)

Horse Spleen

iety. Linder-Horowitz, Ruettinger and Munro (1970) recently reported, for female Fisher rats, the presence of two ferritins in rat heart and rat kidney and that these ferritins differed from rat liver ferritin although, Richter and Lee (1970) found no such difference for female ACI rats. Arora et al (1970) studied the ferritins obtained from a wide variety of human tissues and demonstrated electrophoretic dissimilarities on cellulose acetate and also showed that bone marrow, heart, adrenal and brain each had two 'isoferritins' and concluded that these may have arisen from two different cells of origin or that they may have two different functions within the organ. They also suggest that these differences in mobility, which reflect a difference in protein structure, may be necessary for ferritin to function or that these minor changes could alter the iron binding capacities to suit the function of ferritin within each organ.

At present there does not seem to be general agreement that the ferritin produced by malignant tissues appears to be different from that of normal cells (Richter, 1964, 1965; Richter and Lee, 1970; Lee, Richter and Jackson, 1968; Linder-Horowitz, Munro and Morris, 1970). Several lines of neoplastic human cells in vitro (HeLa, KB, and HEP-2) and certain hepatocarcinoma cells in vivo (Novikoff ascites, Reuber H-35, R-21, and Morris 3924A, 3683F, 5123, 7777, 7793, 7800) have been shown to contain ferritin with a different electrophoretic mobility than the ferritin from normal rat livers, spleen and kidney. It is interesting also that, the most histologically differentiated of the tumours so far studied, Reuber H-139, has a ferritin which is indistinguishable from the normal protein and Richter and Lee (1970) are interested to see if the H-139 cells will eventually produce a 'malignant' ferritin after long-continued transplantation.

In the same vein, it has long been the interest of the biochemist to see if the ferritin of patients suffering from idiopathic haemochromatosis differed from that of a normal patient. The regulation of the iron stores in man is normally controlled by an equilibrium between iron absorption and iron loss (see later section). In haemochromatosis this regulating mechanism is impaired and excessive accumulation of iron occurs as the storage compounds ferritin and haemosiderin. It was suggested that the aetiology of this disease was of structural origin i.e. a pathological alteration of the ferritin molecule itself, and as such is analogous to the molecular diseases affecting haemoglobin. However, Saadi (1962) investigated such a hypothesis by comparing the ferritin isolated from a haemochromatotic and that from a normal patient by starch gel electrophoresis, ultracentrifugation and by fingerprinting of a tryptic hydrolysate and found no difference between the two proteins.

One area of study that is capable of yielding important information on a protein's structure-function relationship is the study of the proteins conformation. By this method it is possible to detect small structural changes that may occur when a protein binds a ligand and these changes may have an important bearing on the mechanism of catalysis.

Conformation of the Protein Moiety of Ferritin and Apoferritin.

Recently the available techniques for protein conformation analysis have undergone considerable refinement and it became the interest of certain workers to see if the iron micelle-core caused the protein shell to adopt a slightly different conformation from that of the apoprotein. Listowsky, Betheil and

England (1967) demonstrated that the optical rotatory dispersion properties of native ferritin were independent of iron content as performed on ferritin fractions of varying iron content obtained by density gradient centrifugation. They confirmed these findings by investigation of the circular dichroism spectra of all of these components and found them to be conformationally indistinguishable (Listowsky, Blauer and Betheil, 1971). They did, however, show by optical rotatory dispersion (ORD) that apoferritin, prepared by chemical reduction of ferritin, appeared to have a more folded structure at pH 4.7. Further, they demonstrated that the protein's ORD properties were unaffected by 10M urea although 3-6M guanidine hydrochloride did alter the conformation and that this appeared to result in dissociation into subunits (Listowsky, Betheil and England, 1967). By circular dichroism(CD) they were able to demonstrate that while ferritin would dissociate in 7M guanidine hydrochloride at pH 7.5, apoferritin would not, unless the pH were reduced to pH 4.5. Analogous behaviour was demonstrated with high concentrations of urea where the protein remained intact at pH 5.0 - 8.0 but dissociated at lower pH values (Listowsky, Blauer and Betheil, 1971). Wood and Crichton (1971) by a combination of ORD and CD demonstrated that at pH 3.2 and above, ferritin has a much lower proportion of ordered structure than apoferritin although the apoferritin used in this study was obtained following chemical reduction with dithionite. These studies, taken in conjunction, therefore raise the question as to the nature of this irreversible conformational change on removal of ferritin iron by chemical reduction and as yet this question remains unanswered although Kopp, Vogt and Maass (1964) claim to have demonstrated that native apoferritin and apoferritin obtained following chemical reduction are not identical by starch gel elec-

trophoresis. These workers found that on electrophoresis chemical reduced apoferritin gave rise to α, β and γ bands whereas the native apoferritin gave only the α band.

Crichton (1969, 1971b) has also studied the conformation of ferritin and apoferritin by considering their respective susceptibilities to proteolysis by a variety of proteolytic enzymes. By such a technique he was able to show that digestion with trypsin caused apoferritin to be cleaved significantly faster than ferritin. This was also the case with chymotrypsin, papain and subtilisin, although cathepsin D and pepsin digested the two proteins at a similar rate. When the digestion was carried out at pH 2.5, however, it was shown that apoferritin was more susceptible to proteolysis by pepsin and he concluded that this may merely reflect the extent of denaturation of the protein at the lower pH value. The unique result obtained with cathepsin D was assumed to reflect the relatively non-specific mode of proteolysis.

Functional Aspects of Ferritin and Apoferritin.

Iron Absorption.

By studying the factors which affect iron absorption one can get some insight into a possible mechanism of iron transport. The body iron content of man and other mammals is regulated by the controlled intestinal absorption of the iron contained in food-stuffs and not as was previously thought by regulated iron losses (for discussion see, McCance and Widdowson, 1937). Normal adult animals absorb only a small fraction of their total dietary iron intake but, under certain physiological and pathological conditions, the absorbed fraction may rise and may even approach unity. The molecular details of the absorptive process and how

the process is controlled, though much studied in the past, are as yet not well understood. The iron present in the diet can be absorbed all along the gastrointestinal tract but, primarily the region of most active absorption is the duodenal region, just below the pyloric sphincter. Hahn, Bale, Ross, Balfour and Whipple,(1943) demonstrated, using radioactive iron, that chronic blood loss resulted in an increase in iron absorption although the amount of ^{59}Fe absorbed was suppressed by pre-feeding with unlabelled iron prior to testing for absorption. As a result of this study and the fact that Granick (1946a) had demonstrated the deposition of ferritin in the mucosal cell following iron administration (Granick, 1946a,b), the 'mucosal block' theory was put forward.

This stated that there was, in the mucosal cell, an acceptor system which could be alternatively saturated or desaturated and that this acceptor could well be apoferritin. The fact that Granick could not detect apoferritin in the mucosal cell, he argued by suggesting that its synthesis was induced by the presence of unbound iron (Granick, 1949, 1954). It was some twenty years before evidence was obtained which contradicted the 'mucosal block' at which time it was shown by a number of workers that an increase in the dose resulted in an increase in the absolute amount of iron absorbed and also that iron deficient humans absorbed more iron than normal patients at all dose levels (Smith and Dannacciulli, 1958; Beutler, Kelly and Beutler, 1962; Gitlin and Cruchaud, 1962; Bannerman, O'Brien and Wilts, 1962). Moderate quantities of ferritin were observed in the duodenal mucosal cells of normal subjects while minimal amounts are present in iron-deficient or haemochromatotic humans (Rosa and Chapin, 1941; Pirzio-Biroli and Finch, 1960). These facts, together with many

others, were incorporated into a new hypothesis on the regulation of iron absorption propounded by Conrad and Crosby (1963). In this, they assumed that there was some form of feedback and that the quantity of iron supplied from the body store to the mucosal cell regulated the quantity of iron that could be absorbed from the gut. Figure 10 shows this hypothesis schematically and it can be seen that when little iron is supplied to the mucosal cell from the body stores, as is the case in iron deficiency, then considerably more can be absorbed and conversely when the body store is heavily loaded with iron, then little can be absorbed.

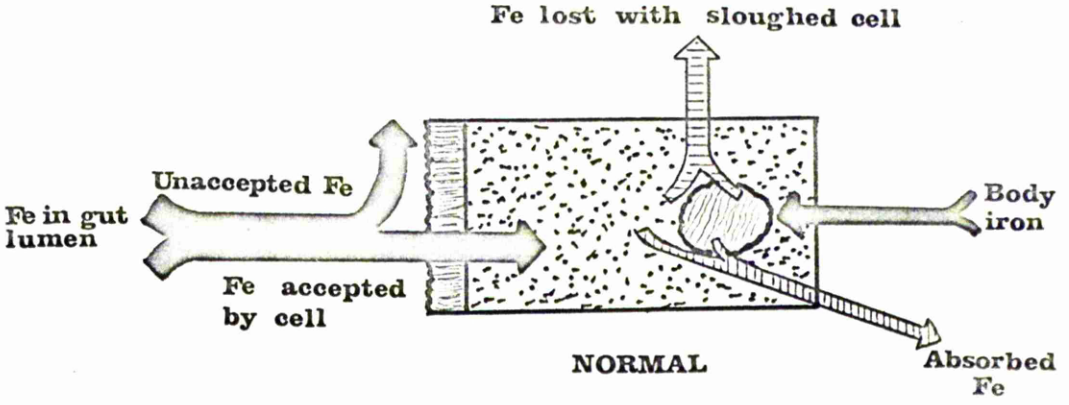
The next stage in the absorption of iron by the body is relatively slow compared to that from the gut to the mucosal cell. This is the transfer of iron from the mucosal cell to the serosal cell. The nature of the iron as it passes through the mucosa is not clear. Some workers believe that the storage form is ferritin while others have been unable to demonstrate this protein in the duodenal epithelial cells. Figure 11 summarises the present view on the uptake of iron from the gut and its eventual deposition as the storage form, ferritin.

With a view to an understanding of the biological role played by ferritin and apoferritin it is important to know the conditions necessary for the protein subunits to associate and also to know the details of how the iron is incorporated into the protein shell to form ferritin.

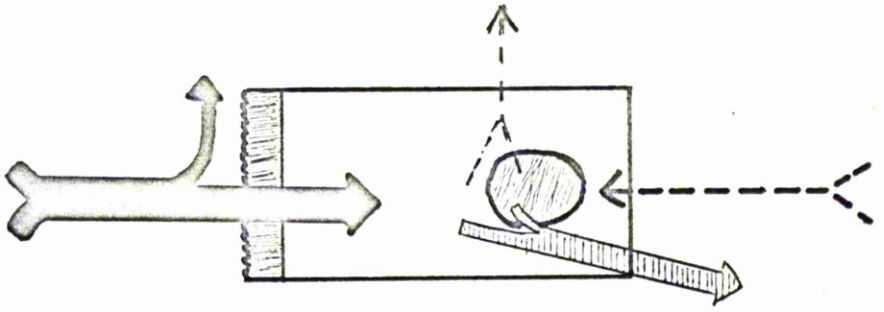
Reassembly and Reconstitution of Ferritin and Apoferritin.

In the preceeding section the proposed method for the transport of iron from the gut to the serosal cell was outlined. It remained only to describe the nature of the interaction of iron

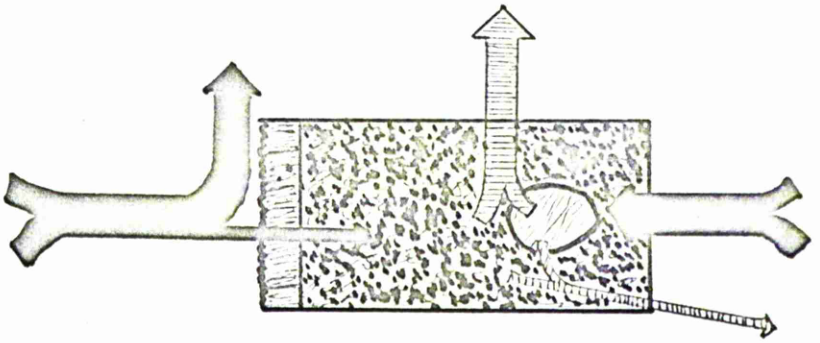
Figure 10. Schematic representation of the hypothesis of Conrad and Crosby (1963) for the regulation of iron absorption. When little iron is supplied to the mucosal cell from the body as in the case of iron deficiency, then considerably more can be absorbed and conversely when the body store is heavily loaded with iron, then little can be absorbed.



NORMAL

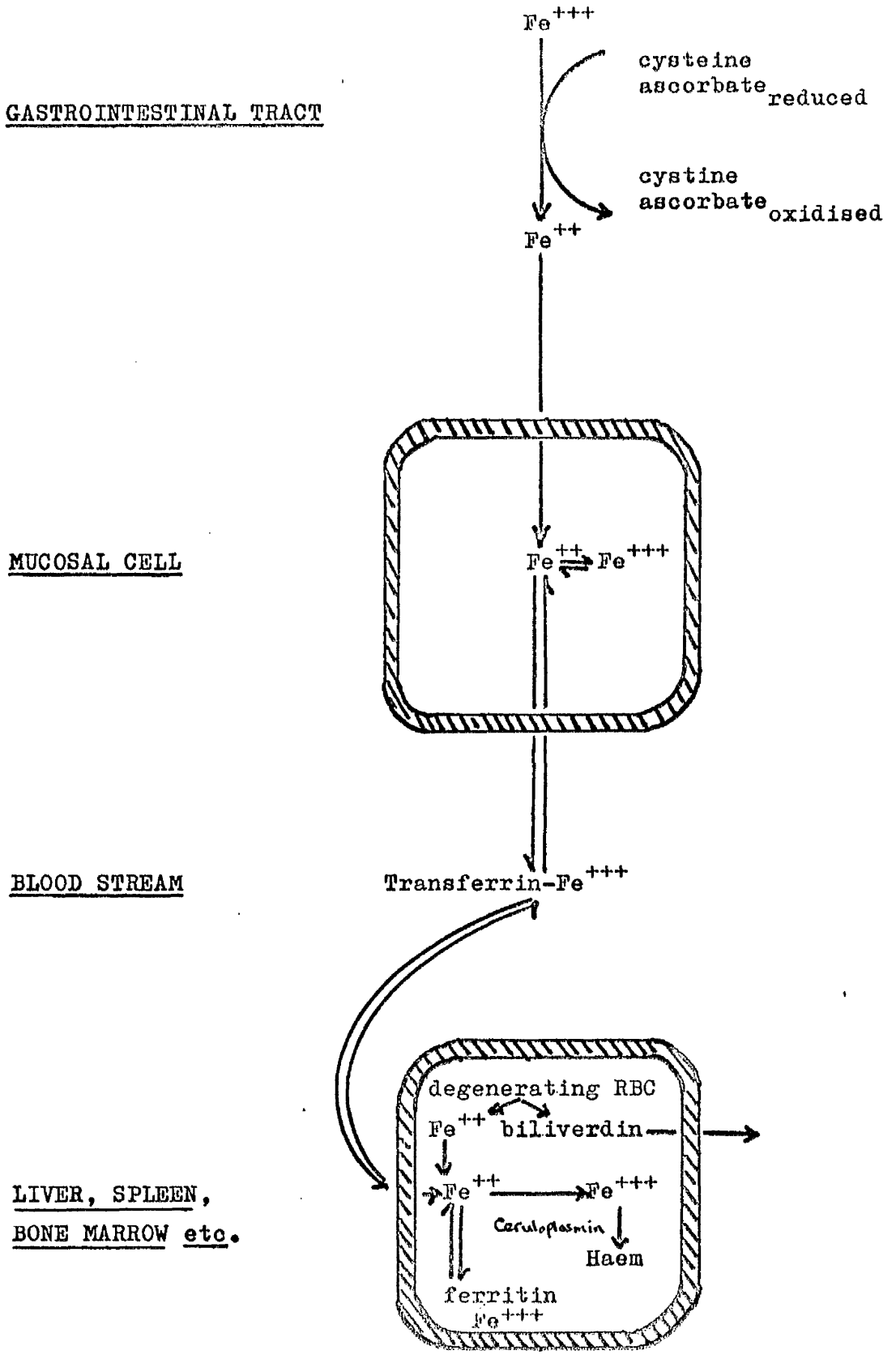


IRON DEFICIENT



IRON LOADED

Figure 11. Pathway for the uptake of iron from the gut into the mucosal cell, the blood stream and eventually into ferritin in those organs concerned with the reticuloendothelial system, bone marrow, liver, spleen etc.


GASTROINTESTINAL TRACT
Fe+++
cysteine
ascorbate reduced
cystine
ascorbate oxidised
Fe++
MUCOSAL CELL
Fe++ ⇌ Fe+++
BLOOD STREAM
Transferrin-Fe+++
LIVER, SPLEEN,
BONE MARROW etc.
degenerating RBC
Fe++
biliverdin
Fe++
Fe+++
Caeruloplasmin
Haem
ferritin
Fe+++

with apoferritin to give ferritin, although it can be said that the details of such an interaction are still not absolutely clear.

In essence there seem to be three viewpoints pervading this topic;

(a) that the apoferritin remains intact during a passive oxidation of the ferrous ions within the interior of the protein shell.

(b) as before, except that the oxidation is catalysed by specific amino acid residues on the protein, and

(c) that the ferritin is formed by the addition of subunits around a preformed iron core.

Harrison and Gregory (1968) demonstrated that it was possible to dissociate both ferritin and apoferritin by treatment with 67% (v/v) acetic acid for 1 hour at $0^{o}C$ and to maintain the protein as subunits in an aqueous glycine buffer at low pH values. When, however, the subunits were dialysed against an aqueous phosphate buffer at a pH around neutrality, they observed spontaneous reassembly of the apoferritin molecules as determined by electron microscopy and ultracentrifugation. This study at least showed that iron cores were not necessary for the formation of the protein shell.

Working on the nature of the product formed by hydrolysis of ferric ions in aqueous solution, some investigators have demonstrated that these are uniformly sized ferric hydroxide micelles which in terms of size, shape, magnetic and Mössbauer properties, stability under physiological conditions, visible and ultraviolet spectra are very similar to ferritin cores (Aasa, Malmstrom, Saltman and Vanngard, 1964; Allerton, Renner, Colt and Saltman, 1966; Spiro, Allerton, Renner, Terzis, Bils and Saltman, 1966; Spiro, Bates and Saltman, 1967; Spiro, Pape and Saltman,

1967). This then led Papo, Multani, Stitt and Saltman (1969) to propose that ferritin is formed by the addition of subunits around such a preformed iron core. The experimental evidence on which this is based is as follows. Incubation of non-crystallisable ferritin, which these workers show by electron microscopy to be apparently ferritin that has lost a few subunits, with apoferritin gives rise to ferritin which is indistinguishable from that of native ferritin. The view that non-crystallisable ferritin is ferritin deplete of a few subunits is, as Drysdale, Haggis and Harrison (1968) have pointed out, not in agreement with the finding of Farrant (1954) who found, following negative staining in two directions, non-crystallisable ferritin to be indistinguishable from that of ferritin and that the few crescent shapes which were visible in both preparations were assumed to be artifacts.

In a recent abstract from Saltman's laboratory (Cepurneek, Hegenauer, Meltzer, Winslow, Spiro and Saltman, 1971) they have described a method for the formation of ferritin from a solution of apoferritin subunits and a solution of natural cores which were obtained from native ferritin. The reconstituted ferritin is indistinguishable from native ferritin by a number of criteria. This work was also done with synthetic micelles with similar findings. These workers have, however, since shown that their product is unstable and very quickly precipitates on storing (P. Saltman, personal communication).

One of the main objections to this model for ferritin biosynthesis is the work of Fineberg and Greenberg (1955) and of Drysdale and Munro (1966) who have demonstrated by radioactive tracer studies that, following induction of ferritin biosynthesis, the components with the highest specific activity were those of

low iron content and that the specific activity decreased with increasing iron content. This then tends to imply that apoferritin or low iron ferritin is the precursor of ferritin. Recently, however, Niederer (1970) argued that this still did not rule out the possibility of Saltman's model if it is assumed that the protein shell of ferritin is in some way stabilised and dissociates into subunits less rapidly than low-iron ferritin. This being the case, the process is complicated by a mixing with the pre-existing liver ferritin pool. Niederer (1970), however, reconstituted ferritin from apoferritin and ferrous ammonium sulphate and, taking samples at different time intervals, demonstrated, although his data is difficult to interpret, that ferritin was formed by a gradual uptake of iron and not as would be predicted on the basis of Saltman's model.

The alternative to Saltman's scheme is an oxidation-reduction system as demonstrated by several investigators (Granick and Hahn, 1944; Harrison, Fischbach, Hoy and Haggis, 1967; Bielig and Bayer, 1955a; Loewus and Fineberg, 1957). Loewus and Fineberg (1957) have shown that incubation with ferrous ammonium sulphate in neutral bicarbonate buffer at 0°C resulted in the formation of ferritin from apoferritin, with about 80% of the original iron content and if the system was first deoxygenated and nitrogen gassed then no ferritin was formed. This was also done under slightly different conditions by Harrison, Fischbach, Hoy and Haggis,(1967) who found ferritin with the appearance of native ferritin. The system in the hands of Pape, Multani, Stitt and Saltman (1968) resulted in a product that was slightly smaller (102 Å) than native (120 Å) and had a smaller central iron core, 48 Å as opposed to the value of 70 Å for the native. However, such differences between reconstituted and native ferritins were

not observed by Harrison et al (1967).

Recently it has been shown in the reconstitution of ferritin from the apoprotein and ferrous ions that the apoferritin can act as an enzyme, a ferroxidase, which actively oxidises ferrous ions to ferric and thereby forms ferritin (Niederer, 1970). In an effort to determine those groups involved in the catalysis, Niederer alkylated sulphydryl groups and found no change. However, he did find that reagents specific for histidine impaired the catalysis and concluded that histidine was at the active site. Since reaction with diazonium-H-tetrazole, which alkylated 10% of the protein's histidine, had no effect on the oxidation he concluded that the reagent was too large to penetrate to the inside of the protein shell where he suggested the 'active' histidine residues were situated.

That apoferritin acts as a catalyst has also been demonstrated by Macara, Hoy and Harrison (1972) and, on the basis of their study, they put forward a mechanism for iron uptake by apoferritin which is basically a model in which the core grows within the protein shell. These workers determined the nature of the product by a variety of techniques and found that it had all the appearances of native ferritin.

The Nature of the Present Study on Ferritin and Apoferritin.

The nature of the present study was to further investigate some structural aspects of the protein and to establish suitable conditions for reconstitution such that the kinetics of the process could be established.

In the structural studies it was intended to re-determine the subunit molecular weight. The inherent difficulties of direct molecular weight determination of a protein subunit by

conventional analytical gel chromatography in dilute aqueous buffers are essentially twofold (Andrews, 1964, 1965). In the normal aqueous buffers employed the interpretation of the proteins chromatographic behaviour can be, in all likelihood, complicated by a polymerisation, ~~to oligomers~~. The other major problem stems largely from the differences in molecular shape and density of the various molecular species employed to calibrate the system. Fortunately, both of these difficulties can be overcome by carrying out the fractionation in the presence of a denaturant. This approach was initially developed by Small and coworkers (Small, Kehn and Lamm, 1963; Cebra and Small, 1967), later by Davison (1968) and in a more recent and extensive study by Fish, Mann and Tanford (1969) and consisted of chromatography in the presence of 6M guanidine hydrochloride. Reduced proteins in high concentrations of guanidine hydrochloride are dissociated to their constituent polypeptide chains and adopt a true random coil conformation, retaining no element of their original native conformation (Tanford, Kawahara and Lapanje, 1967).

A related technique of molecular weight determinations is by polyacrylamide gel electrophoresis in the presence of 1% (w/v) sodium dodecyl sulphate (Maizel, 1966; Shapiro, Scharff, Maizel and Uhr, 1966; Shapiro, Vinuela and Maizel, 1967; Shapiro and Maizel, 1969). This system differs from the latter in that true random coils are not obtained on denaturation, but instead, the reduced proteins are dissociated to constituent polypeptide chains which complex with the detergent. It has been shown for all of the proteins so far studied that each binds a similar fractional amount of sodium dodecyl sulphate (SDS), 1.4 g/g protein, and that the complexes so produced adopt identical hydrodynamic shapes (rods or ellipsoids) (Pitt-Rivers and Impiombato,

1968; Reynolds and Tanford, 1970). Studies of the hydrodynamic and optical properties of these protein-SDS complexes have shown that the length of the complex varies uniquely with the molecular weight (Reynolds and Tanford, 1970; Tiffany and Krimm, 1969).

These two empirical techniques for the determinations of the molecular weights of proteins and polypeptide chains have recently received critical evaluation and have been shown, with little exception, to afford a precision of 5-6% (Fish, Mann and Tanford, 1969; Weber and Osborn, 1969).

Apoferritin was also shown to be dissociated to subunits by treatment with 67% (v/v) acetic acid at $0^{o}C$ for 1 hour and this material remained as subunits following dialysis into dilute glycine-HCl buffer, pH 3.0 (Harrison and Gregory, 1968). This dissociating condition was used as the basis of another method of molecular weight determination by Sephadex gel chromatography.

All of the above denaturants were also used to produce subunits, the molecular weights of which, were determined by sedimentation equilibrium in the analytical ultracentrifuge.

In the structural studies on apoferritin we were interested in the products of cyanogen bromide cleavage and for this reason we attempted to characterize their molecular weights by extending the range for SDS-polyacrylamide gel electrophoresis and also for gel filtration in 6M guanidine hydrochloride.

It was decided to re-investigate the tryptophan content of the protein as this was one of the methods on which the literature value for the subunit molecular weight was based (Harrison and Hofmann, 1961). The method we chose for the quantitative determination was that of Scoffone and coworkers which consisted of treating the protein with 2-nitrophenylsulphenyl chloride (Scoffone, Fontana and Rochi, 1968a,b; Boccu, Veronese, Fontana

and Benassi, 1970). By this method we generated in the intact protein a chromophore, the absorption of which was monitored in the visible region. The tryptophan content was also determined by the two independent spectrophotometric methods of Edelhoch (1967) and Beaven and Schmid, (1957).

Apoferritins from a variety of organs and species (horse spleen and liver, human spleen and liver, human haemochromatotic spleen and liver, rat spleen and liver and pig liver) were examined by quantitative tryptophan determinations and molecular weight estimations by a variety of methods to see if the proteins were organ or species specific, at least with respect to these two criteria.

In an effort to determine the number of subunits per apoferritin molecule we decided to determine the undissociated molecular weight by sedimentation equilibrium in the analytical ultracentrifuge. In this context, electron microscopy was used as a means of determining any symmetry elements in electron micrographs of the undissociated apoprotein and isoelectric focusing was used as a method of demonstrating any microheterogeneity that may exist.

It was also decided to undertake a study of the association-dissociation of the subunit-monomer system. This could be studied both by use of the analytical ultracentrifuge and also by ultraviolet difference spectroscopy. The determination of the dissociation constant, pK, for the dissociation of protons from ionisable side-chain groups is an important application of spectroscopy to protein chemistry. Such determinations are possible if the process of dissociation perturbs the spectrum of one or more of the protein's chromophores. The group ionising need not necessarily be the chromophore itself but instead any group that

interacts with a chromophore and so causes the protein's spectra to change on ionisation. Because such spectral changes are often small a solution of similar absorption properties is used as the reference and measurements are obtained as differences from this reference solution.

This, together with the ultracentrifugal study, provided useful information on the pH stability of the protein, the possible nature of the groups involved in subunit-subunit interaction, the environments of the protein's chromophores and the nature of conformational changes that occurred in the protein.

All of these studies taken together provided a good basis of knowledge on the structural aspects of the protein.

As far as the functional aspects of the study were concerned it was decided to investigate the suggested ferroxidase activity. This term results from the recent work of Osaki and coworkers who observed that ceruloplasmin possesses an extremely potent capacity to oxidise ferrous to ferric ion (Osaki, 1966; Osaki, Johnson and Frieden, 1966). Indeed they find that this reaction is the reaction catalysed most efficiently by the protein and have proposed that the protein be renamed ferroxidase. They speculated that its function is to ensure that serum iron is present in the oxidised valency, thus facilitating its transport in complex with transferrin.

Just as it was gratifying to find a plausible role for ceruloplasmin, so too would it be satisfying to find an enzymatic role for apoferritin.

By studying the catalysis of ferrous iron to ferric, it was hoped to establish suitable assay conditions. The initial studies were involved in the establishment of a suitable buffer system and optimal conditions for pH, substrate concentration and protein

concentration etc.. Suitable criteria were established for the identification of the product, ferritin. These were the extinction at 412 nm, gel electrophoresis (staining for both iron and protein) and electron microscopy. By establishing a specific extinction coefficient for the iron micelle it was possible, using the first of these criteria, to determine the quantity of ferric iron per protein molecule and, from the latter, it was possible to determine the distribution of the iron within the protein molecules.

Once a suitable assay was developed, kinetic parameters were evaluated and these studies provided suitable characterization of the protein's probable function.

A useful study at this stage was the selective chemical modification of a variety of groups and the number of groups modified provided some information on the accessibility of that specific residue. The modified proteins were analysed by sedimentation velocity or column chromatography to determine whether they had remained as monomer or whether the modification had caused the protein to dissociate into subunits. These modifications provided further information on the possible nature of the subunit-subunit interactions. Further, the modified proteins were used in the assay system to determine the effects, if any, that such modifications had on the catalytic function.

By such chemical modifications it was possible to relate function to structure and vice versa.

In his introduction to the 1st. Federation of Biochemical Societies Symposium held in London in 1964, Sanger commented that, "The chemical processes of living matter are almost entirely dependent on the catalytic activity of enzymes, so that the

problems of what enzymes are and how they work are two of the most fundamental in biochemistry. It is clear that this activity must depend on the exact chemical structure of the molecule and particularly on the nature of that part of the enzyme that comes into contact with the substrate and is generally referred to as the 'active site'. In the past, many studies have used indirect approaches to deduce something of the nature of active sites, but these have been greatly hampered by a lack of the knowledge of the chemical structure of the enzymes involved.....

.... The problem will be dealt with mainly from the point of view of the protein chemist; however, there are many other aspects of enzymology that must be considered in any attempt to understand enzymes.".

This then is the subject of the present study.

MATERIALS AND METHODS

General.

Proteins and Peptides. Apoferritin (horse spleen), catalase (bovine liver), cytochrome c (horse heart), glucagon and gramicidin were obtained from Mann Research Laboratories, New York, N.Y. ; alcohol dehydrogenase (liver), chymotrypsinogen A, lima bean trypsin inhibitor, ovalbumin and trypsin from Worthington Biochemical Corporation, Freehold, N.J. ; creatine phosphokinase (rabbit muscle), cytochrome c (horse heart), haemoglobin and insulin from Sigma Chemical Company, St. Louis, Missouri ; ferritin (horse spleen), and transferrin (horse, pooled plasma) from Pentex Inc., Kankakee, Ill. ; alcohol dehydrogenase (baker's yeast), myoglobin (horse heart), and trypsin inhibitor (soya bean) from British Drug Houses Ltd., Poole, Great Britain ; albumin (bovine serum), and γ-globulin (human serum) from Serva, Heidelberg, Germany ; and bacitracin, insulin (bovine pancreas) and ferritin (horse spleen) from Schwarz / Mann, Orangeburg, N.Y..

Reagents Used for Chemical Modification. 2-nitro-phenylsulphenyl chloride and 1,2-cyclohexanedione were obtained from Fluka AG, Buchs SG, Switzerland ; o-methylisourea from Serva, Heidelberg, Germany ; iodoacetic acid from British Drug Houses Ltd., Poole, Great Britain ; tetranitromethane from Aldrich Chemical Company Inc., Milwaukee, Wisconsin, U.S.A. and 5,5'-dithiobis-(2-nitrobenzoic) acid was a gift from Dr. Richard Laursen.

Radiochemicals. Ferrous ammonium sulphate - Fe^{55} and iodoacetic acid - 2 - C^{14} were purchased from the Radiochemical Centre, Amersham, Great Britain.

General Reagents. For the experiments on isoelectric focusing,

the Ampholine carrier ampholytes, range pH 3 - 6, pH 3 - 5, pH 3 - 10, were obtained from LKB-Produkter AB, Bromma - 1, Sweden while the acrylamide (four times crystallised, heavy metal free) was purchased from Serva.

The ferrous ammonium sulphate used in the kinetic studies was obtained from either Fluka AG, Switzerland; Merck, Darmstadt, Germany or British Drug Houses Ltd., Great Britain and the cacodylic acid from Roth OHG, Karlsruhe, Germany.

Ultrapure guanidine hydrochloride used in the spectroscopic studies was purchased from Mann Research Laboratories. All other chemicals were analytical grade, available from commercial sources.

Chromatographic Materials. Sepharose 6B (Lot No. 5073) with a nominal agarose content of 6% was obtained from Pharmacia, Uppsala, Sweden while Bio-Gel A-5m (100 - 200 mesh, control no. 49 62) was purchased from Bio-Rad Laboratories, Richmond, California, U.S.A. Sephadex G-10, G-15, G-75, and G-100 were also obtained from Pharmacia.

Treatment of Data. Much of the routine handling of the data i.e. the calculation of coefficient of linear correlation and rate of regression, standard deviation and $S_{20,w}$ values etc. was facilitated by the use of the Olivetti Programma 101 desk-top computer while most of the data from the analytical ultracentrifuge was processed on a PDP - 8/L computer (Digital Equipment Corporation, Reading, Great Britain) by programs written in FORTRAN 4K by Dr. R. Eason.

Dialysis Cell. In the course of the kinetic studies we desired

some means of continual sampling of both the material being dialysed and the diffusate. For this reason, a dialysis cell was designed and is shown schematically in Figure 12. This was further modified to allow gassing of both materials. The apparatus was also used in determining the extent of modification of dialysis tubing on acetylation.

Preparation of Ferritin and Apoferritin. Much of the work carried out in the study of horse spleen apoferritin was performed on commercially available protein although, many of the experiments were also performed with material isolated from horse spleens in our own laboratory. Since the only ferritin that is commercially available is that from horse spleen, all of our studies on ferritin from other organs and species were carried out with material which we isolated and purified ourselves. In all cases, except for the rat liver and spleen, the isolation was carried out as soon as possible after receiving the fresh tissue. Rat liver and spleen were kindly collected by Mr. W. Melville over a period of about one month and stored at -10^{o}C until these amounted to about 500g. The method used for the isolation and purification was essentially a combination of that of Granick (1943) and of De Groot, Hoenders, Gerding and Bloemendal (1970), but with a few minor modifications. The scheme for the isolation is outlined in Figure 13. In the heat precipitation step the tissue homogenate was stirred continuously, the heating being performed in a large water bath to avoid local overheating. After spinning the heat-coagulated material, the brown supernatant was made 35% (w/v) in ammonium sulphate by the addition of the crystalline reagent (enzyme grade) and left overnight at 0^{o}C with gentle stirring. The precipitate formed was isolated by low speed centrifugation and dissolved in water.

Figure 12. Cross-sectional view of the dialysis cell used in some of the kinetic experiments and also in the assessment of the degree of aceylation of dialysis tubing.

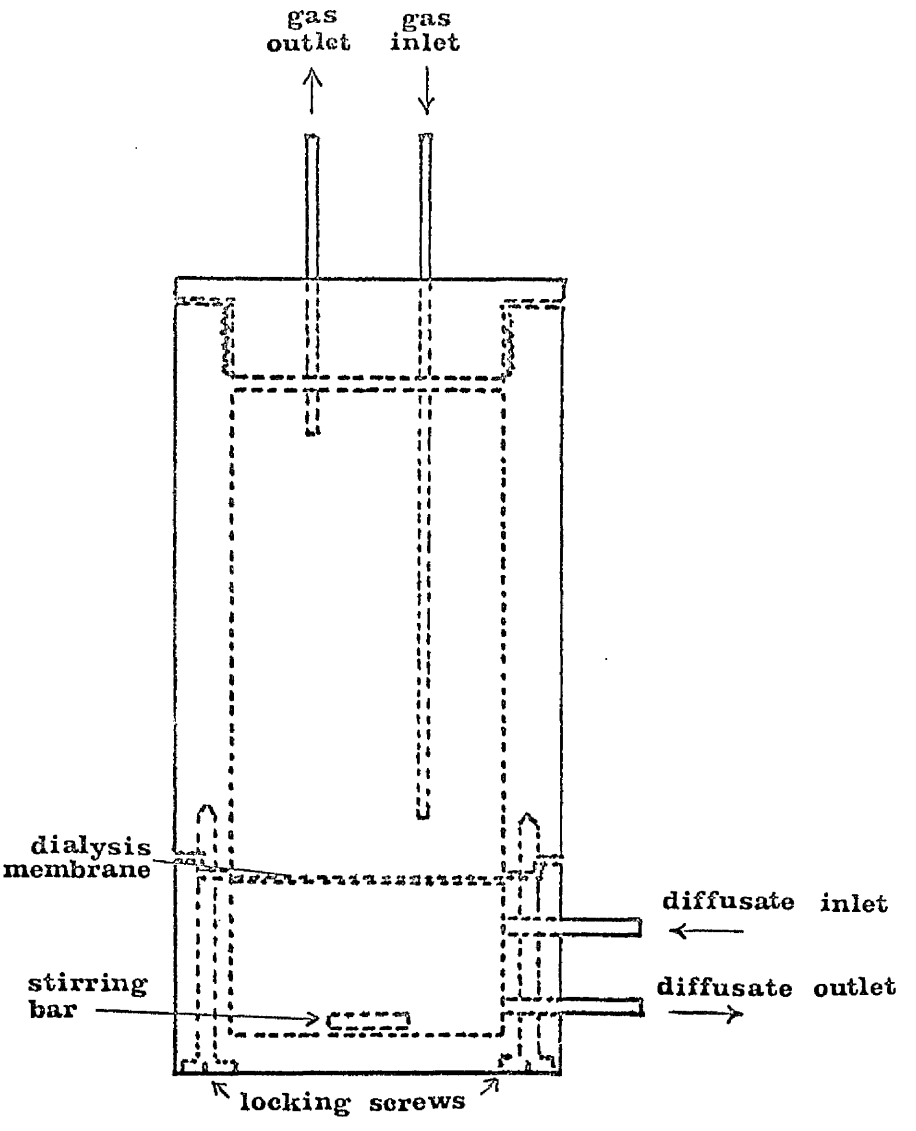
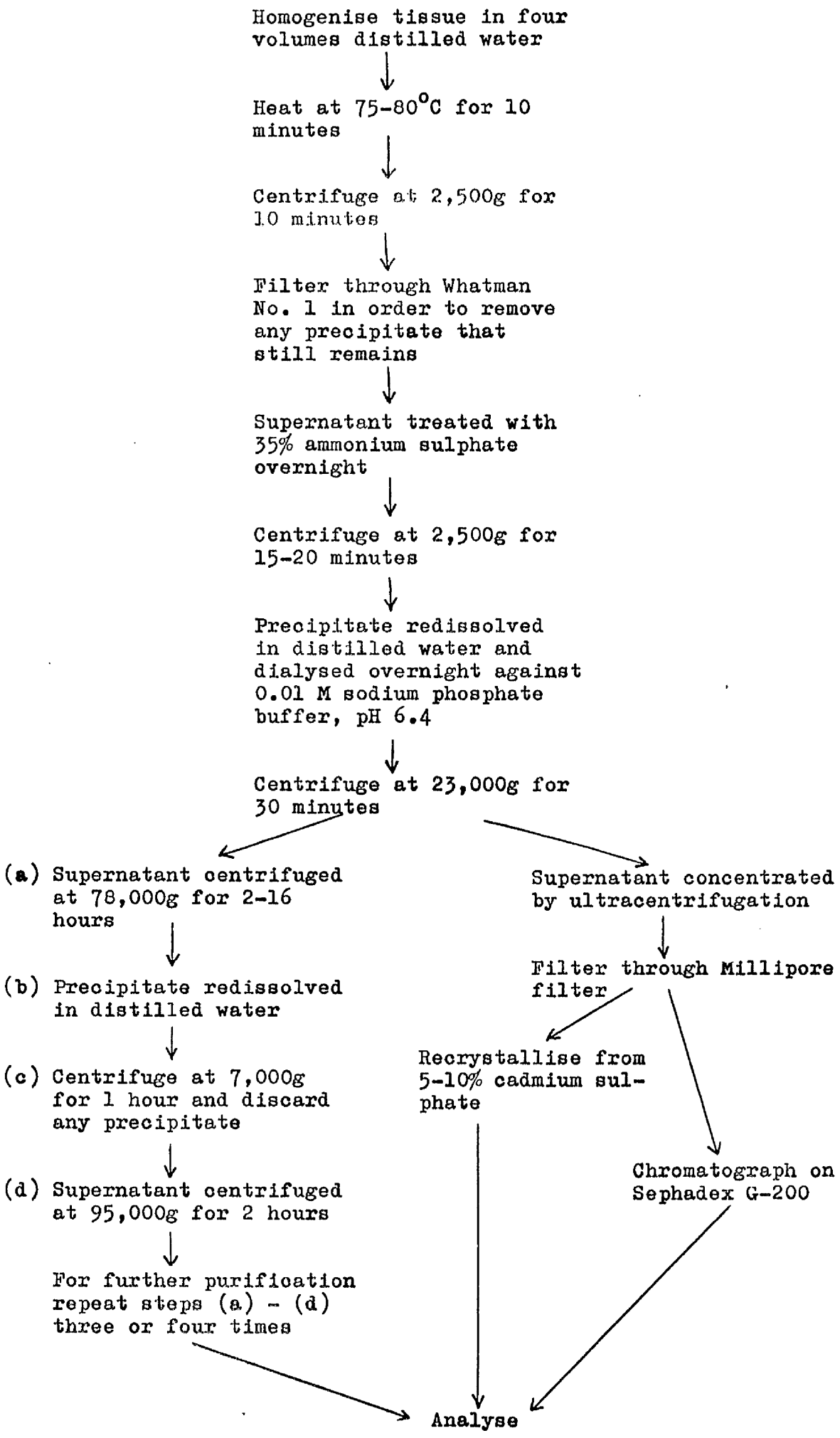

gas outlet
gas inlet
dialysis membrane
diffusate inlet
stirring bar
diffusate outlet
locking screws

Figure 13. Isolation and purification procedures for ferritin as used in this study.


Homogenise tissue in four volumes distilled water
Heat at 75-80°C for 10 minutes
Centrifuge at 2,500g for 10 minutes
Filter through Whatman No. 1 in order to remove any precipitate that still remains
Supernatant treated with 35% ammonium sulphate overnight
Centrifuge at 2,500g for 15-20 minutes
Precipitate redissolved in distilled water and dialysed overnight against 0.01 M sodium phosphate buffer, pH 6.4
Centrifuge at 23,000g for 30 minutes
(a) Supernatant centrifuged at 78,000g for 2-16 hours
(b) Precipitate redissolved in distilled water
(c) Centrifuge at 7,000g for 1 hour and discard any precipitate
(d) Supernatant centrifuged at 95,000g for 2 hours
For further purification repeat steps (a) - (d) three or four times
Supernatant concentrated by ultracentrifugation
Filter through Millipore filter
Recrystallise from 5-10% cadmium sulphate
Chromatograph on Sephadex G-200
Analyse

The material at this stage was usually dark brown but rather turbid and this turbidity was probably due to a small amount of membranous material which had come through from the heat-treatment step. The material was clarified by a short centrifugation at 23,000g. The major problem with any ferritin preparation is the large volume involved and any step which reduces the volume is useful. De Groot et al (1970) described just such an isolation procedure where the volume can be drastically reduced at an early stage in the isolation by merely sedimenting the ferritin by ultracentrifugation. The pellet so obtained can then be further purified by a combination of low and high speed centrifugations. These workers showed by gel electrophoresis that such a procedure gave rise to a highly purified ferritin and that the yield was extremely good. The only modification made of their procedure was the introduction of a dialysis step at the beginning in an effort to remove the large amount of salt present. It was found that by doing this, the process of sedimenting the ferritin was both cleaner and faster.

In some of the earlier experiments with human liver and spleen, the proteins were prepared by Drs. Crichton, Miller and Cumming by the somewhat different method as shown in Figure 13. After concentration by ultrafiltration and removal of any traces of bacterial contamination, these workers either purified the protein by passage through a Sephadex G-200 column, where only the material eluting with the void volume was collected, or by crystallisation from 5 - 10% cadmium sulphate.

Apoferritin was prepared from ferritin by either treatment with sodium dithionite and bipyridyl (Behrens and Taubert, 1952) or, more usually by dialysis against 1% (v/v) thioglycollic acid overnight. The apoprotein so produced was extensively dialysed

against distilled water and then against buffer. The apoferritin was then centrifuged at 23,000g for 20 minutes and either stored at 4°C as a solution in pre-sterilised bottles or as lyophilised material.

Native apoferritin, that is apoferritin that has been isolated without recourse to reduction, was prepared by spinning a stock solution of ferritin at 260,000 g for 2 hours (Macara, Hoy and Harrison, 1972). This treatment causes the heavier ferritin to be sedimented and the supernatant which contained the colourless apoferritin was carefully removed and stored at 4°C as a solution in sterile bottles.

Crystallisation of Ferritin and Apoferritin. Crystallisation of ferritin and apoferritin from a protein solution was achieved by making the solution 5% with cadmium sulphate and leaving undisturbed for 2 days at 4°C. Crystals were obtained by filtration and air-dried.

Amino Acid Analysis. Routinely 1 mg of protein was dissolved in 2 ml of 5.7N hydrochloric acid (constant boiling) and 200 µl of 2-mercaptoethanol added. The resultant solution was transferred to a boiling tube and the contents alternatively degrated and nitrogen gassed several times prior to sealing the tube. This procedure minimised loss of methionine due to oxidation. The sample was then incubated for 20 hours at 110°C. 150 µl of the resultant hydrolysate were taken to dryness by rotary evaporation and the residue repeatedly dissolved in water and re-evaporated in order to completely remove all the hydrochloric acid. The hydrolysate so produced was analysed by the single column system and stepwise elution on either a Jeolco amino acid analyser

(Japan Electron Optics Laboratory Company Ltd., Chiyoda-ku, Tokyo) or on a Unichrom amino acid analyser (Fa, Beckman, Munich, Germany).

Cyanogen Bromide Digestion. A 100-fold molar excess of cyanogen bromide was added to horse spleen apoferritin dissolved in 70% (v/v) formic acid (5 mg/ml). After 20 hours incubation at room temperature, the reaction was stopped by dilution with 10 volumes of ice-cold water. The resulting peptide mixture was re-lyophilised from water several times to completely remove any excess reagent or volatile by-products. Cyanogen bromide digestions were also performed on trypsin, myoglobin and cytochrome c by the same method. In addition, horse spleen apoferritin was digested by cyanogen bromide by a second method. In this case the protein was treated, prior to the addition of cyanogen bromide, for 20 hours at 25^{o}C with a solution of 3mM dithiothreitol in 80% (v/v) formic acid which had been saturated with nitrogen. The digestion and purification were then carried out as described earlier.

End Group Analysis. The N-terminal amino acids of the total cyanogen bromide cleavage mixture were determined by the dansyl method of Gray (1967). The dansyl derivatives of the amino acids were separated and identified by thin layer chromatography on polyamide sheets by the method of Woods and Wang (1967).

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis. The proteins and peptides used in this study as molecular weight markers are listed, together with their molecular weights, in Table 3. The method employed was essentially that of Weber and Osborn (1969) and of Shapiro and Maizel (1969). 0.5 mg of each

protein were prepared for electrophoresis by dissolving in 1 ml of 0.01M sodium phosphate buffer, 1% sodium dodecyl sulphate, 1% 2-mercaptoethanol, pH 7.0 and incubating at 37°C for 4 - 5 hours. The solutions were dialysed overnight against 0.01M sodium phosphate buffer, 0.1% sodium dodecyl sulphate, 0.1% 2-mercaptoethanol, pH 7.0. The samples were then diluted with an equal volume of the same buffer containing 0.005% bromophenol blue and 20% glycerol. The glass gel tubes used in this study (10 or 15 cm) were immersed in hot detergent for two hours, thoroughly rinsed with distilled water, and oven-dried prior to use. In these experiments 10% acrylamide: 0.27% methylenebisacrylamide gels were used and the gel mixture was thoroughly dearated before addition of 0.5% ammonium persulphate. The gels also contained 0.1% sodium dodecyl sulphate and 0.1% 2-mercaptoethanol. Before polymerisation was complete, a few drops of water were carefully applied to the top of the gel solution to ensure a smooth gel surface. The electrophoresis buffer was 0.1M sodium phosphate, 1% sodium dodecyl sulphate, pH 7.0. 100 µl of the sample (corresponding to about 25 µg of protein) were carefully layered on to the top of the gels, under the electrophoresis buffer, and were electrophoresed at 20 ma per gel for about 4 - 6 hours. After electrophoresis, the gels were removed from the tubes by rimming with water and were scanned with a Vitratron densitometer (Vitratron Instruments Ltd., Dieren, Netherlands), in order to determine the distance travelled by the blue marker dye and also to determine the length of the gel prior to staining. These measurements were necessary since the mobilities of the various proteins were expressed relative to bromophenol blue and also because during staining and destaining, the acetic acid causes the acrylamide to swell to some extent. The gels were then stained for 20 minutes with a solution

containing 2.5 g amido black 10B, 454 ml methanol, 92 ml glacial acetic acid and 454 ml distilled water. The stained gels were removed and allowed to destain for 20 - 50 hours in a solution containing 7.5% (v/v) glacial acetic acid and 5% (v/v) methanol. Once the gels were completely destained and the background was colourless, they were once again scanned in the densitometer in order to determine the distance(s) travelled by the protein(s) and peptide(s) and the length of the gel after staining. The electrophoretic mobility is defined as,

$$\frac{\text{distance of protein migration}}{\text{distance of dye migration}} \times \frac{\text{gel length before staining}}{\text{gel length after staining}}$$

This parameter was calculated for each protein and peptide and a plot of logarithm of molecular weight versus electrophoretic mobility was constructed.

Sephadex Gel Filtration. A Sephadex chromatographic column (2.5 × 100 cm) was packed to a height of 95 cm with Sephadex G-100 equilibrated with 0.01M glycine-HCl buffer, pH 3.0. Samples were prepared by dissolving 30 mg of each protein in 1 ml of 67% (v/v) acetic acid and maintained at 0°C for 1 hour. The mixture was dialysed for 16 hours against the 0.01M glycine-HCl buffer and the diffusate was then made 0.5% in blue dextran and 0.1% in tryptophan. A few drops of glycerol were then added and 0.5 ml of the sample was applied by momentarily stopping the column flow and carefully layering under solvent onto the top of the column. The column flow was started again and the flow of buffer was maintained at 25 ml/hour using a Watson-Marlow peristaltic pump (Watson Marlow, Buckinghamshire, Great Britain). The efflu-

ent from the column was passed through the flow cell of an LKB Uvicord II monitoring at 280 nm (LKB Producter, London, Great Britain). Blue dextran was used to measure the void volume (V_o), while tryptophan was used as a measure of the total volume (V_t). Several proteins of known molecular weight were used to calibrate the column.

Another Sephadex chromatographic column (2.5 x 100 cm) was packed to the same height with Sephadex G-75 equilibrated with 1M acetic acid. Samples for Sephadex G-75 gel filtration were prepared by dissolving 30 mg of each protein per ml of 1M acetic acid at room temperature. Blue dextran and glycerol were then added as before and the column runs and calibration were performed exactly as described for the Sephadex G-100 column except that the effluent was collected in 3.6 ml fractions in a BTL Chromofrac fraction collector. Protein was estimated spectrophotometrically at 280 nm in a 1cm light path and blue dextran was monitored at 630 nm.

A third column (2.5 x 100 cm) was packed with Sephadex G-100 equilibrated with 67% (v/v) acetic acid and the column runs and calibration were performed exactly as described earlier. Samples for this column were prepared by dissolving the protein (30 mg/ml) in ice-cold 67% (v/v) acetic acid and incubating at $0^{o}C$ for 1 hour. Following this incubation, the samples were applied to the column as described previously.

Partial peptide fractionation and desalting were routinely carried out on either a Sephadex G-10 column or a G-15 column (2.5 x 50 cm). These were equilibrated with 1M acetic acid or with 0.1M borate buffer, pH 7.0. In the case of the studies on chemical modification, the modified proteins were desalted on a calibrated Sephadex G-75 column (2 x 100 cm) equilibrated with 0.1M borate buffer, pH 7.0. This enabled the low molecular weight reactants

to be removed and the molecular weight of the modified protein to be determined.

Gel Filtration in the Presence of 6M Guanidine Hydrochloride.

Guanidine Hydrochloride Purification. Commercially available guanidine hydrochloride is relatively impure (95%) and, since some of the impurities present are highly absorbing at 280 nm, it is necessary to remove these prior to use. The method used for this study was to dissolve a weighed amount of commercial guanidine hydrochloride in sufficient glass distilled water to give a 6M solution and the pH was decreased to about pH 2.0 - 3.0. Activated charcoal was then added to the brown-coloured solution and the resulting mixture was heated with intermittent stirring to 60^{o}C in a partially closed flask (to minimise solution losses due to evaporation) for about 4 - 6 hours. After heating, the solution was allowed to stand at room temperature overnight and was then filtered through a fine filter paper (Whatman No. 42). The resultant colourless solution was adjusted to the required pH (either 5.0 or 8.6) and an absorption spectrum run to ensure adequate purification. This was adjudged to be the case when the absorption of a 6M guanidine hydrochloride solution at 280 nm was less than 0.1.

Gel Equilibration. The gel filtration media used in these experiments were Sepharose 6B and Bio-Gel A-5m, both with an nominal agarose content of 6%. Pharmacia chromatographic columns (1.5 x 90 cm) which had been silanized before use (treatment with 5% (v/v) dimethyldichlorosilane-toluene at 60^{o}C for 2 hours) were packed to a height of 85 cm with the respective agarose gel which had been equilibrated with 6M guanidine hydrochloride at pH 5.0. At this stage of equilibration great care was exercised to prevent

partial degradation of the agarose beads. To do this, the gel was allowed to equilibrate essentially by slow diffusion of the guanidine into the gel matrix and, after a period of about 20 hours, the mixture was degrated without stirring.

Sample Preparation. The proteins and peptides were prepared for gel filtration by incubation in 6M guanidine hydrochloride, 0.1M 2-mercaptoethanol, pH 8.6 for 6 hours at 37°C. The proteins/peptides thus unfolded and fully reduced were then carboxylmethylated by treatment with iodoacetic acid by the method of Crestfield (1963). Blue dextran (5 mg), tryptophan (1 mg) and 3 - 4 drops of glycerol were then dissolved in 1 ml of the protein solution and, 0.2 ml of this sample was applied to the column. The column effluent was then passed through the flow cell of an LKB Uvicord (LKB Instruments, London, Great Britain) monitoring at 280 nm and the effluent from this was collected in 1 ml fractions in a BTL Chromofrac fraction collector (Baird and Tatlock Ltd., London, Great Britain), thus allowing readings at 630 nm (blue dextran) to be taken manually on a Beckman DB spectrophotometer (Beckman Instruments Ltd., Palo Alto, California, U.S.A.). The flow of eluting solvent was maintained at a rate of 2 ml per hour by use of the difference in height of a solvent reservoir.

Proteins could be recovered by removing the guanidine hydrochloride by extensive dialysis. In the case of small peptides, the dialysis tubing so used had first to be acetylated in an effort to reduce the effective pore size. This was achieved by heating the dialysis tubing with 25% (v/v) acetic anhydride in pyridine at 60°C for 8 hours. The effect of this treatment on the critical pore size was determined by use of the dialysis cell described earlier. The treated membrane was stretched between the two compartments and the lower was filled with distilled water. The

cell was connected to an LKB Uvicord II and flow through the system was allowed to proceed for about 2 hours in which time the base-line was steady. At this time a concentrated aqueous tryptophan solution was added to the top chamber and this event was marked on the recorder. An increase in the recorder reading was noticed and this continued until an equilibrium was reached at which time the recorder reading had reached a plateau. This procedure was also carried out for untreated dialysis membrane.

Quantitative Tryptophan Determinations.

Spectrophotometric Method I. The method used was essentially the spectroscopic method of Edolhoch (1967). Tryptophan determinations were carried out by this procedure for horse liver and spleen, human liver and spleen and human haemochromatotic liver. The protein (0.2 - 1.0 mg/ml) was allowed to unfold in the presence of 6M guanidine hydrochloride, pH 6.50 (Mann, ultra pure) for 18 hours at 37°C. The pH of the guanidine solution was adjusted accurately to pH 6.50 by means of a times-ten scale expander. Duplicate absorption spectra were obtained with a Cary model 15 spectrophotometer. Estimates of the tryptophan and tyrosine content were evaluated as shown below. Including the contribution of cysteinyl residues to the absorption at 280 nm and 288 nm we have,

$$E_{280} = N_{trp}5690 + M_{tyr}1280 + L_{cys}120$$
$$E_{288} = N_{trp}4815 + M_{tyr}385 + L_{cys}75$$

where N, M and L are the number of moles of tryptophan, tyrosine and cysteine per mole of protein. Eliminating M_{tyr} we have,

$$N_{trp} = E_{288}/3103 - E_{280}/10318 - L_{cys}/83.56$$

Thus by measuring the absorption at 280 nm and 288 nm and by using the literature value for L_{cys}, the value of N_{trp} can readliy be evaluated. Once N_{trp} has been determined from experimental data the value of N_{tyr} can be evaluated from either of the equations given earlier.

Spectrophotometric Method II. This method was essentially that of Bencze and Schmid (1957) in which a protein solution (0.1 - 1.0 mg per ml) was quickly adjusted to pH 12.5 and duplicate absorption spectra were run in the region 350 nm - 200 nm on a Cary model 16. A tangent was drawn from the two maxima and the slope of the line so produced was calculated. This could then be compared with the range of values obtained by Bencze and Schmid for model compounds and a value for the ratio tyrosine / tryptophan evaluated. Now, knowing the number of moles of tyrosine per mole of protein from amino acid analysis, the number of tryptophan residues per protein molecule could be easily established.

Chemical Modification Method. A method was recently devised for the conversion of tryptophan to 2-(2-nitrophenylsulphenyl)-tryptophan and the subsequent quantitative evaluation of this modified residue (Scoffone, Fontana and Rochi, 1968a,b; Boccu, Veronese, Fontana and Benassi, 1970). The protein used in this study was fully reduced and carboxymethylated as described earlier. The protein was then dissolved in 50% (v/v) formic acid to give a solution of about 10 mg/ml. To 1 ml of this solution was added 0.5 ml of glacial acetic acid containing 2.5 mg of 2-nitrophenylsulphenyl chloride. The resultant mixture was stirred vigorously and incubated at room temperature for ten minutes after which time the reaction was terminated by the addition of 100 ml of ice-cold acetone containing 2.5 ml 1N hydrochloric acid. The mixture immediately appeared flocculent and was allowed to stand at $0^{o}C$

for about 2 hours. The precipitate was isolated by centrifugation, washed with ice-cold acetone and then dried in a dessicator over concentrated sulphuric acid. To reverse the modification of any cysteinyl residues the protein was then dissolved in 0.1N sodium hydroxide and gassed with nitrogen for 1 hour at room temperature. The protein was then precipitated with ice-cold acetone and purified as before. The protein content of the purified tryptophan-modified protein was determined by taking an aliquot of a solution (about 0.5 mg/ml) for amino acid analysis. The absorbance of the remainder of the solution was measured on a Cary model 15 spectrophotometer at 365 nm. To quantitate the number of tryptophan residues modified per protein molecule, a molar absorptivity of ϵ 4000 was used (Boccu et al, 1970).

Analytical Ultracentrifugation.

Dissociated Apoferritin. Since it is known that apoferritin can be dissociated into subunits by several protein denaturants, it was decided to determine the molecular weight of the subunit obtained from different dissociating conditions.

Dissociation by 6M Guanidine Hydrochloride. Lyophilised apoferritin was dissolved in 6M guanidine hydrochloride, pH 7.0 and incubated at 37^{o}C for 4 hours. The solution was then dialysed overnight at room temperature against the guanidine solution. The sedimentation equilibrium determinations were performed over a protein concentration range of 0.2 - 0.8 mg/ml.

Dissociation by Acetic Acid. An apoferritin solution (0.2 - 1.0 mg/ml) was incubated in ice-cold 67% (v/v) acetic acid for 1 hour at 0^{o}C. The solution was then dialysed extensively against several changes of 0.01M glycine-HCl buffer, pH 3.0.

Dissociation by Alkali. Lyophilised apoferritin was dissolved in

0.01N sodium hydroxide and 0.1N sodium hydroxide and the resultant solutions were dialysed against the respective bases for 16 hours at room temperature. The sedimentation equilibrium runs were performed over a protein concentration range of 0.2 - 0.8 mg/ml.

Dissociation by Sodium Dodecyl Sulphate. Apoferritin dissociated by the anionic detergent sodium dodecyl sulphate was prepared as described earlier for sodium dodecyl sulphate- polyacrylamido gel electrophoresis. The protein concentration range used in this centrifugal study was 0.4 - 0.8 mg/ml.

For all of the dissociated protein solutions described above, the molecular weight determinations were performed with an An-G Ti rotor at a speed of 20,000 revs. per minute.

Both these and the sedimentation equilibrium runs carried out with undissociated apoferritin were performed on a Spinco model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, California, U.S.A.) equipped with electronic speed control and a regulated temperature control unit. The use of the split beam photoelectric scanner accessory, in conjunction with a monochromator and the ultraviolet absorption optical system (Schachman and Edelstein, 1966) permitted the recording of sedimentation patterns at 280 nm and made possible the use of very low concentrations of protein. Whole cell apparent weight average molecular weights ($\overline{M}_{w,app}$) were determined at 20°C.

Undissociated Apoferritin. Before examining the protein by sedimentation equilibrium, we studied the effects, if any, of buffer pH, buffer ionic strength and protein concentration on its sedimentation properties. Protein was dissolved in distilled water and in 0.01, 0.02, 0.05, 0.1 and 0.2M phosphate buffer, pH 6.4 and dialysed against the respective buffer for 20 hours. Sedimen-

tation velocity runs were carried out on each of the samples. Frequently the protein dialysed against distilled water precipitated but it was found that on pelleting this material by centrifugation, enough remained in solution to monitor by ultraviolet optics during ultracentrifugation. To study the effects of pH, we used a 0.01M phosphate buffer at pH 5.5, 6.0, 6.5, 7.0, and 7.5 and the material for centrifugation was prepared as described above. The effect of protein concentration was examined in 0.01M phosphate buffer, pH 6.4 over a concentration range 0.2 - 1.0 mg/ml. In order to avoid problems of adsorption, especially at low protein concentrations, the centrepiece was filled and emptied several times before the final filling. No layering oil was used in these experiments. In all these preliminary studies, the distribution of sedimenting species was plotted as a function of $S_{20,w}$ utilising a FORTRAN 4 program on the PDP 8/L digital computer. Such sedimentation velocity experiments were performed using an AnG-Ti rotor at a speed of 40,000 rpm at $20^{o}C$. Once suitable conditions were established, protein was dissolved in the required buffer at various concentrations (0.2 - 1.0 mg/ml) and dialysed extensively against the same buffer. Sedimentation equilibrium runs were then performed on these samples using the same rotor as above at a speed of 4,000 rpm and a temperature of $20^{o}C$. The time of centrifugation was 4 days. Data obtained from the scanner traces were processed into ln C and r^2 values, using an Olivetti 101, and a plot of ln C versus r^2 was constructed. From such a plot $d\ln C/ dr^2$ could be evaluated and the whole cell apparent weight average molecular weight was then obtained by,

$$\bar{M}_{w,app} = \frac{2RT}{(1 - \bar{v}')\, w^2} \cdot \frac{d\ln C}{dr^2}$$

where $\overline{M}_{w,app}$ is the apparent whole cell weight average molecular weight, w is the angular velocity of the rotor, and $\bar{V}'$ is the effective partial specific volume of the protein.

Isoelectric focusing. The iron of the ferritin was reduced by dialysis against 0.01M phosphate buffer, pH 6.4 , 1% (v/v) thioglycollic acid for 16 hours at 4°C and the apoferritin so produced was dialysed against several changes of 0.01M phosphate buffer, pH 7.2 in order to remove the acid. The protein was then diluted with the same buffer to give a solution of concentration 1-2 mg/ml and the resultant solution was further dialysed against more buffer. In the case of those proteins which had been lyophilised, samples were prepared by dissolving in 0.1M phosphate buffer, pH 6.4 and then dialysed against several changes of 10 mM phosphate buffer, pH 7.2. Such samples were routinely clarified by centrifugation. 3.75 g acrylamide, 0.1 g N,N' - methylenebisacrylamide and 30 µl N,N,N',N' - tetramethylethylenediamine were dissolved in distilled water and made up to 48 ml. 1 ml of the desired ampholine and 1 ml of protein sample were added and the resultant solution stirred gently and dearated for a few minutes. This volume is slightly in excess of that required to fill twenty gel tubes (0.5 x 8 cm) which had been thoroughly cleaned before use. 100 µl of riboflavin (0.5 mg/ml, stored in the dark at 4°C) were then added and the gel tubes filled, making sure that no bubbles formed at the bottom of the tubes. A layer of distilled water was then carefully placed on the top to ensure a flat surface on polymerisation which was effected by exposure to ultraviolet light. Gelling was usually complete in 10 - 20 minutes under the conditions used. The gels were then allowed to equilibrate at 4°C for 1 - 2 hours prior to focusing.

The electrolyte solutions used in this study were 3.5% (v/v) aqueous ethanolamine (cathode) and 2.5% (v/v) phosphoric acid (anode). The cathode was at the bottom of the electrophoresis apparatus. Voltage was applied and was gradually increased to a value of 100 volts. With such an applied potential, the current dropped from an initial value of 2.4 ma to a final value of 0.3 ma as focusing proceded. The voltage was fixed at 100 volts and the gels were electrophoresed for 16 - 20 hours at $4^{o}C$.
The gels were removed from the tubes by rimmimng with distilled water from a fine syringe needle. In order to fix the protein and also to remove the carrier ampholytes, the gels were immersed in several changes of 10% (w/v) trichloroacetic acid for 2 - 4 days after which time they were stored in an aqueous solution containing 7.5% (v/v) acetic acid, 5% (v/v) methanol for a further 24 hours. The gels were then stained with amido black (2.5 g amido black in 908 ml 50% (v/v) methanol, 92 ml glacial acetic acid) for 1 hour and destained by storing in several changes of aqueous solution containing 7.5% (v/v) acetic acid, 5% (v/v) methanol. It was found that the gels were sufficiently destained for photography in about 2 - 3 days.

Dissociation of Apoferritin by Extremes of pH. In this study of the pH stability of apoferritin it was decided to use a universal buffer in an effort to eliminate any problems arising from salt effects. The buffer prepared was essentially a combination of Theorell and Stenhagen's citrate-phosphate buffer and Sørenson's glycine I and II buffers. This buffer was prepared by dissolving 42.03 g citric acid, 12.37 g boric caid, 15.01 g glycine, 11.69 g sodium chloride and 13.6 ml phosphoric acid in 1 litre of distill-

ed water. The buffering capacity of such a solution was in the range pH 1.0 - 13.0. 50 ml of this stock buffer were adjusted to the required pH using concentrated hydrochloric acid or sodium hydroxide and then made up to 250 ml. Protein was dissociated by ice-cold acetic acid as described previously and the resulting solution dialysed for 24 hours at room temperature against universal buffer, 0.01M glycine-HCl buffer or 0.01M glycine-acetate buffer at a variety of pH values. Protein was also dissociated by dissolving in 0.01M glycine-HCl buffer, pH 1.5 and this material was then dialysed for 24 hours against 0.01M glycine-HCl and 0.01M glycine-acetate buffers in the range pH 1.5 - 5.0. After the dialysis, the pH of the various solutions were then redetermined using a Radiometer pH meter, model 25 with a times ten scale expander (Radiometer, Copenhagen, Denmark)

Sedimentation velocity runs were carried out on the various protein solutions using the AnG - Ti rotor at a speed of 40,000 rpm and a temperature of 20^{0}C. Sedimentation coefficients were corrected, where possible, for salt and concentration.

Ultraviolet Difference Spectroscopy. In measuring difference spectra, a solution of apoferritin at the same concentration was used as the reference and measurements were obtained as differences from this reference solution. Because such spectral changes were often small, amplifier drift on warm-up could be significant. For this reason, both the spectrophotometer (in this case a Cary model 16) and the recorder (Honeywell Electronik 194, Honeywell Controls Ltd., Great Britain) were given sufficient time to stabilize. This was adjudged to be the case when the absorption of empty cell compartments (air-air baseline with no cells) reached a constant value. Before each set of experiments

the baseline was adjusted with buffer blanks to zero with the instrumental multipots for the region 400 - 200 nm. The protein solutions in both the sample and reference compartments, prepared as described on the previous page, were made from the same stock solution, the optical density of which was between 0.5 - 1.5. A series of sample and reference solutions, each having different protein concentrations, was prepared and difference spectra were obtained for each pair of matched solutions. A plot of the height of the maximum absorption of the difference peak against the protein concentration was constructed. Prior to obtaining spectra, the protein solutions were routinely clarified by centrifuging for 4 minutes on a Beckman Microfuge 152 (Beckman Instruments Ltd., Palo Alto, California, U.S.A.).

In an effort to improve resolution, slit-widths were never allowed to exceed 0.8 mm. The instrument was fitted with a 0 - 0.1, 0 - 0.2, 0 - 0.5, 0 - 1.0, 0 - 2.0 optical density slide wire on a Cary 1626 Recorder Interface. Since almost all spectra showed evidence for light scattering in the region 400 - 310 nm, they were corrected by the method of Winder and Gent (1971). By this method, a plot of log O versus log λ was constucted for each spectrum and evaluation of the slope of the line obtained allowed the contribution from light scattering to be assessed.

A value of ϵ_{295} = 2381 was used for the molar absorption coefficient for the difference spectrum resulting from the ionisation of tyrosine (Mihalyi, 1968). Values of ϵ_{max} = 1600 and ϵ_{286} = 540 were used for the change in molar absorbance produced by transfer of a tryptophan chromophore from the interior of a protein into water and for the change in molar absorbance resulting from the perturbation of a tyrosine residue respectively i.e. six times that for the free amino acid (Donovan, 1968).

The experiments carried out in the acid region were performed in 0.01M glycine-acetate buffer at various pH values while those in the alkaline region were performed in the universal buffer described earlier. Apoferritin was dissociated by ice-cold acetic acid as described earlier and the resulting solution dialysed against a variety of buffers in the range pH 1.0 - 5.0.

Chemical Modification.

Modification at Cysteine Residues.

Method I. The method employed was essentially that of Canfield and Anfinsen (1963). 50 mg horse spleen apoferritin were dissolved in 5 ml 0.1M tris buffer, pH 8.6 containing 1% (w/v) sodium dodecyl sulphate and 100 µl 2-mercaptoethanol. The solution was gassed with nitrogen for 5 minutes and incubated at 37°C for 4 hours. The protein was then precipitated with 100 ml ice-cold acetone containing 2.5 ml 1N hydrochloric acid. The precipitate obtained following centrifugation was dissolved in dilute acid at pH 2.0. A ten fold molar excess of iodoacetic acid was added and the solution made alkaline by the addition of 30 µl concentrated ammonia. The pH was maintained at pH 8.6 for ten minutes after which time 200 µl 2-mercaptoethanol was added. The modified protein was then precipitated as before, resuspended in acetone and centrifuged again. Finally the protein was dissolved in 1M acetic acid and lyophilised. The entire procedure was repeated with another 50 mg of apoferritin except that the sodium dodecyl sulphate was not added.

The iodoacetic acid used was iodoacetic acid - 2 - C^{14} with a specific activity of 154.045 µCi/mmole. Following modification, an aliquot was hydrolysed and taken for amino acid analysis. Another aliquot was removed, weighed accurately, and taken for

scintillation counting using Packard Insta-Gel as the scintillator. The efficiency of counting C^{14} in Insta-Gel was determined by adding a known standard (toluene - C^{14}) to an aliquot. The efficiency for counting C^{14} in such a scintillator was found to be 79.4%.

Method II. Titration of the protein with 5,5'-dithiobis (2-nitrobenzoic) acid was carried out essentially by the method described by Ellman (1958,1959). Protein was dissolved in 0.05M tris-HCl, 0.4M sodium chloride, pH 8.0 at a concentration of about 1 mg/ml and to this was added a 20-fold molar excess of 5,5'-dithiobis (2-nitrobenzoic) acid dissolved in 0.05M tris-HCl, pH 8.0. The course of the reaction was monitored at 412 nm on a Cary model 16 spectrophotometer. The titration was repeated at pH 9.0 and another with 1% (w/v) sodium dodecyl sulphate present in the mixture. A molar extinction coefficient of 13,600 was used for the liberated thiolate anion (Ellman, 1958, 1959).

Method III. The thiol content of apoferritin was also determined by polarographic titration with phenylmercuric acetate according to the method of Cecil and Snow (1962). A sample of protein was denatured by 1% (w/v) sodium dodecyl sulphate. Before measurements were made oxygen was removed from the solution by evacuation and the oxygen remaining was removed by gassing with nitrogen. The solution was kept deoxygenated by making it 50 mmolar with sodium sulphite, and potassium chloride was added as supporting electrolyte. The electrodes used in this study were a saturated calomel electrode and a dropping-mercury indicator electrode. The solution was titrated with a standard phenylmercuric acetate solution (0.5mM). 10 µl aliquots of titrant were added and the mixture was agitated by a stream of nitrogen. The concentration of uncharged mercurial was determined by measurement of the diffusion current at -0.6 volts. The helpful advice and discuss-

ions from Dr. R. Paterson of the Chemistry Department is gratefully acknowledged and thanks are also due for the provision of the equipment.

Modification at Tryptophan Residues. The method used for the conversion of tryptophan to 2-(2-nitrophenylsulphenyl) - tryptophan has already been described. 2-(2-nitrophenylsulphenyl) - tryptophan was determined spectroscopically using a molar absorptivity of $\epsilon_{365} = 4000$.

Modification at Tyrosine Residues. The method employed for the nitration of tyrosine was essentially that of Sokolovsky, Riordan and Vallee (1966). Protein was dissolved in 0.05M tris buffer, pH 8.0 (1 - 8 mg/ml). Stock tetranitromethane (8.4M) was diluted 1 : 10 with 95% ethanol. 10 - 50 µl of this diluted tetranitromethane was added to the protein solution in 5 µl aliquots with stirring. When the reaction was being monitored spectrophotometrically, 1 mg of protein in 1 ml 0.05M tris buffer, pH 8.0 was in the sample cell while 1 ml of the same buffer was in the reference cell. 10 µl of the diluted nitrating reagent was added quickly to each cell and the recorder switched on. The cells were monitored at 428 nm until the reaction was complete. Determining the extent of nitration spectrophotometrically is complicated because the nitroformate anion produced in the course of the reaction also contributes to the absorbance at 428 nm. However, Bustin (1971) has described a method for determining a corrected value for nitrotyrosine from the spectrophotometric data at 428 nm. He has shown that the nitroformate anion has a wavelength maximum at 350 nm and that the absorption of the anion at 428 nm is 4% of the maximum absorption of this species. He further demonstrated that the molar absorption of the nitroformate at 350 nm is 3.5 times the molar absorbance of nitrotyrosine

at 428 nm. This being the case, we have,

$$\text{Absorbance}_{428} = \text{Absorbance}_{428}^{\text{nitrotyr}} + \text{Absorbance}_{428}^{\text{nitroformate}}$$

$$= \text{Absorbance}_{428}^{\text{nitrotyr}} + 0.04\text{Absorbance}_{350}^{\text{nitroformate}}$$

$$= \text{Absorbance}_{428}^{\text{nitrotyr}} + 0.14\text{Absorbance}_{428}^{\text{nitrotyr}}$$

$$= 1.14\ \text{Absorbance}_{428}^{\text{nitrotyr}}$$

It can be seen from this calculation that the absorption contribution of the nitrotyrosine residues is 87.8% of the total absorption at 428 nm and not 86% as Bustin quoted.

Spectral measurements were performed on a Cary model 16 spectrophotometer. In both cases the modified protein was desalted and purified on a Sephadex G-75 column as described earlier. The purified protein was lyophilised and an aliquot was taken for amino acid analysis.

Modification at Lysine Residues. The method used for the guanidination of lysines was essentially that of Tu, Hong and Solie (1971). Horse spleen apoferritin (50 mg) was dissolved in 20 ml 0.6M o-methylisourea, pH 10.5. The pH was checked after addition of the protein and was readjusted, if necessary, to pH 10.5 by the addition of 1N sodium hydroxide. The reaction mixture was then incubated at 25°C for 3 days after which time it was dialysed extensively against several changes of 0.01M borate buffer, pH 7.4. The protein was then lyophilised and stored. Some modified protein was dissolved in 1M acetic acid and chromatographed in the calibrated G-75 column described previously. The extent of modification was calculated from amino acid analysis which

demonstrated a decrease in the lysine peak with the corresponding appearance of a new peak, homoarginine, which eluted after arginine on the amino acid analyser. The same colour factor for arginine was used throughout. Homoarginine is stable and undergoes little or no destruction during acid hydrolysis (Kimmel, 1967).

Modification at Arginine Residues. The method employed for the conversion of arginine residues to N^5-(4-oxo-1,3-diazaspiro [4.4] non-2-ylidene) - L - ornithine was basically that described by Toi, Bynum, Norris and Itano, (1967) except that the reaction was carried out in 0.1M triethylamine buffer, pH 10.9. 20 mg of protein were dissolved in 2 ml 0.1M triethylamine and the solution was reacted with 10 mg of 1,2 - cyclohexanedione dissolved in 0.5 ml of 0.1M triethylamine at room temperature for 24 hours at pH 10.9. In early studies it was found that lowering the pH to 7.0 in order to stop the reaction, caused the protein to come out of solution. For this reason the reaction was terminated by extensive dialysis against triethylamine buffer at pH 10.0. The pH was then decreased very slowly until the modified protein remained in solution on dialysis against 0.01M borate buffer, pH 7.4. The product was then lyophilised.

The extent of modification was estimated by the decrease in arginine content as measured by amino acid analysis following acid hydrolysis of the modified protein. The decrease in arginyl residues was reflected in the concomitant appearance of some new peaks and these were all found to chromatograph on amino acid analysis with the basic amino acids.

Functional Aspects of Apoferritin.

General. All glassware and cuvettes used in these studies were soaked in concentrated hydrochloric acid and washed with deionised

water to remove trace elements and all solutions were prepared with deionized water. Ferrous iron was determined as the red-coloured Fe^{++}-bipyridyl complex. To 2 ml of 0.5% 2,2-bipyridyl solution in 0.1M buffer, pH 7.4 and 10% (v/v) ethanol was added, 100 µl aliquots of various concentrations of ferrous iron and the resultant solution stirred and allowed to stand for ½ hour. The absorbance of the solutions were then read at 520 nm. A standard curve of A_{520} versus concentration of ferrous iron was plotted and treated by regressional analysis and a value for the molar extinction coefficient was evaluated. The assay was repeated as before except that the reaction mixture and the ferrous salt solutions were 1% (v/v) thioglycollic acid.

Preliminary Assay Experiments. In a preliminary study several buffer systems were used to find the most suitable. These included phosphate, carbonate, bicarbonate, borate, cacodylate-borate, tris-maleate-sodium hydroxide and imidazole. Once a suitable buffer system was obtained, the effect of ionic strength and pH on the solubility of ferrous ammonium sulphate was studied. Thus the ferrous iron content of ferrous ammonium sulphate at a variety of pH values and buffer ionic strengths was established by the quantitative bipyridyl reaction. The results were plotted as percentage ferrous iron remaining in solution against pH , ionic strength etc. A time course for the deposition of FeOOH was also established by use of the bipyridyl reaction. Also found in these preliminary studies was the effect of ferrous ammonium sulphate on the initial pH of the buffer and this was measured at different buffer molarities. In all the experiments on the functional aspects of horse spleen apoferritin, the reactions and incubations were carried out at 25°C.

Identification of Product. Since the product of reconstitution experiments would, if successful, be ferritin we were obliged to establish criteria for the determination of (a) the extent of iron incorporation into the protein and (b) the degree with which the product resembled native ferritin.

Method I. One method for the identification of ferritin is polyacrylamide gel electrophoresis. 6.5 g acrylamide, 0.1758 g N,N'-methylenebisacrylamide were made up to 99 ml with 0.01M borate buffer, pH 6.4. 150 µl N,N,N',N'-tetramethyl-1,2-diaminoethane were added and mixed and dearated for about 10 minutes. 25 mg ammonium persulphate were dissolved in 1 ml of buffer and this was mixed with the acrylamide solution and gels were cast in 10 cm gel tubes. For these conditions, gelling was usually complete in about 10 minutes. Samples, which were routinely reconstituted ferritin that had been extensively dialysed against 0.1M borate buffer, were applied and electrophoresed at 40 ma for about 2 - 4 hours. On completion of electrophoresis, the gels were removed and stained for either protein (with amido black, as for sodium dodecyl sulphate-polyacrylamide gel elctrophoresis) or for iron (2 g potassium ferrocyanide dissolved in 1 litre of 2% hydrochloric acid).

Method II. Samples of apoferritin, native ferritin and reconstituted ferritin were very kindly examined by Mathias Wabl (Max-Planck-Institut für Molekulare Genetik, Berlin) using a Siemens Elmiskop 1 electron microscope. The conditions for microscopy were, 80 kV operating voltage, cooling stage, 200 nm condensor aperture and 50 nm objective aperture. The magnification was x65,000 and photographs were magnified fivefold from the photographic plate. The procedure used for the preparation of the sample was essentially the Valentine technique. A very thin carbon film,

made by indirect evaporation, was floated on the protein solution (concentration 0.02 - 0.5 mg/ml). One minute was allowed for adsorption after which time the thin film was transferred into a 0.5% uranyl acetate solution for one minute. Onto the top of the film, 'holey' carbon film grids were layed down and the combination was then carefully picked up. The grid was then air-dried for a few minutes. Samples were photographed at sections on the grid where the holes of the thick carbon films were covered by the thin film with the specimen on it.

Molar Absorptivity of the Micelle. Reconstitution of ferritin from the apoprotein could be monitored continuously at 420 nm on a Cary 16 spectrophotometer. This, despite being a qualitative approach, allowed the rate of uptake of iron by apoferritin to be determined. The extent of iron incorporation into the protein could be determined only if the specific extinction coefficient of the micelle was known. To determine this, 10 µl of stock ferritin was dissolved in 1 ml of 0.01M acetate buffer, pH 5.0 and the absorbance measured. This was repeated several times in order to evaluate any inherent error and so calculate the mean and standard deviation. Next, 50 and 100 µl of original stock solution were added to 0.95 or 0.90 ml thioglycollic acid respectively. The samples were incubated at 45°C for one hour, after which time they were cooled and clarified by centrifugation if necessary. 10 µl aliquots were then added to 10 ml 2,2-bipyridyl (0.5% (w/v) in 10% ethanol) and absorbance readings (in triplicate) at 520 nm were measured. Again the procedure was repeated several times in order to determine the mean and standard deviation. From this data it was possible to calculate the specific extinction coefficient, $E^{1cm}_{1\%}$, of the micelle.

Enzyme Kinetics. Apoferritin was prepared from ferritin by the thioglycollic acid treatment as described earlier. The apoprotein was then extensively dialysed against several changes of the required buffer at the correct ionic strength and pH.

Effect of Protein Concentration on Reaction Rate. Apoferritin at various concentrations was dialysed extensively against 0.1M borate-cacodylate buffer, pH 5.5. Ferrous ammonium sulphate was then added to the same buffer at a concentration of 10 mg/ml. Two 1 ml aliquots of this solution were added to two 1 ml cuvettes and placed in the spectrophotometer. The machine and recorder were switched on and the baseline was steadied at zero absorbance. 50 or 100 µl samples of protein were then added to the sample cell while the same volume of buffer was added to the reference. The contents of the cells were mixed by quickly inverting and the absorbance was then monitored continuously at 420 nm. Initial velocities (v) were calculated and a plot of v versus protein concentration was constructed. Protein concentration was determined using $E^{1cm}_{1\%} = 9.82$.

Effect of Substrate Concentration on Reaction Rate. The assay system was the same as described above, except that the protein concentration was constant at 1 mg/ml while the concentration of ferrous ammonium sulphate varied in the range 0 - 20 mM. Again the initial velocities, v, were calculated and a plot of v versus substrate concentration constructed. The data was also converted to 1/v and 1/S (where S represents the substrate concentration) and plotted according to the treatment of Lineweaver and Burke (1934).

Effect of pH on Reaction Rate. In this series of experiments the substrate concentration was constant (10 mg/ml) as was the protein concentration (1 mg/ml). 1 ml samples of the protein solution

were extensively dialysed against 0.1M cacodylate-borate buffer at a variety of pH values in the range pH 4.0 - 6.5. Initial velocities were calculated for each assay and a plot of v *versus* pH was constructed.

Additional Experiments. Together these experiments established an assay system and, using this, various samples of lysozyme, β-globulin and bovine serum albumin were tested for ferroxidase activity.

Native apoferritin (prepared as described earlier) and also ferritins of low iron content were also tested for ferroxidase activity using the same assay system.

Each of the pure modified proteins described earlier were also tested for catalytic function by this method. They were assayed in an identical manner to that described earlier and the data was treated similarly.

Some of the functional studies were carried out using the dialysis cell. In these studies, ferrous ammonium sulphate was dissolved in the required buffer, centrifuged if necessary, and placed in a reservoir. The solution was then pumped through the lower chamber of the dialysis cell. When the system was at equilibrium (usually in ½ hour), 2 ml of apoferritin (5 mg/ml) were added to the top chamber. Ferrous iron was determined by the quantitative bipyridyl reaction at 1 minute intervals for 60 minutes. An enzyme blank was obtained by repeating the procedure but in this case, adding 2 ml of buffer in place of the protein solution. Data was plotted as the percentage of ferrous ammonium sulphate oxidised *versus* time of reaction.

RESULTS AND DISCUSSION

Isolation and Purification of Ferritin and Apoferritin.

The protein was isolated from the respective organs and species as described on page 64.

It was frequently found that decantation of the heat-treated supernatant resulted in slight physical disruption of the pellet, thereby causing some of the pellet to remain with the supernatant. This, however, could easily be removed by filtration through Whatman No. 1 filter paper. After precipitation with ammonium sulphate the precipitated protein was spun down at low speed. The supernatant was usually yellow in colour and was discarded. In the event of the supernatant being darker brown in colour, a second ammonium sulphate precipitation was carried out with 50% (w/v) ammonium sulphate and the precipitates combined. Finally, the ferritin was either crystallised or stored as a solution in sterile vials.

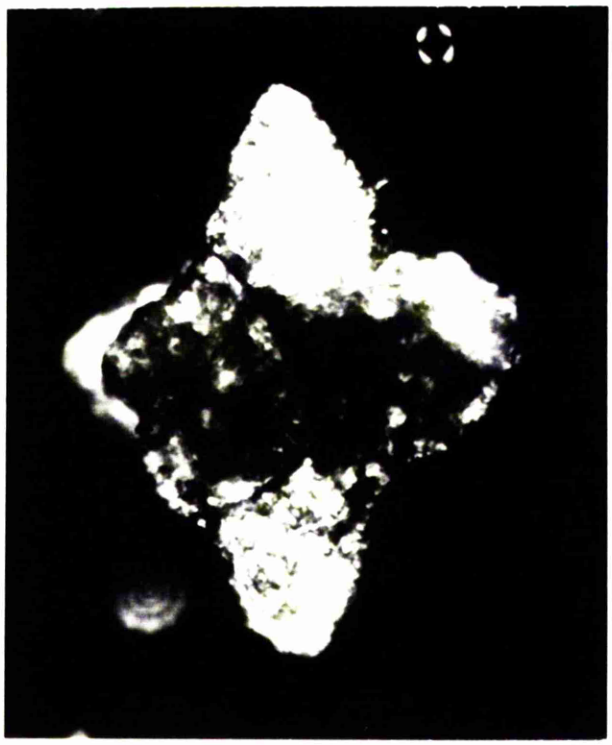
The type of crystals obtained from 5% (w/v) cadmium sulphate were found to be twinned octahedra (tetrahedral pyramids on each face of a cube) as shown in Figure 14. It can be seen that the edges of the crystal form are slightly curved. Apoferritin was obtained from ferritin by the treatment described on page 69. This material is colourless and crystallises from cadmium sulphate as octahedral crystals which are isomorphous with those of ferritin.

The protein isolated by these means was subjected to amino acid analysis and sedimentation and electrophoretic studies.

An aliquot of the protein was hydrolysed as described and subjected to amino acid analysis. The results for such analyses for horse spleen and liver apoferritin and human haemochromatotic liver apoferritin are shown in Table 2. Also shown in this table are the results of amino acid analysis for guinea pig liver apo-

Figure 14. Photomicrographs of typical ferritin (top) and apoferritin (lower) crystals obtained from horse spleen. It can be seen that both ferritin and apoferritin adopt similar crystal forms of twinned octahedra as was shown schematically in Fig. 4.

My thanks are due to members of staff of the Geology Workshop for helpful advice in the taking of these photomicrographs and also for the provision of the equipment.



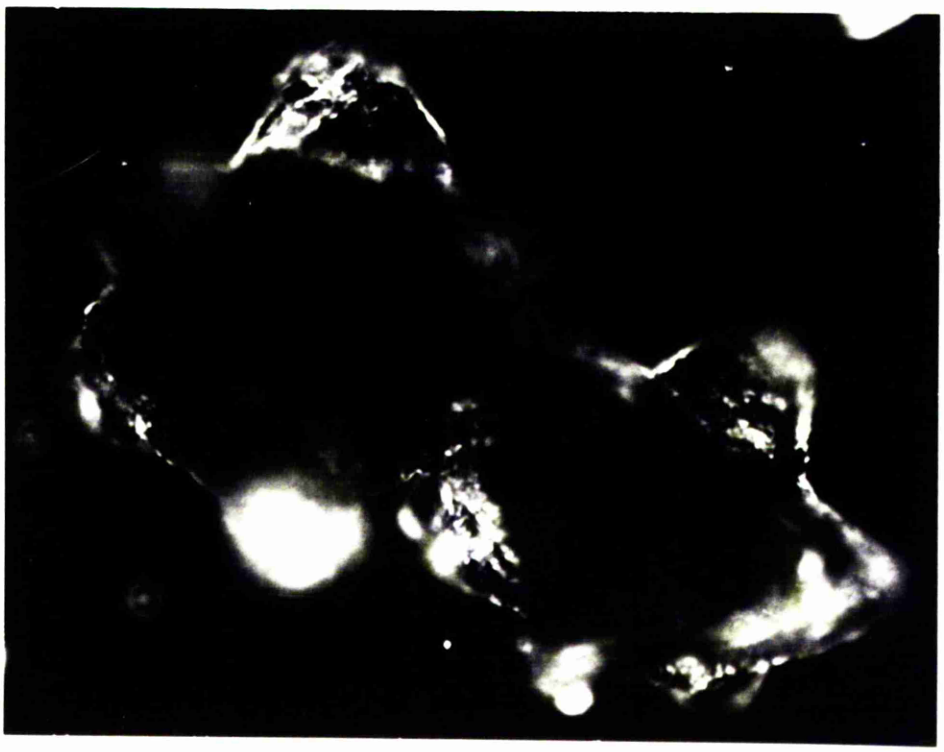


Table 2. Amino acid compositions of apoferritin. The composition of each amino acid is expressed as the number of residues per 18,500 g protein. Horse spleen, horse liver and human haemochromatotic (haem.*) liver were determined in the present study. Guinea pig liver[1] was analysed by Friedberg (1962) and horse spleen[2] was determined by Harrison (1964) and Williams and Harrison (1968). n.d, not determined. [3] In the present study tryptophan was chemically modified and estimated as 2-(2-nitrophenylsulphenyl)-trytophan.

Amino Acid Residue	Horse Spleen	Horse Liver	Human Haem.* Liver	Guinea Pig Liver[1]	Horse Spleen[2]
Cysteic acid	n.d	n.d	n.d	1.03	1.89
Aspartic acid	17.38	17.91	19.16	18.77	17.29
Threonine	5.52	5.63	6.19	5.91	5.53
Serine	8.99	9.02	9.29	7.46	8.61
Glutamic acid	23.79	24.73	23.09	23.21	23.48
Proline	2.57	3.11	2.92	3.33	2.31
Glycine	10.00	10.19	10.10	10.05	9.85
Alanine	13.88	13.38	13.67	12.86	14.00
Valine	6.93	6.93	6.29	6.73	6.97
Methionine	2.77	2.54	2.94	2.66	2.88
Isoleucine	3.47	3.55	2.47	3.70	3.53
Leucine	24.67	24.20	23.38	22.02	24.33
Tyrosine	4.97	4.13	5.02	5.99	5.26
Phenylalanine	7.48	7.65	6.92	6.21	7.13
Histidine	5.62	6.47	5.37	6.73	5.38
Lysine	8.77	8.78	10.43	9.31	8.31
Arginine	9.52	8.97	8.43	8.87	9.10
Tryptophan	2.01[3]	2.10[3]	2.21[3]	1.85	0.83

ferritin (Friedberg, 1962) and horse spleen apoferritin (Harrison, 1964; Williams and Harrison, 1968) performed by other workers and this allows direct comparison to be made. It can be seen that the results for horse spleen apoferritin are in very good agreement with those of Harrison (1964) and Williams and Harrison (1968) except for the value of tryptophan which will be discussed more extensively at a later stage.

From our analysis, horse liver and horse spleen proteins have very similar amino acid compositions, with horse liver perhaps having one extra glutamic acid residue and one less arginine residue. It is interesting to speculate that if this is the case and, further, that if it is the result of a point mutation then the glutamyl residue can only be present as glutamine. Thus, either CAA or CAG is converted to CGA or CGG respectively and, as far as the protein's intrinsic charge is concerned, then this is for all intents and purposes conserved.

The amino acid composition for human haemochromatotic liver apoferritin is also quite similar, with perhaps the following changes; one less glutamic acid, isoleucine and leucine, one more aspartic acid and possibly two more lysyl residues. In order to implicate these changes to the aetiology of the disease and suggest that idiopathic haemochromatosis is analogous to the molecular diseases affecting haemoglobin then it would be necessary to obtain postmortem liver from several different humans and obtain amino acid analysis for purified protein from each. This would allow any variation from individual to individual to be evaluated and the standard deviation for each residue to be calculated. This work is at present in progress but at this stage it is interesting to note that there does seem to be a distinct difference in the lysine content. This is in disagreement with the work of

Saadi (1962) who, on fingerprinting a tryptic hydrolysate, found no difference between the protein obtained from haemochromatotics and that from normal subjects. This obviously merits some further attention.

Guinea pig liver apoferritin has a fairly similar amino acid composition, with a few minor changes. It can be seen that the guinea pig protein appears to have two tryptophan residues which agrees with our analysis for all apoferritins so far considered but is in disagreement with the data obtained by Harrison and Hofmann (1961) for the horse spleen protein using a modified method of Spies and Chambers (1949).

Protein (2 - 5 mg/ml) was either analysed by sedimentation velocity using Schlieren optics or (0.5 - 1.0 mg/ml) by use of the photoelectric scanner. The protein was adjudged pure when it sedimented as a single homogeneous species with a corrected sedimentation coefficient of 17-18 S.

On electrophoresis by the standard method of Davis and Ornstein (1964) both pure ferritin and apoferritin gave rise to a major sharp band. Also observed were two or more minor bands which were assumed to be dimer, trimer etc.. Both ferritin and apoferritin monomer had identical electrophoretic mobilities.

The value obtained for the specific extinction coefficient, $E^{1\%}_{1cm}$ (280 nm), for horse spleen apoferritin was 10.13. Harrison (1964) has reported a variable extinction at 280 nm, $E^{1\%}_{1cm}$ = 8.60 - 9.70, and more recently a value of 9.0 has been used (Macara, Hoy and Harrison, 1972). A somewhat higher extinction has been reported for guinea pig apoferritin, $E^{1\%}_{1cm}$ = 10.1 (Friedberg, 1962) which is in good agreement with that obtained by us. It was found that by using the molar extinction coefficients of model compounds (Sober, 1960; Edolhoch, 1967) then it was poss-

ible to calculate an empirical value for the molar extinction coefficient and hence evaluate $E^{1\%}_{1cm}$ (280 nm). Thus, on the basis of the known content of tyrosine (5 residues, ϵ_m,280 = 1280 or 1330), tryptophan (2 residues, ϵ_m,280 = 5690 or 5600) and cysteine (2 residues, ϵ_m,280 = 120) an empirical value for $E^{1\%}_{1cm}$(280) of 9.80 - 9.83 was obtained which is in good agreement with the value obtained experimentally.

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate is now considered to be one of the most quick, convenient and reliable methods for protein molecular weight determinations. By this method, the molecular weights of the unknown samples are determined by comparison of their electrophoretic mobilities with the mobilities of standard proteins of known molecular weights.

Figure 15 shows typical examples of the result of electrophoresis of a variety of proteins and peptides in the presence of sodium dodecyl sulphate (SDS). It can be seen that a very good separation was obtained for myoglobin, chymotrypsinogen A, pepsin, leucine amino peptidase and bovine serum albumin (Figure 15A-1). Also shown in the figure is a densitometric trace of one of the gels.

The proteins and peptides used as molecular weight markers in this study, together with their molecular weights and relative mobilities, are listed in Table 3. When the relative electrophoretic mobilities were plotted as a function of logarithm of molecular weight, a biphasic relationship was obtained as shown in Figure 16. It can be seen that in the region 12-80,000 a linear relationship was obtained and this was characterised by regressional analysis to give the function:

Figure 15. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Typical gels obtained after electrophoresis, (staining and destaining as described in the text) are shown. A, Gel 1, contains myoglobin, chymotrypsinogen, pepsin, leucine amino peptidase and serum albumin, from left to right. Above this is a densitometric scan of this gel (the sharp peak to the left of centre is an artifact caused by the join of the two microscope slides used to transport the gel in the densitometer). Gel 2, horse spleen apoferritin. B, Gel 1, human transferrin showing the minor component (see text) and horse spleen apoferritin ; Gel 2, ovalbumin and insulin ; Gel 3, bovine serum albumin and insulin.

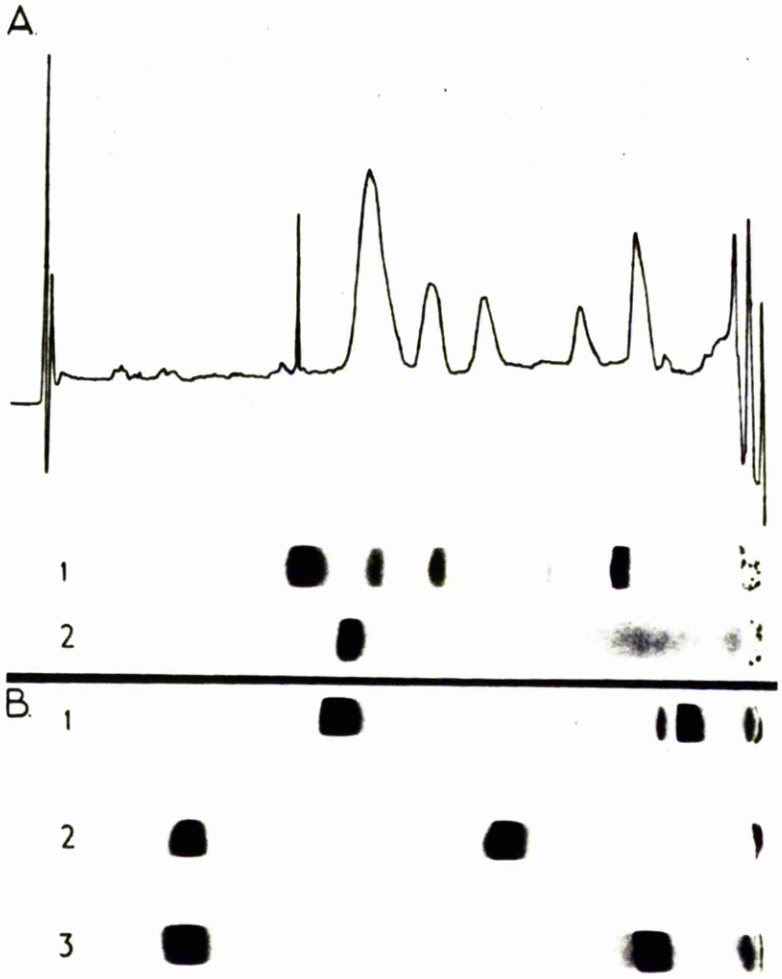
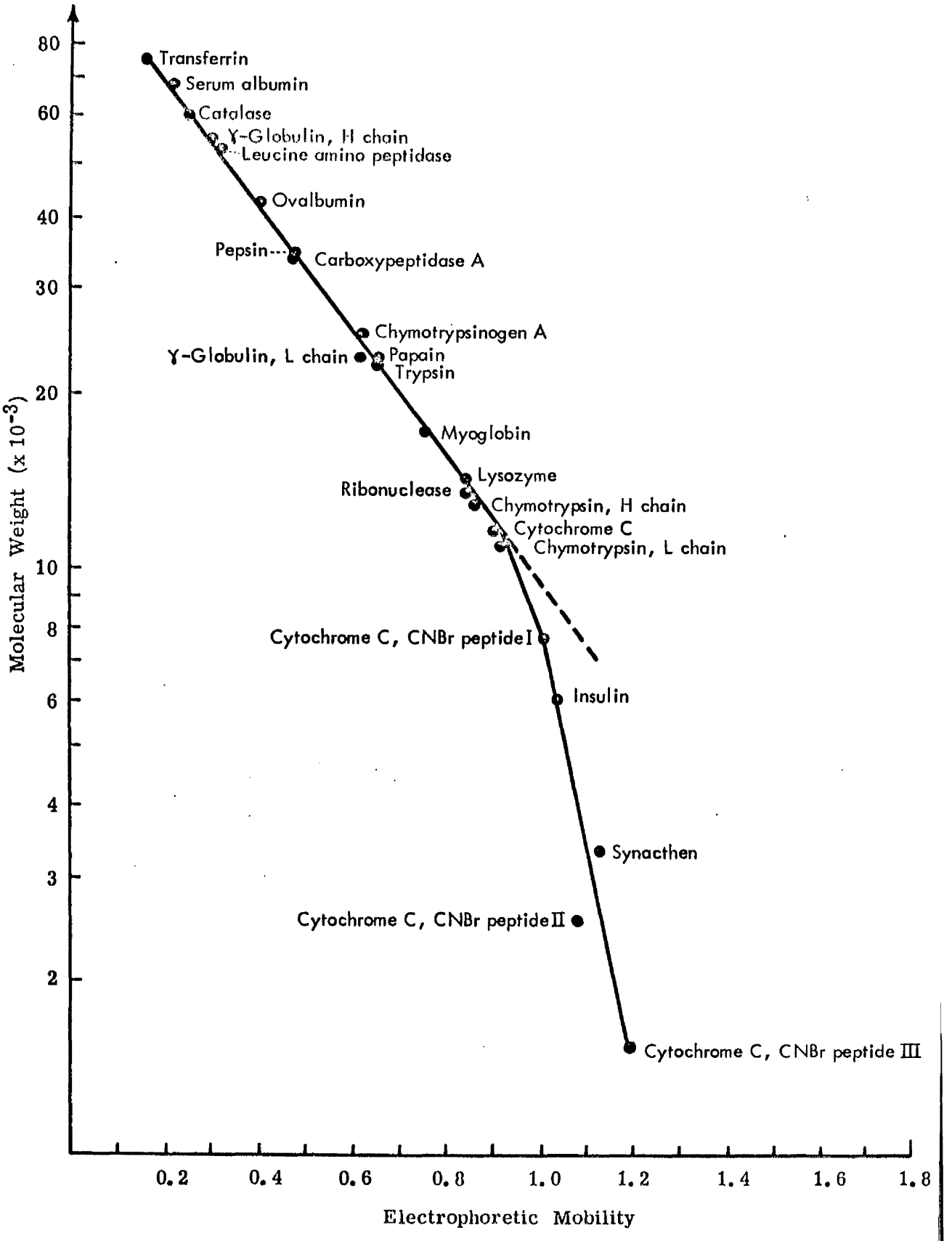

A
1
2
B
1
2
3

Table 3. Sodium dodecyl sulphate-polacrylamide gel electrophoresis. List of molecular weight markers used in this study together with the values for their molecular weight.

Protein	Molecular Weight	Electrophoretic Mobility
Bovine serum albumin	68,000	0.2165
Catalase	60,000	0.2575
γ-Globulin, H chain	55,000	0.2995
Leucine amino peptidase	53,000	0.3200
Ovalbumin	43,000	0.4010
Alcohol dehydrogenase	41,000	0.4130
Pepsin	35,000	0.4790
Carboxipeptidase A	34,600	0.4750
Chymotrypsinogen A	25,700	0.6181
γ-Globulin, L chain	23,500	0.6165
Papain	23,300	0.6500
Trypsin	23,000	0.6480
Myoglobin	17,200	0.7520
Lysozyme	14,300	0.8350
Ribonuclease	13,700	0.8475
Chymotrypsin, H chain	13,000	0.8620
Cytochrome c	11,700	0.8960
Chymotrypsin, L chain	11,000	0.9120
Cytochrome c, CNBr I	7,650	1.0100
Insulin	6,000	1.0330
Synacthen	3,360	1.1270
Cytochrome c, CNBr II	2,530	1.0710
Cytochrome c, CNBr III	1,540	1.2010

Figure 16. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The electrophoretic mobilities of the molecular weight markers used in this study are shown as a function of their respective molecular weights. The relationship so obtained appears to be biphasic and this is dicussed further in the text.


Transferrin
Serum albumin
Catalase
γ-Globulin, H chain
Leucine amino peptidase
Ovalbumin
Pepsin
Carboxypeptidase A
Chymotrypsinogen A
γ-Globulin, L chain
Papain
Trypsin
Myoglobin
Lysozyme
Ribonuclease
Chymotrypsin, H chain
Cytochrome C
Chymotrypsin, L chain
Cytochrome C, CNBr peptide I
Insulin
Synacthen
Cytochrome C, CNBr peptide II
Cytochrome C, CNBr peptide III
Molecular Weight (x 10⁻³)
80
60
40
30
20
10
8
6
4
3
2
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Electrophoretic Mobility

$$\log (M.Wt.) = 5.0712 - 1.1065 \times \text{Electrophoretic Mobility}$$

The coefficient of correlation and rate of regression for this relationship was found to be 0.9990.

Below a molecular weight of about 12,000 this relationship between molecular weight and electrophoretic mobility was no longer valid and the point at which this occurs is known as the 'critical point' or the 'point of inflection'. A new linear relationship between logarithm of molecular weight and electrophoretic mobility was apparent in the region 1,400-8,000 daltons and could be expressed by the function:

$$\log (M.Wt.) = 7.1732 - 3.3237 \times \text{Electrophoretic Mobility}$$

and this was found to have a coefficient of correlation of 0.9066.

The existence of such an inflection and a resultant biphasic relationship was first demonstrated by Dunker and Reuckert (1969) and subsequently by Swank and Munkres (1971) and Williams and Gratzer (1971). These findings helped to explain, in part, the existence of 'anomalous' proteins. Shapiro, Vinuela and Maizel (1967) had demonstrated that lysozyme and notably ribonuclease had lower mobilities than expected. This was also shown in the case of ribonuclease by Dunker and Rueckert (1969) although in the present study and that of Weber and Osborn (1969) and Robertson, Hammerstedt and Wood,(1971) no such anomaly was observed. Since Shapiro et al (1967) used 5% gels and the critical point for these was 20,000 (Dunker and Rueckert, 1969) then this could explain their observations. In the study of Dunker and Rueckert (1969) this explanation seems reasonable for lysozyme but these workers still found ribonuclease to have a decreased mobility. It is known that ribonuclease still has enzymic activity in the presence

of SDS (J.V. Maizel, personal communication) and this suggests that this protein is particularly resistant to unfolding. It is possible that as a result of maintaining at least some of its native structure it is unable to bind the maximum amount of detergent. Since, in such a situation of diminished detergent binding and/or the protein's intrinsic charge becoming significant, this would result in a decreased electrophoretic potential and may be a possible explanation for its anomalous behaviour. Tanford, Kawahara and Lapanje (1967) have demonstrated that the time required for unfolding of a protein by SDS is about 1 hour and since several proteins, notably pepsin and papain (Nelson, 1971) and ribonuclease (J.V. Maizel, personal communication), are known to be relatively resistant to unfolding then perhaps the incubation time should be increased. In the present study this incubation was normally extended to about 5 hours at $37^{o}C$.

The effect of the protein's intrinsic charge on its electrophoretic mobility in this system is as yet unresolved. Just as there have been several well documented reports of variable mobility on altering the protein's intrinsic charge by chemical modification (Swank and Munkres, 1971; Williams and Gratzer, 1971; Tung and Knight, 1971; Arndt and Berg, 1970), so too have there been several which indicate that the protein's mobility is independent of its native charge (Dunker and Rueckert, 1969; Furthmayr and Timpl, 1971; Segrest, Jackson, Andrews and Marchesi, 1971).

In those proteins which have uncommon amino acid compositions like histones (Williams and Gratzer, 1971) or collagen (Furthmayr and Timpl, 1971) or a branched structure like glycoproteins (Segrest, Jackson, Andrews and Marchesi, 1971) then, it would appear that the method is limited and that due caution

should be exercised in the interpretation of the experimental data.

Page and Godin (1969) used columns of Sephadex equilibrated with sodium dodecyl sulphate to determine protein molecular weights although, this system in our own hands did not prove successful. In our experiments using a Sephadex G-200 column (2.5 x 100 cm) equilibrated with 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol we observed that all proteins eluted with the same K_d value irrespective of their molecular weights. This unusual effect has also been noted by other workers (Jacobson and Pfuderer, 1970).

The usefulness of SDS-polyacrylamide gel electrophoresis as a method for molecular weight determinations is further improved by the fact that it is possible to renature proteins from the detergent by simply running on a column of Dowex-2X10 (Lenard, 1971). Also Inouye (1971) recently demonstrated that, by using dansyl derivatives of the proteins, it is possible to detect the protein bands by ultraviolet light without recourse to staining and destaining and further, that this method was sensitive to 0.2 μg of protein.

Until recently the subunit structure and molecular weight of transferrin were not well characterized and, as a further application of the present method, we decided to investigate these properties.

Transferrin is the iron-transporting protein of human serum and it has been shown to specifically bind two atoms of iron per molecule. The finding of a much smaller number of spots as predicted from the lysine and arginine content of a tryptic peptide map of chicken ovotransferrin (Williams, 1962) and human serum transferrin (Jeppsson, 1967) led to the suggestion that the prot-

ein contained two identical subunits. The fact that the protein has two equivalent, non-interacting iron binding sites (Aisen, Liebman and Reich, 1966) lends credence to this view. From SDS-polyacrylamide gel electrophoresis we found that transferrin had an electrophoretic mobility of 0.161 ± 0.006 which corresponds to an extrapolated molecular weight of 76,500 ± 1,500 and we concluded that no subunit structure was present. This value is in close agreement with that determined for the reduced and carboxymethylated protein in 8M urea (Greene and Feeney, 1968) and also for the protein in the presence of 6M guanidine hydrochloride (Fish, Mann, Cox and Tanford, 1969; Aisen, Koenig, Schillinger, Scheinberg, Mann and Fish, 1970). It would thus appear from this study that both human and horse transferrin probably consist of one polypeptide chain and this is in agreement with the results of Elleman and Williams (1970) who identified 34 unique cysteic acid peptides in ovotransferrin (ovotransferrin contains 31 moles of half-cystine per 80,000 g of protein) and also those of Bearn and Parker (1966) who detected only one free N-terminal amino acid, valine.

It would now appear from the work of Phillips and Azari, (1971) that at some stage in its evolution, gene duplication has occurred. They showed that cleavage with cyanogen bromide yielded only three peptides and that the molecular weights of these fragments gave a value of 37,400 which is approximately one-half of the native structure. Recently Palmour and Sutton (1971) have demonstrated that the transferrin isolated from hagfish (Eptatretus stoutii) serum has an apparent molecular weight of approximately 44,000 and that the protein has only one iron binding site. It is interesting to speculate that this lower vertebrate species may represent a primordial form of transferrin and that

gene duplication took place in early vertebrate evolution.

On electrophoresis of transferrin a minor component, constituting about 5-10% of the total protein, was found in all cases for both the human and the horse preparations. This minor band had an average electrophoretic mobility of 0.205 ± 0.004 which corresponds to an estimated molecular weight of 69,000 ± 1,000. It was concluded that this species merely represented an impurity in the preparation and, from the characterised molecular weight of the polypeptide, was most probably serum albumin.

Molecular weights were determined for apoferritins obtained from the following sources:- horse spleen and liver, human spleen and liver, human haemochromatotic spleen and liver, rat spleen and liver and pig liver. The average electrophoretic mobilities are listed, together with the estimated molecular weights, for each in Table 4. Horse spleen ferritin was also dissociated by SDS and the resultant incubation product electrophoresed as before. In the case of ferritin, a narrow band containing iron was observed to travel a short distance into the gel (average electrophoretic mobility, 0.038 ± 0.002).

From Table 4 it can be seen that the differences between the various apoferritins lies within the experimental error and it appears that, for all of the proteins studied, the molecular weight for the polypeptide chain is of the order 18,400 ± 300 which is in marked contrast to the previously accepted value of 23 - 25,000.

The lower value obtained by us (Crichton and Bryce, 1970; Bryce and Crichton, 1971a; Crichton, Miller, Cumming and Bryce, 1972) has, however, since been confirmed by Bjork and Fish (1971).

Because the experimentally derived subunit molecular weight

Table 4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Results for the estimation of the molecular weights of apoferritin from a variety of organs and species.

Protein	Electrophoretic Mobility	Apparent Mol. Wt.
Horse spleen apoferritin	0.731 ± 0.005	18,300 ± 300
Horse liver apoferritin	0.729 ± 0.006	18,400 ± 300
Human spleen apoferritin	0.729 ± 0.008	18,400 ± 400
Human liver apoferritin	0.722 ± 0.008	18,700 ± 400
Human haemochromatotic spleen apoferritin	0.720 ± 0.014	18,800 ± 600
Human haemochromatotic liver apoferritin	0.726 ± 0.014	18,500 ± 600
Rat spleen apoferritin	0.737 ± 0.019	18,000 ± 800
Rat liver apoferritin	0.724 ± 0.014	18,600 ± 600
Pig liver apoferritin	0.736 ± 0.012	18,100 ± 600
Horse spleen ferritin	0.729 ± 0.010	18,400 ± 500

disagreed with that of the literature value it was decided to determine the molecular weight by other techniques. The methods we chose initially were Sephadex gel chromatography and gel chromatography in the presence of 6M guanidine hydrochloride.

Sephadex Gel Chromatography.

Harrison and Gregory (1968) have shown that 67% (v/v) acetic acid is capable of dissociating apoferritin into its constituent subunits and this was chosen as the denaturant for the Sephadex studies.

Sephadex G-75 Gel Chromatography.

Figure 17 shows the plot of relative elution volume (V_e/V_o) against logarithm of molecular weight for the proteins used in this study. It can be seen from the figure that cytochrome c (molecular weight 12,300) does not lie on the straight line and the interpolated value for the apparent molecular weight is 21,870. Figure 18 (a) shows a typical elution profile for albumin and chymotrypsinogen A as monitored continuously at 280 nm (LKB Uvicord). It can be seen by analysing the leading and trailing edges that there is no indication of non-enantiography, whereas, the profile for cytochrome c (Figure 18 b) is skew, indicating an associating-dissociating system, possibly a monomer-dimer interaction and this appears to be the reason for the anomalous behaviour of this protein.

Under similar conditions, horse spleen apoferritin gives an elution profile as shown in Figure 18 (c) and the molecular weights calculated for these two peaks (b) and (c) are 45,400 and 29,700 daltons respectively. Both of these values are considerably in excess of the subunit molecular weight as determined by SDS-polyacrylamide gel electrophoresis. This could be explained

Figure 17. Calibration curve for Sephadex G-75 column operated as described in the text. The proteins used in the calibration were lysozyme (1), cytochrome c (2), chymotrypsinogen A (3), pepsin (4) and bovine serum albumin (5). The molecular weights computed from the experimental data for horse spleen apoferritin are shown. Also shown is the apparent molecular weight for cytochrome c as this does not lie on the linear relationship.

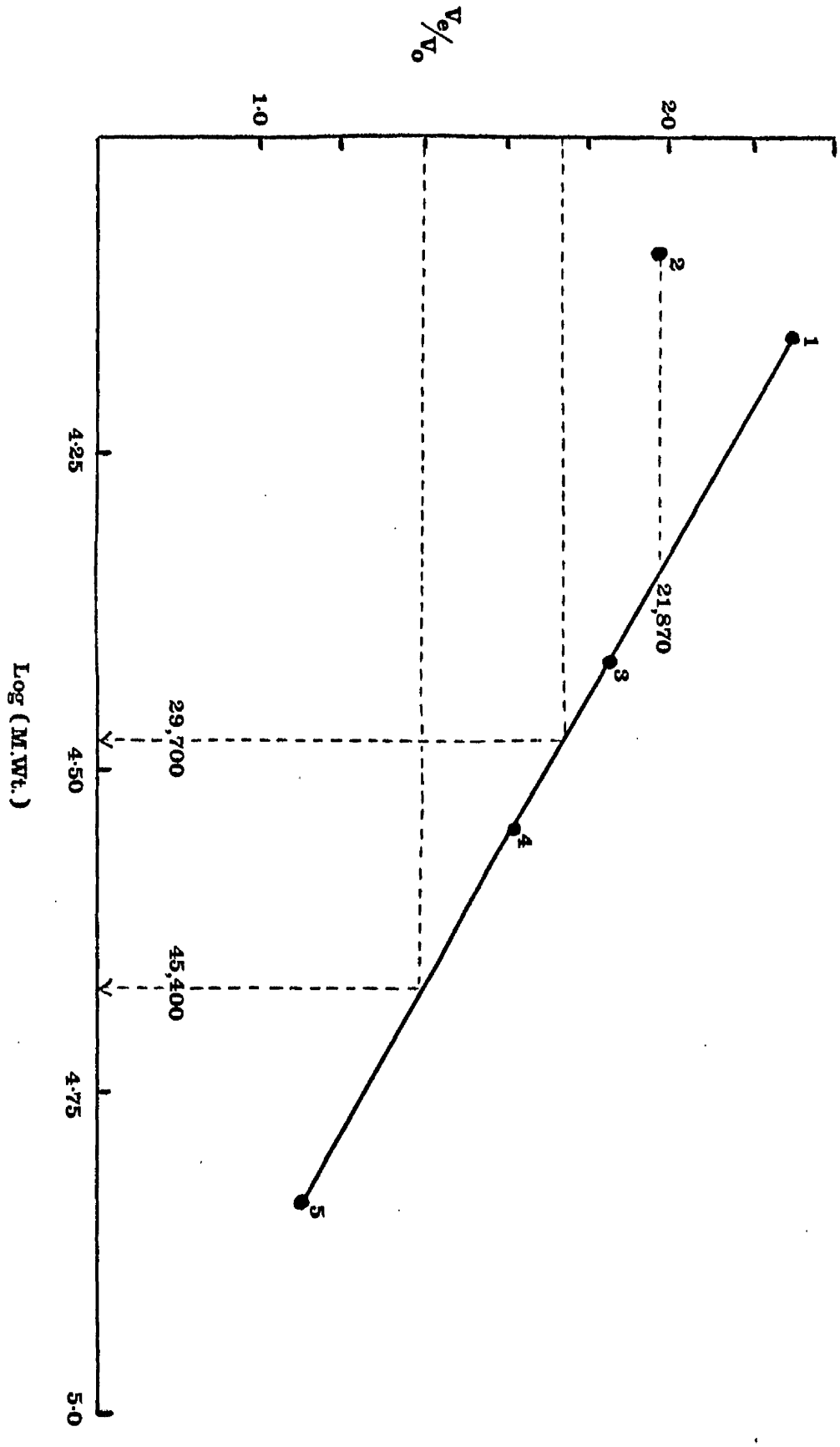
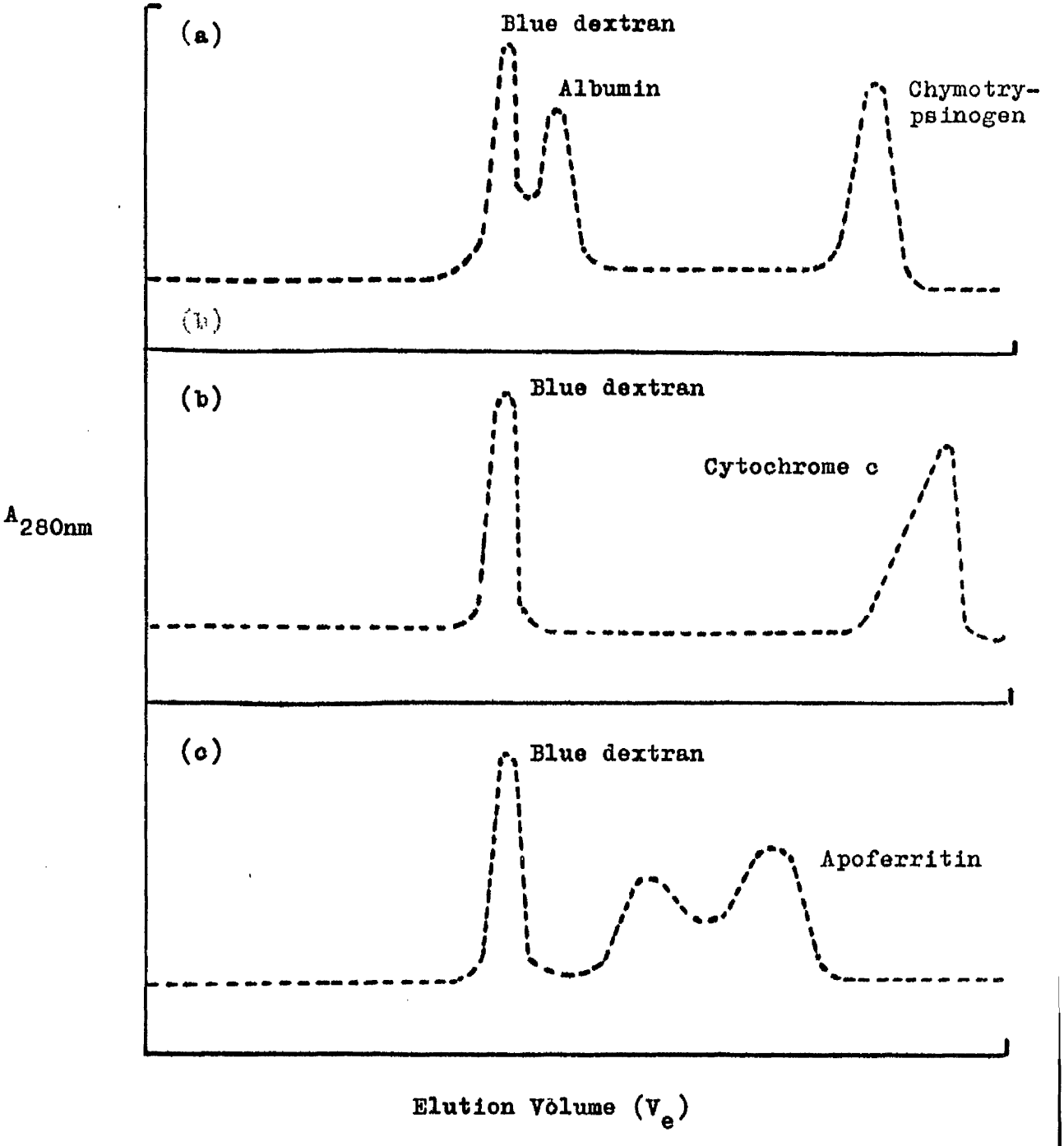

V_e/V_o
1·0
2·0
4·25
4·50
4·75
5·0
Log (M.Wt.)
1
2
3
4
5
21,870
29,700
45,400

Figure 18. Sephadex G-75 gel chromatography. Section (a) shows a typical elution profile for albumin and chymotrypsinogen A . From this elution profile it can be seen that there is no indication of non-enantiography and both of these proteins satisfy the linear relationship obtained for this column. Cytochrome c does not lie on the line and it can be seen from section (b) that the profile for cytochrome c is skew, indicating an associating-dissociating system. Section (c) shows the elution profile for horse spleen apoferritin chromatographed under similar conditions.


(a)
Blue dextran
Albumin
Chymotry-
psinogen
(b)
(b)
Blue dextran
Cytochrome c
(c)
Blue dextran
Apoferritin
A_{280nm}
Elution Volume (V_e)

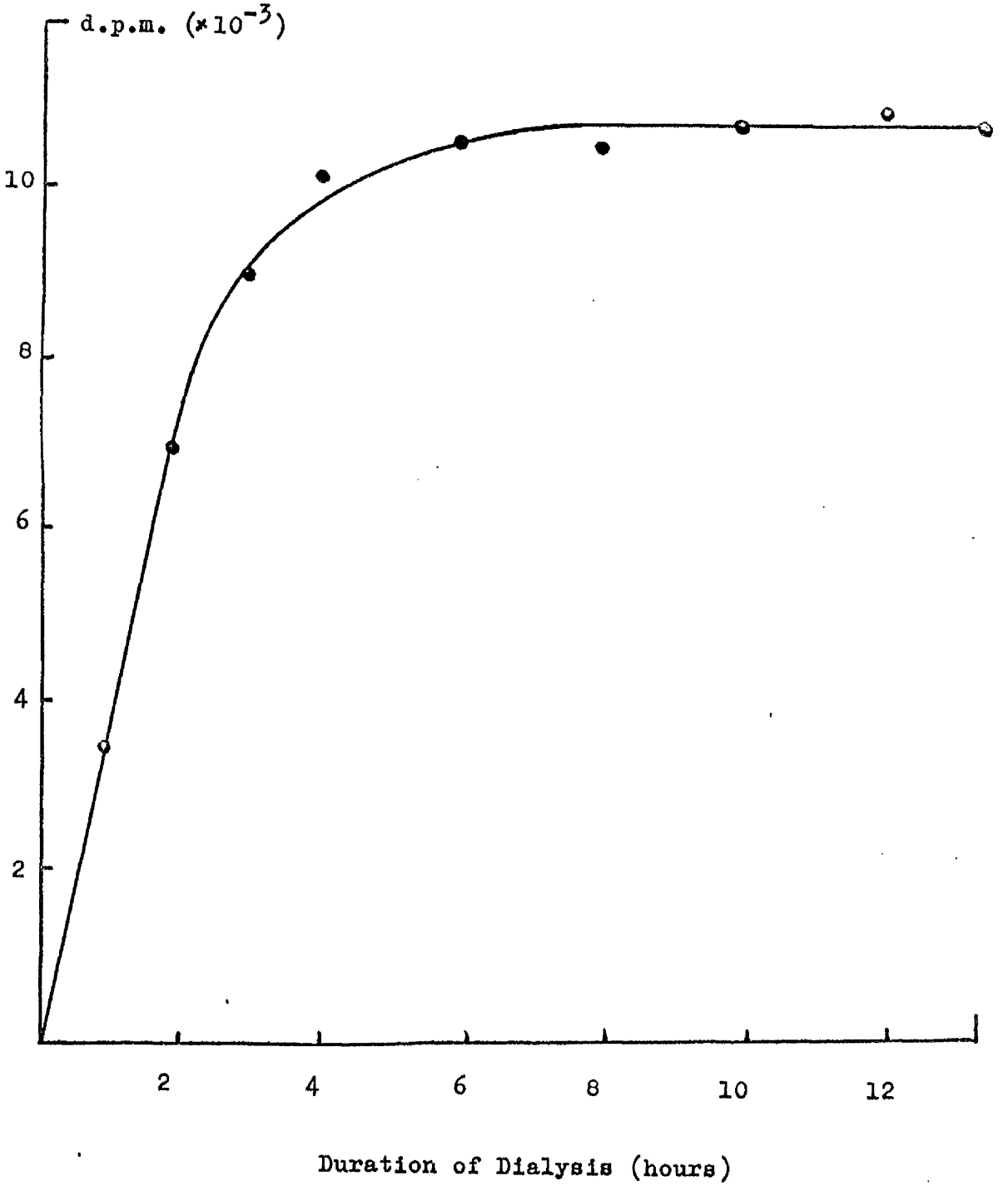
by acetic acid binding or by an associating-dissociating system as appears to be the case for cytochrome c. Candlish and Tristram (1969) recently demonstrated that quite extensive binding occurs between unionised acetic acid and the peptide bond of proteins and that the extent of binding depends on the individual protein.

Since such binding could account for the higher molecular weights obtained for Sephadex gel chromatography it was decided to determine the extent of acetic acid bound to apoferritin. Apoferritin (30 mg) was dissolved in 1 ml of ^{14}C-acetic acid (67%) and maintained at 0^{o}C for 1 hour. The solution was then dialysed against 1M acetic acid and 100 μl aliquots were removed after certain time intervals. Figure 19 shows the results of such an equilibrium dialysis experiment. Equilibrium was attained after about 5-6 hours dialysis and at equilibrium the extent of binding was evaluated by determining the amount of labelled acetic acid inside and outside the dialysis sac. It was found that at equilibrium there was no significant binding of the acetic acid. Protein concentration was determined before and after dialysis.

Because of the relative closeness of the peaks (b) and (c) in the elution profile for apoferritin it was difficult to examine these by frontal analysis. It would appear, however, from the profile and from the apparent molecular weights that if this is an associating-dissociating system then it is not a simple monomer-dimer interaction, but instead, a complex monomer $\longrightarrow$ n-mer $\rightarrow$ m-mer etc. and an unambiguous interpretation of this would be exceedingly difficult.

However, in this context it should also be noted that other workers have found considerably higher molecular weight values

Figure 19. Equilibrium dialysis experiment to determine the amount of acetic acid bound to horse spleen apoferritin. Conditions for the experiment are given in the text.


d.p.m. (×10⁻³)
10
8
6
4
2
2
4
6
8
10
12
Duration of Dialysis (hours)

from Sephadex studies and they have emphasised the need for caution when solely using this technique for molecular weight determinations (Milne and Wells).

Sephadex G-100 Gel Chromatography.

Figure 20 shows the plot of relative elution volume (V_e/V_i) against logarithm of molecular weight for the proteins used in this study. In this case cytochrome c and myoglobin lie off the straight line and again by frontal analysis of their elution profiles it seems possible that this can be explained in terms of an associating-dissociating system, again possibly a monomer-dimer interaction.

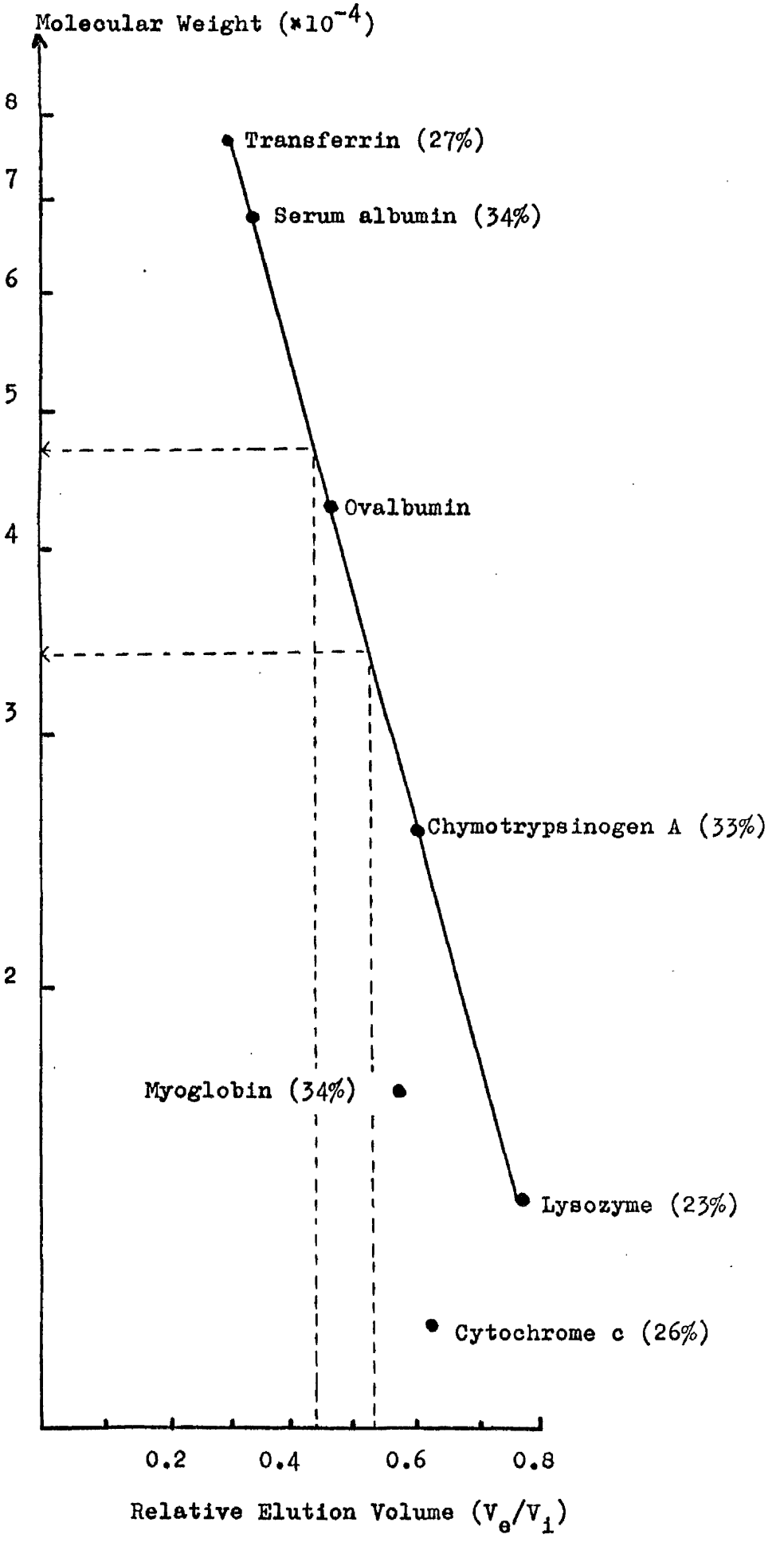
According to Van Holde (1966) globular proteins with about 30% content of hydrophobic residues (leucine, isoleucine, phenylalanine, valine and proline) are likely to exhibit association-dissociation phenomena. However, as most of the proteins used in this study lie close to this dividing line, we cannot deduce such interactions from this empirical rule.

In this study, just as in the case of Sephadex G-75, horse spleen apoferritin had a complex elution profile. Figure 20 also shows the average relative elution volumes for apoferritin and the apparent molecular weights calculated for the two peaks are 47,000 and 34,000. Since equilibrium dialysis experiments again indicated no significant binding of acetic acid to the protein it was concluded that in this study we also had a complex associating-dissociating system.

Sephadex G-100 Equilibrated with 67% (v/v) Acetic Acid.

Harrison and Gregory (1968) had demonstrated that 67% acetic acid produced subunits and, when this material was dialysed

Figure 20. Sephadex G-100 gel chromatography. Calibration of the column was carried out using the molecular weight markers shown. Myoglobin and cytochrome c do not appear to satisfy the linear relationship obtained with the other proteins. The molecular weights obtained for the two peaks of apoferritin are indicated by the hatched lines. The figures in brackets are the contents of hydrophobic residues expressed as a percentage (see text).


Molecular Weight (*10^{-4})
8
7
6
5
4
3
2
Transferrin (27%)
Serum albumin (34%)
Ovalbumin
Chymotrypsinogen A (33%)
Myoglobin (34%)
Lysozyme (23%)
Cytochrome c (26%)
0.2
0.4
0.6
0.8
Relative Elution Volume (V_e/V_i)

against dilute glycine-HCl buffer, pH 3.0, then, the protein remained totally as subunits.

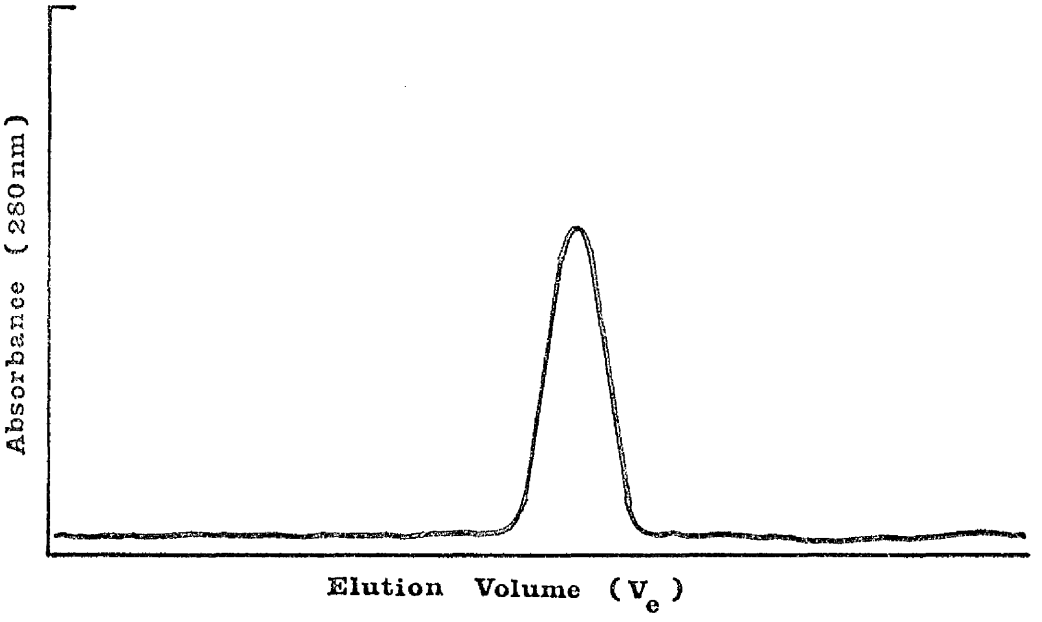
Our Sephadex data did not seem to agree with this study and so we decided to see if 67% acetic acid did, in fact, produce only subunits. A column of Sephadex G-100 equilibrated with this solvent was set up as described. With this column the elution profile for apoferritin was a single guassian peak as shown in Figure 21. It was not possible to determine molecular weights with such a column because the strongly acidic solution hydrolysed the glycosidic linkages in the Sephadex gel matrix. This form of degradation has also been noted by other workers (Fairweather and Jones, 1971). As the hydrolysis was time-dependent we managed to keep the Sephadex exposed to the strong acid for a short period without any noticeable effect.

It seemed, therefore, that 67% acetic acid was capable of producing apoferritin subunits but when this material was dialysed against dilute glycine buffer, pH 3.0 some form of reassociation took place. As this finding disagreed with the observation of Harrison and Gregory (1968) that the protein remains as subunits in dilute glycine-HCl buffer, pH 3.0 it was decided to reinvestigate the system by analytical ultracentrifugation.

This observation of Harrison and Gregory (1968) was in fact shown to be correct (later section) and therefore the disparity could possibly be explained in terms of a concentration effect. At the high concentrations of protein (30 mg/ml) used in the Sephadex studies the equilibrium must be moved in the direction of subunit polymers.

In terms of molecular weight determinations of the apoferritin subunit, Sephadex gel chromatography was therefore found to be impracticable.

Figure 21. Sephadex G-100 gel chromatography. Elution profile obtained for the fractionation of horse spleen apoferritin on a column of Sephadex G-100 equilibrated with 67% acetic acid.


Absorbance (280nm)
Elution Volume (V_e)

Recently, similar elution profiles for horse spleen apoferritin on Sephadex G-100 and Bio-Gel P-100 were observed by other workers, although, they offered no explanation for their findings except that the fraction of lower molecular weight, which elutes from the column ahead of bovine pancreatic ribonuclease (molecular weight 13,000), "most likely represents the fundamental subunit" (Vanecek and Keil, 1969).

It would seem, however, from the arguments given earlier that this is most probably not, in fact, the case.

Gel Filtration in the Presence of 6M Guanidine Hydrochloride.

Piez and coworkers (Piez and Carillo, 1964; Lewis and Piez, 1964) have demonstrated that the hydrodynamic volume of a protein in the random coil conformation is approximately ten times greater than the hydrodynamic volume of its ordered native structure. Essentially this has the effect of reducing the exclusion limit of the gel matrix (based on globular proteins) tenfold. Thus Sephadex G-150, which normally fractionates in the range 5,000 - 400,000 should have an effective exclusion limit of about 40,000 in 6M guanidine hydrochloride and, Sephadex G-200 (5,000 - 800,000) should fractionate proteins up to about 80,000 daltons. This can, in fact, be shown to be the case from the experimental findings of Cebra and Small (1966) who demonstrated that chromatography on Sephadex G-200 equilibrated with 6M guanidine hydrochloride is limited to material of molecular weight less than 100,000.

Agar is a polysaccharide extracted from sea-weed and consists of two components: the major of these is a neutral component, agarose, while the other contains carboxyl and sulphate groups and is called agaropectin. Because of these ionisable groups on agaropectin, considerable adsorption is frequently observed. How-

ever, when this material is removed, the isolated agarose, which is free of ionisable groups, provides a very useful matrix for gel chromatography. Agarose, itself, is a linear polysaccharide consisting of alternate residues of D-galactose and 3,6-anhydro-L-galactose (Araki, 1956). Though there are no covalent cross-linkages stabilising the agarose matrix, it does not seem to be affected by high concentrations of salt provided great care is taken during gel equilibration to avoid hydrolysis of the glycosidic linkages. The advantages of the agarose gel rest undoubtedly on its ability to fractionate high molecular weight substances including viral particles, phages and even cell particles and bacteria.

Because of the lack of well characterized high molecular weight marker substances, the fractionation ranges for the agarose series are somewhat vague. The value quoted for a gel with a nominal content of agarose of 6% is $1x10^4$ - $4x10^6$ and, as this would allow fractionation up to about 400,000 in 6M guanidine hydrochloride, this seemed an ideal choice of matrix for this study.

The experimental procedure for column chromatography, gel equilibration, sample preparation and application and also guanidine hydrochloride purification have already been described (page 75). Recently, Wong, Roxby and Tanford (1971) suggested that the absence of absorption at wavelengths higher than 225 nm was not a good criterion of purity for guanidine hydrochloride. These workers demonstrated the presence of large amounts of non-absorbing impurities in a variety of commercially available guanidine preparations and suggested that constancy of melting point was a more sensitive and useful criterion. In this study we were, however, not concerned with the extraction of enzymatic-

ally active forms of the proteins after fractionation in guanidine hydrochloride, and for this reason the guanidine was adjudged suitable if it had little or no absorption at 280 nm.

In this study all of the proteins and peptides were reduced and carboxymethylated prior to chromatography.

In our studies on the structural aspects of apoferritin we became interested in the purification and characterization of the peptides obtained following cyanogen bromide cleavage. For this reason we decided to try to extend the range for molecular weight estimation by determining the mobilities of cyanogen bromide peptides of well-characterized proteins, cytochrome c , trypsin, and myoglobin (sequences taken from Dayhoff and Eck, 1968).

In our initial studies in this respect with Bio-Gel A-5m we obtained a single peak for insulin. Fish, Mann and Tanford, (1969) also previously noted that insulin "eluted as a broad peak" on a column of similar material. Also the calibration curve obtained by us (Figure 22) compared very favourably with that obtained by these workers. However, when we came to use the Sepharose equivalent (Sepharose 6B), the A and B chains of insulin were found to be easily separated.

Figure 23 shows two typical elution profiles for the same sample on the two different sieving matrices following chromatography under similar conditions. It can be seen that for the molecular weight range 11,000 to 80,000 there is very little difference in the elution pattern. For molecular weights less than 9,000, however, it is apparent that the resolving power of the Sepharose is considerably better than that of the Bio-Gel equivalent. For the characterization of apoferritin cyanogen bromide peptides it was decided, therefore, to use the column of Sepharose 6B.

Figure 22. Calibration curve obtained for a column of Bio-Gel A-5m equilibrated with 6M guanidine hydrochloride. The data was treated according to the theoretical treatment of Porath (1963) and $K_{av}^{\frac{1}{3}}$ was plotted as a function of molecular weight to the 0.555 power.

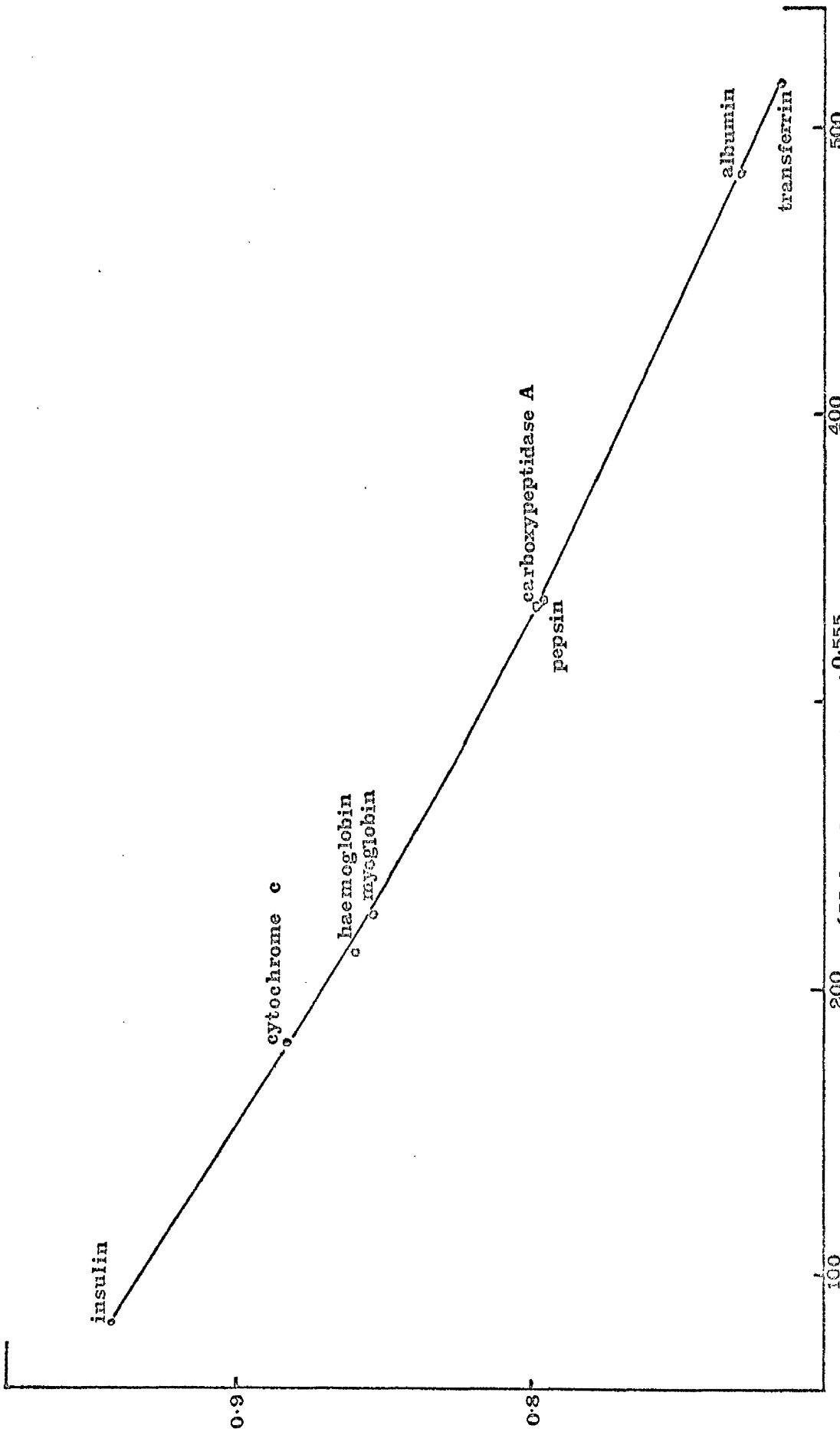
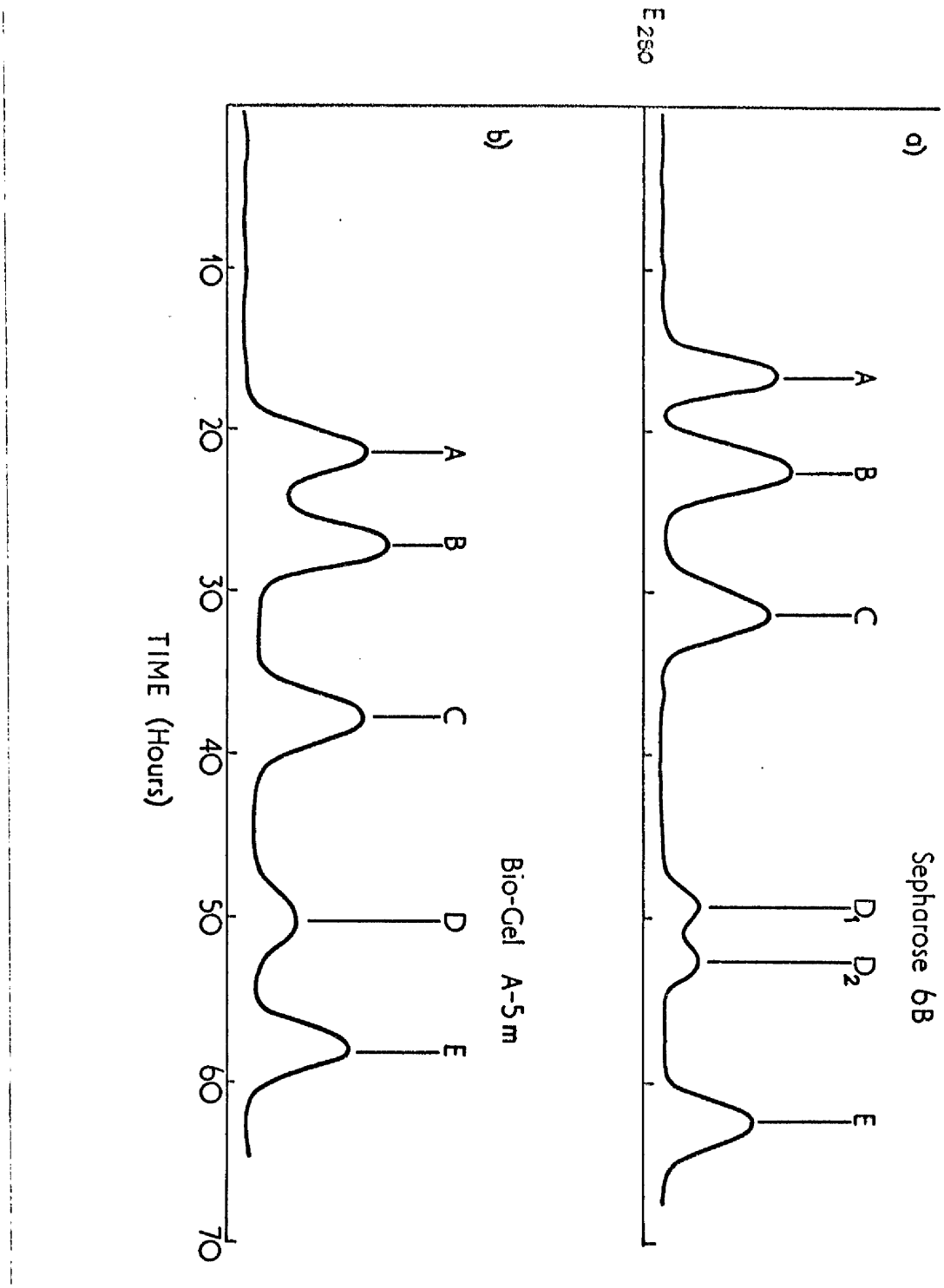

insulin
cytochrome c
haemoglobin
myoglobin
carboxypeptidase A
pepsin
albumin
transferrin
0·9
0·8
100
200
400
500
(Molecular Weight)0·555
$K_{av}^{1/3}$

Figure 23. Effect of gel matrix on the chromatographic resolution. (a) Gel filtration on Sepharose 6B. (b) Gel filtration of an identical sample on Bio-Gel A-5m. A= transferrin; B = alcohol dehydrogenase; C = haemoglobin; D = insulin; D_1 = insulin, B chain; D_2 = insulin, A chain; E = tryptophan.


a)
Sepharose 6B
E 280
A
B
C
D1
D2
E
b)
Bio-Gel A-5m
A
B
C
D
E
10
20
30
40
50
60
70
TIME (Hours)

It is known that Sepharose beads are extremely elastic and that the length of the gel packing is reduced at high flow rates. Early attempts to increase the flow rates of the columns by pumping merely resulted in the development of compression in the gel bed and the consequent diminution of flow rate. Also, at relatively fast flow rates, the resolution of the chromatography was affected. Figure 24 shows the effect of the flow rate on the resolution obtained by chromatography of identical samples of marker substances. From this figure it can be seen that the resolution increases with decreasing flow rate and that adequate resolving power without significant zone broadening due to diffusion can be attained with a flow rate of 2 ml/hr. The flow was maintained at this rate by adjustment of the height of a solvent reservoir.

Treatment of Chromatographic Data.

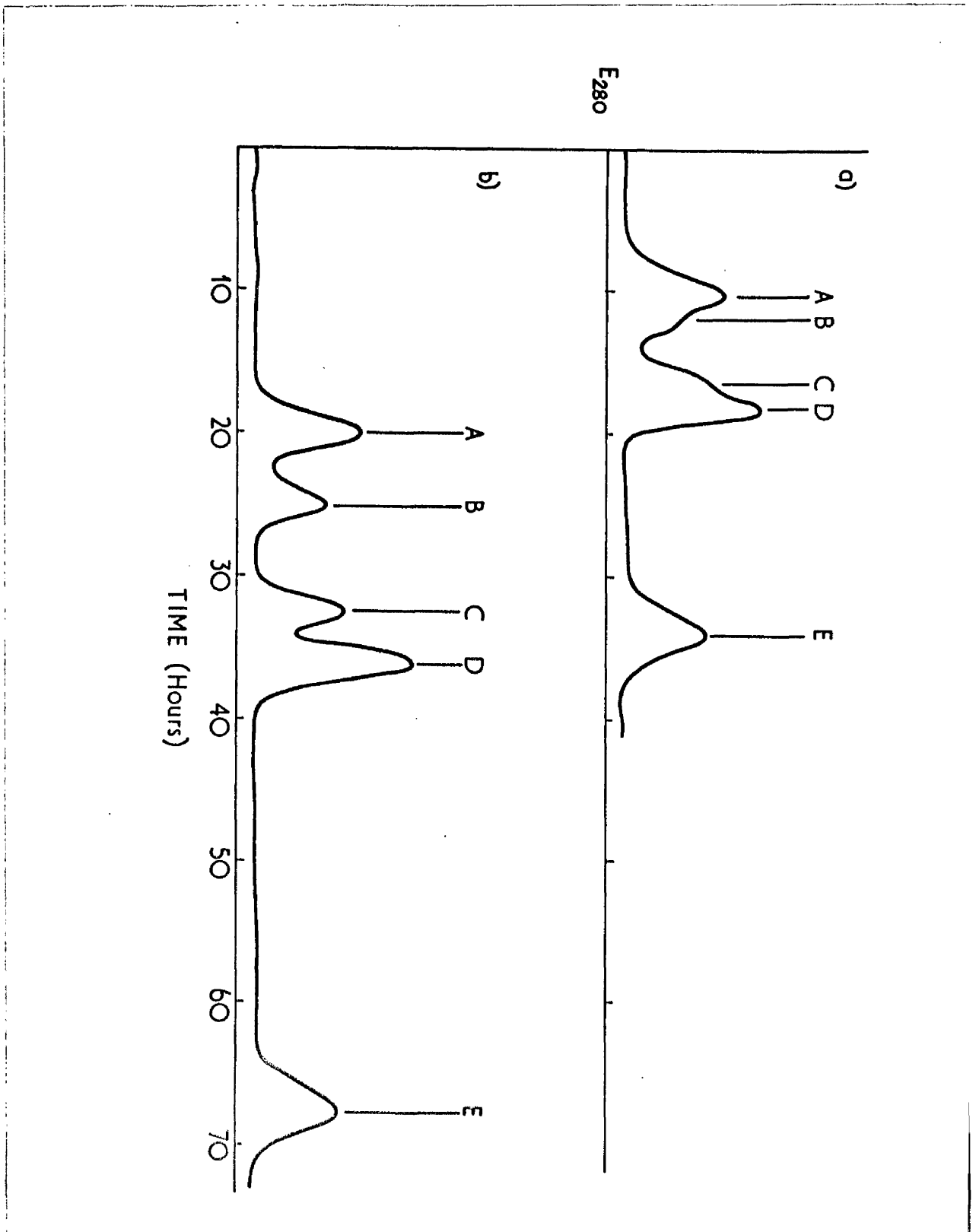
At present there are a number of available methods for the interpretation of gel chromatographic data and at times it is useful to use more than one of these in an effort to obtain as much information as possible.

In all of the studies, we describe the chromatographic behaviour of the protein or peptide by the parameter, K_{av} (Laurent and Killander, 1966);

$$K_{av} = (V_e - V_o) / (V_t - V_o)$$

where V_o, the exclusion volume, is obtained by chromatography of Blue Dextran 2000 (a high molecular weight, coloured polysaccharide), which is completely excluded from the gel matrix. V_t is the total volume accessible to the solvent and in the present experiments this was obtained by chromatography with the amino

Figure 24. Effect of flow rate on the chromatographic resolution. (a) Elution profile obtained following chromatography at 4 ml/hr. (b) Elution profile obtained following fractionation of an identical sample at 2 ml/hr. A = bovine serum albumin; B = ovalbumin; C = horse heart myoglobin; D = cytochrome c; and E = trytophan.


a)
b)
E_{280}
A
B
C
D
E
10
20
30
40
50
60
70
TIME (Hours)

acid tryptophan. V_e is known as the elution volume and is the position at which the protein or peptide elutes following gel filtration. This distribution function , K_{av}, can then be related to the molecular weight in a variety of ways (Determann, 1968).

The most commonly used relationship is the empirical treatment devised by Andrews (1964) in which,

$$K_{av} = a - b.\log M$$

or the data can also be treated according to the theoretical relationship determined by Porath (1963) in which,

$$K_{av}^{\frac{1}{3}} = k_1 - k_2 M^{\frac{1}{2}}$$

where a, b, k_1, k_2 are constants and M denotes molecular weight.

Taking the first of these, Figure 25 shows the plot of the distribution coefficient, K_{av}, against the logarithm of the molecular weight markers used in this study. The protein or peptide can be identified by its number in Table 5.

From this figure it can be seen that a triphasic relationship is obtained. In the region of molecular weight 11,000 - 80,000 there is a region of apparent linearity and this is also the case in the region of molecular weight 1,400 - 8,000. These two linear portions of the curve were characterized by the method of least squares and found to be,

$$\log M = 5.091 - 2.652\ K_{av} \quad \text{(reg. coeff. 1.0008).....1}$$

$$\log M = 5.176 - 2.357\ K_{av} \quad \text{(reg. coeff. 0.9952).....2}$$

respectively.

When the data was plotted according to the treatment of Porath (1963) the results obtained were as shown in Figure 26.

Figure 25. Molecular weight determination by gel filtration on Sepharose 6B in the presence of 6M guanidine hydrochloride. Distribution coefficient (K_{av}) is shown as a function of molecular weight and the linear relationships were computed by the method of least squares analysis. The numbers refer to the proteins and peptides listed in Table 5.

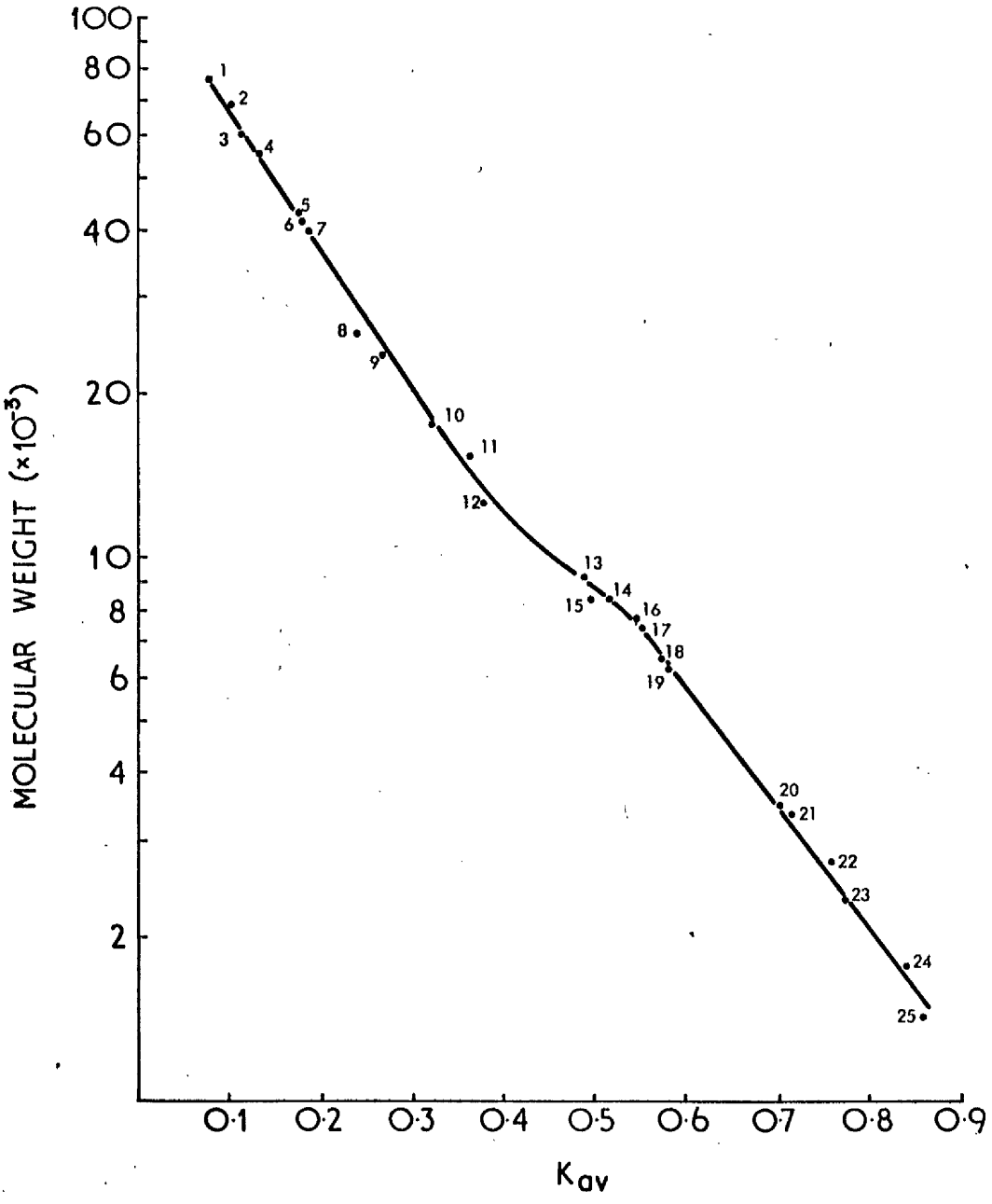
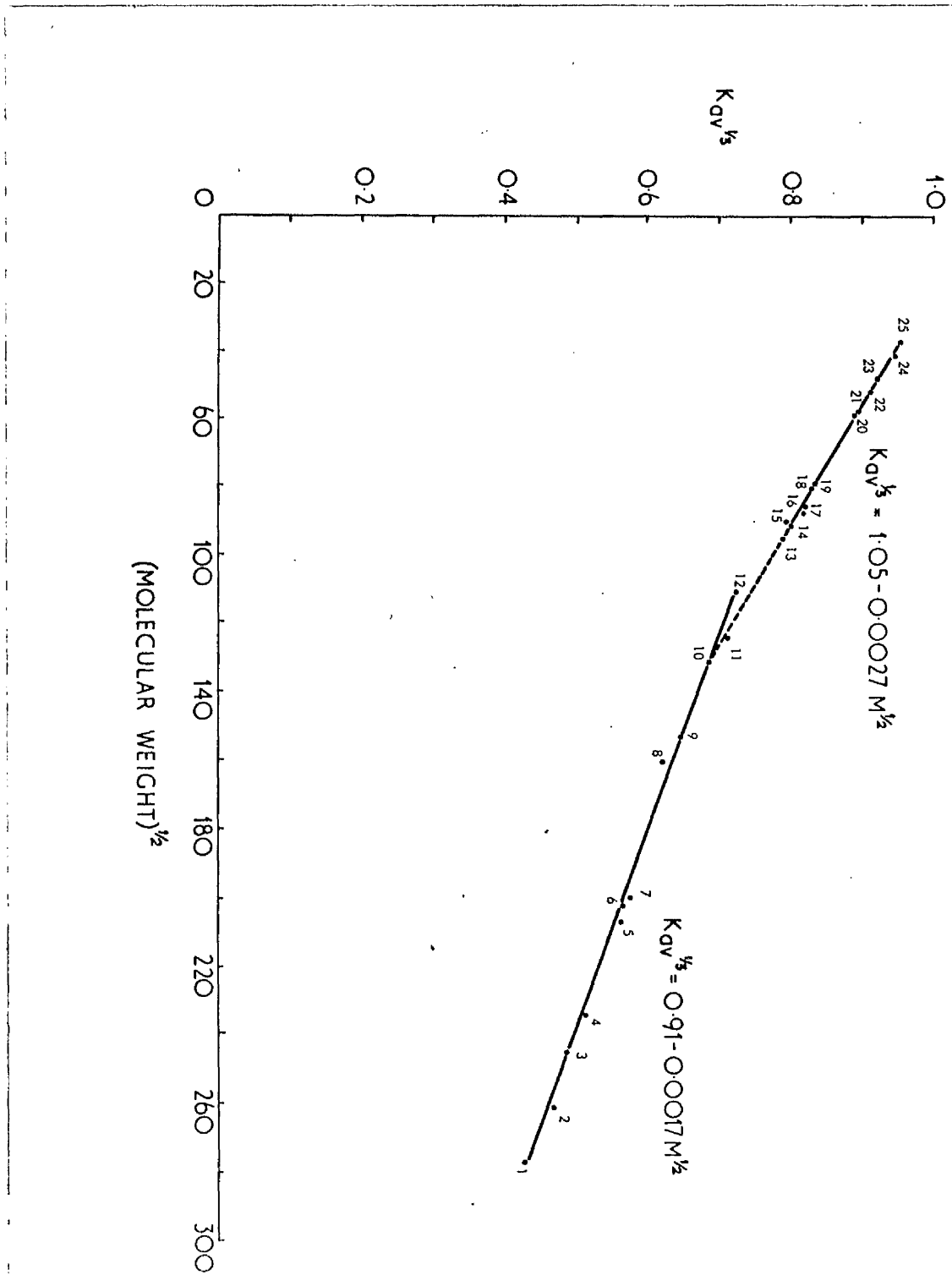

MOLECULAR WEIGHT (×10⁻³)
100
80
60
40
20
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
Kav

Table 5. Gel filtration in the presence of 6M guanidine hydrochloride. List of proteins and peptides used as molecular weight markers in this study.

CNBr, cyanogen bromide peptides

	Protein/Peptide	Mol. Wt.	K_{av}	$(\text{Mol. Wt.})^{\frac{1}{2}}$	$K_{av}^{\frac{1}{3}}$
1	Transferrin	76,600	0.0758	276.77	0.4236
2	Bovine serum albumin	68,000	0.1024	260.77	0.4682
3	Catalase	60,000	0.1144	244.95	0.4857
4	γ-Globulin, H chain	55,000	0.1343	234.52	0.5124
5	Ovalbumin	43,000	0.1768	207.36	0.5616
6	Alcohol dehydrogenase (liver)	41,000	0.1795	202.48	0.5644
7	Creatine phosphokinase	40,000	0.1875	200.00	0.5727
8	Chymotrypsinogen A	25,700	0.2380	160.31	0.6200
9	γ-Globulin, L chain	23,500	0.2673	153.30	0.6445
10	Myoglobin	17,200	0.3218	131.15	0.6855
11	Haemoglobin	15,500	0.3630	124.50	0.7136
12	Cytochrome c	11,700	0.3763	108.17	0.7222
13	Trysin - CNBr I	9,240	0.4893	96.12	0.7882
14	Lima bean trypsin inhibitor	8,400	0.5141	91.65	0.8012
15	Myoglobin - CNBr I	8,211	0.4960	90.62	0.7918
16	Cytochrome c - CNBr I	7,650	0.5492	87.46	0.8191
17	Trypsin - CNBr II	7,568	0.5518	87.00	0.8204
18	Trypsin - CNBr II	6,534	0.5731	80.83	0.8308
19	Myoglobin - CNBr II	6,265	0.5784	79.15	0.8333
20	Glucagon	3,500	0.7021	59.16	0.8889
21	Insulin, B chain	3,383	0.7127	58.16	0.8933
22	Cytochrome c - CNBr II	2,530	0.7579	50.30	0.9118
23	Insulin, A chain	2,090	0.7739	45.71	0.9182
24	Cytochrome c - CNBr III	1,540	0.8470	39.24	0.9462
25	Bacitracin	1,411	0.8563	37.56	0.9497

Figure 26. Molecular weight determination by gel filtration on Sepharose 6B in the presence of 6M guanidine hydrochloride. The figure shows the treatment of the experimentally determined distribution coefficients according to the method of Porath (1963). $K_{av}^{\frac{1}{3}}$ is plotted as a function of molecular weight to the power $\frac{1}{2}$ and the linear relationships were computed by regression analysis as before. The numbers refer to the proteins and peptides listed in Table 5.


$K_{av}^{1/3}$
1·0
0·8
0·6
0·4
0·2
0
20
60
100
140
180
220
260
300
(MOLECULAR WEIGHT)$^{1/2}$
$K_{av}^{1/3}$ = 1·05 - 0·0027 M$^{1/2}$
$K_{av}^{1/3}$ = 0·91 - 0·0017 M$^{1/2}$

From this treatment of the data two linear relationships were obtained, one for the region 1,400 - 9,000 daltons and the other for 12,000 - 80,000 daltons. These were characterized by the method of least squares to be,

$$K_{av}^{\frac{1}{3}} = 1.05 - 0.0027\ M^{\frac{1}{2}} \quad \text{(reg.coeff. 0.9957)} \quad \ldots\ldots 3$$

and $$K_{av}^{\frac{1}{3}} = 0.91 - 0.0017\ M^{\frac{1}{2}} \quad \text{(reg.coeff. 0.9986)} \quad \ldots\ldots 4$$

respectively.

The fact that a linear relationship is obtained from this treatment is a good indication that the proteins do exist in the random coil conformation. Also from these plots it can be calculated by extrapolation that the apparent exclusion limit is in fact 120-150,00 and this agrees reasonably with the value of 400,000 which was expected in this system.

By invoking the concept of two gel populations with different porosities it was hoped to explain the empirical relationships that were obtained. In effect, we are assuming a pore-size distribution in the microstructure of the starting material such that the material with the greater degree of cross-linking was involved in the fractionation of the peptides while that with a somewhat lesser degree of cross-linking was involved in the fractionation of the larger polypeptides. The rational behind this stems principally from the earlier work of Determann and others (Laurent and Killander,1966; Andrews, 1864; Determann and Michel, 1966) who observed that, for a given molecular weight, the K_{av} increases with the decreasing degree of cross-linking over the range of the Sephadex series

From equations 1 and 2 in the general form,

$$\log M = C_1 - C_2 . K_{av}$$

it can be seen, for the region involved in protein chromatography, the absolute value for C_2 is greater than the corresponding value for the linear region involved in peptide fractionation and, from equations 3 and 4 in the general form,

$$K_{av}^{\frac{1}{3}} = C_1 - C_2 \cdot M^{\frac{1}{2}}$$

the converse is true.

Both these findings are consistent with the experimental data obtained by other workers (Andrews, 1964; Determann and Michel, 1966) and favours the view that there are two populations of gel matrix differing only in their porosities.

This view, in itself, could possibly explain the reason for the disparity between the resolution obtained for peptide chromatography by Sepharose and Bio-Gel in that the differences may merely reflect a difference in the method of preparation of the agarose spheres.

Unlike dextrans which are covalently cross-linked, the degree of cross linking in agarose is attributable to hydrogen bonding. Since hydrogen bonds are thought to be labile to high concentrations of guanidine hydrochloride it is, in retrospect, surprising that any such chromatography is possible.

In some earlier experiments with agarose columns equilibrated with 6M guanidine hydrochloride the flow of buffer suddenly stopped. This was assumed to be the result of the generation of fines as a result of the high concentration of salt present. However, if extreme care was taken at the stage of equilibration then the latter was seldom observed. In all cases the flow rate did, however, decrease slowly with time but there was no discernible effect on K_{av} values.

Another method for the interpretation of chromatographic

data was recently described (Afanas'ev, 1970). This was derived theoretically by Afanas'ev from consideration of the surface energy at the protein-solvent interface and the distribution of substances in a two-phase system. The relationship which he found is given by the following equation,

$$\ln\left(1/K_d\right) = \frac{4.84\,(\sigma_1 - \sigma_2)\,N^{\frac{1}{3}}\,V^{\frac{2}{3}}}{RT} \cdot M^{\frac{2}{3}}$$

where K_d is the distribution coefficient, σ_1 and σ_2 are the surface tensions at the macromolecule phase boundary, N is Avogadro's number, V is the specific volume of the macromolecule, R is the gas constant, T is the temperature and M is the molecular weight.

Simplifying this for a given system then,

$$\log\left(1/K_d\right) = A.M^{\frac{2}{3}}$$

where A is a constant.

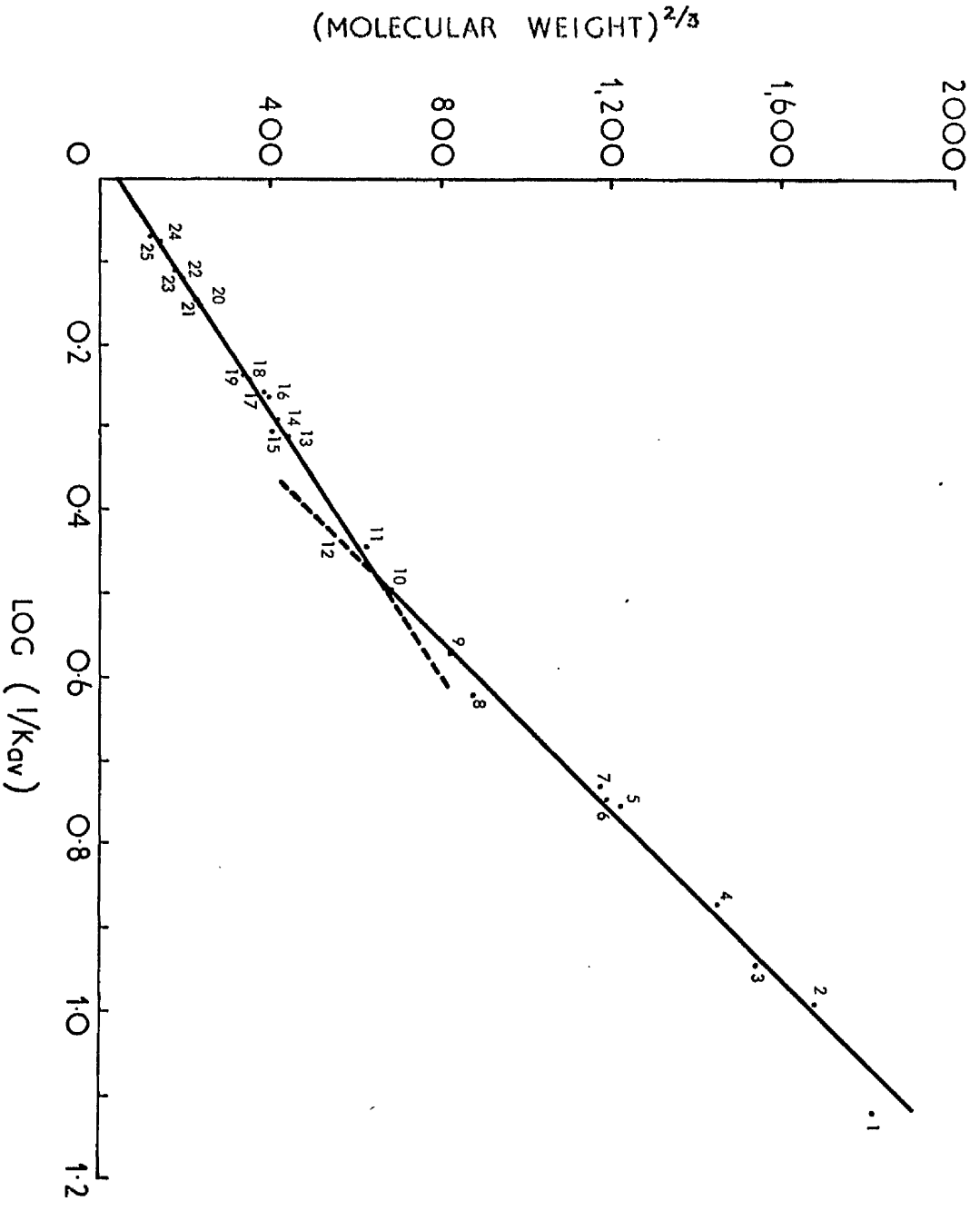
This treatment is unique inasmuch as it defines one point which should be on the line i.e. it would be expected that when a graph is drawn with co-ordinates $M^{\frac{2}{3}}$ and $\log\left(1/K_d\right)$ a straight line passing through the origin would be obtained.

However, when we plot our data in this fashion two linear relationships are once again obtained and neither of these passes through the origin (Figure 27). By supposing the presence of two populations of gel matrix it was found possible to explain the empirical relationships obtained from our data.

<u>For the Gel Population of Lower Porosity.</u>

If we assume that the fraction of the total gel matrix that is involved in protein chromatography is C, then we have;

Figure 27. Molecular weight determinations by gel filtration on Sepharose 6B in the presence of 6M guanidine hydrochloride. The figure shows the treatment of the experimental data according to the method of Afanas'ev (1970). (Molecular weight)$^{\frac{2}{3}}$ is plotted as a function of log $(1/K_{av})$ and the linear relationships were computed by regression analysis as before. The numbers refer to the proteins and peptides listed in Table 5.


(MOLECULAR WEIGHT)$^{2/3}$
2000
1,600
1,200
800
400
0
LOG (1/Kav)
0·2
0·4
0·6
0·8
1·0
1·2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

$$K_{av}\ (\text{empirical}) = \frac{(V_e - V_o)}{(V_t - V_o)} \qquad 1$$

$$K_{av}\ (\text{theoretical}) = \frac{\left[V_e - (1 - C)V_o\right] - C.V_o}{C.V_t - C.V_o} \qquad 2$$

where K_{av} (empirical) is the K_{av} value determined experimentally and K_{av} (theoretical) is the true value for the K_{av} in the gel matrix that is involved in polypeptide gel filtration. Thus from equation 2 above, we have,

$$K_{av}\ (\text{theoretical}) = 1/C. \frac{(V_e - V_o)}{(V_t - V_o)}$$

$$= 1/C.\ K_{av}\ (\text{empirical})$$

Taking logarithms of both side we have,

$$\log\left[1/K_{av}\ (\text{theoretical})\right] = \log\left[1/K_{av}(\text{empirical})\right] - \log\ (1/C)$$

Now from Afanas'ev's equation,

$$A.M^{\frac{2}{3}} = \log\left[1/K_{av}\ (\text{theoretical})\right]$$

hence,

$$M^{\frac{2}{3}} = 1/A.\log\left[1/K_{av}\ (\text{empirical})\right] - 1/A.\log\ (1/C)$$

From regression analysis of our data we obtained,

$$M^{\frac{2}{3}} = 1894.16 \log (1/K_{av}) - 244\ 737$$

hence, $$\log (1/C) = \frac{244.737}{1894.16}$$

$$= 0.1292$$

therefore, $$C = 0.7424$$

Thus from such computations it would seem that about 75% of the gel matrix is responsible for the fractionation of the polypeptide chains.

For the Gel Population of Higher Porosity.

In the present study we used tryptophan as a measure of the total volume accessible to the solvent, V_t. However, if we define the theoretical value for this parameter as V', then we have,

$$K_{av}\ (\text{empirical}) = \frac{(V_e - V_o)}{(V_t - V_o)} \qquad 3$$

$$K_{av}\ (\text{theoretical}) = \frac{(V_e - V_o)}{(V' - V_o)} \qquad 4$$

$$K_{av}(\text{tryptophan}) = \frac{(V_t - V_o)}{(V' - V_o)} \qquad 5$$

from 5,

$$V' = \frac{V_t - V_o + V_o \cdot K_{av}(\text{tryptophan})}{K_{av}(\text{tryptophan})}$$

Substituting this value in equation 4,

$$K_{av}\ (\text{theoretical}) = \frac{(V_e - V_o)}{\left[\frac{V_t - V_o + V_o . K_{av}(\text{tryptophan})}{K_{av}(\text{tryptophan})}\right] - V_o}$$

$$= \frac{V_e - V_o}{V_t - V_o} . K_{av}(\text{tryptophan})$$

$$K_{av}\ (\text{theoretical}) = K_{av}\ (\text{empirical}) . K_{av}\ (\text{tryptophan})$$

Substituting this relationship once again into Afanas'ev's equation we have,

$$M^{\frac{2}{3}} = 1/A.\log\left[1/K_{av}(\text{empirical})\right] + 1/A.\log\left[1/K_{av}(\text{tryptophan})\right]$$

From regression analysis of our data we have,

$$M^{\frac{2}{3}} = 1265.8 \log\ (1/K_{av}) + 43.603$$

Theoretically, the intercept on the y-axis should be the molecular weight of tryptophan to the power $\frac{2}{3}$. Thus we would expect this value to be $(204.2)^{\frac{2}{3}}$, which is equal to 34.68. It can be seen that this value is close to the experimentally determined value of 43.603. This would appear to justify our use of trypto-

phan as a measure of V_t and indicate that there is very little, if any, adsorption.

The value estimated for the molecular weights of various apoferritins by this method are shown in Table 6. The value for the distribution coefficient, K_{av}, represents the mean value and standard deviation of several determinations. Again it can be seen that, for the apoferritins studied, the differences lie within experimental error and it would appear that the molecular weight is of the order of 18,700 ± 500 and this result is in excellent agreement with the value obtained by SDS-polyacrylamide gel electrophoresis.

This value obtained by us (Bryce and Crichton, 1971a,b,c; Crichton, Miller, Cumming and Bryce, 1972) for the molecular weight determined by gel filtration in 6M guanidine hydrochloride has since been confirmed by other workers (Bjork and Fish, 1971).

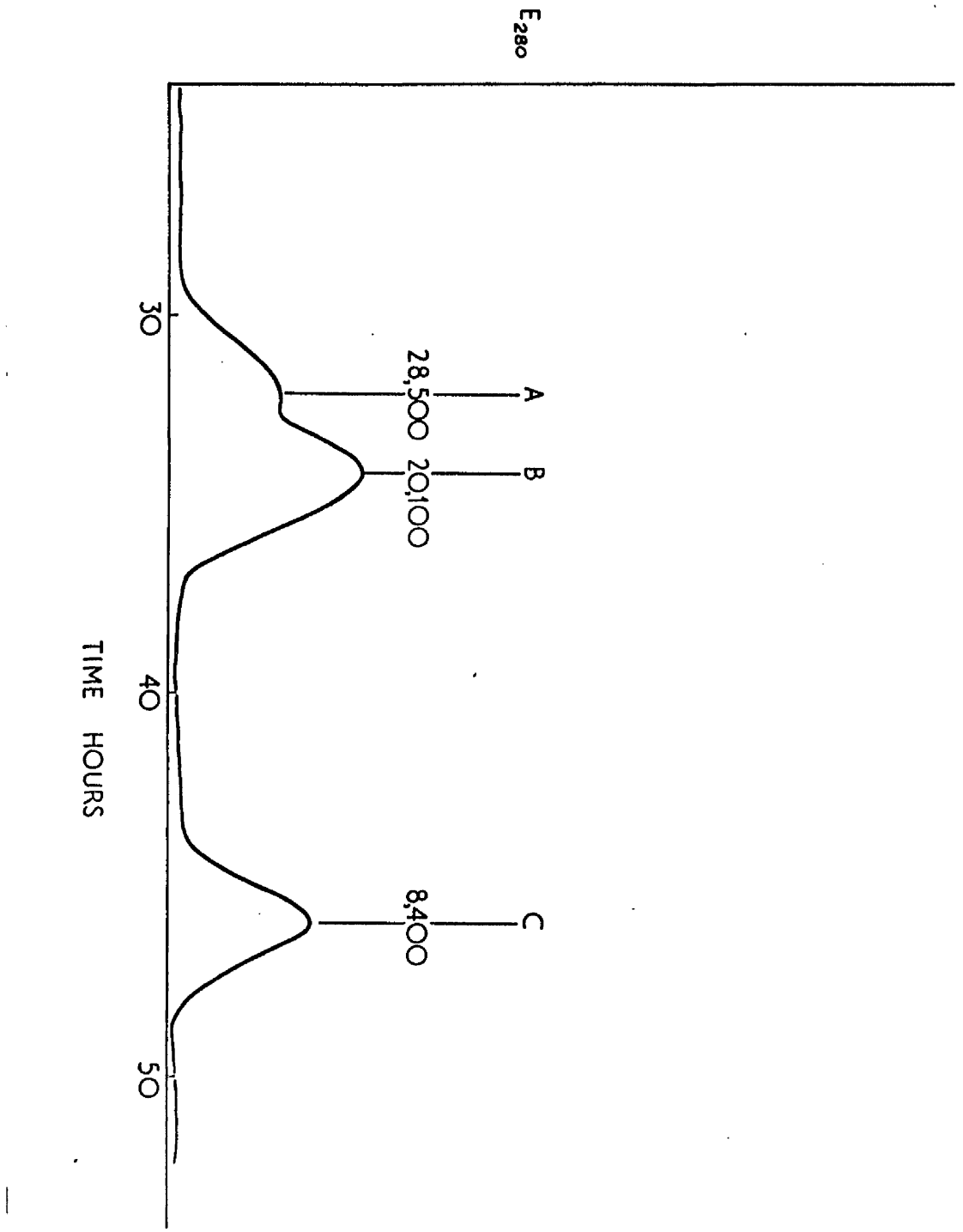
It was also decided to apply the present method to the determination of the molecular weight of the iron binding protein, transferrin. The value obtained for the distribution coefficient of both human and horse transferrin was 0.0758 ± 0.0025 which corresponds to an extrapolated value of 76,600 ± 1,200 which is again in excellent agreement with the value obtained by SDS-polyacrylamide gel electrophoresis and provides further confirmation for the view that transferrin consists of a single polypeptide chain.

An example of the general applicability and potential of this technique can be clearly seen from the results we obtained from the fractionation of lima bean trypsin inhibitor. The elution profile obtained from the fractionation of a commercial preparation of lima bean trypsin inhibitor is shown in Figure 28. All of the detectable trypsin inhibitor activity was associated

Table 6. Gel filtration in the presence of 6M guanidine hydrochloride. Results for the estimation of the molecular weights of various apoferritins.

Protein	K_{av}	Apparent Mol. Wt.
Horse spleen apoferritin	0.309 ± 0.003	18,800 ± 400
Horse liver apoferritin	0.309 ± 0.005	18,800 ± 600
Human spleen apoferritin	0.312 ± 0.004	18,500 ± 500
Human liver apoferritin	0.310 ± 0.004	18,700 ± 500
Human haemochromatotic liver apoferritin	0.311 ± 0.004	18,600 ± 500

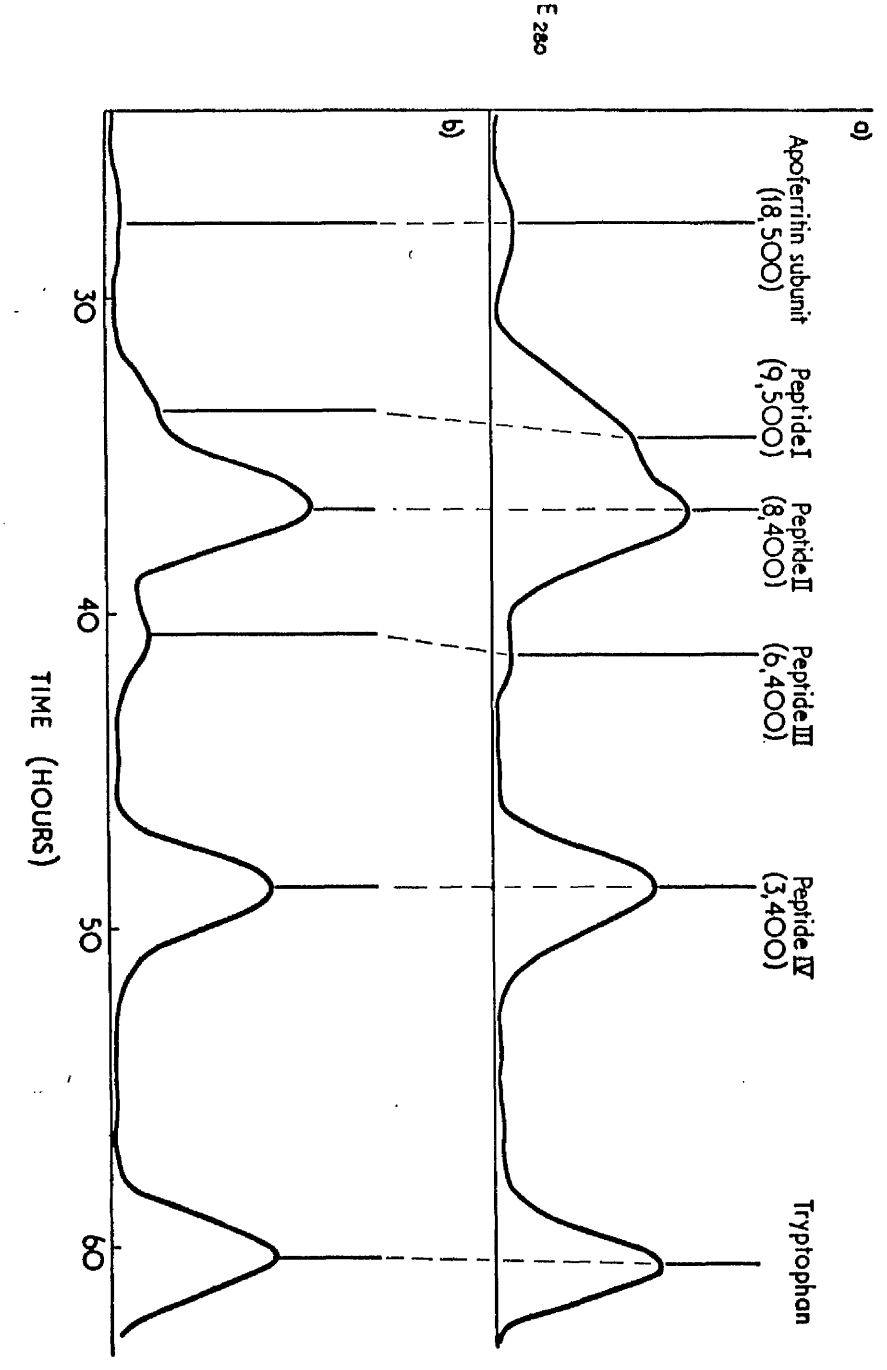
Figure 28. Elution profile obtained from chromatography of a commercial preparation of lima bean trypsin inhibitor on a column of Sepharose 6B equilibrated with 6M guanidine hydrochloride, pH 5.0. The trypsin inhibitor activity is associated with peak C while peaks A and B merely represent impurities


E_{280}
30
40
50
TIME HOURS
28,500
A
20,100
B
8,400
C

with the lower molecular weight species while the higher molecular weight material may merely represent impurities (Jones, Moore and Stein, 1963; Krahn and Stevens, 1971). The apparent molecular weights of the two high molecular weight species were calculated to be 28,500 and 20,100 respectively. It is interesting to note that the difference in molecular weight between these two species is the same order of the molecular weight of the trypsin inhibitor itself (8,400 daltons). This may result from nothing more than chance or else it may represent a precursor-type system in a manner analogous to trypsin-trypsinogen or insulin-proinsulin and for this reason this area probably merits further study.

We also decided to apply this technique in an effort to fractionate the peptides obtained from cyanogen bromide cleavage of apoferritin and thereby obtain estimates of the size of these peptides. The results we obtained for the fractionation of the cyanogen bromide cleavage mixture of horse spleen apoferritin is shown in Figure 29. Also shown is the elution profile of the products of cyanogen bromide cleavage of apoferritin by the second method described on page 71 and it can be seen that treatment with dithiothreitol improves the resolution of the separate peptides. The molecular weights computed for the various peptides were 9,500, 8,400, 6,400 and 3,400. Since the amino acid composition based on a value of 18,500 for the molecular weight of the subunit indicates that there are 3 methionines per subunit then we should expect 4 peptides on digestion with cyanogen bromide. The four peptides that we obtained cannot be combined in any permutation to give us the magnitude of the molecular weight of the subunit. Also the second method of cleavage implied that peptide I (9,500 daltons) probably was not a unique peptide and

Figure 29. Elution profile obtained from fractionation of the cyanogen bromide cleavage products of horse spleen apoferritin on a column of Sepharose 6B equilibrated with 6M guanidine hydrochloride, pH 5.0. (b) Elution profile obtained from the fractionation of the cyanogen bromide cleavage products of horse spleen apoferritin by the modified method of chemical cleavage (see text).


a)
b)
E 280
Apoferritin subunit (18,500)
Peptide I (9,500)
Peptide II (8,400)
Peptide III (6,400)
Peptide IV (3,400)
Tryptophan
30
40
50
60
TIME (HOURS)

that it probably contained an intact methionine.

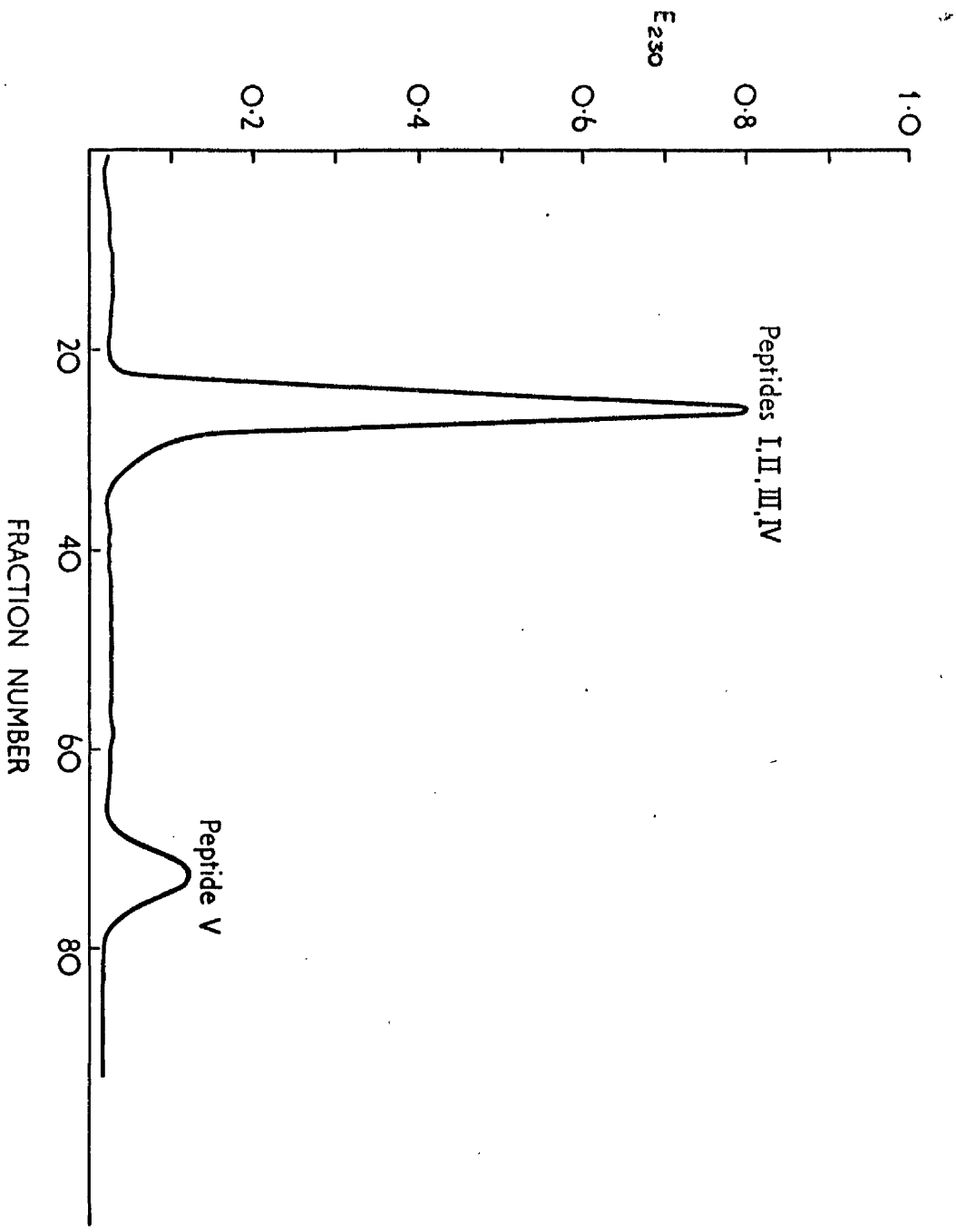
In an effort to rationalise the experimental data we were obliged to assume the presence of an, as yet undetected, peptide. There were two possible reasons for not detecting such a quantity and these were, that it did not contain tryptophan or tyrosine or else that it was sufficiently small to be included under the tryptophan peak.

In early experiments we attempted to dialyse the guanidine hydrochloride from the material by using dialysis tubing that had been treated with acetic anhydride to lower the effective pore size. This treatment had the effect of converting the half-escape time for tryptophan from 1.8 hours to 28 hours. This proved to be a rather troublesome procedure and so it was decided to chromatograph the total cyanogen bromide cleavage mixture on a column of Sephadex G-10 or G-15 equilibrated with 1M acetic acid. By use of these gel matrices the larger peptides I, II, III and IV should be eluted with the void volume and any peptide smaller than 1,500 should be retarded. The elution profile obtained is shown in Figure 30 and the material forming peptide V was purified by rechromatography.

The peptide purified in this manner was hydrolysed and subjected to amino acid analysis. From this the composition was found to be: Asx, 1.1; Glx, 0.98; Gly, 1.05; Leu, 1.0; Phe, 0.7; Ser, 0.7; HSer, present but not determined. The peptide was purified to the extent that contaminating amino acids were present at less than 0.15 moles per mole of peptide. From its composition the molecular weight of the peptide was calculated to be 900 daltons.

Peptide I (9,500 ± 500) can be explained in two ways. The peptide, if it contains an intact methionine, could on cyanogen

Figure 30. Elution profile obtained from chromatography of the cyanogen bromide cleavage products of horse spleen apoferritin on a Sephadex G-15 column (2.5 x 45 cm) equilibrated with 1M acetic acid. Peptides I, II, III, and IV are eluted as one peak with the void volume while peptide V is retarded (see text for peptide nomenclature).


E_{230}
1·0
0·8
0·6
0·4
0·2
20
40
60
80
FRACTION NUMBER
Peptides I, II, III, IV
Peptide V

bromide digestion give rise to either peptide III and IV (6,400 + 3,400) or to peptides II and V (8,400 + 900) as shown in Figure 31. The alignment of these peptides is at present under investigation.

The sum of the four peptides 8,400 ± 400, 6,400 ± 300, 3,400 ± 100 and 900 add up to give 19,100 ± 800 which is in good agreement with the subunit molecular weight. Also the fact that only four peptides were obtained, as predicted, tends to confirm the value for the subunit molecular weight.

This observation was further verified on determining the number of free N-terminal groups in the total cyanogen bromide cleavage mixture. The method chosen was the dansyl method of Gray (1967) and the separation and identification of the dansyl derivatives was performed on polyamide layers as described on page 71. The results are shown schematically in Figure 32. Three dansyl amino acids were detected and these were glutamic acid, glycine and lysine. The finding of only three end groups is compatible with four peptides since it is known that the N-terminal of apoferritin is N-acetylated (Suran, 1966). This result is, however, in disagreement with the study of Crichton (1971a) who found glutamic acid or glutamine , glycine and iso - leucine by the method of Stark and Smyth (1963).

It was said earlier that chromatography in the presence of a denaturant has the advantage over fractionation in normal aqueous buffers in that it eliminates any specific or non-specific aggregation. Recently a technique was developed in which the denaturing solvent was phenol-acetic acid-water (1:1:1, w/v/v) (Pusztai and Watt, 1970). Preliminary studies with such a system did not prove to be successful, although Pusztai himself determined an approximate molecular weight for the apoferritin subunit and found

Figure 31. Possible peptide alignments for the products of cyanogen bromide cleavage of horse spleen apoferritin.

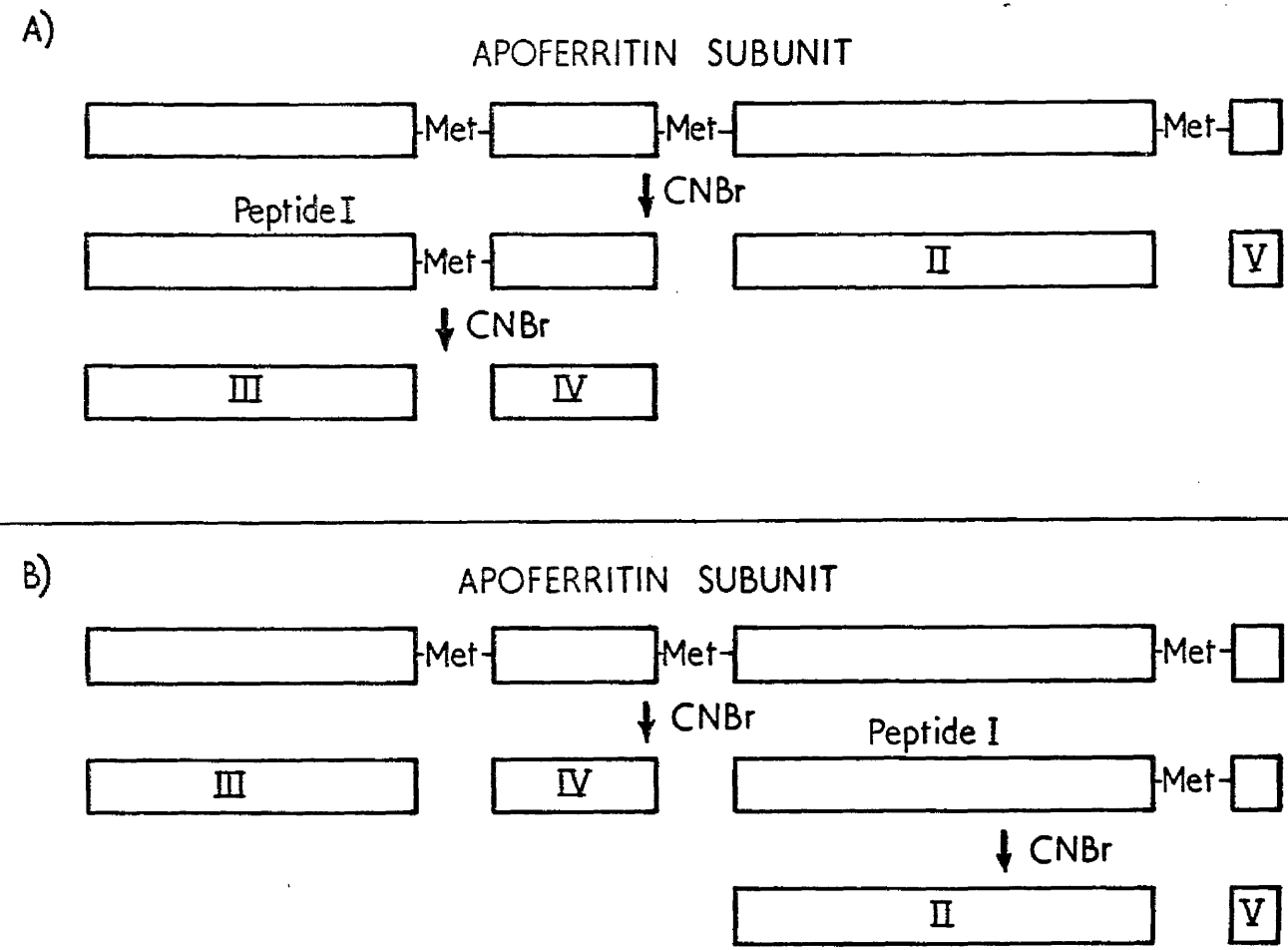
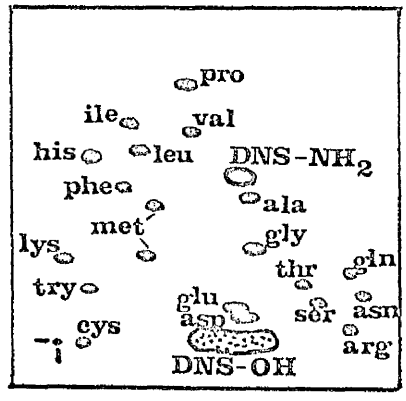
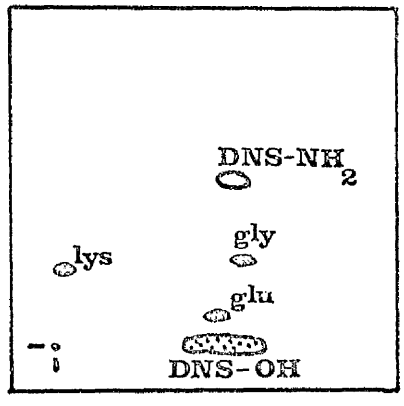

A)
APOFERRITIN SUBUNIT
Met
Met
Met
CNBr
Peptide I
Met
II
V
CNBr
III
IV
B)
APOFERRITIN SUBUNIT
Met
Met
Met
CNBr
Peptide I
III
IV
Met
CNBr
II
V

Figure 32. Upper figure illustrates a typical separation of a mixture of dansyl amino acids on polyamide layer. The solvent systems used in this study were water-formic acid (90%)(100:1.5) and benzene-glacial acetic acid (9:1) according to the method of Woods and Wang (1967). The lower figure shows the results of amino-terminal analysis of the complete cyanogen bromide digest of horse spleen apoferritin using similar conditions.


pro
ile
val
his
leu
DNS-NH2
phe
ala
met
gly
lys
thr
gln
try
glu
asp
ser
asn
cys
arg
DNS-OH


DNS-NH2
gly
lys
glu
DNS-OH

this to be of the order of 19,000 (A. Pusztai, personal communication).

Quantitative Trytophan Determinations.

Another useful method for a molecular weight estimation is to calculate the minimum molecular weight from the amino acid composition and based on the amino acid which appears least frequently in the protein. In horse spleen apoferritin, tryptophan probably represents the amino acid in the smallest amount.

Since hydrolysis of a protein with 6N hydrochloric acid causes almost complete destruction of trytophan, the quantitative estimation of this amino acid is usually carried out by methods which avoid acid hydrolysis. This problem has been circumvented by alkaline hydrolysis (Knox, Kohler, Palter and Walker, 1970), colorimetric procedures (Spies and Chambers, 1949; Barman and Koshland, 1967) and spectrophotometric methods (Goodwin and Morton, 1946; Patchornick, Lawson and Witkop, 1958a,b). Recently, however, several workers have demonstrated that tryptophan can be analysed by standard amino acid analysis following acid hydrolysis of the protein, provided the 6N hydrochloric acid contains 4% thioglycollic acid (Matsubara and Sasaki, 1969) or the hydrolysis is performed in 3N p-toluene-sulphonic acid containing 0.2% 3-(2-aminoethyl)-indole (Liu and Chang, 1971).

The method chosen by Harrison and Hofmann (1961) for the quantitative trytophan determination of horse spleen apoferritin was a modification of the Spies and Chambers (1949) method. These workers used either urea-denatured apoferritin or instead apoferritin that was heat-denatured and partially digested with chymotrypsin and trypsin. The reason for this is that some proteins developed a slightly different colour complex from that ob-

tained with free tryptophan on treating with p-dimethylaminobenzaldehyde (Hamilton, 1960). The result obtained for apoferritin by this method was 21 tryptophan residues per 480,000 g protein and this was used as confirmatory evidence for the existence of 20 subunits of molecular weight 23-25,000. Harrison and Hofmann (1962) also determined qualitatively by staining a tryptic fingerprint of apoferritin with p-dimethylaminobenzaldehyde according to the method of Reddi and Kodicek (1953). By this method they demonstrated the presence of only one tryptophan-containing peptide.

In the present study we decided to redetermine the tryptophan content without having recourse to extensive denaturation and the methods chosen were the spectrophotometric methods of Edolhoch (1967) and Bencze and Schmid (1957) and by chemical modification as described by Scoffone and coworkers (Scoffone, Fontana and Rochi, 1968a,b; Boccu, Veronese, Fontana and Benassi, 1970). In the latter method the protein is treated with 2-nitrophenylsulphenyl chloride. By this method we generate in the intact protein, a chromophore, the absorption of which we can monitor in the visible region. However, this reagent has been shown to react with sulphydryl groups to form mixed disulphides, although, the thiol function can readily be recovered under mild alkaline conditions, with the quantitative release of the nitrothiophenol moiety, thereby allowing accurate tryptophan determinations. The reliability and general applicability of this procedure has been demonstrated for several well characterized proteins (Boccu, Veronese, Fontana and Benassi, 1970) and has the advantage over Koshland's reagent in that multiple reactivity with tryptophan does not occur (Barman and Koshland, 1967; Kondo and Witkop, 1968; Dopheide and Jones, 1968)

The reaction sequence for the conversion of tryptophan to 2-(2-nitrophenylsulphenyl)-tryptophan is shown schematically in Figure 33.

The results obtained for a variety of apoferritins are shown in Table 7.

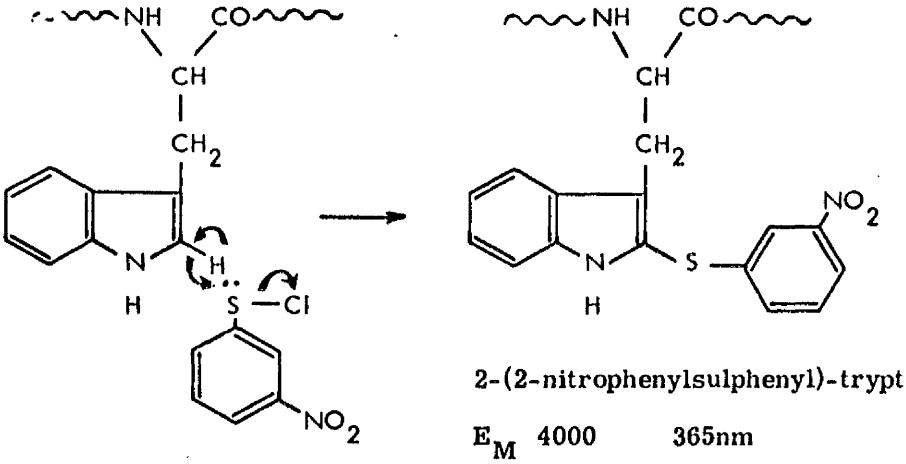
In the case of the spectrophotometric methods, protein was determined by the method of Lowry, Rosebrough, Farr and Randall, (1951) while in the chemical modification the protein content was estimated from amino acid analysis.

It can be seen that the values obtained from the two independent methods are in excellent agreement and that the difference between the tryptophan contents for the various apoferritins lies within experimental error. The average value from all such determinations was calculated to be 55.25 ± 2.43 moles of tryptophan per mole of undissociated apoferritin. This figure is based on a molecular weight of 480,000 daltons so as to be directly comparable with the data of Harrison and Hofmann (1961, 1962) who found 21 moles of tryptophan per mole of undissociated apoprotein by the method described. The value obtained from the present study would imply 2.13 ± 0.09 tryptophan residues per subunit of molecular weight 18,500 and 2.65 ± 0.11 tryptophan residues per subunit of molecular weight 23,000.

In the spectrophotometric method of Edolhoch (1967) it was also found possible to determine the tyrosine content of apoferritin (page 77). The value obtained from such a study was 120 ± 4.9 tyrosine residues per 480,000 g protein or, 4.62 ± 0.19 tyrosine residues per subunit of molecular weight 18,500 or 5.62 ± 0.23 tyrosyl residues per subunit of molecular weight 22,500 which is in very good agreement with the value obtained for this residue by amino acid analysis. This then serves as a very useful check

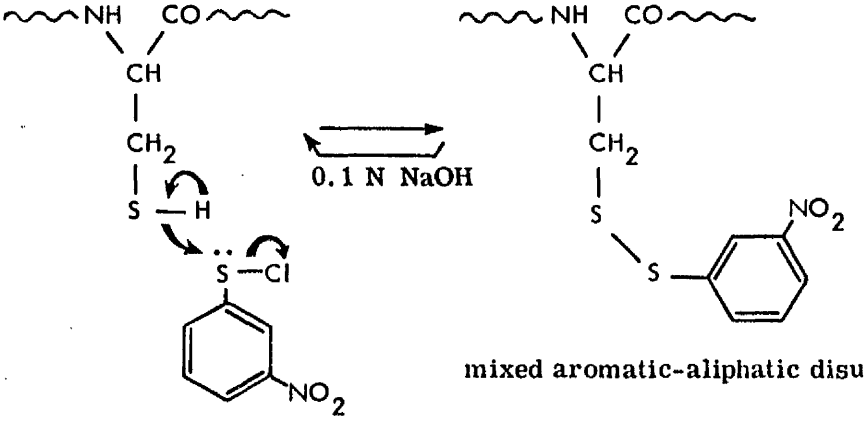
Figure 33. Reaction sequence for the formation of a 2-(2-nitrophenylsulphenyl)-tryptophan residue in an intact protein . It can be seen that the reagent, 2-nitrophenylsulphenyl chloride, also reacts with cysteine residues but this modification can be reversed by treatment with alkali.

Quantitative Evaluation of Tryptophan



2-(2-nitrophenylsulphenyl)-tryptophan

E_M 4000 365nm



mixed aromatic-aliphatic disulphide

Table 7. Quantitative tryptophan determinations for various apoferritins.

Method a refers to the spectrophotometric method of Edolhoch (1967) while Method b refers to the chemical modification method of Scoffone et al (1968).

Human Haem Liver*, human haemochromatotic liver.

n.d, not determined.

Method \ Protein	Horse Liver	Horse Spleen	Human Liver	Human Spleen	Human Haem. Liver*
No. of tryptophan residues/18,500 g protein [a]	2.05	1.99	2.27	2.21	2.21
No. of tryptophan residues/18,500 g protein [b]	2.16	2.03	2.13	n.d	n.d

on the validity of the tryptophan determination.

The tyrosine and tryptophan content of apoferritin were also analysed by the spectrophotometric method of Bencze and Schmid (1957) as described on page 78. A line was drawn tangentially to the two peaks of the absorption spectrum of the protein and the slope of this line (a/b) and the maximum absorption near 280 nm (D_{max}) were determined. Then, using the relationship of Bencze and Schmid (1957),

$$S = 10^3 . (a/b) / D_{max}$$

we find, on substituting these values, that S = +8.31 and therefore from the data of these workers, the molar ratio of tyrosine /tryptophan was found to be 2.5 which is in excellent agreement with all the data

Another criterion that served as a useful check on this data was the value of the specific extinction coefficient, $E^{1\%}_{1cm}(280)$. Based on the argument already presented on page 102 it can be shown that for apoferritin to have 1 tryptophan and 5 tyrosine residues per subunit of molecular weight 18,500 then the value computed for $E^{1\%}_{1cm}(280)$ is 6.74, while for a subunit with 2 tryptophans and 5 tyrosines the value for this parameter is 9.81. The value obtained experimentally for $E^{1\%}_{1cm}(280)$ was found to be 10.13 which is clearly in good agreement with the latter value and once again tends to confirm the view that the subunit contains 2 tryptophan residues and not 1 as was previously accepted (Harrison and Hofmann, 1961, 1962).

Friedberg (1962) estimated the tryptophan content for guinea pig liver apoferritin and obtained a value of 1.85 tryptophan residues per subunit of molecular weight 18,500 by the method of Graham, Smith, Heer and Klein (1947) and this value seems to

agree with our analysis for the protein from both human and horse sources.

Analytical Ultracentrifugation.

As the techniques already described for subunit molecular weight determinations are essentially empirical in their approach it was decided to re-determine the value by a method which was theoretically well-defined. Sedimentation equilibrium is just such a method and it was hoped to apply the theoretical treatment to the data obtained for the apoprotein dissociated by a number of denaturing agents.

In all of the studies on sedimentation equilibrium of the protein subunit and also the undissociated protein the experimental data was treated according to the equation describing the concentration distribution at equilibrium (Yphantis, 1964),

$$M_{\bar{w}}^{app} = \left[2RT / (1 - \bar{v}'\rho).\omega^2 \right] . d\ln C/dr^2$$

where $M_{\bar{w}}^{app}$ is the apparent weight average molecular weight, R is the gas constant, T is the absolute temperature, $\bar{v}'$ is the effective partial specific volume of the solute, ρ is the density of the solution, C is the concentration and r is the distance from the axis of rotation.

In order to avoid problems of adsorption , especially at low protein loading concentrations, the centrepiece was filled and emptied twice before the final filling. No layering oil was used in these experiments.

The partial specific volume, $\bar{v}$, for horse spleen apoferritin was calculated from the amino acid composition by the method of Cohn and Edsall (1943) as described by McMeekin and Marshall (1952) and found to be 0.731 ml per g, whereas the value previous-

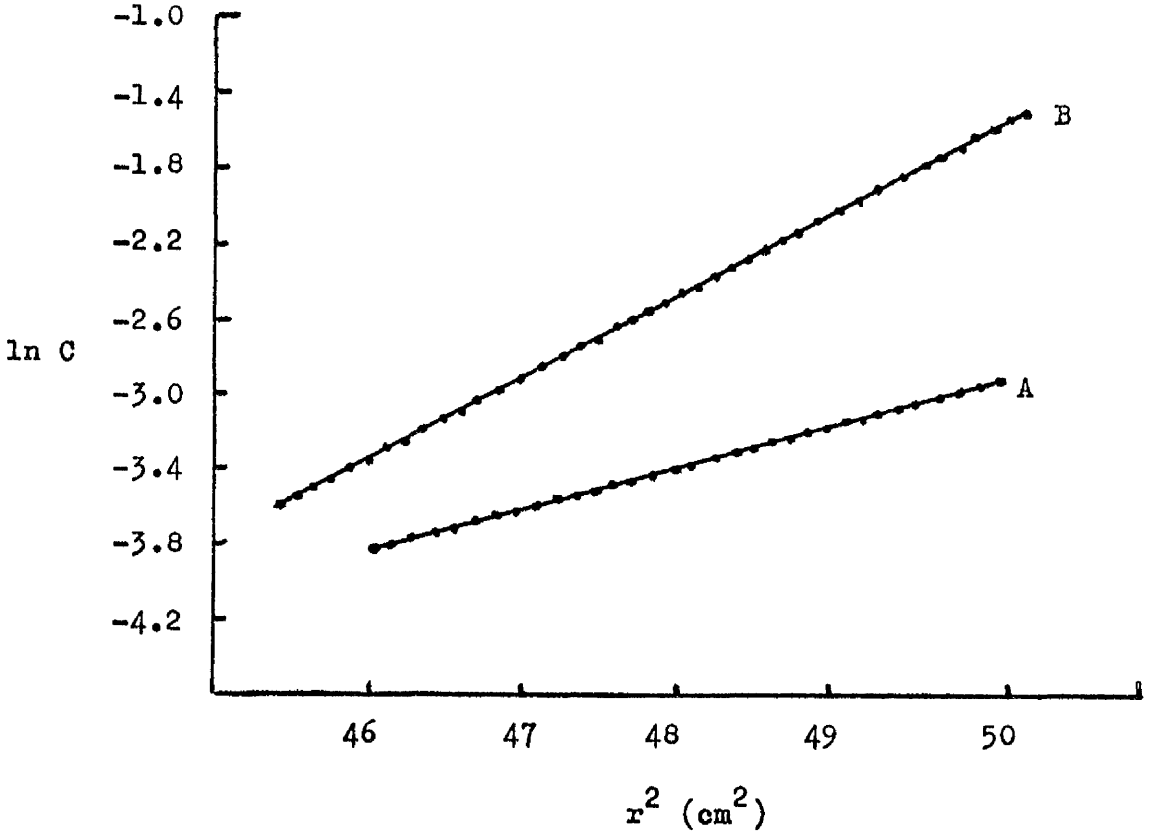
ly published for the partial specific volume from pycnometric estimation was 0.747 ml per g (Rothen, 1944).

Dissociation by 6M Guanidine Hydrochloride.

The data derived from sedimentation equilibrium experiments at 20°C for horse spleen apoferritin were plotted as ln C against r^2 as shown in Figure 34. The rectilinearity of the plot attests to the homogeneity of the dialysed sample. The linear relationship was treated according to the method of least squares (regression coefficient 0.9971) and $d\ln C/dr^2$ was found to be 0.2774. This value could then be substituted into the equation already described. However, one of the major problems in interpreting data with guanidine-dissociated material in the ultracentrifuge is that there is still not complete agreement about the effect of high guanidine concentration on the partial specific volume of proteins. Since 6M guanidine hydrochloride has a high density (1.1455 g/ml) small inaccuracies in $\bar{v}$ produce a relatively large error in the calculated molecular weight. Just as there have been several well documented reports of a 1 to 2% decrease in $\bar{v}$ (Marler, Nelson and Tanford, 1964; Small and Lamm 1966a,b; Kielley and Harrington, 1960; Reisler and Eisenberg, 1969), so too have there been several which indicate no change (Ullman, Goldberg, Perrin and Monod, 1968; Reithel and Sakura, 1963; Yue, Palmieri, Olson and Kuby, 1967; Schome, Brown, Howard and Pierce, 1968; Castellino and Barker, 1968) or even a slight increase (Green and McKay, 1969; Schachman and Edelstein, 1966). From the form of the equation a change in 2% in $\bar{v}$ constitutes a 6% variation in M_{ω}^{app}. Further, there is still some doubt about the extent of preferential binding of guanidine to proteins (Ullman, Goldberg, Perrin and Monod, 1968; Reithel and Sakura, 1963; Castell-

Figure 34. Molecular weight estimation by sedimentation equilibrium in the analytical ultracentrifuge. A, shows the results of a typical sedimentation equilibrium experiment performed with guanidine hydrochloride-dissociated apoferritin . $d\ln C/dr^2$ for this line was calculated to be 0.2774 with a regression coefficient of 0.9971. B, shows the results of a similar sedimentation experiment with acetic acid-dissociated apoferritin that had been dialysed extensively against dilute glycine buffer, pH 3.0. $d\ln C/dr^2$ in this case was computed to be 0.4439 by the method of least squares analysis (regression coefficient = 0.9981).

Both equilibrium runs were performed at 20,000 revolution per minute and 20°C.


-1.0
-1.4
-1.8
-2.2
-2.6
-3.0
-3.4
-3.8
-4.2
ln C
B
A
46
47
48
49
50
r² (cm²)

ino and Barker, 1968; Noelken and Timasheff, 1967).

Since this is the case the results for the computed $\overline{M}_{w}^{app}$ are presented in the form shown in Table 8.

Thus it can be seen that the value for the molecular weight of the subunit of horse spleen apoferritin as determined by sedimentation equilibrium on guanidine-dissociated protein can vary from 15,100 to 21,400.

It is most probable that this subunit represents the smallest submolecular entity physically identifiable with the parent molecule, since the work of Tanford and coworkers has established that higher orders of internal structure in globular proteins (i.e. secondary, tertiary and quaternary) are completely disrupted in this solvent system (Tanford, Kawahara and Lapanje, 1967; Nozaki and Tanford, 1967; Tanford, Kawahara, Lapanje, Hooker, Zarlengo, Salahuddin, Aune and Takagi, 1967; Lapanje and Tanford, 1967; Tanford, Kawahara and Lapanje, 1966).

Recently Bjork and Fish (1971) determined the subunit molecular weight by long-column meniscus depletion sedimentation equilibrium and found it to be 19,200 ± 900 ($\bar{v}$ = 0.747) or 18,200 ± 800 ($\bar{v}$ = 0.733) which is in excellent agreement with the values obtained by us (Bryce and Crichton, 1971a).

Dissociation by 67% Acetic Acid.

A typical plot of ln C *versus* r^2 for protein dissociated by acetic acid followed by dialysis into 0.01M glycine-HCl buffer, pH 3.0 is shown in Figure 34. Again it can be seen that a linear relationship was obtained (regression coefficient 0.9981) which implies a monodisperse macromolecular species. Using the value of 0.4439 obtained for $d\ln C/dr^2$ in this relationship, the apparent weight average molecular weight for horse spleen apoferritin

Table 8. Sedimentation equilibrium experiments on horse spleen apoferritin dissociated by 6M guanidine hydrochloride.

[a] The value for $\bar{v}$ from Rothen (1944)

[b] The value for $\bar{v}$ (Rothen, 1944) with a 1% correction (Kielley and Harrington, 1960).

[c] The value for $\bar{v}$ (Rothen, 1944) with a 2% correction (Small and Lamm, 1966). This is also the value for $\bar{v}$ calculated from amino acid composition (see text).

[d] The value for $\bar{v}$ calculated from the amino acid composition with a 1% correction (Kielley and Harrington, 1960).

[e] The value for $\bar{v}$ calculated from the amino acid composition with a 2% correction (Small and Lamm, 1966).

[f] GuHCl, guanidine hydrochloride.

Extent of preferential binding	Partial specific volume ($\bar{v}$)				
	0.747[a]	0.740[b]	0.731[c]	0.724[d]	0.717[e]
No binding	21,400	20,100	18,700	17,700	16,800
0.05g of GuHCl[f] per g of protein	20,300	19,100	17,800	16,800	16,000
0.10g of GuHCl[f] per g of protein	19,300	18,100	16,800	15,900	15,100

was computed to be 18,400 ± 800 using $\bar{v}$ = 0.731 or 19,500 ± 900 with $\bar{v}$ = 0.747.

The finding of a homogeneous material was in agreement with the observation of Harrison and Gregory (1968) from sedimentation velocity studies. It was shown earlier that this result was not obtained by gel chromatography (page 117). One possible reason for this disagreement is that the association-dissociation phenomena observed in gel chromatography could be concentration dependent and that the high concentrations used for gel filtration experiments shifts the equilibrium in favour ~~in favour~~ of monomer.

Dissociation by 1% Sodium Dodecyl Sulphate.

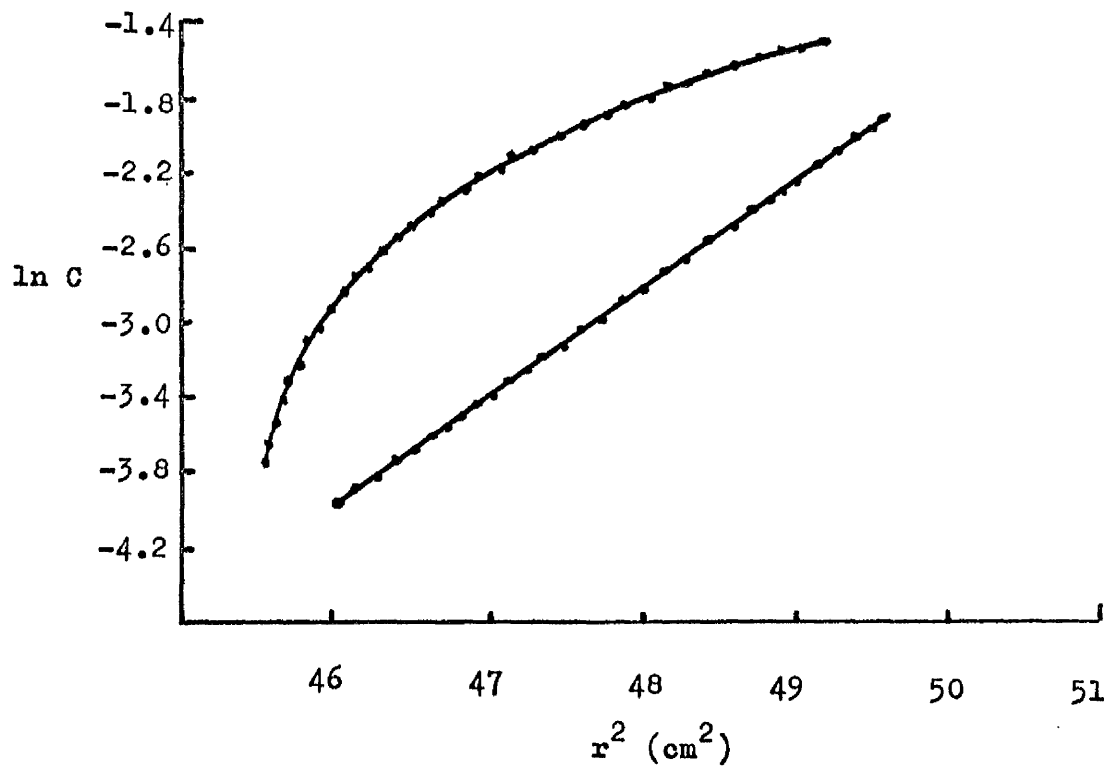
Once again the plot of ln C versus r^2 was rectilinear attesting to the homogeneity of the sample (Figure 35). In one experiment the slope of the linear relationship was shown to be 0.582.

In this method a value for the effective partial specific volume is complicated by the extensive detergent binding. If it is assumed that the binding of SDS for globular proteins is 1.4 g detergent per g of protein, as has recently been suggested (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970), then the resultant $\bar{v}'$ for the protein-SDS complex is 0.8275 ($\bar{v}_{protein}$ = 0.747) or 0.8215 ($\bar{v}_{protein}$ = 0.731) using a value of 0.885 ml/g for the partial specific volume of SDS (Granath, 1953). Substituting these value into the equation we obtain a molecular weight for the protein-SDS complex of 46,000 or 44,500 respectively, which, after correcting for the bound detergent, reduce to values of 19,200 and 18,500 respectively.

Hofmann and Harrison (1963) determined the subunit molecular

Figure 35. Molecular weight estimation by sedimentation equilibrium in the analytical ultracentrifuge. The linear relationship was obtained for apoferritin dissociated by the anionic detergent sodium dodecyl sulphate. The other curve was obtained for protein dissociated by alkali, the upward curvature indicating non-ideal effects.

Both equilibrium runs were performed at 20,000 rpm and 20^{0}C.


-1.4
-1.8
-2.2
-2.6
-3.0
-3.4
-3.8
-4.2
ln C
46
47
48
49
50
51
r² (cm²)

weight by sedimentation velocity and approach to equilibrium . and found this to be 38,000-41,000 at infinite dilution which, after correction for SDS binding (0.5 g/g protein), gave a value for the molecular weight of the subunit of 25-27,000. It is clear that if they underestimated the amount of detergent bound to the protein then the value for the molecular weight of the subunit would clearly be too high. If the accepted value of 1.4 g SDS/g protein is substituted, then their data gives values of 18,100-19,600 ($\bar{v}_{protein}$= 0.747) or 17,500-19,000 ($\bar{v}_{protein}$= 0.731) which are in very good agreement with the present study.

Dissociation by Sodium Hydroxide.

Hofmann and Harrison (1963) observed that apoferritin could be dissociated by treatment with alkali. It was hoped that by the use of such a dissociating condition we could determine the subunit molecular weight without recourse to $\bar{v}$ corrections.

A typical plot of ln C *versus* r^2 for this system is shown in Figure 35 and it can be seen that the relationship is no longer linear.

It is known that plots which concave upwards indicate polydispersity, and those which are concave downwards are an index of non-ideality of the solution. It is thought that in this system we have subunits and monomer, and perhaps intermediates and also products of alkaline hydrolysis of the protein, all forming a very complicated system. Non-ideal effects arise because of steric and electrostatic interaction of species in solution and can be defined in terms of an empirical constant, the second virial coefficient. The redistribution achieved at equilibrium leads not only to a variation in molecular weight throughout the cell but also the magnitude and number of virial coefficients change

throughout the cell. Despite the fact that some workers have managed to derive meaningful data from sedimentation equilibrium experiments on non-ideal, polydisperse substances it was thought that since the present system was very complicated then such complex treatment was not justified (Goldberg, 1953; Manelkern, Williams and Weissberg, 1957; Rowe and Rowe, 1970).

Taking all the sedimentation equilibrium data together, then it can be seen that, as a result of the discrepancy between the measured and calculated $\bar{v}$ for apoferritin, the opposing views on the effect of the various denaturants on the value of $\bar{v}'$ and the uncertainty in the extent of preferential binding of the various denaturants to the protein, then the molecular weight estimations are subject to a rather large variation. Despite this, the apparent subunit molecular weight is still substantially lower than the value previously reported by other workers.

Table 9 summarises the results for the molecular weight determinations of the subunit. It is clear that the polypeptide chain comprising the horse spleen apoferritin molecule has a molecular weight of the order of 18,500. Apoferritins isolated and purified from a variety of other organs and species (horse liver, human spleen and liver, human haemochromatotic spleen and liver, rat spleen and liver and pig liver) were found to be indistinguishable in their subunit molecular weights from the horse spleen protein.

Sedimentation Studies on Undissociated Apoferritin.

In protein structural studies it is often of interest to know the number of subunits that constitute the monomeric protein.

Table 9. Summary of molecular weight obtained for the polypeptide chain of horse spleen apoferritin.

Molecular Weight	Method of determination
18,300 ± 300	SDS-polyacrylamide gel electrophoresis
29,700-34,000	Sephadex gel filtration
18,800 ± 400	Gel filtration in the presence of 6M guanidine hydrochloride
19,100 ± 800	From the sum of the molecular weights of the cyanogen bromide peptides
18,400 ± 800	Quantitative tryptophan determinations
15,100-18,700 (using $\bar{v}$ = 0.731) and 16,800-21,400 (using $\bar{v}$= 0.747)	Sedimentation equilibrium performed on protein dissociated by 6M guanidine hydrochloride
18,400 ± 800 (using $\bar{v}$ = 0.731) and 19,500 ± 900 (using $\bar{v}$ = 0.747)	Sedimentation equilibrium performed on protein dissociated by 67% acetic acid and dialysed into dilute glycine buffer
18,500 (using $\bar{v}$ = 0.731) and 19,200 (using $\bar{v}$ = 0.747)	Sedimentation equilibrium performed on protein dissociated by sodium dodecyl sulphate (one experiment)

To determine this with a protein containing identical, or at least very similar, polypeptide chains is a relatively straightforward procedure in that , one is only required to determine the molecular weight of the subunit and of the undissociated protein and divide one by the other.

The method we chose for the determination of the undissociated apoprotein's molecular weight was sedimentation equilibrium in the analytical ultracentrifuge. Since sedimentation equilibrium suffers from the disadvantage that long time periods are required for the attainment of equilibrium, we performed the initial experiments by sedimentation velocity in an effort to establish conditions i.e. solute concentration, pH, buffer ionic strength etc., such that aggregation to higher oligomers was minimal.

Sedimentation velocity experiments were performed at pH 6.4 and at various initial protein concentrations from 0.25 - 1.5 mg/ml. The data was plotted as the distribution of sedimenting species, G(S), against $S_{20,w}$ computed by a FORTRAN program on the PDP 8/L digital computer and this is shown in Figure 36. At each protein concentration the apoferritin sedimented as a single, major component with a perceptible higher sedimenting species. The latter constituted about 5% of the total material and was assumed from its sedimentation coefficient value of about 27S to be a dimer form of the protein. This was also detected using schlieren optics as shown in Figure 37. Although this was present even at low protein concentrations, it was not considered to be a major source of error in molecular weight determination since it was present in such small amounts.

Extrapolation of $S_{20,w}$ values to zero concentration yielded a value for the sedimentation coefficient at infinite dilution of 17.12 as shown in Figure 38. The concentration dependence obser-

Figure 36. Distribution of sedimenting species, G(S), shown as a function of the sedimentation coefficient, $S_{20,w}$. This data was obtained from the experimental data by use of a FORTRAN 4 program on a PDP 8/L digital computer.

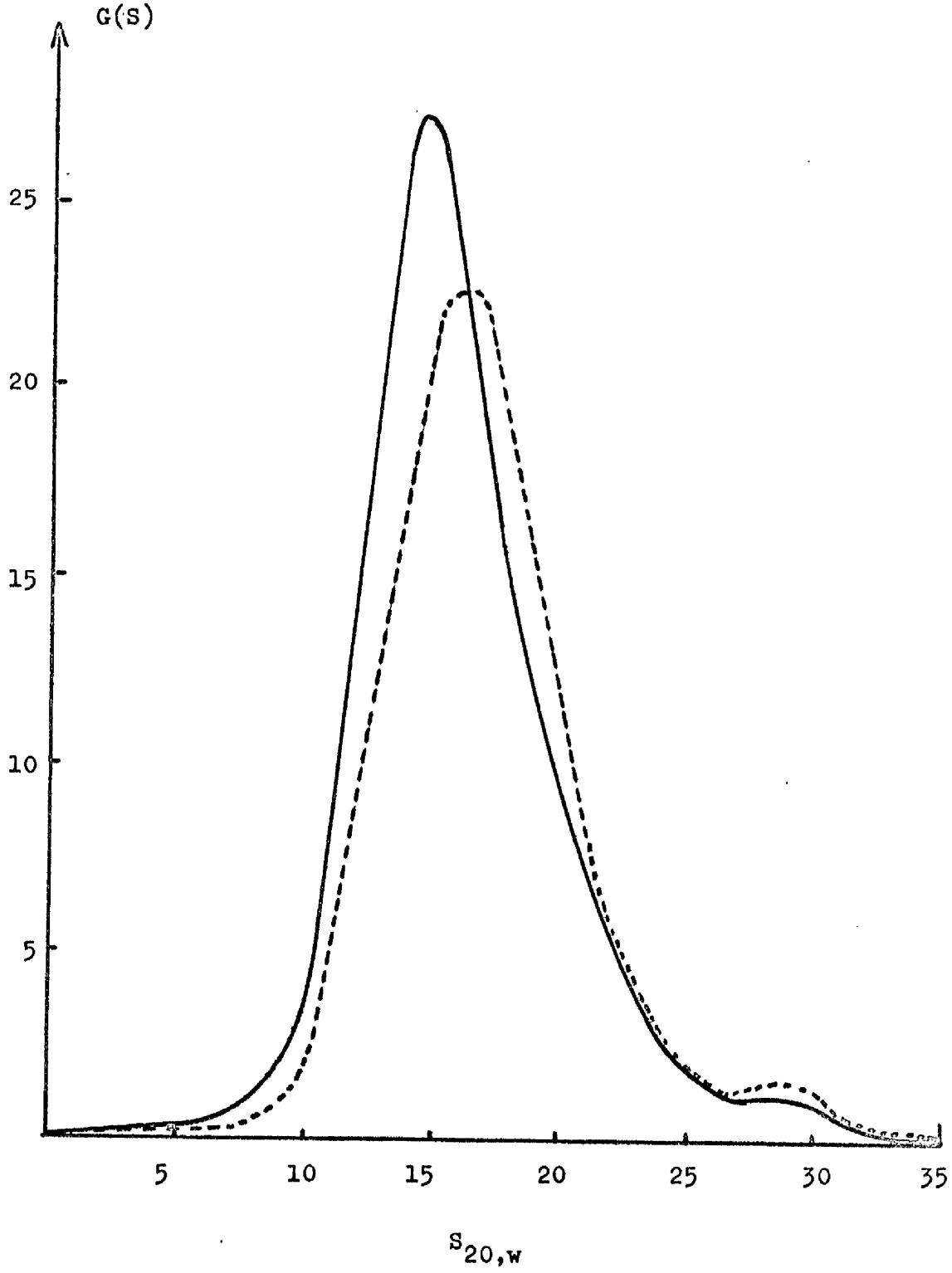

G(S)
25
20
15
10
5
5
10
15
20
25
30
35
$S_{20,w}$

Figure 37. Ultracentrifuge schlieren patterns of horse spleen apoferritin. Sedimentation is from right to left. The protein concentration was 4 mg/ml and the buffer used was 0.05M phosphate, pH 6.4. Centrifugation was carried out at 40,000 rpm.

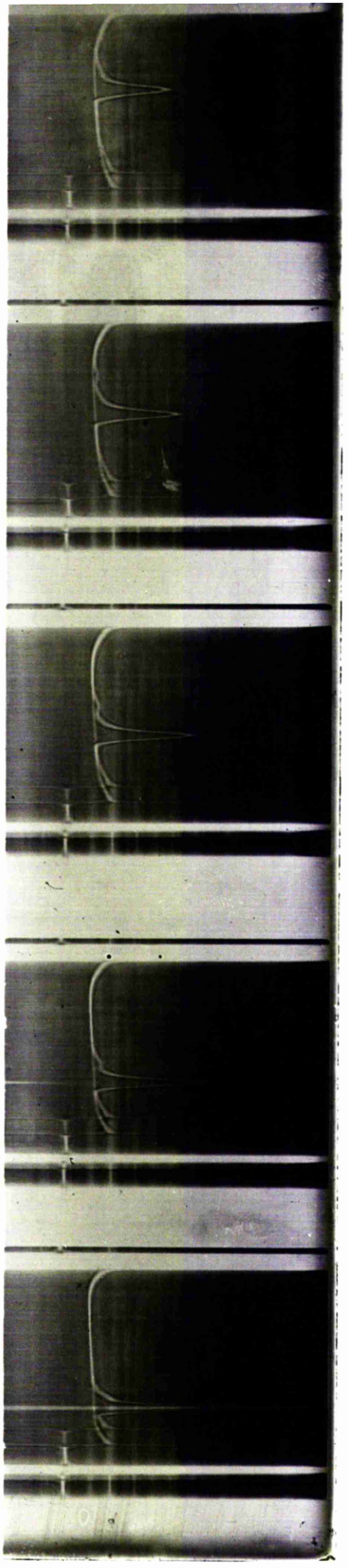
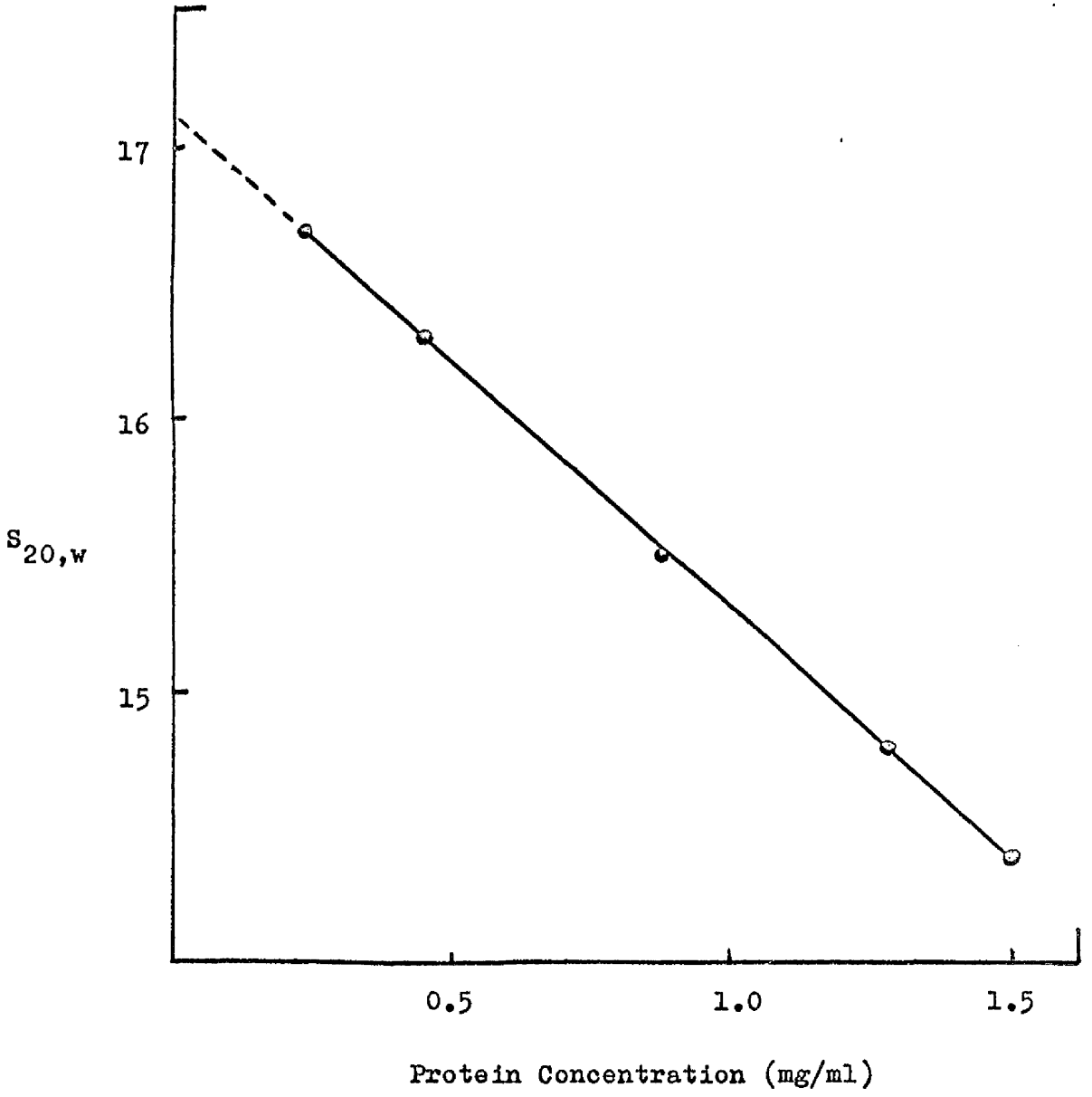


Figure 38. Sedimentation coefficient ($S_{20,w}$) of horse spleen apoferritin as a function of protein concentration. Conditions for the sedimentation velocity runs are given in the text.


17
16
15
$S_{20,w}$
0.5
1.0
1.5
Protein Concentration (mg/ml)

ved is characteristic of compact, globular macromolecules and does not indicate an association-dissociation equilibrium under the conditions employed.

A similar series of experiments was carried out using a variety of phosphate buffers at different ionic strengths (0.01M - 0.2M) and distilled water. In all these studies about 5% higher aggregate was again found and it was apparent that such aggregation was not dependent on ionic strength, at least for the range considered.

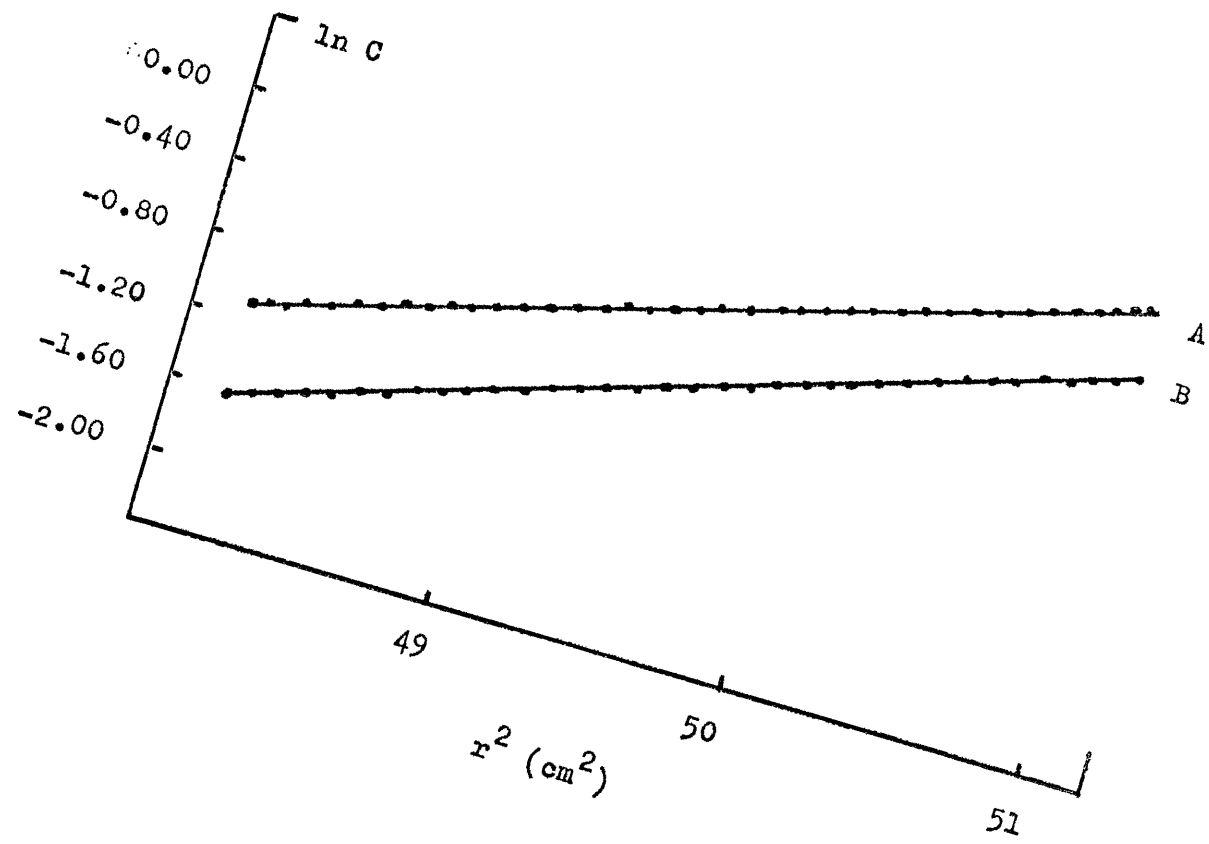
Similarly the data from buffers at a variety of pH values in the range pH 5 - 8 showed no dependence of such oligomers on this parameter and as before the major sedimenting species was homogenous with an $S_{20,w}$ of about 17-18S.

From these preliminary studies, the conditions for sedimentation equilibrium runs were established and these were 0.01M phosphate buffer, pH 6.4 carried out at a variety of protein concentrations to see if $M_{\bar{w}}^{app}$ was concentration dependent.

Figure 39 shows the results of sedimentation equilibrium of apoferritin under these conditions. The linearity of the plot of log C versus r^2 attests to the monodisperse nature of the monomeric species. The data was analysed by the method of least squares (regression coefficient 0.9997) and the value obtained for $d\ln C/dr^2$ was substituted in the equation and the apparent weight average molecular weight computed to be 443,000 using a value of $\bar{v} = 0.731$ or 469,000 using a value of $\bar{v} = 0.747$.

Some sedimentation equilibrium experiments were carried out under the same conditions and the data processed on a Univac 1108 (National Engineering Laboratory, East Kilbride, Scotland) by a FORTRAN program written by Dr. A.B. Barclay. The data for this study was treated according to the mathematical analysis of

Figure 39. Molecular weight estimation of undissociated horse spleen apoferritin by sedimentation equilibrium in the analytical ultracentrifuge. The equilibrium runs were performed at a speed of 4,000 rpm and a temperature of 20°C. Protein concentration was 0.9mg/ml (A) and 0.44 mg/ml (B).


ln C
0.00
-0.40
-0.80
-1.20
-1.60
-2.00
A
B
49
50
51
r^2 (cm^2)

Scholte (1968) and consisted of fitting the best complete polynomial to the $\ln C, r^2$ data. The result obtained from such a study was 430,000 ± 22,000 ($\bar{v}$ = 0.731) for a concentration range 0.07 - 0.93 mg/ml and the $M_{\bar{w}}^{app}$ showed no concentration dependence.

The values obtained in the present study (Crichton, Eason, Barclay and Bryce, 1972) are in excellent agreement with the previously accepted value of 430-465,000 and this represents estimations by a variety of physical techniques (Bjork and Fish, 1971; Harrison, 1959; Rothen, 1944; Richter and Walker, 1967)

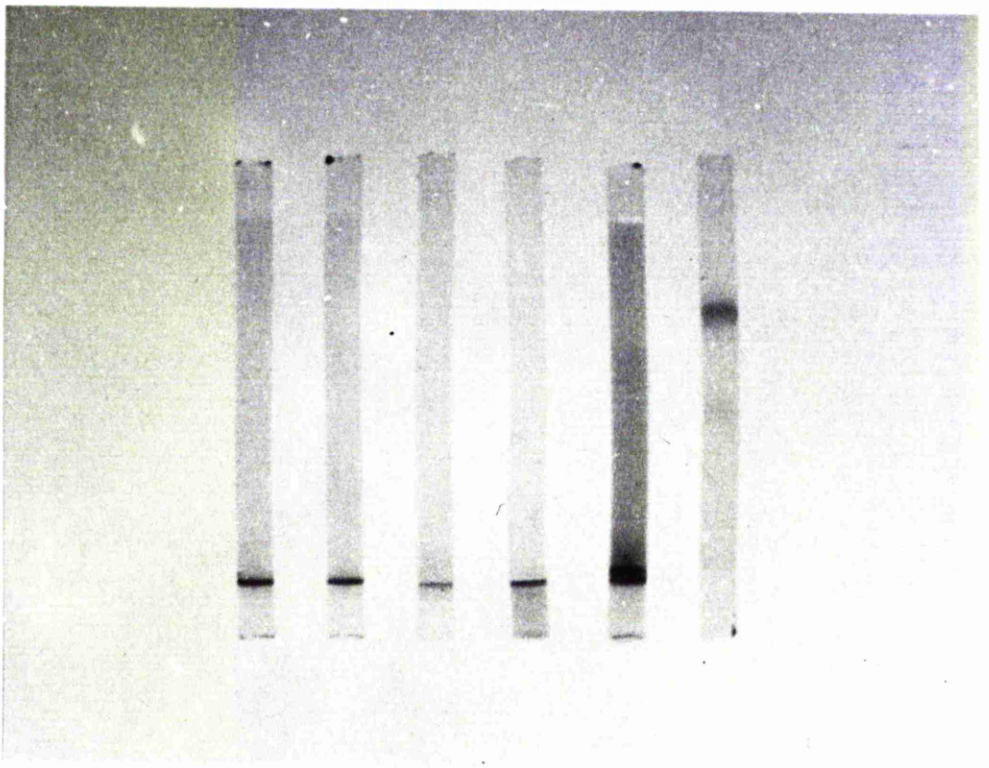
Isoelectric Focusing of Apoferritin.

Before beginning a detailed discussion of a possible subunit structure for apoferritin it is of interest to note that some workers, using isoelectric focusing, observed a polydispersity in apoferritin and concluded that this was most probably a reflection of different types of subunit (Drysdale, 1970; Urushizaki, Fukuda, Matsuda, Niitsu, Yokota and Kitago, 1970; Urushizaki, Niitsu, Ishitani, Matsuda and Fukuda, 1971; Van Kreel, Van Eijk and Leijnse, 1972). As this result is in conflict with some of the physical and chemical investigations carried out in the present study we decided to re-investigate the behaviour of apoferritin by isoelectric focusing.

Since its introduction in 1961 by Svensson (1961) the technique of isoelectric focusing has developed and received wide application. As a technique it is extremely sensitive to small changes in the sample, so much so, that results obtained from this can be easily overinterpreted if fairly extensive precautions are not exercised.

The results obtained for isoelectric focusing in the range pH 3-5 for various apoferritins are shown in Figure 40 (gels a-d).

Figure 40. Isoelectric focusing of horse spleen, horse liver, human spleen and human liver apoferritins in the range pH 3 - 5 are shown in the first four gels repsectively (from left to right). The fifth gel demonstrates the results obtained on isoelectric focusing of a sample of lyophilised horse spleen apoferritin in the range pH 3 - 5. The end gel shows an example of isoelectric focusing of a sample of horse spleen apoferritin in the range pH 3 - 10. In all cases the focusing was carried out as described in the text.



There is only one band in each of the gels for the four different proteins and the isoelectric point of each was approximately the same. In the case of freeze-dried horse spleen apoferritin we frequently observed two bands which may represent native protein and a denatured component (Figure 40, gel e).

The absence of any heterogeneity was also confirmed in the range pH 3-10 as shown in Figure 40, gel f.

The only circumstance in which we observed multiple bands on focusing in this range was with samples which had been lyophilised although, some samples of lyophilised apoferritin also gave only one band. We have observed that once a sample has been lyophilised it is frequently difficult to resolubilise and this was also noted by Hofmann and Harrison (1963). From their study it seems likely that freeze-drying causes some denaturation.

Recently Bjork and Fish (1971) described the presence of two peptides of molecular weight 11-14,000 and less than 5000 respectively, which together accounted for 10% of the total protein in an apoferritin preparation. These workers assume that these peptides may be bone fida subunits, extraneous proteins bound to the apoferritin (although they do not consider this possibility when they evaluate the molecular weight of the undissociated apoprotein) or have resulted from a specific limited cleavage of the protein. In all our experiments with dissociating solvents we have never been able to detect these peptides even with deliberate over-loading of the gels (Crichton and Bryce, 1970; Bryce and Crichton, 1971a). Of all the available electrophoretic techniques the one with the greatest resolution is isoelectric focusing and even with this extremely sensitive technique and deliberate over-loading of the gels we were still unable to detect these components which may, therefore, merely represent denatured apoferritin

or impurities in the preparation.

The earlier investigation of isoelectric focusing of ferritin revealed at least seven common bands and Drysdale (1970) showed that this was not due to a variable iron content since the same pattern was observed with apoferritin. Apart from considerations of buffer-protein interactions, other reasons for the presence of multiple bands on electrophoresis could be (a) application of an impure or denatured protein sample, (b) insufficient washing with trichloroacetic acid to completely remove all the carrier ampholytes or (c) chemical modification of proteins if persulphate is used as the catalyst for polymerisation. With regard to the first of these points it is obvious that if an impure sample is applied, one might reasonably expect multiple bands. Lyophilisation may result in slight denaturation and this could also lead to apparent polydispersity in gels.

With regard to the second possibility, it is obvious that if the synthetic mixtures of aliphatic polyaminopolycarboxylic acids which constitute the carrier ampholytes are not completely removed from the gel prior to staining, these substances can give rise to stained bands on the gel. Thus, only by extensive washing of these gels prior to staining can one avoid this problem which could result in an incorrect interpretation of polydispersity. Also it has been demonstrated that non-specific binding of carrier ampholytes to proteins can occur and the presence of these complexes could also result in a multiple band pattern following focusing (Hayes and Wellner,1969). Recently this point has been stressed by Lee and Richter (1971a,b) who found multiple bands on focusing of ferritin. However, when the sample was refocused it was found that some of the bands were not consistently present and it was concluded that these minor bands probably

represented ferritin-ampholyte complexes. These workers also showed that, on free flow electrophoresis, both ACI rat liver ferritin and Reuber H-35 hepatoma ferritin had one major peak and several minor peaks and since the latter were not observed on standard gel electrophoresis they concluded that they were unlikely to represent distinct 'isoferritins' (Lee and Richter, 1971b).

Despite a few criticisms of the use of ammonium persulphate as a polymerising catalyst, Drysdale (1970) has apparently ruled out the last possibility since similar results were obtained on focusing in a sucrose density gradient (unpublished observation quoted in Drysdale (1970)).

The results discussed here, however, clearly make it unlikely that apoferritin molecules are heterogeneous and we conclude that the polydispersity observed in the earlier studies was an artifact (Bryce and Crichton, 1972).

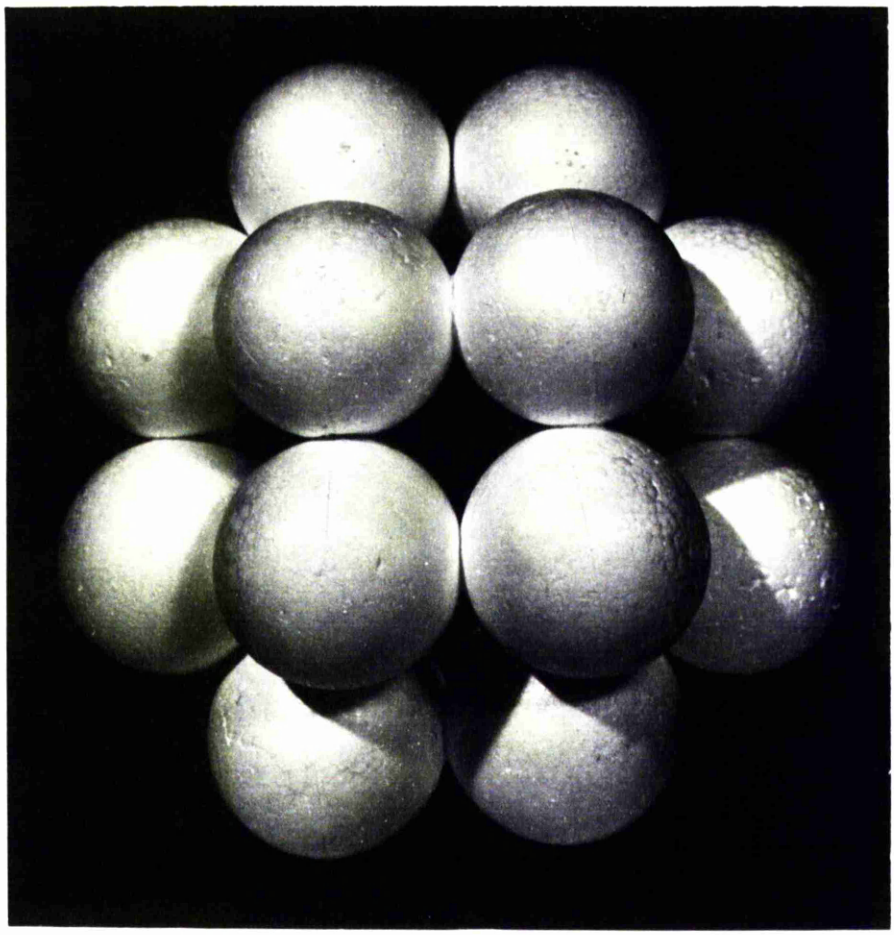
Subunit Structure of Apoferritin.

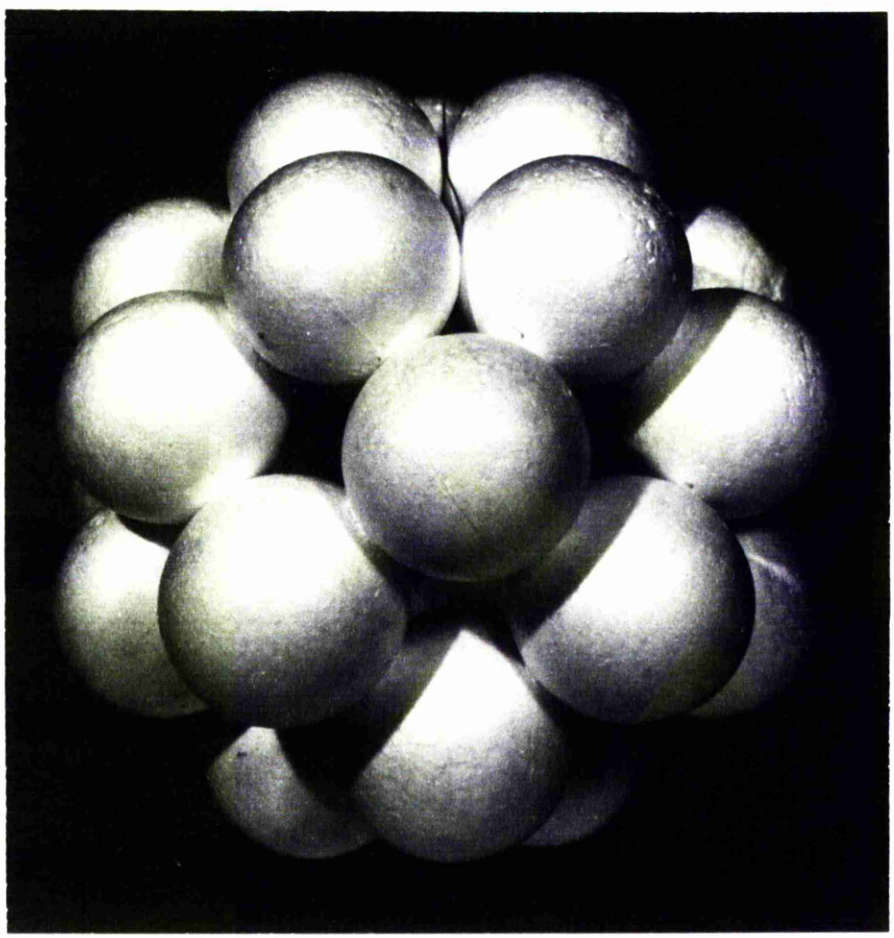
It would appear from these structural studies that the apoferritin molecule, of molecular weight 437,000, consists of 24 identical subunits of molecular weight 18,500. A possible model for such a structure is shown in Figure 41 and is one in which the subunits lie at the vertices of a rhombo-cubo-tetrahedron.

Such a view is in disagreement with the previously accepted concept of 20 subunits and, for this reason, the evidence on which a value of 20 is based merits some comment.

The molecular weight of the subunit was determined by sedimentation-diffusion and approach-to-equilibrium techniques and the value obtained was 25-27,000. As was stated earlier, if the amount of SDS bound to the protein was underestimated then

Figure 41. A model for a possible quaternary structure for horse spleen apoferritin . The structure consists of twenty-four subunits placed at the vertices of a rhombo-cubo-tetrahedron. My thanks are due to Dr. E.J. Milner-White for helpful discussions on possible quaternary structures of oligomers.





this value would be atypically too high.

Although Harrison (1959) demonstated from preliminary X-ray data that apoferritin exhibited 4:3:2 symmetry which is consistent with 24 subunits, a more intensive study revealed a pseudo 5:3:2 symmetry which she interpreted as indicating that apoferritin was composed of 20 subunits and Harrison suggested that these would lie at the vertices of a pentagonal dodecahedron as was shown in Figure 5 (Harrison, 1963).

The problem, however, with such a 20-subunit structure is that the subunits cannot all occupy equivalent positions in the structure. This means that there must be either at least two different types of subunit or else the subunit must be able to exist in two distinct conformations (Hanson, 1968). As we have seen above, there is no undisputed evidence as to the existence of more than one type of subunit.

Further evidence on which a 20-subunit structure was based came from the observation that the number of tryptic peptides obtained agreed well with the theoretical number from the lysine and arginine content based on a molecular weight of 23,000. (Harrison and Hofmann, 1962). However, in view of the study with turnip yellow mosaic virus, some caution should be observed in interpreting such data. In the case of the coat protein of this virus, the expected number of spots based on the lysine and arginine content were obtained from a tryptic fingerprint, despite the fact that 60% of the digest remained at the origin as a ninhydrin negative spot. The reason for this spurious result was that some peptides resulted from chymotryptic cleavage while others represented products of incomplete digestion.

Further evidence in favour of a 20-subunit structure was the quantitative tryptophan determination of 21 residues / 480,000 g

of protein. This work was repeated by several methods in the present study and the value obtained, 52.1 tryptophan residues/ 480,000 g apoferritin, is clearly in disagreement. It can only be stated that the complex and indirect method employed by Harrison and Hofmann (1961, 1962) could lead to inaccurate estimates and the observation that there is only one tryptophan-containing tryptic peptide is not conclusive.

Mainwaring and Hofmann (1968) determined the N-acetyl groups quantitatively and found 19.6 ± 1 / 480,000 g protein. It is possible that the indirect method of estimating this is less accurate than would be required to distinguish between 20 or 24 subunits. This is also the case for the demonstration that carboxypeptidase B liberates 19 moles of arginine and 15 moles of lysine per mole of protein from the C-terminus which has a -lys-arg sequence (Mainwaring and Hofmann, 1968).

More recently, direct evidence in favour of a 20-subunit structure for horse spleen apoferritin came from high resolution electron microscopy (Easterbrook, 1970).

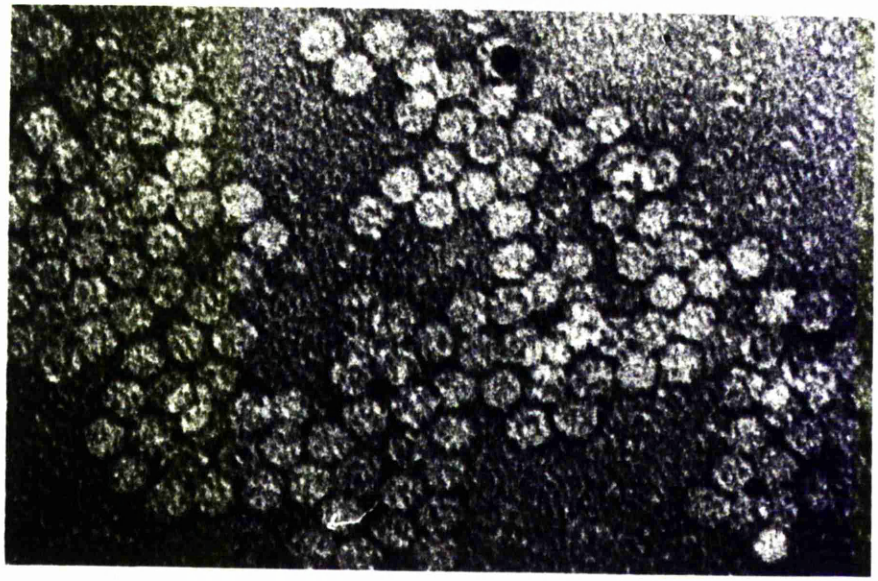
Electron Microscopy of Apoferritin.

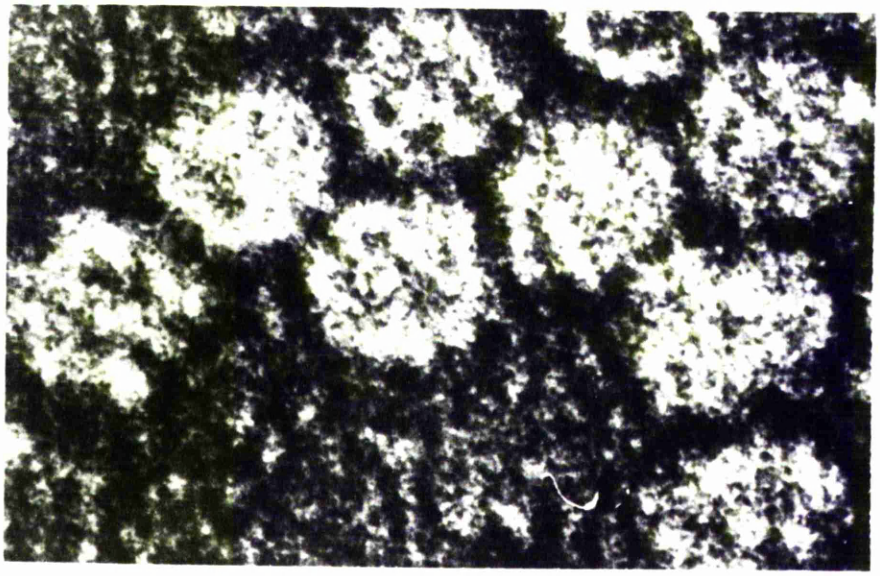
In our studies on the nature of reconstituted ferritin we made use of electron microscopy as a means of identifying product and during the course of these studies we found that the micrographs obtained for apoferritin were sufficiently detailed to allow speculation on the symmetry elements of the observed molecules.

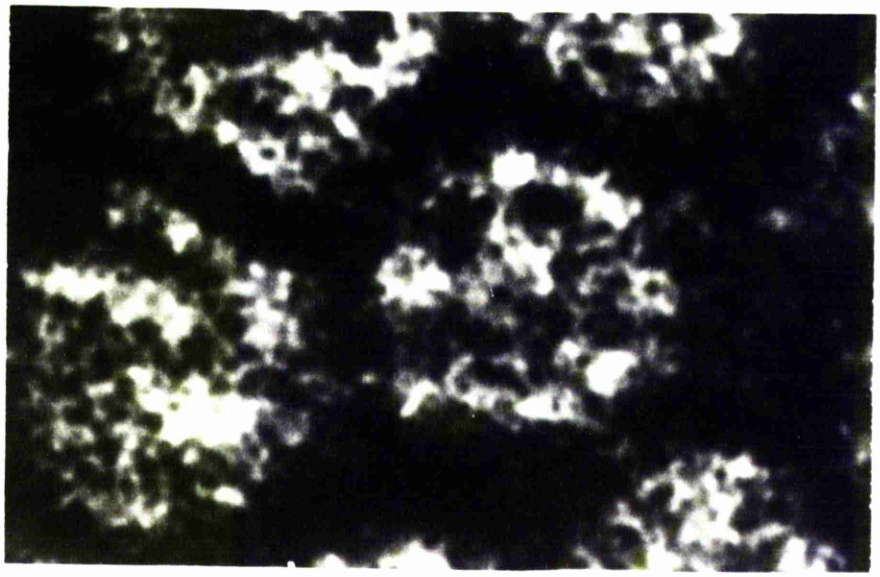
Figure 42 shows an electron micrograph of horse spleen apoferritin. It can be seen from this figure that structures do possess fine detail and in an effort to improve the image contrast and thereby render this fine detail more visible we used the

Figure 42. High resolution electron micrographs of horse spleen apoferritin.

Magnification × 500,000 ; × 1.9 million ; and
× 3.2 million (from top to bottom).







superposition technique of Markham, Frey and Hills (1963).

This was to photograph the object with 1/n times the normal exposure then turn the object through 360/n degrees and re-photograph, again with an exposure of 1/n x normal. This procedure was repeated (n - 1) times and the photographic plate developed. If the object possessed n-fold symmetry then any fine detail present would be intensified while background noise would be diminished. If the object did not possess such a symmetry then the image would appear blurred. In the present study this was repeated for several values of n. Figure 43 shows the results of such a technique and it can be seen that there is strong evidence for a six-fold symmetry which, from the model, can be seen to be consistent with 24 subunits at the vertices of a rhombo-cubo-tetrahedrn.

In the earlier work of Easterbrook (1970) the image which he uses as confirmatory evidence for a 20-subunit structure merits some comment (for reference see Figure 7). If this is to be compared with the model then the central space should be larger than the others, since these are viewed at an angle. This however, is not observed.

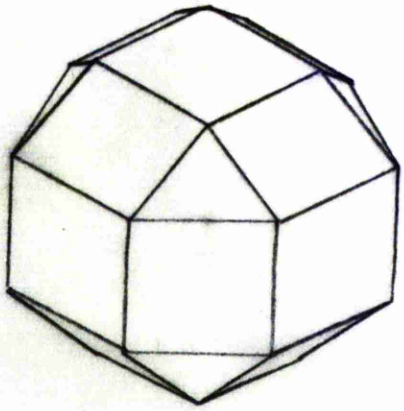
It only remains to stress that such a technique is useful as a rough guide to the subunit arrangement but is not really capable of distinguishing between the two models.

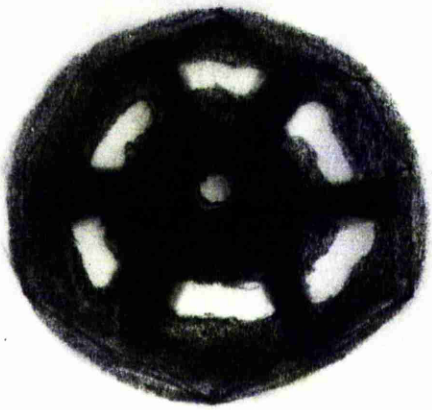
Subunit Arrangement in Apoferritin.

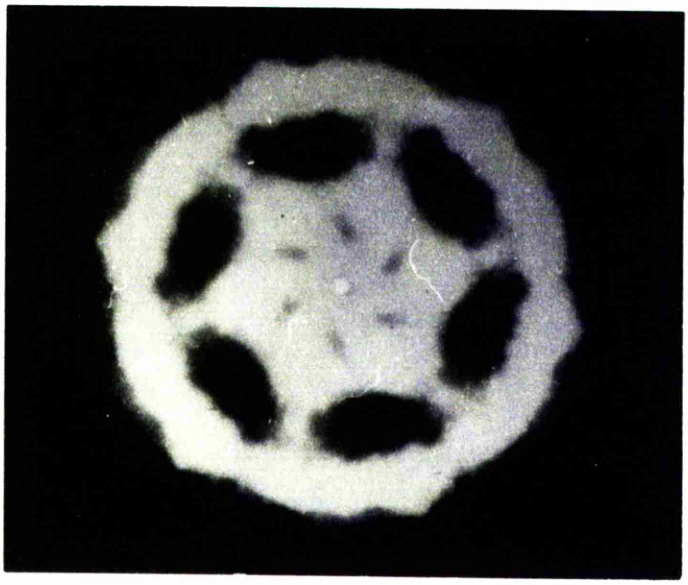
Generalising from the various hypotheses put forward by Crick and Watson (1956) and Casper and Klug (1962) it can be seen that a 24-subunit structure is one in which it is possible to arrange identical units in identical environments to produce a symmetrical structure. Arranging identical units in identical

Figure 43. Possible arrangement of the subunits in the horse spleen apoferritin molecule. The top image is a model of a rhombo-cubo-tetrahedron and the drawing below this is a representation of the appearance of a molecule with subunits at each of the vertices of such a structure. This compares very favourably with an experimentally obtained high resolution electron micrograph of apoferritin that has been subjected to the super-position technique (lower frame) as described in the text (in this case n = 6).

Magnification x 5 million.







environments necessarily produces a symmetrical structure , and there are only a geometrically limited number of kinds of symmetry. The possible three-dimensional arrangements for a 24-subunit structure would be with the subunits at the vertices of a truncated cube, a truncated octahedron or a rhombo-cubo-tetrahedron.

From our studies then, it can be seen that the results strongly favour the concept of a 24-subunit structure for horse spleen apoferritin and cast doubt on the concept of a 20-subunit structure. As Fuller (1969) has pointed out, "for the same reasons to those which favour the construction of macromolecules from a linear arrangement of subunits through identical, equivalent linkages, those macromolecular assemblies which are constructed by the binding of identical subunits in equivalent positions are more efficiently synthesised than those which are produced with little or no internal symmetry". It is a natural consequence that, for a 24-subunit structure possessing 4:3:2 symmetry, it is possible to arrange identical subunits in identical environments whereas, for a 20-subunit structure , as Cornish-Bowden and Koshland (1971) have recently reported, it is impossible to construct a pentagonal dodecahedron from asymmetrical units (which the subunits must naturally be) such that they occupy identical and equivalent environments and one has to resort to a system of quasi-equivalence.

Perhaps an appropriate comment at this stage would be that given by D'Arcy Wentworth Thompson (1942),

"So here and elsewhere an apparently infinite variety of form is defined by mathematical laws and theorems, and limited by the properties of space and number. And the whole matter is a running commentary on the cardinal fact that, under such

foedera Naturai as Lucretius recognised of old, there are things which are possible, and things which are impossible, even to Nature herself.".

Once the protein's subunit structure had been elucidated, we attempted to find out information concerning the pH stability of the monomer, the possible nature of the environment of subunit - subunit contacts and the chemical reactivities of various groups within the protein structure. The reason for this course of action was to obtain a more detailed knowledge of the protein's structure and also to relate these properties to the function of the protein.

The pH Stability of Apoferritin.

Studies at Acid pH.

Initially we chose the technique of sedimentation velocity as a method of monitoring the dissociation of monomer into subunits as a function of pH. Sedimentation velocity runs were carried out as described earlier (page 83). The results for the dissociation and reassociation of the protein are shown as a fuction of pH in Figure 44. It can be seen that as the pH is decreased there is a decrease in the sedimentation coefficient of apoferritin of 17-18S to a value of about 2.5S with a sharp transition between pH 2.8 and 1.6.

From the study of the reassociation of dissociated material

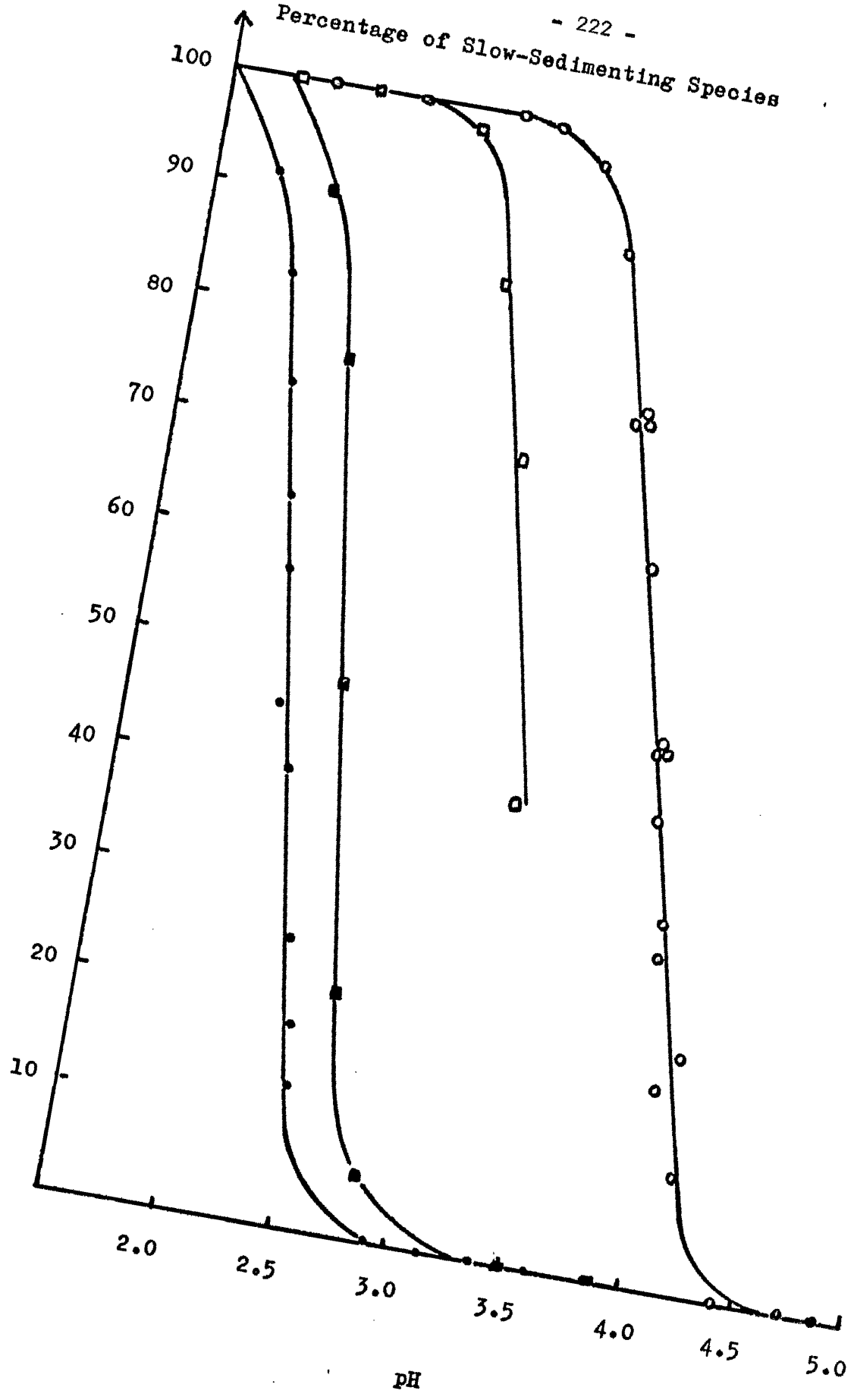
Figure 44. The pH dependence of the dissociation of horse spleen apoferritin. The percentage of slow-sedimenting species was determined from concentration measurements from the scanner traces.

⊕——⊕ Dissociation of protein in 0.01M glycine buffer.

⊡——⊡ Dissociation of protein in universal buffer.

o——o Reassociation of protein in dilute glycine and glycine-acetate buffers.

□——□ Reassociation of protein in universal buffer. At pH values greater than about pH 3.3 the protein precipitated from solution and therefore the extent of reassociation could not be estimated.


Percentage of Slow-Sedimenting Species
100
90
80
70
60
50
40
30
20
10
2.0
2.5
3.0
3.5
4.0
4.5
5.0
pH

it is apparent that the process is completely reversible but the reassociation of subunits to monomer takes place at a more alkaline pH. In the case of dilute glycine and glycine-acetate buffers the reassociation takes place 1.6 pH units higher, in the region pH 3.0 - 4.4.

Below about pH 1.2, a high molecular weight aggregate was present which sedimented very much faster than monomer. It was not possible to estimate the sedimentation coefficient of this material and it was assumed to be acid-denatured protein. It can be seen from the difference in the dissociation profiles of glycine and universal buffers (0.23 pH units) that the stability of the monomer is enhanced at lower ionic strength. This observation may represent a case in which dissociation is enhanced by interaction of salts with ionisable side-chains of the protein, a mechanism which has been frequently suggested for salt-induced dissociation of proteins.

The protein used in these studies was dissociated by 67% acetic acid in the cold for 1 hour. However, when protein which was dissociated by dilute buffer at pH 1.5 was allowed to re-associate, an identical reassociation profile was obtained. This demonstrates that the process is not restricted to acetic acid-dissociated material and this, in itself, eliminates the possibility that the difference in the dissociation and re-association profiles was a result of acetic acid bound to the protein.

In a study of the time-dependence of the system, it was shown that the percentage of subunit did not vary with increasing time of dialysis, so that we can assume that we are dealing with a system at equilibrium.

The data presented in Figure 44 explains the findings of Harrison and Gregory (1968) who demonstrated that treatment with

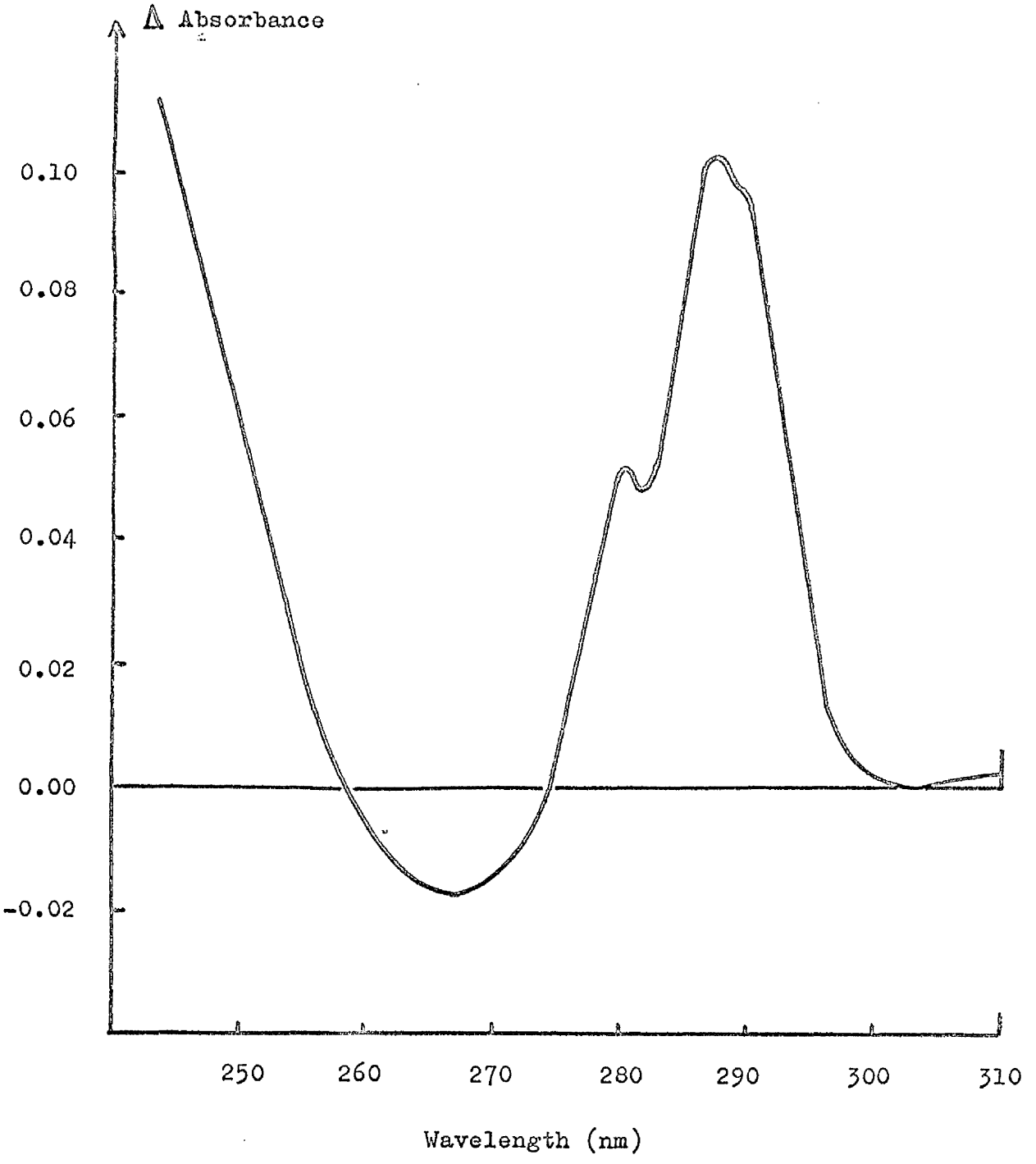
67% acetic acid led to the dissociation of apoferritin into subunits which were stable in glycine buffer at pH 3.0. In all of the studies presented here, no intermediates between oligomer and subunit could be detected but, due to the uncertainty in the absolute value for the sedimentation coefficient of the subunit, it would perhaps be more correct to classify this as 'slow-sedimenting' material.

The acid induced dissociation of apoferritin was also investigated by ultraviolet difference spectroscopy. When a neutral solution of apoferritin was made acid, its spectrum underwent a blue shift (for a shift of the absorption spectrum along the wavelength axis, $\triangle\lambda_{max}$ is taken positive for a red shift and negative for a blue shift). A typical difference spectrum for an apoferritin solution at pH 3.06 relative to a solution at pH 1.63 is shown in Figure 45. The spectral data has been corrected for the effects of light scattering as described on page 85. From this it can be seen that the acid difference spectrum shows wavelength peaks at 280 nm and 287 nm, which is characteristic of a perturbed tyrosine.

When protein subunits associate, chromophores, i.e. tyrosine, tryptophan or phenylalanine, may be 'buried' at the interprotomer surface. This results in an absorption change and the nature of this change may be both qualitatively and quantitatively interpreted, since the difference spectrum identifies the type of chromophore. An example of such a study is the concentration - dependent association of insulin at pH 2.0 (Rupley, Ronthal and Praissman, 1967). The difference spectrum obtained between solutions of 0.03 mg/ml and 26 mg/ml was characteristic of tyrosine and these workers suggested that the formation of insulin dimers affects the environment of one tyrosine residue. An instance in

Figure 45. The acid difference spectrum for a 0.0688% solution of horse spleen apoferritin measured at pH 3.063 relative to a solution at pH 1.634. Measurements were made in 1.0 cm matched cells up to 400 nm and were corrected as in text for scattering contribution

Δ Absorbance, difference in the absorption between the two solutions.


Δ Absorbance
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
250
260
270
280
290
300
310
Wavelength (nm)

which no change in absorption occurs is the $\alpha \rightleftharpoons \beta$ dissociation of glutamic dehydrogenase trimers to monomers (Cross and Fisher, 1965). Light scattering studies showed a considerable difference in the degree of aggregation between 0.1 mg/ml and 1 mg/ml solutions. Cross and Fisher (1965) interpreted this negative result as indicating the absence of chromophores on the surfaces at which association takes place.

It would appear then that the process of dissociation of apoferritin at low pH involves the tyrosine chromophore in some way. Figure 46 shows a plot of the height of the tyrosyl difference spectrum at 287 nm against protein concentration and it can be seen that, for the concentrations used in this study, adherance to Beer's law was observed.

Measurements of difference spectra were therefore made for protein dissociated in dilute glycine and glycine-acetate buffers at a variety of pH values in the range pH 1.5 - 3.0, with protein at pH 1.5 as the reference. The spectra were corrected for light scattering and the difference in molar absorbancy at 287 nm, $\Delta\epsilon_{287}$, was plotted as a function of pH as shown in Figure 47.

As was the case in the sedimentation velocity studies, re-association was performed with protein which had been dissociated either by buffer at pH 1.5 or by protein which had been treated with 67% acetic acid. The data obtained was treated in a similar manner as that from the study of the dissociation and is also shown in Figure 47. Using a value of $\Delta\epsilon_{287} = 540$ for the perturbation of a protein tyrosyl residue, it would appear that when the pH of apoferritin is decreased from pH 3.0 to 1.8, all five of the protein's tyrosine residues are transferred from the interior of the protein into the solvent and the converse is true for the re-association in the range pH 3.0 - 4.4.

Figure 46. Data from the acid difference spectra showing adherence to Beer's law for the concentration range shown. Absorption measurements were corrected for the effects of light scattering.

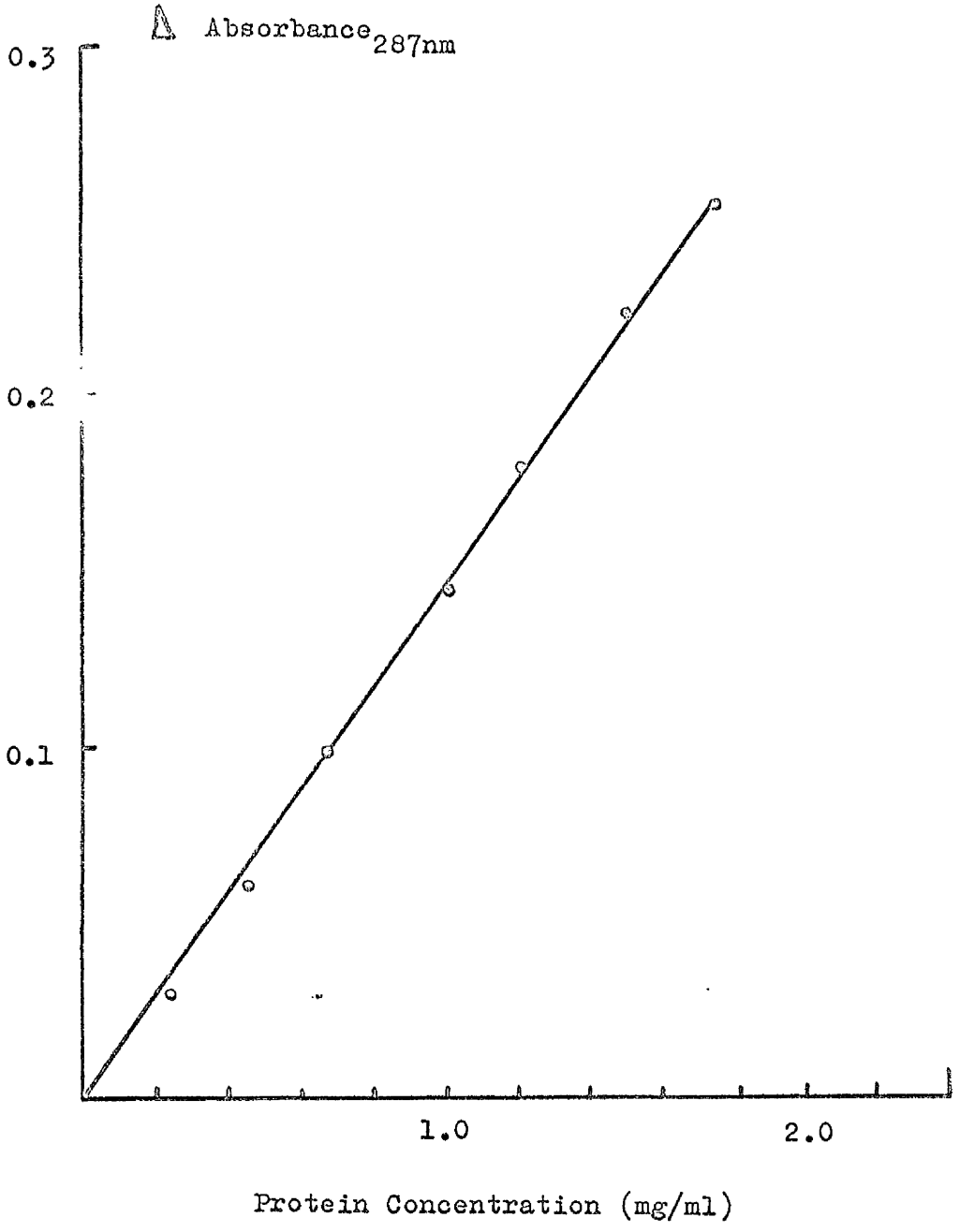
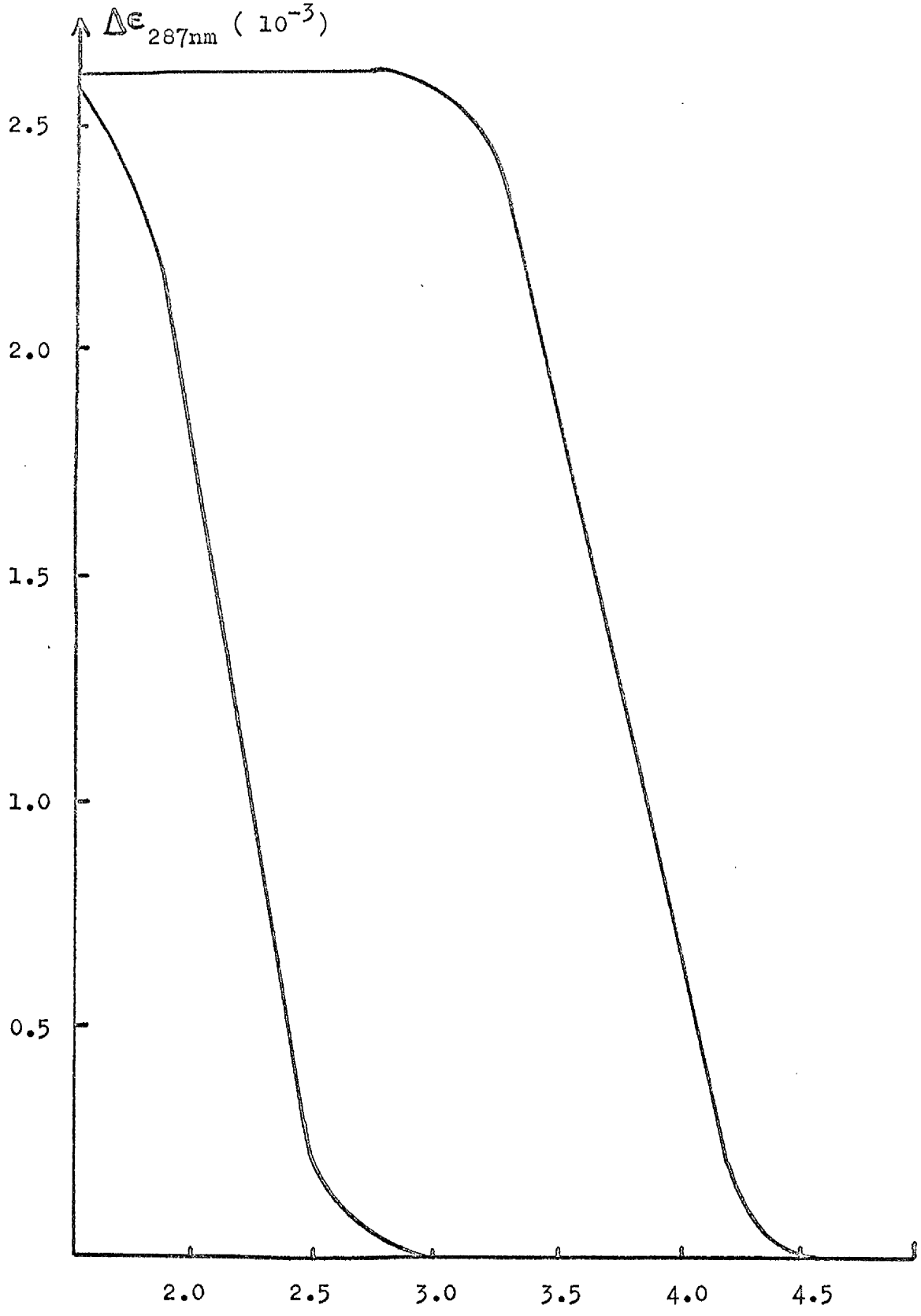

Δ Absorbance287nm
0.3
0.2
0.1
1.0
2.0
Protein Concentration (mg/ml)

Figure 47. The pH dependence of the acid difference spectrum of horse spleen apoferritin. Absorption measurements were corrected for the effects of light scattering. Dissociated apoferritin was dialysed against a variety of buffers at higher pH and the data from the acid difference spectra is shown (transition from pH 3.0 - 4.5)

$\Delta\epsilon_{287nm}$, difference in the molar extinction coefficient of horse spleen apoferritin at 287 nm.


$\Delta\epsilon_{287nm}$ (10^{-3})
2.5
2.0
1.5
1.0
0.5
2.0
2.5
3.0
3.5
4.0
4.5
pH

From a comparison of this data with the sedimentation velocity data, it is clear that the decrease in the sedimentation coefficient exactly parallels the blue shift observed by ultraviolet difference spectroscopy. From these two studies, it seems that on lowering the pH below pH 3.0, the apoferritin subunits undergo a major conformational change causing five tyrosine residues to be brought from a hydrophobic region to an aqueous one.

The only groups ionising in the pH region of interest here (pH 1.0 - 5.0) are carboxyls and, since the ionisation of these causes such major structural changes, it would appear likely that these groups are strongly involved in maintaining the three dimensional structure of apoferritin and perhaps are even involved in subunit-subunit interactions.

According to Wetlaufer (1962), if the equilibrium observed during dissociation involves only two microscopic species, as in the case of carboxyl (-COOH) and carboxylate ion ($-COO^-$), one can calculate the apparent ionisation constant, pK_1 , as follows,

$$pK_1 = pH - \log \left[\alpha/(1-\alpha)\right]$$

where α is the fraction of one species and the value obtained for pK_1 is invariant with α, within experimental error.

However, when a plot of the experimental data was constructed of pK_1 *versus* α, a linear relationship with zero gradient was not obtained, as demonstrated in Figure 48 and this implied that more than two microscopic species were involved. Since this system appeared to be an example of multiple equilibria, we were interested to see if the various reactions occurred independently of one another, or whether they reacted in a co-operative manner. Distinguishing between non-cooperative and cooperative

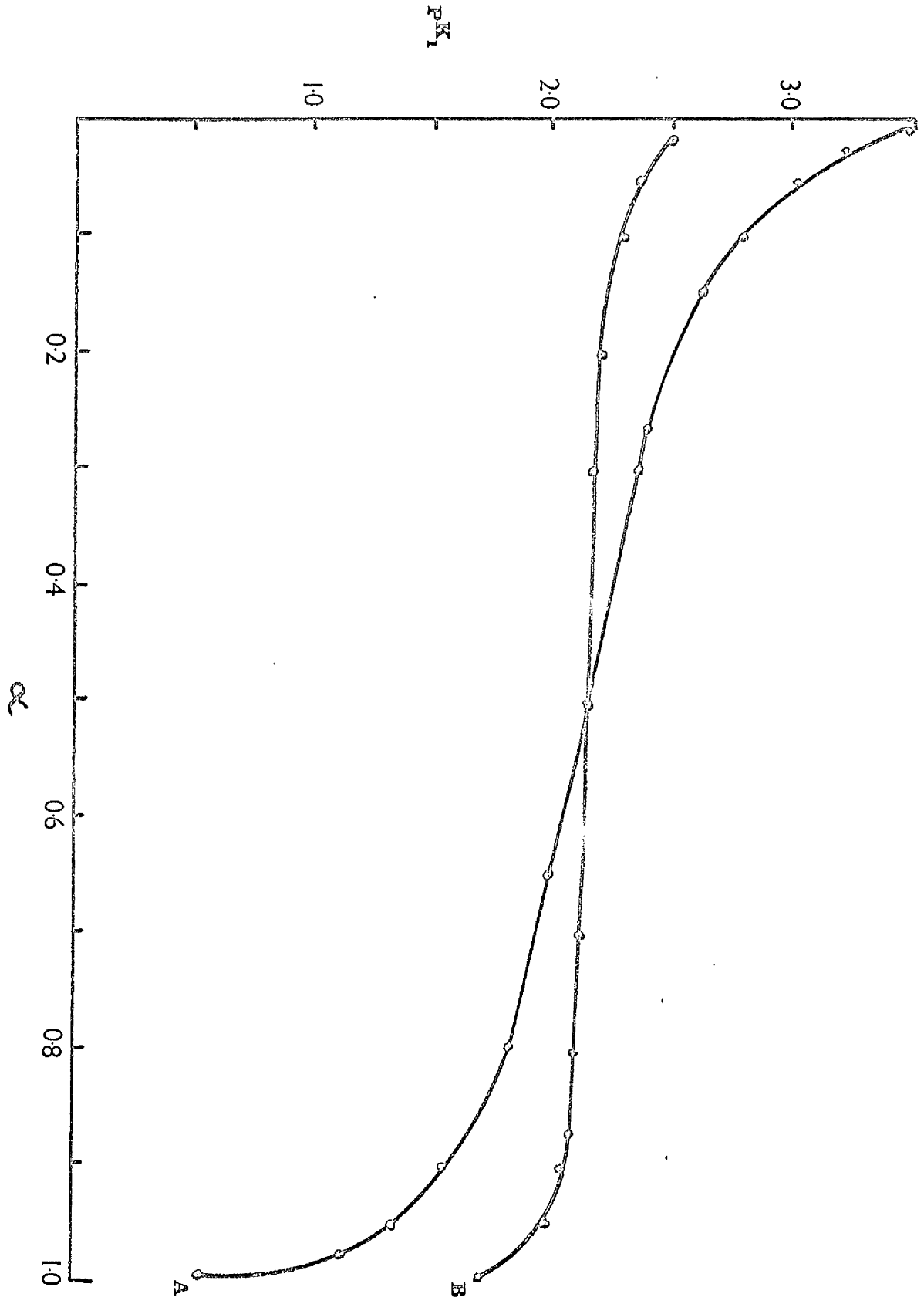
Figure 48. Plots of pK_1 against α, where α is defined as the degree of dissociation.

Curve A was obtained by use of the equation of Wetlaufer (1962),

$$pK_1 = pH - \log (\alpha/(1-\alpha))$$

Curve B was obtained by use of the equation derived in this study (see text),

$$pK_1 = pH - \tfrac{1}{2}\log (\alpha/(1-\alpha))$$


pK1
1·0
2·0
3·0
α
0·2
0·4
0·6
0·8
1·0
A
B

effects can easily be determined by plotting $\log[\alpha/(1-\alpha)]$ *versus* pH and such a graph is known as a Hill plot. When reactions occur independently of one another, the slope of the line in the Hill plot is equal to unity while if co-operativity is expressed then the slope obtained is greater than one.

In the dissociation of chicken egg-white macroglobulin by acid, Donovan, Mapes, Davis and Homburg (1969) found from a Hill plot of their data that the slope was close to unity and they concluded that the titratable groups of the protein which stabilize the subunit association and the conformation are non-cooperative.

When the experimental data obtained for the dissociation of apoferritin from sedimentation velocity studies or ultraviolet difference spectroscopic studies are plotted in such a manner, a relationship was obtained as shown in Figure 49. The reason for the deviation from linearity at the extremes is that the errors in these readings are quite significant, while the data in the central region of the plot is the most accurate. From this plot the gradient was found to be 2.07 which indicates some form of co-operativity.

For such co-operativity in a general titration system we have;

$$R_1H \underset{}{\overset{k_1}{\rightleftharpoons}} R_1^- + H^+$$

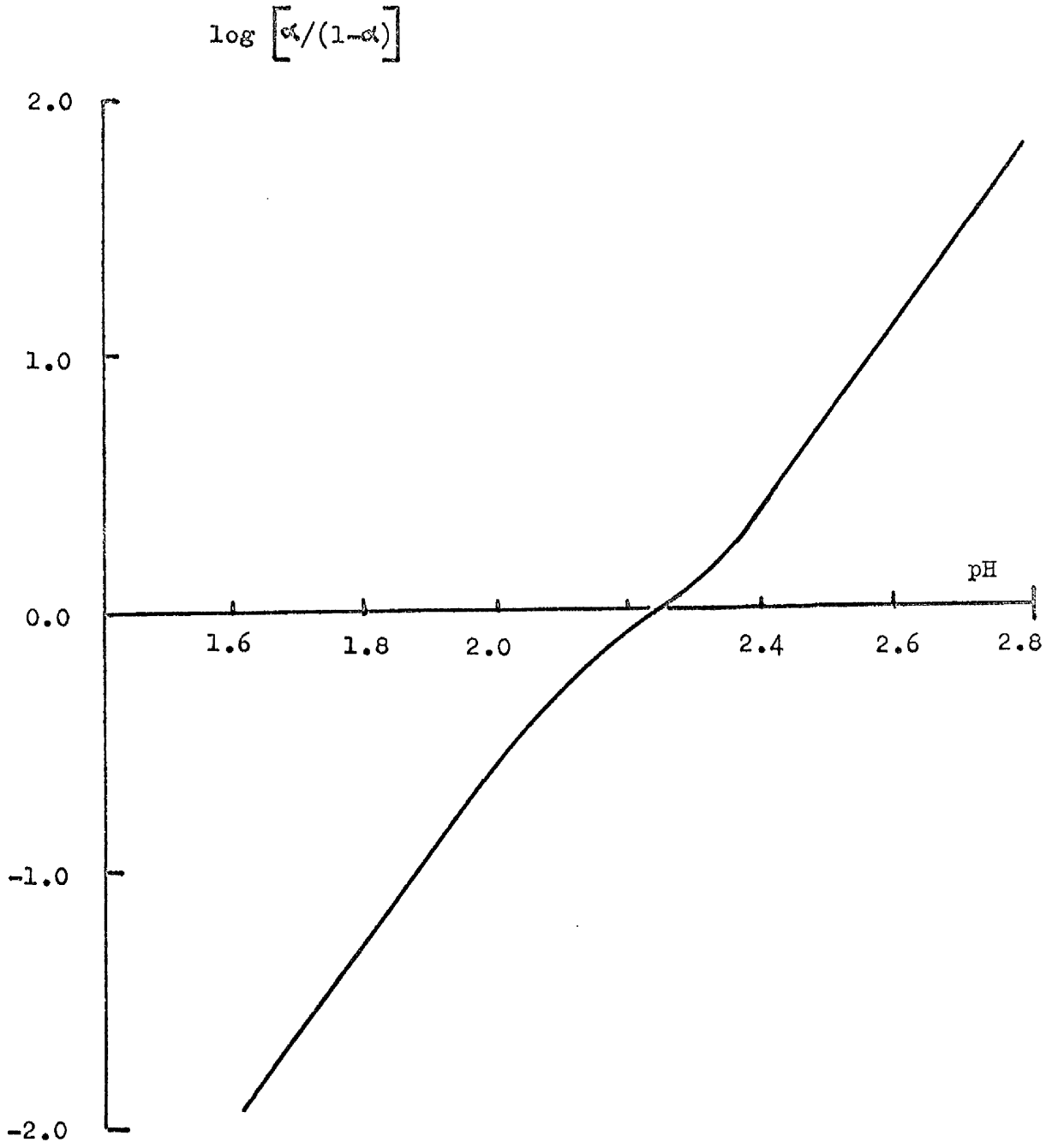
$$R_2H \overset{k_2}{\rightleftharpoons} R_2^- + H^+$$

$$\vdots \qquad\qquad \vdots \qquad\qquad \vdots$$

$$R_nH \overset{k_n}{\rightleftharpoons} R_n^- + H^+$$

now, $$k_1 = [R_1^-].[H^+] / [R_1H]$$

Figure 49. The pH dependence of the dissociation and acid difference spectrum of horse spleen apoferritin. The data plotted in this form is known as a Hill plot. α is the fraction of slow-sedimenting material or the fraction of the total difference spectrum of tyrosine at 287 nm.


log [α/(1-α)]
2.0
1.0
0.0
-1.0
-2.0
pH
1.6
1.8
2.0
2.4
2.6
2.8

and $$K_{overall} = k_1 \cdot k_2 \cdot \cdots \cdot k_{n-1} \cdot k_n$$

$$= [H^+]^n \cdot \prod_{i=1}^{n} \left\{ [R_i^-] / [R_iH] \right\}$$

If we define α as the fraction of deprotonated species, then it follows that;

$$\alpha/(1-\alpha) = \prod_{i=1}^{n} \left\{ [R_i] / [R_iH] \right\}$$

$$\therefore \quad k = \left\{ \alpha / (1-\alpha) \right\} \cdot [H^+]^n$$

i.e. $$-\log k = -\log\left\{\alpha/(1-\alpha)\right\} - n \cdot \log H^+$$

$$\log \alpha/(1-\alpha) = n \cdot pH - pK$$

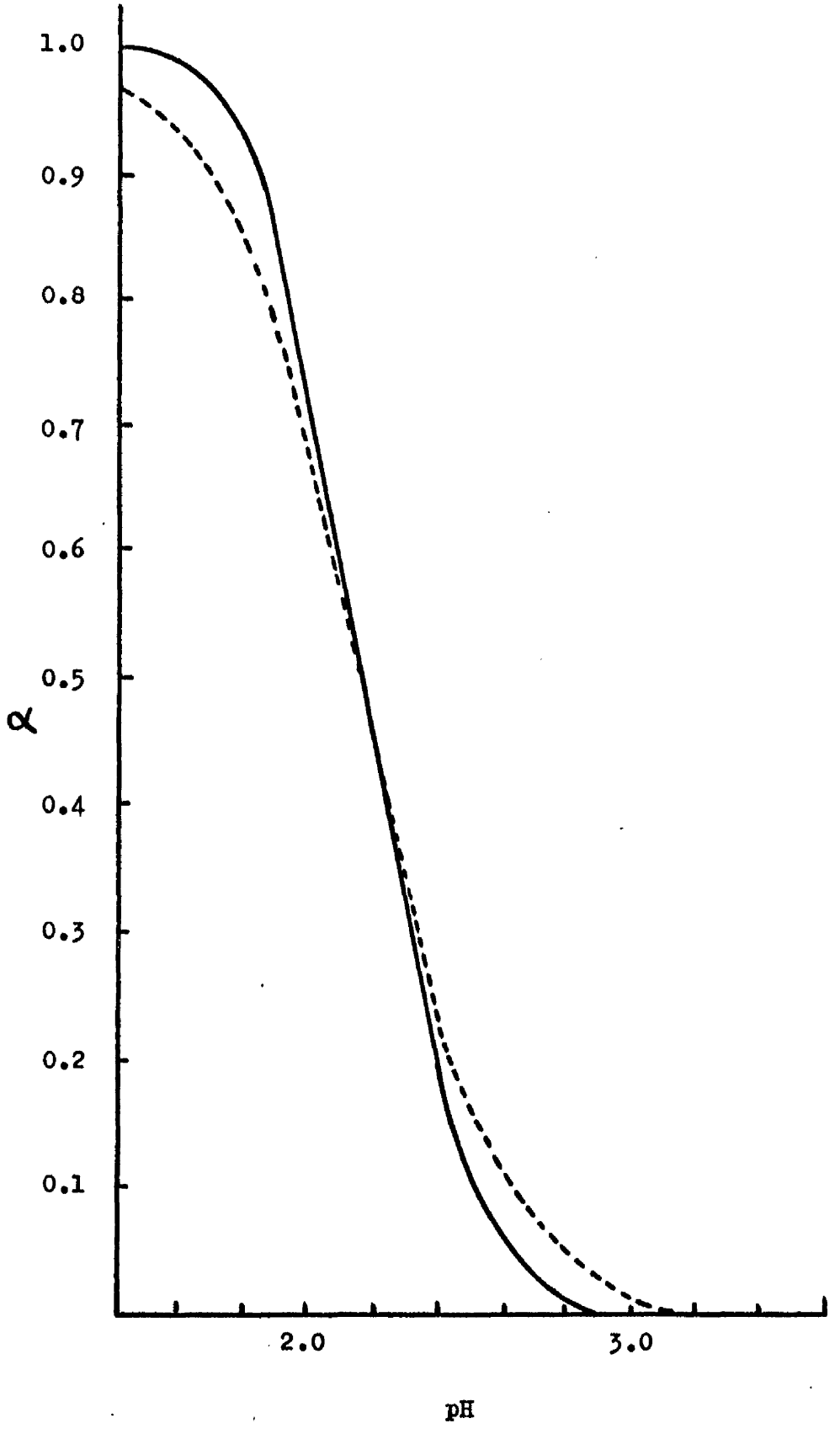
From above it can be seen that n represents the number of groups titrated. This means that the slope of the curve in the Hill plot represents the number of groups being protonated. This would indicate that the dissociation of apoferritin into subunits by acid is caused by the protonation of two carboxyl groups.

Using the theoretically derived equation above, it was possible to plot a theoretical dissociation profile for a given pK and it can be seen from Figure 50 that this fits the experimental data very well. Also by using this equation, it can be seen that pK_1 is invariant with α, within experimental error (Figure 48).

This then suggests that there are at least two carboxyl groups involved in stabilising the quaternary structure of apoferritin

Figure 50. Comparison of a theoretically derived dissociation profile (dotted line) with that obtained from the experimental data (full line).

α is the fraction of slow-sedimenting material or the fraction of the total difference spectrum of tyrosine at 287 nm.


1.0
0.9
0.8
0.7
0.6
0.5
α
0.4
0.3
0.2
0.1
2.0
3.0
pH

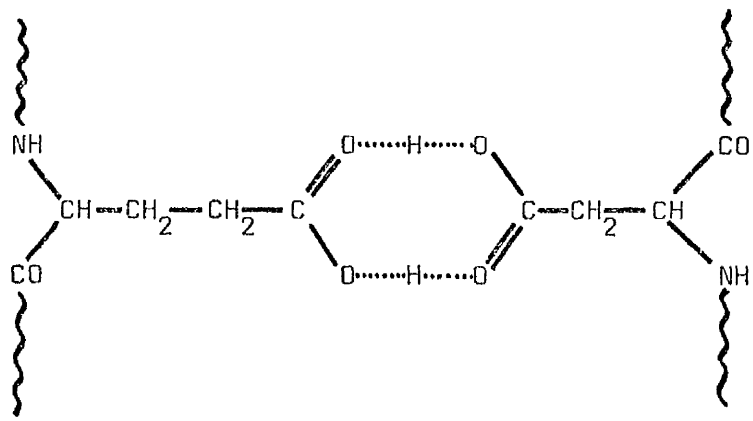
and from the dissociation profiles, the apparent pK values are 2.16 which is very low compared to the values of 3.6 for α-amino groups and 4.73 for β and γ-amino groups (Tanford and Epstein, 1954).

It has been shown that hydrogen bonding of a tyrosine and carboxyl group (Figure 51) can cause a decrease in the pK of the acid function but this would only amount to about 0.3 of a unit (Laskowski and Scheraga, 1954). It would also be possible to have a carboxyl-carboxyl double hydrogen bond system (Figure 51) and this would lower the pK yet again. On the back titration of the protein's tyrosine residues it was found that the apparent pK was 3.78 which is much closer to the expected pK of an normal carboxyl group. This is to be expected since the dissociated protein forms a more 'open' structure, as witnessed from the environment of the tyrosine residues, and since the interaction which imposes the restraint on the protonation of these carboxyl groups in the native structure is no longer present, then it follows that the pK observed will be close to the inherent pK of that titratable group.

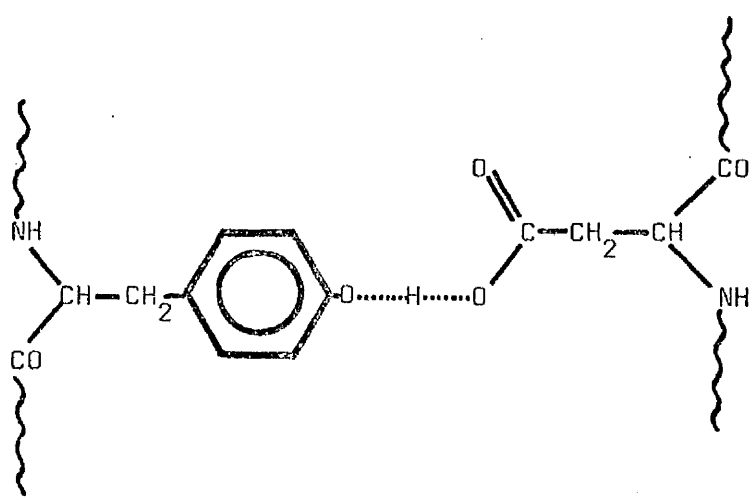
With protein at pH 1.5 still as the reference, we analysed the environment of the tyrosine residues in the region pH 0.0 - 1.5. It was found that on decreasing the pH, the acid-induced denaturation blue shift was partially lost which suggested that in this pH region some refolding and/or re-association was taking place and this is in agreement with the study by sedimentation velocity in which a fast sedimenting denatured product was observed.

For the acid dissociation of apoferritin we observed another conformational change in the region pH 3.0 - 3.5. In this study we used protein at pH 3.0 as the reference and protein in the region pH 3.0 - 4.0 as the sample. As shown in Figure 52, the diff-

Figure 51. Possible hydrogen bond systems in proteins.

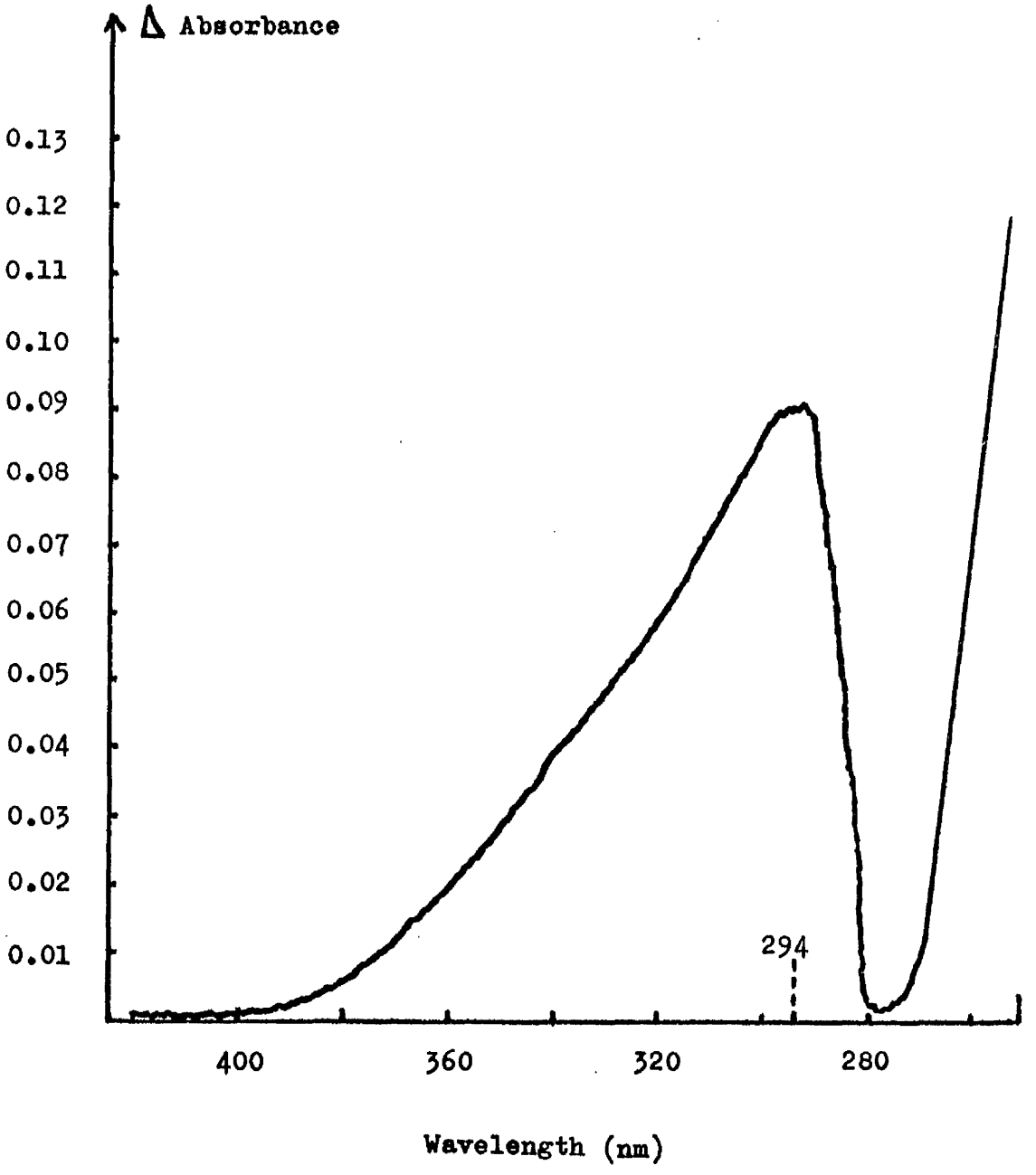


carboxyl-carboxyl hydrogen bond



tyrosyl-carboxyl hydrogen bond

Figure 52. The acid difference spectrum for a solution of horse spleen apoferritin measured at pH 3.241 relative to a solution at pH 2.977. Measurements were made in 1.0 cm matched cells up to 400 nm and were corrected for the effects of light scattering.


Δ Absorbance
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
294
400
360
320
280
Wavelength (nm)

erence spectrum obtained had a wavelength peak at 294 nm which is characteristic of a perturbed tryptophan residue. The perturbation of this tryptophan was studied at a variety of pH values and the molar absorbance changes at 294 nm were plotted as a function of pH as shown in Figure 53.

Using $\Delta\epsilon_{294} = 1600$ (Donovan, 1964, 1968) for the change in molar absorbance produced by the transfer of a tryptophan residue from the interior of a protein into water, it can be seen that one tryptophan residue is involved in such a transfer in apoferritin.

The carboxyl function being titrated in this region had an apparent pK = 3.25 and the protonation of this causes a minor conformational change in the environment of one tryptophan residue and it would appear that this is not involved in subunit-subunit interactions since no observable change in the sedimentation coefficient is apparent in this region. It is apparent, however, that the transition is very sharp and this can be tentatively explained in terms of a phenomenon which is referred to as an 'ionisation explosion' (Scheraga, 1961). This manifests itself as an abnormal steepening of the titration curve in the region in which the carboxyl groups ionize. It has been demonstrated, at least for bovine serum albumin, that such a titration anomaly can be accounted for by carboxyl-carboxyl acetic acid dimer-type bonds (Loeb and Scheraga, 1956) and perhaps this is the explanation in the present study.

On re-association, the data is a little more complex to interpret and the spectra shown in Figure 54 will help to explain this. It can be seen that up to about pH 4.0 the only group being perturbed is tyrosine. However, at higher pH values the spectra have a long shoulder in the region 320 nm to 290 nm. In

Figure 53. The pH dependence of the perturbation of a tryptophan residue of horse spleen apoferritin. Measurements were corrected for the effects of light scattering.

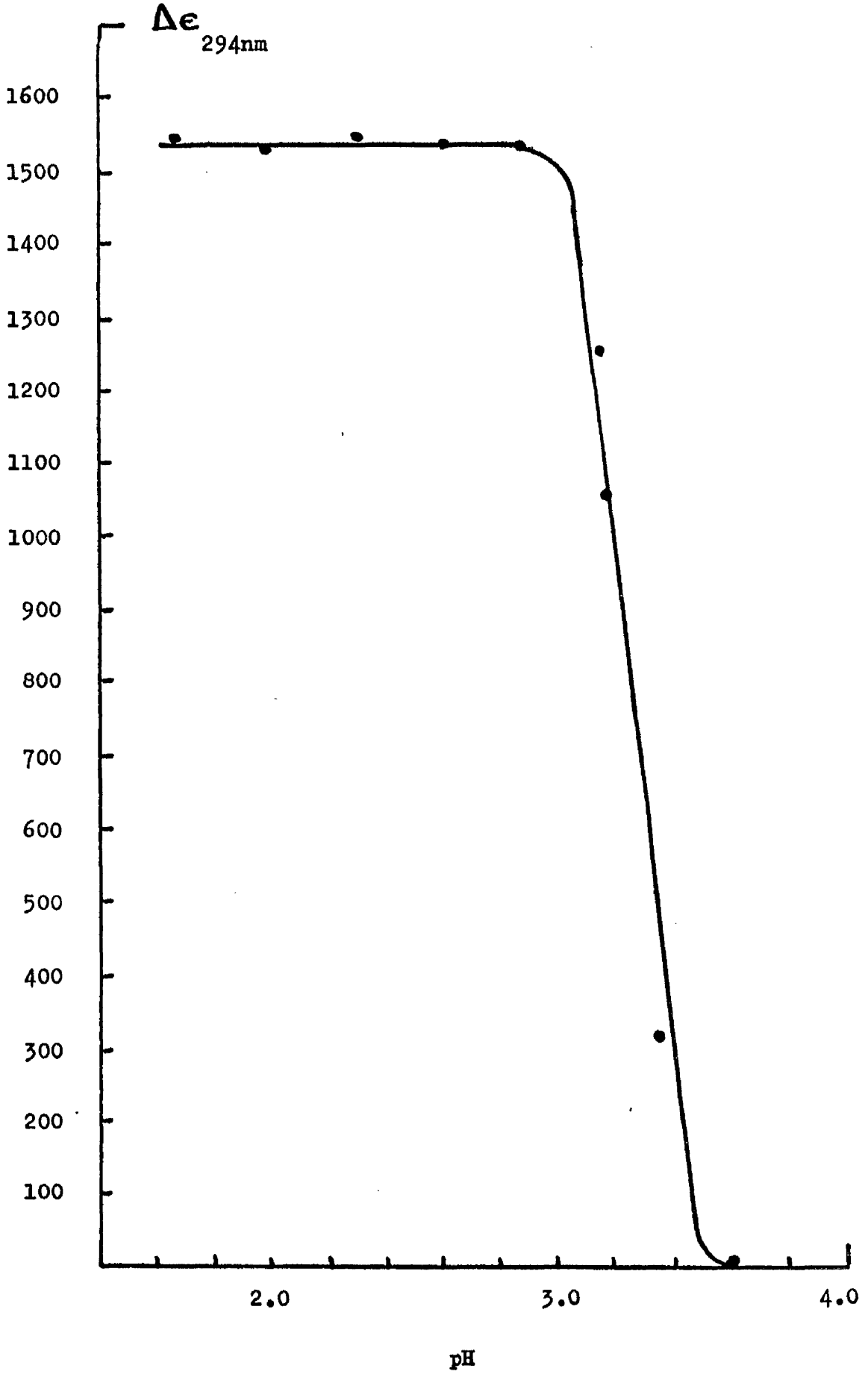

Δε 294nm
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
2.0
3.0
4.0
pH

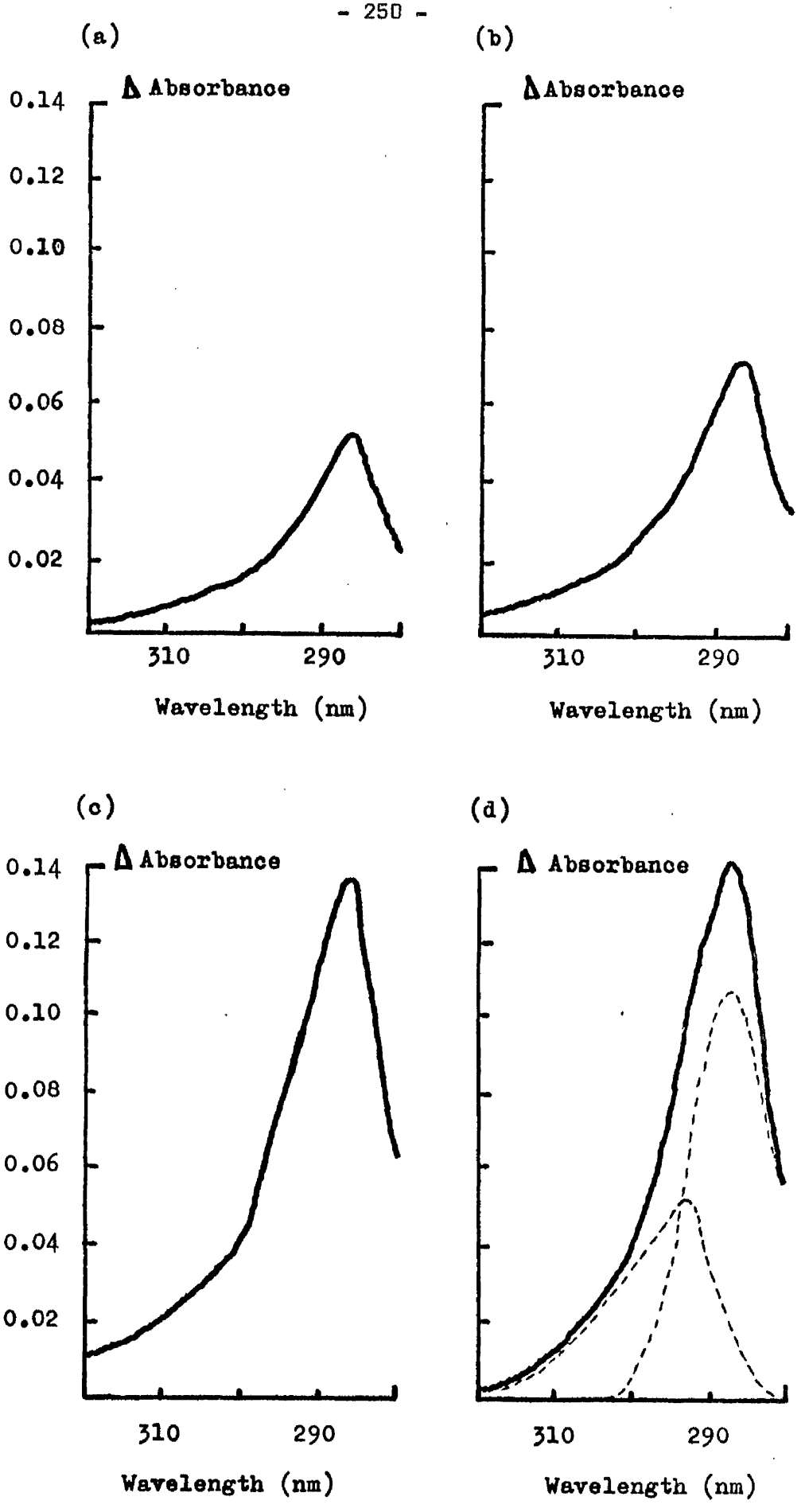
Figure 54. The pH dependence of the perturbation of tryptophan and tyrosine residues in horse spleen apoferritin. Measurements were carried out in 1.0 cm matched cells relative to a solution at pH 1.553. All protein samples had been dissociated by acid and then dialysed back into glycine-acetate buffers at higher pH values.

(a) Protein sample pH 3.77

(b) Protein sample pH 4.12

(c) Protein sample pH 4.47

(d) Theoretical spectra calculated for the perturbation of five tyrosine residues and one trytophan residue . Dotted lines represent the absorption contribution from each of these.


(a)
Δ Absorbance
0.14
0.12
0.10
0.08
0.06
0.04
0.02
310
290
Wavelength (nm)
(b)
Δ Absorbance
310
290
Wavelength (nm)
(c)
Δ Absorbance
0.14
0.12
0.10
0.08
0.06
0.04
0.02
310
290
Wavelength (nm)
(d)
Δ Absorbance
310
290
Wavelength (nm)

this region we were, in fact, monitoring the perturbation of the tryptophan residue discussed earlier. Using the molar extinction coefficients for the perturbation of 'buried' tyrosine and tryptophan residues, then it is possible to construct a theoretical spectrum for a protein with 1 tryptophan and five tyrosines as shown in Figure 54. It can be seen from this that the agreement between the theoreical curve and the experimental curve at pH 4.5 is very good.

Using the equations derived by Herskovits and Sorensen (1968),

$$\Delta\epsilon_{291-293}(\text{protein}) = a.\Delta\epsilon_{291-293}(\text{trp}) + b.\Delta\epsilon_{291-293}(\text{tyr})$$

$$\Delta\epsilon_{286-288}(\text{protein}) = a.\Delta\epsilon_{286-288}(\text{trp}) + b.\Delta\epsilon_{286-288}(\text{tyr})$$

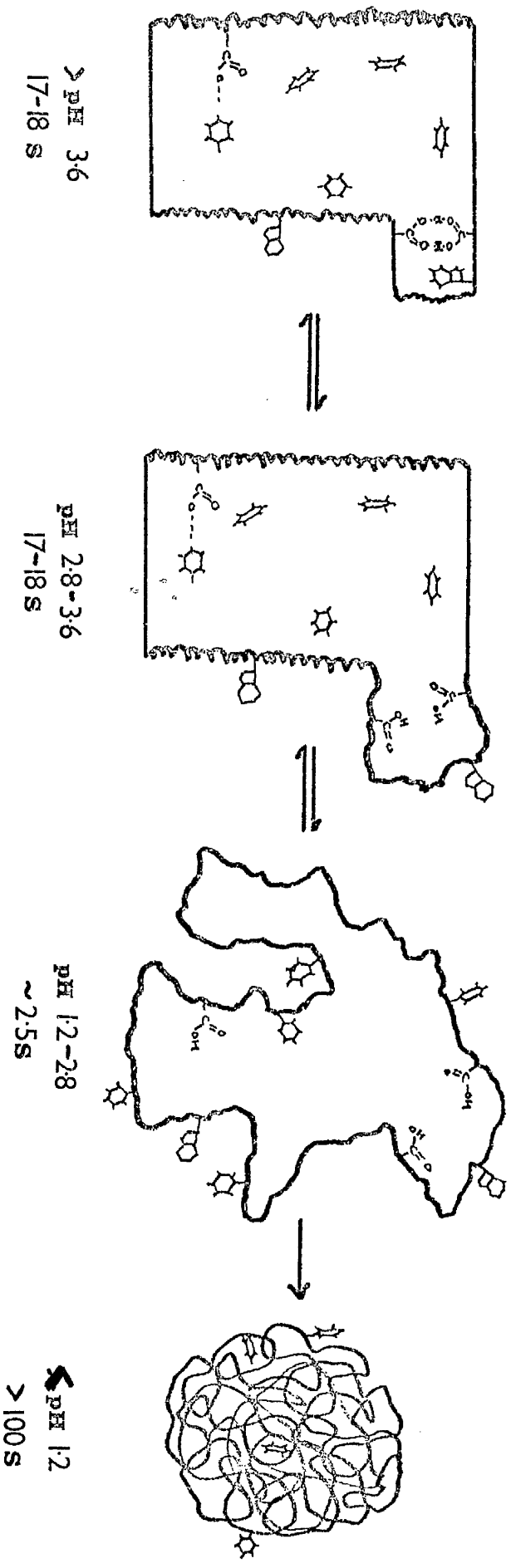
where a and b represent the apparent number of exposed tryptophan (trp) and tyrosine (tyr) residues in the protein, it was possible to calculate the region of pH in which the tryptophan in the dissociated form of the protein was 'buried', in the native form, on re-association. This was found to be pH 4.0 - 4.5.

To summarize this data we will make use of Figure 55 in order to avoid confusion by the rather large number of different effects caused by changing the pH in the region pH 0.0 - 5.0. This figure schematically represents the various forms in which the apoferritin molecule can exist.

Studies at Neutral pH.

Apoferritin was studied under neutral conditions in the range pH 5.0 - 9.0 by sedimentation velocity in the analytical ultracentrifuge. The sedimentation coefficients were corrected

Figure 55. Summary of the pH dependence of the dissociation, the perturbation of the tyrosine residues and the perturbation of a tryptophan residue of horse spleen apoferritin.


> pH 3·6
17-18 s
pH 2·8-3·6
17-18 s
pH 1·2-2·8
~ 2·5 s
pH 1·2
< 100 s

for temperature and concentration and the results obtained are shown in Table 10. It can be seen that, within the limits of experimental error, the protein's sedimentation properties are constant in this pH region and the only species detectable was monomer.

In one experiment to try to detect the presence of a small amount of subunit, the protein concentration used was 20 mg/ml and the cell was scanned at 230 nm at which wavelength, the optical density of apoferritin is increased approximately five-fold. In this experiment we would have been able to detect at least 0.05 optical density units of non-sedimenting species and this would have represented approximately one-twothousandth of the protein material applied. When the solution was sedimented, no residual non-sedimenting material was found as shown in Figure 56a. That we did not detect subunit does not prove that an equilibrium does not exist, but rather that the equilibrium lies well in the direction of the monomer.

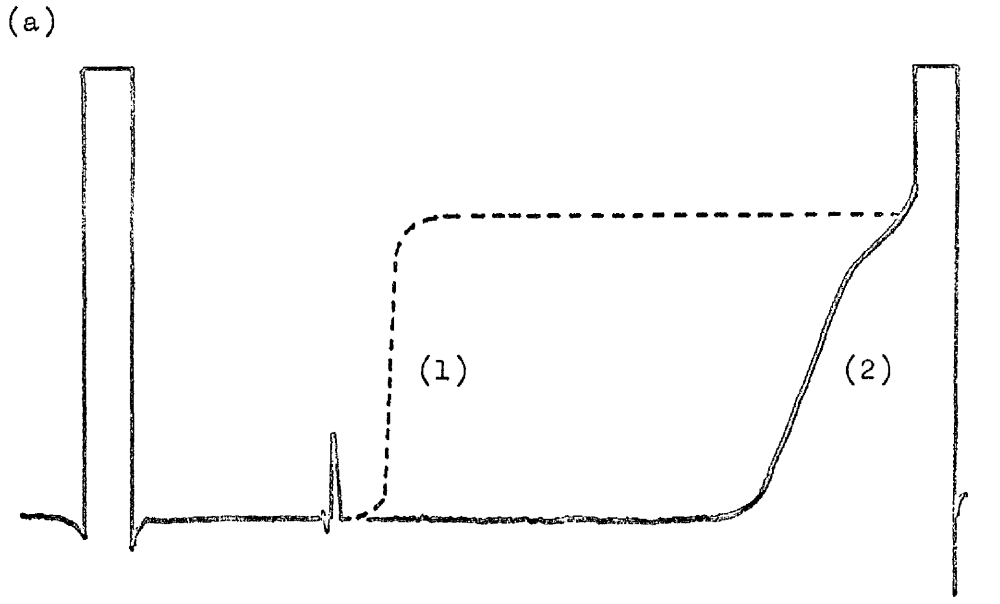
This experiment was also performed by gel filtration on a calibrated Sephadex G-100 column, using the same protein concentration and monitoring at 280 nm and 233 nm. The results are shown in Figure 56b and once again no subunit was detectable.

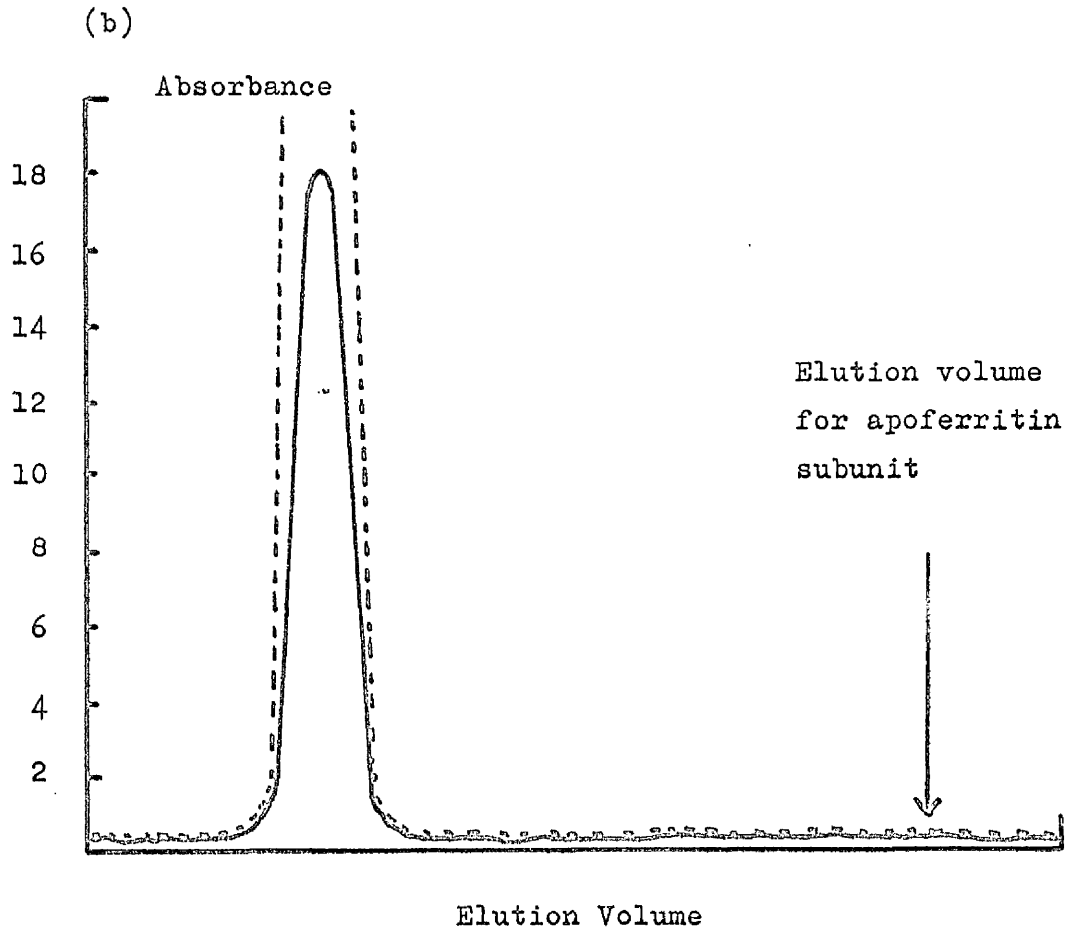
Ultraviolet difference spectroscopy was applied to apoferritin in this pH region and the results were negative. This finding does not, however, rule out any minor conformational changes that do not affect the environments of the various chromophores. It does, however, imply that the titration of the imidazole groups of apoferritin in this pH range does not give rise to some form of charge perturbation on the chromophores as was observed for pancreatic ribonuclease (Donovan, 1965). In this study Donovan (1965) demonstrated a pH dependent tyrosine difference spectrum in the region pH 5-9. However, the $\Delta\epsilon$ for the change was abnormal-

Table 10. Sedimentation coefficients obtained for horse spleen apoferritin in the range pH 5 - 9. These values were corrected for concentration and temperature. The conditions for the runs are given in the text.

pH	Sedimentation coefficient
5.03	17.1
5.37	16.8
5.75	16.4
6.11	16.7
6.55	16.5
6.82	17.1
7.09	16.9
7.40	17.0
7.83	17.0
8.00	16.6
8.66	16.9
8.88	16.8
9.02	16.5

Figure 56. Studies on horse spleen apoferritin at neutral pH.
(a) Typical scanner trace from sedimentation velocity run on the analytical ultracentrifuge. Boundary (1) represents the start of the run while the more diffuse boundary (2) represents the end. Protein concentration was 20 mg/ml and the cells were scanned at 230 nm.
(b) Typical elution profile obtained from the chromatography of a solution of horse spleen apoferritin on a column of Sephadex G-100 equilibrated with 0.1M borate buffer, pH 7.0. Protein was monitored at 280 nm (full line) and 233 nm (dotted line).


(a)
(1)
(2)


(b)
Absorbance
18
16
14
12
10
8
6
4
2
Elution volume
for apoferritin
subunit
Elution Volume

ly low and he suggested that the dissociation of protons from imidazole groups produced a direct electrostatic perturbation of a phenolic chromophore.

Studies at Alkaline pH.

Sedimentation velocity experiments were carried out with protein in the region pH 9 - 13.5 and the sedimentation coefficients obtained were corrected for concentration and temperature. The fraction of the slow-sedimenting material of the total was expressed as a percentage and the values obtained were plotted as a function of pH as shown in Figurd 57. The apparent sedimentation coefficient for slow-sedimenting species at pH 13.0 was found to be 0.02 which suggested, that at this pH, hydrolysis was probably occurring. Since sedimentation velocity was not a critical test for such hydrolysis, we resorted to gel chromatography on a calibrated Sephadex G-75 column. By this method we were able to detect products of alkaline hydrolysis at pH values in excess of pH 12.2 - 12.4 while in buffers below this value no hydrolysis was apparent. In order to avoid complications occurring from such products of hydrolysis, we studied the re-association of subunits from pH 12.0 downwards and the results are shown in Figure 57. It can be seen from this figure that the process of dissociation is reversible and that the re-association follows the pH dependent dissociation exactly.

Also shown in this Figure are the results of an alkaline dissociation study carried out by Williams and Harrison (1968) and it can be seen from comparison, that the difference between the two studies is approximately 0.44 pH units. One possibility for the disparity was the ionic conditions for the run, in the present study universal buffer was used, while Williams and Harri-

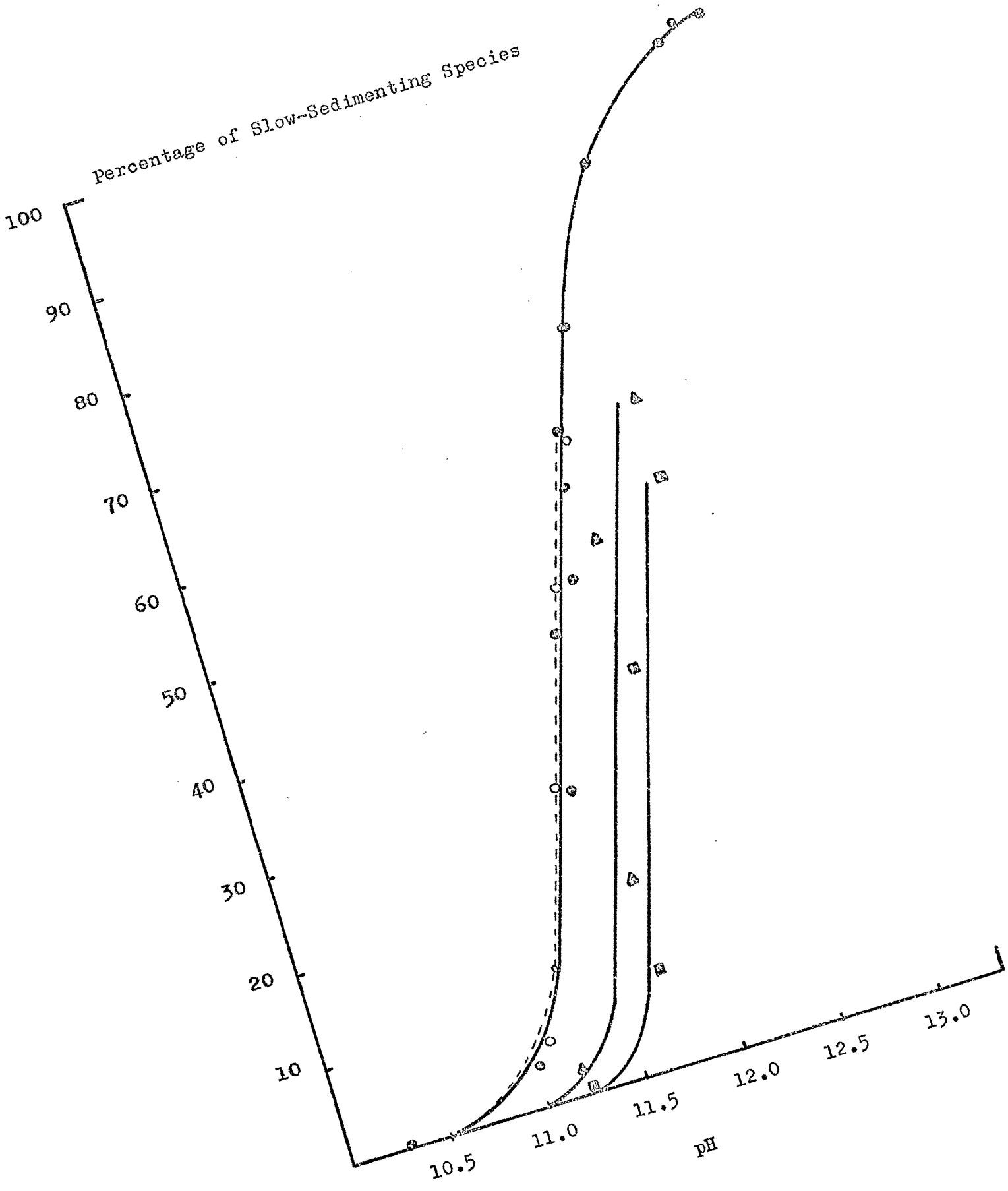
Figure 57. The pH dependence of the alkaline dissociation of horse spleen apoferritin. The percentage of slow-sedimenting species was determined from concentration measurements from the scanner traces.

●——● Dissociation of apoferritin in universal buffer.

○——○ Reassociation of apoferritin from protein which had been dissociated at pH 12.0 and then dialysed into universal buffer at a variety of lower pH values.

■——■ Results of Williams and Harrison (1968) for the dissociation of apoferritin in 0.02M borate buffer.

▲——▲ Results of Williams and Harrison (1968) plotted as percentage of subunit to monomer (see text).


Percentage of Slow-Sedimenting Species
100
90
80
70
60
50
40
30
20
10
10.5
11.0
11.5
12.0
12.5
13.0
pH

son (1968) employed 20 mM sodium borate buffer, but this has not as yet been investigated. It should be noted, however, that the data of these workers was obtained for a dimer-enriched solution of apoferritin. Since these workers showed that the dimers were resistant to high pH values and, since the equilibrium that we were investigating was monomer to subunit, then a better comparison of their data would be obtained by estimating the fraction of subunits to monomer plus subunits. When this is done, the agreement between the two studies is much better with a $\Delta pK_{app} = 0.26$.

In a similar manner to the acid dissociation study, ultraviolet difference spectroscopy was employed to the study of a variety of apoferritin solutions in the range pH 9.0 - 13.5. By use of this technique it was found possible to titrate spectrophotometrically all of the tyrosyl residues of apoferritin.

Spectra were obtained for apoferritin solutions at a variety of pH values using protein at pH 7.5 as the reference solution. Alkaline difference spectra were obtained, as shown in Figure 58, with a wavelength maxima at 294 nm which is characteristic of tyrosine. The difference in the molar absorption coefficient at 294 nm was plotted as a function of pH as shown in Figure 59. Using values of $\Delta\epsilon_{294} = 2357$ for tyrosine and $\Delta\epsilon_{294} = 830$ for tryptophan for the alkaline difference spectra (Mihalyi, 1968) it can be calculated that all five tyrosine residues have been deprotonated and from the apparent dissociation coefficient, $pK_{app} = 11.8$, it can be seen that all of them have abnormally high pK values, the normal being about 9.6 (Tanford and Epstein, 1954).

This is further confirmation that all of the tyrosyl residues are inaccessible to solvent and can be titrated only after unfolding of the polypeptide chain.

From a comparison of the data of the spectrophotometric

Figure 58. Typical alkaline-induced ultraviolet difference spectrum for horse spleen apoferritin measured at pH 12.92 relative to a solution at pH 7.52.

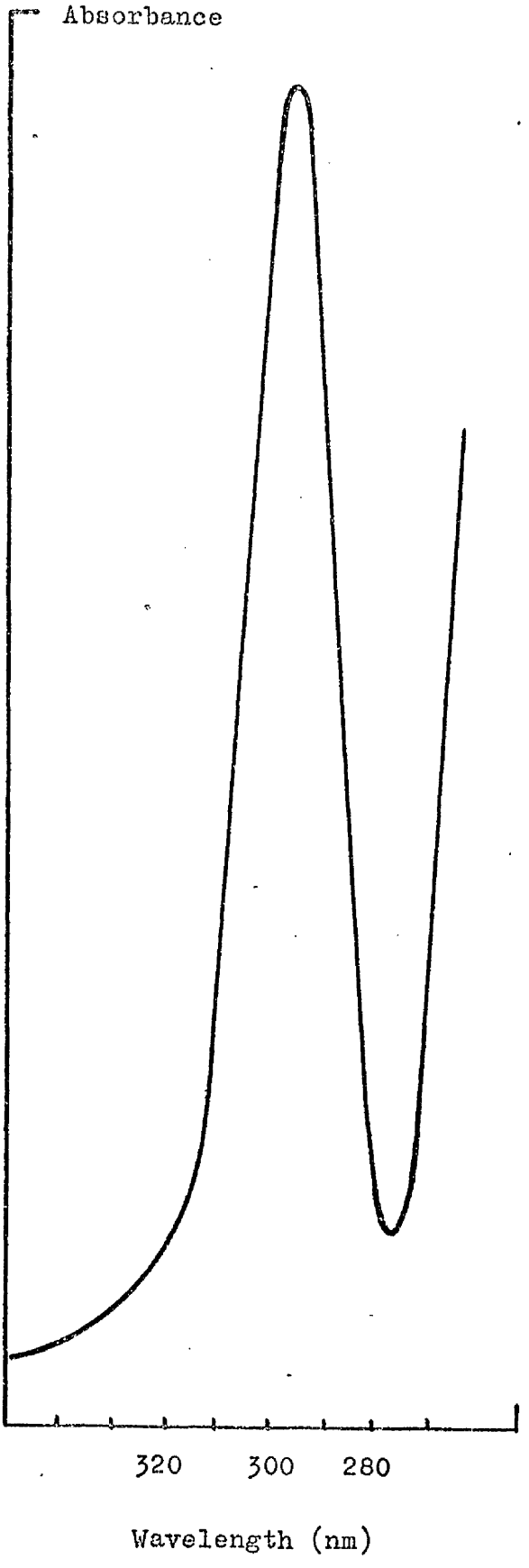
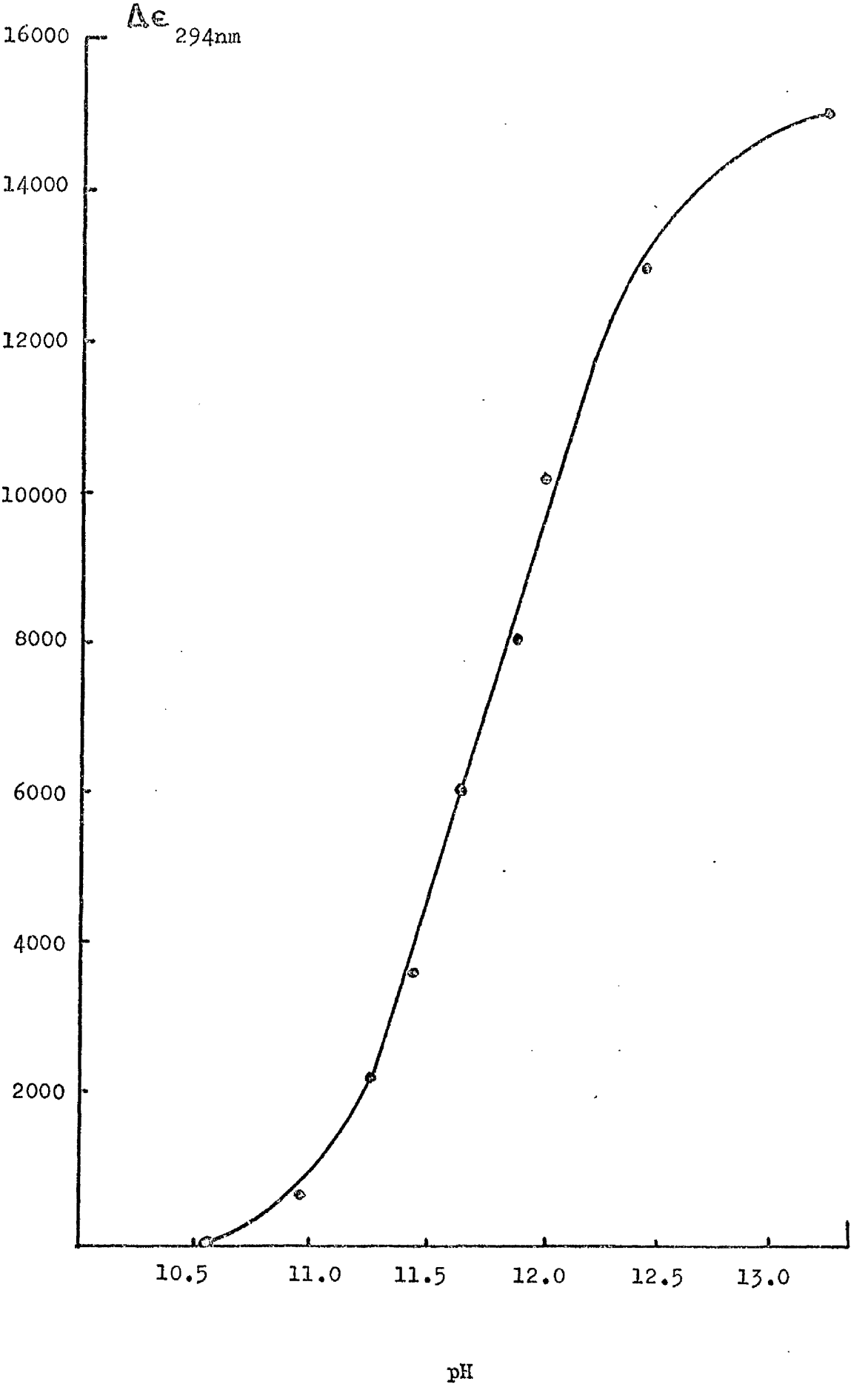

Absorbance
320
300
280
Wavelength (nm)

Figure 59. Spectrophotometric titration of the phenolic groups of horse spleen apoferritin.


Δε 294nm
16000
14000
12000
10000
8000
6000
4000
2000
10.5
11.0
11.5
12.0
12.5
13.0
pH

titration study and the alkaline dissociation study, it can be seen that, just as in the case of the acid-induced conformational change , the two parallel each other very well. Thus it would seem reasonable to assume that the dissociation of monomer to subunit at alkaline pH values is caused by a major pH dependent conformational change which results in the cleavage of inter and intra-molecular bonds.

From the apparent pK of the change it should be possible to speculate on the nature of the groups involved in such bonding, although such extrapolations must obviously be made with caution. The pK_{app} obtained from this study was 11.8 and this would suggest anomalous tyrosyl residues (involved in hydrogen bonding) or salt linkages with ϵ-amino groups of lysine (pK 10.53) or even the guanido groups of arginine (pK 12 - 12.5). As will be seen later two lysyl residues and one arginyl residue per subunit are found to be refractive to chemical modification and it is interesting to speculate that these groups may well be involved in the maintainance of the protein's quaternary structure.

Chemical Modification of Apoferritin.

In the three-dimensional structure of a protein, the side chain groups of some amino acid residues may be present on the surface in contact with the solvent medium and not interacting with the other side chain groups. Others may be buried in the internal region and not accessible to the solvent medium. Still

others may be on the surface but blocked by the interaction with the other amino acid side chain groups and, thus, not be available for chemical modification. A detailed study of the reactivity of the amino acid residues may give valuable information, not only about the state of the particular amino acid residue, but also about the folding of the polypeptide chain of the protein.

Modification at Tryptophan Residues.

The protein was modified by 2-nitrophenylsulphenyl chloride exactly as described earlier (page 78) and the extent of modification was calculated by use of the molar extinction coefficient, $\Delta\epsilon_{365} = 4000$, for 2-(2-nitrophenylsulphenyl)-tryptophan. The reaction sequence for the modification is shown on page 176 and the amino acid analysis of the modified apoferritin is shown in Table 11.

It can be seen that the only group affected by the reagent is tryptophan and this is quantitatively converted to 2-(2-nitrophenylsulphenyl)-tryptophan. The modified protein was chromatographed on a calibrated Sephadex G-75 column and the elution profile obtained is shown in Figure 60 . From this it can be seen that the protein elutes with the void volume and none was present in the region expected for the subunit. The modified protein was also analysed by sedimentation velocity in the analytical ultracentrifuge and gave a homogeneous sedimenting material with a corrected sedimentation coefficient, $S_{20,w} = 17.4$ which is indicative of apoferritin monomer From these two studies it can be seen that modification of the two tryptophan residues does not cause the protein to dissociate into subunits.

From the studies of the acid dissociation of apoferritin it was evident that one tryptophan was inaccessible to solvent while

Table 11. Amino acid composition of tryptophan-modified horse spleen apoferritin. The amino acid residues are expressed as the number of residues per 18,500 g of protein.

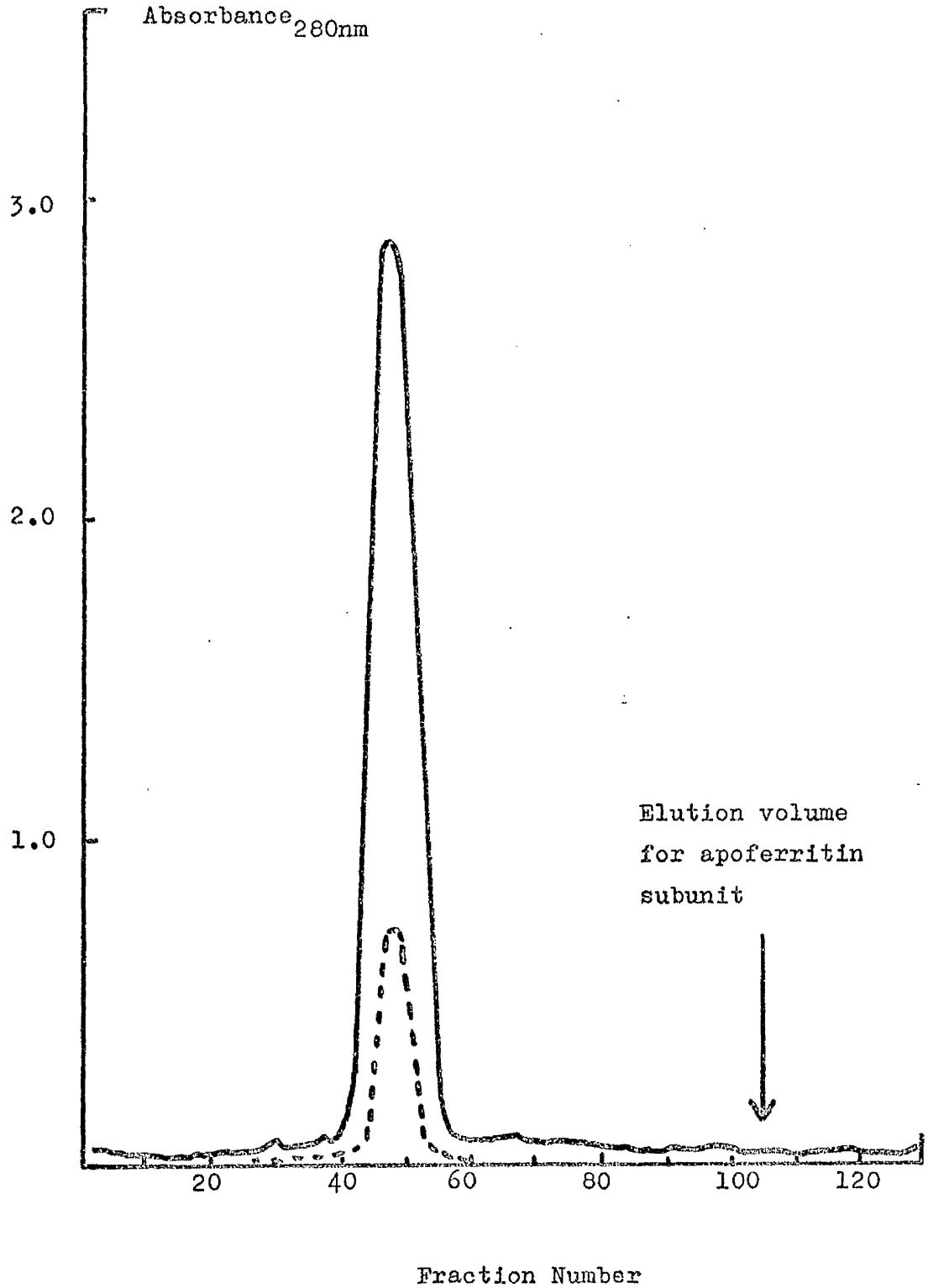
n.d, not determined.

Nps-tryptophan, 2-(2-nitrophenylsulphenyl)-tryptophan.

Amino Acid Residue	Control	Modified
Cysteic acid	n.d	n.d
Aspartic acid	17.47	17.63
Threonine	5.23	5.21
Serine	8.77	8.99
Glutamic acid	23.91	23.77
Proline	2.16	2.51
Glycine	9.95	10.04
Alanine	14.04	14.05
Valine	6.92	6.97
Methionine	2.84	2.22
Isoleucine	3.17	2.87
Leucine	24.94	25.11
Tyrosine	5.09	5.04
Phenylalanine	7.62	7.81
Histidine	5.89	5.87
Lysine	8.86	8.92
Arginine	9.72	9.66
Nps-tryptophan	0.00	2.03

Figure 60. Typical elution profile obtained from the chromatography of tryptophan-modified horse spleen apoferritin on a column of Sephadex G-75 equilibrated with 0.1M borate buffer, pH 7.0. Protein was monitored at 280 nm and also 365 nm (dotted line).

Modification was achieved by treatment with 2-nitrophenylsulphenyl chloride.


Absorbance 280nm
3.0
2.0
1.0
Elution volume
for apoferritin
subunit
20
40
60
80
100
120
Fraction Number

the other was completely accessible. The conditions under which chemical modification of tryptophan by sulphenyl halides was carried out did, however, assure adequate unfolding of the polypeptide chain i.e. 50% acetic or formic acid (pH~1.9). At this pH the 'buried' tryptophan would be normalized and could easily be modified. Once modified, the protein can, on raising the pH, re-associate to give apoferritin monomers.

Although the nitrophenylsulphenyl group introduced does not greatly change the hydrophobic nature of the tryptophan side chain it does constitute a significant steric factor. As yet, we have not investigated whether this steric factor is significant enough to exclude the modified tryptophan from the 'buried' region that the group occupies in the native structure. This could probably be determined by ultraviolet difference spectroscopy in the acid pH region using the difference spectrum of 2-(2-nitrophenylsulphenyl)-tryptophan as a measure of the minor conformational change in the region pH 3.0 - 3.5.

It has been observed by some workers that the presence of a nitro-phenylsulphenyl-tryptophan does cause major conformational changes (Parikh and Omenn, 1971; Atassi, Perlstein and Habeeb, 1971). In staphylococcal nuclease the modified tryptophan lies between two major stretches of α-helix, and although the modification causes major disruption of α-helix, as witnessed by circular dichroism, the specific activity of the protein is not significantly altered (Parikh and Omenn, 1971) whereas in lysozyme the nitrophenylsulphenyl-modified protein, which again has a significant conformational change, is reported to have no enzymic activity (Atassi, Perlstein and Habeeb, 1971).

It will be seen later that the nitrophenylsulphenyl-modified apoferritin does retain full activity and this would certainly

suggest that tryptophan is not involved directly in the catalytic function of apoferritin, but there may be present a small, as yet undetected, conformational change and this is being investigated at present.

Modification at Tyrosine Residues.

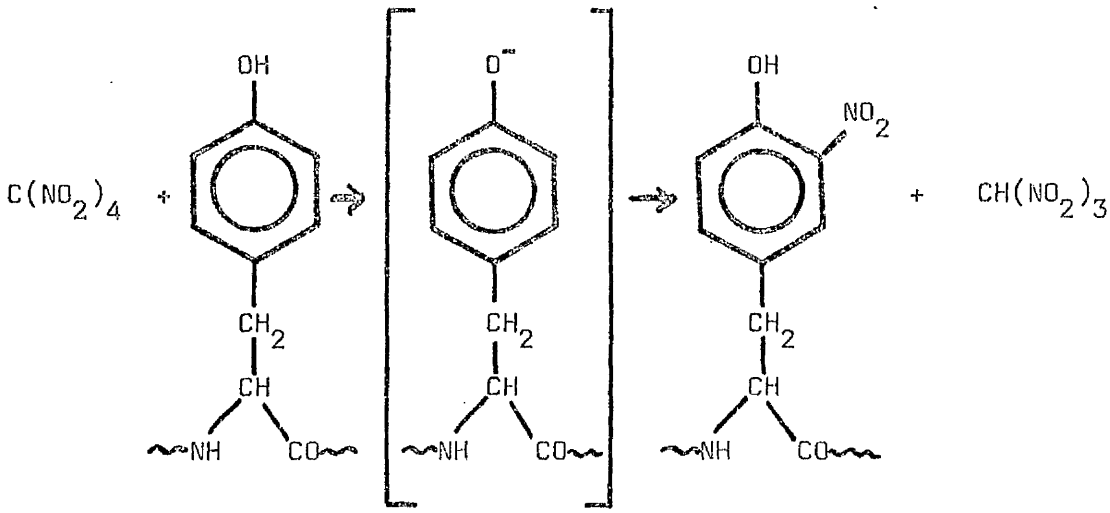
Since the first studies on the use of tetranitromethane as a selective agent for modification of tyrosine residues in proteins (Sokolovsky, Riordan and Vallee, 1966), the technique has been used in a great number of studies to determine the accessibility of the tyrosine residues to modification (Cuatrecasas, Fuchs and Anfinsen, 1968, 1969; Christen and Riordan, 1968; Irie and Sawada, 1967).

The reaction of tyrosine with tetranitromethane is shown in Figure 61. It has been shown that the reagent can also react with cysteine as shown in the figure and, in one case, a tryptophan residue was modified (Cuatrecasas, Fuchs and Anfinsen, 1968).

Apoferritin was treated with tetranitromethane as described on page 88 and the course of the reaction was monitored at 428 nm. The results are shown in Figure 62. Using an $\epsilon_{428} = 4200$ (Sokolovsky, Riordan and Vallee, 1966), a value of 1.03 was calculated for the number of tyrosine residues orthonitrated.

An aliquot of the modified protein was hydrolysed and the amino acid composition of the resultant hydrolysate was determined. Nitrotyrosine was observed to elute on amino acid analysis just after phenylalanine as shown in Figure 63 and the ninhydrin colour factor was determined from a commercial sample of nitrotyrosine. The results of amino acid analysis indicated the presence of 0.96 nitrotyrosyl residues and 3.89 tyrosyl residues per subunit and it can be seen from a comparison between this and the

Figure 61. Reaction sequence for the nitration of tyrosine and cysteine residues by tetranitromethane.



$$R'SH + C(NO_2)_4 \longrightarrow R'SNO_2 + CH(NO_2)_3$$

$$R'SNO_2 + R'SH \longrightarrow R'SSR' + H^+ + NO_2^-$$

Figure 62. Nitration of horse spleen apoferritin by tetranitromethane monitored spectrophotometrically. Nitration was carried out at pH 8.0 and the protein concentration was 1.54 mg/ml.

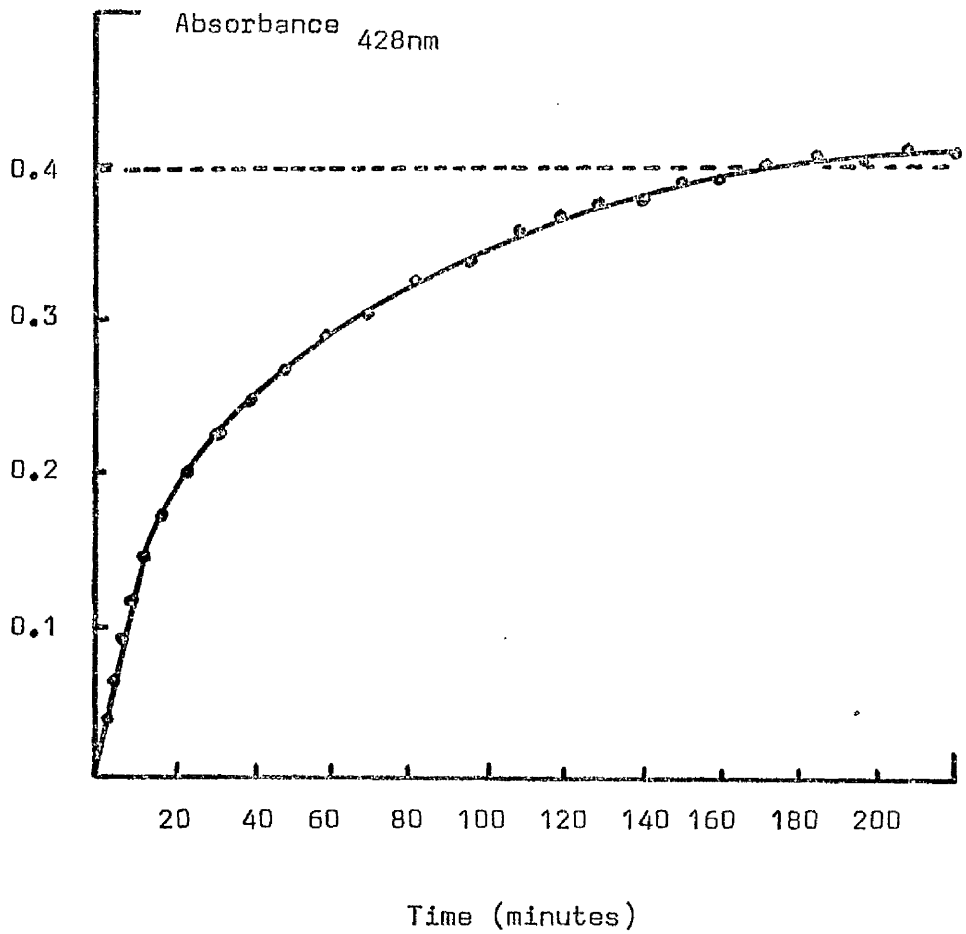
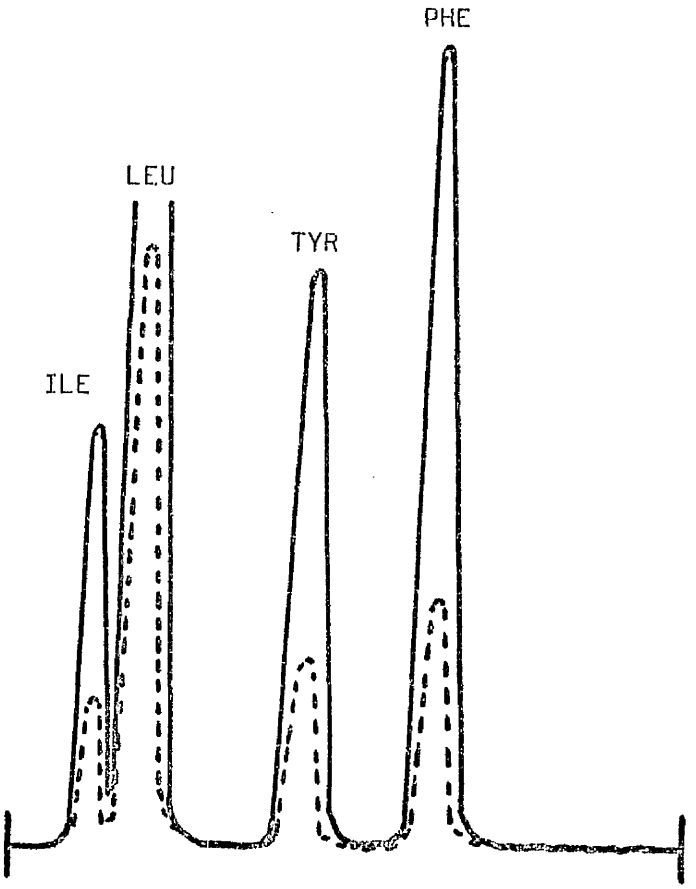
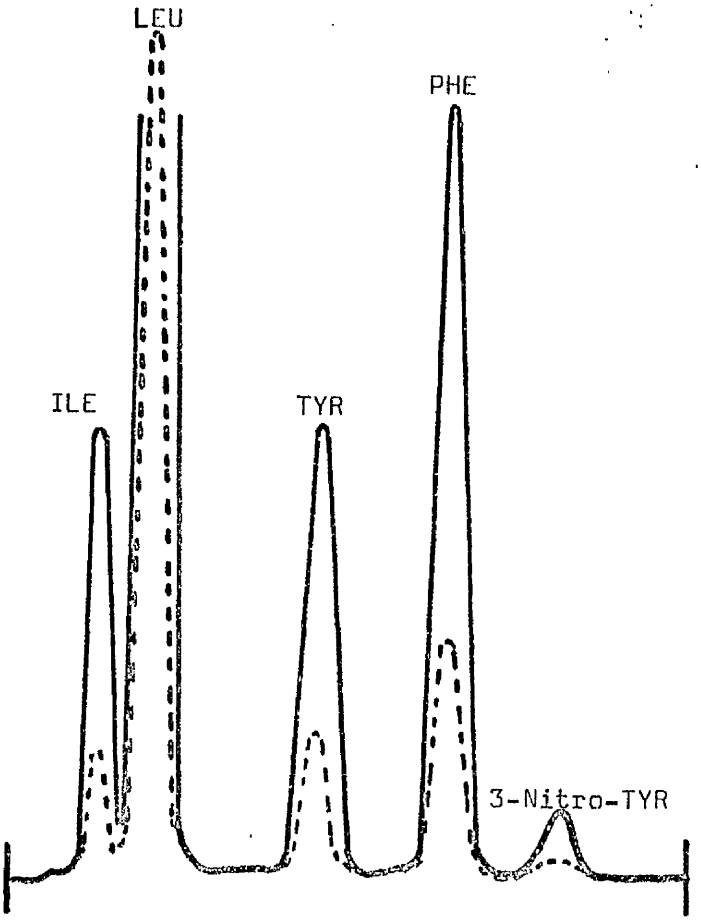

Absorbance 428nm
0.4
0.3
0.2
0.1
20
40
60
80
100
120
140
160
180
200
Time (minutes)

Figure 63. Analyses of the neutral amino acids in untreated horse spleen apoferritin (upper trace) and tetranitromethane-treated apoferritin (lower trace). The peaks in order of elution represent, isoleucine, leucine, tyrosine, phenylalanine and 3-nitrotyrosine.

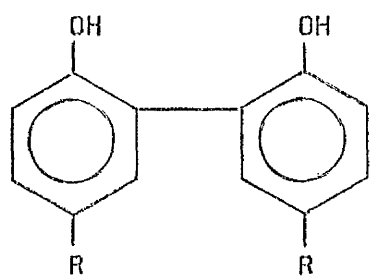

PHE
LEU
TYR
ILE


LEU
PHE
ILE
TYR
3-Nitro-TYR

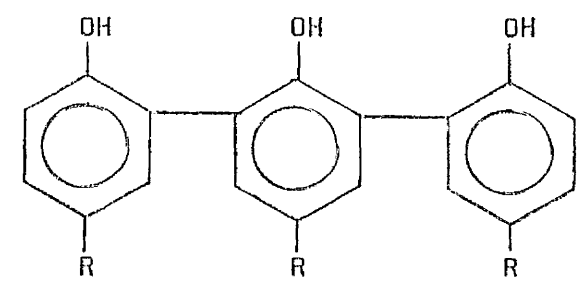
control that the amount of nitrotyrosine plus tyrosine in the modified protein is equal to the amount of tyrosine in the untreated protein within the limits of the experimental error as shown in Table 12..

The degree of modification determined by spectral measurements agrees very well with the results of amino acid analysis and because of the relatively limited reaction time, formation of 3,5-dinitrotyrosine was not detected.

It has been reported in some instances that nitration of proteins caused intermolecular cross-linking asa result of covalent cross-links formed between tyrosine rosidues (Doyle, Bello and Roholt, 1968; Boesel and Carpenter, 1970; Vincent, Lazdunski and Delaage, 1970). Two tyrosine residues linked by biphenyl bonds gives 'dityrosine' [2,2'-dihydroxy-5,5'-di-(2-carboxy-2-aminoethyl)biphenyl] which is compound I below, while three tyrosines give [2,2',2"-trihydroxy-5,5',5",-tri-(2-carboxy-2-aminoethyl)terphenyl] , compound II, and these compounds have been detected following treatment of tyrosine with tetranitromethane (Williams and Lowe, 1971)



(I)



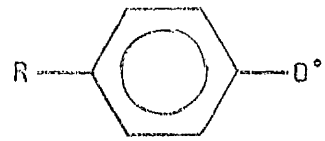
(II)

These probably arise as a result of a condensation reaction between phenoxide anions,

Table 12. Amino acid composition of tyrosine-modified horse spleen apoferritin. Amino acid residues are expressed as the number of residues per 18,500 g of protein.

n.d, not determined.

Amino Acid Residue	Control	Modified
Cysteic acid	n.d	1.83
Aspartic acid	17.27	17.73
Threonine	5.20	5.55
Serine	9.01	8.51
Glutamic acid	23.82	23.79
Proline	2.31	1.57
Glycine	9.60	10.05
Alanine	13.55	13.67
Valine	7.02	6.28
Methionine	2.83	2.85
Isoleucine	3.44	3.62
Leucine	24.93	24.99
Tyrosine	5.02	3.92
Phenylalanine	7.61	7.45
Histidine	5.88	5.39
Lysine	8.69	8.21
Arginine	9.77	10.06
Tryptophan	n.d	n.d
Nitrotyrosine	0.00	0.96



which have been implicated in the mechanism of the nitration reaction (Bruice, Gregory and Walters, 1968).

To test if such cross-linking had occurred as a result of nitration of horse spleen apoferritin we examined the protein by sedimentation velocity in the analytical ultracentrifuge. When a sample of the nitrated apoferritin was sedimented in the ultracentrifuge there was no slow-sedimenting material, indicating that nitration of one tyrosine residue had not caused the protein to dissociate. Although the majority of the protein sedimented with a sedimentation coefficient of 16.9 (indicating monomer), there was a continuous concentration-dependent association phenomena observed. As the protein was being centrifuged, oligomers sedimented fast to the bottom of the cell. This caused a redistribution in the monomer species (about 10% of the total) and some material aggregated to higher oligomers and this equilibrium-type situation continued for the duration of the run. This has unfortunately not, as yet, been studied by gel chromatography which would perhaps provide more readily interpretable data for this re-distribution. This aspect of the modification is currently being investigated.

The reactivities of tyrosyl residues are, in part, a reflection of their exposure to the solvent, particularly in the nitration of the protein with tetranitromethane because of the relative mildness of the reagent. It would appear then from the nitration of apoferritin that one tyrosine is accessible to the reagent. However, it will be remembered that the results from the spectro-

photometric titration studies, both in the acid and alkaline region, led to the view that all the tyrosine residues were inaccessible to solvent (page 224 & 262). Myers and Glatzer (1971) have, however, recently stated that caution should be applied in interpreting such data and that, the accessibility of tyrosine to tetranitromethane cannot always be taken as a measure of the exposure of this residue to the polar solvent in which the protein is dissolved. On nitration of subtilisin with tetranitromethane, a reagent whose action is presumably dependent on the environment of the tyrosine residues, these workers have obtained evidence that in some cases phenolic groups in apolar locations can be preferentially nitrated.

The tyrosine nitrated in apoferritin may well be an example of such a situation and this would be a possible solution for the cause of the apparent disparity between the two studies.

Modification at Lysine Residues.

The conversion of protein amino groups to guanidino groups by o-methylisourea salts (Hughes, Saroff and Corney, 1949; Roche, Margue and Baret, 1954; Bello, 1955) has been shown to be both highly selective (Chervenka and Wilcox, 1956; Klee and Richards, 1957) and potentially useful in the study of chemically modified proteins and in sequence studies.

It has been demonstrated that, on modification at pH 10.5, lysine residues are converted quantitatively to homoarginine residues and little or no other modification was observed (Hughes, Saroff and Corney, 1949; Roche, Margue and Baret, 1954; Plapp, Moore and Stein, 1971) although modification can occur to a minimal extent at the α-amino group of the N-terminal amino acid (Kimmel, 1967) and also at a cysteinyl residue of papain when

carried out at pH 7.0 as shown in Figure 64 (Banks and Shafer, 1972). Also shown in Figure 64 is the reaction sequence for the guanidination of lysine residues by o-methylisourea.

The most direct and precise method for the estimation of the extent of guanidination is to determine the homoarginine content of the protein hydrolysate. This amino acid is stable to acid hydrolysis and on amino acid analysis it elutes as a symmetrical but slightly broad peak which elutes after arginine. In the present study homoarginine was assumed to have the same colour factor as arginine.

The guanidination of lysine residues has been found useful for studying the environment and essential nature of lysine side chains in proteins and it has been shown in a great many cases to extensively modify the lysine residues with little or no effect on the protein's enzymic activity (Chervenka and Wilcox, 1956; Shields, Hill and Smith, 1959).

A further use of this modification procedure comes in sequence studies since trypsin is unable to cleave the peptide bond at homoarginine (Shields, Hill and Smith, 1959). This not only facilitates the ordering of the possible peptide alignments but also provides larger peptides which are useful, both for the automatic protein sequenator of Edman and Begg (1967) and the peptide sequencer of Laursen (1971).

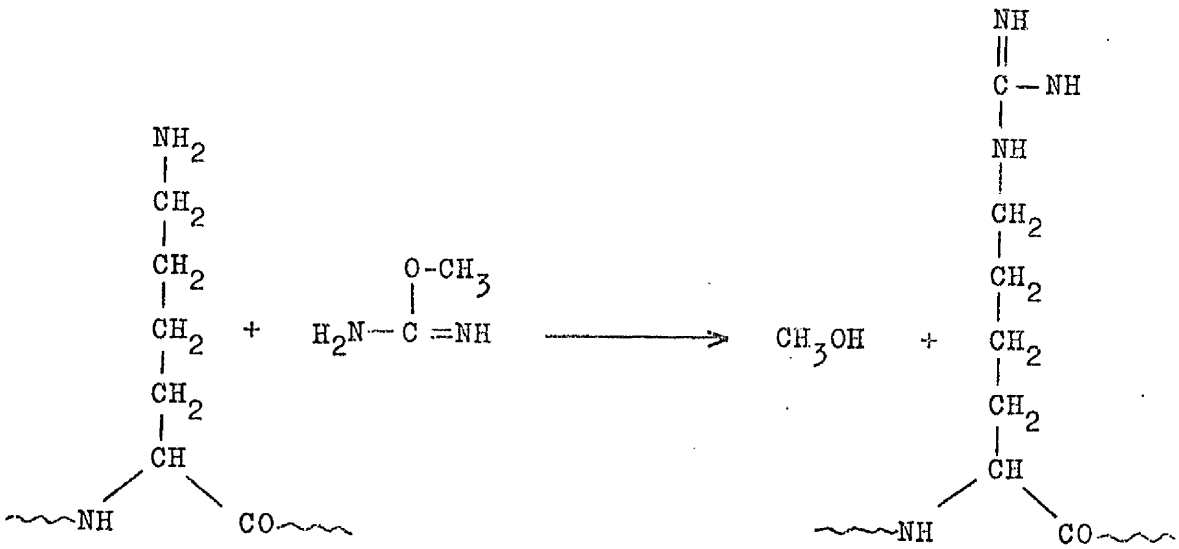
In the present study, the apoferritin was reacted with o-methylisourea as described on page 89 and the reaction was terminated by extensive dialysis.

On amino acid analysis of the hydrolysate of the pure, modified protein, a decrease in the lysine content was observed with a corresponding appearance of homoarginine as shown in Figure 65. The amino acid composition was calculated and is shown in Table

Figure 64. Guanidination of lysine residues in a protein by treatment with o-methylisourea at pH 10.5. The amino acid residue formed by this reaction is homoarginine. (A).

o-methylisourea can also react with cysteine residues to form O-methylcysteine (see text). (B)

A



B

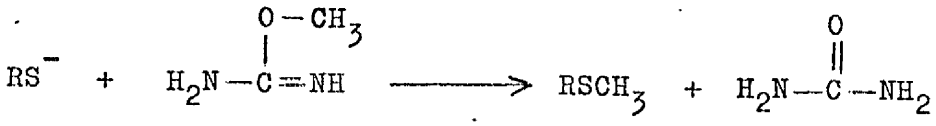
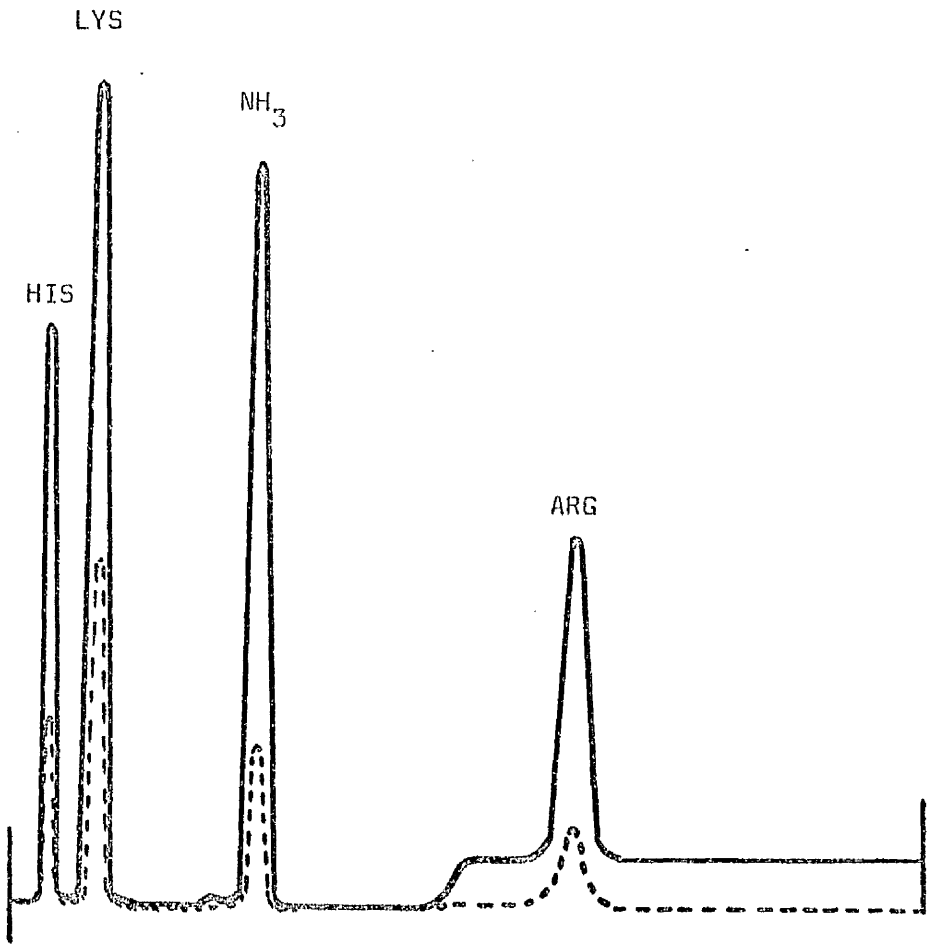
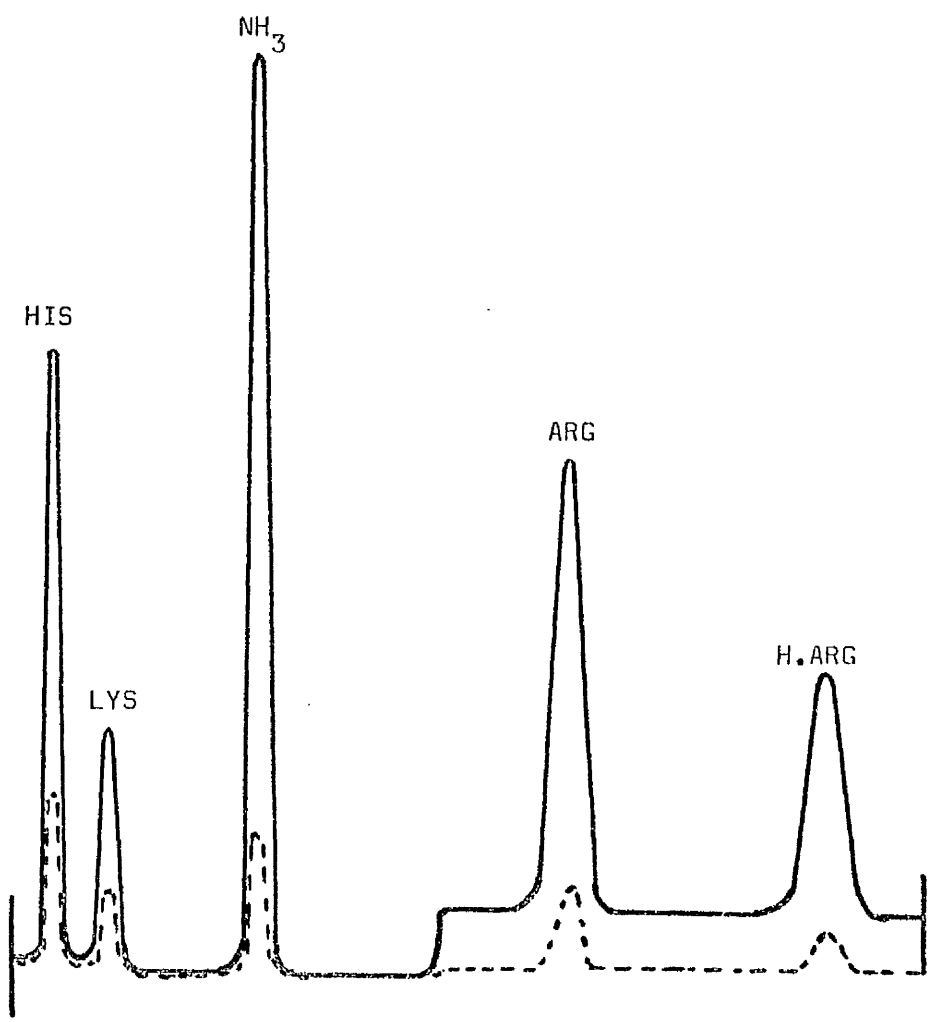


Figure 65. Analyses of the basic amino acids in untreated horse spleen apoferritin (upper trace) and O-methylisothiourea -treated apoferritin (lower trace). The peaks, in the order of elution, represent histidine, lysine, ammonia, arginine and homoarginine.


LYS
NH3
HIS
ARG


NH3
HIS
ARG
LYS
H.ARG

13. From this it can be seen that lysine was the only group modified and the sum of the lysine plus homoarginine was always found to be equal to the lysine content of the untreated protein, within the limits of the experimental error

In the present study, no guanidination of the N-terminal α-amino acid would be expected as this group is N-acetylated. The modified protein was chromatographed on a calibrated Sephadex G-75 column and a typical elution profile obtained is shown in Figure 66. From this it can be seen that the protein elutes as a single homogeneous species and no subunits or intermediates were present. That the modified protein remained as monomer was further verified by the presence of a single sedimenting species with a sedimentation coefficient, $S_{20,w} = 17.0$ when subjected to sedimentation velocity in the analytical ultracentrifuge.

From these studies, it can be seen that seven of the nine lysine residues in apoferritin can be guanidinated by o-methylisourea and that the modification of these groups does not cause the monomer to dissociate into subunits.

It is quite probable that the groups reacting most readily with this reagent are likely to be the most available spatially and, that unavailability of the remainder could be due to their involvement in strong salt bridges although the modified amino groups could also participate in ionic or hydrogen bonding with only slightly less effectiveness than the primary amino groups.

From the study of the alkaline dissociation of apoferritin it was suggested that salt linkages could be involved in the maintainance of the native structure and the pK for these was shown to be about 11.8.

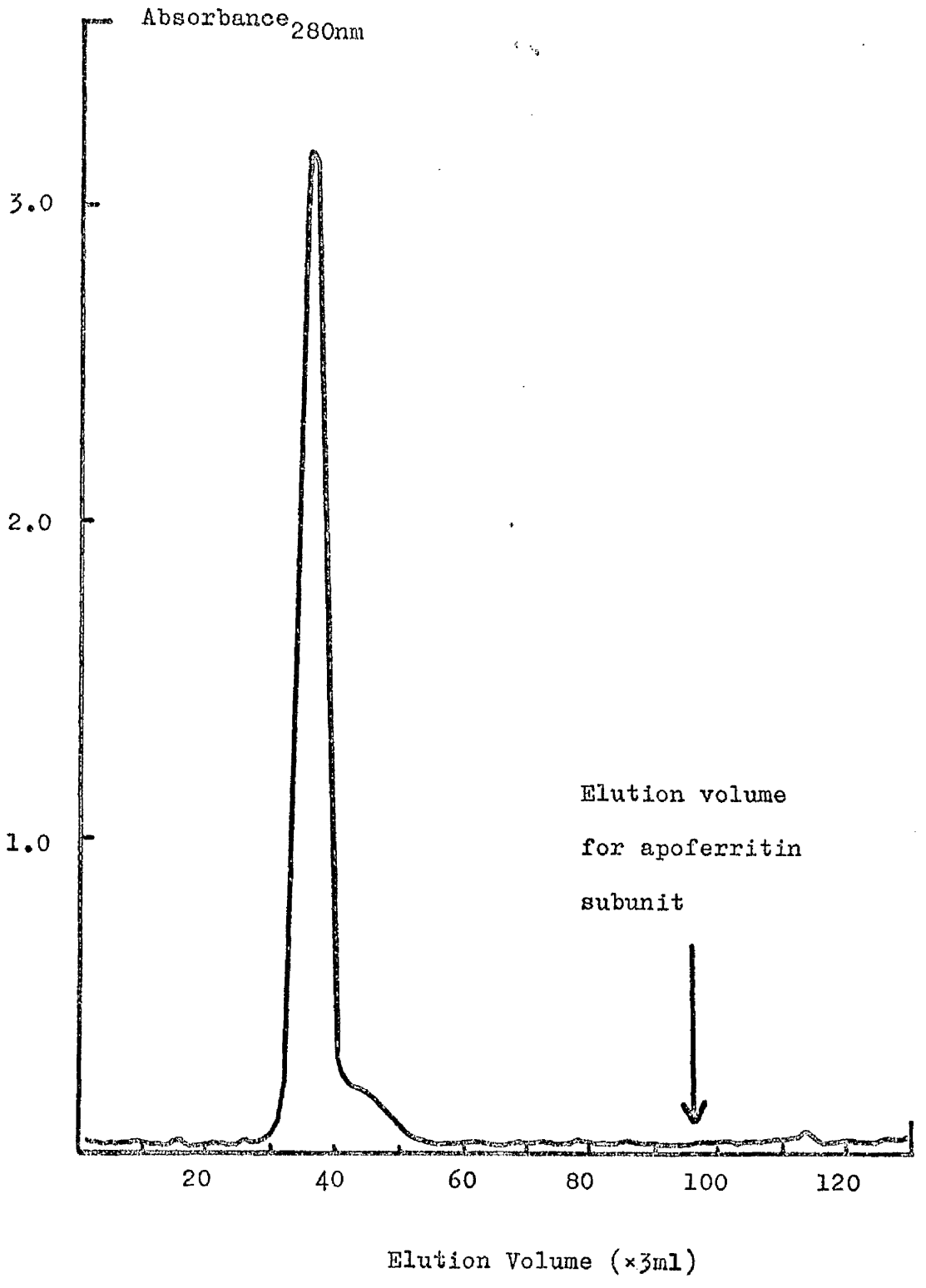
The present modification by o-methylisourea was carried

Table 13. Amino acid composition of lysine-modified horse spleen apoferritin. Amino acid residues are expressed as the number of residues per 18,500 g of protein.

n.d, not determined.

Amino Acid Residue	Control	Modified
Cysteic acid	n.d	n.d
Aspartic acid	17.73	17.69
Threonine	5.55	5.60
Serine	8.47	8.78
Glutamic acid	23.55	23.87
Proline	2.31	2.64
Glycine	10.05	9.94
Alanine	13.67	13.90
Valine	6.33	6.60
Methionine	2.41	2.26
Isoleucine	3.35	2.93
Leucine	24.49	24.87
Tyrosine	5.11	4.58
Phenylalanine	7.07	7.38
Histidine	5.49	5.67
Lysine	8.77	1.92
Arginine	10.02	9.84
Tryptophan	n.d	n.d
Homoarginine	0.00	6.66

Figure 66. Typical elution profile obtained from the chromatography of lysine-modified horse spleen apoferritin on a column of Sephadex G-75 equilibrated with 0.1M borate buffer, pH 7.0. Protein was monitored at 280 nm.
Modification was achieved by treatment with o-methylisou[redacted]-urea (see text).


Absorbance 280nm
3.0
2.0
1.0
Elution volume
for apoferritin
subunit
20
40
60
80
100
120
Elution Volume (×3ml)

out at pH 10.5 at which value no dissociation was observed and therefore if such salt-linkages occur, then, at this pH, they would be expected to be intact. It is possible, therefore, that either one or both of the lysine residues that are refractive to modification by o-methylisourea are involved in stabilization of the native structure by a salt linkage. An interesting extention of this study would be to guanidinate the apoferritin in the presence of a denaturant, say 1% sodium dodecyl sulphate, and see if all the lysine groups are modified. If so, it would be interesting to see if the protein subunits were then able to reassociate to monomer. However, this aspect of the modification has, unfortunately, not as yet been investigated.

The catalytic activity of the modified protein will be discussed at a later stage.

Modification at Arginine Residues.

Since the pK for arginine groups in proteins is very high (>12) this amino acid normally occurs in the protonated form and this, together with the resonance stabilization of the guanido group, make it a relatively difficult group to modify specifically. The amino groups of arginine residues can condense with carbonyl groups to give Schiff bases but these reactions are normally reversed on acidification with the regeneration of arginine. However, it has been demonstrated that the Schiff bases can be stabilized if heterocyclic rings are formed during the course of the reaction. An example of this is the reaction of 1,2-cyclohexanedione with arginine giving a final product of N^5-(4-oxo-1,3-diazaspiro [4.4] non-2-ylidene)-L-ornithine which is stable to acid hydrolysis (Figure 67).

The reaction of cyclohexanedione with apoferritin was carri-

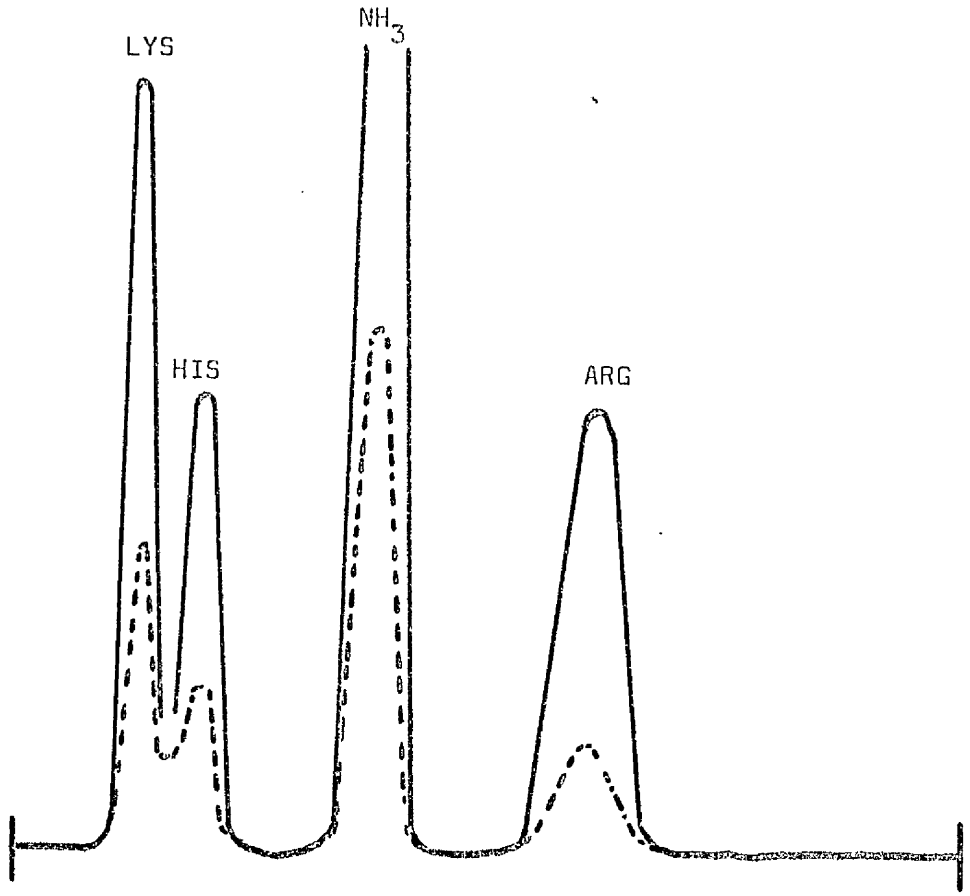
Figure 67. Reaction sequence for the formation of N^5-(4-oxo-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine residues by the reaction of 1,2-cyclohexanedione with arginine residues.

ed out at pH 10.9 as described on page 90.

A sample of the modified protein was hydrolysed and the hydrolysate was analysed by ion-exchange chromatography on a Jeol-co amino acid analyser. The results for this analysis are shown in Figure 68 and it can be seen that the arginine content of the modified protein is significantly lower than that of the control. The amino acid composition of the modified protein was calculated and is shown in Table 14.

In order to determine the elution position for N^5-(4-oxo-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine on amino acid analysis by the standard method, we modified a small amount of free arginine by the same procedure. The amino acid analysis of the sample was then determined routinely and the elution profile for the basic amino acids is shown in Figure 69. No material was found on the long column as would be expected for acidic and neutral amino acids. On the short column, a peak corresponding to unreacted arginine was found and also a peak in the position of ornithine which is reported to be a by-product of the reaction caused by alkaline degradation of the N^5-(4-oxo-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine (Toi, Bynum, Norris and Itano, 1967). Two small peaks were observed in the region of ammonia and these have not, as yet, been identified. The N^5-(4-oxo-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine elutes in a position almost identical to that of lysine. For this reason of the complexity of products on arginine modification we have, as yet, been unable to quantitatively identify the products of the reaction and at present we can only say that nine of the ten arginine residues are apparently reactive with 1,2-cyclohexanedione, while one residue appears to be refractive. Measurements for the other amino acid residues in apoferritin indicate no significant alter-

Figure 68. Analyses of the basic amino acids in untreated horse spleen apoferritin (upper trace) and 1,2-cyclohexanedione-treated apoferritin (lower trace). The peaks, in order of elution, represent lysine, histidine, ammonia, arginine and ornithine.


LYS
NH3
HIS
ARG

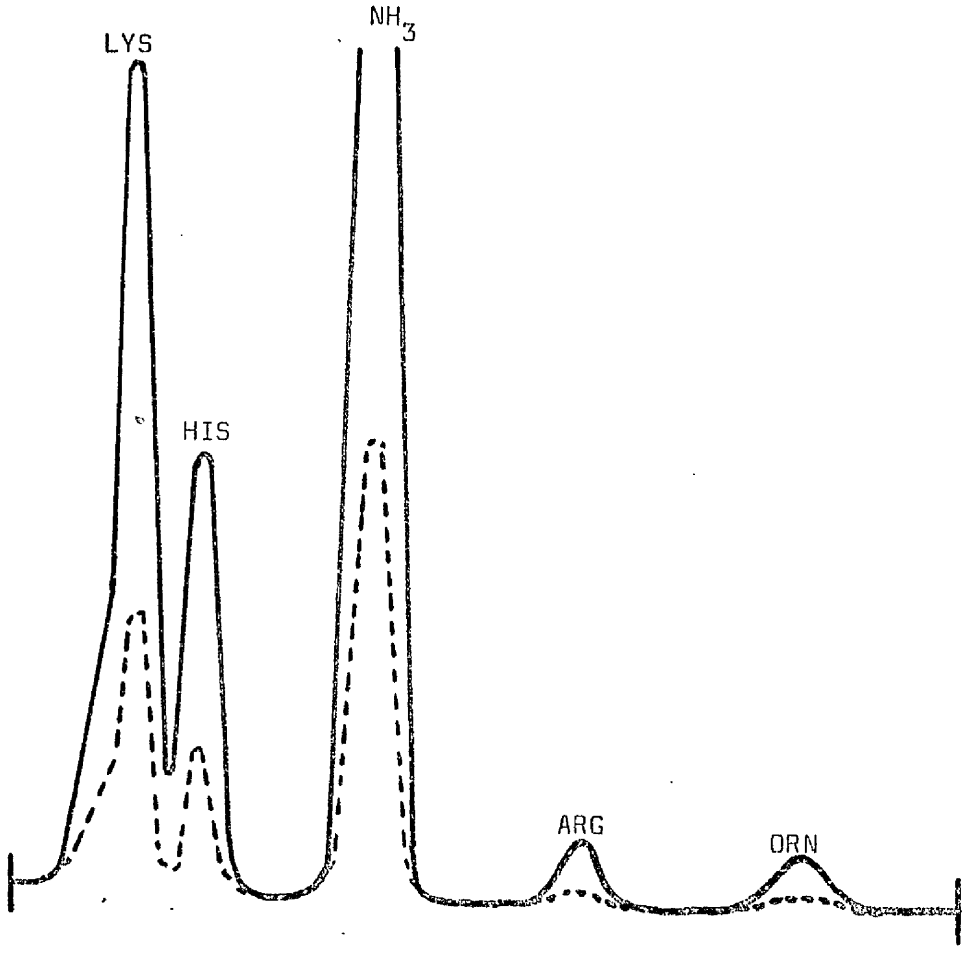
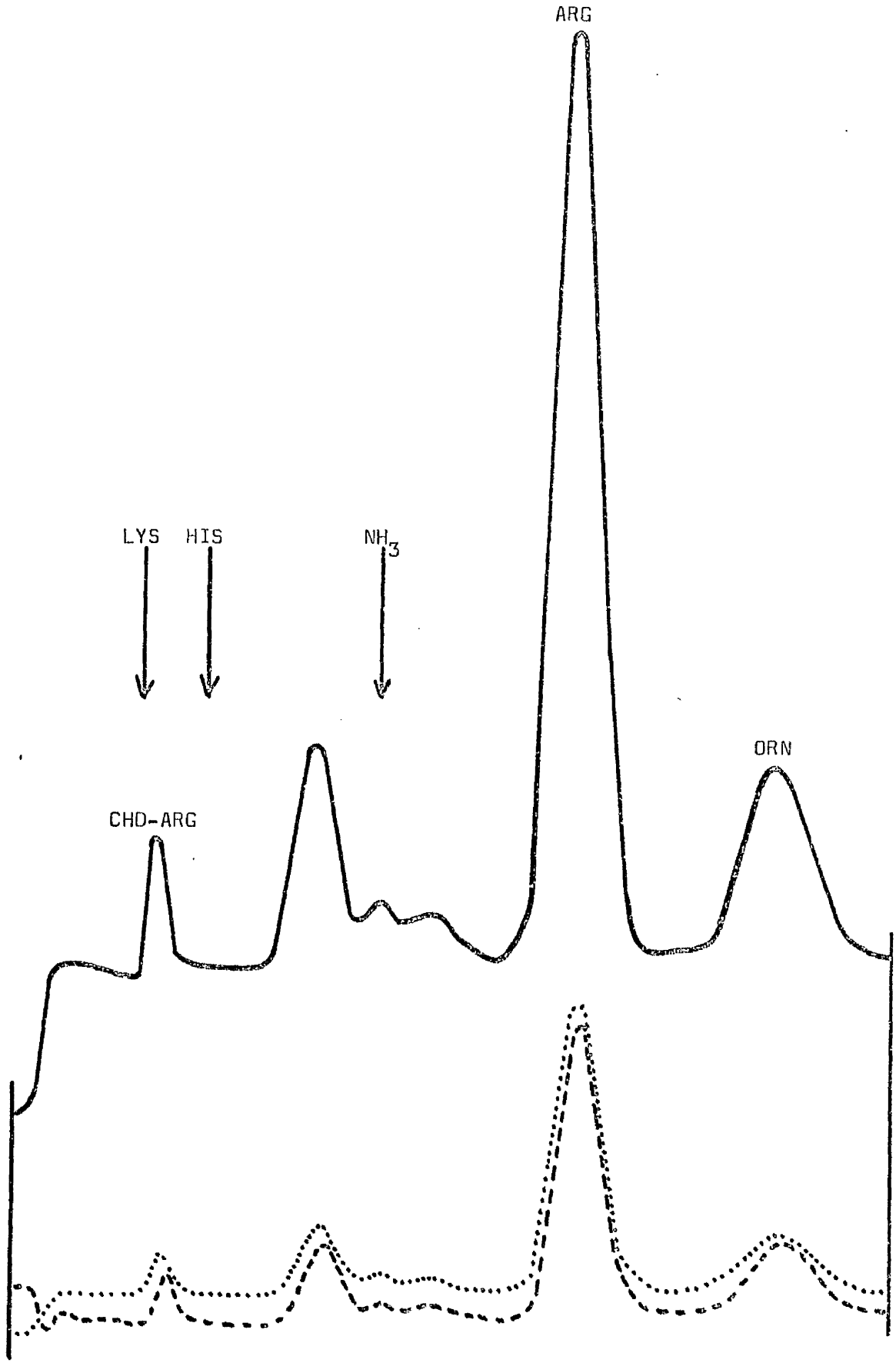

LYS
NH3
HIS
ARG
ORN

Table 14. Amino acid composition of arginine-modified horse spleen apoferritin. Amino acid residues are expressed as the number of residues per 18,500 g of protein.

n.d, not determined.

Amino Acid Residue	Control	Modified
Cysteic acid	n.d	n.d
Aspartic acid	17.55	17.94
Threonine	5.52	5.55
Serine	9.02	8.75
Glutamic acid	23.36	23.99
Proline	2.44	2.89
Glycine	9.90	9.81
Alanine	13.90	13.78
Valine	6.93	6.17
Methionine	2.64	2.19
Isoleucine	3.11	2.85
Leucine	24.87	24.89
Tyrosine	5.14	5.02
Phenylalanine	7.38	7.63
Histidine	5.67	5.59
Lysine	8.54	n.d
Arginine	10.01	0.86
Tryptophan	n.d	n.d
Ornithine	0.00	0.87

Figure 69. Analysis of the basic amino acids in the product of the reaction of free arginine with 1,2-cyclohexanedione. The elution positions for known amino acids are indicated. The peaks which have been identified are, in order of elution, N^5-(4-oxo-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine (CHD-ARG), unreacted arginine (ARG) and ornithine (ORN).


ARG
LYS
HIS
NH3
ORN
CHD-ARG

ation in any of these groups.

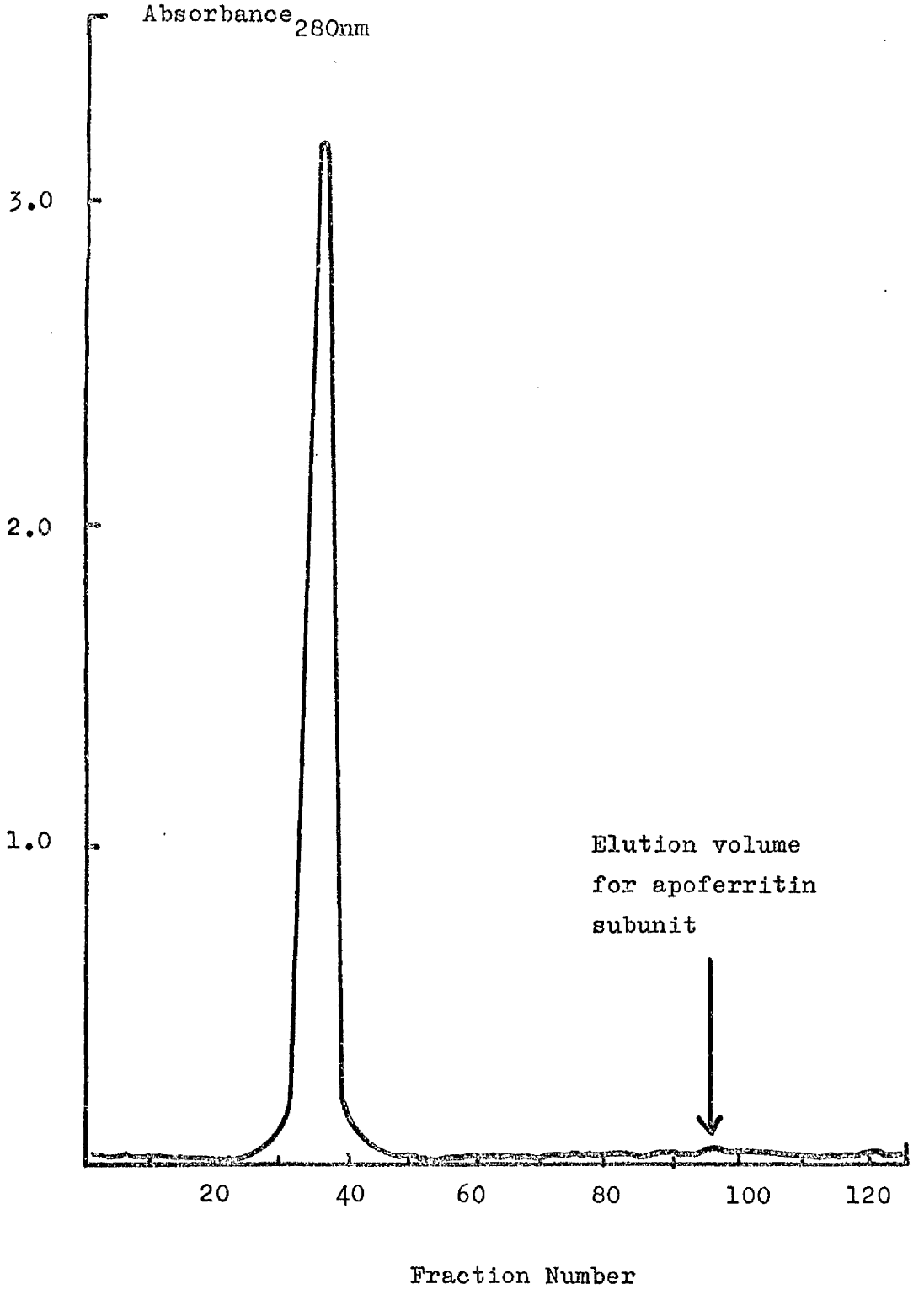
It can be seen that the reaction appears to be specific for the guanido group although, some workers have obtained a partial loss of cysteine, possibly as a result of the strong alkaline conditions used (Kassell, 1967). This side reaction has also been observed by other workers who demonstrated that the use of 0.2M sodium hydroxide as the solvent for modification of arginine by cyclohexanedione caused the destruction of disulphide bonds in certain disulphide-containing proteins (Habeeb and Habeeb, 1971). They also showed that when the reaction was carried out in 0.1M triethylamine at pH 10.9, no such reactions occurred and for this reason this solvent was chosen for the present study.

The pH for the reaction was 10.9 and it can be seen from Figures 57 and 59 (pages 260 & 265 respectively) that at this pH value the conformation of apoferritin will probably still be representative of the native state. If this is the case, then it would appear that nine of the ten arginine residues are accessible to the reagent while one residue is refractory. As with the case of the two refractive lysyl residues, this arginine could be inaccessible to the modifying reagent because it is at the surface of contact of subunit-subunit interaction, possibly in the form of a salt linkage.

The modified protein was analysed by a combination of gel chromatography on a calibrated Sephadex G-75 column and sedimentation velocity in the analytical ultracentrifuge and found to exist as a homogeneous species which corresponded to apoferritin monomer (Figure 70).

The catalytic activity of the arginine-modified protein was examined and this will be discussed at a later stage.

Figure 70. Typical elution profile obtained from the chromatography of arginine-modified horse spleen apoferritin on a column of Sephadex G-75 equilibrated with 0.1M borate buffer, pH 7.0. Protein was monitored at 280 nm. Modification was achieved by treatment with 1,2-cyclohexanedione.

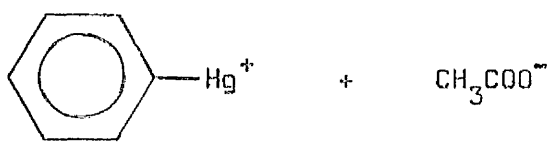

Absorbance 280nm
3.0
2.0
1.0
Elution volume
for apoferritin
subunit
20
40
60
80
100
120
Fraction Number

Modification at Cysteine Residues.

By Polarographic Titration.

By making use of the fact that free sulphydryl groups (-SH) will react with mercury salts to form mercaptide bonds, it has been shown possible to estimate the thiol content by polarographic titration.

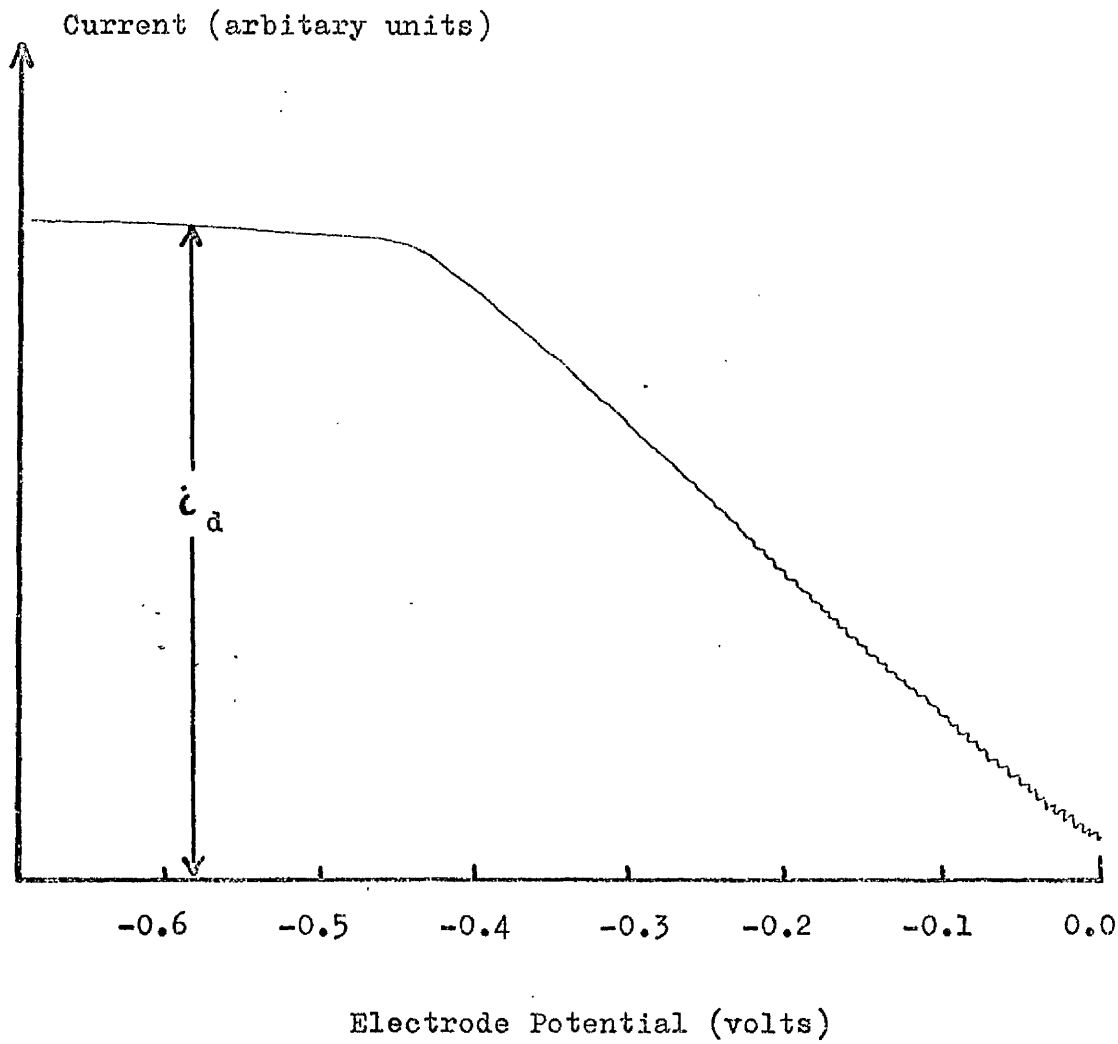
Essentially, this method consists of maintaining a solution of phenylmercuric acetate at an applied potential. The heavy metal salt ionises to give,



and the phenylmercuric ions ($PhHg^+$) migrate to the cathode and thereby cause a current known as the diffusion current. If, however, any material is present that is capable of complexing with the $PhHg^+$ ions then the diffusion current is decreased. More phenylmercuric acetate can be added until the complexing material is saturated and at that point the diffusion current increases sharply.

A typical polarographic wave is shown in Figure 71. The potential was gradually increased and the corresponding value of the current was recorded automatically. The discharge of each ion species is accompanied by a 'wave' represented by an increase of current until a constant current, the diffusion current (i_d), is reached. The height of the wave, i_d, is proportional to the concentration in the solution of the ion being discharged. The potential at the centre of the rising part of the wave is referred to as the half-wave potential and serves to identify the ion.

Figure 71. Schematic representation of a typical polarographic wave. i_d represents the diffusion current.


Current (arbitary units)
i_d
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
Electrode Potential (volts)

It can be seen that at the applied potential used in these experiments, -0.6 volts, we were within the plateau region.

The theory of this method has been outlined by Cecil and Snow (1962).

The results of the experiments for horse spleen apoferritin are shown in Figure 72. Also shown in this figure are the predicted titration curves on the basis of 2 and 3 free sulphydryl groups per subunit. It can be seen that in the present study, we were unable to titrate polarographically any sulphydryl groups and this suggests that the 1 cystine found on amino acid analysis is present as cystine in the native structure of the protein. Alternatively the thiol groups could be refractive to treatment with phenylmercuric acetate.

By Reaction with 5,5' - Dithiobis(2-nitrobenzoic) Acid.

The scheme for the reaction of sulphydryl groups with 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) is outlined in Figure 73.

A solution of apoferritin was incubated at pH 8.0 or 9.0 with DTNB under the conditions described on page 87 and the reaction was monitored at 412 nm. The reaction was allowed to proceed for 4 hours at 25^{o}C after which time there was still no significant increase in the absorbancy (0.016 moles/18,500 g protein). the protein was incubated in 1% SDS and the procedure repeated. Again there was no liberation of thiolate ion, indicating that there were no reactive sulphydryl groups.

5,5'-dithiobis-(2-nitrobenzoic) acid, Ellman's reagent, was designed as a specific reagent for thiol groups (Ellman, 1959) in 1959 and since that time it has proved eminently successful as shown by the numerous papers describing its use. It has been used for the determination of the total, the protein-bound and non-

Figure 72. Determination of thiol content of horse spleen apoferritin by polarographic titration. The solution contained protein at a concentration of 0.09 mg/ml, sodium dodecyl sulphate (1%), potassium chloride (35mM) and sodium sulphite (50mM). The capacity of the polarographic cell was 4 ml. Titrant (phenylmercuric acetate, 0.5 mM) was added in 10 µl aliquots.

●——● Results obtained from the present study.

O——O Theoretically derived results based on a thiol content of two cysteine residues per 18,500 g protein (see text)

Δ——Δ Theoretically derived results based on a thiol content of three cysteine residues / 18,500 g protein (see text)

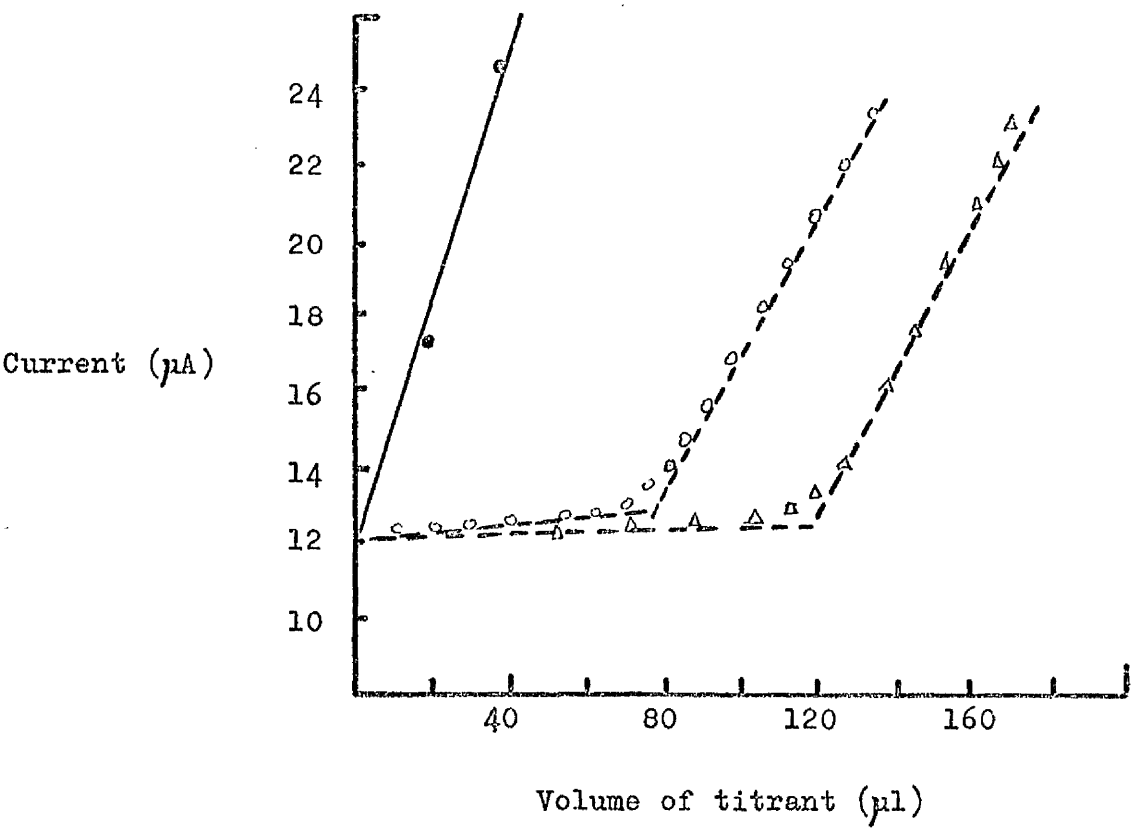
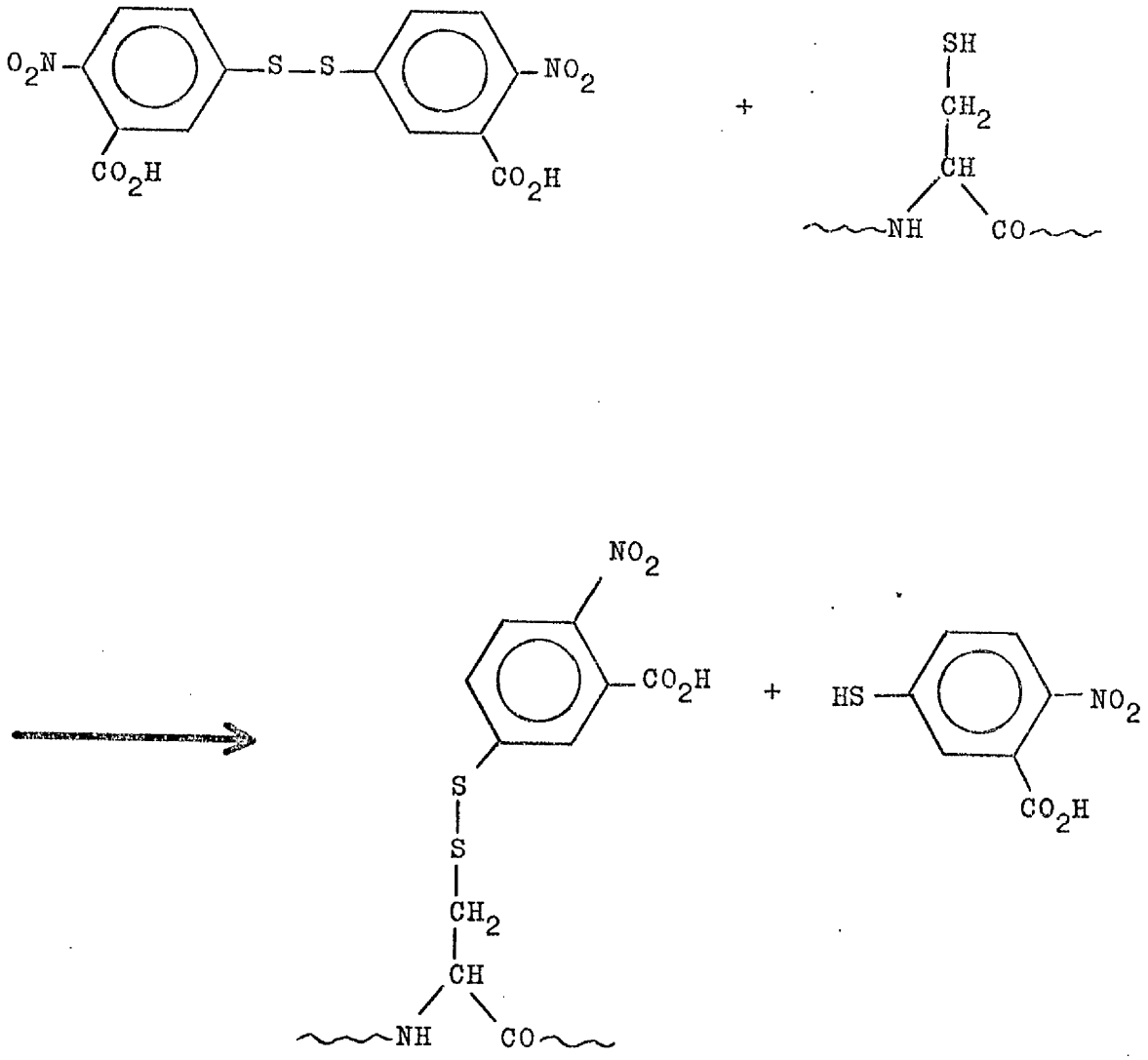

Current (μA)
24
22
20
18
16
14
12
10
40
80
120
160
Volume of titrant (μl)

Figure 73. Reaction of cysteinyl residues with 5,5'-dithiobis-(2-nitrobenzoic) acid.



protein bound sulphydryl groups (Sedlak and Lindsay, 1968) and also for the determination of disulphide groups in proteins (Cavallini, Graziani and Dupre, 1966; Zahler and Cleland, 1968). The technique was further developed for the location of thiol groups with differing reactivities at various levels of denaturants which render originally 'masked' thiol groups accessible to the reagent (Malkin and Rabinowitz, 1967; Younathan, Paetkau and Lardy, 1968; Eagles, Johnson, Joynson, McMurray and Gutfreund, 1969).

To ensure that the reagent was still functional we reacted it with glutathione and observed an absorption change at 412 nm. The change in molar absorption coefficient was evaluated and agreed well with the value indicated by Ellman (1959).

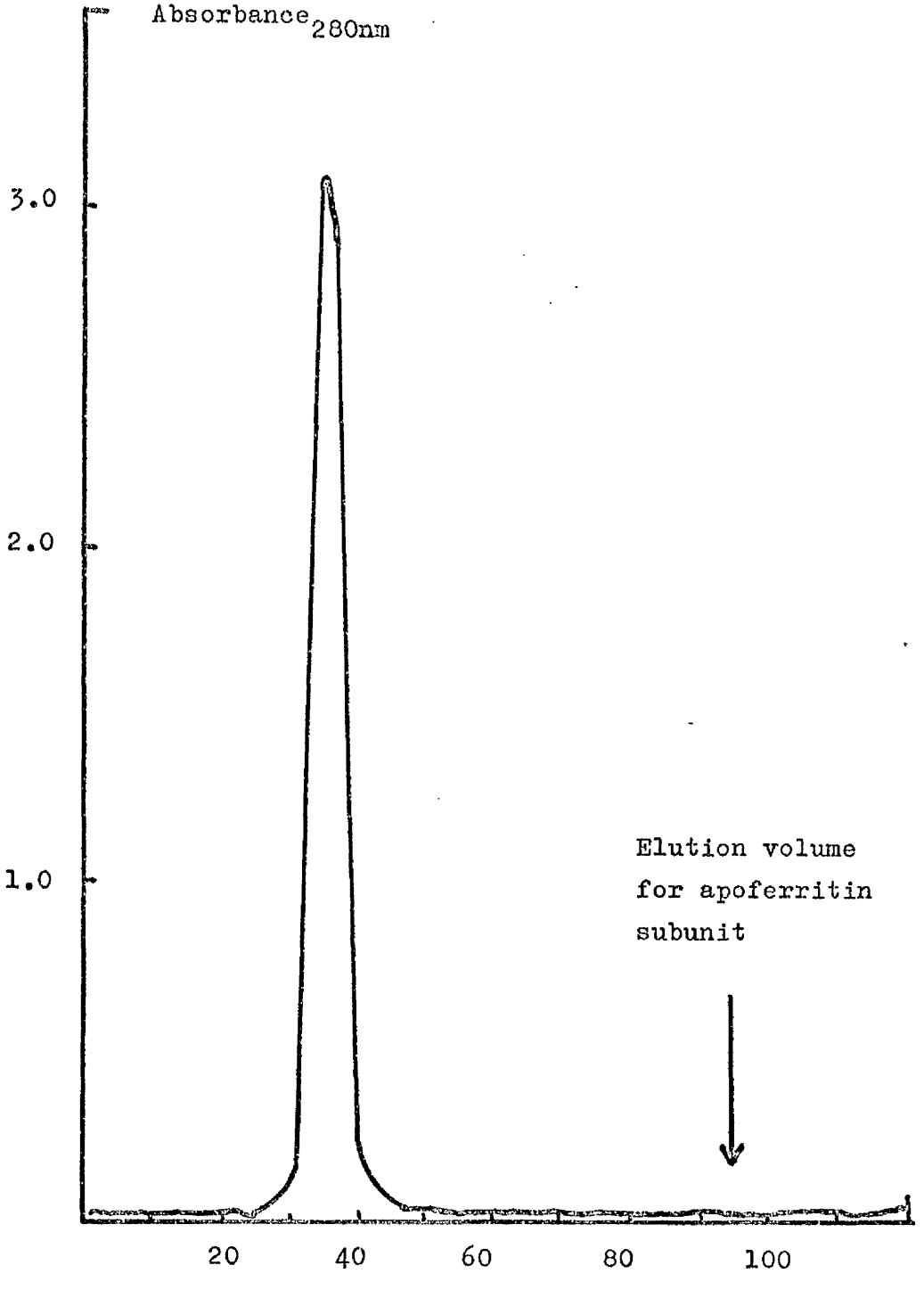
The result for apoferritin strongly suggested the presence of a disulphide bond and if there was another sulphydryl group then it would have to remain inaccessible to the reagent even after SDS-denaturation. Alternatively, the protein had either two or three sulphydryl groups and all of these would have to remain 'buried' on denaturation.

By Reaction with Iodoacetic Acid.

Apoferritin that had been reduced by 2-mercaptoethanol was reacted with iodoacetic acid - 2 - C^{14} in the absence and in the presence of sodium dodecyl sulphate as denaturant as described on page 86.

The purified labelled protein was run on a calibrated Sephadex G-75 column and the eluant was monitored at 280 nm. Fractions were collected and examined for radioactivity and the results of such a study are shown in Figure 74. Protein concentration was determined by amino acid analysis and by use of a

Figure 74. Typical elution profile obtained from the chromatography of carboxymethylated horse spleen apoferritin on a column of Sephadex G-75 equilibrated with 0.1M borate buffer, pH 7.0. Protein was monitored at 280 nm.
Carboxymethylation was achieved by treatment with iodoacetic acid at pH 8.6.


Absorbance 280nm
3.0
2.0
1.0
Elution volume
for apoferritin
subunit
20
40
60
80
100
Fraction Number

specific extinction coefficient, $E^{1\%}_{1cm}(280) = 9.81$. Thus, by knowing the protein concentration of a sample of carboxymethylated apoferritin and also the specific activity of the labelled iodoacetic acid, it was possible to calculate the number of groups carboxymethylated. The value for the number of groups modified was found to be the same, both in the absence and presence of denaturant, and this was 3.05 ± 0.07 residues per 18,500 g of protein.

A typical amino acid elution profile for carboxymethylated apoferritin is shown in Figure 75. It can be seen that a peak corresponding to S-carboxymethylcysteine is present and this lies just ahead of aspartic acid. No cystine peak can be observed. Another new peak elutes after alanine and prior to the normal elution position of cystine and this was identified as 3-monocarboxymethylhistidine.

The amino acid composition was determined for the modified apoferritin and the results are shown in Table 15. It can be seen that, the loss of cystine is balanced by the appearance of two residues of S-carboxymethylcysteine per 18,500 g protein and also the decrease in the histidine content by about one residue is matched by the appearance of a single residue of 3-monocarboxymethylhistidine, using the same colour factor for 3-monocarboxymethylhistidine as for histidine.

Between them, these residues account for the three carboxymethylated residues found by radioactive labelling studies.

The reaction sequences for the formation of these carboxymethylated amino acids are shown in Figure 76.

It has been demonstrated from studies with molecular models that in acetyl-L-histidine the 1-position is markedly hindered sterically, both by the carboxyl group and the α-acetylamino

Figure 75. Analysis of the acidic and neutral amino acids in carboxymethylated horse spleen apoferritin. Modification was carried out by treatment with iodoacetic acid at pH 8.6. The peaks, in order of elution, represent carboxymethylcysteine (not present in the control), aspartic acid (ASP), threonine (THR), serine (SER), glutamic acid (GLU), proline (PRO), glycine (GLY), alanine (ALA), 3-monocarboxymethylhistidine (not present in the control) and valine (VAL). No cystine peak was observed in the modified protein. The histidine peak of the modified protein (not shown here) was found to be lower than that of the control.

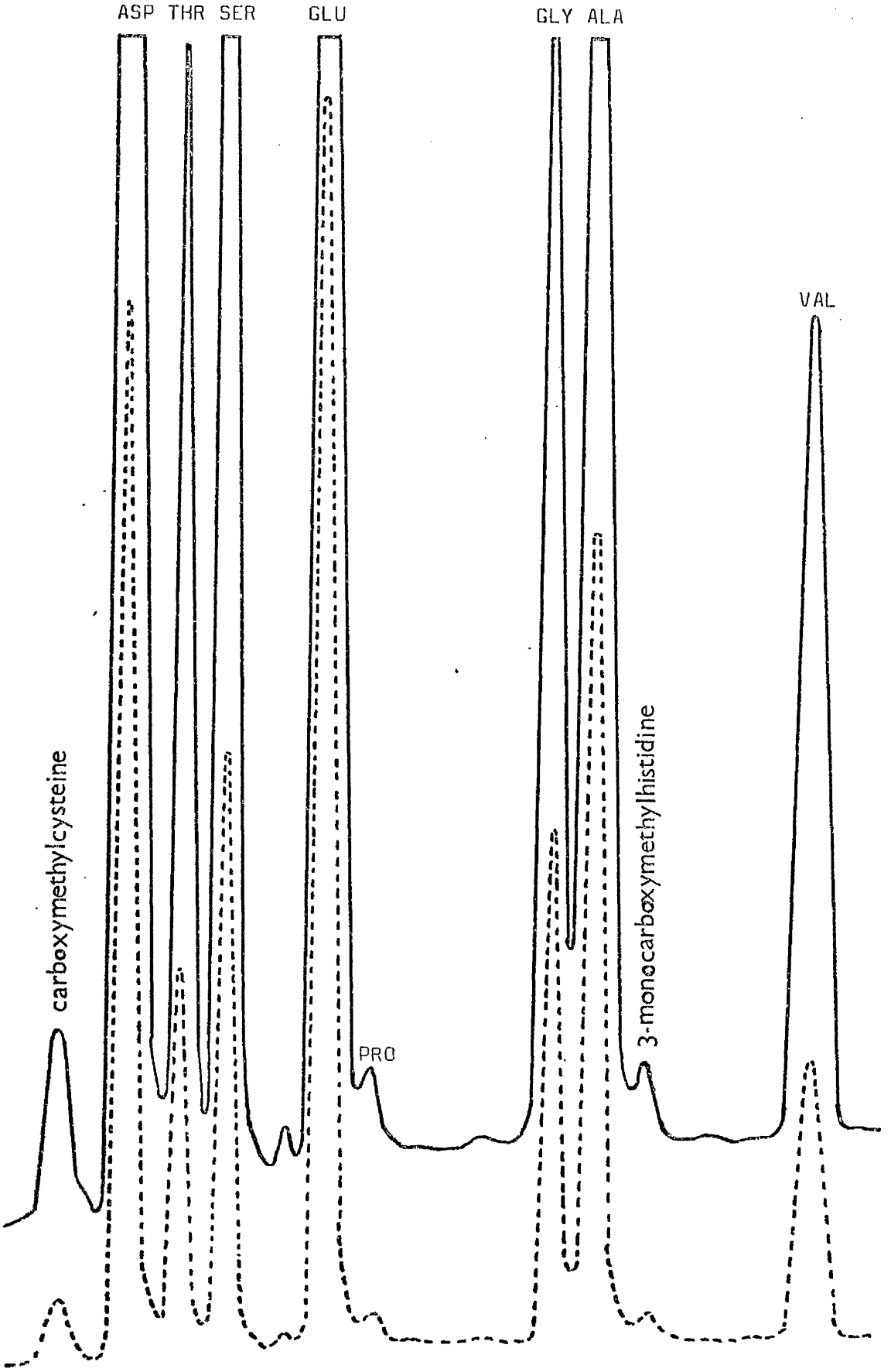

ASP
THR
SER
GLU
GLY
ALA
VAL
carboxymethylcysteine
PRO
3-monocarboxymethylhistidine

Table 15. Amino acid composition of carboxymethylated horse spleen apoferritin. Amino acid residues are expressed as the number of residues per 18,500 g of protein.

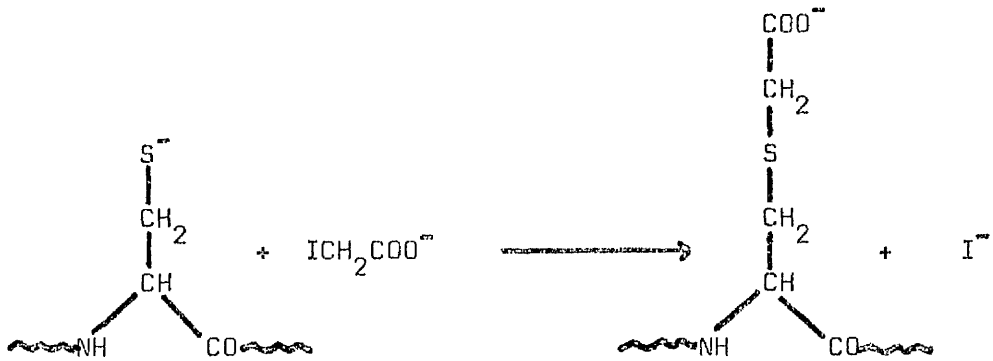
n.d, not determined.

S-CM-cysteine, S-carboxymethylcysteine

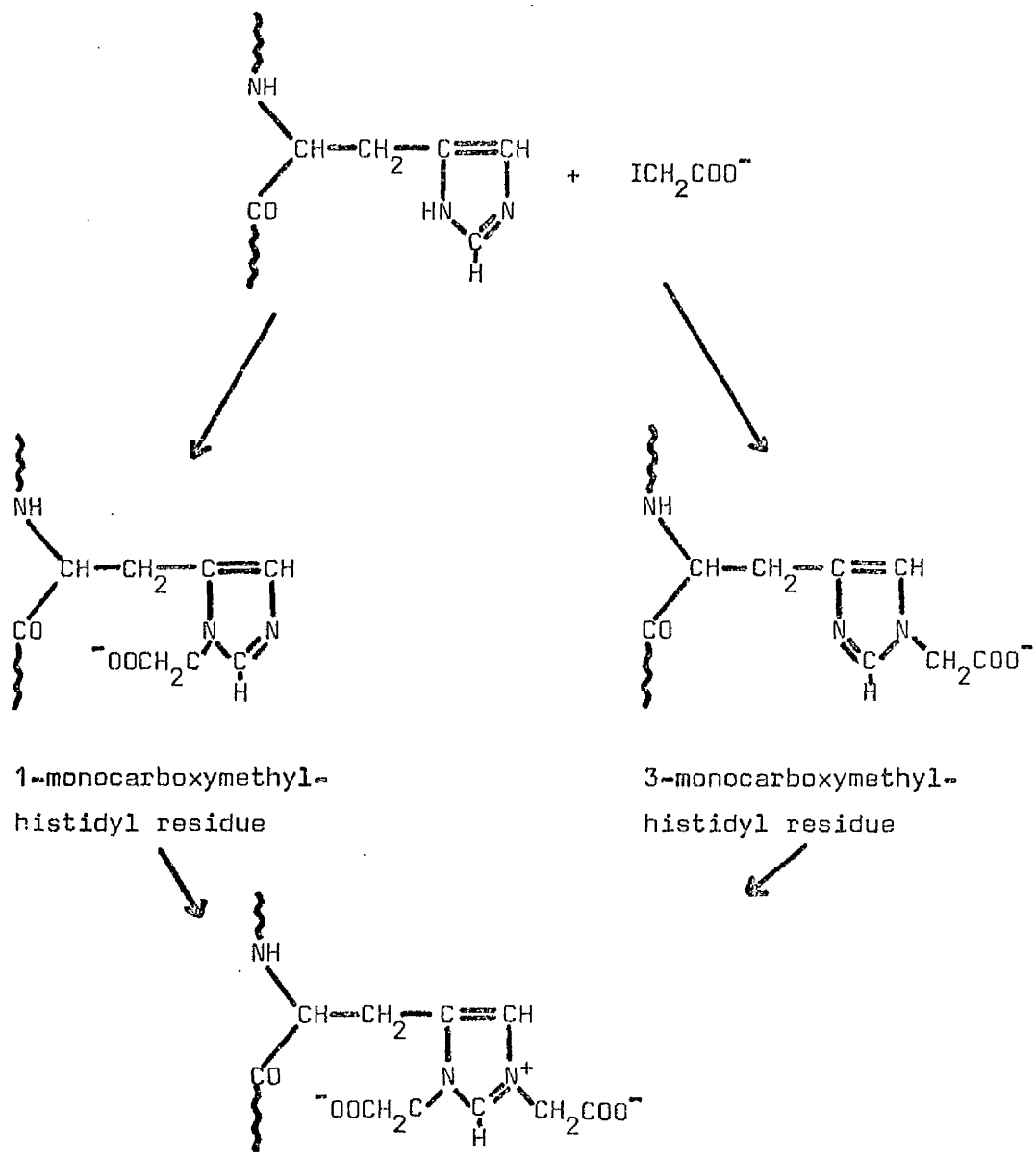
3-CM-histidine, 3-mono-carboxymethylhistidine.

Amino Acid Residue	Control	Modified
Cysteic acid	n.d	n.d
Aspartic acid	17.50	17.39
Threonine	5.21	5.57
Serine	8.99	8.93
Glutamic acid	23.73	24.04
Proline	2.41	3.25
Glycine	9.70	9.88
Alanine	13.88	14.11
Valine	6.91	6.30
Methionine	2.65	2.38
Isoleucine	2.95	3.75
Leucine	24.77	24.85
Tyrosine	4.94	4.80
Phenylalanine	7.11	6.83
Histidine	5.81	5.07
Lysine	8.55	8.21
Arginine	10.11	9.24
Tryptophan	n.d	n.d
S-CMcysteine	0.00	2.05
3-CM-histidine	0.00	1.02

Figure 76. Reaction of cysteinyl and histidyl residues with iodoacetic acid.



S-carboxymethyl-
cysteinyl residue



1-monocarboxymethyl-
histidyl residue

3-monocarboxymethyl-
histidyl residue

1,3-dicarboxymethylhistidyl residue

group. For this reason the 3-isomer would be expected to be formed in the greatest amount and this is observed, both in other studies (Crestfield, Stein and Moore, 1963a,b) and in the present one with apoferritin.

Since the modification rection was carried out at pH 8.6 most, if not all, of the histidine residues would be in the basic form. To avoid carboxymethylation of histidine groups in proteins at pH 8.6, some workers have restricted the concentration of alkylating agents to a small molar excess (Cole, Stein and Moore, 1958; Sela, White and Anfinsen, 1959). This allows the more reactive sulphydryl groups to be preferentially alkylated. In the present case it would appear that in apoferritin there exists a very reactive histidine. It would also seem reasonable to assume two ½cystine residues and this is consistent with the data of Williams and Harrison (1968) who found 1.89 residues / 18,500 g protein. It is also in very good agreement with the data of Mazur,Litt and Shorr (1950) who deduced 1.92 residues / 18,500 g protein from studies with protein modified by iodoacetamide at pH 7.4. The fact that there are two ½ cystine residues per subunit and that these could exist in the native structure of apoferritin in the form of a disulphide bond provides an explanation for being unable to titrate any free sulphydryl groups both polarographically and by titration with DTNB, and this seems a reasonable interpretation of the data of these two earlier studies.

Again the pure modified protein was analysed by sedimentation velocity in the analytical ultracentrifuge and by column chromatography in the calibrated column of Sephadex G-75 and was found to exist solely as the monomer and, that no dissociation into subunits occurred as a result of carboxymethylation.

Functional Aspects of Apoferritin.

Preliminary Experiments.

Before studying the nature of the protein's catalytic activity it is necessary to consider some of the fundamental aspects of the chemistry of iron salts.

Iron has 26 electrons and, according to the electronic theory of valency, these would be arranged in the following way,

$$(1s)^2 \ , \ (2s)^2 \ , \ (2p)^6 , (3s)^2 (3p)^6 \ , \ (4s)^2 \ , \ (3d)^6$$

The most stable form of the iron ion in solution is the ferric state, Fe^{+++}, in which the two (4s) electrons and one (3d) electron are lost, leaving five unpaired electrons in the d-level, which is exactly one for each orbital and this gives a stable configuration to the outer shell. Ferrous ion, Fe^{++}, results from the loss of two (4s) electrons, leaving an outer shell of $(3s)^2(3p)^6(3d)^6$ in the third layer and this is also relatively stable. However, owing to the limited solubility of ferric salts in aqueous solvents it was necessary to select a suitable buffer system in which the solubility product for both the ferrous and ferric salt was not exceeded and also one in which rapid autoxidation was minimal, thereby allowing the catalytic oxidation of ferrous iron to be conveniently monitored

Although ferrous ammonium sulphate is less soluble than ferrous sulphate, it is less easily oxidised by air and for this reason was preferred as the starting material in the present investigation. Since the autoxidation of ferrous ion is pH dependent, we carried out a preliminary study on this aspect, prior to an investigation for a suitable buffer. The percentage of ferrous iron remaining in solution was determined by the quantitative

bipyridyl reaction using an experimentally derived molar extinction coefficient of 8158. The values obtained were plotted as a function of pH as shown in Figure 77. From this it can be seen that ferrous iron is stable in acid solution up to about pH 6.0. On increasing the pH the ferrous ion is slowly oxidised to ferric iron until at pH 9.0 and above, no ferrous iron exists. This result is in very good agreement with that found by Conrad (1970) for ferrous chloride. From this study we decided to use buffer at pH 6.0 and the various buffers investigated are listed in Table 16 together with the observed solubility of ferrous ammonium sulphate in each of these.

It was shown, as might have been expected, that phosphate buffers could not be used and this is most probably because all metallic phosphates, except those of the alkali metals, sodium, potassium etc., are insoluble in water.

When ferrous ammonium sulphate was dissolved in 2% sodium carbonate, a heavy precipitate was formed. In this case the ferrous carbonate was, presumably, rapidly oxidised by air to ferric carbonate which hydrolysed at once to hydrated ferric oxide as follows;

$$2Fe_2(CO_3)_3 + 6H_2O \longrightarrow 2Fe_2O_3.3H_2O + 6CO_2$$

This was also observed in neutral bicarbonate buffers and could be explained in the same way.

Imidazole and imidazole containing sodium thiosulphate and potassium iodate also proved to be unsatisfactory in that a precipitate of ferric oxide was observed on addition of ferrous ammonium sulphate.

Tris-maleate-sodium hydroxide also proved unsatisfactory, although in this case the ferrous ammonium sulphate was oxidised

Figure 77. Solubility of ferrous iron as a fuction of pH. Ferrous iron was determined as the red-coloured Fe^{++}-bipyridyl complex at 520 nm (see text).

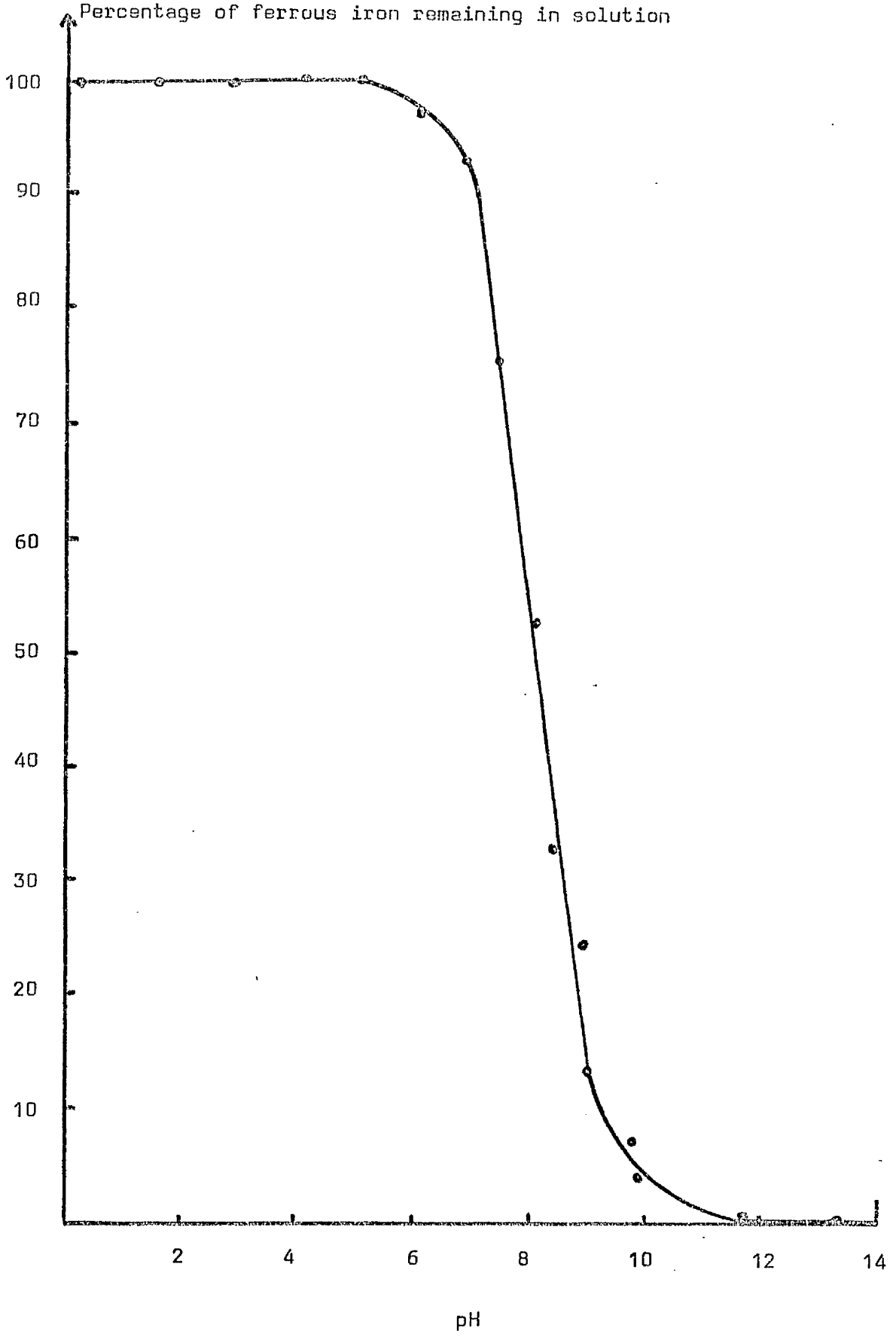

Percentage of ferrous iron remaining in solution
100
90
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
pH

Table 16. List of solvents tested for suitability for use in assay system. Using these the solubility of ferrous ammonium sulphate was tested and the results are shown.

\+ soluble.

\- insoluble.

Solvent system	Solubility
distilled water	+ + + +
0.1M phosphate buffer	- - - -
0.001M phosphate buffer	- -
0.1M sodium carbonate	- - - -
0.02M bicarbonate buffer	- - -
0.1M imidazole buffer	- - -
0.1M imidazole buffer, 50mM potassium iodate, 200mM sodium thiosulphate	- - -
0.01M tris-maleate-sodium hydroxide buffer	+
0.1M borate buffer	+ +
0.02M borate buffer	+ + +
0.1M borate-cacodylate buffer	+ + + +
0.02M borate-cacodylate buffer	+ + + +

to a ferric salt which was soluble. However, it was shown by the quantitative bipyridyl reaction that the oxidation of ferrous ammonium sulphate in this system was extensive.

In the case of 100 mM borate buffer there was a slight initial precipitate of ferric oxide on addition of ferrous ammonium sulphate as shown in Figure 78 but it was found that this could be conveniently removed by centrifugation and the colourless solution that resulted could be used in an enzyme assay system. Protein (~10 mg/ml) was extensively dialysed against 100 mM borate buffer, pH 6.0. 100 µl were added to 1 ml of ferrous ammonium sulphate solution (~5 mg/ml) and the reaction was monitored at 420 nm as shown in Figure 79. It can be seen that there is an initial hyperbolic increase in the optical density indicating that some oxidation was occurring. However, when the process was repeated with lysozyme and bovine serum albumin, similar effects were noticed as shown in Figure 80. Since it seemed unlikely that both these proteins should express a similar ferroxidase activity, the apparent catalysis was most likely to be non-enzymic.

An indication as to a possible explanation of these results came from a study on the effect of ionic strength on the solubility of ferrous ammonium sulphate. The results of this study are shown in Figure 81 and it can be seen that the solubility decreases with increasing molarity. One possible explanation is that on dissolving ferrous ammonium sulphate the pH may decrease and the solubility would be higher at acid pH. As the molarity increases, the buffering capacity increases and so the solution does not become so acid.

On testing this it was, in fact, found that the pH of borate buffer was lowered on dissolving crystalline ferrous ammonium sulphate and this demonstrates another important aspect of the

Figure 78. Solubility of ferrous iron in 100 mM borate buffer, pH 6.0 as a fuction of time. A biphasic relationship is observed, with an initial fast deposition of FeOOH followed by a very slow oxidation of the ferrous iron.
Ferrous iron was determined as the red Fe^{++}-bipyridyl complex at 520 nm (see text).

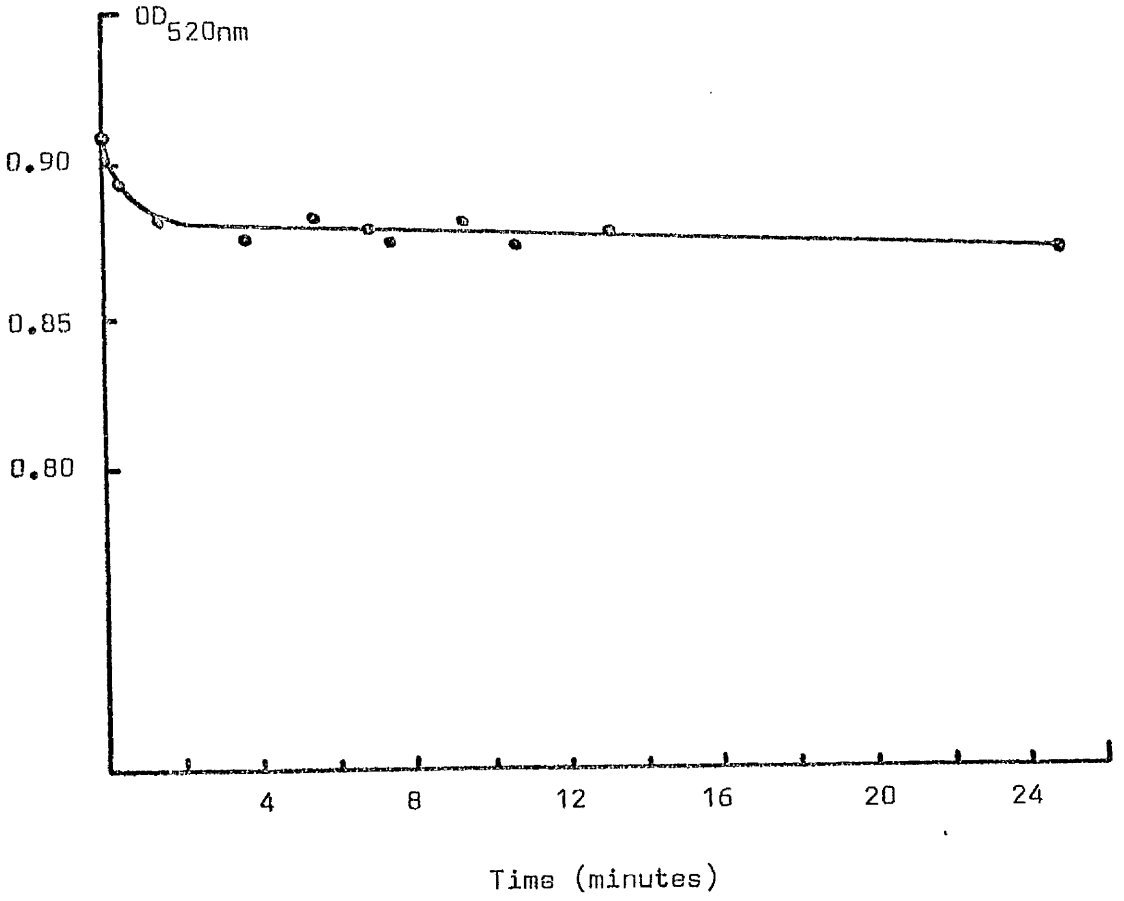

OD_{520nm}
0.90
0.85
0.80
4
8
12
16
20
24
Time (minutes)

Figure 79. Oxidation of ferrous iron caused by the addition of a solution of horse spleen apoferritin (2 mg/ml) to a solution of ferrous ammonium sulphate in 100 mM borate buffer, pH 6.0.

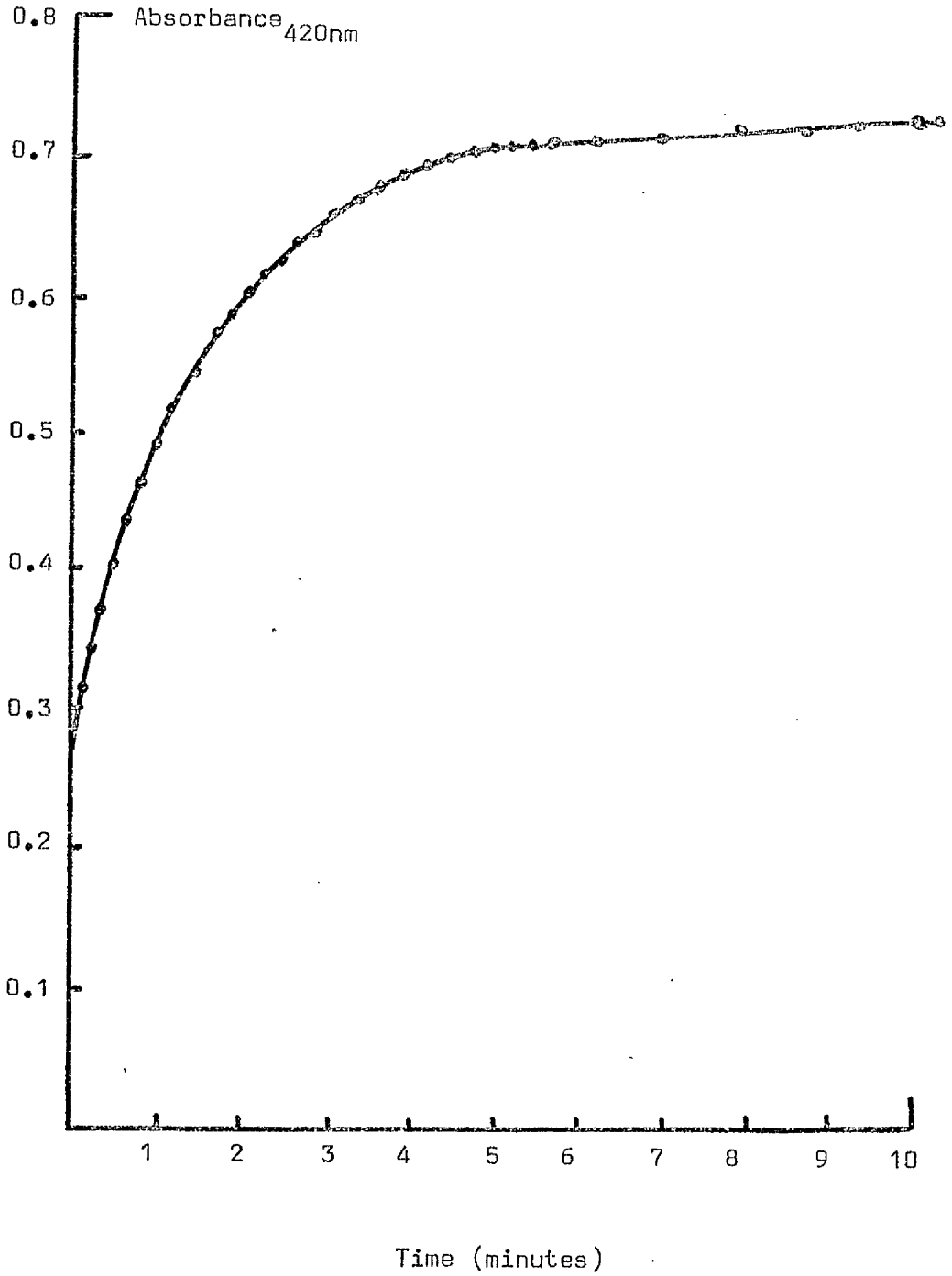

0.8
Absorbance 420nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
Time (minutes)

Figure 80. Oxidation of ferrous iron caused by the addition of a solution of albumin (A, 4 mg/ml) or lysozyme (B, 4 mg/ml) to a solution of ferrous ammonium sulphate in 100 mM borate buffer, pH 6.0.

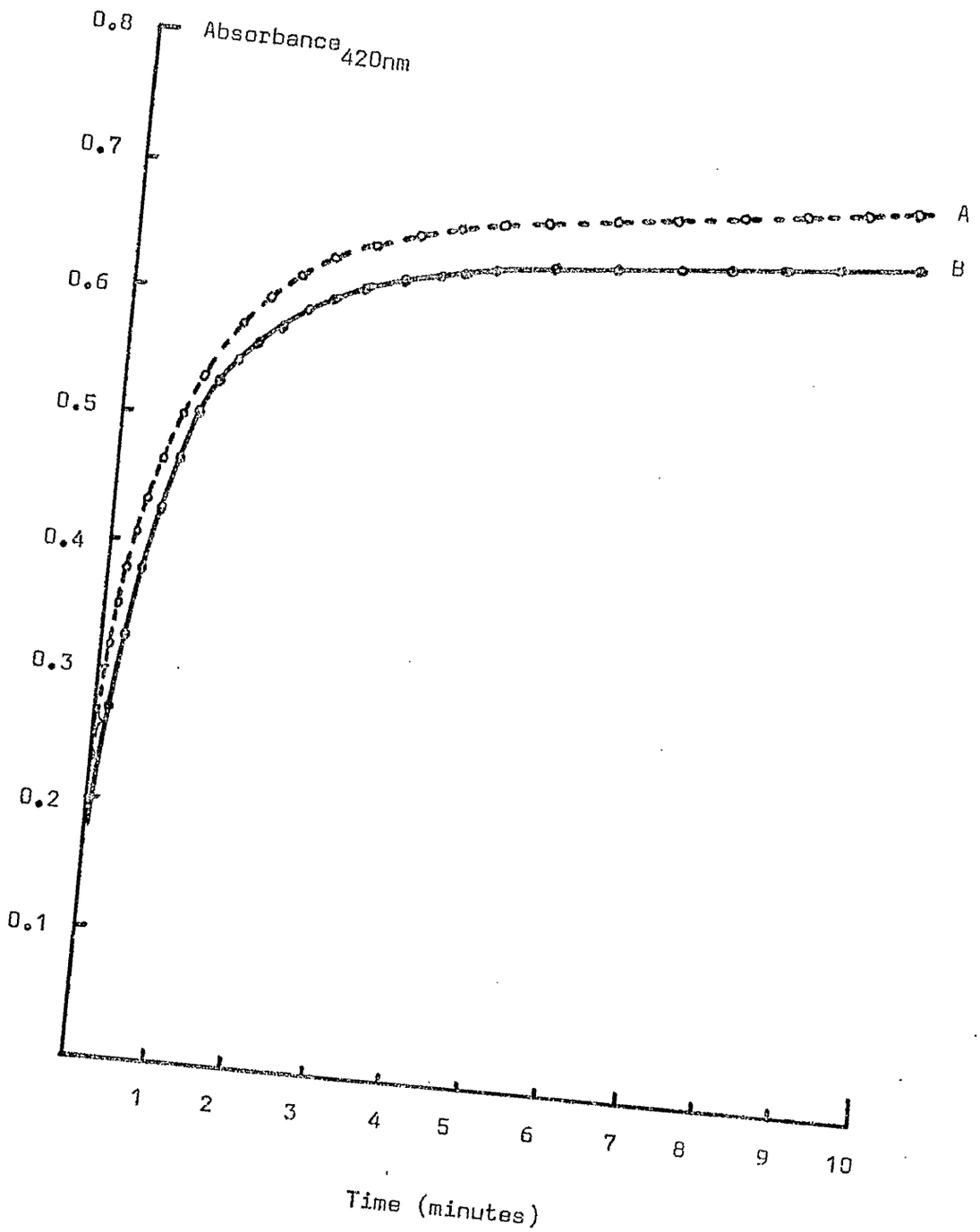
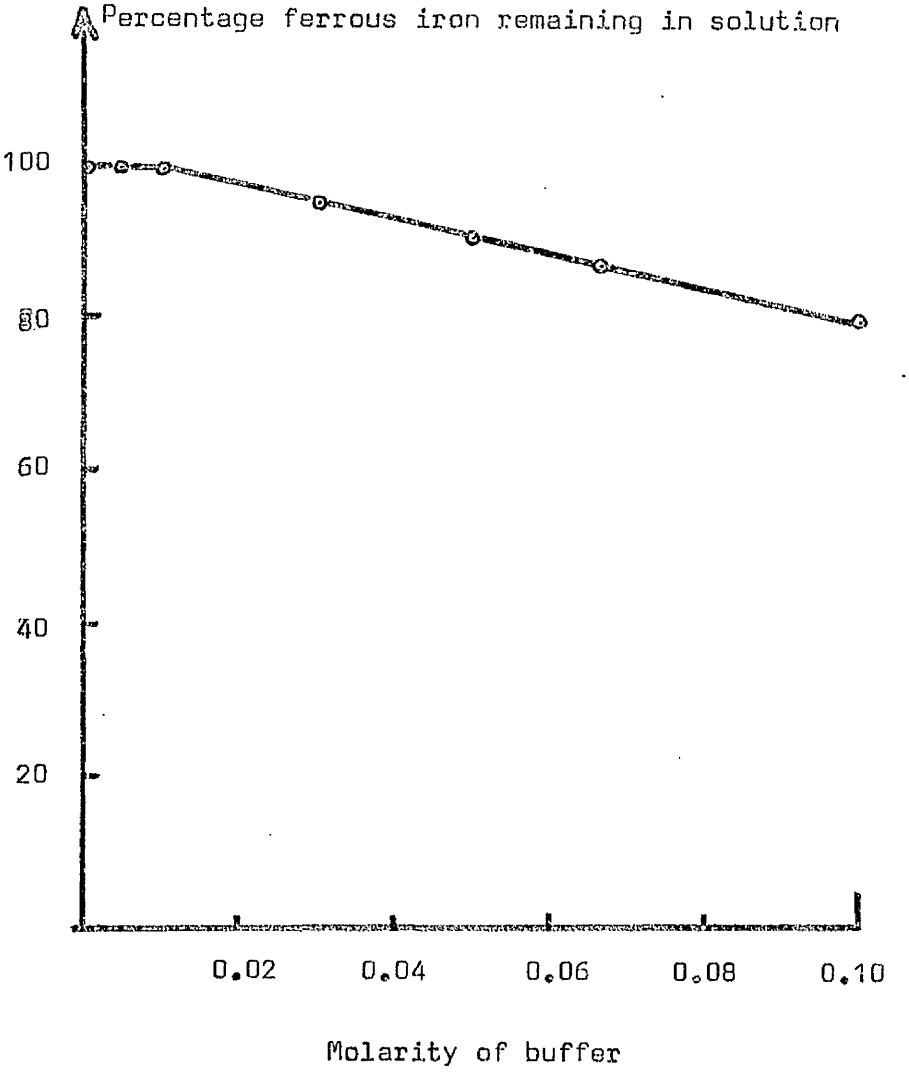

0.8
Absorbance 420nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A
B
1
2
3
4
5
6
7
8
9
10
Time (minutes)

Figure 81. Effect of the molarity of borate buffer on the solubility of ferrous iron. Ferrous iron was determined as the red-coloured Fe^{++}-bipyridyl complex at 520 nm (see text).


Percentage ferrous iron remaining in solution
100
80
60
40
20
0.02
0.04
0.06
0.08
0.10
Molarity of buffer

chemistry of the metal .

In general, reactions in aqueous solutions are reactions of complex ions. For example, a solution of ferric chloride is really a solution containing hydrated ferric and chloride ions in a number of complexes formed from the ferric and chloride ions viz,

$$FeCl^{+2}\ ,\ FeCl_2^{+},\ FeCl_3\ ,\ FeCl_4^{-}\ ,\ FeCl_5^{-2}\ ,\ FeCl_6^{-3}$$

In addition, reactions in aqueous solutions are complicated since the chloride and ferric ion have a hydration shell attached, and therefore even these are complex ions. Thus to understand the reactions of ferric chloride, we must understand the factors that determine the structure and reactivity of complex ions. It is accepted that with increasing charge and decreasing size of the cation, the hydration energy increases (Speakman, 1962). The effective radius for ferric cation is quite small, 0.6 Å, and thus the hydration energy is high.

$$Fe^{+3} + 6H_2O \rightleftharpoons Fe^{3+}(H_2O)_6$$

With such a high bond energy , the electrons concentrate between the Fe^{+++} and oxygen atoms. This shift of electrons makes the hydrogen atom more positive than in normal water and leads to repulsion of the proton by the positively charged ferric ions, coupled with a weakening of the OH bond, hence the following hydrolysis occurs;

$$(H_2O)_5\cdot Fe\overset{3+}{:}\ddot{O}:H \rightleftharpoons (H_2O)_5\cdot Fe:\ddot{O}:^{+2} + H^+$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad H$$

This then is a possible explanation as to why solutions containing iron yield acid solutions whereas solutions of sodium salts (for which the cation hydration energy is low) do not hydro-

lyse, but yield neutral solutions. It would appear then, that 100 mM borate buffer did not have sufficient buffering capacity to cope with this effect and for this reason we attempted to find another buffer system in the region pH 4 - 8 which would have sufficient buffering capacity to resolve this problem. The buffer system chosen was 100mM borate-cacodylate.

With this buffer system we carried out preliminary investigations on the effect of the buffer pH and ionic strength on the oxidation of ferrous ammonium sulphate and the extent of the changes of the buffer pH caused by dissolving the ferrous ammonium sulphate as described earlier (page 91).

Figure 82 shows the amount of ferrous ammonium sulphate in solution at a variety of pH values as a function of time. From this figure it can be seen that at pH 7.4 the rate of oxidation of ferrous iron is very high while at pH values lower than about pH 5.5 there is virtually no oxidation taking place. In Figure 83 these data are plotted as percentage of ferrous ammonium sulphate in solution as a function of pH. It will be seen later that the initial velocity can be measured within about 4 minutes of dissolving ferrous ammonium sulphate and therefore the amount oxidised is not significant until values in excess of about pH 6.0.

The effect of ferrous ammonium sulphate (F_2AS) on the initial pH of the buffers at a variety of pH values is plotted in Figure 84. It can be seen that virtually no change in buffer pH occurs up to about pH 7.0 while at higher values the pH decreases significantly. Thus it would appear that 100mM borate-cacodylate buffer has significant buffering capacity in the region pH 3.0 - 7.0 to counteract the inherent acidity of the ferrous and ferric hydrated ions.

Figure 82. The effect of pH on the solubilty of ferrous iron in 100mM borate-cacodylate as a function of time. Ferrous iron remaining in solution was determined as the red-coloured Fe^{++}-bipyridyl complex at 520 nm.

—— borate-cacodylate buffer, pH 4.656

×——× borate-cacodylate buffer, pH 5.222

Δ——Δ borate-cacodylate buffer, pH 5.380

□——□ borate-cacodylate buffer, pH 5.556

o——o borate-cacodylate buffer, pH 5.919

Δ——Δ borate-cacodylate buffer, pH 6.391

▣——▣ borate-cacodylate buffer, pH 6.888

⊕——⊕ borate-cacodylate buffer, pH 7.402

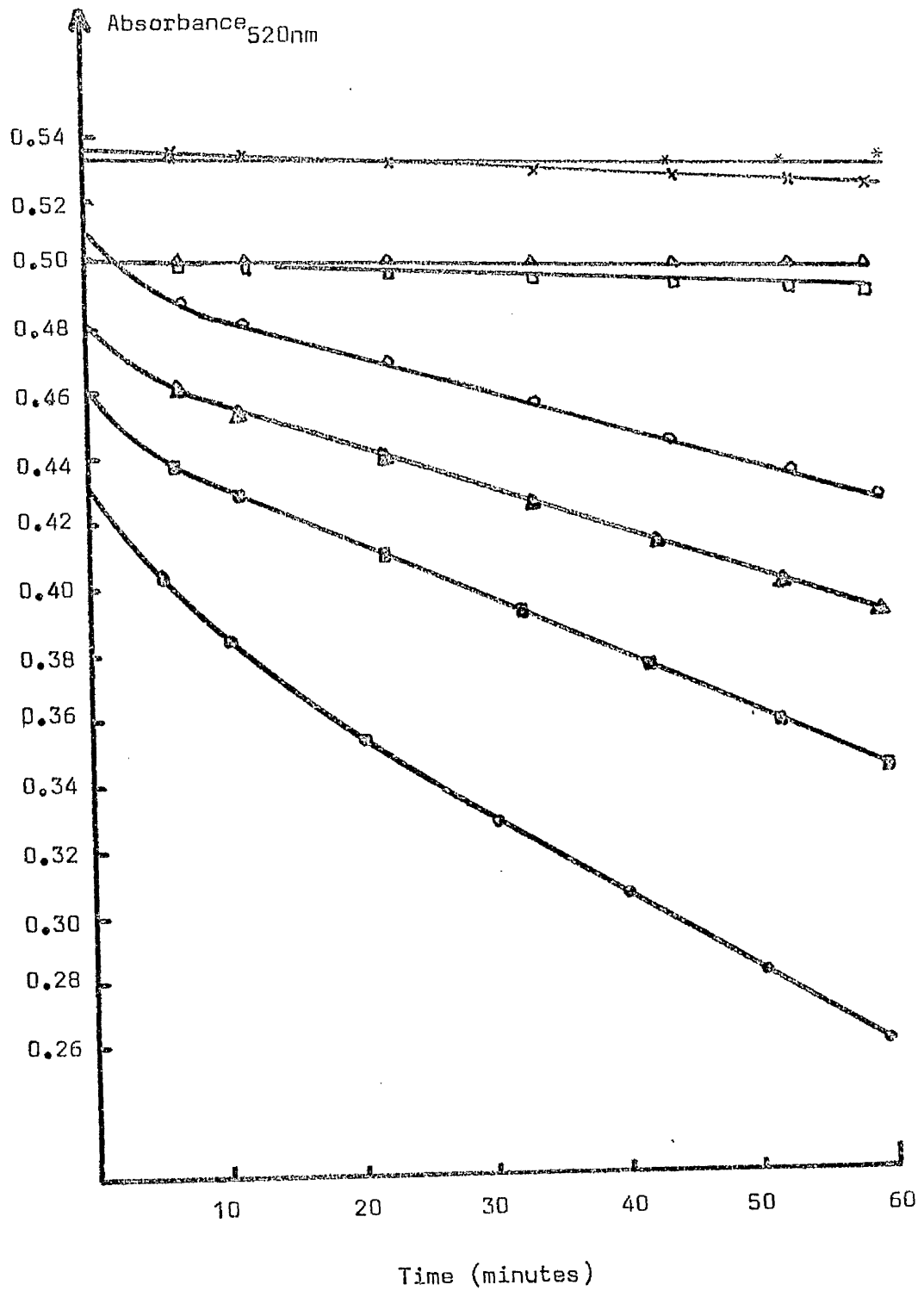

Absorbance 520nm
0.54
0.52
0.50
0.48
0.46
0.44
0.42
0.40
0.38
0.36
0.34
0.32
0.30
0.28
0.26
10
20
30
40
50
60
Time (minutes)

Figure 83. The effect of pH on the solubility of ferrous iron in 100mM borate-cacodylate buffer as a function of time. Ferrous iron remaining in solution was determined as the red-coloured Fe^{++}-bipyridyl complex at 520 nm (see text).

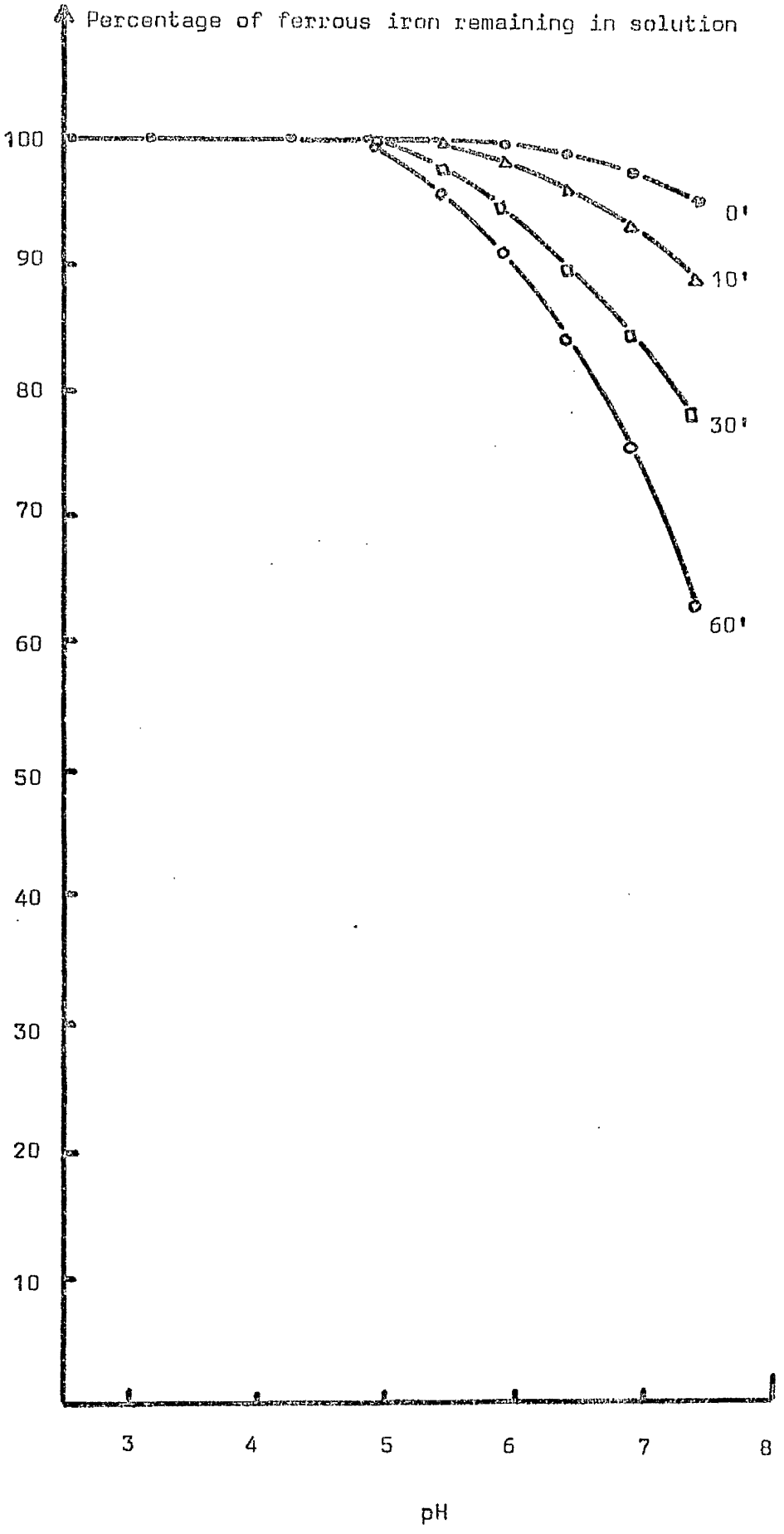
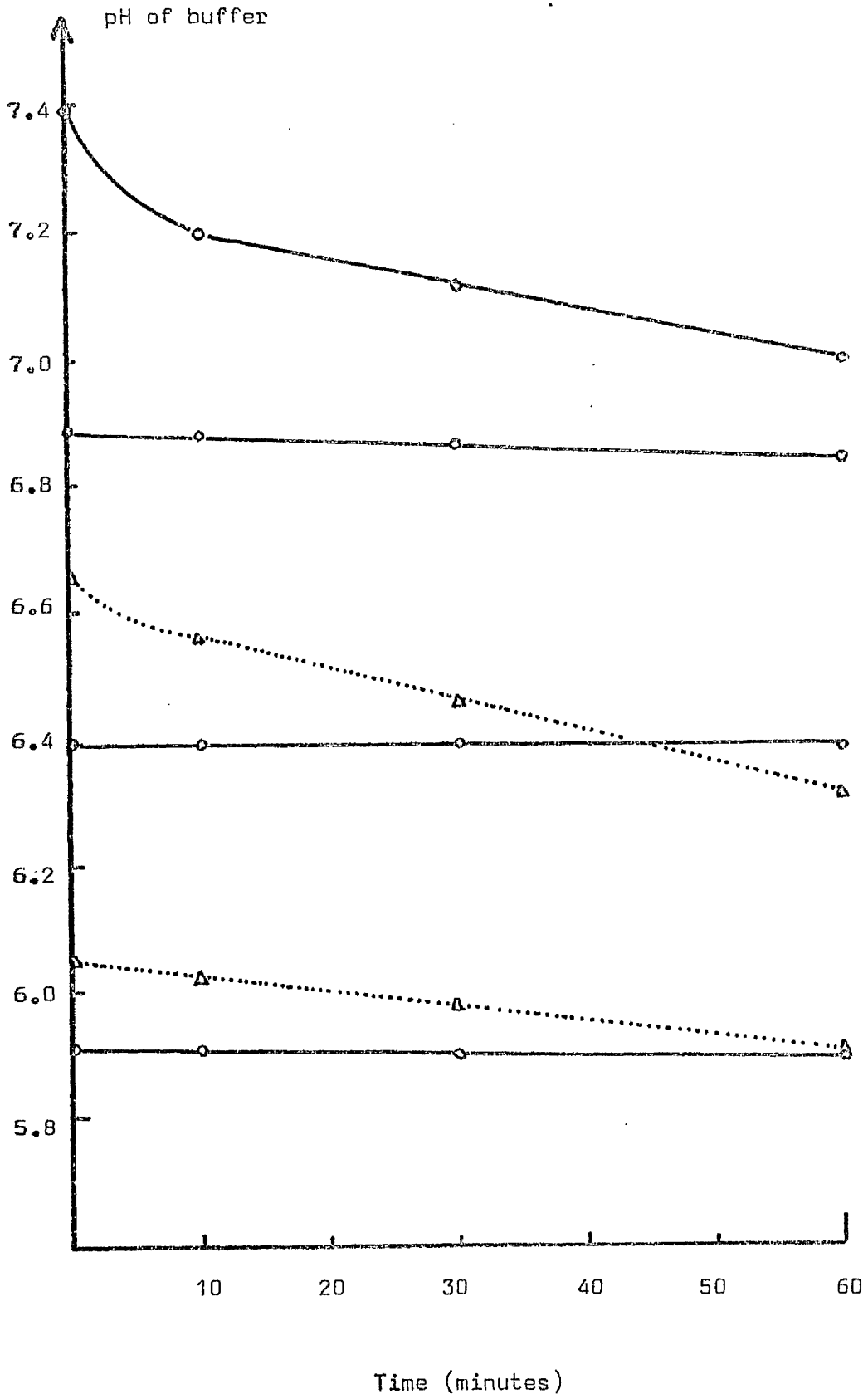

Percentage of ferrous iron remaining in solution
100
90
80
70
60
50
40
30
20
10
0'
10'
30'
60'
3
4
5
6
7
8
pH

Figure 84. The effect of the addition of ferrous ammonium sulphate on the initial pH values of a variety of borate-cacodylate buffers. Accurate pH values were determined by use of a Radiometer pH meter, model 25 equipped with a times-ten scale expander (Radiometer, Copenhagen, Denmark).

o——o These curves represent data obtained for 100mM borate-cacodylate buffers. The pH values for the buffers were (from the top down) 7.402, 6.888, 6.391 and 5.919. Data was also obtained for buffers at pH values 5.556, 5.380, 5.222, 4.656, 4.226 and 3.168 but these are not shown. The pH's of these buffers did not change significantly.

Δ····Δ These curves represent data obtained for 20mM borate-cacodylate buffers. The pH values for the buffers were (from the top down) 6.663 and 6.062.


pH of buffer
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
10
20
30
40
50
60
Time (minutes)

Identification of Product.

A quick and reliable test for identifying ferritin is to electrophorese a sample on polyacrylamide gel and stain duplicate gels with either amido black for protein or with potassium ferrocyanide for iron. When this is done for ferritin the iron-containing band is in an identical position as the protein zone. The results of such an electrophoresis for the reconstituted ferritin of these studies is shown in Figure 85.

This method effectively only tells us that the protein contains some iron but does not, however, give us any indication of the distribution of iron within the protein i.e. it would not be possible by this method to distinguish between a sample in which 50% of the material contained 'full' ferritin and 50% apoferritin and a sample of 'partially-filled' ferritin which contained about 50% of the total iron possible. For this reason it was important to monitor on some form of distribution of the ferritin molecules, be it on a molecular weight basis as in sedimentation velocity studies in the analytical ultracentrifuge or, by direct visualisation by use of the electron microscope.

By the method employed for electron microscopy it is possible to differentiate between ferritin and apoferritin as demonstrated in Figure 86. The ferritin molecules can be seen to have characteristic dense, particulate cores and peripheral electron-lucent shells while apoferritin molecules appear to have no such cores and in some molecules there is even a marked substructure as was discussed earlier on page 213. From these electron micrographs it is possible to determine the approximate size of the core and of the protein shell and these were found to be approximately 70 Å and 120 Å respectively.

It has been found that by using the following equation it

Figure 85. Identification of reconstituted ferritin by gel electrophoresis. Duplicate gels were stained for protein by amido black (gel, right) or for iron by potassium ferrocyanide (gel, left)

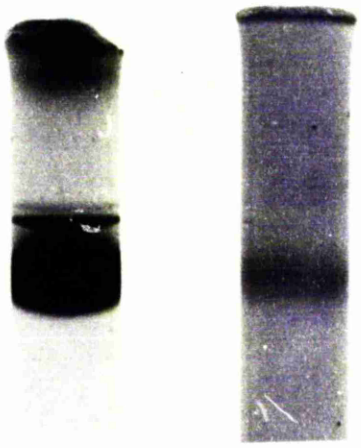
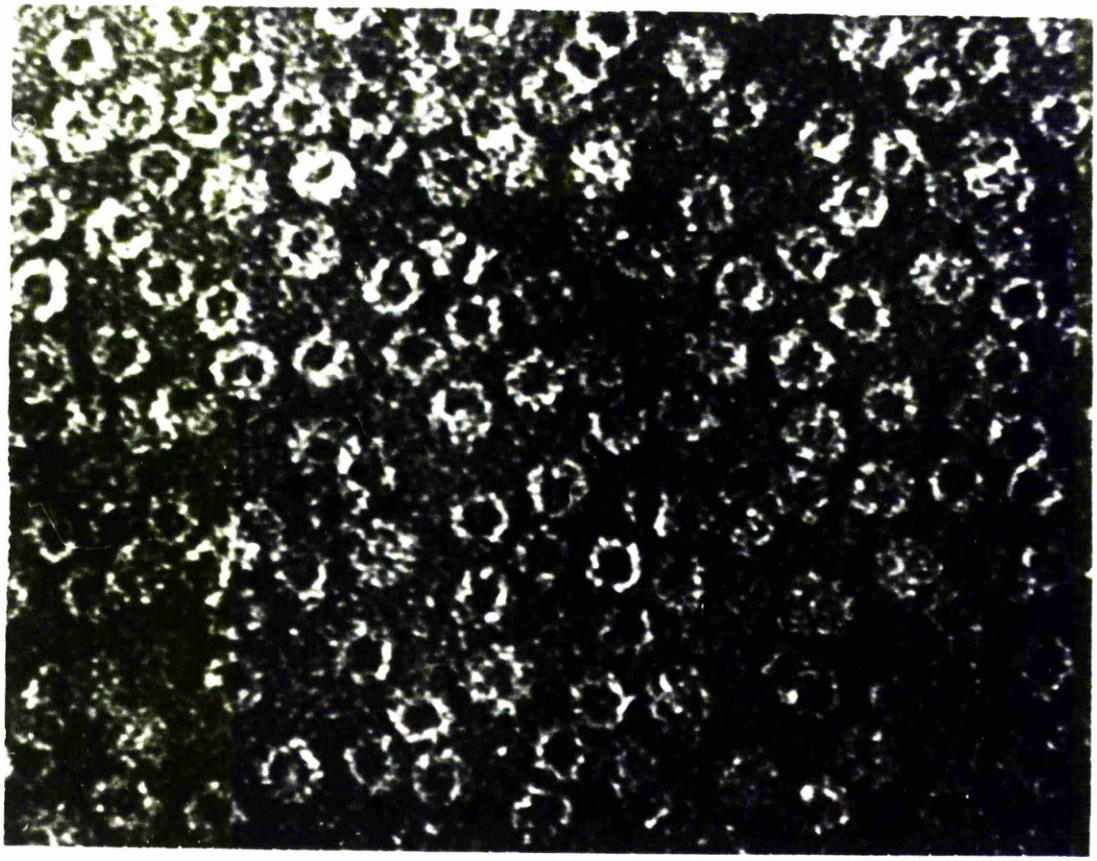
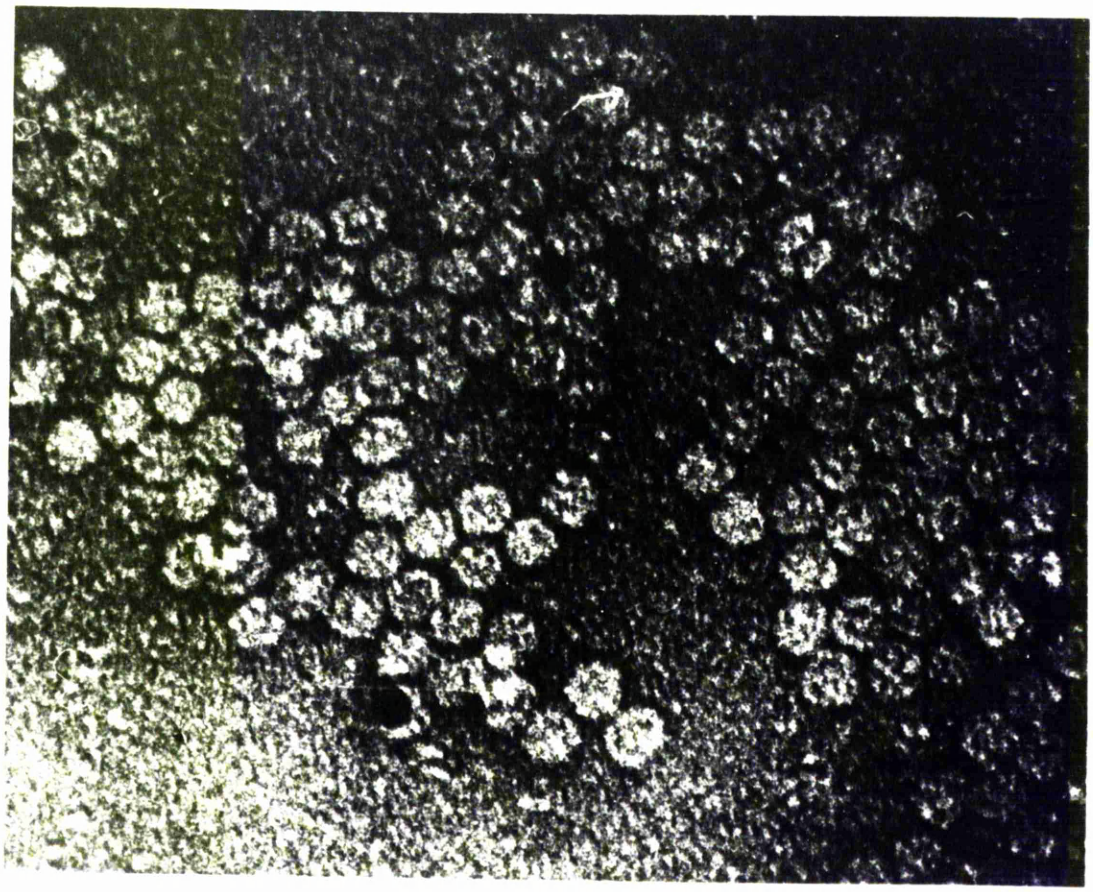


Figure 86. Differentiation of ferritin and apoferritin by electron microscopy. Top frame: ferritin molecules with characteristic dense, particulate core and peripheral shell. Lower frame: Apoferritin molecules with some evidence for detailed substructure.

Magnification x 700,000





is possible to derive these two parameters theoretically,

$$4/3\pi R^3 = M/\mathcal{N}\,(\bar{v}_2 + \delta_1 v_1^o)$$

(Tanford, 1966), where $\bar{v}_2$ is the partial specific volume of solute, v_1^o is the partial specific volume of the solvent, δ_1 is the effective solvation and is assigned a value of 0.19 g/g of dry protein, $\mathcal{N}$ is Avogadro's number, M is the molecular weight of the protein and R is the radius of the particle.

Thus by substitution of M = 18,500 and $\bar{v}_2$ = 0.731 into the equation we can calculate the radius of the apoferritin subunit (R_{sub}). With reference to Figure 87 we can calculate the radius of the apoferritin molecule and the micelle,

$$R_{total} = R_{sub} + R_{sub} / \sin 22^o30'$$

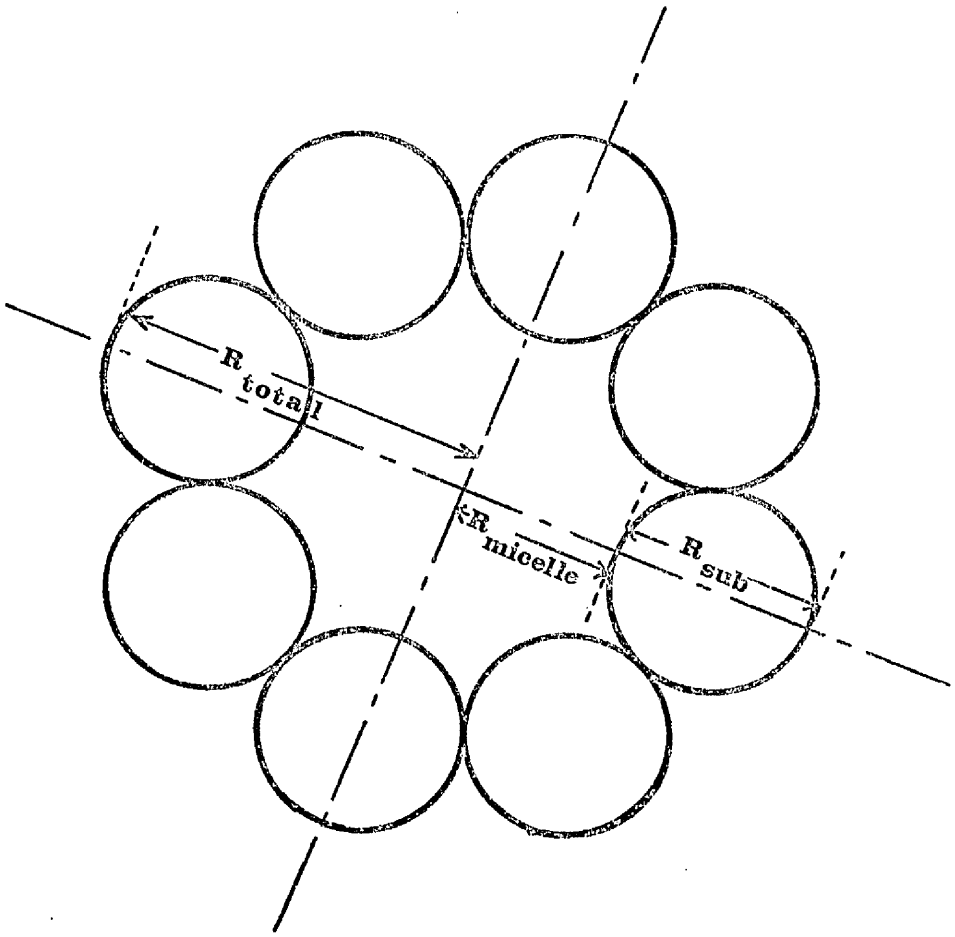
$$R_{micelle} = R_{sub} / \sin 22^o30' - R_{sub}$$

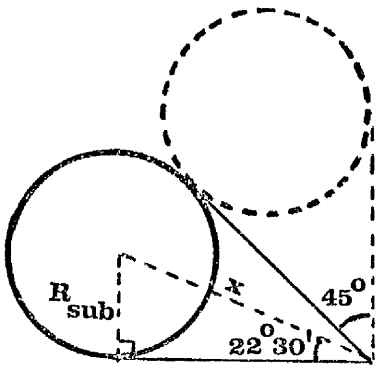
By this process, the values obtained for these two parameters are 134 Å and 60 Å which are in good agreement with those obtained experimentally.

Electron microscopy of the product of reconstitution gave micrographs as shown in Figure 88 and it is clear that the product is ferritin i.e. that the micelles formed are within the protein shell. In the reconstituted samples, no significant differences could be found between the dimensions of the reconstituted ferritin and native ferritin as was observed by Pape, Multani, Stitt and Saltman, (1968).

In order to quantitatively interpret the data obtained by monitoring the reconstitution process at 420 nm, it is necessary to know the specific extinction coefficient of the micelle , $E_{1cm}^{1\%}$.

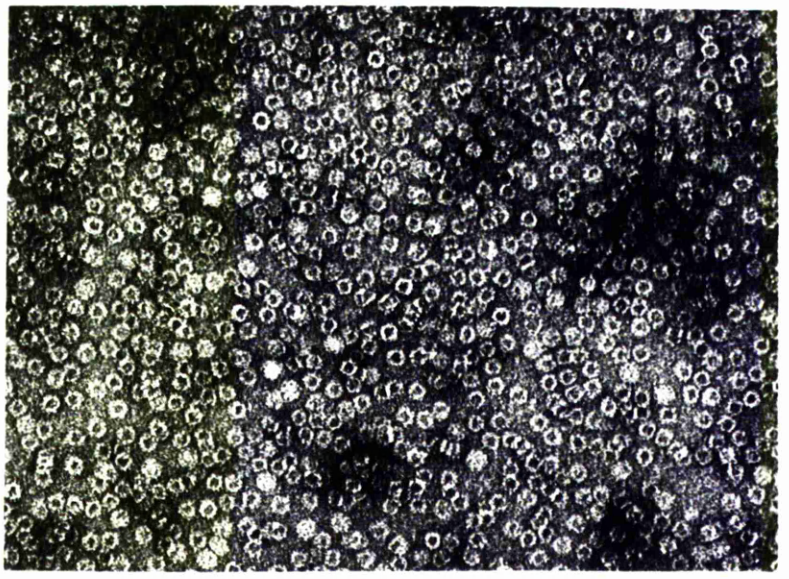
Figure 87. Model for the determination of the theoretical values for the radius of the apoferritin molecule and for the radius of the iron micelle. The model used is that with the subunits placed at the vertices of a rhombo-cubo-tetrahedron.

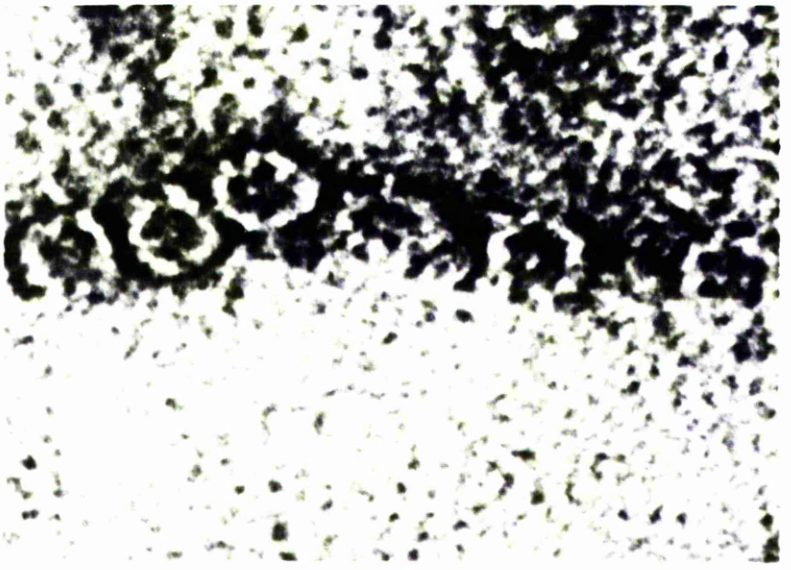


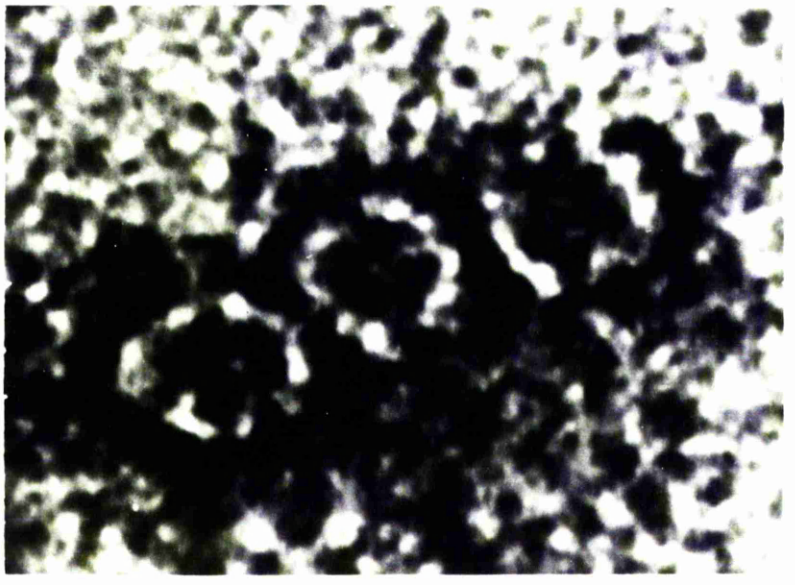


$x = R_{sub}/\sin 22^{\circ}30'$

Figure 88. Electron micrographs of the product obtained from reconstitution experiments. Top frame: magnification 2×10^5, middle frame: magnification 1×10^6, lower frame: 2×10^6.







The method for determining this parameter was described earlier (page 93) and its value was estimated to be 61.4 ± 1.5. This represents the mean value and standard deviation of ten experiments. This value is in disagreement with that of 100 cited by Macara, Hoy and Harrison, (1972).

Enzyme Kinetics.

Effect of Protein Concentration.

The effect of apoferritin concentration on the initial velocity was investigated as described earlier on page 94. Typical results from such studies are shown in Figure 89 and the initial velocity was measured for each concentration of protein. From such a curve it can be seen that the progress of the reaction is in the form of a rectangular hyperbola. Studies were carried out at concentrations of apoferritin in the range 0.2 - 4 mg/ml. When the initial velocities were plotted as a function of protein concentration a linear relationship was obtained as shown in Figure 90.

It is a general rule that the initial velocity of an enzyme catalysed reaction shows a first order dependence on the concentration of enzyme and this generality has been demonstrated for a great many enzymes with widely different specificities.

Deviations from this rule do occur and the best example of this would possibly be pepsin for which the velocity of digestion was shown to be roughly proportional to the square root of the concentration of pepsin. However, Northrop (1920) showed that this was only true for an impure preparation of pepsin and was not the case with the highly purified enzyme. He also demonstrated that on addition of products of peptic digestion the velocity decreased at high enzyme concentrations as a result of the pres-

Figure 89. Uptake of ferrous iron by apoferritin as a function of time. The progress of the reaction is shown for two different concentrations of apoferritin and hyperbolic relationships were obtained as shown.

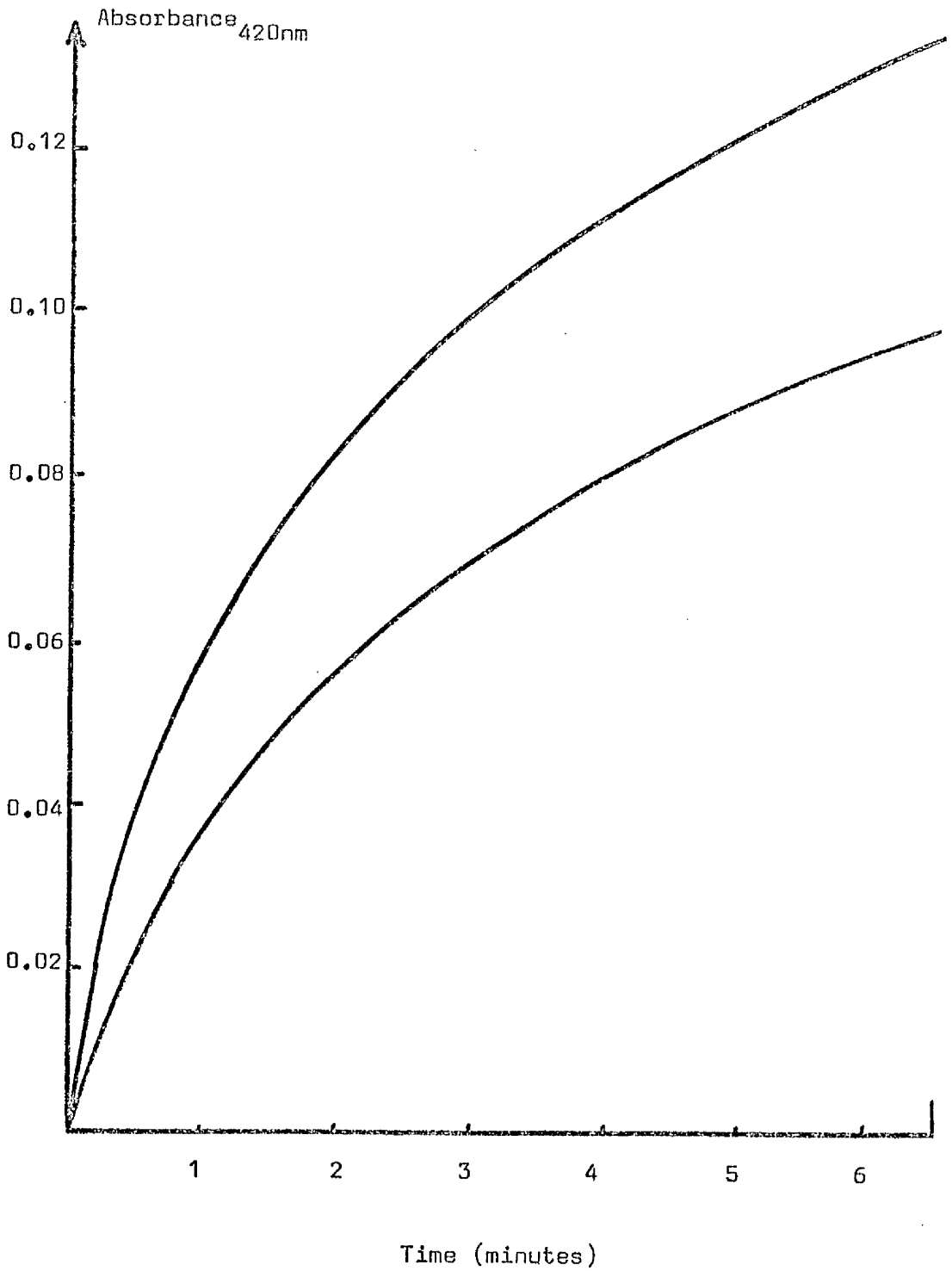
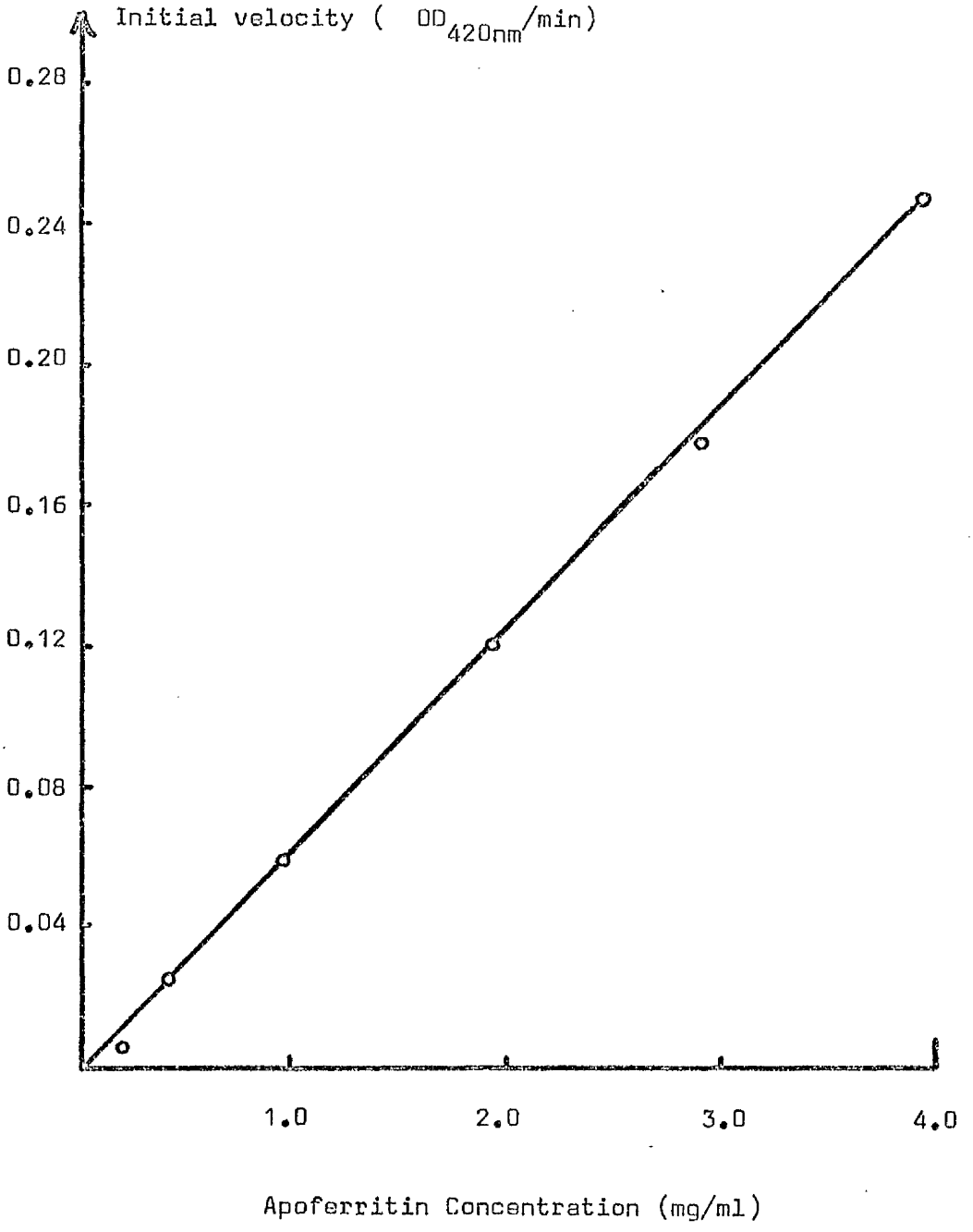

Absorbance 420nm
0.12
0.10
0.08
0.06
0.04
0.02
1
2
3
4
5
6
Time (minutes)

Figure 90. The effect of apoferritin concentration on the initial velocity of iron uptake, v. Conditions were as described in the text.


Initial velocity (OD_{420nm}/min)
0.28
0.24
0.20
0.16
0.12
0.08
0.04
1.0
2.0
3.0
4.0
Apoferritin Concentration (mg/ml)

ence of inhibitors which combined with the pepsin. The majority of other exceptions have, however, been shown to be the effect of the enzyme preparation on the pH of the solution.

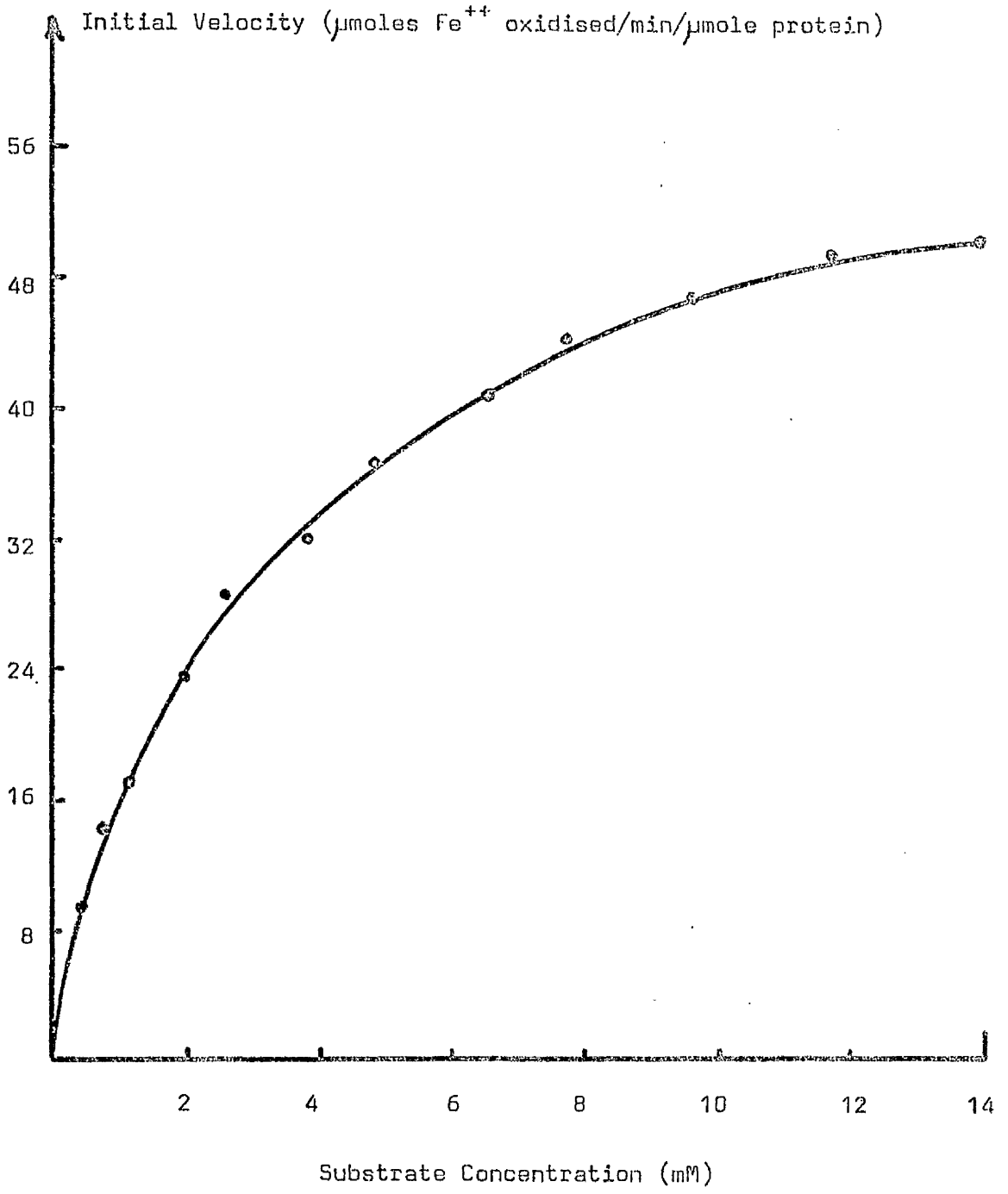
In the only study on the kinetics of apoferritin ferroxidase activity, Macara et al (1972) observed a rather complex relationship between initial velocity and apoferritin concentration. These workers explained this by stating that at low protein concentrations the process was complicated by competative formation of γ-FeOOH outside the protein, while at higher concentrations the decrease in initial velocity, v, implied that the protein concentration was not rate-determining. If, however, one recalculates their data it is found that at the high protein concentrations, say 2.6 mg/ml, which is equivalent to 5.9 μM, then, the number of ferrous atoms per protein molecule is only about 250. From their data on the effect of substrate concentration on initial velocity it can be calculated that the initial velocity is still increasing even when a 1000-fold molar excess of substrate is present. It would appear then that what these workers were observing, at least in the region of protein concentration 0.8 - 2.6 mg/ml, was a reaction in which both the substrate and protein concentration affect the reaction rate.

Effect of Substrate Concentration.

The effect of substrate concentration on the initial velocity was investigated as described on page 94.

The data obtained from such a study was plotted as initial velocity against ferrous iron concentration as shown in Figure 91. The curve so obtained was observed to have the form of a rectangular hyperbola which is typical for an enzyme catalysed reaction defined by the Michaelis-Menten equation (Michaelis and Menten,

Figure 91. Effect of the concentration of ferrous ammonium sulphate on the initial velocity, v, of ferrous iron uptake. The conditions for the assay are given in the text.


Initial Velocity (μmoles Fe++ oxidised/min/μmole protein)
56
48
40
32
24
16
8
2
4
6
8
10
12
14
Substrate Concentration (mM)

$$v = V_{max}[S] / (K_m + [S])$$

where v is the initial velocity, V_{max} is the maximum velocity, $[S]$ is the substrate concentration and K_m is the Michaelis constant.

From this treatment it is possible to estimate approximate values for the two kinetic parameters, V_{max} and K_m, where K_m is the substrate concentration at half the maximum velocity and these values are sufficient to explicitly define the rate law for the reaction.

The values obtained for these constants were 58 μmoles ferrous iron oxidised/ min/ μmole protein and 2.62mM respectively

This method for the determination of these kinetic constants is, however, subject to considerable error since it involves the determination of an asymptote which is an exceedingly difficult task. For this reason, several workers have investigated a variety of linear transformations of the Michaelis-Menten equation and the best known of these is the double reciprocal form of Lineweaver and Burk (1934). Re-arranging the Michaelis-Menten equation we have,

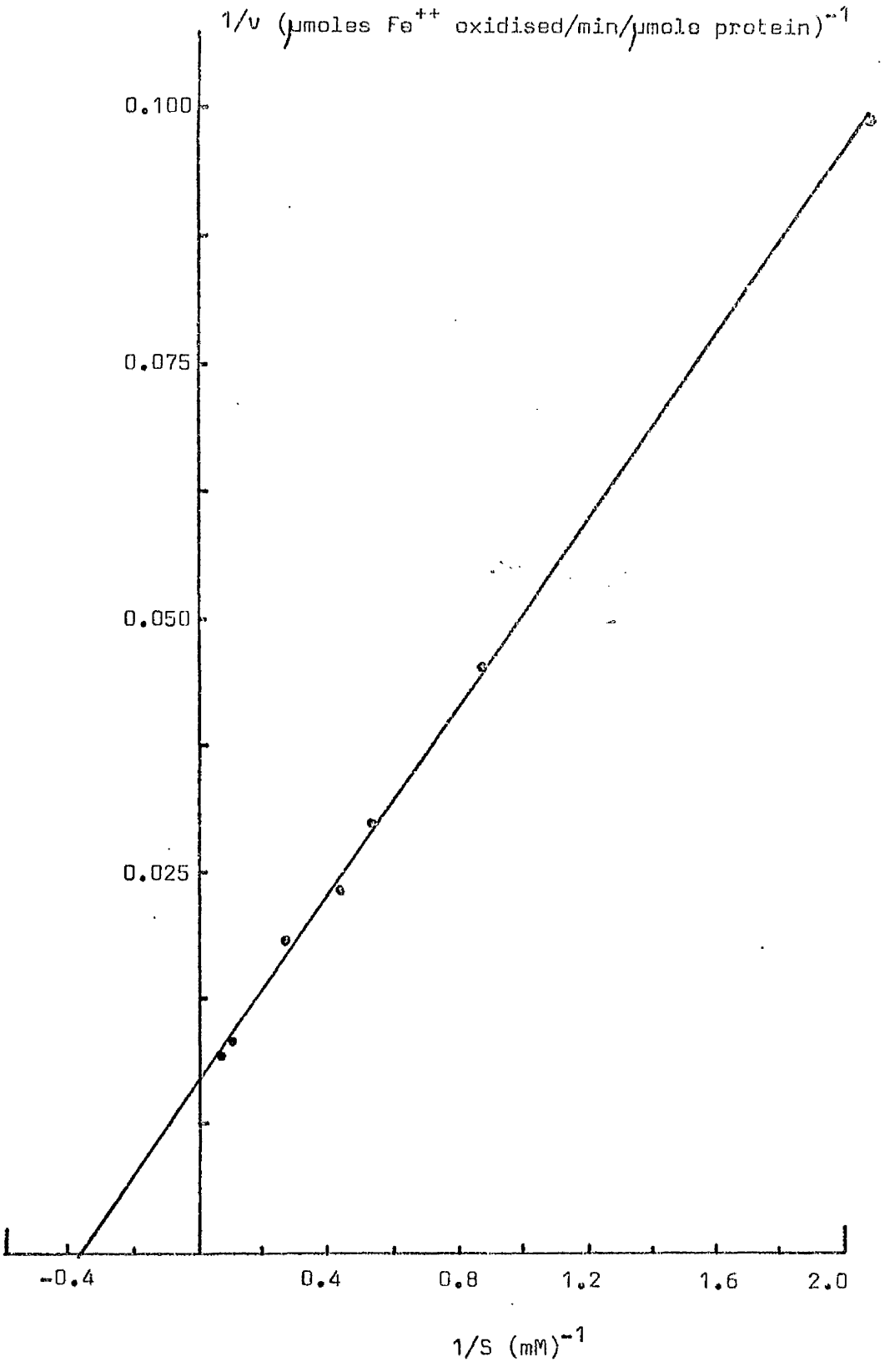
$$1/v = K_m / V[S] + 1/V_{max}$$

and thus a plot of 1/v versus $1/[S]$ should yield a straight line with gradient K_m / V_{max} and an intercept on the ordinate of $1/V_{max}$

When the experimental data is plotted in this form, a straight line was obtained as shown in Figure 92. The data of this figure was subjected to unweighted linear regressional analysis and the resultant coefficient of correlation was found to be 0.9991.

There have been numerous reports recently that significant error can be introduced when linear regression is applied to such

Figure 92. Lineweaver-Burke plot for horse spleen apoferritin.


1/v (μmoles Fe++ oxidised/min/μmole protein)-1
0.100
0.075
0.050
0.025
-0.4
0.4
0.8
1.2
1.6
2.0
1/S (mM)-1

a problem. The reason for this objection results from the inherent form of the double reciprocal equation which causes the data corresponding to high concentrations of substrate being crowded together near the ordinate and as a result, an unweighted least squares fit of the data is based much too heavily on these data points. The data in the reciprocal form can be treated by applying properly chosen weighting factors (Johansen and Lumry, 1961; Wilkinson, 1961)or it can be analysed by making least square fits directly to the Michaelis-Menten form of the curve (Cleland, 1963; Hanson, Ling and Havir, 1967; Atkins, 1971a,b). In the present study, however, the data fit the assumed relationship extremely well as demonstrated by the correlation coefficient and therefore recourse to further refining by computational and iterative methods was not required.

The values obtained for V_{max} and K_m from this study were 61.53 μmoles ferrous iron oxidised / min / μmole apoferritin and 2.823mM respectively.

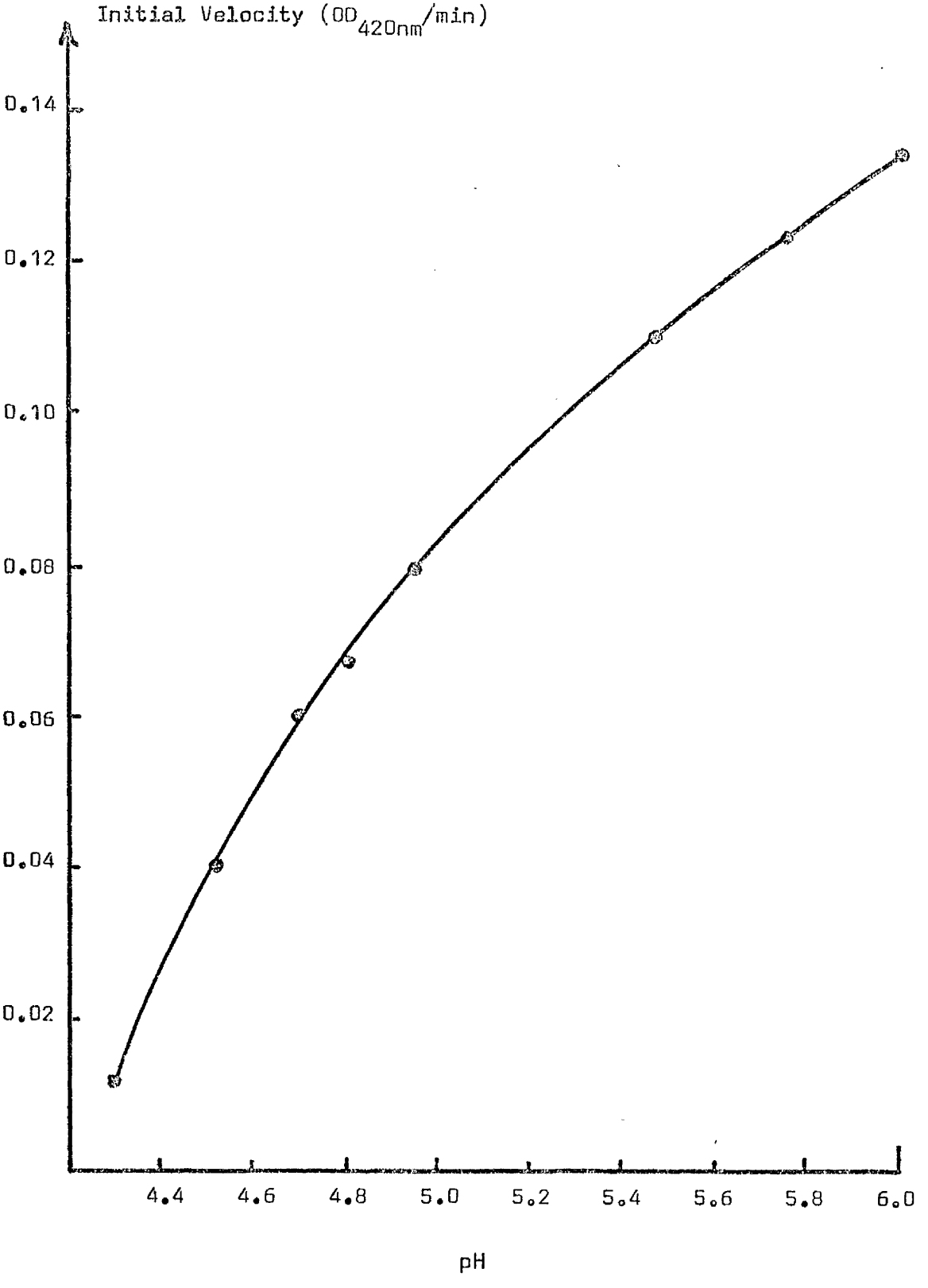
In the study on the kinetics of apoferritin ferroxidase activity Macara et al (1972) observed a sigmoidal progress curve and they interpreted this in terms of a crystal growth model. However, in the present study and that of Niederer (1970) this sigmoidal relationship could not be detected.

Effect of pH

The effect of pH on the initial velocity was investigated as described earlier on page 94. The data obtained was plotted as initial velocity versus pH as shown in Figure 93 and it can be seen that a smooth curve is obtained.

In such an investigation the characteristic type of curve is a fairly symmetrical inverted parabola with the maximum represent-

Figure 93. The effect of pH on the initial velocity,v, of ferrous iron uptake. The conditions for the assay are given in the text.


Initial Velocity (OD_{420nm}/min)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
4.4
4.6
4.8
5.0
5.2
5.4
5.6
5.8
6.0
pH

ing the pH optimum.

This value can vary significantly from enzyme to enzyme with the range of pH 1.5 - 2.0 for pepsin to pH 9.7 - 9.9 for arginase, although in some cases, notably xanthine oxidase, catalase and bacterial dehydrogenase (Dixon and Thurlow, 1924; Michaelis and Pechstein, 1913) independence of pH over a wide range has been found and thus these proteins have no observable pH optima.

In the present case we can consider the experimental curve obtained as representing the lower region of the parabola. The remainder of the curve, and hence the pH optimum, could not, however, be determined because the reaction is complicated by rapid autoxidation at near neutral pH.

In all of these kinetic studies of Macara et al (1972) and of those in the present study, the progress of the reaction was followed by formation of ferric oxide hydrate. In some studies, however, with the dialysis cell we monitored the oxidation by following the loss of ferrous iron, making use of the quantitative bipyridyl reaction. The results of this study are shown in Figure 94 and were found to be similar to the previous studies .

In the study of Macara et al (1972), using an imidazole buffer, pH 7.4 containing sodium thiosulphate and potassium iodate, bovine serum albumin was shown to be capable of oxidising ferrous iron although the product was soluble. In Niederer's study he found that the opposite was true i.e. that serum albumin slightly inhibited ferrous iron oxidation and this was also true for lysozyme.

We decided to re-investigate this property in the present assay system and the results for a variety of proteins are shown in Figure 95. It is clear that the only protein capable of oxid-

Figure 94. Uptake of ferrous iron by apoferritin as a function of time. The progress of the reaction was monitored by the decrease in ferrous iron concentration as determined by the quanitative bipyridyl reaction and again a hyperbolic relationship was obtained as shown.

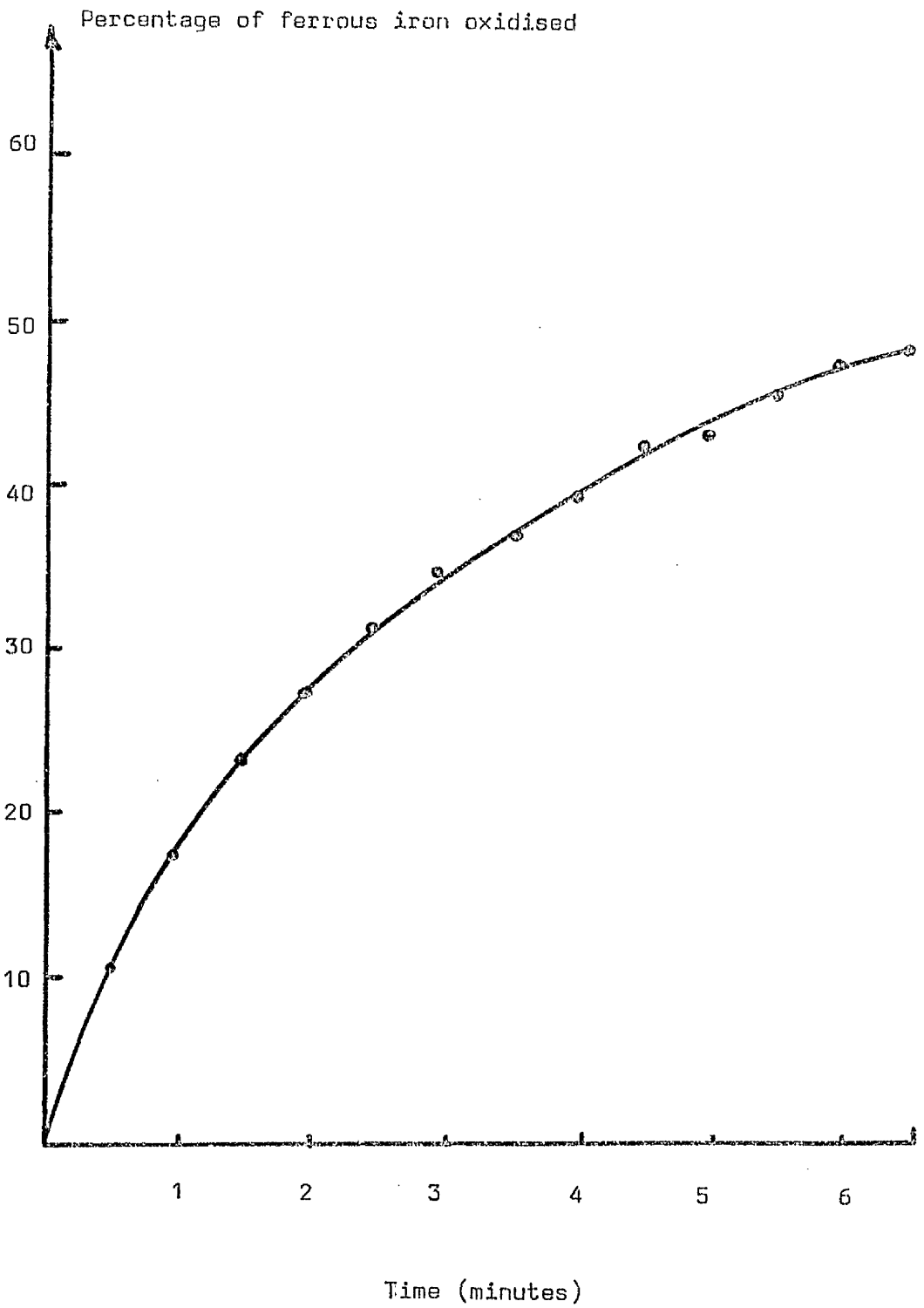
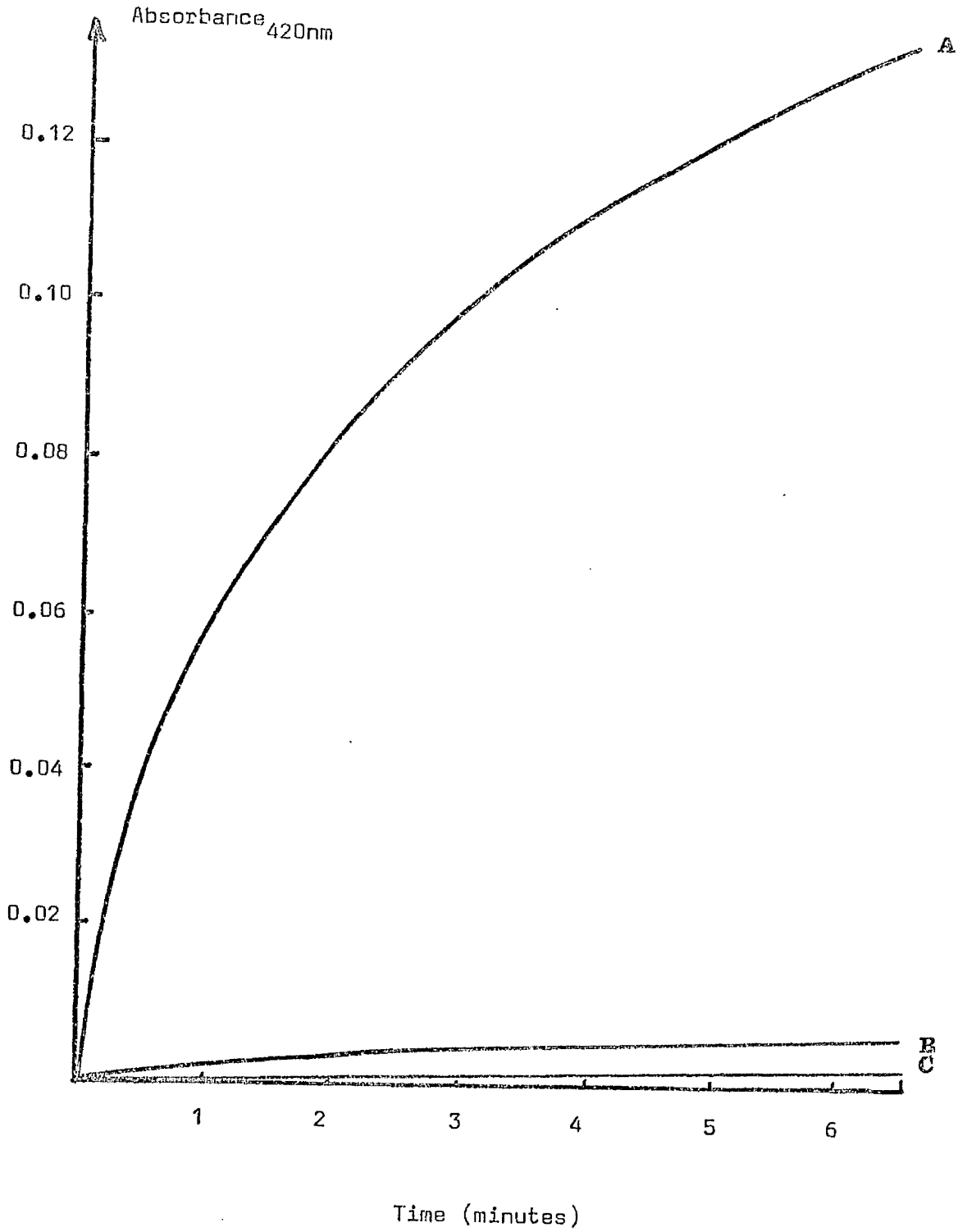

Percentage of ferrous iron oxidised
60
50
40
30
20
10
1
2
3
4
5
6
Time (minutes)

Figure 95. The effect of various proteins on the oxidation of ferrous iron in 100mM borate-cacodylate buffer. A, horse spleen apoferritin (2.273μM), B, bovine serum albumin (13.8μM) and lysozyme (26.64μM).


Absorbance 420nm
0.12
0.10
0.08
0.06
0.04
0.02
A
B
C
1
2
3
4
5
6
Time (minutes)

ising ferrous iron, under the conditions used, was apoferritin and no non-specific oxidation or catalysis was observed for the other proteins. This has the obvious advantage in that attempts to determine slight changes in the kinetics of chemically modified apoferritin would be possible in this system but would not be in the system described by Macara et al (1972).

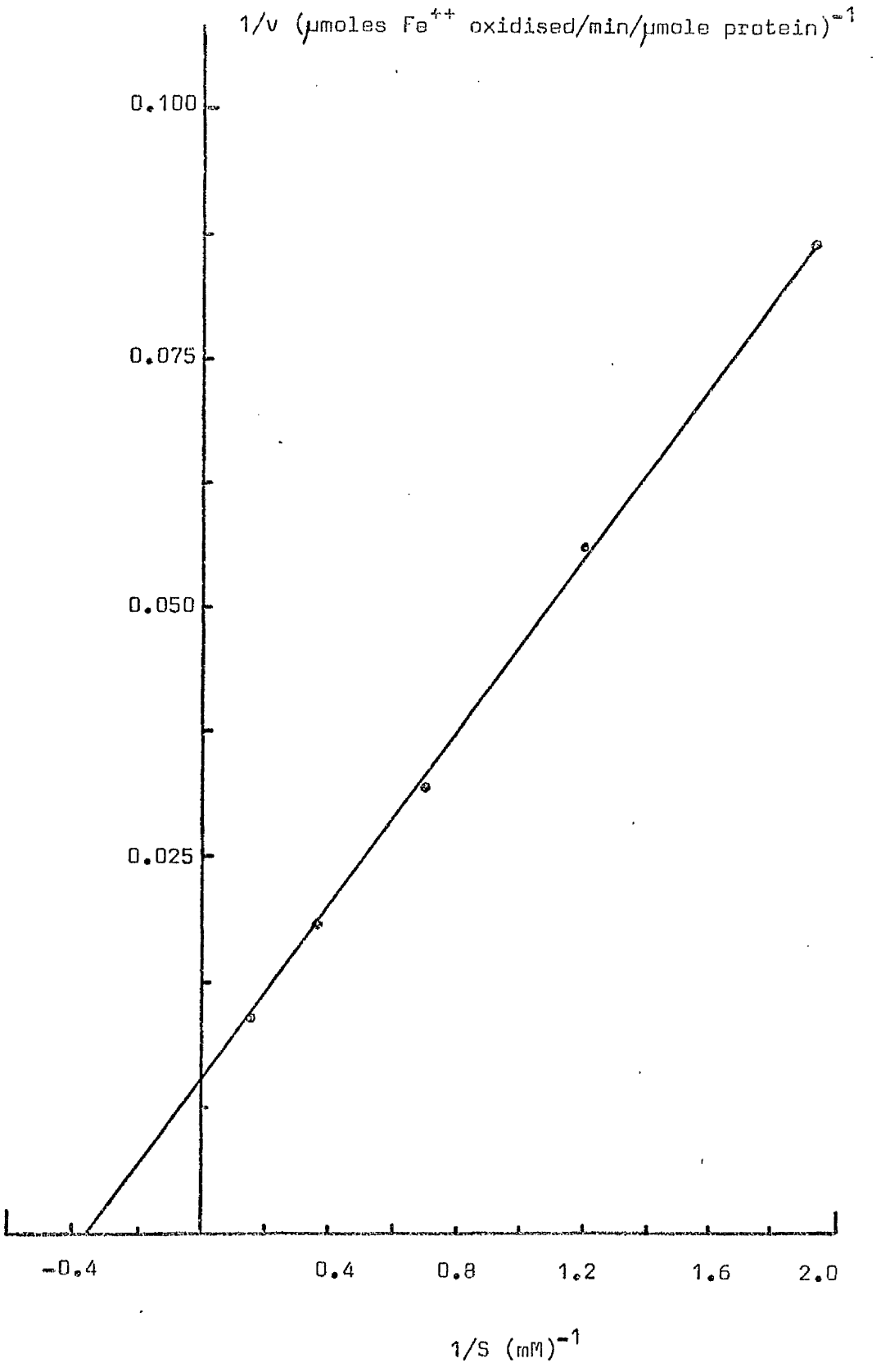
Native apoferritin was isolated as described on page 70 and this material was assayed by the same method. The results are shown in Figure 96 and the kinetic constants , V_{max} and K_m , were determined. These were found to be 67 μmoles ferrous iron oxidised / min / μmole protein and 2.84mM respectively. These values do not differ from the corresponding values for apoferritin obtained from the chemical reduction of ferritin and therefore chemical reduction of the iron from ferritin does not appear to cause any apparent irreversible change in the protein's structure.

From these studies it was found that suitable assay conditions were 100mM borate-cacodylate buffer, pH 5.5 with substrate at a concentration of 25mM and protein at about 1 mg/ml (Crichton and Bryce, 1972)

It is clear from the value of V_{max} that, on comparison with other enzymes, the specific activity of apoferritin is not very high although it should be remembered that the conditions are not optimal. This is also apparent from the very high value for K_m and possible explanations for this are discussed at a later stage.

Another protein has recently been shown to demonstrate a ferroxidase activity, both in vivo and in vitro, and this is the

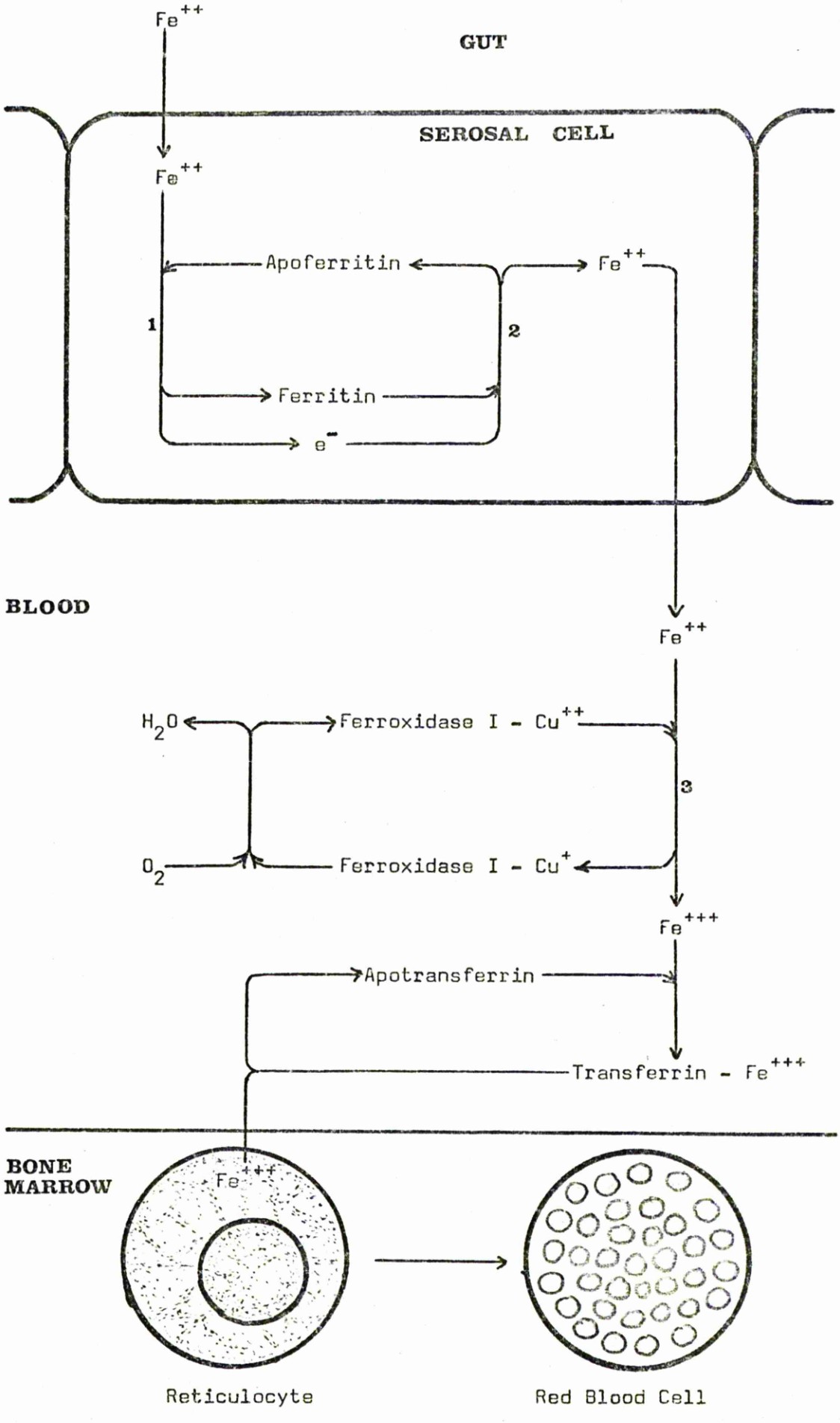
Figure 96. Lineweaver-Burke plot for native horse spleen apoferritin.


1/v (μmoles Fe++ oxidised/min/μmole protein)-1
0.100
0.075
0.050
0.025
-0.4
0.4
0.8
1.2
1.6
2.0
1/S (mM)-1

copper-containing glycoprotein, ceruloplasmin (Osaki, 1966,1967; Osaki, Johnson and Frieden, 1966, 1971; Osaki and Johnson, 1969; Ragen, Nacht, Lee, Bishop and Cartwright, 1969; Lee, Roesser, Nacht and Cartwright, 1970; Roesser, Lee, Nacht and Cartwright, 1970). It is found in mammalian serum, has a molecular weight of 155,000 and binds 8 molecules of copper. It was shown that ceruloplasmin oxidises a number of polyphenols and polyamines but it was not certain that this catalytic activity represented biological function. However, Osaki and co-workers (1966, 1967, 1969, 1971) demonstrated that it had an extremely potent capacity to oxidise ferrous to ferric ion. Since iron is absorbed in the reduced state, but is bound to transferrin in the oxidised state, it has been proposed that this oxidation represents the biological role of ceruloplasmin and for this reason these workers have proposed that the protein be re-named ferroxidase or by its systematic name of ferrous-O_2-oxidoreductase. Recently it has been called ferroxidase I since Topham and Frieden (1970) have purified and identified a non-ceruloplasmin ferroxidase from human serum and suggest that it be called ferroxidase II.

With ceruloplasmin in mind and the present observation that apoferritin also exhibits a ferroxidase activity it is interesting to speculate on a possible route from iron absorbed from the gut to the formation of haemoglobin and this is shown schematically in Figure 97. Thus, ferrous iron is absorbed into the serosal cell where it is oxidised and stored by apoferritin to give ferritin. The iron, when required, can perhaps be removed by a proposed 'ferriductase' system to yield ferrous iron which can pass through the cell membrane and be oxidised by ceruloplasmin to give ferric iron which can combine with apotransferrin to give transferrin. The transferrin then contributes its iron directly

Figure 97. Possible route from the ferrous iron absorbed from the gastrointestinal tract to its incorporation into haemoglobin.
Enzyme system 1 would most possibly be apoferritin itself.
Enzyme system 2 may perhaps be the ferriductase system found by Osaki and Sirivech (1970).
Enzyme system 3 is ceruloplasmin (ferroxidase I).


Fe++
GUT
SEROSAL CELL
Fe++
Apoferritin
Fe++
1
2
Ferritin
e-
BLOOD
Fe++
H2O
Ferroxidase I - Cu++
3
O2
Ferroxidase I - Cu+
Fe+++
Apotransferrin
Transferrin - Fe+++
BONE MARROW
Fe+++
Reticulocyte
Red Blood Cell

to the reticulocyte cells of the bone marrow and it is these cells that eventually produce the young red blood cells that make haemoglobin.

Such a ferritin reducing enzyme has been demonstrated in a number of different mammalian liver homogenates and Osaki and Sirived (1971) have shown its requirement for NADH and FMN. They also demonstrated that its reaction is not inhibited by 1mM allopurinol and suggest, therefore, that it is probably not liver xanthine dehydrogenase.

Just as in the case of ceruloplasmin's peripheral activity with polyphenols and polyamines, Mazur and coworkers demonstrated that ferritin exhibited biological activity in catalysing the oxidation of adrenaline and also it had a vasodepressor activity, but these activities were not really considered to be major biological functions of ferritin. In fact in the oxidation of adrenaline it appears that the catalysis is due solely to the iron and not the protein since they observed that apoferritin had no activity and that inorganic iron and other iron-containing proteins, oxidised cytochrome c, methaemoglobin and ferricytochrome c, were just as active, if not more so.

These workers, however, did demonstrate that ferritin's catalytic activity, both for vasodepressor activity and oxidation of adrenaline, was dependent on the sulphydryl content of the protein. Thus they showed that its vasodepressor activity is associated with free sulphydryl groups (which we did not find in the present study) and when these are oxidised the protein becomes inactive while oxidation of the sulphydryl groups of ferritin appears to increase the proteins ability to oxidise adrenaline. Saltman, Fiskin and Bellinger (1956) , however, showed that uptake of iron by ferritin is not affected by iodoacetic acid or iodo

acetamide, which implies that sulphydryl groups or not a necessary requirement for function, although, they do stress that the inability of these reagents to inhibit iron uptake could be attributed to the poor permeation of these compounds through the cell membrane.

Since this problem of permeation does not exist for the in vitro assay system established earlier (page 361) then we decided to specifically modify groups in an effort to elucidate which residues were important for function and which were unimportant.

A number of chemical modifications of various residues of apoferritin have already been described (page 267-327) and the products of each of these was assayed for ferroxidase acivity.

Tryptophan-modified Apoferritin.

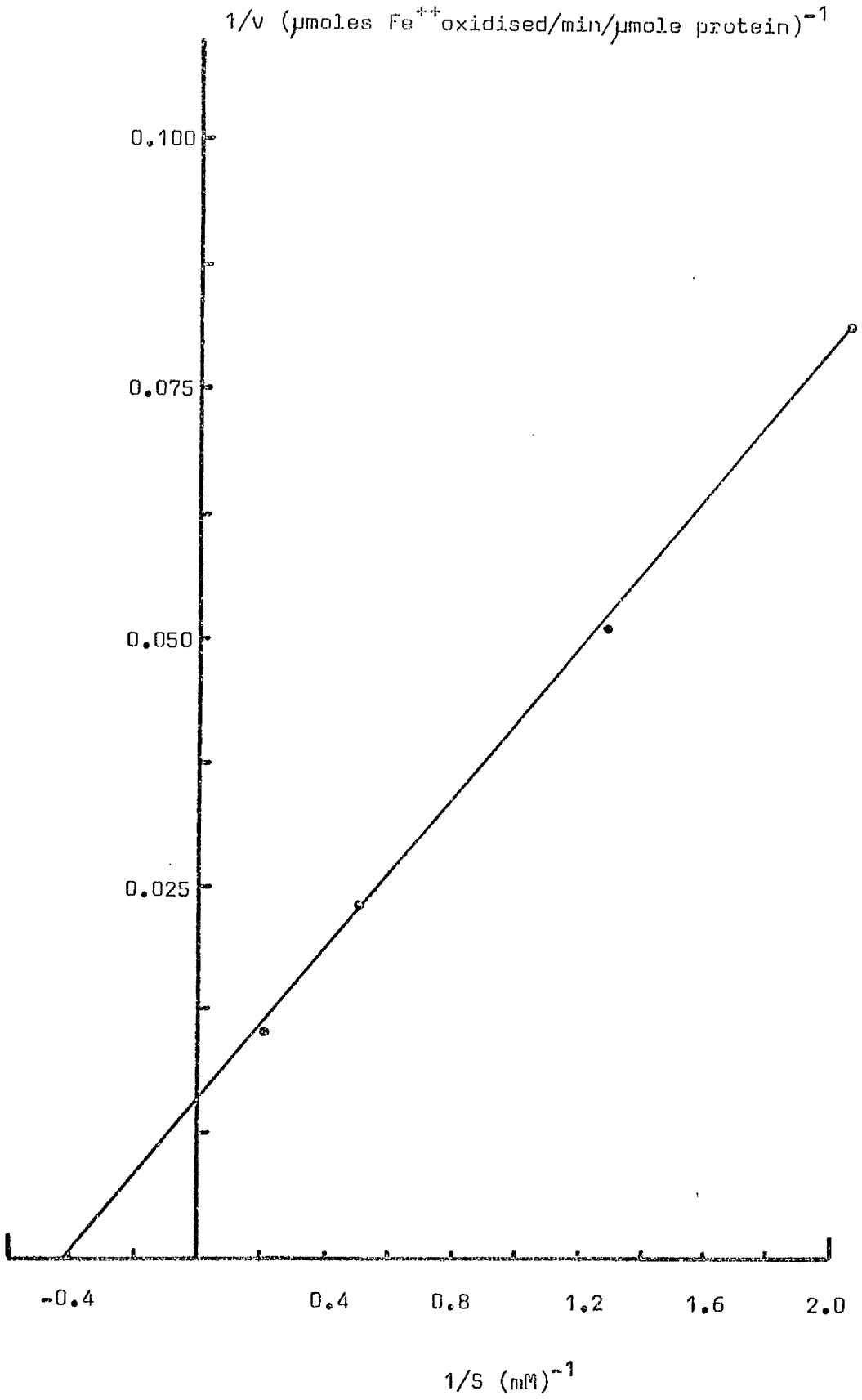
For apoferritin modified by 2-nitrophenylsulphenyl chloride the assay was carried out exactly as described for native apoferritin and apoferritin obtained by chemical reduction of ferritin, the conditions being 100mM borate-cacodylate buffer, pH 5.5 with protein at a concentration range of approximately 1 mg/ml and substrate concentration in the range 0.5 - 20mM.

The results are shown in Figure 98 and the kinetic constants V_{max} and K_m were found to be 63 μmoles ferrous iron oxidised / min / μmole protein and 2.32mM respectively. These values are not significantly different from unmodified apoferritin and it would appear from this that the two tryptophan residues in apoferritin are not required for catalytic function.

Tyrosine-modified Apoferritin

Apoferritin modified by tetranitromethane was assayed for ferroxidase activity in the same way and the results are shown

Figure 98. Lineweaver-Burke plot for tryptophan-modified horse spleen apoferritin. Modification of the protein was achieved by treatment with 2-nitrophenylsulphenyl chloride.


1/v (μmoles Fe++ oxidised/min/μmole protein)-1
0.100
0.075
0.050
0.025
-0.4
0.4
0.8
1.2
1.6
2.0
1/S (mM)-1

in Figure 99. The kinetic parameters V_{max} and K_m were computed to be 58 μmoles ferrous iron oxidised / min / μmole protein and 2.71mM respectively and again these are not significantly different from the corresponding values for the unmodified protein. In the case of tyrosyl-modified apoferritin it was found that only one residue was modified (page 274) and it seems that this single tyrosine residue is not involved in catalytic function of apoferritin.

Lysine-modified Apoferritin.

A similar study was carried out on apoferritin modified at lysine residues by o-methylisourea. The results for the assay are shown in Figure 100 and from this it was possible to calculate V_{max} and K_m for the modified protein. The values obtained for the parameters were found to be 55 μmloes ferrous iron oxidised / min / μmole protein and 2.44mM respectively and these values are in good agreement with those for the unmodified protein. In the case of protein modified by o-methylisourea the modification was found to be quite extensive, in that, seven of the nine lysine residues were converted to homoarginine. It was shown earlier that these seven residues are probably not involved in the maintainance of the quaternary structure but instead, are situated on the exterior of the protein and are easily accessible to solvent. From the present study it would also appear that modification of these residues causes no change in the catalytic activity of apoferritin and therefore it would seem reasonable to assume that these groups are not involved either directly or indirectly in the protein's ferroxidase activity.

Arginine-modified Apoferritin.

Figure 99. Lineweaver-Burke plot of tyrosine-modified horse spleen apoferritin. Modification of the protein was achieved by treatment with tetranitromethane.

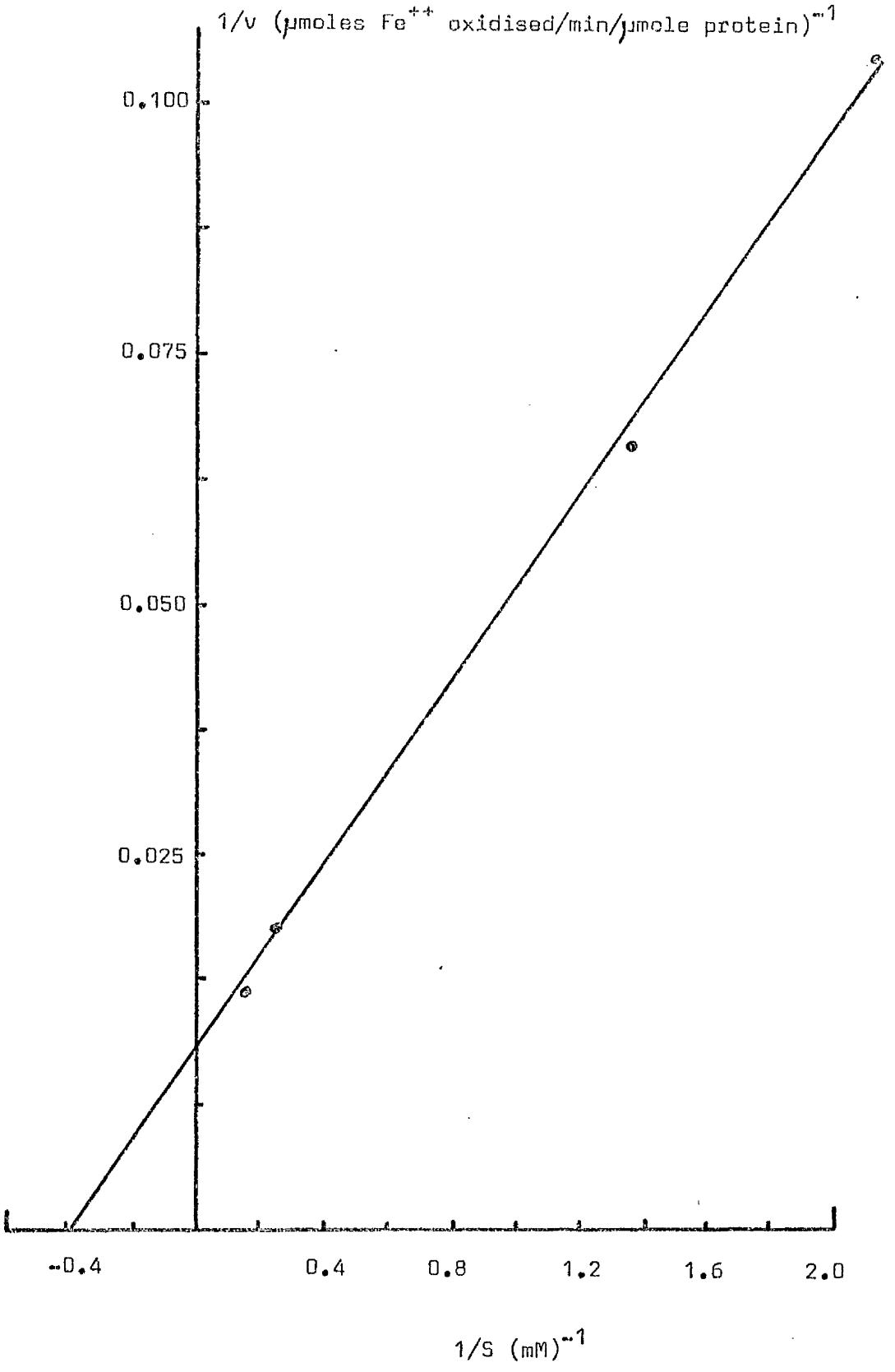
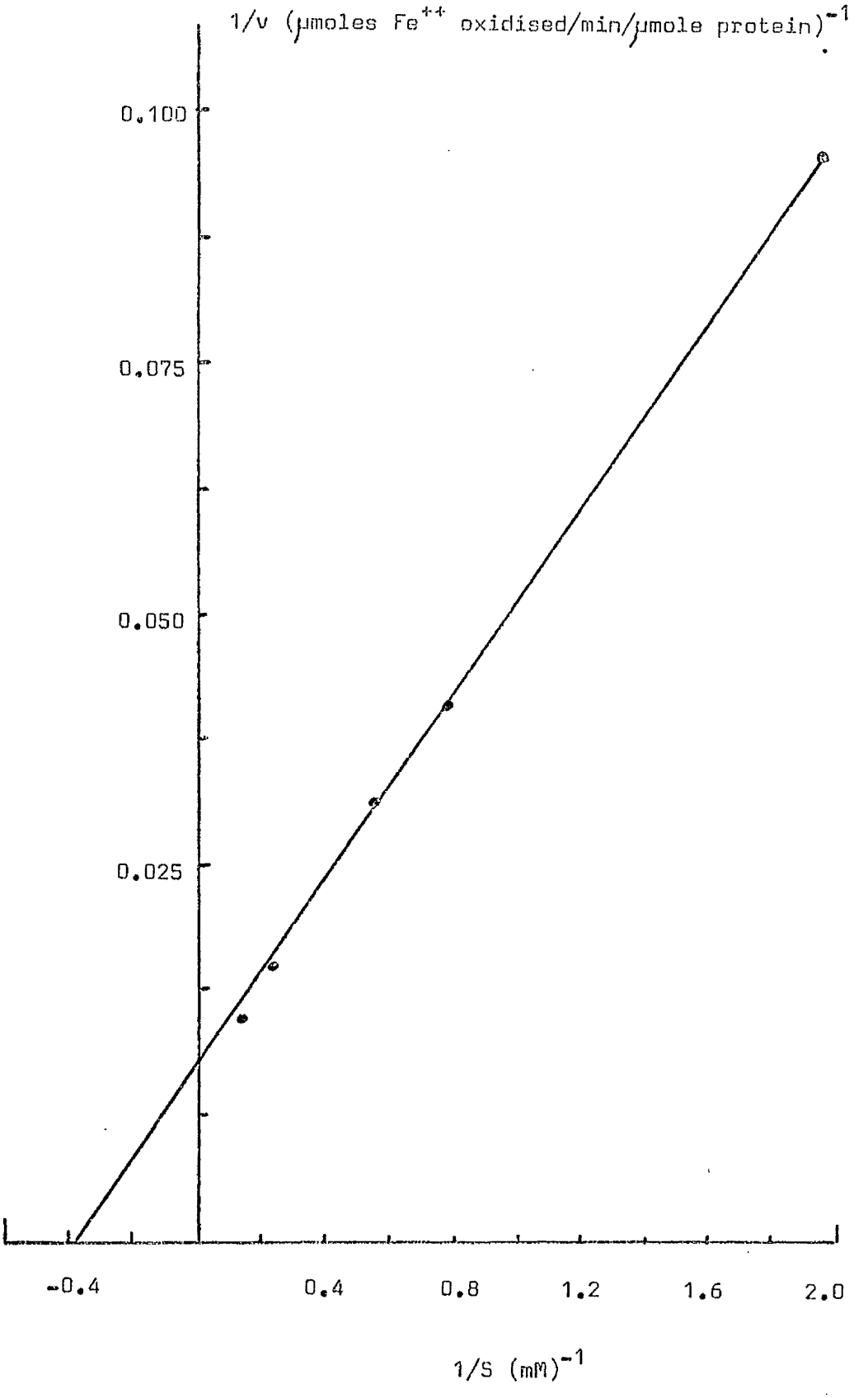

1/v (μmoles Fe++ oxidised/min/μmole protein)-1
0.100
0.075
0.050
0.025
-0.4
0.4
0.8
1.2
1.6
2.0
1/S (mM)-1

Figure 100. Lineweaver-Burke plot for lysine-modified horse spleen apoferritin. Modification of the protein was carried out by treatment with o-methylisourea.


1/v (μmoles Fe++ oxidised/min/μmole protein)-1
0.100
0.075
0.050
0.025
-0.4
0.4
0.8
1.2
1.6
2.0
1/S (mM)-1

Apoferritin that had been modified by 1,2-cyclohexanedione was assayed for ferroxidase activity and the results are shown in Figure 101. V_{max} and K_m were found to be 57 μmoles ferrous iron oxidised / min / μmole protein and 2.3mM and these value are again similar to the values for these constants for unmodified apoferritin. Just as in the case of the lysine modified protein, apoferritin treated with 1,2-cyclohexanedione causes extensive modification in that 9 of the 10 arginine residues are modified. The fact that the catalytic activity is not impaired, is a good indication that these 9 arginine residues are not involved in the proteins catalytic function.

Carboxymethylated Apoferritin.

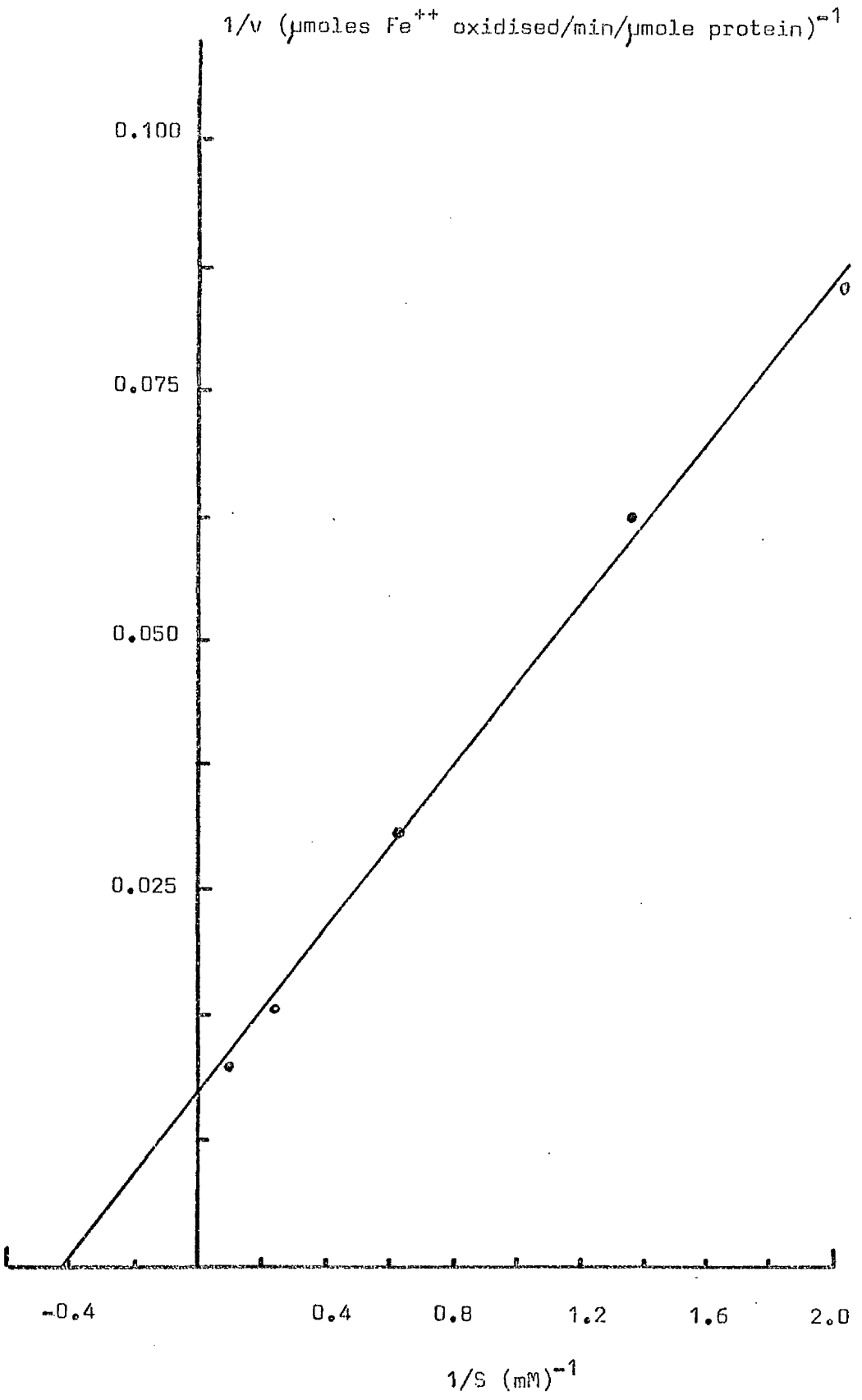
Protein that had been carboxymethylated at two ½ cystine residues and one histidine residue was assayed for ferroxidase activity. Unlike the other modified apoferritins, the carboxymethylated protein had no catalytic activity.

Similar results were obtained by other workers who observed in in vivo studies that treatment of apoferritin with iodoacetamide suppressed iron incorporation (Mazur, Green and Carleton, 1960).

This observation then raised the interesting question as to whether the activity of the protein was lost as a result of cysteinyl modification or whether it was a result of carboxymethylating one histidine residue. To resolve this problem, one need only attempt to modify in turn each group specifically and note the effect, if any, on the catalytic function.

In the present study this approach has, unfortunately, not as yet been undertaken, but it would seem from the work of Niederer (1970) that it is the histidine residue that is import-

Figure 101. Lineweaver-Burke plot for arginine-modified horse spleen apoferritin. Modification of the protein was achieved by treatment with 1,2-cyclohexanedione.


1/v (μmoles Fe++ oxidised/min/μmole protein)-1
0.100
0.075
0.050
0.025
-0.4
0.4
0.8
1.2
1.6
2.0
1/S (mM)-1

ant for function. In his study he alkylated sulphydryl groups with iodoacetamide at a variety of pH valuesfrom pH 4.5 - 8.0 and found that these did not have a significant effect on catalytic activity. When he included zinc ions ,Zn^{++}, in the assay mixture , the ferroxidase activity of apoferritin was abolished and he suggested that the loss of activity could be due to the zinc ions preferentially binding to a histidine residue at the 'active site'. He also reacted apoferritin with diazonium-H-tetrazole which is known to be specific for histidine residues and found that only 10% of the histidines (about 0.6 of a residue per subunit) reacted and that the resultant modified protein was still active. He suggested, however, that the active histidines could be situated on the inside surface of the protein shell and that diazonium-H-tetrazole could not penetrate the intersubunit spaces.

Further evidence for the suggestion that a histidine residue is at the active site of apoferritin comes from the <u>in vivo</u> studies of Coleman and Matrone (1969). These workers investigated the effects of a zinc diet on the iron stores in the liver since it had been shown that zinc toxicity resulted in a condition resembling iron deficiency anaemia. In their study they demonstrated that the toxicity does not appear to be associated with the synthesis of apoferritin from $[^{14}C]$-amino acids but instead, it appears to interfere with iron micelle formation. It was demonstrated that on iron-induced ferritin synthesis, the amount of ferritin protein/liver/100 g body wt. is almost identical in control animals and animals with a zinc-supplemented diet. However, when they determined the amount of iron/protein they found a very significant difference between the two. In view of the present study and that of Niederer (1970), one

explanation is that the Zn^{++} ions bind to the histidine thereby limiting the catalytic activity, which would result in ferritin with a limited iron micelle.

Another piece of rather indirect evidence for the view of an active histidine, although quite relevent, comes from the studies on ferroxidase I (ceruloplasmin). With this protein, Vasiletz, Shavlovsky and Neifakh (1972) have demonstrated that while tyrosyl residues may be important in the maintenance of the native structure, histidine residues seem to be directly involved in the protein's catalytic activity.

GENERAL DISCUSSION

The present study was concerned with an approach to the elucidation of the structure and function of apoferritin, the emphasis being placed on how these two properties of the protein were inter-related. The problem was approached using a variety of physical, chemical and kinetic methods and it will be the purpose of this general discussion to attempt to integrate all of the results from the various approaches.

In order to find out about the biological role played by apoferritin, we were interested in the interaction between the protein and all of the other components of the system that would be required to promote some form of catalysis, and to try to elucidate how such a catalysis may arise. It is an important prerequisite to have some information on the exact chemical structure of the molecule, particularly on the nature of those amino acid residues that constitute the catalytic 'active-site' and also on the types of interaction which are important in maintaining the structural integrity of the protein molecule. It is the results of such physical and chemical methods that provide a framework for interpreting the forces responsible for enzyme-ligand binding and for enzymic catalysis.

It was found from a variety of studies that the apoferritin molecule appears to have a molecular weight of approximately 440,000 and consists of twenty-four apparently identical subunits of molecular weight 18,500 and this was in disagreement with the view that apoferritin consisted of twenty subunits of molecular weight 23-25,000. This disparity has, however, already been discussed quite extensively (page 209) and it only remains here to point out one important functional aspect of the value of the number of subunits that constitute the apoferritin molecule, and

that is, the process of self-assembly.

As Casper and Klug (1962) pointed out, "self-assembly is a process akin to crystallisation and is governed by the laws of statistical mechanics..... It is the transition from a state in which protein subunits are randomly arranged in space to a state in which they are highly ordered, that virus assembly is like crystallisation. The driving energy for this process is provided by the formation of inter-subunit bonds. The order in the final structure is a necessary consequence of the statistical mechanical compulsion to form the maximum number of the most stable bonds."

In such assemblies it has been demonstrated that grades of organisation are essential and, if a multisubunit particle can be synthesised from sub-assemblies which then associate, then the complex molecule can be built with a much higher degree of efficiency (Crane, 1950). Quite recently, Rosen (1970) extended these ideas by a theoretical statistical treatment for sub-assembly processes. By this method he derived an equation for determining the total number of finished particles which had been correctly synthesised and this was,

$$v_n = p^{r1 + r2 + \ldots\ldots r_n}.(M/L)$$

where v_n is the total number of correct particles, p is the probability that two units will be put together correctly, r_i is the number of units produced at the $(i - 1)$th stage, **N** is the number of sub-assembly stages, M is the size of the particle and L is the number of subunits. He argued that for the most efficient system then the term $p^{r_1 + r_2 + \ldots\ldots r_n}$ must be maximised and since $p<1$ this would mean that the exponent must be minimised. From elementary number theory this is achieved by decomposition of the

exponent into prime numbers. For a twenty-four subunit structure this would give,

$$v_n = p^{2+2+2+3}.(M/24) = p^{9}.(M/24)$$

This theory, however, is much more suited to linear assemblies and it is felt that for closed structures like that of apoferritin or for spherical viruses, some form of correction factor for geometrical considerations is lacking. Despite this, it is clear that the process of self-assembly by sub-assembly is a more efficient process than by a step-wise addition of single units in which case,

$$v_n = p^{24}.(M/24)$$

In this context, a twenty subunit structure is not so susceptible to sub-assembly process and this would be a much more difficult structure to synthesise from the nascent polypeptide chains that make up the subunits.

Despite this, there are claims for twenty-subunit structures for chicken liver glutamate dehydrogenase (Frieden, 1962; Rogers, Geiger, Thompson and Hellerman, 1963), fibrinogen (Koppel, 1966), mammalian dihydrolipoyl transacetylase (Ishikawa, Oliver and Reed, 1966) and low density lipoprotein (Pollard, Scanu and Taylor, 1969; Pollard and Devi, 1971), although the evidence is not very conclusive. The dihydrolipoyl transacetylase from E. coli is, however, thought to contain twenty-four identical subunits (Wilms, Oliver, Henney, Mukherje and Reed, 1967; Henney, Wilms, Muramutsu, Mukherje and Reed, 1967). This enzyme is one of three which constitutes in E. coli a complex system that catalyses a lipoic acid oxidative decarboxylation of pyruvic acid and these are pyruvate dehydrogenase, dihydrolipoyl transacetylase and dihydrolipoyl

dehydrogenase. The other analogous class of complex, namely that involved in the oxidative decarboxylation of α-ketoglutamate has also been shown to consist of three enzymes, α-ketoglutamate dehydrogenase, dihydrolipoyl transuccinylase and dihydrolipoyl dehydrogenase. De Rosier, Oliver and Reed (1971) have recently shown that the dihydrolipoyl transuccinylase has octahedral symmetry (4:3:2) and suggest that this enzyme is also composed of twenty-four identical subunits.

From the present study and from the data of other workers it is clear that the apoferritin monomer is a very stable structure. It is stable in the range pH 3-10 and at temperatures up to 80^{o}C and also is relatively resistant to chemical denaturants in that 10M urea does not cause dissociation into subunits. This stability has an important direct bearing on the functional aspect of the protein. It was seen earlier that Pape, Multani, Stitt and Saltman (1968) proposed that ferritin was formed by the addition of subunits around a preformed iron micelle (page 53). The fact that the monomer is so stable and resistant to dissociation would tend to contadict such a view. Also from the sedimentation velocity study described on page 254 it was seen that, if an equilibrium existed between monomer and subunit then it would have to lie very much in the direction of monomer. Also some *in vivo* studies on the biosynthesis of ferritin lend support to the idea that newly synthesised ferritin is ferritin of least iron content (Fineberg and Greenberg, 1955; Drysdale and Munro, 1966) suggesting that apoferritin, or a molecule of low iron content, is the precursor of ferritin. Niederer (1970) has, however, suggested that if iron stabilises the ferritin molecules such that 'full' ferritin dissociates only slowly then, the nascent subunits when interacting with the pre-existing liver ferritin pool would

be incorporated into iron poor ferritin preferentially. The conclusive experiment to disprove Pape's model would be to demonstrate a redox system in which apoferritin took up iron in a gradual way to form 'full' ferritin. The experiments of Niederer (1970) and of Macara et al (1972) and also those described in the present study demonstrate such a redox system and support the view that ferritin is synthesised by the gradual oxidation of ferrous iron within the apoferritin protein shell.

As redox reactions are involved not only in essential energy storage processes but also in the biosynthetic and degradative pathways, then such reactions are of vital importance to biological systems. The enzymes which catalyse redox reactions can be classified into several different groups on the basis of the mechanism of reaction and some of these can be understood in terms of basic organic chemistry. Essentially these sub-classes are ; nicotinamide adenine dinucleotide enzymes (using nicotinamide adenine dinucleotide as co-enzyme), flavo-enzymes (using FMN or FAD as co-enzyme), metalloflavoenzymes (in addition to flavin prosthetic group, also contains one or more metal ions), hydroxylases (catalyse hydroxylations using molecular O_2) and oxygenases (catalyse reactions in which both oxygens are incorporated into the substrate).

Oxidation-reduction reactions may be regarded as being made up of two half reactions involving electrons. The measure of the tendency of any species to accept electrons, that is, to become reduced, is known as its reduction potential. The standard reduction potential for ferric/ferrous is +0.77 while for O_2/peroxide is + 0.30. The ferric/ferrous system refers to aqueous solutions and the ions are surrounded by water molecules. When, however, six cyanide ions ligand the metal ion, as in the case ferricyanide

/ferrocyanide, the reduction potential is decreased and in the case of cytochromes where the iron atom is liganded to haem the potential may be even further reduced. It is known that a change in redox potential of a system can influence the rate of reaction in which the system is involved and it is probable that the changes in reduction potentials of some metal ions brought about by the special ligand systems found in enzymes are a major factor in the activity of these enzymes. Udenfreund and co-workers studied the ability of a ferrous/O_2 system to hydroxylate substrates. They found, that on increasing the pH, the reaction became more rapid; at higher pH values the redox potential of the Fe^{++}/Fe^{+++} became more negative. When EDTA was added it complexed with the metal ions and the redox potential dropped from 0.77 to 0.14 and this caused the reaction to increase. Thus, a change in the oxidation-reduction potential can influence the energy of activation, and therefore the reaction rate. In our *in vitro* assay system we were concerned with Fe^{++}/Fe^{+++} in aqueous solvents and it is quite feasible that *in vivo* the metal ions are liganded in some way. Thus, by analogy, this would decrease the reduction potential and perhaps increase the rate of the reaction and this would help to explain the low values obtained for V_{max} and the relatively high value of K_m.

In the studies of the functional aspects of apoferritin we assumed molecular oxygen to be the electron acceptor and this perhaps giving rise to hydrogen peroxide although this has not, as yet, been shown to be the case. In general, however, it is found that enzymes which utilise molecular oxygen as an electron acceptor contain either flavins or metal ions (or both). A one-electron transfer would give rise to the oxygen radical and the oxidised form of the metal. The oxygen radical then accepts a

second electron to yield the peroxide oxidation level. In the apoferritin assay system it remains to determine if such a process occurs and also to determine the effect, if any, that the presence of reduced FMN has on the reaction rate.

In the chemical modification studies it was seen that histidine was a likely candidate for the source of the protein's catalytic activity. The imidazole group of histidine has been shown to be important as an essential proton-transfer agent in a great many other enzymes including the transaminases, ribonuclease A, fructose diphosphate aldolase and certain glycosidases. However, it remains to modify histidine residues of apoferritin specifically, perhaps with diazonium-H-tetrazole or with diazosulphanilic acid, and note the effect on the protein's function. As Gutfreundt and Knowles (1962) pointed out, "With a complete knowledge of the structure and dynamic behaviour of protein molecules during catalysis, workers will be in a position to design and study much more realistic models of enzyme reactions than has been possible up to now.".

SUMMARY

The nature of the present study was to further investigate the structural and functional aspects of horse spleen apoferritin.

The protein was dissociated under a number of conditions and the molecular weight for the subunit was determined by a combination of sedimentation equilibrium, polyacrylamide gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulphate and gel filtration in the presence of 6M guanidine hydrochloride. The value obtained from these studies, 18,500, was in disagreement with the previously determined value of 23,000.

The molecular weight for the undissociated apoprotein was calculated by sedimentation equilibrium and was found to be 445,000 which was in excellent agreement with the literature values. This then led us to suggest that the protein consists of twenty-four subunits and not twenty as was assumed from earlier studies and this view has had direct support from a preliminary electron microscopic study.

Because of our interest in the cyanogen bromide peptides we attempted to characterise their molecular weights by extending the range for sodium dodecyl sulphate-polyacrylamide gel electrophoresis and for gel filtration in the presence of 6M guanidine hydrochloride. For the sodium dodecyl sulphate system, although there was an apparent linearity of the calibration in the region below about 10,000, the slope of the line was so steep as to make the application of this region to molecular weight determinations impracticable. It was, however, found possible to

determine peptide molecular weights in 6M guanidine and this technique was applied successfully to the determination of the molecular weights of the cyanogen bromide peptides of apoferritin. The molecular weights obtained, 8,400, 6,400, 3,400 and 900 add up to give a molecular weight of 19,100 which is in reasonable agreement with our previously determined value for the subunit molecular weight.

It was also decided to reinvestigate the tryptophan content of the protein as this was one of the methods on which a value of 23,000 for the subunit molecular weight was based. The method employed consisted of treating the protein with 2-nitrophenylsulphenyl chloride which generates in the intact protein a chromophore the absorption of which can be monitored in the visible region. The value obtained, 2.03 tryptophan residues per 18,500 g protein, was in disagreement with the previously accepted value of 1 residue per 23,000 g protein. However, this value of two was further confirmed independently by two well-established spectrophotometric methods.

Apoferritin was isolated and purified from a variety of organs and species and for the proteins studied, horse liver and spleen, human liver and spleen, human haemochromatotic liver and spleen, rat liver and spleen and pig liver, these were found to be indistinguishable in their subunit molecular weight and tryptophan content.

It was recently reported that apoferritin exhibits polydispersity when subjected to isoelectric focusing on polyacrylamide gels. This was interpreted as indicating a hetero-

geneity at the level of the subunit and since this was in disagreement with our view that the subunits were identical we reinvestigated. Our results of isoelectric focusing indicate that the protein from a variety of sources is homogeneous and does not exhibit any polydispersity.

In an ultracentrifugal study on the pH stability of horse spleen apoferritin it was found that in the range pH 3 - 10.5 the protein exists solely as monomer (17 - 18 S) while at pH 1.6 - 3.0 and at pH 10.5 - 13.0 monomer to subunit transitions were apparent. Also in this study it was noted that at pH 0.0 - 1.0 irreversible denaturation of the protein occurred and at values in excess of pH 12.6 alkaline hydrolysis resulted. When protein was dissociated at low pH, either directly or by ice-cold 67% acetic acid, and then dialysed back against various buffers of higher pH, the acid dissociated transition, although completely reversible, was shifted to the range pH 3.2 - 4.6.

These pH transitions were further studied by the technique of ultraviolet difference spectroscopy. For the acid transition it was found that on lowering the pH below 3.0 , all of the proteins tyrosines residues were perturbed and this perturbation was studied as a function of pH. It was found that this tyrosyl perturbation exactly paralleled the acid dissociation and reassociation of apoferritin. The profile of the transition was analysed mathematically and compared with theoretically derived equations. From such a treatment it was found that some degree of cooperativity was present and this is discussed in terms of hydrogen bond systems. Also found from these studies was a conformational change in the range pH 3.1 - 3.4 as demonstrated by

perturbation of a tryptophan residue. In the alkaline spectroscopic titration all five tyrosyl residues titrated with apparent pK values of about 11.8 and as such were adjudged to be 'buried'.

On the chemical modification of apoferritin with 1,2-cyclohexanedione, nine of the ten arginyl residues were converted to N^5-(4-oxo-1,3-diazaspiro[4.4]non-2-ylidene)-L-ornithine while treatment with o-methylisourea converted seven of the nine lysine residues to homoarginine. From this it was assumed that the groups modified were relatively accessible to solvent while the one refractive arginine and the two refractive lysines were most probably inaccessible and may even be involved in inter-subunit binding by salt linkages. On nitration of the protein with tetranitromethane only one tyrosine was found to react and this was demonstrated both spectrophotometrically and by amino acid analysis. Treatment of apoferritin with labelled iodoacetic acid at pH 8.6 caused three residues to be carboxymethylated and these were shown by amino acid analysis to be two residues of cysteine and one residue of histidine.

It was recently suggested that apoferritin was capable of catalysing the oxidation of ferrous iron. On studying this, it was found possible to demonstrate a ferroxidase activity and further, an assay for this sytem was established. The effect of protein concentration, substrate concentration and pH on the initial velocity were investigated and it was found that the protein exhibited the normal Michaelis-Menten relationship and the kinetic parameters V_{max} and K_m were determined. By studying a variety of proteins it was shown that only apoferritin exhibit-

ed ferroxidase activity. Native apoferritin, tryptophan-modified apoferritin, tyrosine-modified apoferritin, lysine-modified apoferritin and arginine-modified apoferritin were assayed and shown not to differ significantly from the unmodified horse spleen protein. Carboxymethylated apoferritin was, however, found to have no catalytic activity and this is discussed in the light of studies by other workers which tend to implicate histidine in the process of iron incorporation into ferritin.

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