

CAERULOPLASMIN: THE ENIGMATIC COPPER PROTEIN *

STUART H. LAURIE and EDWIN S. MOHAMMED

*School of Chemistry, Leicester Polytechnic, P.O. Box 143, Leicester, LE1 9BH
(Gt. Britain)*

(Received 9 April 1980)

CONTENTS

A. Introduction	279
B. Metabolism	280
C. Physical and chemical properties	282
(i) Isolation and lability	282
(ii) Amino acid composition	285
(iii) Carbohydrate composition	287
(iv) Copper content	287
(v) Physical properties	287
D. Properties of the Cu ions and their binding sites	289
(i) Stoichiometry and physical properties	289
(ii) Nature of the Cu binding sites	292
(iii) Redox potentials of the Cu sites	293
(iv) Interaction with anions	293
E. Caeruloplasmin as an oxidase	296
(i) General oxidase activity	296
(ii) Influence of ions	298
(iii) Oxidase activity towards natural substrates	300
(iv) Antioxidant activity	303
(v) Does Cp have an oxidase or oxidase-related role in vivo?	304
F. Caeruloplasmin as a transport form for Cu	305
G. Wilson's disease	306
Note added in proof	307
References	308

A. INTRODUCTION

Caeruloplasmin is the intensely blue coloured, copper-containing glyco-protein of the α_2 -globulin fraction of mammalian blood. When Holmberg and Laurell [1] first separated and named this protein they could not have suspected the intensive research and controversy that would ensue from their report. Various facets of the protein have been the subject of a number of previous reviews. For example, its oxidase activity, particularly towards iron, has been reviewed by Frieden and co-workers [2,3]; comparisons with other

* No reprints are available.

blue copper-containing proteins have been made [4,5]; recently a short review of its possible physiological roles has appeared [6]; numerous non-critical summaries have appeared in various bio-inorganic texts. The position prior to 1964 is illuminated by several chapters in the text 'The Biochemistry of Copper' [7], the discussions from this symposium report are as enlightening as the main papers.

Any worker in the field of copper biochemistry will, invariably at some stage, have to consider Cp * and its properties. Despite the intense research activity over the past 30 years he or she will find that the physiological role(s) of Cp is not known with any certainty (it has even been suggested that it may have no role [8]), and neither have the structure, copper content and the nature of the copper-binding sites been established with any certainty. Paradoxically, Holmberg and Laurell, in a series of papers from 1947 to 1951 [1,9–12], determined and appeared to have settled some of the above properties, although the physiological role eluded them.

Our involvement with copper biochemistry over the past few years, particularly in relation to Wilson's disease [13,14], has forced us to consider the role of this enigmatic protein and shown the need for a comprehensive review, understandable to both the chemist and biochemist; this we have set out to do covering the literature up to the end of 1978.

B. METABOLISM

Cp accounts for 90–95% of the total copper in human blood plasma. Significantly, the copper is firmly bound and does not undergo exchange with the remaining Cu in the plasma. Only 70% of the total body Cp is accounted for by the plasma content, the remainder is extravascular [15]. The serum Cp level varies considerably in healthy humans and also varies with age. An analysis [16,17] of 309 healthy individuals gave an average adult Cp level of 310 mg dm^{-3} serum with amounts varying between 100 and 500 mg dm^{-3} .

The Cp level is known to vary significantly in a number of diseases and disorders. It always increases, perhaps by a factor of 2 to 3, during pregnancy, and varies to a lesser extent in many chronic infections, some of which are indicated in Table 1. These disease-related changes in Cp level are the reason for its being classified as an 'acute phase reactant', the clinical significance is uncertain.

The biosynthesis of Cp occurs within the liver and it is here that the Cu is incorporated into the apoprotein [27]. This is demonstrated by the intravenous injection of copper-64 labelled copper(II) compounds which shows

* Abbreviations used: Cp, caeruloplasmin; PPD, *p*-phenylenediamine; EDTA, disodium dihydrogen ethylenediaminetetraacetate; DEAE, diethylaminoethyl cellulose; TRIS, tris(hydroxymethyl)aminomethane; ESR, electron spin resonance; CD, circular dichroism; ORD, optical rotatory dispersion; IR, infrared; MW, molecular weight.

TABLE 1

Pathological disorders and other states which show changes in caeruloplasmin levels

Disorder/state	Change in Cp level ^a	Ref.
Biliary cirrhosis	+	18
Chronic liver disease	+	18
Hepatic necrosis	—	20
Hodgkin's disease	+	17
Hypoproteinaemia	—	24
Kwashiorkor	—	25
Menkes' disease	—	24
Myocardial infarction	+	19
Nephrosis	—	23
Pregnancy	+	26
Schizophrenia	+	21
Wilson's disease	—	22

^a +, increase in level above normal; —, decrease in level.

[28] that Cu undergoes a rapid transfer (half-life 8–10 min) from the blood to the liver, the radioactivity then re-emerges into the blood stream as Cp bound Cu. Marceau and Aspin [29] found copper-67 labelled Cp to disappear from rat plasma with an average half-life of 13 h, this is significantly longer than the 5–7 min. recorded for the more easily dissociable Cu–albumin fraction in blood plasma. Figures for the *in vivo* turnover of Cp seem highly variable, for example, a half-life of 56 h has been reported for rabbit plasma [30]. Significantly (*vide infra*) the half-life appears to be dependent upon the carbohydrate moiety, particularly sialic acid; removal of the latter by neuroaminidase reduced the half-life in rabbit plasma to less than 0.5 h [31].

Synthesis of the apoprotein is reportedly independent of Cu status and its presence in the plasma of Cu deficient rats has been demonstrated [32]. These workers also demonstrated the inability of the circulating apoprotein to incorporate Cu ions, the incorporation only occurring at the time of synthesis [33]. Other reviewers have cast doubt on these findings citing the observation made by Owen [34] that Cp could exchange its Cu atoms with free ionic Cu in the presence of a reducing substrate. In his work Owen [34] used the artificial substrate PPD, so although his observations had no direct relevance to the plasma situation it could be a key step in any release of Cu from Cp in various tissues [35]. More recently, Vierling et al. [36] have measured the kinetics of incorporation of ⁶⁴Cu and ⁶⁷Cu into Cp in healthy humans and those with a hepatic Cu overload (sufferers from biliary cirrhosis and Wilson's disease). They conclude that Cu is incorporated into Cp by two routes, a slower route probably involving initial equilibration with a hepatic storage pool and a more rapid route which presumably by-passes this pool. The latter route is impaired in primary biliary cirrhosis. A cautionary

note must be added here, for in this study [36], as in many others, the Cp level was determined from the oxidase activity of serum, other serum constituents may also contribute to the overall activity (*vide infra*).

C. PHYSICAL AND CHEMICAL PROPERTIES

(i) *Isolation and lability*

In their original work Holmberg and Laurell [1] isolated Cp from human sera by a combination of ammonium sulphate precipitation, dialysis and precipitation by organic solvents. Various methods of separation with different results were obtained by the earlier methods. Thus, Broman [37] after fractionating human serum on hydroxylapatite followed by extensive dialysis found two forms of Cp. The minor fraction had the same absorption spectrum and oxidase activity (towards dimethyl-PPD) as the major Cp component. Significantly in this work, Broman added EDTA to Cp solutions to suppress significant oxidase activity of extraneous Cu ions. Later Morell and Scheinberg [38] obtained four Cp fractions from the pooled serum of 9109 blood donors and found differences in the histidine content of the different Cp fractions. With a milder treatment and a smaller number of pooled sera Sober et al. [39] also found heterogeneity as the Cp they separated contained between 2 and 3 different Cp fractions.

With some foresight Sanders et al. [40] reported a method of separating Cp from Cohn fractions which deliberately avoided the use of denaturing agents. In the first of a number of important papers by Deutsch [41] the crystallisation of Cp was described after separation (from out-dated blood-bank plasma, *vide infra*) by DEAE chromatography, dialysis and selective precipitation from $(\text{NH}_4)_2\text{SO}_4$ solutions; the characteristics of the final product were entirely compatible with those reported by others. This method of separation and purification described by Deutsch was subsequently used by a large number of workers (certainly this paper [41] is the one most often referred to in Cp studies). Although Deutsch carefully pointed out the sensitivity of Cp, particularly in the isolated form, these observations have frequently been ignored. For example, Deutsch found that aqueous solutions of the purified protein lost Cu even on storage in the cold and concomitantly developed electrophoretic-active minor components. The presence of Cl^- ion was also found essential for protein stability in solution. A more rapid route to a relatively high yield of homogeneous, crystalline, Cp, this time from Cohn-Fraction IV-1, was also published by Deutsch and his group [42]. In an important comprehensive, but frequently overlooked, paper Kasper and Deutsch [43] reported, in some detail, the physicochemical characteristics and *lability* of the Cp products from the above preparations [41,42]. The apoprotein was also obtained by, the now standard method of, dialysis against cyanide solutions (this causes reduction of Cu(II) to the more mobile Cu(I) which is stabilised in aqueous solution as the cyano complex). An attractive route to the apoprotein by reduction

with ascorbic acid followed by treatment with the chelating agent *N,N*-diethylthiocarbamate [44] was found to cause some protein aggregation. The most significant feature of Kasper and Deutsch's paper [43], however, was the establishment of the sensitivity of Cp to its environment. Thus, contact with solutions of common buffers such as barbitol, TRIS, EDTA, or chelating or reducing agents produced other electrophoretic-active components. Even prolonged DEAE chromatography was found to cause notable changes; the possibility that the much favoured hydroxyapatite chromatography medium could cause similar changes was also suggested. It is noteworthy that Holmberg and Laurell [1] reported problems in their separation due to adsorption of Cp onto other proteins and that in 1959 Hjerten [45] had used Cp as an example of how artefacts may be generated from adsorption chromatography.

Kasper and Deutsch [43] also observed that old preparations of Cp showed a loss of blue colour and the presence of components of high ionic mobilities. These observations tally with their findings of small amounts of *N*-terminal amino acid lysine as well as the predominant *N*-terminal valine, others [46] have also reported fractional amounts of other *N*-terminal amino acids. The lability of Cp was also revealed by Witwicki and Zakrzewski [47] who found that unmasking reagents for thiol group determination led to a parallel loss of the blue colour and oxidase activity, and that the determination of the number of thiol groups was influenced by the concentration of TRIS buffer, a finding reminiscent of Kasper and Deutsch's observations [43]. Huber and Frieden [48] reported that storage of Cp at -20°C was unsatisfactory but was satisfactory at -80°C . The finding of two crystal types [49] after extensive chromatography and dialysis is no doubt a result of the lability of the protein. Likewise, the procedure described by Morell et al. [50] for growing crystals from aqueous solutions after 1–3 days at room temperature, not surprisingly, was found to lead to artefacts attributed to protein aggregation. Aware of these problems Broman and Kjellin [51] developed a rapid, cyclic, semi-continuous separation in which 'destructive' methods were deliberately avoided, their product appeared to be homogeneous. The lability of Cp towards various reagents and storage is attributed [43] to changes in Cu valency, in amounts of Cu lost, or both, and do not appear to be reversible.

The general consensus is that many of the earlier reports of heterogeneity (including the many on two types of Cp proteins which differ in their properties [37,38,41,42,52–54]) arise from the lability of the protein and the procedures used and is not due to any genetic polymorphism. In support of this Deutsch and Fisher [55], using seemingly pure crystalline Cp, found 3 fractions after further chromatography on hydroxyapatite. Two of the fractions were present in small amounts (3% and 12% of total), the 12% component had many identical chemical and physical properties of the main (Cp) component but from its mobility was probably an aggregate. The other fraction appeared to be subunits.

Another source of heterogeneity, not often recognised as such, is the carbohydrate moiety. The amount of carbohydrate present in Cp has been measured as between 5 and 8% by weight [41,43,56]. Its influence, particularly that of the sialic acid residues, on physiological properties has already been pointed out [31]. Furthermore, Ryden [57] has shown that Cp components as reported by Broman [37] to differ only in their carbohydrate content. Removal of the terminal sialic acid residue appears to have no effect on the oxidase activity or the absorption of 610 nm visible radiation [37,57].

Not unrelated to the problem of heterogeneity is that of reports on the subunit composition of Cp. Poulik [58] first reported the presence of several polypeptide chains from urea-starch gel electrophoresis, since then there have been reports of between 2 and 8 subunits for Cp from different species [59–61]. These subunits were produced in dissociating media and had molecular weights in the range 17 000–80 000. Later Ryden [62] showed that commercial samples of human Cp contained fragments which were absent if the protein was isolated from fresh serum. Since trypsin was observed to hydrolyse Cp into several fragments Ryden proposed that the fractions present in the commercial samples resulted from proteolytic hydrolysis. Human Cp appeared more sensitive to proteases than that from other species. Nevertheless, many of the earlier workers were well aware of possible artefacts (e.g. see ref. 63) and a single polypeptide chain, as proposed by Ryden [62], of ca. 1050 amino acids would be the largest so far known for a plasma protein. This complex situation arises with other blue Cu-proteins, for example, Reinhammer [64] has reported similar differences in terms of purity and properties for both laccase and stellacyanin preparations.

Using fresh preparations Ryden [65] re-examined the behaviour of the protein in the dissociation medium 6 mol dm⁻³ guanidine HCl, using hydrodynamic (viscosity), gel electrophoresis and *N*-terminal amino acid analysis. Again, his results were consistent with a single polypeptide chain structure for human Cp, although gel filtration did show small and variable amounts of a 17 000 molecular weight component, which is typical of degraded samples. Contrary to this Freeman and Daniel [66] propose a tetrameric structure, L₂H₂, for Cp with molecular weights of 16 000 (L) and 53 000 (H). They found that 80% of the original oxidase activity could be restored by re-association of the 4 fragments in the presence of Cu ions thus apparently ruling out proteolytic fragmentation. However, it should also be pointed out that their Cp was purified from commercial samples and was stored at -20°C (cf. comments made earlier [48]). Kasper and Deutsch [43,67] had in fact earlier pointed out that a loss of Cu resulted in the appearance of electrophoretic variants and that concomitant with the Cu loss was a conformational change resulting in new antigenic determinants. A further complication arises from the finding by Carrico et al. [68] of an apo form in human serum which was immunologically similar to Cp, contained 1 ESR-active Cu atom per molecule, but had no oxidase activity. The authors claim the protein is neither a subunit of Cp nor an artefact. The presence of the apoprotein, again with a minor

Cu content, has been reported in the serum of rats fed on Cu-deficient diets [69]. The Cu content itself, however, could be an artefact since Cp is known to bond extraneous Cu ions which require chelating agents for their removal (*vide infra*).

In a later physicochemical study Ryden and Bjork [70] reiterated those aspects which require careful consideration in the handling and separation of Cp, i.e. (i) proteolysis, (ii) a slow loss of Cu and some precipitation (aggregation), (iii) heterogeneity of the carbohydrate moiety. In their case Ryden and Bjork [70] purified Cp from either normal or retroplacental serum, the latter requiring no delipidation step so that time-consuming dialysis and other concentration steps could be avoided. All separations were carried out at -4°C . These authors found however, that, contrary to other claims [48], storage at -90°C still resulted in irreversible changes in Cu content and conformation. It should be fairly obvious that in assessing differing claims as to the properties of Cp the above factors must be strongly borne in mind; too often they appear to have been ignored. Furthermore, claims for homogeneity must rest on more than a single property, i.e. not just the ratio of the absorbances at 610 and 280 nm.

(ii) Amino acid composition

The analyses from several sources are listed in Table 2, the data are presented in the form of percentage composition rather than the more usual 'number of residues per mol' because of the different molecular weights used. Considering that several corrections are always required in amino acid analyses the agreement between the different analyses is good. They all show a very high acidic amino acid content (aspartic and glutamic acids) which is consistent with the isoelectric pH of 4.4. The tryptophan content is also relatively high compared to other proteins.

There is some disagreement over the number of cysteine groups. Kasper and Deutsch [43] found only one out of a total of 15, the remaining 14 being assigned to intramolecular disulphide (cystine) linkages. On more recent evidence inaccessible cysteine groups could also be present, such as those bonded to the prosthetic Cu ions. Seemingly, in agreement with this Witwicki and Zakrzewski [47] found only one residue accessible to alkylation in the native protein, this increased to two in the presence of excess urea and then to three with the further addition of EDTA. The unmasking of the thiol groups coincided with the bleaching of the blue colour and loss of oxidase activity. In contrast Ryden and Eaker [77] found no accessible cysteine in the native protein of fresh preparations, some alkylation did take place, however, with older preparations. Under denaturing conditions they also found three cysteine residues, accessible to alkylation, out of a total of fifteen, in agreement with the earlier workers [47]. Ascorbate reduced Cp also contained just three cysteine residues [77], a result which contradicts a suggestion [78] that one of the disulphide bridges functions as a two electron acceptor during oxidase reaction.

TABLE 2

Chemical composition of caeruloplasmin

(1) Amino acids

	Percent of total amino acid residues			
	[43]	[71]	[75]	[70]
Aspartic acid	11.4	11.6	11.9	11.9
Threonine	7.3	6.8	7.4	7.2
Serine	5.8	5.6	6.0	5.8
Glutamic acid	11.0	11.0	11.0	11.2
Proline	4.8	5.7	5.0	4.3
Glycine	7.2	7.1	7.4	7.2
Alanine	4.7	4.7	4.8	4.6
Valine	5.6	6.2	6.0	5.8
Methionine	2.2	2.3	1.6	2.5
Isoleucine	4.7	4.8	5.1	5.0
Leucine	6.7	6.7	6.7	6.6
Tyrosine	6.0	5.5	4.7	6.0
Phenylalanine	4.6	4.8	5.5	4.6
Lysine	5.7	6.2	6.4	6.2
Histidine	3.6	4.0	4.1	3.7
Arginine	3.9	4.0	3.7	3.9
Tryptophan	2.3	1.8	1.9	2.0
Half-cysteine	1.2	1.3	0.8	1.4
Cysteine	0.08	—	—	0.3
Total residues found	1184	1002	1276	1065

(2) Carbohydrates

	Mol per 134 000 g		
	[31]	[70]	[76]
Glucosamine	17	15.7—19.2	18
Mannose	9.1	14.2	} 36
Galactose	9.7	12.3	
Fucose	—	1.6	2
Sialic acid	9.8	8.6	9
Total residues found	45.6	~54	55

(3) Copper

Mol per 134 000 g
6.0—6.6 [70], 6.8 [43], 5.8 [51], 5.7—6.1 [72], 7.2 [73]
6.0 [49] ^a , 5.9 [74] ^a ,

^a After removal of ca. 1 mol Cu by chelex-100 treatment.

(iii) Carbohydrate composition

The differences in values reported for the number of carbohydrate groups (see Table 2) is not unexpected [57] from the nature of the hydrolytic procedure used. The only reasonable agreement is on the sialic acid groups, the analysis of which requires a relatively mild hydrolysis. Jamieson [76] found a mannose : galactose ratio of 3 : 2 in both the native protein and glycopeptide derivatives in contrast with an earlier report [79] which gave the ratio as 1 : 2. Attempts at obtaining pure glycopeptides have not been particularly successful [57,76,80]. The overall conclusions appear to be that sialic acid forms the terminal residue [81], that asparagine is the amino acid responsible for binding the carbohydrate chain to the polypeptide chain [76], and that the number of oligosaccharide chains in the protein is ca. 9. These results are in line with those from other glycoproteins [82]. Quite clearly work in this area is difficult but, in view of the likely physiological importance of the carbohydrate moiety, further work is clearly desirable. To compound the difficulties a link between a high carbohydrate content and a low sulphur amino acid recovery has been claimed [64].

(iv) Copper content

The many reports on the Cu composition give values in the range of 0.27–0.32% by weight (see Table 2). The number of Cu atoms in the protein molecule thus varies between 8.0 (for a 160 000 MW) and 5.5 (for a 132 000 MW). The more recent analytical figures tend toward the lower composition of 0.27%. A most significant paper is that by Huber and Frieden [48], who found the Cu content to decrease from 0.32₅% to 0.28₄% on treatment of protein solutions with the metal-chelating resin chelex 100. The removal of this ca. 1 atom Cu from the protein had no effect on the colour or enzymic properties of the protein. Taking this 1 'loosely bound' Cu into account means a Cu content of either 7 atoms (160 000 MW) or 6 atoms (132 000 MW). This latter figure, which has become the more acceptable, however, does not appear to satisfy magnetic measurements, as we shall see. Subsequently Cp has been shown [74] to have a plethora of binding sites not only for more Cu(II) ions, but also for Co(II), Ni(II) and Zn(II) ions. All these ions can be removed with chelex 100. The problems of extraneous metal ion contamination was recognised earlier by Broman [37] and by Morell et al. [50]. Metal ion contamination could easily arise from reagents used during the protein separation or from some Cp proteolysis and would explain the higher analytical figure of 0.32% for the Cu content given in earlier reports.

(v) Physical properties

One of the most vexing questions concerning Cp is its molecular weight. As Table 3 shows a wide range of values have been reported. Most of the

TABLE 3
Physical parameters of caeruloplasmin

Molecular weight	Method of determination ^a	A_{610}/A_{280} ^b	$S_{20,w}$ ^c	Other parameters	Ref.
150 000	a, b			$M, 2.7 \cdot 10^{-5}$ (pH 7.4)	1,53
150 000 (142 000)	a, b				83
160 000—	a, b	0.045	7.08	$\bar{v}, 0.713$ (20°C); $D, 3.76 \cdot 10^{-7}$; $M, 4.72 \cdot 10^{-5}$ (pH 7.01)	65 ^d 43
155 000	c			$\bar{v}, 0.714$ (20°C); see ref. 51	
143 000	c	0.041			59
132 000 ± 4000	d			$\bar{v}, 0.7149$	84
134 000	e	0.045		$\bar{v}, 0.715$	65
134 000 ± 3000	e	0.048— 0.049	7.25	$D, 4.46 \cdot 10^{-7}$	70
160 000—	f	0.045	7.04		71
161 000					
122 000—	b	0.045	7.07		66
126 000		0.047		$M, 3.73 \cdot 10^{-5}$ (pH 7.6)	31

$\bar{v}$, Partial specific volume ($\text{cm}^3 \text{g}^{-1}$); D , diffusion coefficient ($\text{cm}^2 \text{s}^{-1}$); M , electrophoretic mobility ($\text{cm}^2 \text{volt}^{-1} \text{s}^{-1}$) in a phosphate buffer solution, room temp., pH ca. 7, ionic strength 0.10 mol dm^{-3} .

^a a, Sedimentation velocity; b, diffusion; c, modified (Archibald) form of approach to sedimentation equilibrium; d, crystal measurements; e, sedimentation equilibrium; f, light scattering; ^b Ratio of 610 and 280 nm absorbances. ^c Sedimentation coefficient at 20°C in water, Svedberg units. ^d Recalculated using a different $\bar{v}$ value.

earlier workers reported values between 150 000 and 160 000. Then Magdoff-Fairchild et al. [84] obtained a value of 132 000 ± 4000, since then others [65,70] have reported similar values. A fairly recent report [71] again gives a molecular weight of 160 000, but the relatively low amino acid content (see Table 3) that was found in this case appears contradictory to the high MW. A recent value [66] of 122 000–126 000 seems well outside the range found by others. Most of the older work relied on the use of sedimentation techniques. These have been criticized [66,70] because of (a) possible errors in the diffusion coefficient (e.g. Kasper and Deutsch [43] obtained a value of $3.8 \cdot 10^{-7} \text{cm}^2 \text{s}^{-1}$ which differs significantly from that of others [40,70,73,85] who found values in the range $4.4\text{--}4.6 \cdot 10^{-7} \text{cm}^2 \text{s}^{-1}$), (b) the extrapolation procedures required, and (c) the probability of impurities in many cases (e.g. proteolytic fragments). As Ryden and Bjork [70] point out the presence of just 10% of a dimeric form would raise the apparent MW by 10% when determined by the 'approach to equilibrium' method. It is

often stated or implied that such problems do not apply to the crystallographic method employed by Magdoff-Fairchild et al. [84]. However, extrapolation of properties from dilute solutions to the solid state were required in terms of the partial specific volume of the protein and the water content of the crystals. A small change in the latter property would quite significantly alter any calculated MW. Furthermore, the crystals used in this work had been supplied by Morell et al. [50] who had slowly crystallised the protein over several days at room temperature (some protein aggregates were noted). Nevertheless a figure of 134 000 seems to be the currently accepted value [86], giving a Cu content of 6–7 atoms per Cp molecule. Further confirmation of the MW does seem to be still required.

Other parameters used as tests for protein purity (see Table 3) are the sedimentation coefficient ($S_{20,w} = 7.1 \pm 0.1$ S) and the ratio of the absorbances at 610 and 280 nm, A_{610}/A_{280} . The optimum value for the latter appears to be ≥ 0.045 . Several lines of evidence point to the Cp molecule being elongated in shape. Vasiletz et al. [87] from low angle X-ray scattering of freshly prepared Cp give approximate dimensions of $6.5 \times 6.5 \times 13$ nm for an ellipsoidal shape. Kasper and Deutsch [43] report an axial ratio of 11 : 1 based on sedimentation and diffusion studies with the prosthetic Cu groups playing an important role in both the secondary and tertiary structures of the protein. Likewise, Ryden and Bjork [70] find their hydrodynamic data to be consistent with an elongated shape for the Cp molecule.

A detailed examination of porcine Cp by ORD and CD spectroscopy [88] is interpreted in terms of a large β conformation content with some random coiling, similar conclusions were reached with human Cp. Freeman and Daniel [89] also find a large β conformation content for human Cp from IR and CD measurements. Reduction and/or removal of Cu did not cause disaggregation but led to some unfolding [88] as originally claimed by Kasper and Deutsch [43].

D. PROPERTIES OF THE Cu IONS AND THEIR BINDING SITES

(i) Stoichiometry and physical properties

The different types of Cu ions found in copper proteins have been well reviewed [4,5]. In brief, there are three classes of Cu generally recognised. Type I refers to the Cu(II) ions responsible for the blue colour of these proteins, and hence referred to as the 'blue Cu' ions, they are characterised by an intense absorption near 600 nm with an extinction coefficient (ϵ) of 3000–6000 per Cu atom and an unusually small nuclear hyperfine coupling constants, $A_{\parallel}$, in its ESR spectrum (see Fig. 1 for a typical Cu(II) ESR spectrum). Type II Cu ions are often referred to as the 'non-blue Cu(II)' because of their relatively weak absorption in the visible region (ϵ 100–400), they also give a less intense but broader ESR spectrum than type I Cu and have a larger $A_{\parallel}$ constant. Type III refers to the ESR inactive Cu atoms which

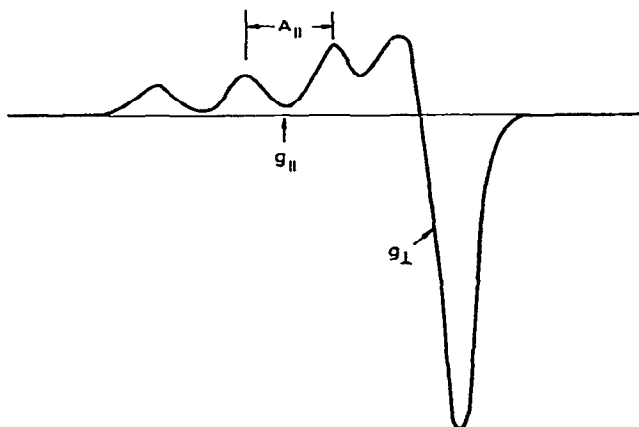


Fig. 1. Typical ESR spectrum (X-band) of Cu(II) at 77 K showing the different parameters. $A_{\perp}$ is calculated from $A_{\parallel}$.

are also responsible for an intense absorption around 310–350 nm, $\epsilon > 3000$. The similarity in properties of the blue Cu ions from different Cu proteins has led others to consider the evolutionary development of these proteins [3,90]. Cp, like the other Cu-containing oxidases, laccase and ascorbate oxidase, contains all three types of Cu ions; their spectroscopic properties are shown in Table 4.

Measurement of the temperature dependence of the magnetic susceptibility [91] of Cp has established that $44 \pm 2\%$ of the Cu ions are paramagnetic and magnetically dilute (types I and II). This figure is substantiated by integrations of the ESR spectrum [92,93]. Earlier conflict over the number of types I and II ions can be attributed to variations in ESR properties with the history of the Cp preparation [93]. In a very careful study Deinum and Vanngard [93]

TABLE 4

Physical properties of the Cu ions in caeruloplasmin

Type	Number	ESR parameters [94]				UV/vis spectrum	
		$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}^a$	$A_{\perp}^a$	Absorbance maxima (nm)	ϵ
I	1	2.215	2.06	92	10	610	5500
I	1	2.206	2.05	72	10	610	5500
II	1	2.247	2.06	180	25	no measurable spectrum	
III	3/4	ESR inactive				330	~3300

ϵ , extinction coefficient ($\text{mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$)

^a Gauss.

obtained a 2 : 1 ratio for type I : type II ions, a figure supported by the ESR—pH dependence study of Gunnarsson et al. [94] and by the study of the effect of the addition of nitric oxide on the ESR and optical absorption properties of Cp [95]. There thus appears to be 2 type I and 1 type II Cu ions, with the two type I Cu ions in slightly different environments [93]. Two separate studies [89,96] on the absorption and CD spectra over the range 300–900 nm show the presence of six electronic transitions all of which arise from the type I Cu sites.

Having accounted for 3 of the Cu ions this leaves either 3 or 4 diamagnetic Cu ions. This raises a problem which has frequently been overlooked by previous reviewers e.g. [3,5]. Comparison is often made with laccase whose Cu ions are well delineated [5]. However, the laccase molecule contains only 4 Cu ions (1 type I, 1 type II, 2 type III) and there is good evidence that the 2 type III Cu ions are in sufficiently close proximity to each other to be antiferromagnetically coupled, i.e. they constitute a diamagnetic Cu(II) dimeric unit. That such a unit also exists in Cp has been definitely stated [3,5], based on the evidence from the interaction of the odd electron molecule nitric oxide with fully reduced Cp [97]. NO induces a seven hyperfine line ESR spectrum centred around $g = 4$; such a spectrum would be expected from the addition of one electron to a Cu^{II} diamagnetic pair. The authors of this work [97], however, categorically stated that they could not specifically state which of the three Cu types was responsible for these transitions at the $g = 4$ field. Furthermore, in a more detailed study [96], the same group observed a weak $g = 4$ signal in frozen solutions of native Cp in the absence of nitric oxide. This signal intensified on the addition of NO and concomitantly a hyperfine pattern appeared. The fact that this signal is observed in oxidised Cp makes any assignment very uncertain.

In laccase the diamagnetic Cu(II) ions are associated with the 330 nm absorption band and also form a single two electron reduction site [98]. In contrast the redox titration behaviour of Cp is complex, for example, the 310 nm band in this protein can be removed by modification of the protein's structure as well as by reduction [93]. These same authors [93] also found the rate of reduction of the protein to differ drastically depending on the method of preparation and the buffer media. Whilst in phosphate buffered solutions only 0.5 electron per Cu ion is required to remove the 610 and 330 nm absorption bands, Carrico et al. [99] find that in other media 1.0 electron per Cu is required. This latter figure is also supported by the work of Deinum and Vanngard [93]. The reason for the different behaviour in phosphate media is not known.

The consumption of 1.0 electron per Cu means, of course, that all the Cu ions in the native protein must be in the +II oxidation state; this raises problems concerning the stoichiometry. The more recently acceptable figure of 6 Cu ions per 134 000 MW protein means that 50% of the total Cu is accounted for by types I and II while the more acceptable figure is $44 \pm 2\%$. This also leaves an odd number (3) of Cu ions to be included among the

diamagnetic type III group, and according to the above they must all be in the +II oxidation state. It is plausible that a Cu(II) ion may have a very weak and barely detectable ESR spectrum (that of type II is fairly weak) particularly if the Cu(II) ion can undergo charge transfer or electron delocalisation to a neighbouring group e.g. a disulphide group [78]. Ryden and Bjork [70] have proposed that the odd Cu ion belongs to a fourth type of Cu group which is ESR non-detectable and simply required by stoichiometry. The presence of 7 Cu ions per molecule provides a better fit to the physical data: 3 type I and II Cu ions accounts for 43% of the total Cu leaving an even number of type III ions (2 pairs of Cu(II) dimers?). The various possibilities are summarized in Table 4.

(ii) Nature of the Cu binding sites

Type I

The spectroscopic properties of the 'blue Cu' ions are atypical of those of the vast majority of low molecular weight tetragonal or square planar Cu(II) complexes, in that (a) the extinction coefficient of the 610 nm band is very much larger, (b) there is also a multitude of other weak absorption bands in the visible region, and (c) the ESR hyperfine coupling constant $A_{\parallel}$ is unusually small. In order to account for these unusual properties nearly every conceivable permutation of ligand type and geometry has been proposed. The weight of data now strongly favours a tetrahedral geometry involving coordination via the imidazole nitrogen atoms of histidine residues and the sulphur atoms of cysteine and/or methionine residues [100–102]. The combination of tetrahedral geometry and Cu–S bonds is necessary to explain the intense 610 nm absorption band ($RS(\sigma) \rightarrow Cu(d)$ charge transfer) and the small $A_{\parallel}$ value. Such an environment also minimises the energy required for electron reduction to Cu(I). More definitive evidence comes from the X-ray diffraction analysis of plastocyanin [103] which shows the type I Cu to have a tetrahedral environment of 2S atoms from cysteine and methionine and 2 N atoms from histidine residues. A very similar environment appears probable in azurin [104,105]. There is both chemical and physical evidence for the involvement of cysteine [48,106] and histidine residues [43,107–109] with the type I Cu ions in Cp. However, Nylen and Pettersson [110] have disputed the conclusions of Bannister and Wood [108] claiming the photochemical technique they used was not selective for histidine. Using the histidine-specific reagent diethylpyrocarbonate Nylen and Pettersson [110] surprisingly found that carbethoxylation of the imidazole groups had no effect on the 610 nm absorption but did influence the ESR spectrum attributable to type II Cu; the process was fully reversible.

Type II

Measurement of the spectroscopic properties of this Cu are difficult because of the far more intense spectra generated by the type I Cu ions. However,

it is now fairly clear from the spectra obtained (e.g. ESR spectrum, Table 4) that this Cu ion closely resembles the well known low molecular weight tetragonal type Cu(II) complexes. The type II Cu of superoxide dismutase has been shown to be bonded to 4 N atoms of 4 histidine residues [111]. Evidence for a similar environment in the case of Cp comes from the ESR modulation experiments of Mondovi et al. [109] and from more conventional ESR measurements [96,112] which indicate an environment of 3 or 4 N atoms around the Cu ion. In these experiments the spectrum of the type I Cu ions was removed by 'bleaching' these ions with nitric oxide.

Type III

With the uncertainty in the nature of the oxidation states and the stoichiometry of the Cu ions within this class virtually nothing is known about their environment. Intuitively, one would expect the involvement of thiol groups (cysteine and methionine) or the disulphide cysteine, either as bridging ligands between Cu(II) centres in the diamagnetic dimers or by stabilising Cu(I) centres. Certainly charge-transfer between Cu and S centres would account for the 330 nm absorption band associated with the type III Cu ions. Similar ideas were advocated earlier by Byers et al. [78] but fell into disfavour over reports on the cysteine stoichiometry [77], but there appear to be no logical alternatives at the present time. Some evidence for the presence of diamagnetically coupled Cu(II) centres in Cp comes from anaerobic redox measurements [99,113].

(iii) Redox potentials of the Cu sites

In their pioneering studies Holmberg and Laurell [1] established that the Cu ions must have a relatively high electrode potential associated with their reduction. This has been borne out by the work of Deinum and Vannigard [93] who showed the reduction of the type I Cu ions to be biphasic with estimated mid-point potentials of 490 and 580 mV for these two Cu ions. This difference in redox behaviour between the 2 type I ions has been observed by others [113,114]. No values have been obtained for the potentials of the other Cu ions in Cp, although those of the type III centres are also probably high [99].

(iv) Interaction with anions

A large number of ions, particularly anions, have been found to influence the oxidase activity of Cp. This section is included here, however, because in most cases the effect of added ions can be attributed either to direct interaction with the Cu ions or to indirect interaction via conformational changes. Many of the earlier workers recognised the influence that anions exerted on oxidase activity (see ref. 115 for references). One of the most significant of these studies is that of Curzon and Speyer [115] who showed that, among the

inorganic anions studied, those that are good monodentate ligands, e.g. N_3^- and CN^- , are the most effective oxidase inhibitors. Multidentate ligands on the other hand, e.g. amino acids, 1,10-phenanthroline, 8-hydroxyquinoline, had very little inhibitory effect. Curzon [116] in fact had already established that maximal inhibition with azide occurred at a 1 : 1 N_3^- : Cu ratio. These observations and the finding of an inverse correlation between anion size and inhibitory power [115] are consistent with the Cu site(s) involved being inaccessible to the outer aqueous environment, a factor which had been independently established from proton relaxation studies [117]. Not all inhibitory anions need be good Cu ligands, unsaturated carboxylates

containing the moiety $\text{>C}=\overset{\textstyle |}{\text{C}}\cdot\text{CO}_2\text{H}$ were also found [116] to be good oxidase inhibitors. Kasper [118] established that a direct interaction occurred between the inhibitory ions N_3^- , NCO^- and NCS^- and type I Cu, as evident from the reduction in the 610 nm absorption of Cp. The inhibition produced by N_3^- and NCO^- is completely reversed by their removal, whilst that of NCS^- is only partially reversible. Inhibition by CN^- involves the complete dissociation of one Cu ion (by reduction) and is not reversible, the Cu ion removed is said to be [118] type I. A more quantitative study of the same anions by Byers et al. [78] established 1 : 1 and 2 : 1 anion : Cp complex formation for N_3^- and NCS^- , and 1 : 1 complexes only for NCO^- and CN^- . The latter two anions appear to enhance reduction and the authors [78] concluded that they bind to different type Cu ions than N_3^- and NCS^- which from spectroscopic changes appear to bind to one of the type I Cu centres. Fluoride ion, which is also an oxidase inhibitor, had no effect on the 610 nm band and was found [78] not to compete with azide ion. Although this latter finding appears to confirm a conclusion reached by Andreasson and Vanngard [119] that fluoride binds to the type II Cu ion, in many other respects this study [119] differs in results. For example, using a combination of visible absorption and ESR spectroscopies only 1 : 1 complexes were found [119] with N_3^- and NCS^- ions. Furthermore, both N_3^- and F^- were found to bind to the type II Cu ion, in contrast to the findings of Byers et al. [78], and those of Falk and Reinhammer [120]. The difference in findings can be attributed to the low inhibitor concentration used by Andreasson and Vanngard [119] whilst other studies have all used high concentrations in order to enhance the effect of these anions. Conformation of the binding of F^- to type II Cu has recently come [112] from studies on the ESR spectral changes induced by F^- on both native and NO treated Cp.

The binding constants for various anions are given in Table 5. Those reported by Gunnarsson et al. [121], based on kinetic measurements of the inhibition of oxidase activity, differ from those calculated from spectroscopic changes [78], which involve the 'resting' native protein. Apart from F^- the reduced protein appears to bind the anions (particularly N_3^-) far more strongly than does the oxidised form. For the oxidised form the binding constants are very much greater than those for the aqueous Cu^{2+} ion.

TABLE 5
Binding constants for anions to caeruloplasmin

Anion	Oxidised Cp		Reduced Cp log K_1 ^b
	log K_1	log K_2 ^a	
N_3^-	3.4, ^a ~ 0 ^b	1.7	5.7
NCS^-	2.7 ^a	1.0	—
NCO^-	1.55, ^a ~ 0 ^b	0	3.8
CN^-	1.3 ^a	0	—
F^-	1.4 ^b	—	2.0
Cl^-	1.5 ^b	—	1.9
I^-	1.4 ^b	—	1.9

$K_1 = [Cp.A]/[Cp][A]$, $K_2 = [CpA_2]/[CpA][A]$.

^a From ref. 78 using 0.25 mol dm⁻³ sodium acetate solution, pH 5.5, 15°C. ^b From ref. 121 using 0.05 mol dm⁻³ sodium acetate solution, pH 5.5, 25°C.

The changes induced by neutral salts on the secondary structure on enzymes has been known for some time [122,123], and the general enzymic inhibitory order observed as $CH_3CO_2^- < Cl^- < NO_3^- < Br^- < I^- < NCS^- < ClO_4^-$. The possible influence on the secondary structure of Cp was neglected until Herve et al. [124] examined the influence of anions in the above series on the ultra-violet CD spectrum of Cp, and concluded that the anions exerted their inhibitory action in two ways. First, by complexation with Cu ions which is detectable at low anion concentrations and modifies the symmetry of the local sites; second, by interaction with the protein molecule leading to subtle conformational changes which are only detectable by CD at higher anion concentrations. The changes in the CD were interpreted in terms of increased disorder and the formation of some α -helix in the polypeptide chain. The same authors later reported [125] on the effect of anions on the CD and visible absorption spectra and found that at high concentrations N_3^- , NCO^- and NCS^- gave rise to similar changes i.e. a decrease in absorption at 610 nm and enhanced absorption at ca. 400 nm. The decrease at 610 nm is attributed to the breaking of a Cu^{II} -S (cysteine) bond via anion-induced protein conformational changes rather than by direct interaction with the type I Cu ion. This preference is based on rather tenuous inferences from isosbestic points. The new band at 400 nm, also observed by others [78], is assigned to a charge-transfer transition from a new metal-protein bond, whilst Byers et al. [78] assign it to a $Cu(II)$ -anion charge transition. It should be noted that in this study [125] large anion : Cp ratios were used and the spectral changes often appeared time dependent.

Mention has been made already of the interaction of nitric oxide, NO, with Cp [96,97,112]. NO is an odd electron molecule that can act as a single electron donor or acceptor. Initial interaction with excess NO occurs [96] at the type I Cu centres, over a 2 h incubation period reduction first

occurs at one of the type I Cu centres (termed Ib) by electron transfer ($\text{NO}-\text{Cu(II)} \rightarrow \text{NO}^+-\text{Cu}^{\text{I}}$) followed by a slower reduction at the other type I Cu(Ia) and is accompanied by conformational changes as shown by differences in the type II CD signal. These changes are reversible (by flushing the samples with oxygen). Type Ia Cu is claimed [124] to be the anion-sensitive Cu and at low temperatures (as used in the ESR studies [97]) is the preferential site for NO interaction, in contrast to the behaviour at room temperature [97]. This temperature dependence has also been found with NO treated ascorbate oxidase [126].

E. CAERULOPLASMIN AS AN OXIDASE

(i) General oxidase activity

All the Cu containing proteins are capable of participating in oxidation–reduction reactions, and Cp is no exception. Those blue proteins containing only type I Cu (e.g. stercorocyanin) can act as oxidising agents and then be re-oxidised back to the blue Cu(II) state by molecular oxygen, their properties are therefore more consistent with an electron-carrier function in a redox process [127].

Holmberg and Laurell [1,10] were the first to establish the oxidative properties of Cp. They showed that Cp could catalyse the oxidation, by O_2 , of a variety of polyphenols, aromatic polyamines and other reducing substrates. Among these *p*-phenylenediamine (PPD) was found to be the most active. The general oxidase activity is weak compared to other oxidases, e.g. laccase of plant origin, but is greater than that of simple Cu(II) salts and complexes, which also differ in that toxic H_2O_2 is the normal product from these as compared to H_2O from Cp and other oxidases. It was also established [1,10] that the oxidase activity is inhibited by the inorganic anions CN^- , N_3^- , NCO^- and NCS^- , and that the apoenzyme is inactive. Since then the coloured PPD and its derivatives have been used to determine Cp activity and its stoichiometry in serum. A recent report outlines the optimum assay conditions [128].

Non-linear Michaelis–Menten kinetics found in earlier studies for Cp catalysed oxidation reactions were shown by Young and Curzon [54] to result from the accumulation of radical products. The addition of a radical scavenger resulted in more normal linear kinetic behaviour for a wide range of substrates. The Michaelis–Menten parameters from this very thorough study [54] are given in Table 6. The interesting feature of these results is the small range of the ν_{max} values indicating the rate-determining step to be relatively independent of the nature of the substrate. The K_m values are interpreted as being consistent with substrate binding to certain of the protein side-chains. Others have reached similar conclusions [107,129].

Carbomethoxylation of imidazole groups in Cp was found [110] to have no effect on the electronic absorption spectrum but did change the ESR spectrum and the oxidase activity, thereby implicating type II Cu in the oxidase

TABLE 6
Kinetic parameters of caeruloplasmin substrates [54]

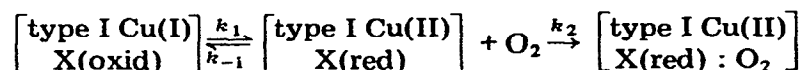
Substrates	$\nu_{\max}$ ($\text{e}^- \text{Cu}^{-1}$ min^{-1})	K_m ($10^{-6} \text{ mol dm}^{-3}$)
<i>p</i>-Amino compounds		
<i>N</i> -Acetyl- <i>p</i> -phenylenediamine	3.42	12 300
<i>p</i> -Aminophenol	3.53	1540
<i>p</i> -Anisidine	4.05	6140
2-Chloro- <i>p</i> -phenylenediamine	5.35	241
<i>NN'</i> -Di- <i>s</i> -butyl- <i>p</i> -phenylenediamine	6.05	620
2,5-Dichloro- <i>p</i> -phenylenediamine	3.94	740
<i>NN</i> -Diethyl- <i>p</i> -phenylenediamine	3.18	556
<i>NN'</i> -Dimethyl- <i>p</i> -phenylenediamine	4.33	164
<i>NN</i> -Dimethyl- <i>p</i> -phenylenediamine	5.13	203
Durenediamine	6.00	171
<i>N</i> -Ethyl- <i>N</i> -(2-hydroxyethyl)- <i>p</i> -phenyl- enediamine	5.49	110
<i>N</i> -Ethyl- <i>N</i> -2-(<i>S</i> -methylsulphonamido)- ethyl- <i>p</i> -phenylenediamine	6.06	87.2
2-Methoxy- <i>p</i> -phenylenediamine	6.20	161
<i>N</i> -(<i>p</i> -Methoxyphenyl)- <i>p</i> -phenylenediamine	6.50	20.6
2-Methyl- <i>p</i> -phenylenediamine	5.49	213
2-Nitro- <i>p</i> -phenylenediamine	6.93	1260
<i>p</i> -Phenylenediamine	4.44	292
<i>p</i> -Phenylenediamine-2-sulphonic acid	3.83	2620
<i>N</i> -Phenyl- <i>p</i> -phenylenediamine	4.78	47.7
<i>NNN'</i> -Tetraphenyl- <i>p</i> -phenylenediamine	5.06	197
Catechols		
L-Adrenaline	2.29	2550
Catechol	8.98	282 000
3,4-Dihydroxyphenylacetic acid	a	<250 000
3,4-Dihydroxyphenylalanine (dopa)	a	~20 000
3,4-Dihydroxyphenethylamine	7.54	2850
4-Methylcatechol	6.81	60 300
L-Noradrenaline	2.71	2810
5-Hydroxyindoles		
5-Hydroxyindol-3-ylacetic acid	1.50	8340
5-Hydroxytryptamine	5.68	908
5-Hydroxytryptophan	1.78	16 300
5-Hydroxytryptophol	2.87	5100
Other compounds		
<i>m</i> -Aminophenol	4.03	199 000
<i>o</i> -Aminophenol	3.60	2880
Ascorbate	4.07	5200
<i>NN</i> -Dimethyl- <i>m</i> -phenylenediamine	4.02	3050
<i>NN</i> -Dimethylaniline	a	<25 000
<i>p</i> -Methoxyphenol	a	<700 000
<i>m</i> -Phenylenediamine	5.60	36 000
<i>o</i> -Phenylenediamine	1.30	2950
Pyrogallol	10.80	57 900

TABLE 6 (continued)

Substrates	$\nu_{\max}$ ($\text{e}^- \text{Cu}^{-1} \text{min}^{-1}$)	K_m ($10^{-4} \text{ mol dm}^{-3}$)
Quinol	5.64	65 700
Resorcinol (100 mM)	} Activity undetectable	
Gentisic acid (100 mM)		
Aniline (2 mM)		
Mercaptoethanol (100 mM)		

$\nu_{\max}$ and K_m from standard Lineweaver—Burke plots. ^a $\nu_{\max}$ not determinable because of limited solubility and/or K_m .

reaction. Reduction by the most potent reducing agent, the hydrated electron, was found [130] to proceed by a bimolecular process, interpreted as the electron first attaching itself to a residue on the protein surface (peptide bonds or disulphide groups, rate constant $k > 10^{10} \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$) followed by internal transfer to an imidazole residue and ultimately to a type I centre ($k = 9 \cdot 10^2 \text{ s}^{-1}$). The authors state that milder reductants do not necessarily proceed via the same pathway. Cognizant of the inaccessibility of type I Cu to external ligands (including solvent water) a cooperative type mechanism for the oxidation for Cp and laccase by molecular O_2 has been advanced [130] as



where, X = type II or III site. $K = k_1/k_{-1} \ll 0.1$. In this mechanism the molecular O_2 reacts with a solvent accessible redox site, i.e. a type II Cu(I) or a half-reduced type III dimer, which is in electron exchange equilibrium with the type I site. Oxidation of reduced type I sites by O_2 was found to obey true first order kinetics [131].

(ii) Influence of ions

Anions

Certainly those anions which are known to bind to Cp also inhibit oxidase activity, however it seems that all anions, including Cl^- , are inhibiting at high concentrations. Curzon and Speyer [115] found that many of the inhibitors reported earlier, e.g. saturated aliphatic carboxylates, hydrazines, 1,10-phenanthroline, borate and various psychoactive drugs, were either totally ineffective or only weakly active if stimulatory Fe and reaction products were removed.

Among the most potent inhibitors are the anions CN^- and N_3^- . The former acts by the reduction and removal of Cu ions while the inhibition by the

latter has been shown to parallel the formation of the 1 : 1 Cp : N_3^- complex [119] in which N_3^- binds to the type II Cu [112,119] (see section D (iv)). Azide inhibition, which is reversible in contrast to that of CN^- , can also be overcome by the addition of Cl^- , CH_3CO_2^- and EDTA; the latter not acting as a chelating agent but by some non-specific mechanism similar to that of the acetate ion. Parallel Lineweaver—Burke plots were obtained [116] with different N_3^- concentrations suggesting that N_3^- inhibits by binding either to an enzyme-substrate complex or to some intermediate form of the enzyme during catalysis. Studies [121] on steady-state behaviour in the presence of N_3^- , NCO^- , F^- , Cl^- or Br^- are consistent with ternary complexes of the type enzyme—substrate—inhibitor being kinetically insignificant. The inhibition by N_3^- and NCO^- is attributed to strong interaction with the reduced form of Cp (see Table 5), whilst halide ions interact with both the oxidised and reduced forms. Andreasson and Vanngard [119] found some zero-time oxidase activity which was independent of N_3^- concentration, which they interpret as an initial oxidase reaction involving type I Cu (from spectroscopic changes) followed by azide inhibition at some later state, e.g. the binding of O_2 . This latter conclusion had been implied in Curzon's work [116] and confirmed by the stopped-flow measurements of Manabe et al. [132]. These inhibition studies all point to type II Cu being the active site in oxidase activity for both Cp and laccase [127,133,134].

Unsaturated aliphatic carboxylates resemble the inorganic anions in that their inhibition is also consistent with the ternary enzyme—substrate—inhibitor complex having no kinetic significance [135]. In contrast to the inhibitory inorganic anions, however, these carboxylates bind with approximately the same affinity to both the oxidised and reduced forms of Cp.

Cations

The influence of metal ions on oxidase activity was first reported by Broman [37], who found that extraneous Cu(II) ions enhanced the oxidase activity of Cp towards dimethyl—PPD. Fe(II) is also a strong stimulator of the oxidase activity of Cp [136,137]. Since these metal ions have been shown to bind to Cp [74], not surprisingly, their enhancement of oxidase can be stopped by the addition of chelating agents. Peisach and Levine [138] found that the stimulatory effect depended on the substrate used, thus with Cu(II) *ortho* related substituents were found to be required (e.g. as in *o*-phenylenediamine, catechol and epinephrine), whilst with Fe(II) *para* related substituents appeared necessary (e.g. as in PPD, hydroquinone and *p*-aminophenol). The metal ion enhancement of the oxidase activity of laccase was observed to be less than that of Cp.

On the other hand, high concentrations of Cu(II) and Fe(II), and the metal ions Co(II), Ni(II), Mn(II) and Zn(II), are found to be inhibitory [74,139]. The inhibition by Co, Ni and Zn roughly parallels their binding affinity for Cp [74]. The concentrations required for inhibition, however, ($>10^{-2}$ mol dm $^{-3}$) are very much greater than the physiological concentra-

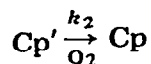
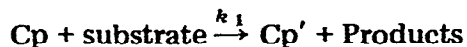
tions of 'free' metal ions, so that *in vivo* they are likely to be either weakly stimulatory or to be too dilute to have any effect.

(iii) *Oxidase activity towards natural substrates*

Ascorbate as substrate

Frieden and co-workers at one time considered [140,141] the possibility that Cp might be the mammalian equivalent of the plant enzyme ascorbate oxidase, which catalyses the reaction $2 \text{AH}_2 + \text{O}_2 \rightarrow 2 \text{A} + 2 \text{H}_2\text{O}$ where, AH_2 is ascorbate anion and A is dehydroascorbate. Cu(II) salts can also oxidase ascorbate but lead to H_2O_2 as a product as distinct from ascorbate oxidase and Cp, and they are inhibited by chelating agents, including albumin protein, so such activity can have no physiological significance. The Cp catalysed oxidation of ascorbate differs in many respects from that of ascorbate oxidase [140,141] including a much smaller turnover rate for the substrate. Nevertheless, Osaki et al. [142] have calculated that, with the relatively high concentration of Cp normally found in human serum, half of the total ascorbate would be oxidised in ca. 3 min. Despite the claim [140] that Cp was an authentic mammalian ascorbate oxidase doubts remained as to its physiological significance since serum ascorbate levels remain fairly constant and the Cp catalysis is inhibited by both citrate ion and apotransferrin at the concentrations at which they are found in human serum. The inhibition by apotransferrin is consistent with the finding that low concentrations of Fe(II) ion are required for ascorbate ion oxidation [143,144]. The overall evidence therefore precludes ascorbate oxidase activity as the role for Cp *in vivo*.

The kinetics of the ascorbate oxidation have been measured [129] by the stopped-flow technique utilising the absorbance changes at both 610 and 340 nm. The reduction process follows second-order kinetics in both Cp and substrate and the overall reaction can be simply represented by the two reactions

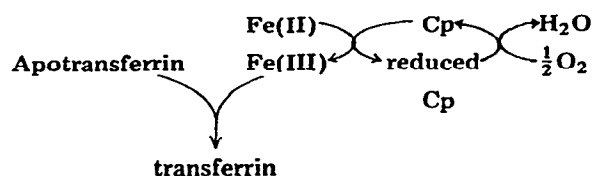


where, Cp and Cp' are the oxidised and reduced forms of the protein respectively, k_1 is the second-order reduction rate constant, and k_2 is an apparent first order rate constant for the reoxidation of Cp by O_2 . This interpretation is consistent with earlier conclusions by the same group that an enzyme—substrate complex in the classical sense has no kinetic significance.

Iron(II) as substrate

Although low Fe(II) levels stimulate Cp oxidase activity, Osaki et al. [142] have shown that the Cp catalysed oxidation of Fe(II) by O_2 has a higher molecular activity than other Cp substrates ($550 \text{ mol Fe(II) min}^{-1}$

$\text{mol}^{-1} \text{Cp}$). Following more detailed studies Osaki et al. [145] concluded that the physiological role of Cp is the catalysis of Fe(II) in serum and hence renamed Cp as ferroxidase (ferro- O_2 oxidoreductase E.C.1.12.3.1). Iron entering the bloodstream from the gastrointestinal tract is believed to be in the Fe(II) state and must therefore be oxidised to Fe(III) for incorporation into the iron transport protein transferrin. The Florida group [145] found the non-enzymic route for Fe(II) oxidation in serum to be insufficient to account for the rate of Fe(III) –transferrin formation. The ferroxidase activity may be represented by the scheme



Kinetic studies [146] show an initial rapid reaction between Cp and Fe(II) . ($k = 1.2 \cdot 10^6 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$) followed by a slower electron transfer from Fe(II) to a Cu(II) centre (probably type I since the 610 nm absorbance decreases). The rate determining step appears to be a conformational change in the protein just before reoxidation as judged from the large entropy change of $-96 \text{ kJ mol}^{-1} \text{ K}^{-1}$. This latter observation seems common to most Cp substrates, as is the v_{max} value and the biphasic (non-linear kinetics) behaviour [146].

The attractiveness of the hypothesis of a ferroxidase role for Cp is that it provides a molecular link between the metabolism of the elements Cu and Fe. Copper deficiency is known to influence Fe metabolism in animals and since more than 90% of the Cu in the blood of mammals is in the form of Cp, a ferroxidase role would be of obvious importance. In support of this Cp was found [147] to mobilise Fe from liver under physiological conditions, whereas Cu bound to albumin was ineffective. These authors [147] further point out that the liberated Fe would generate a very steep Fe concentration gradient between iron storage cells and the capillary systems resulting in a rapid iron movement out of the cells. An *in vivo* relationship has also been demonstrated [145,148] between Cp injected into Cu-deficient animals and the subsequent rise in plasma Fe levels.

One major drawback to the ferroxidase role for Cp is that the majority of Wilson's disease patients have depressed Cp levels and yet show no symptoms of Fe deficiency or defects in iron transport [149]. Topham and Frieden [150], however, claim to have found a second ferroxidase enzyme (ferroxidase II) in human serum, which they say would account for the normal Fe metabolism in Wilson's disease patients. Others [151] have not been able to find this second enzyme in fresh, undialysed, serum from either healthy humans or from Wilson's disease patients. Citrate ion can also function as a ferroxidase in blood serum [151, 152]. The ability of

citrate to catalyse the oxidation of Fe(II) is not unexpected since Fe(III) citrate complexes are thermodynamically highly stable and this should increase the oxidation potential of Fe(II) making the oxidation step more favourable. Citrate and ferroxidase II are similar in that both are initially associated with β -lipoprotein fractions in the serum, both display no oxidase activity towards PPD, and their ferroxidase activities are not inhibited by N_3^- ; thereafter their properties differ markedly [150,152]. Frieden and Hsieh, however, have expressed some doubts [3] over ferroxidase II being a protein. Other ferroxidase active molecules present in serum are apotransferrin [153] and apoferritin [154,155], probably for the same reason as advanced above for the citrate ion, i.e. enhanced thermodynamic stability of the Fe(III) ion. Schen and Rabinovitz [156] also reported a non-Cp source, as yet unidentified, of ferroxidase activity in serum. Clearly the nature and significance of this second source of ferroxidase activity is far from certain.

However, Roeser et al. [157] have demonstrated that deficiency induced in swine by feeding on a Cu deficient diet could be alleviated by administration of Cp but not by simple Cu salts — at least not until their Cu became incorporated into Cp. More importantly, they claimed from a very limited study, to have detected iron deficiency in a small number of patients of Wilson's disease who had severely depressed Cp levels (<5% of normal). Non-Cp ferroxidase activity appeared to remain unchanged. The link with very low Cp levels could explain why iron deficiency had previously been overlooked, although in a survey of 230 patients approximately 60% had Cp levels $\leq 16\%$ of normal [149]. Moreover, these low levels are normally maintained throughout a patient's life, so the oversight still remains surprising. If iron deficiency is linked with very low Cp levels this raises a further dichotomy in that Nature would appear to be overproducing Cp to the extent that 90% of the serum Cp is surplus to the ferroxidase requirement! One further cautionary note should be added — *in vitro* demonstrations of ferroxidase activity frequently use aqueous Fe(II) ions as substrate, but *in vivo* very little of this simple ion exists (the transition metal ions are predominantly in the form of chelates, see ref. 158). Furthermore, as intimated above, the properties of a metal ion, e.g. its redox properties, are well known to be dependent upon its coordination environment.

Biogenic amines as substrates

Administration of centrally active drugs, such as D-lysergic acid diethylamide (LSD), to animals has been found to elevate 5-hydroxytryptamine and depress catecholamine levels in the brain [159]. Since Cp can use noradrenaline and 5-hydroxytryptamine as substrates [160], and cognizant of Cp's oxidase activity towards amines, Barrass and Coult [161] decided to investigate the effect of LSD on Cp levels. Administration of LSD led to the inhibition of the Cp oxidation of 5-hydroxytryptamine but resulted in acceleration of the Cp oxidation of noradrenaline and dopamine. Some of the drugs used in the treatment of mental illness were also found to affect

the Cp oxidation of these substrates. Thus, tranquillisers of the phenothiazine class accelerated their oxidation, whilst antidepressant drugs (other than monoamine oxidase inhibitors) inhibited their oxidation. These substrates are important neurotransmitters and consequently Barrass and Coult [161] proposed that Cp, or some enzyme with similar properties, may be of importance as a fine control on the levels of these substances in the regions of the brain in which they act as neuro-transmitters. The true physiological significance of these observations is still to be established. Enhanced oxidation of dopamine by Cp, or a Cp-like enzyme, has been invoked to explain the lower brain dopamine levels found in Parkinson's disease [162]. Normal motor function in man may depend on neurochemical equilibria involving the three substrates mentioned above and other transmitter substances, and Parkinson's disease may well be associated with disturbances in such equilibria. Barrass et al. [162] propose that 3-hydroxy-4-methoxyphenylethylamine, produced endogenously by aberrant methylation of dopamine, interacts with Cp to enhance dopamine oxidation.

The rate of oxidation by Cp of the tranquillising drugs of the phenothiazine class was found [163] to be enhanced by the presence of natural reducing agents. The activation of the oxidation of dopamine and dihydroxy-phenylalanine (dopa) by the phenothiazine derivatives is attributed to their acting as cycling intermediates between Cp and catecholamine [163]. Oxidase activity towards other biological amines has also been reported [164–167] but their *in vivo* significance is yet to be established.

(iv) Antioxidant activity

The oxidation properties of Cp could also have a potential role in removing toxic (free-radical) products formed as by-products in metabolic processes. It has been known for some time that human blood serum contains a potent lipid antioxidation inhibitor (see ref. 168 and references therein), and recently Cp has been shown to possess such anti-oxidant activity [168,169]. The Cp used in these studies was not particularly pure ($\sim 83\%$ from the A_{610}/A_{280} ratio) and the antioxidant activity was relatively weak. Interestingly, the lipid antioxidation was found [168] to be induced by the presence of ascorbate and Fe(II) ions, and the antioxidation by Cp to be inhibited by azide. The parallels with the oxidase properties of Cp are obvious and raise the possibility that the antioxidant activity results from the ferroxidase properties of Cp. A second source of antioxidant activity in serum is apotransferrin [169]. Although Cu salts and apoCp were found [168] to be inactive, the possible influence of trace amounts of Cu and Fe on the antioxidant activity was not investigated. Gutteridge [170,171], however, has found that both Cu(II) and Cu(I) can catalyse lipid oxidation.

Gutteridge [6] has pointed out that the 'acute phase reactivity' of Cp resembles other alpha-globulins, in that its level is raised whenever there is tissue damage, and that during inflammatory states two distinct antioxidant

roles could be speculatively assigned to Cp. These are, first, the prevention of released iron (as a result of cell damage) acting as a free radical catalyst, as in the formation of superoxide radical ion: $\text{Fe(II)} + \text{O}_2 \rightarrow \text{Fe(III)} + \text{O}_2^-$. Second, by a direct inactivating role against free radicals produced by phagocytosing white cells and which may escape into the extracellular fluids which do not contain the protective enzymes catalase and superoxide dismutase.

There are a number of reports which show that plasma Cu and Cp levels are raised in rheumatoid arthritis patients [172–174] and a review of the significance of these changes in levels has recently appeared within this journal [175]. It is tempting to postulate that Cp has an anti-inflammatory (antioxidant) role in rheumatoid arthritis but this would be as speculative as its lipid antioxidant role at present. Furthermore, a recent report [176] suggests that the elevated Cp levels claimed for rheumatoid arthritis patients arises because of the inclusion of data from female patients with naturally elevated Cp levels from the taking of oral contraceptives. Inclusion of this group is claimed to significantly distort the figures.

(v) Does Cp have an oxidase or oxidase-related role in vivo?

To these authors the most definitive evidence for an oxidase role for Cp *in vivo* comes from the observation [177] of a typical Cu(II) ESR signal ($g \sim 2$) in both serum and intact blood which steadily decreased when the blood or serum was kept under anaerobic conditions but which returned to near its original intensity when subsequently exposed to air. Since $\geq 90\%$ of the Cu(II) in blood is present in Cp this signal can only be attributed to Cp itself. Another multiplet at $g \sim 4.3$, assigned to the Fe in transferrin, remained unaltered during these experiments. The question then arises as to what is/are the natural substrate(s)? This is complicated by the lack of specificity in the oxidase activity of Cp, although it is a less efficient enzyme than its counterpart laccase. Frieden and co-workers [143] have divided the substrates into three groups as shown in Fig. 2. Groups 1 and 2, the latter including the biogenic amines, are considered as true substrates, while those in group 3 are considered to be pseudosubstrates because they react via some intermediate substrate (e.g. A in Fig. 2 could be Fe), this group includes ascorbate and NADH for example, and probably the antioxidant substrates.

Since Fe(II) is the substrate with the lowest K_m values and because of evidence linking Cp with Fe metabolism then, not unnaturally, a ferroxidase role has been assigned to Cp. As already pointed out this raises a major problem as to the overabundance of Cp for this role. A further major question which this role raises is why should Nature design such a complex oxidant in terms of its size, and the number of Cu ions and their different properties, for such a relatively simple one-electron oxidation process? The possibility of some other ferroxidase active source in blood serum also still remains.

The mechanism of the oxidase reaction is still obscure. The 'entatic'

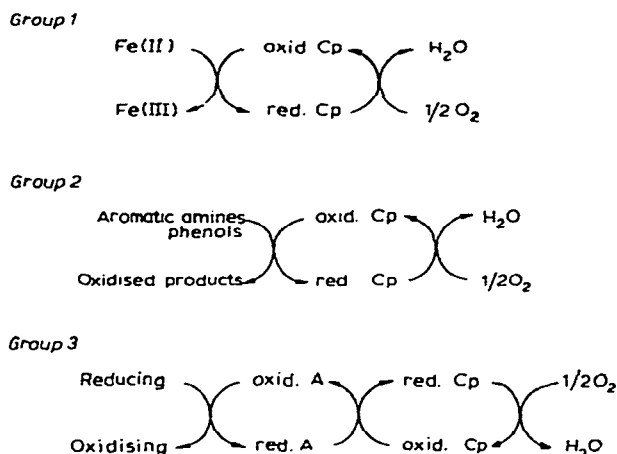


Fig. 2. Classification [143] of the various substrate groups and how they react, oxid = oxidised form, red = reduced form, A = intermediate substrates.

nature of the type I Cu sites and the partial reduction of their associated spectral properties during oxidation would appear to favour them as the primary redox-active sites. Inhibition studies, however, point to the type II Cu as being the substrate binding site; these observations support a cooperative type of interaction between the different Cu sites. Although the 310 nm absorption band associated with the type III Cu ions decreases concomitantly with the 610 nm band of the type I Cu ions during oxidation, it is now apparent that this can arise from protein conformational changes which means the function of the type III sites remains unknown.

F. CAERULOPLASMIN AS A TRANSPORT FORM FOR Cu

Broman [178] was one of the first to raise the possibility of Cp acting as a specific carrier system for transporting Cu from the liver to cells. The transport medium for Cu in blood is known to be albumin protein rather than Cp because of the lack of exchange between Cp-bound Cu and ionic Cu *in vivo* [33]. Early evidence for a tissue Cu transport function for Cp came from ^{64}Cu distribution studies in rats [179] and the serendipitous observation [35] that Cp could accept electrons from the cell respiratory system, in particular from cytochrome c. The reaction with cytochrome c was shown to occur under anaerobic conditions in the presence of a substrate, such as succinate, reduced cytochrome c or NADH. Under aerobic conditions the oxidised form of Cp was found to inhibit the electron-transport system [35].

Two reports point to Cp providing the Cu required for incorporation into cytochrome c oxidase [29,180]. Marceau and Aspin [181] compared the fate of ^{64}Cu -albumin and ^{67}Cu labelled Cp after intravenous injection into rats. While both radioactive isotopes eventually became attached to intra-

cellular proteins the ^{67}Cu was found to be tightly bound in cytochrome *c* oxidase while the ^{64}Cu was only loosely bound and readily exchangeable, supporting the contention that only Cp provides the functional, intrinsic, Cu ions of cytochrome *c* oxidase. Cp transfer of Cu to superoxide dismutase was also observed [181,182]. Although somewhat sceptical of the validity of the work of Marceau and Aspin, Frieden and co-workers [3,183] have come to similar conclusions. Using rats fed on Cu deficient diets to reduce their cytochrome *c* oxidase level Hsieh and Frieden [183] observed that the cytochrome *c* oxidase activity was enhanced after addition of Cp to the diet, but that Cu in other forms only increased the enzymic activity after incorporation of the Cu into Cp had occurred. These authors conclude that the evidence strongly points to Cp having a physiological role as a transport form providing Cu for intracellular enzymes, while the other recognised transport form, the Cu—albumin complex in blood, is involved in the transport of newly absorbed Cu from the gut to the liver.

The observation by Owen [34] that Cp can release its Cu atoms under physiological conditions in the presence of a reducing substrate offers a possible mechanism for the transfer of Cu from Cp to cytochrome *c* oxidase within the cells where the necessary substrates are present. However, the results of Holtzman and Gaumnitz [32,69] and Marceau and Aspin [29] show that both ^{14}C and ^{64}Cu labelled Cp disappear from rat plasma at the same rate. These findings are supported by double-labelling experiments [182] and measurements on the disappearance of the oxidase activity of Cp from human plasma [33]. These results all point to Cp being catabolized at the time of Cu release in the cells. The double-labelling experiments of Marceau and Aspin [182], in fact, show that the Cu does not remain attached to the catabolized Cp fragments.

A multifunctional role for Cp *in vivo* in terms of oxidase action and Cu transport would certainly overcome some of the obstacles raised previously over an oxidase role only, i.e. the relatively high concentration of Cp and its complexity.

G. WILSON'S DISEASE

There are several, excellent, reviews on Wilson's disease which cover the involvement of Cp [149,184,185], but so fundamental is the role of Cp in this disease to the proposals concerning its physiological status that it is worthwhile reiterating some of the more relevant features. Wilson's disease or hepatic lenticular degeneration results from a genetically determined inborn error in Cu metabolism causing the accumulation of fatal amounts of Cu in body tissues. Provided diagnosis is early enough patients may survive, with complete reversal of all neurological disorders, by a constant regime of the drug D-penicillamine. This drug enhances Cu excretion and maintains the patient in a negative Cu balance. One of the more consistent biochemical features of the disease is a low (although variable) blood Cp level. From the

combined data on 230 patients, Sass-Kortsak and Bearn [149] found that 60% had Cp levels of less than 50 mg dm^{-3} (cf. ca. 310 mg dm^{-3} for healthy humans [16,17], see section B), in some of these the Cp was barely detectable, approximately 80% had levels below 100 mg dm^{-3} and 4% had, at the time of diagnosis, near normal levels. Physical, chemical and immunological measurements show that Cp from Wilson's disease patients is identical to that from healthy humans [186–188]. However, the possibility of genetically determined differences in the sialic acid moiety has been pointed out [31] and a hypothesis has recently been made [189] that Cp homeostasis is controlled by the sialic acid moiety. Obviously, further work on the physiological significance of the carbohydrate content of Cp is of great importance. *

The studies on Cu distribution, and the clinical and biochemical changes in Wilson's disease are very comprehensive and any conclusions as to the physiological properties of Cp must be consistent with these studies. This is where many of the proposals appear to flounder. To quote from Walshe [185], who has done so much of the pioneering work on this disease: "The fact is that patients with Wilson's disease, provided their copper balance is controlled with the chelating agent penicillamine, can live a normal life and, if children show normal growth rates, without any signs of failure of haem synthesis, breakdown of the electron transfer chain or abnormally high levels of biologically active amines in the circulation. In view of this observation it is difficult to believe that Cp is essential for iron mobilization, for cytochrome synthesis or for enzymatic destruction of amines in the plasma. The only point at which this protein would appear to have a bearing on the pathogenesis of the disease is in the control of copper balance and hence copper toxicity but even this is difficult to envisage when it is remembered that a significant percentage of patients have caeruloplasmin concentrations which approach or even enter the normal range".

NOTE ADDED IN PROOF

A number of papers have appeared in the medical literature, mainly by M. Foster and co-workers, in which direct use of the Cp ESR signal in blood serum has been made to assess Cp levels in human disease states (see J.A. Green, T. Pocklington, A.A. Dawson and M. Foster, *Br. J. Cancer*, 41 (1980) 356 and references therein; S. Cannistraro and P.L. Indovina, *Phys. Med. Biol.*, 24 (1979)197). Unfortunately, no definitive answer is given to the ferroxidase role of Cp in these studies. No really significant advances have been noted over the period from late 1978 to early 1980. A number of papers report on increased Cp levels in cancer patients (e.g. M.C.M. Mateo et al., *Biomedicine*, 31 (1979)66; N. Morita et al., *Igaku Kenkyu (Japan)*, 49 (1979)277; G.S. Andrews, *J.Clin. Pathol.*, 32 (1979)325). A direct positive

* The significance of the carbohydrate moiety of glycoproteins has recently been summarized: R.D. Marshall, *Biochem. Soc. Trans.*, 4 (1979) 800.

correlation between Cp levels, from radial immunodiffusion, and total Cu in serum has been established (B. Mallet et al., *Pharm. Biol.*, 13 (1979)325). R.A. Loevstad (*Gen. Pharmacol.*, 10 (1979)147) has found that Cu(II) ions enhance the in vitro oxidation of catecholamine by Cp. A correlation between serum antioxidant activity and serum Cp level was observed in a number of pathological states except, interestingly, in Wilson's-disease patients (L.M. Cranfield et al., *Ann. Clin. Biochem.*, 16 (1979)299). Cp has also been postulated to have a protective anti-inflammatory role (C.W. Denko, *Agents Actions*, 9 (1979) 333). A detailed spectroscopic study establishes the blue Cu centres of Cp to be structurally identical to those in the other blue Cu proteins, and shows that structural changes at these centres occur on anion binding (J.H. Dawson et al., *J. Am. Chem. Soc.*, 101 (1979)5046).

REFERENCES

- 1 C.G. Holmberg and C.B. Laurell, *Acta Chem. Scand.*, 2 (1948) 550.
- 2 E. Frieden, in M. Friedman (Ed.), *Protein-Metal Interactions*, Plenum Press, New York, 1974, p. 1.
- 3 E. Frieden and H.S. Hsieh, in A. Meister (Ed.), *Advances in Enzymology*, Wiley, London, 1976, p. 187.
- 4 T. Vanngard, in E. Ehrenberg, E.C. Malmstrom and T. Vanngard (Eds.), *Magnetic Resonances in Biological Systems*, Pergamon, Oxford, 1967, p. 213.
- 5 J.A. Fee, *Struct. Bond.*, 23 (1975) 1.
- 6 J.M.C. Gutteridge, *Ann. Clin. Biochem.*, 15 (1978) 293.
- 7 J. Peisach, P. Aisen and W.E. Blumberg (Eds.), *The Biochemistry of Copper*, Academic Press, New York, 1966.
- 8 J. Peisach, P. Aisen and W.E. Blumberg (Eds.), *The Biochemistry of Copper*, Academic Press, New York, 1966, p. 571.
- 9 C.G. Holmberg and C.B. Laurell, *Acta Chem. Scand.*, 1 (1947) 944.
- 10 C.G. Holmberg and C.B. Laurell, *Acta Chem. Scand.*, 5 (1951) 476.
- 11 C.G. Holmberg and C.B. Laurell, *Acta Chem. Scand.*, 5 (1951) 921.
- 12 C.G. Holmberg and C.B. Laurell, *Scand. J. Clin. Lab. Invest.*, 3 (1951) 103.
- 13 B. Sarkar, A. Sass-Kortsak, R. Clarke, S.H. Laurie and P. Wei, *Proc. R. Soc. Med.*, 70, suppl. 3 (1977) 13.
- 14 S.H. Laurie and D.M. Prime, *J. Inorg. Biochem.*, 11 (1979) 229.
- 15 T.A. Waldmann, A.G. Morell, R.D. Wochner, W. Strober and I. Sternlieb, *J. Clin. Invest.*, 46 (1967) 10.
- 16 D.W. Cox, *J. Lab. Clin. Med.*, 68 (1966) 893.
- 17 A. Sass-Kortsak, in H. Schwiegk, (Ed.), *Handbuch der inneren Medizin*, Springer, Verlag, Berlin, 1974, p. 627.
- 18 I.H. Scheinberg and I. Sternlieb, *Pharmacol. Rev.*, 12 (1960) 355.
- 19 N.R. Rowell and A.J. Smith, *Brit. Med. J.*, 2 (1959) 459.
- 20 J. Briggs and J.M. Walshe, *Lancet*, 2 (1962) 263.
- 21 R.G. Heath, B.E. Leach, L.W. Byers, S. Martens and C.A. Feighley, *Am. J. Psychiatry*, 114 (1958) 683.
- 22 I.H. Scheinberg and D. Gitlin, *Science*, 116 (1952) 484.
- 23 G.E. Cartwright, C.J. Gubler and M.M. Wintrobe, *J. Clin. Invest.*, 33 (1954) 685.
- 24 D.J. Danks, P.E. Campbell, B.J. Stevens, V. Mayne and E. Cartwright, *Pediatrics*, 50 (1972) 188.

- 25 M.E. Lahey, M. Behar, F. Viteri and N.S. Scrimshaw, *Pediatrics*, 22 (1958) 72.
- 26 I.H. Scheinberg, C.D. Cook and J.A. Murphy, *J. Clin. Invest.*, 33 (1954) 963.
- 27 C.A. Owen and J.B. Hazelrig, *Am. J. Physiol.*, 210 (1966) 1059.
- 28 A. Sass-Kortsak, *Adv. Clin. Chem.*, 8 (1965) 1.
- 29 N. Marceau and N. Aspin, *Am. J. Physiol.*, 222 (1972) 106.
- 30 P. Aisen, A.G. Morell, S. Alpert and I. Sternlieb, *Nature (London)*, 203 (1964) 873.
- 31 A.G. Morell, R.A. Irvine, I. Sternlieb, I.H. Scheinberg and G. Ashwell, *J. Biol. Chem.*, 243 (1968) 155.
- 32 N.A. Holtzman and B.M. Gaumnitz, *J. Biol. Chem.*, 245 (1970) 2354.
- 33 I. Sternlieb, A.G. Morell, W.D. Tucker, M.W. Greene and I.H. Scheinberg, *J. Clin. Invest.*, 40 (1961) 1834.
- 34 C.A. Owen, *Proc. Soc. Exp. Biol. Med.*, 149 (1975) 681.
- 35 C.F. Brown and J.B. White, *J. Biol. Chem.*, 236 (1961) 911.
- 36 J.M. Vierling, R. Shrager, W.F. Rumble, R. Aamodt, M.D. Berman and E.A. Jones, *Gastroenterology*, 74 (1978) 652.
- 37 L. Broman, *Nature (London)*, 182 (1958) 1655.
- 38 A.G. Morell and I.H. Scheinberg, *Science*, 131 (1960) 930.
- 39 H.A. Sober, F.S. Gutter, M.M. Wyckoff and E.A. Peterson, *J. Am. Chem. Soc.*, 78 (1956) 751.
- 40 B.E. Sanders, O.P. Miller and M.N. Richard, *Arch. Biochem. Biophys.*, 84 (1959) 60.
- 41 H.F. Deutsch, *Arch. Biochem. Biophys.*, 89 (1960) 225.
- 42 H.F. Deutsch, C.B. Kasper and D.A. Walsh, *Arch. Biochem. Biophys.*, 99 (1962) 132.
- 43 C.B. Kasper and H.F. Deutsch, *J. Biol. Chem.*, 238 (1963) 2325.
- 44 A. Morell and I.H. Scheinberg, *Science*, 127 (1958) 588.
- 45 S. Hjerten, *Biochim. Biophys. Acta*, 31 (1959) 216.
- 46 H.E. Schultze and A. Mahlung, *Bibliotheca Haematol.*, 12 (1961) 197.
- 47 J. Witwicki and K. Zakrzewski, *Eur. J. Biochem.*, 10 (1969) 284.
- 48 C.T. Huber and E. Frieden, *J. Biol. Chem.*, 245 (1970) 3973.
- 49 J.A.J. Trip and J. Van Dam, *Clin. Chim. Acta*, 36 (1972) 561.
- 50 A.G. Morell, C.J.A. Van Den Hamer and I.H. Scheinberg, *J. Biol. Chem.*, 244 (1969) 3494.
- 51 L. Broman and K. Kjellin, *Biochim. Biophys. Acta*, 82 (1964) 101.
- 52 T. Kaya, *J. Biochem.*, 56 (1964) 122.
- 53 R. Richterich, A. Tempereri and H. Aebi, *Biochim. Biophys. Acta*, 56 (1962) 240.
- 54 S.N. Young and G. Curzon, *Biochem. J.*, 129 (1972) 273.
- 55 H.F. Deutsch and G.B. Fisher, *J. Biol. Chem.*, 239 (1964) 3325.
- 56 H.E. Schultze and G. Schwick, *Clin. Chim. Acta*, 4 (1959) 15.
- 57 L. Ryden, *Int. J. Protein Res.*, 3 (1971) 191.
- 58 M.D. Poulik, *Nature (London)*, 194 (1962) 842.
- 59 W.N. Poillon and A.G. Bearn, *Biochim. Biophys. Acta*, 127 (1966) 407.
- 60 H. Mukasa, S. Kajiyama, K. Sugiyama, K. Funakubo, M. Itoh, Y. Nosoh and T. Sato, *Biochim. Biophys. Acta*, 168 (1968) 132.
- 61 K. Simons and A.G. Bearn, *Biochim. Biophys. Acta*, 175 (1969) 260.
- 62 L. Ryden, *F.E.B.S. Lett.*, 18 (1971) 321.
- 63 W.N. Poillon and A.G. Bearn, in J. Peisach, P. Aisen and W.E. Blumberg (Eds.), *The Biochemistry of Copper*, Academic Press, New York, 1966, p. 525.
- 64 B. Reinhammer, *Biochim. Biophys. Acta*, 205 (1970) 35.
- 65 L. Ryden, *Eur. J. Biochem.*, 26 (1972) 380.
- 66 S. Freeman and E. Daniel, *Biochemistry*, 12 (1973) 4806.
- 67 C.B. Kasper and H.F. Deutsch, *J. Biol. Chem.*, 238 (1963) 2343.
- 68 R.J. Carrico, H.F. Deutsch, H. Beinert and W.H. Orme-Johnson, *J. Biol. Chem.*, 244 (1969) 4141.
- 69 N.A. Holtzman and B.M. Gaumnitz, *J. Biol. Chem.*, 245 (1970) 2350.

- 70 L. Ryden and I. Bjork, *Biochemistry*, 15 (1976) 3411.
- 71 O. Nakagawa, *Int. J. Protein Res.*, 4 (1972) 385.
- 72 C.B. Kasper, *Biochemistry*, 6 (1967) 3185.
- 73 I.M. Vasiletz, K.A. Moshkov and V.P. Kushner, *Mol. Biol.*, 6 (1972) 246.
- 74 D.J. McKee and E. Frieden, *Biochemistry*, 10 (1971) 3880.
- 75 J.T. Edsall and P.F. Spahr, personal communication, quoted in ref. 7, p. 517.
- 76 G.A. Jamieson, *J. Biol.Chem.*, 240 (1965) 2019.
- 77 L. Ryden and D. Eaker, *F.E.B.S. Lett.*, 53 (1975) 279.
- 78 W. Byers, G. Curzon, K. Garbett, B.F. Speyer, S.N. Young and R.J.P. Williams, *Biochim. Biophys. Acta*, 310 (1973) 38.
- 79 H.E. Schultze, *Scand. J. Clin. Lab. Invest.*, 10 (1957) 135.
- 80 W.E. Marshall and J. Porath, *J. Biol. Chem.*, 240 (1965) 209.
- 81 A.G. Morell, C.J.A. Van Den Hamer and I.H. Scheinberg, *J. Biol. Chem.*, 241 (1966) 3745.
- 82 R.G. Spiro, *Annu. Rev. Biochem.*, 39 (1970) 599.
- 83 K.O. Pederson, in R. Stoops (Ed.), *Les Proteines: Rapports et Discussion*, Institut Intd. de Chimie Solvay, Bruzelles, 1959, p. 19.
- 84 B. Magdoff-Fairchild, F.M. Lovell and B.W. Low, *J. Biol. Chem.*, 244 (1969) 3497.
- 85 P. Andrews, *Biochem. J.*, 96 (1966) 595.
- 86 F.J. Carver and E. Frieden, in *Metal Ion Transport and Storage*, British Biophysical Society and Inorganic Biochemistry Discussion Group Meeting, Sheffield, England, 1979.
- 87 I.M. Vasiletz, R.L. Kayushina, I.P. Kuranova, K.A. Moshkov, M.M. Shavlovsky and S.A. Nevfakh, *Biochimika*, 18 (1973) 972.
- 88 Y. Hibino, T. Samejima, S. Kajiyama and Y. Nosoh, *Arch. Biochem. Biophys.*, 130 (1969) 617.
- 89 S. Freeman and E. Daniel, *Biochim. Biophys. Acta*, 534 (1978) 132.
- 90 L. Ryden and J-O. Lundgren, *Nature (London)*, 261 (1976) 344.
- 91 P. Aisen, S.H. Koenig and H.R. Lillenthal, *J. Mol. Biol.*, 28 (1967) 225.
- 92 A. Ehrenberg, B.G. Malmstrom, R. Aasa and T. Vanngard, *J. Mol. Biol.*, 5 (1962) 450.
- 93 J. Deinum and T. Vanngard, *Biochim. Biophys. Acta*, 310 (1973) 321.
- 94 P.O. Gunnarson, U. Nylén, G. Pettersson, *Eur. J. Biochem.*, 37 (1973) 47.
- 95 R. Wever, F.X.R. Van Leeuwen and B.F. Van Gelder, *Biochim. Biophys. Acta*, 302 (1973) 236.
- 96 F.X.R. Van Leeuwen and B.F. Van Gelder, *Eur. J. Biochem.*, 87 (1978) 305.
- 97 F.X.R. Van Leeuwen, R. Wever and B.F. Van Gelder, *Biochim. Biophys. Acta*, 315 (1973) 200.
- 98 B.R.M. Reinhammer and T. Vanngard, *Eur. J. Biochem.*, 18 (1971) 463.
- 99 R.J. Carrico, B.G. Malmstrom and T. Vanngard, *Eur. J. Biochem.*, 20 (1971) 518.
- 100 E.J. Solomon, J.W. Hare and H.B. Gray, *Proc. Natl. Acad. Sci., U.S.A.*, 73 (1976) 1389.
- 101 A.R. Amundsen, J. Whelan and B. Bosnich, *J. Am. Chem. Soc.*, 99 (1977) 6730.
- 102 U. Sakaguchi and A.W. Addison, *J. Am. Chem. Soc.*, 99 (1977) 5189.
- 103 P.M. Colman, H.C. Freeman, J.M. Guss, M. Murata, V.A. Norris, J.A.M. Ramshaw and M.P. Venkatappa, *Nature*, 272 (1978) 319.
- 104 H.A.O. Hill, B.E. Smith, C.B. Storm and R.P. Ambler, *Biochem. Biophys. Res. Commun.*, 70 (1976) 783.
- 105 E.T. Adman, R.E. Stenkamp, L.C. Sieker and L.H. Jensen, *J. Mol. Biol.*, 123 (1978) 35.
- 106 N.S. Ferris, W.H. Woodruff, D.B. Rorabacher, T.E. Jones and L.A. Ochrymowycz, *J. Am. Chem. Soc.*, 100 (1978) 5939.
- 107 P.O. Gunnarsson, G. Pettersson and I. Pettersson, *Eur. J. Biochem.*, 17 (1970) 586.

- 108 W.H. Bannister and E.J. Wood, *Eur. J. Biochem.*, 11 (1969) 179.
- 109 B. Mondovi, M.T. Graziani, W.B. Mims, R. Oltzik and J. Peisach, *Biochemistry*, 16 (1977) 4198.
- 110 U. Nylen and G. Pettersson, *Eur. J. Biochem.*, 27 (1972) 578.
- 111 J.S. Richardson, K.A. Thomas, B.H. Rubin and D.C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 1349.
- 112 J.H. Dawson, D.M. Dooley and H.B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 4078.
- 113 R.J. Carrico, B.G. Malmstrom and T. Vanngard, *Eur. J. Biochem.*, 22 (1971) 127.
- 114 E. Walaas, O. Walaas and R. Lovstad, in J. Peisach, P. Aisen and W.E. Blumberg (Eds.), *The Biochemistry of Copper*, Academic Press, New York, 1966, p. 537.
- 115 G. Curzon and B.E. Speyer, *Biochem. J.*, 105 (1967) 243.
- 116 G. Curzon, *Biochem. J.*, 100 (1966) 295.
- 117 W.E. Blumberg, J. Eisinger, P. Aisen, A.G. Morell and I.H. Scheinberg, *J. Biol. Chem.*, 238 (1963) 1675.
- 118 C.B. Kasper, *J. Biol. Chem.*, 243 (1968) 3218.
- 119 L.E. Andreasson and T. Vanngard, *Biochim. Biophys. Acta*, 200 (1970) 247.
- 120 K.E. Falk and B. Reinhammer, *Biochim. Biophys. Acta*, 285 (1972) 84.
- 121 P.O. Gunnarsson, U. Nylen and G. Pettersson, *Eur. J. Biochem.*, 27 (1972) 572.
- 122 J.C. Warren, L. Stowring and M. Morales, *J. Biol. Chem.*, 241 (1966) 309.
- 123 J.C. Warren and S.G. Cheatum, *Biochemistry*, 5 (1966) 1702.
- 124 M. Herve, A. Garnier, L. Tosi and M. Steinbuch, *Biochim. Biophys. Acta*, 405 (1975) 318.
- 125 M. Herve, A. Garnier, L. Tosi and M. Steinbuch, *Biochim. Biophys. Acta*, 439 (1976) 432.
- 126 F.X.R. Van Leeuwen, R. Wever, B.F. Van Gelder, L. Avigliano and B. Mondovi, *Biochim. Biophys. Acta*, 403 (1975) 285.
- 127 B.G. Malmstrom, B. Reinhammer and T. Vanngard, *Biochim. Biophys. Acta*, 205 (1970) 48.
- 128 N.H. Schuster, C.A. Cusworth and R. Condron, *Med. Lab. World*, 27 (1978) 401.
- 129 P.O. Gunnarsson, U. Nylen and G. Pettersson, *Eur. J. Biochem.*, 37 (1973) 41.
- 130 M. Faraggi and I. Pecht, *J. Biol. Chem.*, 248 (1973) 3146.
- 131 M. Goldberg and I. Pecht, *Biophys. J.*, 24 (1978) 371.
- 132 T. Manabe, N. Manabe, K. Hiromi and H. Hatano, *F.E.B.S. Lett.*, 16 (1971) 201.
- 133 R. Malkin, B.G. Malmstrom and T. Vanngard, *Eur. J. Biochem.*, 10 (1969) 324.
- 134 R. Malkin, B.G. Malmstrom and T. Vanngard, *F.E.B.S. Lett.*, 1 (1968) 50.
- 135 P.O. Gunnarsson and G. Pettersson, *Eur. J. Biochem.*, 27 (1972) 564.
- 136 G. Gurzon, *Biochem. J.*, 79 (1961) 656.
- 137 W.G. Levine and J. Peisach, *Biochim. Biophys. Acta*, 77 (1963) 602.
- 138 J. Peisach and W.G. Levine, *J. Biol. Chem.*, 240 (1965) 2284.
- 139 G. Curzon, *Biochem. J.*, 77 (1960) 67.
- 140 S. Osaki, J.A. McDermott and E. Frieden, *J. Biol. Chem.*, 239 (1964) 3570.
- 141 E. Frieden, S. Osaki and H. Kobayashi, *J. Gen. Physiol.*, 49 (1965) 213.
- 142 S. Osaki, J.A. McDermott, D.A. Johnson and E. Frieden, in J. Peisach, P. Aisen and W.E. Blumberg (Eds.), *The Biochemistry of Copper*, Academic Press, New York, 1966, p. 559.
- 143 J.A. McDermott, C.T. Huber, S. Osaki and E. Frieden, *Biochim. Biophys. Acta*, 151 (1968) 541.
- 144 S. Osaki, *J. Biol. Chem.*, 241 (1966) 5053.
- 145 S. Osaki, D.A. Johnson and E. Frieden, *J. Biol. Chem.*, 241 (1966) 2746.
- 146 S. Osaki and O. Walaas, *J. Biol. Chem.*, 242 (1967) 2653.
- 147 S. Osaki and D.A. Johnson, *J. Biol. Chem.*, 244 (1969) 5757.
- 148 H.A. Ragan, S. Nacht, G.R. Lee, C.R. Bishop and G.E. Cartwright, *Am. J. Physiol.*, 217 (1969) 1320.

- 149 A. Sass-Kortsak and A.G. Bearn, in J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson (Eds.), *The Metabolic Basis of Inherited Diseases*, McGraw-Hill, New York, 1978, p. 1098.
- 150 R.W. Topham and E. Frieden, *J. Biol. Chem.*, 245 (1970) 6698.
- 151 D.M. Williams, D.D. Christensen, G.R. Lee and G.E. Cartwright, *Biochem. Biophys. Acta*, 350 (1974) 129.
- 152 G.R. Lee, S. Nacht, D. Christensen, S.P. Hansen and G.E. Cartwright, *Proc. Soc. Exp. Biol. (N.Y.)*, 131 (1969) 918.
- 153 G.W. Bates, E.F. Workman, M.R. Schlabach, *Biochem. Biophys. Res. Commun.*, 50 (1973) 84.
- 154 C.F.A. Bryce and R.R. Crichton, *Biochem. J.*, 133 (1973) 301.
- 155 T.G. Macara, T.G. Hoy and P.M. Harrison, *Biochem. J.*, 126 (1972) 151.
- 156 R.J. Schen and M. Rabinovitz, *Clin. Chim. Acta*, 13 (1966) 537.
- 157 H.P. Roeser, G.R. Lee, S. Nacht and G.E. Cartwright, *J. Clin. Invest.*, 49 (1970) 2408.
- 158 P.M. May, P.W. Linder and D.R. Williams, *J. Chem. Soc., Dalton Trans.*, (1977) 588.
- 159 J.D. Barchas and D.X. Freedman, *Biochem. Pharmacol.*, 12 (1963) 1232.
- 160 E. Walaas, R. Lovstad and D. Walaas, *Biochem. J.*, 92 (1964) 18p.
- 161 B.C. Barrass and D.B. Coult, *Biochem. Pharmacol.*, 21 (1972) 677.
- 162 B.C. Barrass, D.B. Coult and R.M. Pinder, *J. Pharm. Pharmacol.*, 24 (1972) 499.
- 163 R.A. Lovstad, *Biochem. Pharmacol.*, 23 (1974) 1045.
- 164 R.A. Lovstad, *Acta Chem. Scand.*, 26 (1972) 2832.
- 165 J.K. Hampton, I.J. Rider, T.J. Goka and J.P. Prestlock, *Proc. Soc. Exp. Biol. Med.*, 141 (1972) 974.
- 166 B.C. Barrass, D.B. Coult, R.M. Pinder and M. Skeels, *Biochem. Pharmacol.*, 22 (1973) 2891.
- 167 B.C. Barrass, D.B. Coult, P. Rich and K.J. Tutt, *Biochem. Pharmacol.*, 23 (1974) 47.
- 168 D.J. Al-Timimi and T.L. Dormandy, *Biochem. J.*, 168 (1977) 283.
- 169 J. Stocks, J.M.C. Gutteridge, R.J. Sharp and T.L. Dormandy, *Clin. Sci. Mol. Med.*, 47 (1974) 223.
- 170 J.M.C. Gutteridge, *Anal. Biochem.*, 82 (1977) 76.
- 171 J.M.C. Gutteridge, *Biochem. Biophys. Res. Commun.*, 77 (1977) 379.
- 172 L. Heilmeyer, W. Keiderling and G. Stuwe, *Kupfer und Eisen als Korpereigene Wirkstoffe*, Fischer, Jena, 1941, p. 1.
- 173 L.O. Plantin and P.O. Strandberg, *Acta Rheum. Scand.*, 11 (1965) 30.
- 174 W. Niedermeier and J.H. Griggs, *J. Chron. Dis.*, 23 (1971) 527.
- 175 J.R.J. Sorenson, *Inorg. Perspectives Biol. Med.*, 2 (1978) 1.
- 176 D.D. Bajpayu, *Ann. Rheum. Dis.*, 34 (1975) 162.
- 177 S. Onori, S. Cannistraro, P.L. Indovina and L. Sportelli, *International Conference on ESR of Transition Metal Ions in Inorganic and Biological Systems*, Nottingham, England, 1979.
- 178 L. Broman, in J. Peisach, P. Aisen and W.E. Blumberg (Eds.), *The Biochemistry of Copper*, Academic Press, New York, 1966, p. 499.
- 179 C.A. Owen, *Am. J. Physiol.*, 209 (1965) 900.
- 180 M.H.K. Shokeir and D.C. Shreffler, *Proc. Natl. Acad. Sci. U.S.A.*, 62 (1969) 867.
- 181 N. Marceau and N. Aspin, *Biochim. Biophys. Acta*, 293 (1973) 338.
- 182 N. Marceau and N. Aspin, *Biochim. Biophys. Acta*, 293 (1973) 351.
- 183 H.S. Hsieh and E. Frieden, *Biochem. Biophys. Res. Commun.*, 67 (1975) 1326.
- 184 G.W. Evans, *Physiol. Rev.*, 53 (1973) 535.
- 185 J.M. Walshe, in P.J. Vinken and G.W. Bruyn (Eds.), *Metabolic and Deficiency States of the Nervous System*, North-Holland, Amsterdam, Oxford, 1976, p. 379.
- 186 N.A. Holtzman, M.A. Naughton, F.L. Iber and B.M. Gaumnitz, *J. Clin. Invest.*, 46 (1967) 993.
- 187 R.J. Carrico and H.F. Deutsch, *Biochem. Med.*, 3 (1969) 117.
- 188 S.A. Neifakh, I.M. Vasiletz and M.M. Shalovsky, *Biochem. Genet.*, 6 (1972) 231.
- 189 G.L. Fisher and M. Shifrine, *Oncology*, 35 (1978) 22.