THE COPPER PROTEINS

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INTRODUCTION

The presence of copper in plant and animal tissues has been known since 1816 and 1833 respectively(1,2), but until the 1920's it was usually assumed to be there by accident. Nevertheless in 1847 HARLESS (3) had detected copper in snails and had shown that it existed in combination with the blood proteins in these invertebrates. Between 1920 and 1930 nutritional studies established that copper was an essential trace metal and was necessary for haemopoiesis. Since that time a large number of copper-containing proteins have been isolated from animal, plant and microbial sources. Some of these proteins have well defined functions such as enzyme activity or the ability to transport oxygen, while others have no known function.

The nutritional aspects of copper have been reviewed by UNDERWOOD(4), but it is convenient to summarize present knowledge of copper deficiency and copper toxicity here. The copper content of the whole human body is 100-150 mg., but infants have two to three times more copper per unit of body weight. The majority of the body copper is found in liver, heart, kidney, hair, brain, bones and muscle, while spleen, lungs, and especially endocrine glands contain relatively smaller amounts. The liver appears to act as the main storage organ, and its copper content may be taken as a fairly reliable index of copper status.

Copper is relatively poorly absorbed. Depending on the other constituents of the diet, 75-90% of dietary copper (as ^{64}Cu) appears in the faeces in 3-5 days along with a little in the urine. The kidneys do not appear to be an important route of copper excretion, but on the other hand, the bile tends to be relatively rich in copper. There is no good estimate of the daily dietary requirement for copper; most authorities quote 1-2 mg./day.

Copper deficiency in animals manifests itself in a number of ways: anaemia, depressed growth, bone disorders, depigmentation of hair, fibrosis of the myocardium, and diarrhoea have all been recorded, but not all in the same species at the same time. Copper has long been known to be toxic, both acutely and chronically, but again no reliable figures are available for the effects and tolerated doses in humans. In animals about 4-10 times the normal requirement over a period appears to do no harm, and in rats, toxic effects are not seen until about 100 times the normal requirement is given. Excessive ingestion of copper in animals leads to passive accumulation in the tissues, especially the liver. Up to a certain point this is tolerated without apparent ill effects, but beyond this level there may occur a catastrophic liberation of a high proportion of the copper into the blood. Haemolysis, jaundice, and usually death ensue.

A number of laboratories are engaged in the study of the copper proteins. Besides satisfying the fundamental curiosity of biochemists there is the hope that biochemical studies may throw some light on copper deficiency and copper toxicity, as well as on the problem of Wilson's disease (see below).

For the sake of convenience the copper proteins will be discussed in three sections: (1) mammalian copper proteins; (2) haemocyanins; and (3) plant and microbial copper proteins. Some of the properties of the copper proteins are set out in Table I. This list is not exhaustive: the copper proteins continue to be discovered, while on the other hand, in one or two cases, a protein thought to be a copper protein has turned out to be merely contaminated with copper. The table does include all those copper proteins that have been studied in any detail.

THE MAMMALIAN COPPER PROTEINS

Ceruloplasmin. Normal plasma contains a copper protein which was isolated and crystallized by HOLMBERG and LAUREL(5) in 1947-8. It was called ceruloplasmin because of its blue colour. It has a molecular weight of 160,000 and contains 8 atoms of copper per molecule, and it also has oxidase activity. In electrophoresis it moves with the alpha-globulins, and almost all of the copper in mammalian plasma is in the form of ceruloplasmin. Interest in ceruloplasmin was stimulated when in 1952(6) it was shown that ceruloplasmin was deficient or even absent from the plasma of patients with Wilson's disease(7,8,9). In 1911 WILSON(10) described: "... progressive lenticular degeneration, a familial nervous disease associated with cirrhosis of

TABLE I

Properties of some of the copper proteins

Protein	Source	M.Wt.	%Cu	State of Cu	Cu atoms /mol.	Colour (abs. max.)
Ceruloplasmin	serum	160,000	0.34	$\frac{1}{2}$Cu+ /$\frac{1}{2}$Cu++	8	blue (610mu)
Erythrocuprein	rbc's	33,500	0.38	Cu++	2	blue-green (655mu)
Haemocuprein	rbc's, liver	30,000	0.35	Cu++?	2	blue
Hepatocuprein	liver	30-40,000	0.35	Cu++?	2	blue-green (660mu)
Cerebrocuprein	brain	35,000	0.30	Cu++?	2	blue-green (660mu)
Amine oxidase	liver mitochondria	250,000	0.05-0.1	Cu++	4	colourless/ pink
Cytochrome oxidase	mitochondria	?	?	Cu++?	2?	(830mu)
Uricase	liver	120,000	0.06	?	1	?
Dopamine beta-hydroxylase	adrenals	290,000	0.10	Cu++	4-7	colourless
Haemocyanin	arthropods	0.4-1x10^6	0.15-0.19	Cu+	ca.20	blue (570mu)
Haemocyanin	molluscs	2-9x10^6	0.23-0.26	Cu+	200-400	blue (570mu)
Tyrosinase	fungi	130,000	0.20	Cu+	4	colourless
Laccase	lac tree	130,000	0.33	Cu+/Cu++	4	blue (615mu)
Ascorbate oxidase	plants, bacteria	140,000	0.34	6Cu+/2Cu++	8	blue (608mu)
Galactose oxidase	fungi	75,000	0.09	Cu++	1	pink-yellow
Azurin	bacteria	14,600	0.45	Cu++	1	blue (625mu)
Plastocyanin	chloroplast	21,000	0.58	Cu++	2	blue (597mu)
Stellacyanin	lac tree	20,000	?	?	1	blue (604mu)

the liver...": the name 'hepatolenticular degeneration' is still commonly used for the disease. Two years later it was reported(11) that there was an excess of copper in the liver in this disease, and later it was shown that this applied to the brain too. In 1948 it was suggested(12) that the sulphydryl chelating agent British Anti-Lewisite (BAL) might be used therapeutically in Wilson's disease.

Ceruloplasmin does not bind copper irreversibly, since the copper atoms can be exchanged for radioactive copper under suitable conditions. It is also possible to prepare copper-free, or apo-ceruloplasmin, and this can be reconstituted by adding copper, to give native ceruloplasmin once again. In fact the eight copper atoms in a molecule of ceruloplasmin are not identical: four of them appear to be loosely bound (exchangable with ^{64}Cu), while four are more tightly bound (dialysable only after chymotryptic digestion). Probably four are Cu++ and four are Cu+ (see ref. 13): nevertheless the Cu+ atoms of ceruloplasmin do not bind oxygen as do those of haemycyanin.

Perhaps the most notable feature of ceruloplasmin is its oxidase activity towards a variety of polyamines and polyphenols, and possibly ascorbic acid. However, despite a great deal of experimental work on the enzymic properties of ceruloplasmin, the physiological significance of this activity remains completely obscure. Ceruloplasmin could represent a copper transport system in the plasma which just by chance happens to have oxidase activity. However, some patients with Wilson's disease, controlled with penicillamine, appear to lead a normal life with no detectable copper and no detectable ceruloplasmin in the serum. On the other hand, though ceruloplasmin has a number of oxidase activities, the substrates for oxidation so far studied can occur rarely, if ever, in the plasma, with the exception of ascorbic acid. The range of compounds that can be oxidised in presence of ceruloplasmin is very wide. **p**-Phenylenediamine (PPD) is a good substrate, and a number of other, similar compounds have been compared with it. In order, with respect to ease of oxidation, some of these compounds are: PPD; N,N-dimethyl-PPD; N-methyl-PPD; p-aminophenol; hydroquinone; N-N-dimethyl-MPD; MPD (MPD=**m**-phenylenediamine). The reaction sequence that has been suggested for these oxidations is complex and involves free radical formation and conversion of the Cu++ of ceruloplasmin to Cu+. Finally the Cu+ is reoxidised by molecular oxygen to Cu++. Other compounds whose oxidation is catalysed by ceruloplasmin include several reducing agents, hydrosulphite, hydroxylamine, thioglycolate, and hexacyanoferrate.

The significance of ceruloplasmin as an ascorbic acid oxidase is both obscure and controversial. It is fairly well established that ceruloplasmin can catalyse ascorbate oxidation, but the demonstration is difficult for two reasons. Firstly the activity is low in comparison with true ascorbate oxidases from plants, and secondly free cupric ions (with which preparations of ceruloplasmin may be contaminated) also catalyse the oxidation of ascorbate; indeed, under certain conditions they do so more efficiently than does ceruloplasmin (see discussion in ref. 14). Such ascorbate oxidation in the serum might in any case be considered biologically wasteful, since the energy released presumably could not be used for phosphorylation. On the other hand, it has been shown(15) that the product of ascorbate oxidation, dehydroascorbate, penetrates brain and eye tissues, and erythrocytes more rapidly than ascorbate. This might conceivably represent some sort of transport system, but set against this must be the very low ascorbate oxidase activity of ceruloplasmin, despite its relatively high concentration in serum.

We are left, then, with only a very vague idea of the function of ceruloplasmin. (It might be that ceruloplasmin is an evolutionary relic, in the sense that most, if not all, animals have a copper protein in the blood, and in some of them (molluscs and arthropods) this has evolved into a respiratory carrier for oxygen (haemocyanin, see below). Furthermore, some recent work has suggested that lack of ceruloplasmin may not be the primary lesion in Wilson's disease. The original hypothesis(16) was reasonable: failure to sythesize the protein led to the plasma copper remaining diffusible, with the result that it entered the tissues and accumulated. In liver, excess copper produced necrosis and fibrosis, in kidney, deficient tubular reabsorption, in brain, degeneration of the basal ganglia and consequently the characteristic motor disturbances, and finally in the eye, the pigment rings which are diagnostic of the disease. Unfortunately there is poor correlation between ceruloplasmin concentration and the duration and severity of Wilson's disease(16), and, indeed, in a few cases, the blood ceruloplasmin level appears to be almost normal(17,18).

Wilson's disease is inherited in a recessive manner, and the heterozygotes can be identified by the fact that, like the homozygotes, they show delayed incorporation of copper into ceruloplasmin(19). It was a possibility therefore that the ceruloplasmin present in some patients with Wilson's disease was structurally abnormal and could not bind copper properly. In a recent study however(20), tryptic peptide maps were made with ceruloplasmin isolated

from an individual with Wilson's disease whose blood ceruloplasmin level was close to the normal. The peptide maps were normal, which would seem to rule out the synthesis of an abnormal protein in this genetic trait. Thus it may be that we will have to look to the other copper proteins of mammals for an explanation of the defect in Wilson's disease.

Other Mammalian Copper Proteins. Our knowledge of the small copper proteins of the body, namely erythrocuprein, haemocuprein, hepatocuprein, and cerebrocuprein, goes little farther than what is summarised in Table I. As is seen, these are all similar in copper content, colour, and size, and in addition they are all characterised by a lower than normal absorption in the ultraviolet (280mu). In this latter respect they are abnormal compared with almost all other proteins in having a very low content of aromatic amino acids. For example, amino acid analysis of highly purified human erythrocuprein showed that tyrosine and tryptophan were absent(21). That these soluble tissue copper proteins are nevertheless different proteins is shown by their distinct chromatographic behaviour and by their immunological properties.

These copper proteins account for a considerable proportion of the tissue copper. Cerebrocuprein, hepatocuprein, and erythrocuprein each account for about 60% of the total tissue copper of brain, liver, and erythrocytes respectively. These proteins have no known function, nor has any enzyme activity been attributed to them so far. They may be present as copper storage depots and in addition may be important in picking up any free copper in the cell. For a number of reasons free copper cannot be allowed to accumulate in the tissues of a normal individual. While some enzymes need copper for activity, others are strongly inhibited by copper ions. Copper ions form fairly stable chelates with amino acids and could therefore interfere with the process of protein synthesis. In addition copper ions can combine non-specifically with carboxyl groups and sulphydryl groups of proteins, possibly altering the overall properties of the proteins considerably. No doubt in copper toxicity we are observing such derangement of these processes.

PORTER(22) has recently partially purified a new tissue copper protein from the liver mitochondria of newly born infants, which he has called "neonatal hepatic mitochondrocuprein". This contained more than 3% copper, and was distinct from the other liver copper proteins including cytochrome oxidase, and probably performs a storage function for copper in the immature animal analogus to that of ferritin for iron storage. It is interesting that the liver of the newly born infant contains a much higher concentration of copper than other tissue, and also that the mitochondrial fraction of new born liver may contain up to 30 times as much copper as the mitochondrial fraction from adult liver. In fact a newly born infant is similar to a patient with Wilson's disease, in that he has a high liver copper and also lacks the normal amount of ceruloplasmin.

Cytochrome oxidase(23,24), the terminal electron acceptor of the electron transport chain, is a haem-copper-protein, is almost universal in cells, and is located in the mitochondria. It seems probable that the unit of cytochrome oxidase contains one cytochrome **a** and one cytochrome **a3**, and that each cytochrome is associated with one copper atom. Kinetic studies indicate that the cytochromes as well as the copper atoms participate as electron carriers in the electron transport chain. Such kinetic experiments do however require very careful interpretation. That cytochrome oxidase is an integral component of the mitochondrion makes its isolation and purification all the more difficult. As a result there has been much discussion and controversy, not only as to whether preparations are contaminated with extraneous copper, but also whether the copper does in fact participate in electron transport.

Some of the amine oxidases are somewhat better characterised, and these, like cytochrome oxidase, are widely distributed in animals, plants, and bacteria(25). The general reaction that they catalyse is:

$$R\text{-}CH_2NH_2 + O_2 + H_2O \longrightarrow R\text{-}CHO + NH_3 + H_2O_2.$$

Amine oxidases have been classified into mono- and di-amine oxidases, but recently this classification has been criticised on the basis that "diamine oxidase" acts on many monoamines and *vice versa*(26). It seems probable that all amine oxidases will turn out to be copper proteins(25) but another cofactor is always present, either pyridoxal phospate or a flavin.

The so-called "diamine oxidase" from pig kidney proved to be the same protein as histaminase(27), in other words the one protein attacks **both** diamines and histamine. In a similar way "monoamine oxidase" acts on both alkylamines and arylamines(28). The function of monoamine oxidase seems to be breakdown of amines of high biological activity, both naturally occuring and pharmacological. The most important substrates are tryptamine derivatives (5-hydroxy-tryptamine) and catechol amines (adrenaline, dopamine). Diamine oxidase on the other hand is concerned with histamine breakdown and with breakdown of various biologically active diamines.

Unlike most of the other copper proteins, amine oxidases are either colourless (hog kidney diamine oxidase) or pink (monoamine oxidases from various sources). Nevertheless the copper appears to be in the cupric state, and in the case of histaminase at least, is probably bound to the pyridoxal prosthetic group(28). Clearly a great deal of work remains to be done before our knowledge of the structure and mechanism of action of both cytochrome oxidase and amine oxidases is anywhere near complete.

THE HAEMOCYANINS

Haemocyanins are high molecular weight copper proteins which are found in the blood of some, but not all molluscs and arthropods (29,30). They have the property, like haemoglobins, of combining reversibly with oxygen, but unlike the haemoglobins they are dissolved directly in the blood or or haemolymph rather than being held in the corpuscles. In most of the specimens of haemolymph that have been examined, haemocyanin has been found to account for 90-98% of the protein present.

TABLE II
Comparison of Haemoglobin and Haemocyanin

Property	Haemoglobin	Haemocyanin
Metal	Iron (Fe++)	Copper (Cu+)
Porphyrin	yes	no: copper bound directly to protein
M. Wt.	67,000	400,000-10,000,000
Colour	red	blue
Structure	2 pairs identical subunits	half, and eighth-to-twelfth mols. may be obtained
Occurence	erythrocytes	dissolved in blood

Since haemocyanins and and haemoglobins have the same function, that is, oxygen transport in the blood, it is instructive to compare the two proteins, As Table Two shows, apart from being oxygen-carrying metallo-proteins, they could hardly be more different—a striking example of convergent evolution presumably. However it should be mentioned that some investigators have thrown doubt on this oxygen-carrying function of haemocyanins. It is pointed out(31) that in some crustaceans the haemocyanin content of the blood at certain moulting stages is lowered to levels such that the function of the pigment is completely acessory. Furthermore, PILSON(32) found a great variation in the haemocyanin content of the molusc, **Haliotis** which was unrelated to weight, sex, or reproductive activity. Thus it is possible that haemocyanins represent some sort of food or copper reserve which just happens to be capable of carrying oxygen. It is clear that many questions concerning haemocyanin remain to be resolved. Apart from doubts about the function of the protein, there is in addition very little knowledge about the synthesis of the protein or the mode of attachment of the copper. It is interesting that haemocyanins have been reported to possess enzymic activity in addition to their oxygen-carrying ability. The activities that have been observed are catalase, polyphenoloxidase, and lipoxidase(33).

Several of the properties of haemocyanins have been examined in great detail. Particular attention has been given to the oxygen-carrying ability, the subunit structure and the mode of attachment of the copper atoms. It may be hoped that information obtained about haemocyanins will be of use in relation to the study of other copper proteins.

It has long been known that during the oxygenation of haemocyanin, when the colour of the compound changes from colourless to deep blue, one molecule of oxygen is bound for every two atoms of copper. The oxygen dissociation curves for haemocyanin are largely homologous with those of other respiratory pigments (though, of course, they differ in detail), and have shapes from hyperbolic to sigmoid. The shape of the curve depends on a number of factors including the stage of purification, the pH, and the presence or absence of certain ions, especially calcium and magnesium. There has been some controversy as to whether there is any interaction between the oxygen-binding sites on the same molecule. Recent reports would seem to indicate that there is a negative interaction. Since in the largest molecular weight haemocyanins there are several hundred copper atoms, and since the molecules are capable of splitting into subunits under certain conditions (see below), any interaction must surely be very complex indeed. It has been found that thiocyanate and thiourea(34), and more recently ethyl isocyanide(35) cause the reversible expulsion of oxygen from haemocyanin. Further study of such reactions may lead to a better understanding of the oxygenation reaction.

The subunit structure of haemocyanins has long attracted the attention of the biochemists and biophysicists. Early ultra-centrifuge studies on the molecular weight of haemocyanins revealed that, depending on the conditions of pH and ionic composition, the molecule could undergo dissociation into subunits. Generally,

the components of the highest molecular weight always exist in the pH region around the isoelectric point. On either side of this region smaller units are formed, but units of different sizes may co-exist at a particular pH. Thus in the case of the haemocyanin from the snail, **Helix pomatia,** over the pH range 4.7-7.0 (the stability region) a single high molecular weight species is present, from pH 4.6-3.6, and from pH 7.4-8.1, whole and half molecules are present, and outside these regions eighth or tenth molecules are formed. It seems fairly certain that in the blood of marine invertebrates, which has a pH of 8.2-8.3, the haemocyanin molecules are undissociated, due to the presence of Ca++ and Mg++ in the haemolymph. These ions have the property of extending the stability region of the whole molecules, so that to this extent the process of dissociation is probably un-physiological. One may speculate that these animals, instead of evolving blood corpuscles to contain the respiratory pigment, have evolved giant molecules which have somewhat similar properties. For example they would not be able to diffuse out of the blood system into the tissues. It has been possible to demonstrate something of the subunit structure in the electron microscope. VAN BRUGGEN and his coworkers(36) showed that the molecule of **H. pomatia** haemocyanin at pH 6.0 was a cylinder of length 335A and diameter 300A, and had a five-fold axis. This five-fold axis may explain why one-tenth molecules can be obtained under certain conditions.

Perhaps the most interesting feature of haemocyanin is its copper atoms, which most workers consider to be in the univalent or cuprous state in both oxygenated and deoxygenated haemocyanin. The reasons for believing this to be the case come from a number of sources. For example, the absorption spectrum of haemocyanins is not like that of copper proteins known to contain cupric copper, most, if not all of which absorb at 610mu. Furthermore copper can be removedf rom haemocyanin with KCN to give a colourless protein, and the copper can then be restored by the addition of cuprous, not of cupric, compounds. However, this evidence is not completely satisfactory, and some investigators have suggested that in the oxygenated molecule the copper is half cuprous and half cupric(37).

Related to the question of valency of the copper is the problem of how the copper is joined to the protein. Since all attempts to demonstrate the presence of a porphyrin or similar cofactor have failed, it is assumed that the copper is linked directly to the amino acid side chains of the protein. Nevertheless, at present it is impossible to state which of the amino acids in the protein bind the copper atoms. The copper is certainly very tightly bound, and is only removed by cyanide or by agents which cause denaturation of the protein molecule. Many compounds capable of chelating copper have been tried, but, with the exception of cyanide, none has removed a significant proportion of the copper. The residues most likely to be implicated in the binding of copper would seem to be cysteine (-SH) and histidine (imidazole). There is little evidence to support the idea of copper binding by cysteine sulphydryl groups, while evidence from titration curves(33) and from photooxidation experiments(38) suggest that histidine residues are involved, though not necessarily exclusively, in copper binding. It may be said at this point that little is known of the mode of binding of copper in **any** copper protein. Certainly evidence obtained with haemocyanin may provide valuable clues about copper binding in other copper proteins. If only for this reason, the investigation of haemocyanins represents a very fruitful field of study.

THE PLANT AND BACTERIAL COPPER PROTEINS

The copper proteins of plant and microbial origin comprise a group of enzymes, tyrosinase, ascorbate oxidase, laccase, and galactose oxidase, a component of the photoynthetic system, plastocyanin, along with several proteins of unknown function, such as stellacyanin from the Japanese lac tree, the blue copper protein from mung bean seedlings, and azurin from bacteria. No doubt many more such proteins will be discovered. It is proposed to limit the present discussion to the first group, the plant copper enzymes, since these have been most studied. Details of some of the other proteins appear in Table I.

Tyrosinase. This protein has long been known to contain copper(39). It can catalyse two different aerobic oxidation reactions: (a) hydroxylation of tyrosine ("cresolase activity"), and (b) dehydrogenation of a number of **o**-dihydric phenols or catechols ("catecholase activity"). Because of this latter activity it has also been called polyphenol oxidase. In fact, tyrosinase does not only occur in plants and fungi. The tyrosinase that has been studied the most is that from mushrooms, and it is for this reason **only** that it is included in this section. Tyrosinases are also known from insects and from mammals. Tyrosinase is the enzyme responsible for the formation of pigments, melanins, in animals throughout the phylogenetic scale. These include the pigments of skin, eyes, and feathers: thus tyrosinase has been isolated from skin and from melanomatous tissue. Tyrosinase is clearly

a very important enzyme—its action is connected with both medical and social problems.

The relationship between the two types of activity of the enzyme is the subject for considerable controversy. Some workers consider that both activities are contained within the same protein molecule, whereas others think that it is the products of catecholase action that are responsible for the hydroxylation activity(40). A further suggestion worthy of consideration is that the enzyme is composed of subunits, some having largely cresolase activity and some having largely catecholase activity. The resolution of this controversy must await further work with the enzyme. Nevertheless, depending on the source and isolation procedure used, the enzyme may turn out to have either a high ratio of catecholase to cresolase activity, or a low one.

Tyrosinase is unusual among the copper proteins in being colourless. Examination of the spectrum reveals a strong absorption of 280mu due to the high content of aromatic amino acids, and a weak absorption at around 340mu.

TABLE III
Comparison of Spectral Properties of some Copper Proteins

Protein	State of Cu	280mu	340mu	570mu	610mu
Tyrosinase	Cu+	+++	+/—	—	—
Haemocyanin	Cu+	++	—	—	—
Oxyhaemocyanin	Cu+/Cu++?	++	+	+	—
Ceruloplasmin	Cu+/Cu++	++	+	—	+
Erythrocuprein	Cu++	+	—	—	+

Furthermore there is no difference between the absorption spectrum of the enzyme and that of the copper-free apo-enzyme. If the spectrum of tyrosinase is compared with the spectra of other copper proteins (Table III) it is seen that only proteins containing Cu++ appear to absorb in the 610mu region of the spectrum. Tyrosinase seems to contain only Cu+, but it is important to perform the valency determination on freshly prepared material, since on storage, there is partial oxidation of the copper with a corresponding loss in enzymic activity(41).

Ascorbate Oxidase. This enzyme, isolated from many plant tissues (apple, potato, spinach), catalyses the aerobic oxidation of ascorbic acid to dehyroascorbic acid(42). It has never been detected in animal tissues. Solutions of the enzyme are blue, but the colour is bleached when ascorbate is added, oxygen is absorbed, and then, when all the ascorbate has been oxidised, the colour returns. The copper-free apoenzyme prepared by dialysis against cyanide is also colourless. The active copper enzyme can be regenerated by the addition of Cu+ but not of Cu++ salts. Experiments indicate that the copper exists in the native enzyme in a mixed valence state where Cu++:Cu+ = 3:1. Thus the copper in the enzyme seems to be somewhat similar to that in ceruloplasmin, and it is likely that only the Cu++ is involved in enzymic activity. (It will be remembered that ceruloplasmin also shows ascorbate oxidase activity).

It is interesting that removal of the copper with cyanide leads to an apo-enzyme containing 10 free sulphydryl groups, whereas the native enzyme contains no sulphydryl groups. This might seem to indicate that the copper is bound to sulphydryl groups. However, this does not appear to be the case, since if the apo-enzyme is treated with p-chloromercuribenzoate, which blocks sulphydryl groups, the enzyme activity and copper may still be restored. This conclusion is strengthened by the finding that if the native enzyme is denatured (i.e. the protein chain is opened out) 10-12 sulphydryl groups are revealed. It looks therefore as though these sulphydryl groups have nothing to do with the binding of copper, but instead have some part to play in stabilising the structure of the protein. It has been suggested that the copper might be bound to histidine + alpha-amino groups, and/or histidine + carboxyl groups in the protein.

The function of the ascorbate oxidase in plants is uncertain. It is possible that there is a multienzyme system in plant respiration involving glutathione reductase, dehydroascorbate reductase, and ascorbate oxidase(43) (Fig. 1). It has long been known that plant tissues contain glutathione reductase and dehydroascorbate reductase, but the latter is absent from the mitochondria. From the results obtained with pea seedlings(43)it it seemed possible that about 25% of the total respiration might proceed by this route.

Laccase. If thin layers of the latex from the Japanese lac tree, **Rhus vernicifera,** are exposed to moist air they darken and dry to give a lustrous translucent material which forms the basis of a furniture finish. Investigation of the latex(44,45) led to the isolation of a copper enzyme, laccase, which catalyses the oxidation of certain hydroquinones and semiquinones. The semiquinones then react non-enzymically to

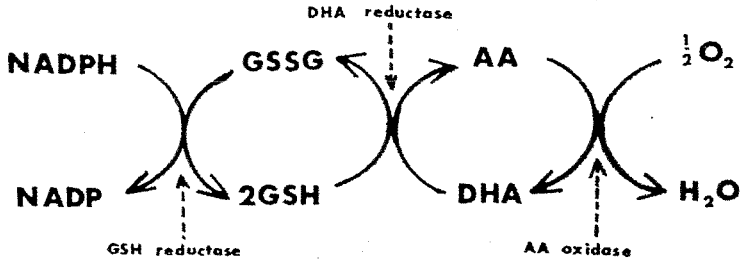

Fig. 1. Possible terminal oxidative pathway in pea seedlings. GSH, GSSG are reduced and oxidised glutathione, AA, DHA are ascorbic acid and dehydro-ascorbic acid, respectively.

produce the corresponding quinones. The semi-quinone formation is the result of a one electron transfer from the substrate to copper of laccase to produce cuprous copper which is then re-oxidised to cupric copper by molecular oxygen.

Laccase has since been found in many plant tissues. It is essentially an extracellular enzyme, and the darkening and hardening of lac tree latex is a result of the action of the enzyme, in the presence of oxygen, on "laccol", which is not a single compound, but a whole group of similar compounds. These compounds may be regarded as the true substrates of the enzyme. The basic structural feature of the whole molecule is that it is a 1,2-diphenol with a long unsaturated hydrocarbon chain at position 3 of the phenol ring. Nevertheless, laccase also catalyses oxidation of a number of simpler aryl diphenols and diamines, as well as coniferyl alcohol, which is a percursor of the lignin of woody plants. Thus laccase may be connected with the process of wood formation, and probably also with wound repair in plants. It is interesting that the wood rotting fungus, **Polyporus versicolor**, produces laccase and uses it to break down the lignin of woody tissue.

Galactose Oxidase. This is a copper enzyme produced by fungi, and has been isolated from **Polyporus circinatus.** The reaction that it catalyses is the oxidation of **D**-galactose at carbon 6 to give D-galactohexodiadose(13). The enzyme is one of the few copper proteins whose copper is removed by strong chelating agents or by hydrogen sulphide.

SOME CONCLUSIONS

In the average text-book of biochemistry one may find perhaps half a page out of, say, the thousand pages in the book devoted to copper proteins. On the other hand, wherever one looks in living organisms one finds copper proteins of one sort or another. It is not the purpose of the present author to criticise this state of affairs, but rather to redress the balance by showing what an interesting group of compounds the copper proteins form, and what a wealth of material there is for study.

No attempt will be made to summarise the foregoing discussion, because in itself it represents but a summary of a considerable amount of work that has been performed. Instead, one or two conclusions can be drawn which represent general principles in the field of copper proteins:

(1) Copper though itself toxic, is found as a component of proteins from almost all life forms.

(2) Copper proteins are almost always blue in colour if they contain cupric copper, but colourless if they contain cuprous copper. Intermediate cases are however encountered which make it difficult to apply this rule to all copper proteins.

(3) Copper proteins are nearly always associated with oxygen in some way, either as oxygen carriers or as oxidase enzymes in which molecular oxygen participates in the enzyme reaction. This is presumably a reflection of the ability of copper atoms to change from Cu++ to Cu+ and back again.

(4) Though the sulphydryl groups of cysteine residues in proteins would appear to be likely candidates for binding copper, only in one case (galactose oxidase) has this been demonstrated, and in most other cases there is actually evidence **against** the participation of sulphyryl groups.

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