STUDIES ON THE ACTIVE CENTER IN CANCER MAGISTER HEMOCYANIN

by

Constance Simo, A.B.

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.....
(Professor in charge of thesis)

[redacted]

.....
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GENERAL DISCUSSION

INTRODUCTION

Hemocyanins are copper-containing respiratory pigments found in a wide variety of invertebrate species. Early studies of the protein, discovery of its oxygen-binding ability in 1867 and proposal of the name "hemocyanin" in 1878 have been reviewed elsewhere (57, 101). The reaction between oxygen and hemocyanin is a particular example of a large class of reactions, which do not involve the formation of chemical bonds in the conventional sense, so-called reversible associations. The mechanisms of oxygen binding, by which respiratory proteins function in O_2 transport and storage in the animal kingdom, remain unknown even where the whole protein and the active site have been highly characterized as in hemoglobins and myoglobins. Hemocyanins present additional problems, however, as both the oxidation state of the copper and its mode of binding to the protein are unknown. Approaches to the study of this molecule conveniently fall into four categories. One deals with physical aspects, molecular weights and conformation of intact aggregates and subunits. The other three deal with chemical aspects, such as the reaction of hemocyanin with oxygen, attempts to determine the ligands of copper and attempts to determine the valence of copper.

I Physical Characterization of the Protein and its Subunits

A. Molecular Weight.

Hemocyanins were among the first proteins to be studied in detail by physical methods. For many years the survey of

molecular weights of hemocyanins from over sixteen species showed a large range of values which weren't always reproducible (28, 101). Finally Svedberg and co-workers showed that the molecules can undergo reversible associations depending on conditions of protein concentration, ionic strength and pH (124). Components of highest molecular weight, e.g. 4×10^6, S_{20} w 60, exist in a pH range which includes the isoelectric point. Smaller, homogeneous components, e.g. 25, 16 and 5S in crustacea, appear on the alkaline side of the isoelectric point (121). They can be reversibly formed within a pH stability range of 4-9, with slight variation according to species.

The presence of divalent cations, 10^{-4} M strength is necessary to insure the full extent of reversibility of subunits to the intact aggregate (127, 128). In the presence of 0.01 M Ca(II) or Mg(II) the dissociation of Helix pomatia molecules doesn't begin until pH 11 (129), where under normal conditions an irreversible denaturation would have occurred. In a separate study (130), the presence of Mg(II) ions in 0.01 M concentration stabilized the 58S pH 7 structure of squid (Loligo pealei) hemocyanin to pH 10, where otherwise a 19S component would have appeared at pH 8. This influence of divalent cations may be similar to that found for Ca(II) with alpha amylases (37). Ca^{++} is a stabilizer of alpha amylases and its participation in the enzymatic activity has recently been ascribed to the

metal producing "intramolecular cross links similar in function to disulfide bridges, which confer . . . the structural rigidity required for effective catalytic activity" (37).

The molecular weights (m.w.) of hemocyanins ($0.5\text{-}4.0\times10^6$) are higher than those found for other copper proteins, generally about 1.5×10^5, or less (40). They vary according to species, from 0.78×10^6 in lobster plasma to 6.7×10^6 in snail plasma. Some aggregates have been reported with molecular weights of 10 million (32). It has been suggested that these molecules may represent "the limit of size which has stability without developing the heterogeneity of structure displayed by true corpuscles and cells" (102). They are exceeded in molecular size only by the viruses. The copper content varies from 0.15 to 0.25% by weight (40).

Evidence that the molecular weights of these proteins may be simple multiples of a unit molecular weight has accumulated since this was first suggested by Svedberg and Heyroth in 1929 (120). The high resolution techniques of electrophoresis and starch gel chromatography have been applied mainly to arthropod hemocyanins and at pHs on the alkaline side of their isoelectric points (84, 139). From five to fifteen distinct protein zones have been found in various arthropod sera using borate-buffered starch gel, in the region of pH 8 (84). This suggests that the alkaline dissociation products, having ultracentrifugal homogeneity, may not be identical with respect to charge since the number of zones exceeds the number of sedimentation components.

B. Tertiary Structure

Electron microscope studies on gastropod and crustacean hemocyanins (127, 128, 129) at neutral pH have revealed cylindrical aggregates with diameters from 80-100 Å and heights of about 140 Å, depending on the number of parallel layers of subunits. Dissociation is believed to occur by the separation of these layers into shorter, hollow right cylinders. The observed projections of the 16S component of crustacean hemocyanin, at pH 8.2, are compatible with a model consisting of eight subunits lying on the corners of a cube. The 23S component would be a dimer of two of these cubes (127). The most recent sedimentation analyses of a hemocyanin (squid) reveal the principal stable association products of an 11S, m.w. 385,000, pH 10 component to be a dimer, 19S, m.w. 770,000, pH 8.9 and a decamer, 58.7S, m.w. 3,750,000, pH 7 (130, 131). Information about the symmetry of each component, from frictional ratios, is consistent with a split of the decamer, along a plane perpendicular to the cylinder axis, into five 19S structures, each of which yields two 11S structures. Combining van Bruggen's view of the 11S monomer as a cube with the total copper content of the aggregate allows estimation of an average number of copper atoms per cube. The copper content of the decamer is .26 weight % of 3.75×10^6 or 9.75×10^3. If one assumes uniform distribution of the copper and each of the ten 11S components of the decamer to be a cube, the interesting value

of sixteen copper atoms per cube results. Two coppers comprising an active site, for example, may be regularly distributed at each of the eight corners or faces of a cube subunit. There have been only two positive reports of subunit activity. The activity of a subunit of an arthropod hemocyanin was measured by reversibility of deoxygenation(95). It was observed to decrease in proportion to the length of time the solution was maintained in a deoxygenated state. Two stable dissociation products of a decapod hemocyanin were reported to reversibly combine with oxygen (130).

There is no alteration, detectable by sedimentation analysis, in either tertiary or quarternary structure of hemocyanins, upon removal of all the copper using cyanide (86, 181). Recent optical rotary dispersion data confirm this and also indicate that no appreciable change in protein conformation occurs on oxygenating hemocyanin (38, 131). There may however be some fine structural alteration, so far undetected by these methods, upon cycling the protein between fully oxygenated and deoxygenated states. Synthetic reversible oxygen-carrying chelates have been observed to deteriorate to 60 and 70% of their original activity upon repeated cyclings (133). While optical rotation is a phenomenon which depends on fine symmetries of a molecule (78), there is evidence that it is not as sensitive to some structural changes in copper proteins as optical and electron spin resonance (ESR) spectroscopy. More drastic denaturations of

laccase and ceruloplasmin, by urea, were necessary to bring about changes in optical rotation comparable to the loss of color and appearance of a Cu(II) ESR signal (82). On the other hand, there is evidence that the oxygen carrying property of synthetic chelates (133) may in part depend upon the existence of holes or crevices in the crystal lattice which are large enough to accomodate the oxygen molecule. This has also been suggested for hemoglobin (24).

II The Reaction with Oxygen

A. The Statistical Ratio, Cu/O_2

The generally accepted stoichiometric ratio between O_2 bound and copper in oxygenated hemocyanin (HC) is 1:2. This is based on three independent series of chemical analyses, all made before 1941. Using a Van Slyke constant volume apparatus, KCN to release dissolved oxygen and pyrogallol or hydrosulfite to reabsorb it, and electrolytic separation of copper with subsequent titration by thiosulfate, a simple ratio of one copper atom to one oxygen atom was obtained for each of thirteen species tested (103, 50, 98). Rawlinson (98) reported from 2.04-2.34 or an average of 2.12 atoms Cu/molecule O_2 for a crystallized HC from crayfish. These are of course statistical ratios and cannot be used as exclusive evidence for the participation of two copper atoms per active site. The ratio would be the same, for example, if one half of the total copper were functioning for structural stability, while only one copper is associated with an oxygen

molecule at each active site. A very important part of the study of hemocyanins therefore resides in the analyses of the oxygenation reaction.

B. The Oxygenation Reaction

The combination of a respiratory pigment with molecular oxygen can be approximately described by the Hill equation:

$$y = \frac{(p/P_{50})^n}{1+(p/P_{50})^n} = \frac{K(x)^n}{1+K(x)^n}$$

where "y" is the degree of oxygenation, "p" is the oxygen pressure in mm Hg, "P_{50}" is the oxygen pressure at half saturation, $x = pO_2$, "K" is a constant and "n" is the sigmoid coefficient, a measure of interaction between oxygen-combining sites. This equation was empirically arrived at by A.V. Hill in 1910 (56), in an attempt to explain the differences among observed oxygen equilibrium curves on the basis of aggregations of "molecules" of hemoglobin, each with a minimum molecular weight of 16,600. His primary reaction for the oxygenation of hemoglobin was: $Hb_2 + 2O_2 \xrightarrow{K'} Hb_2O_4$ from which he derived the Hill equation:

$$I \qquad K' = \frac{Hb_2O_4}{(Hb_2)(O_2)^2} = \frac{y}{(1-y)(O_2)^2}$$

II $K'(pO_2)^2 - K'(y)(pO_2)^2 = y$ or

$K'(pO_2)^2 = y + K'(y)(pO_2)^2$ therefore

$K'(pO_2)^2 = y(1 + K'(pO_2)^2)$

III
$$\frac{K x^n}{1 + K x^n} = \frac{K' (pO_2)^2}{1 + K' (pO_2)^2} = y$$

The ratio between the number of bound O_2 molecules and the number of single active sites is expressed in "n". The shape of the curve represented by the general equation III, when "y" is plotted as a function of "x", is determined by the order of the equation, the value of "n". If "n" equals one, the resulting curve is a section of a rectangular hyperbola and each oxygen molecule combines independently. If "n" is greater than one, the curve becomes sigmoid and cooperative interaction exists among the oxygen-combining sites. The log form of equation I allows rapid determination of "n" from experimental data.

$$K' (pO_2)^n = \frac{y}{1-y} \quad ; \text{ if } x = \log K'(pO_2) \text{ and } y = \log \frac{y}{1-y}$$

in $y = mx + b$

then $b + n \log (K' (pO_2)) = \log \frac{y}{1-y}$

Hills equation is an oversimplification of the oxygen equilibrium with Hb and in fact doesn't fit the best data very well near 0 or 100% oxygenation. Although it was originally based on false assumptions of equilibria between colloidal hemoglobin aggregates and oxygen, it has worked so well because an identical equation results as an approximation of an exact expression relating proportions of oxygen bound groups on a single multivalent macromolecule (68).

The oxygen equilibria for hemocyanin sera show great variety in both P_{50} and "n" values with species, and are highly dependent on specific conditions for individual hemocyanins. The P_{50} values vary from 1 mm to about 16 mm among differing species and with pH for any particular one (102, 107). A high CO_2 pressure or decreased pH tends to dissociate oxygenated hemoglobin, promoting gas exchange in cells. This increase of P_{50} with decreased pH is known as the Bohr effect and in the case of hemoglobin may be related to the acidity of an imidazole (134). If similar data for hemocyanin are compared, it will be seen that a plateau of highest P_{50} values falls in the neutral region and oxygen affinity increases demonstrably on both acid and alkaline sides (105, 107). This would account for disagreement on the point of whether hemocyanins exhibit a positive, the absence of or a reverse Bohr effect, especially in cases where measurements were confined to the physiological range (106) or one side of it (102).

The equilibria for hemocyanins of selected species of cephalopods and gastropods conform to Hill's equation taking "n" equal to 1, 2 or 4 "only at certain pH values" (102). In fact, the value of "n" not only varies significantly with pH for the same hemocyanin, but in several cases changes above 50% saturation at a single pH (107). This dependence of "n" on degree of oxygenation was observed but not recognized in earlier studies (105), either because measurements weren't made in the region of 50% saturation or the results were described as not conforming to Hill's equation. From the data available it appears that "n" increases more sharply above $\log \frac{y}{1-y} = 0$ (105,107). In alkaline pHs, "n" ranges from 1.5→4.1 at pH 8.1, and may even decrease at acid pHs (105). In other words, the influence of oxygen on the interaction of active sites seems greater when the molecule is reversibly dissociated. These may be active sites either within the same subunit, or between subunits, in which case "n" would have the original Hill meaning. In this latter case one would have to propose that oxygen concentration influences the state of aggregation. This is not altogether unlikely since the state of aggregation is very sensitive to pH and there is evidence that oxygenation makes some hemocyanins more acidic. Oxygenation decreased the dissociation constant of a divalent acid or basic group in Homarus by about 0.8 to 0.9 pH units. This was not observed with Limulus, (112).

This would also be consistent with the absence of or a small change in "n" at 50% oxygenation and pHs at or less than neutral.

The presence of Ca(II) ions in the ratio of about 70 to 140 moles per mole of crayfish hemocyanin has recently been shown to maximize the "n" and minimize P_{50} (107). In this case "n" refers to the interaction of active sites within the same molecule, since the Ca(II) concentrations (.03-.06 M) are well within the range preventing dissociation of subunits until pH 9. In the presence of Ca(II) a reverse Bohr effect is seen sharply on both sides of pH 6, in these studies on crayfish HC, while in the absence of Ca(II) there is a plateau region of highest P_{50} between pHs 6 and 8. It is likely that the use of calcium will allow greater distinction between two pH dependent phenomena, the state of aggregation and the ionization of ligands at the active site. These are aspects of the oxygen equilibrium reaction which have only recently been reopened and it remains to be seen whether a predictable behavior of both "n" and P_{50} values with varying pH and divalent cation concentration are characteristic of a particular type of hemocyanin.

The interaction of hemocyanin with gases other than oxygen has not been studied in such detail. In the course of much early work on hemocyanin, it was observed that the deoxy protein, in the presence of H_2 or N_2 , remains colorless when exposed to inert gases such as methane, ethane and

acetylene (29). There is recent evidence that a colorless carbon monoxide complex is formed (132), while two reports that such a compound does not exist have appeared in the literature within the past five years (58, 138). A green solution was reported to result from the reaction of deoxy hemocyanin with nitric oxide, NO (29). This matter has been pursued in the present thesis.

C. Physiological Function of Hemocyanins

Hemocyanin containing bloods vary in oxygen binding capacity according to the organism in which they occur. That the bloods of cephalopods function physiologically as oxygen carriers has been established by observation of color change as it passes through the gills, and analyses of oxygen and carbon dioxide content in arterial and venous bloods of squid and octopus (104). The blood of these most active marine invertebrates, belonging to the highest class of mollusc, contains sufficient hemocyanin to enable combination of 100 ml with 4-5 ml of oxygen (102). In less active classes the concentrations of hemocyanin are smaller and it is not completely certain that they function in oxygen transport. It has been suggested that the large protein molecule may serve as food storage (141) or be influenced in its role of oxygen transport during moulting by a changed *in vivo* calcium concentration (80, 107).

There also seems to be no clear rule about the distribution of hemocyanin and hemoglobin. Some snails, gastropoda,

another class of molluscs, use one, some the other and one species, Busycon, uses hemocyanin as a respiratory pigment yet has myoglobin in some of its muscles (6). On the whole these properties appear to be related to the respiratory problems which confront different kinds of animals.

III Ligands of Copper

The copper in copper proteins, such as laccase, ceruloplasmin, erythrocuprein and hemocyanin, is not easily removable by dialysis between pH 5 and 8 (40). This is one feature which distinguishes copper enzymes and proteins from a metal ion activated enzyme, e.g. carboxypeptidase. The bonding in copper proteins must also be distinguished from the strong but dissociable bonding between copper and a non specific protein, e.g. serum albumin. No conclusions about accessibility of the copper or the pKs of bonding groups in hemocyanins can be drawn from the fact that the copper becomes dialyzable at pH 4. The failure of bulky copper complexing agents or ion exchange resins to produce the apo-protein at neutral or slightly alkaline pHs indicate that the copper is inaccessible. Failure of attempts to obtain a copper containing peptide by proteolytic enzymes (57) suggest the copper bonding may involve the protein's tertiary structure. An irreversible dissociation of subunits begins below pH 4 making it difficult to determine whether the copper is released from groups with pKs in this region or by the dissociation of subunits bound by groups with pKs

in this region.

It has generally been accepted, since 1950, that the copper is directly bound to a section of protein and not a prosthetic group of a porphyrin type. Early attempts to isolate a prosthetic group by alkaline degradation resulted in varied reports of black and green Cu-polypeptide products. In 1940 (98) dark green homogeneous crystals, containing all the copper, were obtained reproducibly by alkaline degradation (10% KOH, 60° C, 30 min) of crystalline crayfish hemocyanin. There was no report on the reversibility of the pH effect. Since a crystallized hemocyanin or degradation product may represent an altered, not a physiological material, this criterion has lost much of the importance formerly attached to it. If a functional hemocyanin could be reconstituted from a well-characterized green degradation product, however, such crystals would bear reexamination by ESR. Since Cu(II) ions combine easily with many peptides at high pHs, most degradation products are believed to be artifacts, probably fragments containing copper sulfide. These earlier studies were reviewed with complete bibliographies in 1945 (28), 1950 (102) and 1962 (42).

In the past decade there has been a renewed attempt to identify the ligands of copper at the active site, using specific blocking agents and modern titration and spectrophotometric techniques. Certain investigators of long experience have committed themselves to the presence or

absence of copper-nitrogen or copper sulfur-bonding on the basis of their observations. The presence of nitrogen or sulfur at the active site therefore remains to be established.

The kinetics of the removal of copper from Limulus and Busycon hemocyanins were studied in 1954 (35). Where "R" is a copper binding group on the protein:

$$\text{For} \quad RCu_2 = RCu + Cu(I); \quad k_1 = \frac{(Cu_2)}{(Cu(I))\ (RCu)}$$

$$\text{and} \quad RCu = R + Cu(I); \quad k_2 = \frac{(RCu)}{(R)\ (Cu(I))}$$

$$\text{then} \quad 2Cu(I) + 2CN^- = 2CuCN$$

The concentrations were calculated by spectrophotometric and chemical analyses and the association constants k_1 and k_2 determined were of the order of 10^{17} - 10^{19}. Although the only copper amino acid complex with a constant in this range is cuprous cysteine (1.5×10^{19}), the evidence is inconclusive since a pair of amine groups could probably bind copper with similar strength (15).

The visible absorption spectrum of oxy HC was compared with that of Cu(II) bovine albumin in 1955 (69). The copper is bound to the albumin by a single thiol group with possible involvement of carboxyl groups since the methylated protein shows reduced binding while the acetylated one shows no loss

of ability to interact with copper (68). Oxyhemocyanins have characteristic bands at 340 and 575 mu and the cupric albumin absorbs at 375 mu and broadly at 550. A model in which each copper as Cu(I) is bound to a cysteine thiol group at the active site was proposed. This model may explain the nondialyzability of the copper and, if partial oxidation of the metal occurs in the presence of oxygen, may also account for the higher extinction (5-10 times greater per gram copper) which distinguishes hemocyanin from the simple Cu(II) protein complex. The enhanced extinction of Prussian blue arises from charge transfer transition between Fe(II) - Fe(III) (108).

The Cu(I) - S linkage was challenged in 1958 on the basis of evidence obtained with group specific replacement and blocking agents (75). Reagents such as silver ions or mercuric chloride which can displace copper in the albumin complex failed to do so in Helix hemocyanin. A reconstitution of the hemocyanin spectrum was also obtained in this study by Lontie upon addition of cuprous copper to an apo protein in which the SH groups had been blocked by N-ethylmaleimide or PCMB*. Since recovery of hemocyanin activity was measured by optical density at 340 mu and not recycling, it is unsure whether a quantitative reconstitution of the native protein had actually been made. On the other hand the same investigators were the first to restore an apo hemocyanin to 100% physiological activity (74) by refinement

*parachloromercuribenzoate

of techniques used by Kubowitz (72). Curiously, the involvement of SH groups is not completely ruled out by this observation even in the case of 100% functional reconstitution of the blocked apo protein. The necessity of unfolding both the native and apo proteins with 8M urea in order to get reproducible amperometric titrations of thiol groups (86) implies that sulfur groups are well masked in both materials. The release of a highly stabilized Cu(I) copper may be accompanied by a masking rearrangement or reaction of the ligand atoms, reversible under reducing conditions. The apo-protein can be reconstituted either by anaerobic combination with Cu(I) (74) or addition of Cu(II) followed by reduction with dithionite (118).

The involvement of two imidazole groups in the copper binding was suggested (75) as an alternative to the Cu(I) sulfur linkage. This would be consistent with titration curves and stability regions obtained for normal and copper free Helix hemocyanin. The apo protein dissociates by sedimentation analyses around pH 6.5, about 1.3 units lower than the native HC, a difference which also appears in the titration curves. The critical negative charge is reached sooner in the copper-free material, in the region where imidazole groups lose protons. However, just as the pH of a free amino acid is altered (decreased) in a metal complex (30, 64) it also may be influenced by neighboring groups. This makes direct extrapolation to histidine residues difficult.

The earlier dissociation of the apo protein is particularly interesting from another aspect. Since no major conformational change occurs simply upon removal of the Cu (86, 13), it implies that the subunit binding groups are very close to, if not identical with, some copper binding groups. Dissociation doesn't begin until pH 8 when there are groups with pH s altered by the presence of copper. The junction between subunits is more labile to increasing hydroxyl ion concentration in the absence of copper.

Amperometric titration of thiol groups in native and apo Cancer magister hemocyanin gave further apparent evidence to make the uniform binding of all active coppers through sulfhydryl less probable (86). The appearance of about five new SH groups corresponding to the absence of about 20 copper atoms in the apo protein gave an average of one SH group for each 4.2 atoms of copper removed. The significance of this ratio is somewhat in doubt, however, since denaturation of both the native and apo protein with 8M urea was necessary to make it reproducible. Any copper released from the active sites by this treatment of the native protein could then mask SH groups not involved at the active site. The seriousness of an error from this source is not great, however, since the difference found is not large. A more serious source of error is the formation of disulfide bonds from free SH groups during the action of urea. This

phenomenon, which might also occur in the absence of O_2 (17) would prevent a true comparison. It was fully described only after the hemocyanin work was done and protection of these groups is now made in amperometric titrations of hemoglobins.

The observations which indicate that sulfur is at the active site are as indirect as those which eliminate it from the active site or point to nitrogen. The most recent speculations about the ligands of copper in hemocyanin are, perhaps even more indirectly, based on analogies between the spectra of Cu(I) and Cu(II) models and that of hemocyanin. R.J.P. Williams (138) concluded that HC contains a cuprous copper bound to two sulfur atoms from consideration of 1) the known absorptions of Cu(I) thiol, oxygen and dipyridyl complexes, 2) the comparative polarizability of sulfur compared to nitrogen and oxygen and 3) the stereochemistry of Cu(I). The sulfur, oxygen and nitrogen model complexes all have strong absorbancies between 300 and 600 mu, with the Cu(I)-S bands occuring at the shorter wavelengths and the Cu(I)-O bands at the longer wavelengths. Cu(I)-(SR) models absorb in a range of $3.0\text{-}3.25 \times 10^{+4} cm^{-1}$ (300-340mu), Cu(I)-dipyridyl in a range of $1.2\text{-}3.0 \times 10^{+4}$ cm^{-1} (400-500mu) and Cu(I)-O_2 in a range $1.6\text{-}1.9 \times 10^{+4} cm^{-1}$ (500-600 mu). The UV maximum of oxyhemocyanin, generally placed at 340 mu but quoted by Williams at 375mu, with a copper molar extinction of 6,000 M^{-1} cm^{-1} (138) was attributed to Cu(I)-S interaction

primarily on the basis of these model complex spectra.

The band at 575 mu may arise from the proximity of a partially oxidized cuprous copper to S, N or O, since a similar band appears in a Cu(II) protein complex with thiol (69) and possibly oxygen bonding (68) and falls in a range characteristic of charge transfer from Cu(I) to nitrogen (15). The extinction per gram atom of copper for this band in hemocyanin is 600 M^{-1} cm^{-1}, a bit high for known d-d transitions and a bit low for charge transfer transitions (15). The high extinction for the UV band of oxy HC characterizes it as a charge transfer type. Although Cu(I) -S transitions appear to give rise to similar bands, it would have to be postulated that the transition is possible only in the presence of oxygen, or from a partially oxidized cuprous, since it is absent in the deoxy material. An interesting alternative explanation of this band is that it arises from a charge transfer to the oxygen molecule itself. There is good reason to believe that the oxygen is partially reduced or peroxidic when associated with hemocyanin. Benzidine and Ti(IV) tests for peroxide (70), analogy with a hemoglobin model (69) and analogy with synthetic reversible oxygen carrying chelates (133) indicate a partial displacement of metal electrons toward the O_2. The O_2^- ion in solution gives rise to an absorption at 340 mu (55). Assignment of sulfur as a ligand from spectral features alone is therefore impossible. Two other ligand field considerations,

polarizability of ligands and steric requirements, would be consistent with and possibly strengthen the case for copper-sulfur bonding. The copper in both deoxy and oxy hemocyanin appears to be cuprous in a conventional sense (87). Sulfur atoms, being most polarizable, nitrogen next and oxygen least, could provide the necessary stability to a copper in this lowest oxidation state. A preference of small cations in the first transition series for nitrogen or oxygen over sulfur has been observed and is attributed to the large size of the sulfur atom causing steric hindrance in octahedral coordination (138). Cu(I) complexes, however, show virtually no tendency to hexacoordinate and are tetrahedral when tetracoordinated.

IV Valence of Copper

It is evident from the foregoing that the valence of copper in oxyhemocyanin is directly related to the problem of the unknown ligands of copper. The most recent direct (70, 87) and indirect (69, 133) evidence, already quoted, requires partially oxidized Cu(I) atoms or perhaps two or four coppers, some Cu(I) and others Cu(II), at the active site in oxyhemocyanin.

The copper in deoxy HC is believed to be uniformly monovalent, principally because of successful reconstitutions of the apo protein using cuprous chloride under hydrogen (41, 74). The absence of a visible spectrum is occasionally quoted as additional evidence, but water of hydration is

known to affect the visible spectrum. For example, completely anhydrous cupric sulfate is colorless. Recovery of functional native protein has been measured by OD at 340 mu, upon saturation with oxygen, (41, 74) and by recovery of original extent of catalase activity (41). Unless special mention is made, it is assumed that the reconstituted material is fully reversible to oxygen association, taking loss of activity through surface denaturation into account if the cycling is done using vacuum.

With the exception of experiments with ESR, all attempts to measure the valence of copper in oxyhemocyanin directly, by redox potential, magnetic susceptibility, valence specific agents and polarography have either failed or been inconclusive.

Redox potentials of solutions of oxy HC were measured in 1933 (23) and again in 1940 (98). Since the development of steady potentials after each addition of oxidant took from 1-2 hrs., the electrochemical reaction probably involved atoms of the protein other than the metal. The approximate E^{o}, redox potential of hemocyanin (540 mV) is higher than that of other copper proteins (300-400 mV) (138). As with a number of known altered redox potentials of specifically complexed coppers (15) this increase indicates steric hindrance and/or a high degree of unsaturation in the ligands around the copper. Some examples of this are Cu(I)-imidazole, 255mV; Cu(I)-2 imidazoles or 2 NH_3, 345mV in comparison with

167mV for oxidation of a hydrated (unchelated) Cu(I) to Cu(II).

No significant difference in the magnetic susceptibility of the oxy and deoxy protein was observed in 1941 (100). The doubtful value of this approach was realized at the time. Despite the difference of 1.73 Bohr magnetons between Cu(I) and Cu(II), the relative solubility of the protein and consequently its copper content is comparatively so low that a valence change from plus 1 to 2 would, in this experiment, have changed the mass susceptibility by only 0.03 10^{-6}/gram. This different was not significant on the torsion balance used (99).

Polarographic reduction of a metal has been used to distinguish Cu(I) from Cu(II) in simple complexes. No clear stepwise change in the wave could be observed with hemocyanin however (69). It was concluded that "evidently the copper, whatever its valence, is held very strongly by the protein, or is sufficiently embedded in the macromolecule that electrons dropping from the mercury electrode are unable to reach it" (69).

The same workers attempted to measure the quantity of Cu(I) copper present in acidified oxyhemocyanin by 2 2' biquinoline, which forms a pink complex only with the monovalent copper. Glacial acetic acid, necessary to the biquinoline test was believed to "displace the Cu(I) from the protein" (69). The results indicated 50% (Cu(I) and 50% Cu(II) in the oxy-protein. Expression of

100% of the copper as the biquinoline complex was reported for the deoxygenated material. This result was also obtained by Felsenfeld in 1959 (36). He proposed a cuprous-cupric pair bridged by O_2 at each active site. It was later shown that denaturation of oxyhemocyanin by glacial acetic acid results in a strongly Cu(II) ESR spectrum at room temperature (87). A possible explanation of the 1955 results may be that on the average 50% of the cuprous copper released by acid in the presence of biquinoline is quickly bound. In the absence of oxygen this average would also be significantly increased.

Another chemical observation (117), a positive biuret test upon addition of dilute alkali alone to oxyhemocyanin, would be consistent with a cupric-containing active center structure. Deoxygenated HC under the same conditions gives no coloration.

In 1956 (137) Williams questioned the validity of these chemical methods and, therefore, the necessity of a cupric at the active center in the oxy-protein. He suggested that a cuprous-cuprous structure, with some contribution by a $(Cu(II)O_2^-..Cu(I))$ form would be consistent with the existing spectral and paramagnetic data. Indeed, there is no paramagnetism detectable by ESR in either the native oxy- or the deoxy-protein at room temperature (87). At liquid nitrogen temperature, the observed signal never amounts to more than 1% of the total Cu present (118).

The contribution of a mixed valence resonance form to the stability of an oxygenated active site, however, offers an attractive explanation of extinction of the long wavelength band of oxyhemocyanin. Enhancement of extinctions has, in a few specific cases such as Prussian blue (108), been attributable to a mixed valence structure. In view of the spin resonance data, the lifetime of this mixed valence form would have to be so short or the cupric signal arising from it sufficiently broadened by some mechanism as to render it undetectable.

The mixed valence structure might arise by the transfer of an electron to oxygen, as proposed in the resonance hybrid models of both Klotz (69):

$$Cu^{++} \,..O_2^{-}..Cu^{+} \rightleftharpoons Cu^{+} \,..O_2^{-}..Cu^{++}$$

and Manwell (83):

$$Cu^{+} ..O_2.. \; Cu^{++} \rightleftharpoons Cu^{++} - O_2^{-}.. \; Cu^{+} \rightleftharpoons Cu^{+} ..O_2^{-} - Cu^{++} \rightleftharpoons Cu^{++} - O_2^{=} \,..Cu^{++}$$

An alternative is that it arises without an actual valence change in the oxygen, but through either an electron excitation within the metal orbitals, d-d transition (138), or a charge transfer mediated by the ligand O_2 (82). Both would have a ground state representation of $Cu^{+} ..O_2.. \; Cu^{+}$. The resolution of each of the 575 and 340 mu bands into two components at 77° K (85) may be additional evidence for the participation of two copper atoms in the oxygen binding center.

That there may be two copper atoms with slightly different ground state energies in the active site is discussed in detail in a later section (Experimental II D.).

The absorption spectra of trivalent copper in aqueous solution (Cu(III) periodate) have been recently analysed (140). A 540 mu band (ϵ = 250 $M^{-1}cm^{-1}$) was attributed to a d-d transition while both 415 (ϵ = 11,100 $M^{-1}cm^{-1}$) and 365 mu (ϵ = 4,590 $M^{-1}cm^{-1}$) bands were attributed to electron transitions from oxygen to copper. It is possible that this valence state may also contribute to the unusual oxidation state of copper in oxyhemocyanin, especially since all models of the active center to date assume that oxygen exists as O_2 in solution (see General Discussion II C.). In summary, it is doubtful that the actual extent of the partial oxidation will be described until the Cu ligands are identified and a more quantitative theory of chemical bonding (62) can be applied to the problem.

The experiments reported in the following sections were undertaken in order to confirm and expand upon some characterizations of hemocyanin and to attempt to form a true methemocyanin to permit observation of the active site by ESR.

EXPERIMENTAL

I Confirmation and Further Characterization of Physical Properties of the Active Center in Cancer magister Hemocyanin

A. Determination of the Cu/O_2 Ratio Using the Oxygen Electrode.

1. Aim:

One molecule of oxygen combines stoichiometrically with two atoms of copper during the oxygenation of hemocyanin. The chemical and manometric evidence upon which this ratio is based was published before 1941 (50, 98, 103) and has been summarized in the Introduction. Recent improvements in polarographic techniques have allowed direct determinations of oxygen tensions in solutions of oxyhemoglobin (21) and suspensions of mitochondria (89). Experiments were carried out to confirm the statistical ratio and determine an extinction per active site or $(O_2)M^{-1}$ at 575 mu from polarographic measurements of oxygen concentration.

2. Materials and Methods:

a. Preparation and Storage of Hemocyanin. Serum from Cancer magister was collected by inverting live crabs after removal of their major claws. The pH of this serum varied from 6.8-7.2. Two types of impurities, a lipid-like film and an easily-sedimented foam coagulate, were immediately removed by centrifugation in the Servall at 4°C and 17,000 rpm (32,700 x g) for 20 min, followed by filtration through a

thin layer of buffer washed glass wool. The hemolymph was then combined with an equal volume of .1M Tris buffer pH 6.8, .02M Ca(II), and concentrated by centrifugation in a Spinco rotor "21" at 21,000 rpm, 4°C, for 15 hrs or in a Spinco rotor "50" at 50,000 rpm, 4°C, for 5 hrs in a Spinco Model L centrifuge. The resulting dark blue gelatinous pellets were taken up in 5-10 ml of .05M Tris buffer at pH 6.8 containing 0.01M Ca . The resulting clear concentrate generally contained from 2.5-3.0 mM copper.

Deionized, distilled water with conductivity not higher than .8 uMoh or 5 ppm NaCl (0.1mM), Sigma high purity 7-9 Tris and reagent grade $CaCl_2$ were used throughout. The fresh concentrate gave a single peak in the Spinco Model E analytical ultracentrifuge with S_{20w}25,mw950,000 and 5% of a smaller component. These values were comparable to those found for hemocyanin from the same source (86). This preparation differs from that reported only in the use of Tris buffer and omission of dialysis of the final material against several changes of buffer. The use of Tris allows fortification of buffer solutions with at least .01M Ca(II), recently shown to stabilize subunits and maximize the protein's interaction with oxygen (107). Dialysis was omitted because this did not affect sedimentation results while it minimized both surface denaturation and removal of physiological salts.

Fresh concentrates were used for final determinations in every experiment reported. The storage of hemocyanin is difficult at best. A catalase activity has been reported to increase with the *in vitro* age of the protein (41). In our experience, a fine sediment appeared, accompanied by a noticeable loss of blue color after storage of the concentrate for one week at 4°C. This is probably due to bacterial action. With the exception of microfiltration, the usual measures taken to prevent bacterial action without denaturation, have been unsatisfactory in the case of hemocyanin. Thawing material stored at -20°C resulted in the formation of a fine precipitate and expression of up to 10% of the total copper present in the supernate as cupric by ESR. Storage under toluene, which has been used for this protein (36), resulted in an increased percentage of the total copper expressed as cupric by ESR. The low temperature ESR cupric signal of fresh concentrated hemocyanin accounts for less than 1% of the total copper present by chemical analyses. Contact with toluene increases this to 5% and with chloroform to 10 and 20%. The aged untreated concentrate yields a clear dark blue supernatant with less than 1% total copper as cupric by ESR, after centrifugation in a refrigerated Servall at 17,000 rpm for 20 min. A successful sterile filtration (pore size .45 microns) and storage of a diluted hemolymph has been reported (38) but could not be reproduced even with the aid of prefiltration techniques and advice of bacteriology department staff members.

b. The Oxygen Electrode. A Beckman model 777 oxygen analyser was used to measure oxygen tensions of solutions in a specially designed water cooled vessel, Fig. 1, a. Two significant improvements in polarographic technique are combined in this simple setup. A replaceable teflon, oxygen permeable, membrane separates the oxygen sensor from the sample. This minimizes contamination of the electrodes by sample solutions. The vessel is designed to house a small flat disc magnet which insures rapid and complete mixing and maximum stability during a run. The sample chamber was kept at 0° C by a circulating water methanol mixture from a Lauda Kryomat. When one of the vessel openings was not being used for gas flow or sample addition, sample temperature was measured directly by insertion of a thermometer with a ground glass collar.

The instrument's oxygen readout was calibrated to register 159mm, the partial pressure of oxygen in air at sea level, at the beginning of and several times during each experiment. Chemical zero, obtained by flushing pre-purified nitrogen (Industrial Air, O_2 less than 3 ppm) through the dry sample chamber or using a glucose oxidase system, was greater than electrical zero by no more than 1 mmHg. The limits of accuracy according to the manual are 1% full scale with constant temperature and 5% full scale with shifting temperature. These are equivalent to 2.5 mm or .007 mM O_2 and 12.5 mm or 0.03 mM O_2 on a scale in which 250 mm would

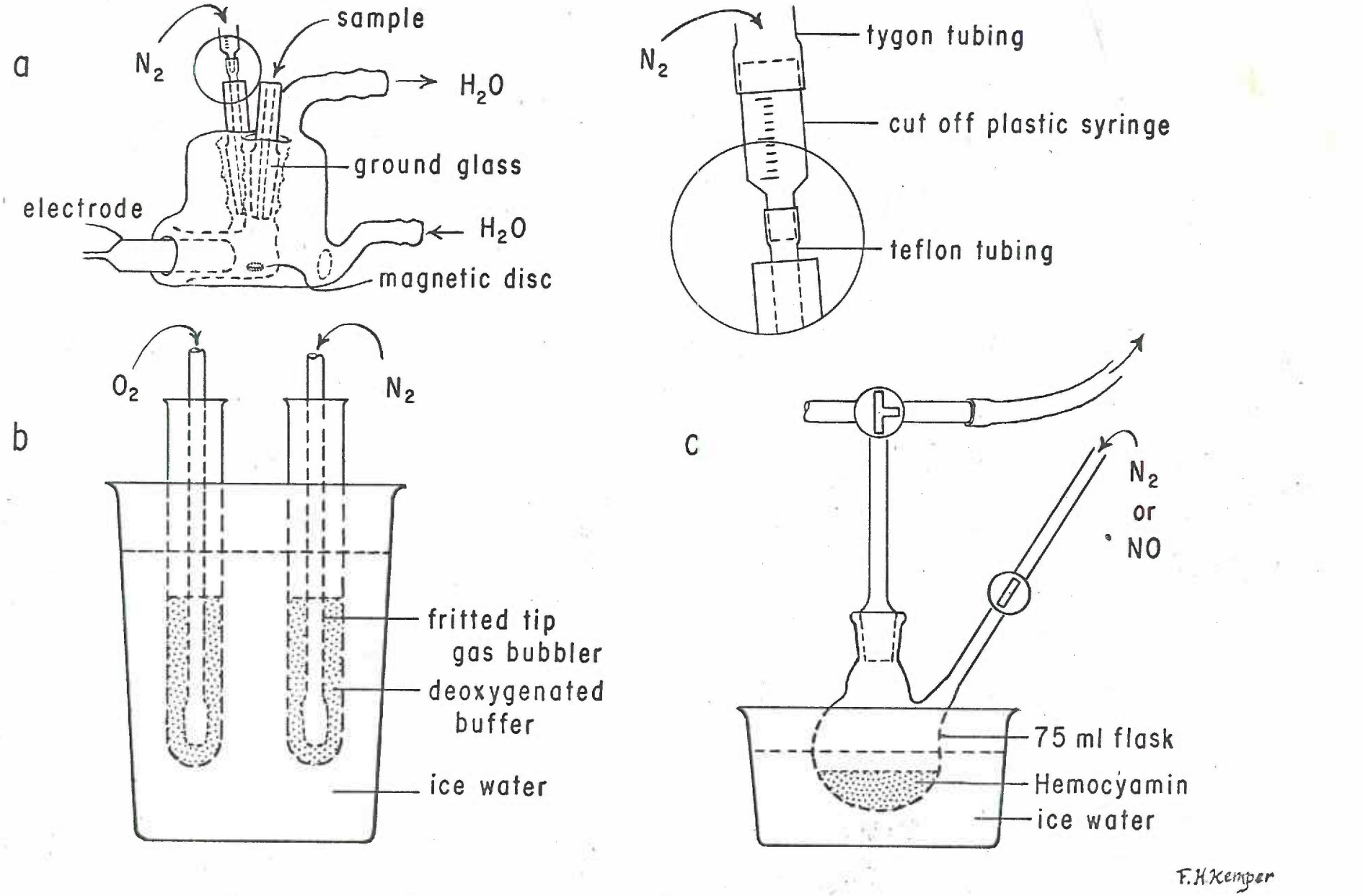
Figure 1

Diagrams of:

a. oxygen electrode vessel

b. buffer-gas equilibration setup

c. hemocyanin gas equilibration vessel


a
N_2
sample
H_2O
ground glass
electrode
H_2O
magnetic disc
N_2
tygon tubing
cut off plastic syringe
teflon tubing
b
O_2
N_2
fritted tip
gas bubbler
deoxygenated
buffer
ice water
c
N_2
or
NO
75 ml flask
Hemocyamin
ice water

F. H Kemper

give a full 100 division deflection. The observed discrepancies between calculated and recorded oxygen tensions in blank experiments fell between 3 mm or 0.0086 mM and 8 mm, 0.029 mM. This sensitivity is an order of magnitude less than that recently obtained by a modified Clark oxygen electrode circuit (33) routinely used to measure changes of oxygen uptake of .3 ul O_2 or 0.01 mM. Polarographic determination of Hill's coefficient would be possible with such a modified circuit since the partial pressure of half saturation, P_{50}, for hemocyanins is about 10 mm Hg (107, 109). Increments of 3 mm between 1 and 20 mm could not be measured accurately using the Beckman instrument. In the concentration range of interest in the present experiments, the Beckman oxygen analyser allowed determinations of at least 3% accuracy.

The oxygen concentration bound to a known concentration of hemocyanin was read directly from a plot of (O_2) in solution, as measured by the oxygen electrode, against (O_2) total, calculated from a known addition of fully oxygenated buffer at 0° C.

$$(O_2)_{total} = (O_2)_{bound} + (O_2)_{solution}$$

Deoxygenated hemocyanin was prepared in a tonometer vessel, Fig. 1, c., which allows gentle swirling of the protein solution at 0° C, under a stream of gas. It was used in all

preparations of deoxygenated hemocyanin, since repeated evacuation and flushing with nitrogen resulted in surface denaturation. The oxygenated and deoxygenated buffers, 0.05 M Tris pH 6.8 0.01 M Ca were prepared by flushing oxygen and prepurified nitrogen through fritted glass bubblers, Fig. 1, b., for no less than an hour at 0°C. Micro syringes, 0.01 ml, kept at 0°C and flushed with the appropriate gas were used for all transfers.

The capacity of the sample chamber, Fig. 1, a., was 3 ml. It was fitted for flushing with N_2 as shown. For the determination of a single point on a graph, 0.5 ml deoxygenated hemocyanin and an aliquot of anaerobic buffer were added and stirred under a slow stream of nitrogen bubbles until a stable base line was established. The addition of varied amounts of oxygenated buffer brought the total sample volume to 3 ml in each case. The change in oxygen concentration registered by a Bausch and Lomb "vari chart" recorder reached a plateau in less than one minute. This value minus the base line value gave $(O_2)_{solution}$ for a specific (O_2) total or volume of oxygen saturated buffer added. A control, in which the .5ml deoxy hemocyanin was replaced by .5m. deoxygenated buffer, was also recorded at each $(O_2)_{total}$ value. Ten observations of $(O_2)_{total}$ concentrations between .182mM (0.25 ml oxygenated buffer) and 1.82 mM (2.5 ml oxygenated buffer at 0°C) constituted a single experiment. The millimolar equivalence of oxygen in the aliquots of oxygenated buffer was based on the

solubility of O_2 in water at 0°C, 48.9 ml/l, from International Critical Tables, 1928, volume 3, page 257. This is equivalent to 2.181 mM .

The range of $(O_2)_{total}$ was chosen so that the lowest value, .182 mM, equivalent to 63.5 mm, was over five times P_{50}, 10mm or 0.028 mM. Values for $(O_2)_{solution}$ less than 100mm were discarded in determinations of the best fitting line by the method of least squares. In order to further minimize errors resulting from extrapolation, concentrations of hemocyanin were used which would allow the $(O_2)_{bound}$ value, which is the $(O_2)_{total}$ at 0mm $(O_2)_{solution}$, to fall in a region close to the origin. The range of accuracy of $(O_2)_{bound}$ values at this intercept was never less than 0.01 mM as estimated by the S_b value from least squares (5).

The functional copper was estimated in two ways. Total copper present by chemical analyses was assumed equal to functional copper only in fresh samples which gave virtually no cupric ESR signal. Difference spectra between oxy and deoxy hemocyanin at 575 mu on these samples gave an extinction coefficient for the functional copper. Chemical determinations of total copper in fresh concentrates and copper EDTA standards by the biquinoline method (36) and the bathocuproein sulfonate method, as performed by Marie Vanneste, gave values within 0.01-0.03 mM or 1-2% of one another.

3. Results and Discussion:

a. The Cu/O_2 Ratio. An example of a typical experiment is shown in Table I, a. and Fig. 2 a and b, and a summary of ratios is made in Table I, b. The extinction per mole functional copper at 575 mu is 413 ± 37, at pH 6.8 in the presence of 0.01M Ca(II). Care was taken to check this value at several dilutions of hemocyanin since it has been noticed that Beer's Law, linear correspondence between concentration and optical density, is not strictly true for concentrations above 2.0 mM copper. This may be due to the difficulty of maintaining 100% O_2 saturation at these concentrations. Determinations of absolute values were always made on a Zeiss spectrophotometer or a Cary Model 14, with a fixed photomultiplier tube attachment.

Variation in the extinction of copper in oxygenated hemocyanin at 575 mu, against a buffer blank, $e_{575} = 600 \pm 50\ M^{-1}cm^{-1}$ was observed in different preparations over two years. The failure of Beer's Law under certain conditions has been repeatedly noted in the literature [101), most often ascribed to alterations in a scattering effect rather than changes in true light absorption and never systematically studied. Since the equilibrium between the protein, its subunits and oxygen is sensitive to slight changes in pH and divalent cation concentration, a working extinction coefficient was established for the hemocyanin stock solutions of different preparations. The results from three separate polarographic determinations of oxygen bound to

Figure 2

Polarographic determination of oxygen bound to hemocyanin:

a. typical recordings at single value (O_2) total : 1.27 mM

b. determination of a single value of (O_2) bound by the method of least squares

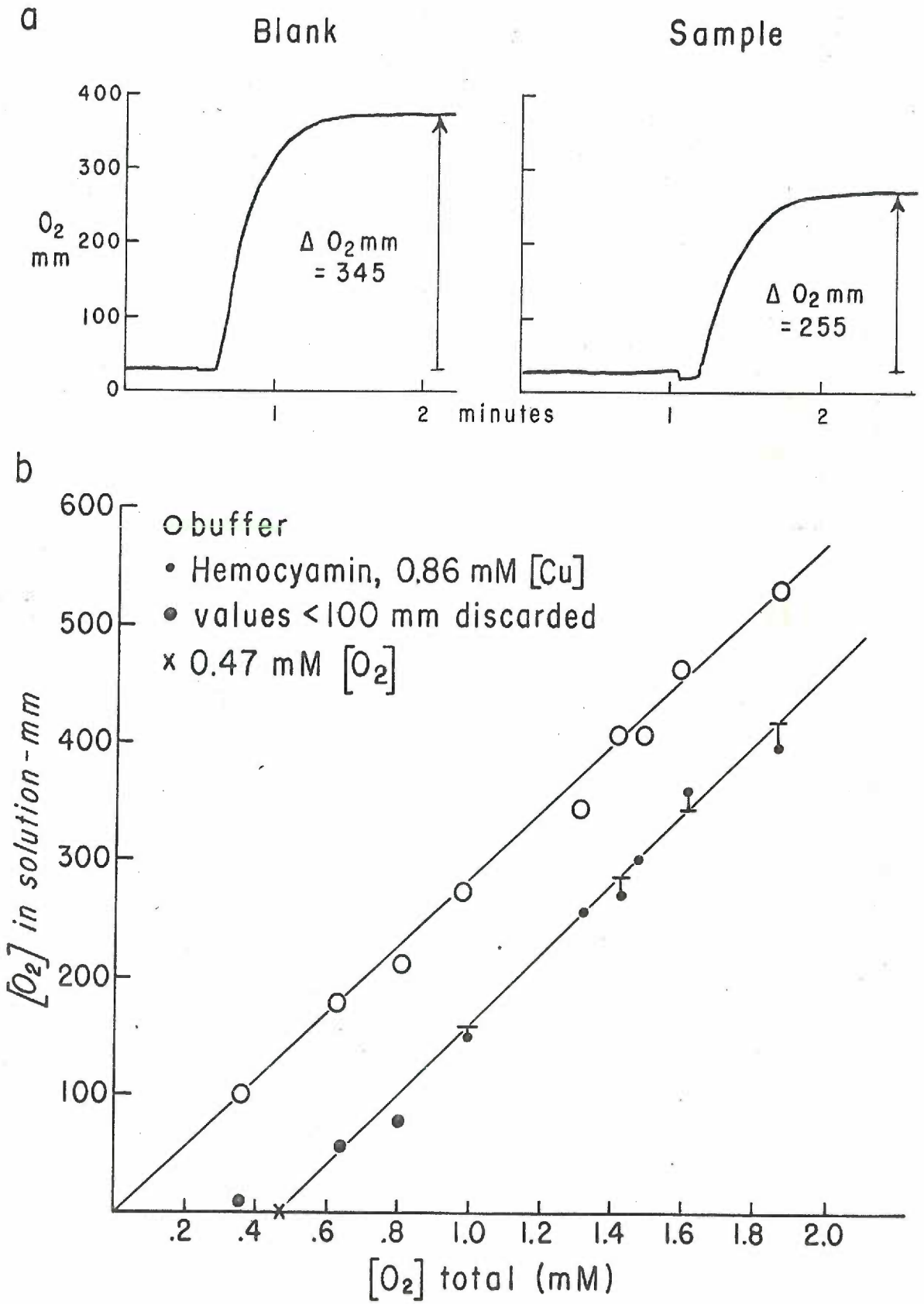

a
Blank
Sample
400
300
200
100
0
O_2 mm
Δ O_2 mm = 345
Δ O_2 mm = 255
1
2
minutes
1
2
b
600
500
400
300
200
100
$[O_2]$ in solution - mm
○ buffer
• Hemocyamin, 0.86 mM [Cu]
● values < 100 mm discarded
× 0.47 mM $[O_2]$
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
$[O_2]$ total (mM)

TABLE I

Polarographic determination of (O_2) bound to hemocyanin:

a. typical experiment for the determination of a single value of (O_2) bound

b. summary of values and average ratio Cu/O_2

TABLE I

a.

	(O_2) total (mM)	Δ mm	anaerobic buffer (ml)	deoxy HC (ml)	oxygenated buffer (ml)	total volume (ml)
Blank	.364	100	2.5	0	0.5	3
HC	.364	10	2.0	0.5	0.5	3
B	.618	180	2.15	0	0.85	3
HC	.618	57.5	1.65	0.5	0.85	3
B	.80	215	1.9	0	1.1	3
HC	.80	72.5	1.4	0.5	1.1	3
B	.98	275	1.65	0	1.35	3
HC	.98	150	1.15	0.5	1.35	3
B	1.27	345	1.25	0	1.75	3
HC	1.27	255	0.75	0.5	1.75	3
B	1.45	410	1.0	0	2.0	3
HC	1.45	300	0.5	0.5	2.0	3
B	1.63	467	0.75	0	2.25	3
HC	1.63	365	0.25	0.5	2.25	3
B	1.82	525	0.5	0	2.5	3
HC	1.82	395	0	0.5	2.5	3

b.

functional Cu	(O_2) bound	ratio
0.86 mM	0.47 mM	1.83 ± .01
0.32 mM	0.19 mM	1.70 ± .01
0.32 mM	0.16 mM	2.0 ± .01

Average 1.84 Cu/O_2

hemocyanin copper, Table I b., show an average of one oxygen molecule bound per 1.84 functional coppers. The various actual bindings of oxygen at the active sites which would be consistent with this statistical ratio are shown in Fig. 3. Clearly two possibilities, Fig. 3 d. and e., are less likely than the others. Considering only cases a., b. and c., the statistical ratio would mean that there can be no more than two and no less than one copper at the active site. If it could be shown, by polarographic determinations on partially denatured hemocyanin, that loss of activity also occurs in pairs, it might strengthen the case for total copper being functional. This is considered in Experimental Section II D., 3c, and Gen. Disc. I A., 1.

b. The Extinction for Functional Cu and the Active Site. The $\epsilon_{max\ 575}^{bound\ (O_2)}$ $M^{-1}cm^{-1}$ was determined to be 1,262 100 for oxyhemocyanin, pH 6.8 0.01M Ca^{++} at room temperature against buffer and 737 ± 20 against deoxyhemocyanin. Fully oxygenated samples from polarographic determinations of the statistical ratio were used in these measurements. Samples were transferred directly from the electrode chamber to optical cells and the optical density at 575mu was read after a few minutes' allowance for temperature equilibration between solution and cell. Variation in ϵ_{max} may be the result of slight temperature differences at the time of reading, or differences in scattering. Beer's Law has been observed to not hold accurately for some

Figure 3

Possible distributions of oxygen at the active site, which would give a statistical ratio of 2 Cu/O_2 .

Possible distributions of copper and oxygen consistent with a statistical ratio of 2:1

a.	b.	c.	d.	e.
...Cu Cu-O_2	Cu^{O_2}Cu	$^{O_2}_{Cu}$ $^{O_2}_{Cu}$	O_2 O_2 O_2 O_2 Cu Cu	O_2 O_2 O_2 Cu Cu O_2
..Cu Cu-O_2	Cu^{O_2}Cu	$^{O_2}_{Cu}$ $^{O_2}_{Cu}$	Cu Cu	Cu Cu
..Cu Cu-O_2	Cu^{O_2}Cu	Cu Cu	Cu Cu	Cu Cu
..Cu Cu-O_2	Cu^{O_2}Cu	Cu Cu	Cu Cu	Cu Cu

Actual ratio at the active center:

Cu/O_2

1/1	2/1	1/1	1/2	1/4

Cu non-bonding/Cu bonding

1/1	0/1	1/1	3/1	7/1

Possible distributions if the mechanism of oxygen binding is not uniform:

Cu^{O_2}Cu
Cu^{O_2}Cu
Cu^{O_2}Cu
Cu^{O_2}Cu
Cu..Cu-O_2
Cu..Cu-O_2
Cu..Cu-O_2
Cu..Cu-O_2

or

$\begin{bmatrix} Cu & Cu \\ Cu & Cu \end{bmatrix}$-$O_2$

$\begin{matrix} (Cu & Cu)\text{-}O_2 \\ Cu & Cu \\ O_2 & O_2 \end{matrix}$

hemocyanins beyond the range of 5% protein (101, 105), equivalent to only 0.1 mM Cu of Cancer magister. Concentrations around 0.4 and 0.8mM Cu were used in these experiments. The ϵ_{max} per active site values are, as expected, approximately twice those found for ϵ_{575} M^{-1} cm^{-1} copper. The extinction per active site will be of greater interest in the future, once the number of coppers and the source of the absorption at 575 mu are known. A value of ϵ_{340} can also be calculated from this value and will be discussed in a later section. In the near future, an extinction per active site at 575 mu, based on a measured bound oxygen concentration and calculated for the 340 mu absorption may become more useful than that per mole copper, when comparing hemocyanin with synthetic reversible oxygen carrying chelates. Considering both the position and probability of a transition associated with O_2 binding activity may help classify hemocyanin with particular model chelates, whose structure and mechanism of O_2 interaction are better known.

e. The Cu/CO Ratio. The ϵ_{575} for functional copper had immediate use in the experiments of Walter Vanneste (132) on the binding of radioactive CO by hemocyanin. Once the value was established for a stock solution showing no cupric ESR signal, the total functional copper in actual samples could be read from their difference spectra at 575 mu.

In brief, this experiment involved equilibration of a

deoxy hemocyanin sample and a hemoglobin control, in duplicate, with radioactive CO at four known partial pressures, four values between 100 and 800 mm Hg. Samples were then transferred to small glass bottles fitted with vaccine caps, containing ferric cyanide, and equilibrated in a tonometer, after one designed by Dr. Pitcairn, for one hour to insure full release of that $C^{14}O$ bound into the gas phase. The gas above each sample and Hb control was then completely transferred into dry and evacuated matching vessels containing plastic-stilbene scintillation beads, and the radioactivity was counted. Each pair of vessels had a correction factor for true total volume, relative to that pair with the lowest total volume. From a plot of counts per min. on the ordinate against pCO in mm Hg, abscissa, a value for cpm/ (CO) for a known concentration of hemoglobin was obtained by extrapolation to the y intercept, pCO at 0 mm Hg, using the method of least squares. This was directly proportional to the extrapolated value of cpm at 0 mm CO for the parallel determination on hemocyanin and gave the number of moles of $C^{14}O$ bound by HC. The concentration of functional copper in each of three experiments divided by the observed value of CO bound gave ratios of 2.3, 2.46 and 2.05.

In order to bring this ratio closer to two, sources of error which lead to a loss of radioactive CO or an overestimation of functional copper must be looked for. Consider-

ing the extreme care in operation and the sensitivity of the method of (CO) determination, and the difficulty in obtaining reproducible extinctions for hemocyanin, already described, over-estimation of functional copper is the more probable source of error. In any case, the existence of a hemocyanin CO complex was firmly established for the first time by these experiments. The controversy over the existence of such a compound has persisted from the earliest work on the protein to the present day. Solutions of oxy-hemocyanin treated with CO become colorless (72, 109). A ratio of 2 coppers per CO was reported in 1934 (109) on the basis of determinations with a Van Slyke apparatus and by absorption of CO into an ammoniacal cuprous chloride solution. The affinity of HC for CO was estimated to be 1/20 that of HC for O_2, on the basis of oxygen dissociation curves determined in the presence of CO. The P_{50} determined in the Vanneste experiment was 73 mmHg. Rawlinson, in 1940 (98), found no evidence of CO binding to HC using an iodine pentoxide method of CO determination (10). More recently, within the past two years, the non-existence of a HC-CO complex has been referred to twice (58, 138).

B. Characterization of the Triplet State in Native and Apo Hemocyanin

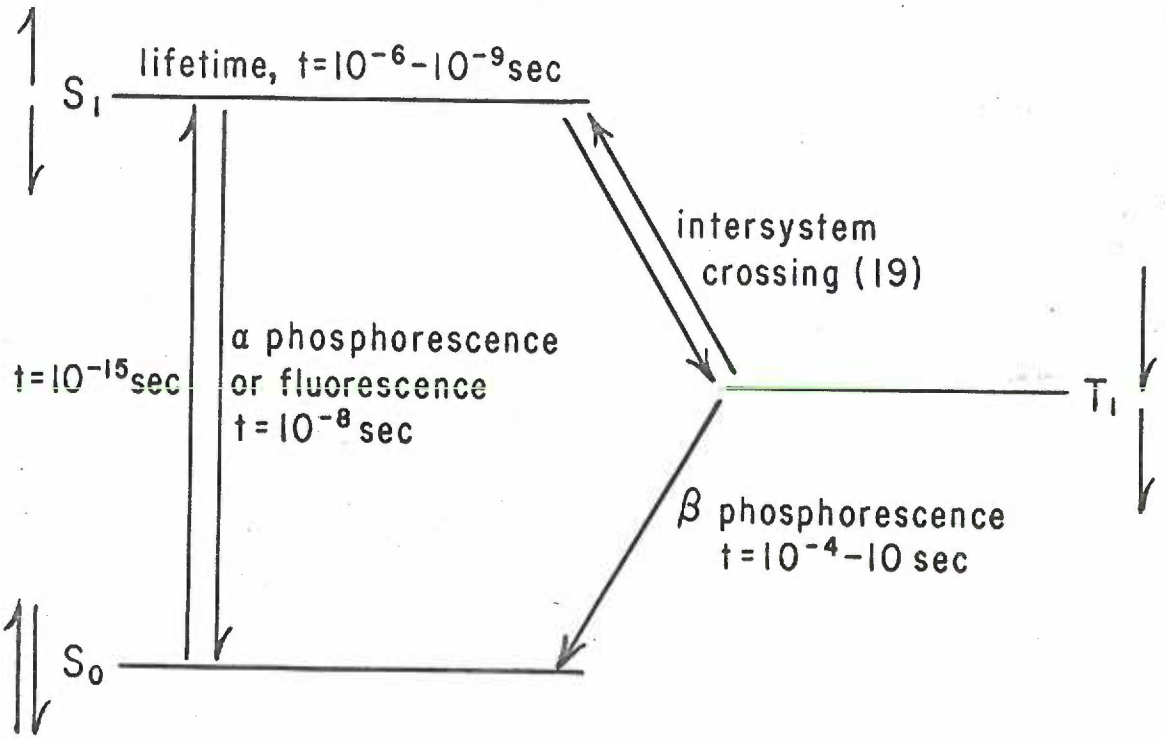
1. Aim:

All protein solutions absorb ultra violet (uv) light with a λ max at 280 mu, characteristic of trypotophan residues (111). This absorption may cause various physical changes. Coagulation of protein solutions occurs upon uv, broad band, irradiation at the isoelectric point (4). The formation of low molecular weight substances and a correlation between increase of light absorption and increase in concentration of aggregated albumin in uv irradiated solutions have been reported (111). Irradiation by wavelengths of 280 mu and shorter, at room temperature and $0^{o}C$, resulted in an inhomogenous solution of lower molecular weight substance in hemoglobin and a splitting of Helix hemocyanin into two $\frac{1}{2}$ molecules (122, 123). More recently, triplet states of several aromatic amino acids, albumins and D amino acid oxidase were characterized by ESR (116).

Triplet states are generally the lowest excited electronic state of a molecule (94), have lifetimes in fluid solvents of the order of 10^{-4} sec., several orders greater than that of excited singlets, and their chemical behavior is usually characteristic of biradicals (Fig. 4). Formation of the triplet state, which involves a change in total electron spin momentum, is less favored than energy transfer processes in which total spin momentum is conserved (94).

Figure 4

Schematic comparison of features of the triplet excited state, T_1, with the singlet, S_1, and ground, S_0, states.



↑↓ paired electrons at the same energy level

↑ ↓ paired electrons at different energy levels

↓ ↓ unpaired electrons at different energy levels

The triplet state, however, has been postulated as an intermediate in energy transfer in chemical (19) and biochemical (116) systems.

The following experiments were undertaken to characterize the triplet state of a protein more precisely, by photoactivation curves, and to observe the quenching effects of oxygen and copper, both involved in the activity of hemocyanin and both well characterized quenchers of aromatic hydrocarbon triplets (52, 90, 94).

2. Materials and Methods:

All ESR spectra were obtained using a Varian V- 4500 EPR spectrometer equipped with a V FR-2200 Field dial magnetic field control unit and with a 100 kc modulation unit. The microwave circuit was modified (9) to allow low power and power saturation studies, described in Experimental Sections II, B and D. A Hewlett-Packard 431B power meter was used to measure the incident power in milli watts (mW). For these determinations, the incident power was always 25 mW, equivalent with our klystron to an attenuation of about 10 decibels (db).

The first derivative of the resonance absorption curve was recorded by a Varian F80 X Y recorder and triplet life times were recorded by a Sanborn Model 320 Recorder. For the measurement of half life, the field was positioned at the trough of the triplet signal, that portion farthest away from the base line, and the path of the beam was interrupted

after a stable line on the Sanborn recorder was obtained. Ordinarily, the recorder was set for full deflection at 1 milli volt (mV) chart speed at 2 mm/sec. Occasionally, for life times less than 5 seconds, the chart speed was 10 mm/sec and for small signal heights (low triplet concentrations) full deflection was set at .5 mv. An example of the semi log plot of decreasing signal height or triplet concentration with time is shown in Fig. 5. Every decay curve thus plotted appeared to be first order for at least 3/4 of the full life time. The value of $t_{\frac{1}{e}}$ is readily obtained from the plot by reading the time in seconds necessary for a decrease of the signal height to $\frac{1}{e}$ of any chosen value.

Samples contained in quartz tubes, 3 mm average internal diameter, were placed in a liquid nitrogen quartz dewar which was inserted into a Varian 4531 multipurpose cavity from the top. This dewar holds sufficient liquid nitrogen to keep a sample at -196°C for two hours. Although noise and artifacts, from the boiling liquid nitrogen, are greater in this than in the variable temperature dewar, the quartz used in its construction will not suffer uv damage whereas uv irradiation of the quartz variable temperature dewar may result in a contaminating signal at g=2. A Bausch and Lomb High Intensity Monochrometer, fitted with a 200 W, OSRAM UV-visible mercury source, 200 mu—700 mu, was used for irradiation of samples in the cavity. The monochrometer

Figure 5

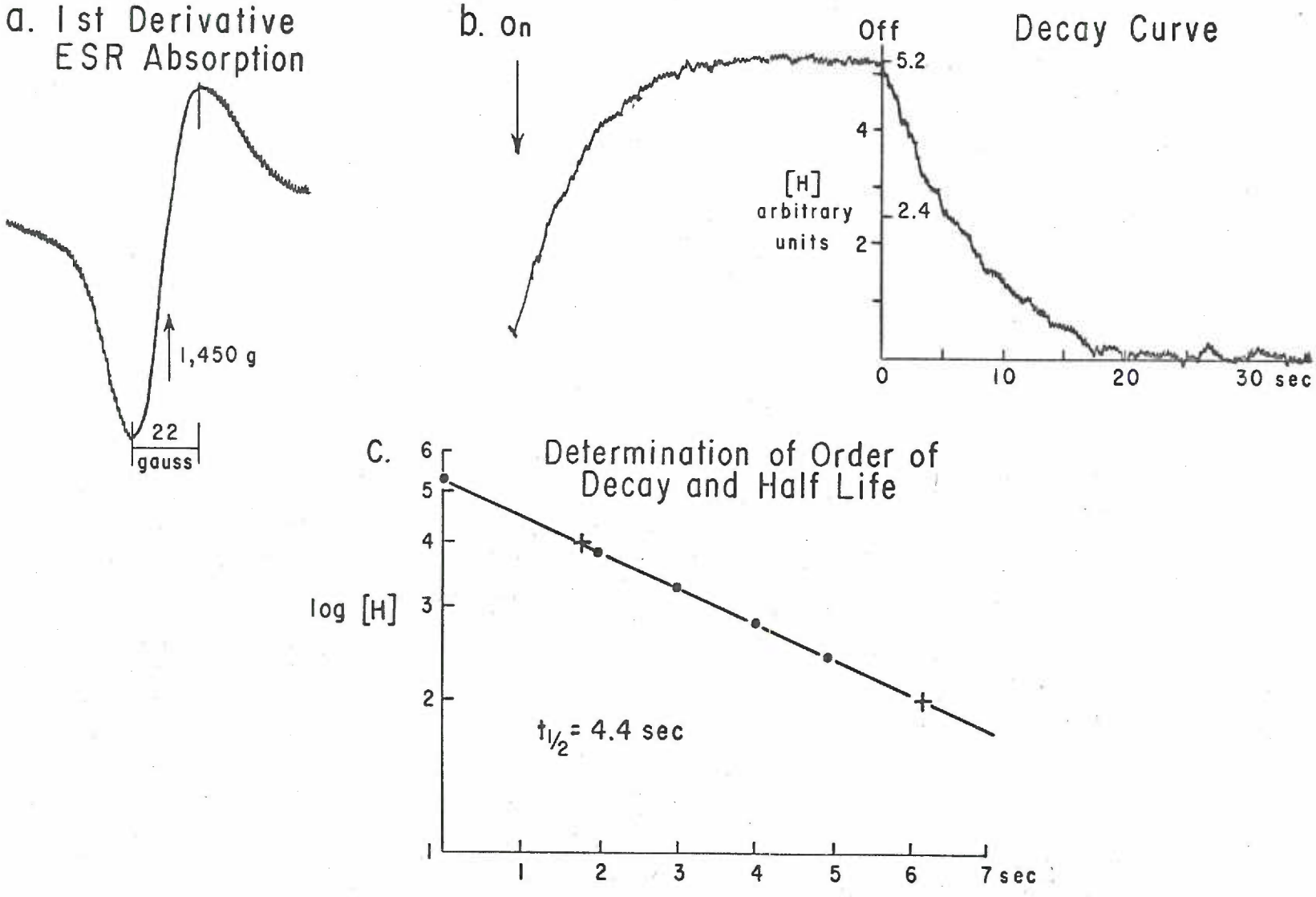
Determination of triplet half life in tryptophan:

a. typical derivative ESR spectrum of tryptophan triplet absorption produced by irradiation of 290 mu

b. typical decay curve, Sanborn recorder, for signal in a. (above)

c. Semi log plot signal height (arbitrary units) vs. time (seconds) of the curve in b. (above)

THE TRYPTOPHAN TRIPLET



entrance to exit slit ratio was set at 3.6:2.0.

Since the dispersion of the uv-visible lamp is 7.4 mu/mm the exit slit should be 1.35 mm in order to pass a band of 10mu. The image of the entrance slit at the exit slit is 56% the size of the entrance slit. It is therefore necessary to multiply the width of the exit slit by 1.78 to obtain the proper width of the entrance slit. Using this ratio, only a single wavelength can pass through the system at its full intensity. Others, at 5 mu on either side of the setting in the case of a 10 mu band width will appear at 50% intensity. An exit slit of 2 mm gives a band width of 14.8 mu or $\pm$7.4 mu range at each setting. This was the narrowest exit slit which gave sufficient power for triplet formation in every experiment. The power in mV for every tenth wavelength, including and between 210 and 340 mu was read from a voltmeter in circuit with an Eppley thermopile 6163. The 3 mm window width of the thermopile, the 6 cm distance from the monochrometer exit lens and the monochrometer slit settings simulated the conditions of sample irradiation in the ESR cavity. The correction factor for this thermopile, 0.0592 mv/mw cm^{-2}, allowed conversion of the mV to mW/cm^2. This value multiplied by the area of radiance on the quartz ESR tube, the flux, gives energy in watts which can be converted to Kilocalories (Kcal). The observed Kcal divided by Kcal/mole photons at that wavelength gives the number of einsteins (E) or moles of photons

incident on the sample at that wavelength. A sample calculation is shown in Appendix II. The curve resulting from such a calculation at every mV reading is shown in Fig. 6. Triplet concentration in a sample, or signal height (SH) after a steady state is reached, divided by the E value of the activating wavelength results in a quantum yield value for the activation spectrum.

Solutions of oxy and deoxy native and apo-hemocyanin were prepared by equilibration of the protein with O_2 or N_2 in a tonometer, Fig. 1, c., at 0°C for no less than an hour. The gases were obtained from Industrial Air. The water-pumped nitrogen, with less than 3 ppm O_2 was further purified by passage through a wash solution of vanadyl sulfonate in the presence of amalgamated zinc (88) and a .2 M phosphate buffer solution pH 7.0 containing nitrophenol indicator. Apo-hemocyanin was prepared by dialysis, at 4°C, of a 20 ml portion of a freshly prepared Spinco concentrate containing about .1mM protein and 2.4 mM copper, against two changes of two liters of .05 M Tris buffer pH 6.8, .01 M in Ca and .01 M in CN^-. $Ca(CN)_2$ decomposes in cold water while the CuCN is insoluble. Three two-liter washings with fresh buffer were considered sufficient to remove the cyanide. Native concentrate against which the apo-hemocyanin would be compared was dialyzed for the same length of time in the absence of cyanide. The resulting apo-protein, .1 mM in protein and .08 mM in Cu, could be

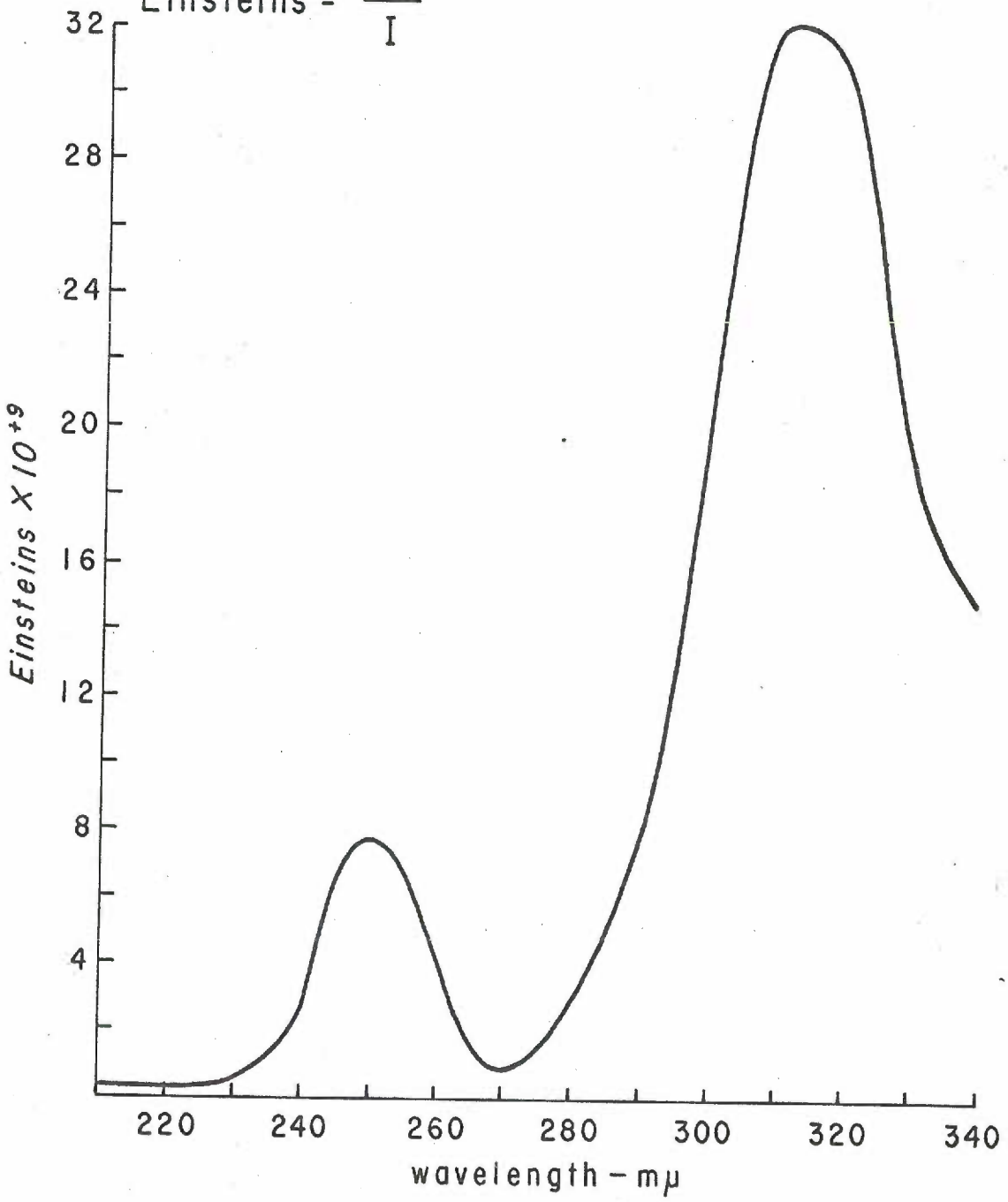
Figure 6

Spectrum of einstein values from the Bausch and Lomb high intensity monochrometer with entrance/exit slit ratio of 3.6/2.0. These einstein values were used in determination of triplet photoactivation spectra of hemocyanin Figures 7 and 8, and tryptophan, Fig. 9, obtained under the same conditions of illumination.

$$\text{I} \quad \frac{\text{Kcal}}{\text{Mole of light at } \lambda} = \frac{281.37 \times 10^3}{\text{Å}}$$

II Actual Kcal from mv = mv x 24.32×10^{-7}

$$\text{Einsteins} = \frac{\text{II}}{\text{I}}$$



restored to 86-90% activity upon dialysis against buffer solution containing cupric sulfate followed by the anaerobic addition of 2 mg/ml dithionite and exposure to air. Activity was measured by optical density at 575 mu on the basis of protein concentration and by loss of epr cupric signal. The activity as measured by optical density was reversible in the absence of oxygen, with some loss in activity, not quantitated, upon 2 to 3 repeated cyclings. The apo - protein could also be reconstituted in the conventional way, (41) by anaerobic combination with Cu(I).

All native and apo-protein solutions were passed through columns of Sephadex G75, which greatly retards the passage of all molecules under 50,000 molecular weight, before use in the ESR studies of the triplet state. This was done to minimize the possibility of a triplet signal arising from or being quenched by contaminating paramagnetic species.

Deionized distilled water with conductivity not less than 0.5 uMho or greater than 0.8 uMho, equivalent to 3-5 ppm NaCl (0.1 mM NaCl) was used throughout. The Sephadex was equilibrated with .05 M Tris buffer containing 0.01 M Ca^{++} at pH 6.8. Columns of 100 ml and 300 ml capacity were used and samples no greater than 10% total capacity were slowly and directly applied. Buffer was eluted at the rate of .5 ml per minute to minimize dilution of the protein on the column. The optical density of the eluate

at 280 mu was automatically read and recorded by a Gilson Medical Electronics spectrometer in conjunction with a Texas Instruments "recti riter" recorder. A minimum of two hours in the 100 ml column and three in the 300 ml column from the time of application to that of collection, allowed a discreet boundary and recovery of 90% of the protein only slightly diluted. The eluted protein was collected in 2 ml batches, every 10 min., by a Buchler automatic refrigerated fraction collector, which kept the column and all samples at 4°C.

Triplet life-times were measured in both aqueous solutions and organic suspensions of HC, which gave clear hydrocarbon glasses upon freezing. Measurement and comparison of signal heights produced by irradiations at different wavelengths are valid only in the transparent rigid solutions. The degree of scattering which would vary uniformly with wavelengths (inversely proportional to λ^4) and not uniformly from sample to sample makes triplet concentration comparisons impossible in cracked and opaque frozen aqueous solutions. The composition and specific features of a wide variety of low temperature glasses have been recently summarised (119). For these experiments, a glass was required which did not denature the protein, remained rigid during irradiation and was transparent throughout the visible and quartz ultra violet region. Alcohols and mixtures of alcohols are best suited

to meet these requirements (91). The results of testing several different solvents for the hemocyanin system are summarized below:

Methanol, an ether, isopentane, alcohol mixture (91) and isopentane each combined 1:1 with the protein-aqueous phase caused denaturation at room temperature and formed cracked and cloudy glasses at low temperature, 77°K. Propylene glycol combined 2:1 with the aqueous phase, formed a clear glass with many cracks. Ethylene glycol, 2:1, formed a clear glass with few cracks, but the triplet half life of the apo and native Hc in the presence and absence of oxygen differed greatly from that observed in aqueous solutions. Only glycerol, 1:1, formed a clear glass and exhibited triplet half lives comparable to those found in the aqueous solutions. Recycling of the hemocyanin O_2-binding ability was checked, even though the glycerol: oxyhemocyanin solution was clear and blue, because a Helix hemocyanin has been reported to dissociate in solutions containing glycerol (124b). If dissociation occured in these solutions, it did not significantly affect the activity.

3. Results and Discussion:

a. Source of the Triplet. All triplet spectra appeared only upon irradiation of the protein with uv light less than 340 mu. They were recorded with the field centered at 1.45 kg and 250 gauss equivalent to a full sweep of the

x axis. Modulation amplitude of 1,000, equivalent to 4 gauss in our instrument, was used throughout. A representative signal is shown in Fig. 5, a. The signal corresponds in both position of center, 1.45 kg, and half line width, 20-22 gauss, to that recently reported by Shiga and Piette for tryptophan (116). A 200 W high pressure PEK-Lab Co. mercury lamp, with cut-off filters but no monochrometer, was used in their study and there was no report of samples in which air had been excluded compared with those in which it had not. The half life of their tryptophan standard in 6N HCl was 4.1 seconds and that of the tryptophan-like triplet signal, from four proteins studied, ranged from 2.6-3.7 seconds. For a discussion of the order of quenching, see the following section, b, and General Discussion II A. A mixture of .003M tryptophan in buffer and an equal volume of glycerol, (0.0015M final), was the standard for the hemocyanin series. The triplet signal was virtually identical to that reported for the 6N HCl solution in every respect including half life. The half life, 4.4 seconds, was unaffected by the presence or absence of oxygen. The half life of the tryptophan-like triplet from native and apo, oxy- and deoxyhemocyanins in buffer glycerol solutions ranged from 1.7-4.8 seconds.

A signal arising from tyrosine, with a center at 1,280 gauss, ½ line width of 32 gauss, could be observed in 290 mu irradiated standard solutions, .0015 M in buffer-glycerol, but not in irradiated protein solutions. This was also observed for bovine serum albumin, ovalbumin and the apo- and holo-enzymes of D amino acid oxidase in the only other such study of triplet states in proteins (116). Reasons, advanced at that time, for the dominance of the tryptophan-like triplet included 1) the high quantum yield of tryptophan standards, also noted in glycerol standards, as compared with tyrosine and phenylalanine, 2) some quenching effect due to hydrogen bond formation by the OH group of tyrosine and 3) an energy transfer process from tyrosine to tryptophan for which some evidence had been obtained. The correspondence of the protein signal with a tryptophan signal, therefore, doesn't necessarily mean that other uv absorbing amino acids have not interacted with the light.

There may be an interesting correlation between the calculated energy gap, ΔE from the highest filled and the lowest unfilled level, for substances absorbing in the short uv, and these triplet phenomena. The theoretical lowest transition energy ΔP which is ΔE in units of β, has been calculated for a number of heterocyclic compounds of biological interest (125). It has been expressed in units of β, the resonance integral of the LCAO method (7).

Absorptions at the longest ultra violet λ max characteristic of a compound, correspond to transitions involving conjugated electrons, π-π^* transitions (97), which may be involved in the triplet activity. In Table II, a selection from the data of Szent-Georgi and Isenberg (125), it can be seen that substances having their longest wave length uv absorption band toward the visible region of the spectrum, riboflavin and DPNH, have a smaller energy gap between the lowest empty energy level and the highest filled one, than those substances absorbing in the uv only. A short energy gap means either that the lowest empty level is low, the substance is a good acceptor, or that the highest filled band is high, and the substance is a good donor. Colored substances therefore tend to be better donors and/or acceptors than uv only absorbing substances. The relative size of the π-π^* energy gap for the uv only absorbing substances, however, may be related to their triplet activity in a similar manner. That is, triplet activity may be viewed as an intra molecular variation of the same phenomenon, which gives rise to colored substances, the transfer of electrons between two molecules. This idea would be consistent with the results observed for triplets in proteins. Tryptophan may be the dominant source of the observed triplet, not only because it has a lower energy gap than the other aromatic amino acids, but

TABLE II

Energy gap between ground and excited states in some biochemicals taken from the data in (125). See text, p. 48.

TABLE II

A Selection of Data

From (125)

	λ_{max} (mu)	ΔP energy gap in units of β	$\lambda \Delta P$
Histidine	205	1.820	371
Phenylalanine	208	1.901	396
Tyrosine	244	1.792	401
Tryptophan	280	1.397	390
Riboflavin	450	0.839	378
DPNH	340	1.213	412

The value of β in these calculations corresponds to 3.26eV or 75 Kcal/mole.

also because others may be less stable, due to structural features, and the energy released during their decay is sufficient to reinforce the tryptophan triplet. The shorter the energy gap in a uv only absorbing substance, the more likely its triplet state will be detectable. The detectability will depend on a concentration effect when it is alone, and both a concentration and reinforcement effect when mixed with other similar substances.

b. Quenching, a Discussion. The mechanism of triplet quenching in general and by paramagnetic species in particular, has been the subject of some systematic studies in recent literature (52, 77, 94). Quenching of a triplet may be very complete and/or efficient, preventing its observation. The quenching may be incomplete and/or less efficient, allowing detection of signals with smaller triplet concentrations and in the case of second order mechanisms, (see below) shortened half lives. Porter and Wright (94) were the first to point out the possible relevance of the relatively forbidden triplet transition to energy transfer in biological systems. The following discussion is designed to provide a background of terminology and considerations sufficient to understand the subsequent description of the triplet state in hemocyanin.

Mechanisms of first order decay proceed by an intramolecular alteration, with a possible dependence on energy transfer to the solvent. Such interaction with the solvent

is unlikely in cases of aromatic hydrocarbon triplets, since, although these rates decrease with increasing solvent viscosity (napthalene: 1.2×10^4 sec^{-1} in hexane and 1.0 sec^{-1} in rigid glass), they reach maximum values in the gas phase. A significant structural difference between the nuclear configuration of a ground state and the excited states of a molecule such as benzene, has been proposed to account for the full range of observed solvent viscosity dependence of first order decay rates (94). The "Dewar" structure of benzene are:

Evidence for such altered nuclear configurations in excited states has been reported (26). Energy transfers between triplet and solvent molecules undoubtedly occur, but can be considered of secondary importance. Resistance to a changing of angles between atoms in a triplet molecule, offered by the viscosity and/or polarity of the solvent, is of primary importance in first order decay rates. A first order quenching effect, or retardation of both the formation and decay of triplets with solvents of increasing viscosity, is characterized by a lowered steady state triplet concentration and an <u>extended</u> life time.

The second order decay of triplets involves intermolecular quenching between identical triplets or by some paramagnetic species and will show a quencher concentration dependence. Unlike that in first order processes, quenching efficiency here refers to a decrease in half life, even to the point of rendering an activation known to occur, undetectable (76). In a second order process, then, quenching results in a shortened life time and either no change or a decrease in steady state triplet concentration.

A number of mechanisms have been proposed for specific cases of second order triplet quenching, but they are of two basic types: 1. chemical reactions, or those involving electron or H atom transfer and chemical alteration of the quencher, and 2. non chemical reactions, in which the quenching molecule truly acts as a catalyst for the radiationless transition from T_1 to S_o (52). Quenching by paramagnetic gases (79) and ions (76) is believed to occur via a reversible complex formation between triplet and quencher. The evidence for the existence of such a complex is indirect. Quenching efficiencies of different metal and metal chelate quenchers were better understood in terms of their properties, when the quenching mechanism was proposed to depend on the formation of such a complex. Examples of such properties are, the suitability for charge transfer interactions, the degree of hydration and the degree of unsaturation (76).

In these and other studies of triplet activity of aromatic hydocarbons, at room temperature, optical density changes were directly related to concentrations of reacting (76) or non reacting (52) species. This allowed actual determination of rate constants according to specific reaction schemes. There is no standard triplet concentration for ESR measurements, however, and actual decay rates, based on first or second order processes, comparable to those found in the literature could not be calculated.

Triplet decay was sufficiently retarded in these frozen solutions so that decay curves such as seen in Fig. 5, b., were commonly obtained at a 2 mm/sec chart speed. The use of a Computer of Average Transients in conjunction with the Sanborn recorder (116) may have improved the reproducibility of the absolute value of a life time, since the exact point of zero triplet concentration was often difficult to read. A qualitative understanding of the differences between amino acid solutions in the presence and absence of copper and oxygen and of the differences between apo and native hemocyanin solutions in the presence and absence of oxygen was possible from comparison of half lives and triplet photoactivation curves.

c. Quenching, the Effect of Copper. In a preliminary experiment, six alpha amino acids, .0015 M in 1:1 buffer glycerol solutions, were examined for evidence of triplet

state formation upon uv irradiation. The monochrometer was set at 290 mu with both slits wide open, 6/6, allowing a band of 44 mu to pass. The ESR incident power, cavity and dewar were the same as those used in the protein triplet detections. The amino acids, arginine HCl, histidine HCl cysteine HCl, creatine, tyrosine, tryptophan and phenylalanine were obtained from Cal. Biochem. Co. and the pH s of .003M solutions of these in .05M Tris buffer pH 6.8, .01M in Ca , were adjusted to near neutrality, pH 6-7.5, by the addition of acid or base. A parallel series, containing equimolar amounts of amino acid and Cu(II) ions (.003 M amino acid and .003M $CuSO_4 \cdot 5H_2O$) was prepared and handled in the same manner. Triplet signals comparable to those reported (116) were observed in the tryptophan and tyrosine solutions, but none could be detected in the solutions of phenylalanine or any other tested amino acid. In 6N HCl, phenylalanine gives rise to a signal at 1300 gauss with half line width of 63 gauss (116).

A strong phosphorescence was exhibited by the frozen phenylalanine and creatine solutions, a weak phosphorescence by histidine and none by arginine and cysteine. Since this was a preliminary experiment to determine possible sources of triplet signals pertinent to the protein study, no further attempt was made to study those solutions which phosphoresced but had undetectable signals. This could

easily be done by varying the magnetic field center by increments of 250 gauss between 800 and 2000 gauss with modulation from 3.8-8.0 gauss. If this were systematically repeated during irradiation by different uv wavelengths between 220 and 330 mu, it is likely that the right combination would allow detection of a triplet signal.

All signals disappeared in the presence of equivalent concentrations of Cu(II). Since only frozen solutions were being observed, random encounter of molecules was not possible. The interaction between Cu(II) and an amino acid, at room temperature, which led to the total quenching at low temperature, could have been either first or second order. Comparisons were possible between frozen solutions of comparable protein samples, however, since the quenching was not complete. The amino acid-Cu(II) reactions were not studied further, by varying the cupric/amino acid ratio, for example.

The half lives of the oxy- and deoxyhemocyanin triplets in glycerol were relatively unaffected by the presence of copper, Table III. The NO complex was an exception. It should be noticed that triplet photoactivation spectra were recorded on three different occasions and only on the glycerol mixtures marked iii, iv and v in Table III. This notation is carried over to Figs. 7 and 8 and corresponds to the chronological order of the original data.

TABLE III

Triplet quenching: the effect of oxygen and copper on the half lives of hemocyanin in various solutions. All values were obtained from the triplet ESR spectrum produced by irradiation at 290 mu. Each column, i, ii, iii, iv and v contains data obtained in the same experiment. Triplet photo activation spectra were obtained for the glycerol mixtures OG, NG, AOG and ANG in three separate experiments (iii, iv and v).

TABLE III

(All numbers represent $t_{\frac{1}{2}}$ in seconds)

	i	ii	iii	iv	v
OW		2.6	1.7		
NW		4.7	4.8		
AOW		4.0	3.7		
ANW		4.3	4.8		
OG	1.5	1.4	3.4	3.2	1.8
NG	2.5	4.2	4.4	3.9	4.7
AOG		4.2	3.7	4.8	2.2
ANG		5.0	3.8	4.0	5.2
OE	2.5	4.0			
NE	12.2	3.6			
AOE		4.1			
ANE		4.2			
NOG					2.1
ANOG					4.3

Key

O: oxyhemocyanin
N: deoxyhemocyanin
A: apohemocyanin
W: aqueous buffer
G: dilution 1:1 with glycerol
E: dilution 1:2 with ethylene glycol
NO: HC-NO complex

All triplet spectra of the apo-protein contained more area than those of the native protein, Fig. 7, indicating that, in the presence of copper, the total triplet concentration which could be produced was decreased. When the total area under a photoactivation spectrum is decreased it will be referred to as a "concentration quenching" effect. Concentration quenching was not always accompanied by a predictable change in half life.

The differences in the shapes of the spectra of native and apo-protein, Fig. 7, were much greater than those differences existing between oxy and deoxy samples, Fig. 8. The correspondence between triplet concentration and signal height at 275 and 290 mu was therefore reliable in considering the quenching effect of oxygen, Table IV, while it is not in the case of copper, Table V. While there is a triplet concentration quenching in the presence of copper, Fig. 7, the overall effect was not as drastic as that seen in the Cu(II)-amino acid experiments. The concentration quenching may result from some paramagnetic features of copper at the active site in oxyhemocyanin, since it was greater in the oxygenated samples than in the deoxygenated ones, Fig. 7. The absence of an effect on half lives in either oxy or deoxy-samples corresponds to the absence of some paramagnetic quenching features at the active site, Table V.

Figure 7

Triplet photoactivation spectra of hemocyanin:

Quenching effect of copper.

A. in the presence of oxygen

Oxygenated apo-hemocyanin - - - -
Oxygenated native hemocyanin ——————

B. in the absence of oxygen

Deoxygenated apo-hemocyanin - - - -
Deoxygenated native hemocyanin ——————

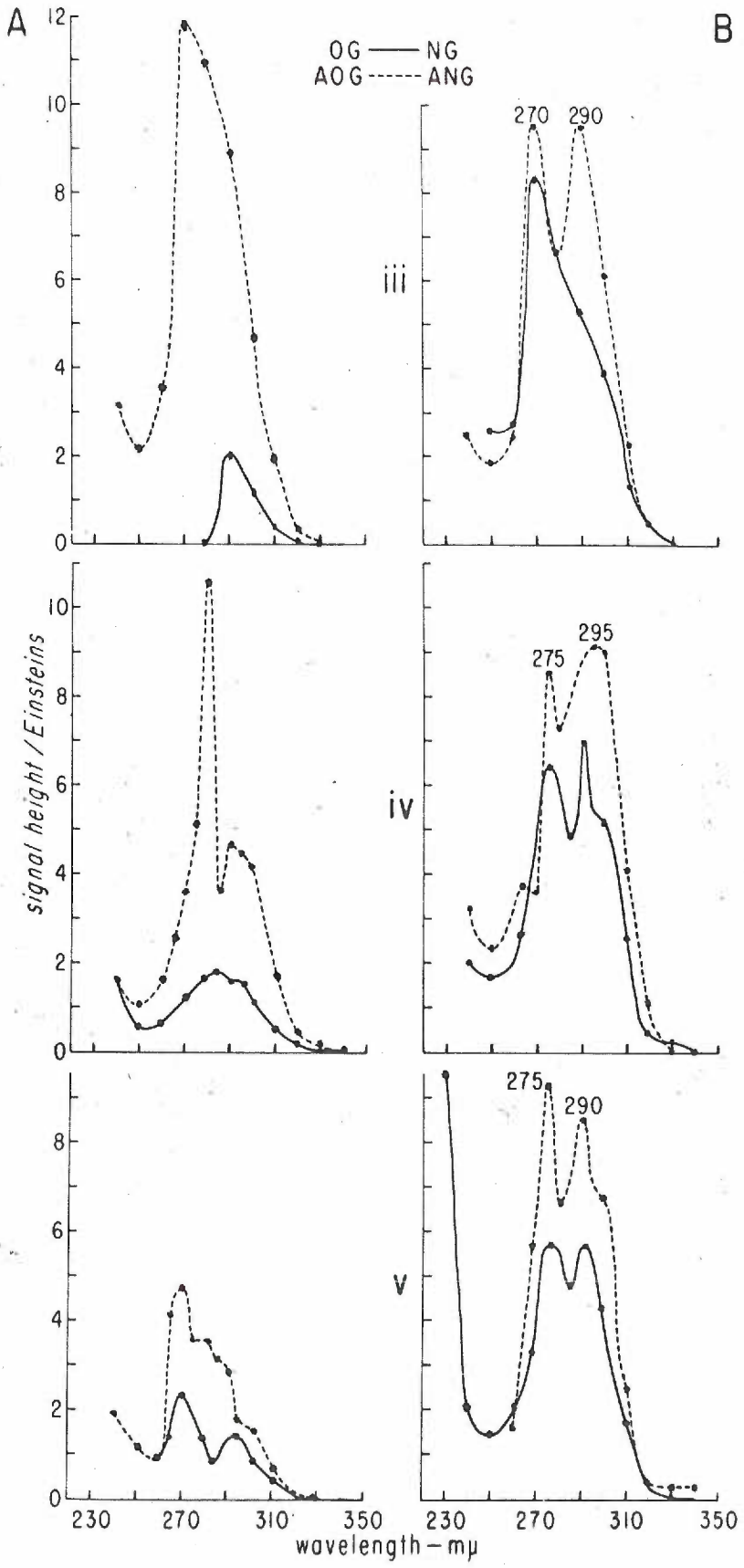

A
B
OG —— NG
AOG ---- ANG
iii
iv
v
270
290
275
295
275
290
signal height / Einsteins
wavelength – mμ
0
2
4
6
8
10
12
230
270
310
350

Figure 8

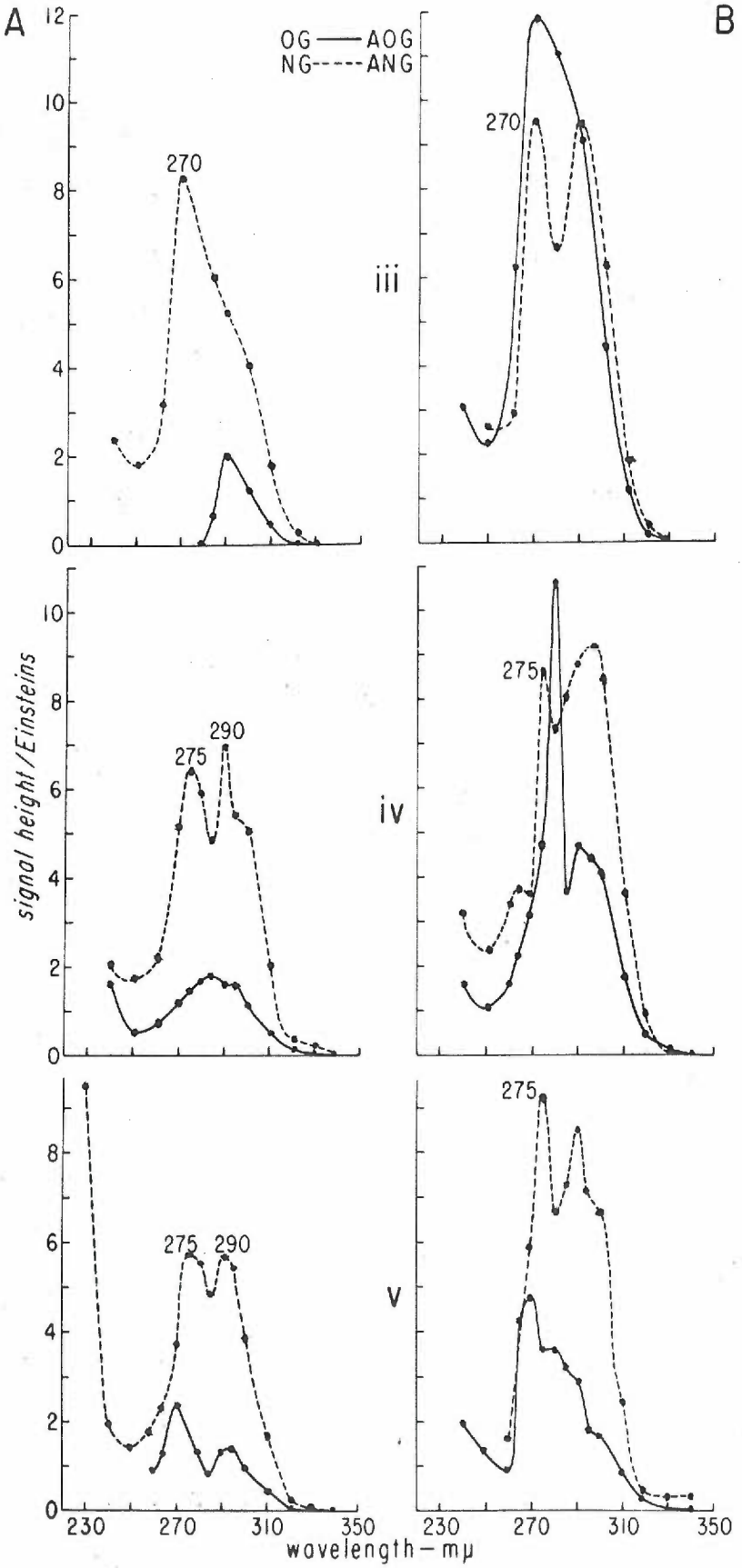
Triplet photoactivation spectra of hemocyanin:
Quenching effect of oxygen.

A. in the presence of copper

Deoxy native hemocyanin - - - -
Oxy native hemocyanin ————

B in the absence of copper

Deoxygenated apo-hemocyanin - - - -
Oxygenated apo- hemocyanin ————

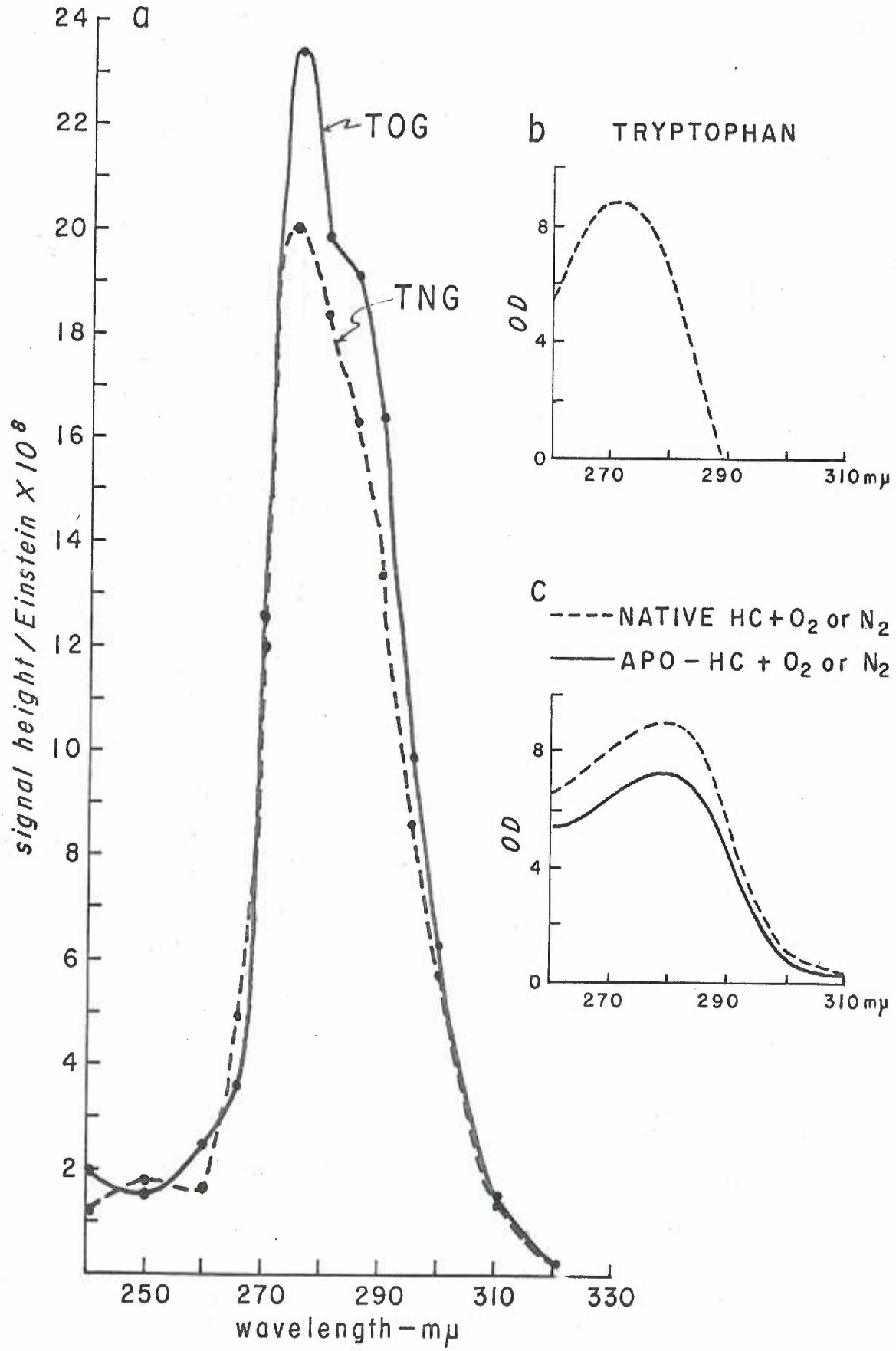

A
B
OG —— AOG
NG ----- ANG
270
270
iii
275
290
iv
275
275
290
v
signal height / Einsteins
wavelength – mμ
0
2
4
6
8
10
12
230
270
310
350

d. Quenching, the Effect of Oxygen. No first or second order quenching process appears to occur between tryptophan and dissolved oxygen at low temperature, since the orders of the decay and half lives were exactly the same in the presence and absence of oxygen. In fact, from the photoactivation curve, Fig. 9, a., triplet formation at 290 mu is made more probable in the presence of oxygen. The presence of oxygen increases triplet concentration produced by irradiation at 290 mu, indicating some sort of interaction between dissolved oxygen and the free amino acid.

Comparison of signal heights over a range of activating wavelengths was possible only in the glycerol mixtures which formed the least altered frozen solutions of minimum and comparable scattering properties. The uv absorption spectrum of a .003 M solution of tryptophan in water was recorded, Fig. 9, b., for comparison of max with that of maximum yield of triplet formation seen in the photoactivation curve. Neither tris buffer nor glycerol could be used to simulate the ESR sample solution because use of either one as a reference resulted in too great a loss of uv light to allow measurement of a spectrum. The correspondence between the triplet peak at 275 mu with a shoulder at 290 and the room temperature absorption at 275 with a bulge at about 300 mu is very close, Fig. 9, a. & b.

Figure 9

The triplet photoactivation spectrum of tryptophan (a), compared with the tryptophan and hemocyanin uv absorption spectra, (b) and (c) respectively.


a
TOG
TNG
signal height / Einstein X 10⁸
wavelength – mμ
b
TRYPTOPHAN
OD
270
290
310 mμ
c
----NATIVE HC + O2 or N2
——APO – HC + O2 or N2
OD
270
290
310 mμ

This indicates that there is no transfer of absorbed uv light to another, possibly longer wavelength, at which triplet activation actually occurs in the free amino acid.

This was not strictly true in solutions of both native and apo proteins in the presence or absence of oxygen, as seen in Fig. 8. While there is a peak in every case corresponding to the uv maximum, 275-280 mu, of native and apo HC in the presence of N_2 or O_2, there is in every case a second peak and/or shoulder in the region of 290-300 mu. This may arise either directly from light absorbed at these wavelengths, or are energy transfer from the stronger, shorter wavelength λ max or a combination of these two, the latter reinforcing the former.

The most peculiar feature of the activation curves of the tryptophan-like signal from proteins, Fig. 8, in comparison with those curves of the free amino acid, is that even the curves of the deoxygenated proteins exhibit the shoulder and/or peak at 290 mu while it is absent in the deoxy free amino acid, Fig. 9, a. Remembering that the protein curves were obtained from signals with field position, half life and signal heights corresponding to the tryptophan triplet, it becomes unlikely that we are observing an energy transfer process to another site giving rise to the 290 mu peak. A second, different, triplet producing site would be expected to have signal characteristics different from those of tryptophan. Such processes may be

occuring but could not account for the observation. A more likely explanation is that tryptophan residues, even in the deoxygenated proteins, have a permanent environment which simulates that of the free amino acid in the presence of oxygen. The nature of the interaction of dissolved oxygen with the free amino acid which facilitates the second triplet transition of slightly lower activation energy, 290 mu, within the same molecule is difficult to guess. Alterations in the symmetry of a molecule, caused by vibrations due to temperature or oscillations arising from charge transfer interactions, have been used in explanations of increased favorability of energy transfers or electronic transitions (115) and may be appropriate here. To my knowledge, the triplet photoactivation spectra of tryptophan, Fig. 9a, and hemocyanin, Figs. 7 and 8, represent the first attempt to obtain such data for any material.

Data from Fig. 8 and Table III were collected for Table IV in order to examine the effect of oxygen on triplet production in both native and apo-hemocyanin. It can be seen in Fig. 8 and Table IV that activation over the whole range of wavelengths produces considerably less triplet concentration in all native samples containing oxygen than in those which do not. It can also be seen that whatever triplet is produced in these samples decays faster in the presence of oxygen. This may, in part, account

TABLE IV

Quenching: the effect of oxygen on the hemocyanin triplet maxima and half lives,

A. in the presence of copper

B. in the absence of copper

TABLE IV

		275 mu ($SH/E \times 10^8$)	290 mu ($SH/E \times 10^8$)	$t_{\frac{1}{2}}$ (seconds)
A.				
iii				
	NG	8.30	5.25	4.4
	OG	0.00	2.00	3.4
iv	NG	6.40	7.00	3.9
	OG	1.40	1.60	3.2
v				
	NG	5.70	5.65	4.7
	OG	1.40	1.60	1.8
B.				
iii				
	ANG	9.50	9.50	3.8
	AOG	11.90	9.50	3.7
iv				
	ANG	8.60	9.75	4.0
	AOG	5.00	4.60	4.8
v				
	ANG	9.25	8.50	5.2
	AOG	1.40	1.65	2.2

TABLE V

Quenching: the effect of copper on the hemocyanin triplet maxima and half lives,

A. in the presence of oxygen

B. in the absence of oxygen

TABLE V

		275 mu (SH/E x 10^8)	290 mu (SH/E x 10^8)	$t_{\frac{1}{2}}$ (seconds)
A.				
iii				
	OG	0.00	2.00	3.4
	AOG	11.90	9.50	3.7
iv				
	OG	1.40	1.60	3.2
	AOG	5.00	4.60	4.8
v	OG	1.40	1.60	1.8
	AOG	1.40	1.65	2.2
B.				
iii				
	NG	8.30	5.25	4.4
	ANG	9.50	9.50	3.8
iv	NG	6.40	7.00	3.9
	ANG	8.60	9.75	4.0
v	NG	5.70	5.65	4.7
	ANG	9.25	8.50	5.2
C.	NOG	0.00	0.90	2.1
	ANOG	6.50	6.25	4.3

for the lowered average triplet concentration.

The presence of oxygen does not have such a consistent effect in the apo-protein samples, Table IV B. A higher steady state triplet concentration in the deoxy samples is accompanied by both an increase (v) and a decrease (iv) of the corresponding half life. In one case, (iii) triplet production and half lives are almost identical in the presence and absence of oxygen. All apo samples used in these experiments were from a stock solution of G75 treated protein, prepared three weeks earlier.

If the triplet activation curves of the apo-protein are compared with one another, Fig. 8, series B, there appears to be a decrease in total possible triplet production by the oxygenated apo-protein with the age of the sample. The time which elapsed (two days) between the recording of each set, however, compared with the age of the stock solution (three weeks), makes it unlikely that an aging process affecting the triplet activity was going on at a rate indicated by the spectra. The more likely possibility is that this apparent progression is accidental and the differences in the spectra arise from different orientation and aggregation of subunits at the time of freezing. This would be consistent with the only known difference between native and apo-protein, the greater ease of subunit formation in the apo-protein (75).

One effect of oxygen which is noticeable only by comparison of signal heights over the full range, Fig. 8, is

that the presence of oxygen in both native and apo-proteins appears to facilitate triplet producing transitions between 270 and 290 mu, while there is a sharp drop in quantum efficiency between these wavelengths in all deoxy samples. Particularly clear examples of this are iii B and iv A and B. Any decrease at wavelengths between 270 and 310 mu is very reliable since the einstein values of the monochrometer, Fig. 6, increase steadily in this range.

In all such comparisons, it is important to keep the energy profile of the monochrometer in mind. Errors in comparisons of relative quantum efficiency, SH/E, will be greatest at the wavelengths having lowest einstein values. In these experiments small differences in signal height at activating wavelengths less than 240 mu and around 270 mu are easily made significant upon division by the small einstein value. It should be noticed that this consideration which may make reproducible comparison between activity at 270 and 290 mu difficult, improves the reliability of observations at activating wavelengths of 290 mu and higher since these are characterized by higher einstein values, or more effective moles of light, at a particular wavelength. In other words, differences in signal heights at these wavelengths had to be substantial and easily reproducible in order to be divided by a large number and still appear as a shoulder or peak in the triplet activation curves.

A few tentative conclusions can be made in summary. The triplet activity of hemocyanin appears to be modified to always allow activation of the protein tryptophan-like triplet at 290 mu. This is only seen in the photoactivation spectrum of tryptophan in the presence of oxygen. There are two sources of concentration quenching. The concentration quenching due to both oxygen and copper can be seen in Fig. 8, A.; that due to copper alone can be seen in Fig. 7, A. The presence of copper did not affect the half life in any regular way. The presence of oxygen consistently decreased the half life only in the native protein. The variation in spectral shapes and the effect of oxygen on the half life of triplets produced in the apoprotein may be due to some aspect of subunit formation which was not under control.

II Attempts to Produce Methemocyanin

In 1933 (23) a colorless product, which reversibly turned green-blue when exposed to air, was reported to be a methemocyanin. It was obtained by oxidation of deoxy-horseshoe crab hemocyanin by $KMnO_4$ or K_3MoCN at neutral pH. By following the potassium permanganate or potassium molybdicyanide oxidation of hemocyanin electrometrically, it was found that one equivalent of oxidizing agent was reduced for each atom of copper (23). This, and the altered oxidizing power of the methemocyanin itself, equivalent to a change of one electron per copper, prompted the labelling of the new compound "methemocyanin", to correspond with methemoglobin. In 1941 Rawlinson (100) made magnetic susceptibility measurements on this product and found no change in the valence state of hemocyanin copper in the presence of these oxidizing agents. This observation and the fact that the "methemocyanin" had the same oxygen carrying properties as native hemocyanin, led to a consensus that groups unrelated to the physiological activity had been oxidized and the product was not a true methemocyanin. It has been suggested (36) that the failure of sufficiently strong oxidizing agents to convert active site cuprous to cupric may be due to 1) an unusual stability furnished by the protein; the Cu(I)-protein equil. constant for association equals 10^{18} (35), and 2) inaccessibility of the active sites.

The effect of hydrogen peroxide on Limulus and Busycon hemocyanins was studied in 1959 (36), and the formation of a true methemocyanin of Busycon was reported at that time. This material 1) had two cuprous, Cu(I) oxidized for every active site destroyed, 2) did not lose Cu(II) on an IR-120 cationic exchange resin and 3) could be regenerated to active hemocyanin upon reduction by potassium ferrocyanide or, notably, excess peroxide. This study gave further evidence of copper ions being located in pairs, or multiples of two. At the point at which one half of the Cu(I) ions were oxidized, a little more than one half of the O_2-bonding activity was still evident at 340 mu. The reaction was followed by measurement of active site concentration from OD at 340 mu and cuprous ion concentration by the biquinoline reaction. The usual biquinoline method used in measurement of cuprous copper in hemocyanin (69) and tyrosinase (63, 71) was modified by addition of EDTA to protect cupric ions from possible reduction by sulfhydryl groups when the protein is denatured by the glacial acetic acid (36). The high incidence of a "double hit" even at low peroxide concentrations was an indication of the proximity of coppers. One peroxide molecule always oxidized both Cu(I)s of an active site since 0.5 equiv. H_2O_2 per mole copper oxidized about $\frac{1}{2}$ total Cu(I), or two oxidizing equivalents always oxidized 2 Cu(I).

The recent applications of ESR technique to studies of the valence of copper in hemocyanin (87) and other copper

proteins (81, 82), make the formation of a true methemocyanin of very great interest. Blue oxyhemocyanin does not give rise to a significant cupric signal at room temperature (87) or liquid N_2 temperature (118), even though only one half of the total functional copper in oxyhemocyanin can be chemically determined to be Cu(I), (36, 69). A true methemocyanin, with oxidized copper atoms at the active site and reducible to native hemocyanin, would permit observation of functional copper atoms by ESR. Some information about the identity of neighboring atoms might also be obtained if the resulting cupric signal showed evidence of hyperfine splitting. Interaction of an unpaired electron with the magnetic moments of the nuclei of nearby atoms results in hyperfine splitting in both organic (135) and inorganic (93) free radicals.

The experiments to be described in the following section were undertaken in an attempt to characterize the functional copper in its cupric state by ESR. In addition to observation of the effect of H_2O_2 on Cancer magister and Busycon hemocyanins and observation of the cupric ESR signal of the Cu(II)-apo hemocyanin complex, attempts were made to photooxidize the functional copper by high intensity monochromatic irradiation and to introduce an unpaired spin at the active site by the substitution of NO for O_2.

A. Experiments on the reaction of *Cancer magister* and *Busycon caniculatum* deoxyhemocyanins with H_2O_2.

1. Aim:

These experiments were of two types. In one, deoxyhemocyanin was combined with excess hydrogen peroxide without continued nitrogen bubbling. In a previous study (36) this was observed to prevent the formation of a methemocyanin from *Busycon*. In these experiments we were interested in detecting a HC-H_2O_2 complex which may form during this catalase-like activity of hemocyanin. In the second type of experiment, deoxy hemocyanin was combined with excess H_2O_2, under conditions described as leading to the formation of methemocyanin. Reproduction of the chemical and optical evidence for a methemocyanin and observation of this material by ESR were of primary interest in these experiments.

2. Materials and Methods:

a. Catalase-like Activity of *Cancer magister* Hemocyanin. The *Cancer magister* hemocyanin was collected, concentrated and stored as described in Experimental section I, A, 2. A concentration range around 2.5 mM copper was used for these experiments, since it was suitable to observations both by ESR and optical density at 575 mu. Deoxygenated hemocyanin samples were prepared in a tonometer, Fig. 1, c., transferred to quartz Thunberg cuvettes, 3 ml total volume, flushed with N_2 and combined with a known amount of H_2O_2 in the side arm. Adjustments of pH were made by dialysis against 0.05 M

phosphate buffer, pH 4.5-7.0 or 0.05 M Tris buffer, pH 8.0-9.0. No adjustments to 1.01 M Ca were made in these experiments.

Hydrogen peroxide solutions were standardized in two ways, either by titration with a standardized solution of potassium permanganate or with a standardized solution of thiosulfate in the presence of potassium iodide.(126). The iodometric method has an advantage over the permanganate because the titration can take place in the presence of glycerol or salicylic acid, sometimes used as preservatives in commercial H_2O_2 preparations (126). Baker and Adamson reagent grade, 30% hydrogen peroxide was used. Fresh 3% stock solutions, having a measured concentration of 1.012 ± 0.01 M were periodically made from this concentrated reagent.

Several observations, low temperature ESR and optical spectra, were made on deoxyhemocyanin within 300 m seconds of its being mixed with excess amounts of H_2O_2. The rapid flow apparatus was the same as used in studies of intermediates in oxidative phosphorylation (12), a modification of the aparatus of Bray (14). In these experiments, two five ml syrihges, one containing deoxy HC, the other a H_2O_2 solution, were positioned in front of a mechanically-driven stage. The two solutions were mixed in a lucite chamber and ejected into a low temperature ESR tube of special design containing isopentane at liquid N_2 temperature. The duration of contact between the two solutions was regulated by both the

total volume delivered and the speed at which the motor-driven stage depressed the barrels simultaneously. For these experiments a 0.4 ml total volume was necessary for observations of both low temperature optical and paramagnetic spectra. Using 30 cm of #10 teflon tubing (35 ul total volume) and setting the motor from 10-40% of its maximum torque, contact times from 210-21 msec, respectively, were obtained. The steadiness and duration of the pressure on the syringe barrels was automatically recorded on the screen of a Tektronix type 535A oscilloscope and photographed by a Polaroid Land camera mounted on the screen. The ESR unit was the same as described in Experimental section I, B, 2., without the Field dial and microwave circuit modifications. The first derivative of absorption was measured by a Varian strip chart recorder. Samples were placed in quartz tubes, approximately 3 mm internal diameter, in a variable temperature dewar for low temperature studies and in a quartz cuvette, 0.9 x 0.5-0.01 inch for room temperature studies. A 2 mM Cu-EDTA standard was used at both room and low temperatures.

Optical spectra at low temperature were made with a modification of the spectrometer system used by Bonner and Estabrook (13), employing a Cary Model 14 scattered transmission accessory and a Cary 1462 photomultiplier tube. Samples in ESR tubes were mounted in a holder of special design, Fig. 10, a., and inserted into liquid N_2 in a dewar

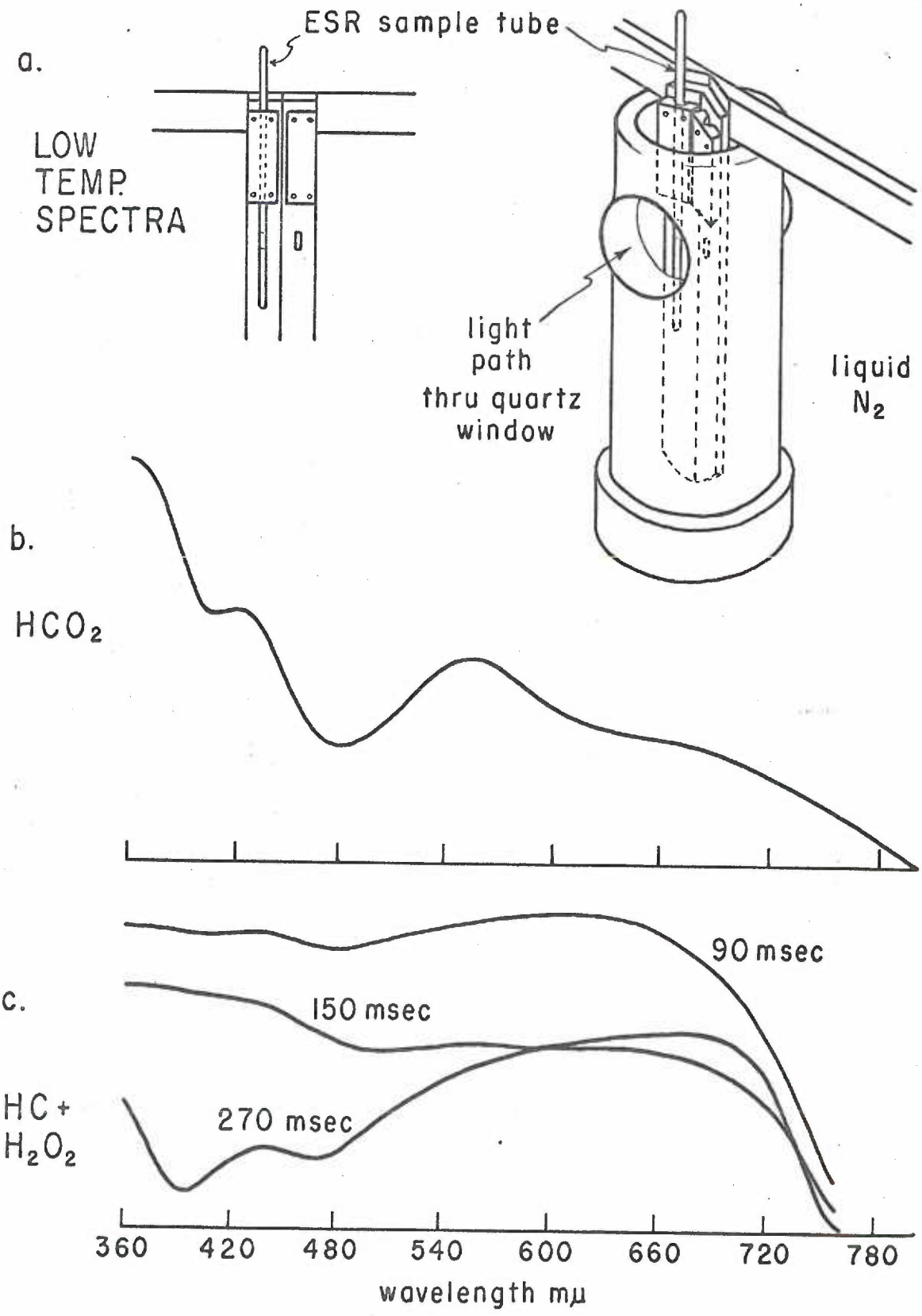
Figure 10

Low temperature optical spectra ($77^{\circ}K$)

a. Sample holder and dewar for liquid nitrogen, modifications for the Cmry model 14 scattered transmission attachment.

b. Low temperature spectrum of oxyhemocyanin.

c. Low temperature spectrum of deoxyhemocyanin in the presence of excess hydrogen peroxide for 90, 150 or 270 msec, at room temperature.



3 mM HC + 168 mM H_2O_2 or ~ 100 equivalents H_2O_2 per active site

with quartz windows.

b. Methemocyanin. In these experiments, both Cancer magister and Busycon hemocyanins, either .8 mM or 1.6 mM total copper, were combined with varying amounts of H_2O_2. The Busycon hemocyanin was prepared by centrifuging the clear purple-blue serum in the Spinco Model L, # 40 rotor at 40,000 rpm for 5 hours. The clear pellet was taken up in a minimal amount of 0.05 M Tris buffer, pH 6.8 containing 0.01 M Ca. About 13 ml of serum, 0.6 mM copper, per whelk was collected by cutting away the horn foot and draining.

The deoxyhemocyanins were prepared in the tonometer vessel, Fig 1, C and 2.5 ml was transferred, using N_2-flushed syringes to test tubes containing 0.5 ml aliquots of H_2O_2 solutions under N_2. Nitrogen bubbling was continued for at least five minutes and the mixture was allowed to stand at 0° C under N_2 for 30 min in every case. The continued flushing with N_2, after H_2O_2 addition, was necessary to obtain chemical and optical evidence for the oxidation of the copper. The "methemocyanin" of both Cancer magister (118) and Busycon (36) is reduced by excess H_2O_2 unless any O_2 generated is driven off. This is not the case for Limulus (36), which may indicate that its catalase-like activity is less. It was also observed (36) that the methemocyanin formed from the reaction of peroxide with deoxy Limulus serum was not reversible upon reduction with either

ferrocyanide or excess peroxide.

Six mixtures of H_2O_2 and HC, ranging from 0.2 to 2.0 equivalents of H_2O_2 per atom of copper, plus a water control, constituted an experiment. For example, 2.5 ml of a 1.92 mM Cu HC combined with 0.5 ml 9.6 mM H_2O_2 gave a final solution of 3 ml containing 1.6 mM total Cu and 1.6 mM H_2O_2 or 2 equivalents H_2O_2 per Cu.

Conventional biquinoline determinations of total Cu and EDTA modified biquinoline detns. of Cu(I) were made on each sample. The EDTA modified determinations were found to be very sensitive to the concentration of EDTA. The reported procedure (36) involved the addition of 0.2 ml of 0.2 M EDTA to an aliquot of working solution, bringing the total volume to 2 ml with water and adding 2 ml of the biquinoline acetic acid reagent. In our experience this resulted in a rapid and easily observable loss of the purple color, or oxidation of the Cu(I) to Cu(II). By using 0.1 ml of a 0.01 M EDTA solution with 0.5 ml concentrated HC, or an EDTA excess of only ten times that of total copper, stable chemical determination of 50% total copper as Cu(I) could be obtained in the native oxygenated material. Optical density was recorded at 340 mu. Dilutions of each working solution were made by adding 0.1 ml to 4 ml oxygenated 0.2 M PO_4 buffer pH 6.8.

3. Results and Discussion

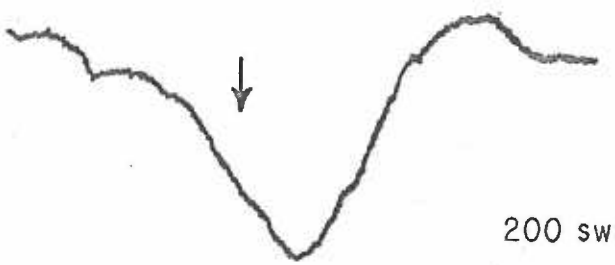
a. Catalase-like activity of Cancer magister. The decomposition of H_2O_2 by hemocyanin, at about the rate of

1 uM H_2O_2/ uM Cu/ min, has been reported for several species (36, 41). When deoxy hemocyanin 2.7 mM copper was combined with 6 mM H_2O_2 at pH 4.5, 6.5 and 8.5, no cupric ESR isgnal could be detected at room temperature, Fig. 11. These spectra were recorded at the same settings as the 2 mM copper standard and an oxidation of as low as 15% of the total copper (.4 mM) would have been detected. A cupric ESR signal from Cancer magister deoxy-HC solution 2.5 mM in Cu and 4.0 or 10.0 mM in H_2O_2, has been observed at room temperature (87). Neither the technique of combining the deoxy protein with peroxide nor what fraction of the total copper was represented in the Cu(II) ESR signal were reported. In the experiments reported here, no cupric signal representing less than 15% total copper would have been detected. The methemocyanin Cu(II) signal (87) was also reported to develop slowly, while no more than 10 minutes elasped between mixing and recording in these experiments. When H_2O_2 was added to deoxy-HC in the same combining ratio 4-5 equivalents per mole copper, in a nitrogen filled thunberg cuvette, 86-90% of the OD 575 mu was recovered within 4 minutes. The low temperature optical spectrum for oxy HC, pH 6.5 is shown in Fig. 10, b. The deoxy protein gave no apparent absorption in this region except that due to scattering. When H_2O_2 was combined with deoxy hemocyanin in the ratio of 6:1, 8:1 and 10:1 equivalents per mole copper, various low temperature spectra

Figure 11

Room temperature ESR spectra of deoxy-hemocyanin and excess hydrogen peroxide at three different pHs.

ROOM TEMPERATURE ESR SPECTRA DEOXY HC-H_2O_2

		[Cu] mM	[H_2O_2] mM	sweep units	gain units
pH 4.5		2.7	6	200	500
pH 6.5		2.7	6	200	500
pH 8.5		2.7	6	200	500
Cu(EDTA)$_2$		2.0		200	500

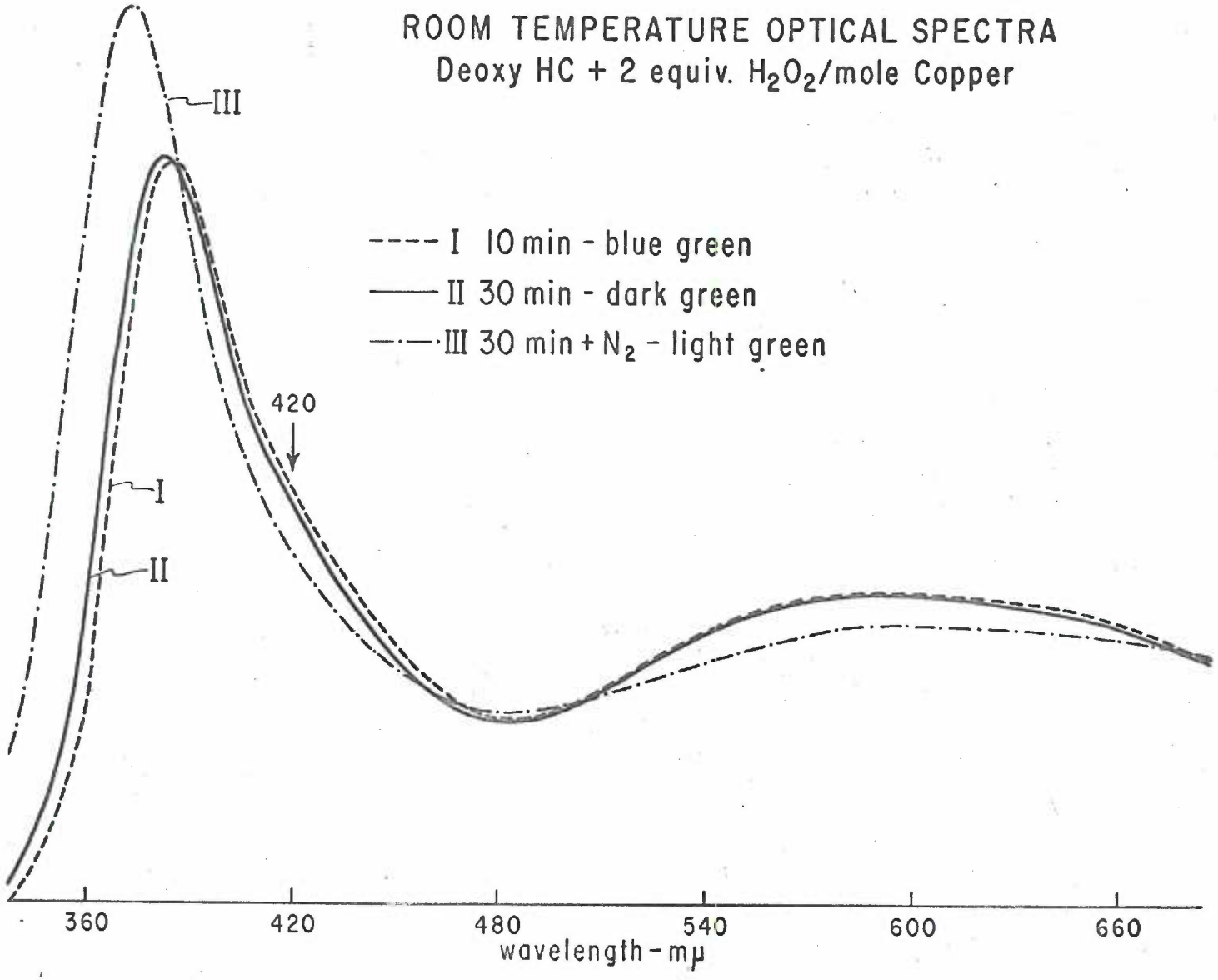
200 sweep units = modulation amplitude 0.98 gauss

having a shoulder at 650 mu were observed but were difficult to reproduce. Only when the ratio was increased to 100:1 were the spectra reproducible and showed a definite change with time, Fig. 10, c. The ratio of 100 equivalents per active site was also used by Chance (18) in his early observations of complex I between peroxidase and H_2O_2. A spectrum identical to that of oxyhemocyanin, Fig. 10, b., developed if contact between deoxy solutions and H_2O_2 was longer than .5 seconds. Those combinations between deoxy-HC and 4 equivalents peroxide per mole Cu under nitrogen, which were used in the room temperature optical studies, also gave low temperature spectra identical to that of oxyhemocyanin.

Room temperature optical spectra of the Cancer magister HC combined with an excess of H_2O_2, without the continued bubbling of N_2 to remove any O_2 formed, were consistent with the low temperature spectra, Fig. 10, c., in both the existence of a shoulder at 650 and the diminished absorbance at 340 mu, Fig. 12. In these experiments, 2 equivalents of deoxygenated H_2O_2/ mole copper were anaerobically combined with deoxy hemocyanin. The mixture was flushed with nitrogen for about 10 seconds and allowed to stand at 0°C under N_2 for 10 and 30 minutes. There was an easily observable color change from blue green, at 10 min (Fig. 12, a.), to a deep green at 30 min (Fig. 12, b.). If this solution is once again vigorously flushed with N_2, it turns paler green

Figure 12

Room temperature optical spectra of *Cancer magister* deoxyhemocyanin and excess hydrogen peroxide. No nitrogen bubbled through solutions after addition of peroxide, see text p. 72.


ROOM TEMPERATURE OPTICAL SPECTRA
Deoxy HC + 2 equiv. H_2O_2/mole Copper
---- I 10 min - blue green
—— II 30 min - dark green
—·— III 30 min + N_2 - light green
III
I
II
420
360
420
480
540
600
660
wavelength - mμ

and the spectrum of oxyhemocyanin develops more strongly Fig. 12, c.

The spectral evidence suggests that under the appropriate conditions an addition compound between hemocyanin and H_2O_2 is formed. It has a spectrum distinct from both deoxy and oxyhemocyanin. The copper extinction at 370 mu is 260 $M^{-1}cm^{-1}$. There is no development of an ESR signal accompanying its formation. By chemical determination of Cu(I), however, the deoxy-excess H_2O_2 mixture, under N_2, has about 60% total copper as Cu(II), Fig. 3. Future studies on the kinetics of the formation of the complex and its decay to oxyhemocyanin should provide an interesting comparison for the H_2O_2addition compounds with peroxidase and and catalase, first described by Chance (18).

The spectral evidence presented here for a rapidly decaying complex between deoxyhemocyanin and $H_2O_2$is considered to be only a preliminary observation. In the low temperature optical spectra, both the relative amplitude and position of peaks are subject to doubt because of the varied scattering properties among frozen samples and the large slit width, from 2.2-2.4 mm, necessary for recording. Considerations of ratios and techniques of combining deoxy HC with peroxide and the general location of absorptions unique to the complex, described here, may serve as a guide to studies using the more rapid and sensitive room temperature spectrometer-flow system, recently described by Gibson (44).

b. Methemocyanin. The chemical and optical evidence for a methemocyanin of Busycon are plotted in Fig. 14. It was possible to obtain from 40-50% loss of active site concentration upon addition of an excess of one equivalent of H_2O_2 per mole of Cu in three separate determinations. The significant difference between optical and chemical determinations of active site concentrations below 1 equiv/Cu, represents data from only one experiment. It was not observed by Felsenfeld and may be due to some error in handling solutions. Our main interest lay in obtaining ESR spectra of the material which appeared to be methemocyanin according to optical and chemical evidence. The ESR spectra of the Busycon HC mixtures with an excess of one equivalent of H_2O_2 per copper never exhibited a Cu(II) signal corresponding to more than 0.04 mM or 2% of the total copper. Approximately 90% of the original optical density, at 340 mu, could be recovered by reduction of the Busycon methemocyanin with dithionite, 1 mg/ml, followed by equilibration with oxygen.

The results of biquinoline determinations of Cu(I) in Cancer magister are plotted in Fig. 13. The value of 80% Cu(II) copper was obtained for the oxygen flushed deoxy-HC excess H_2O_2 mixtures in four determinations. This is a value 30% above that expected for oxyhemocyanin and corresponds to a loss of at least 15% of the active site. The addition of small amounts of catalase to the Cancer

Figure 14

The titration of Busycon hemocyanin with hydrogen peroxide. Nitrogen bubbled through solutions after addition of peroxide, see text p. 66. The values of OD_{340} below 1.0 equiv H_2O_2 were determined only once.

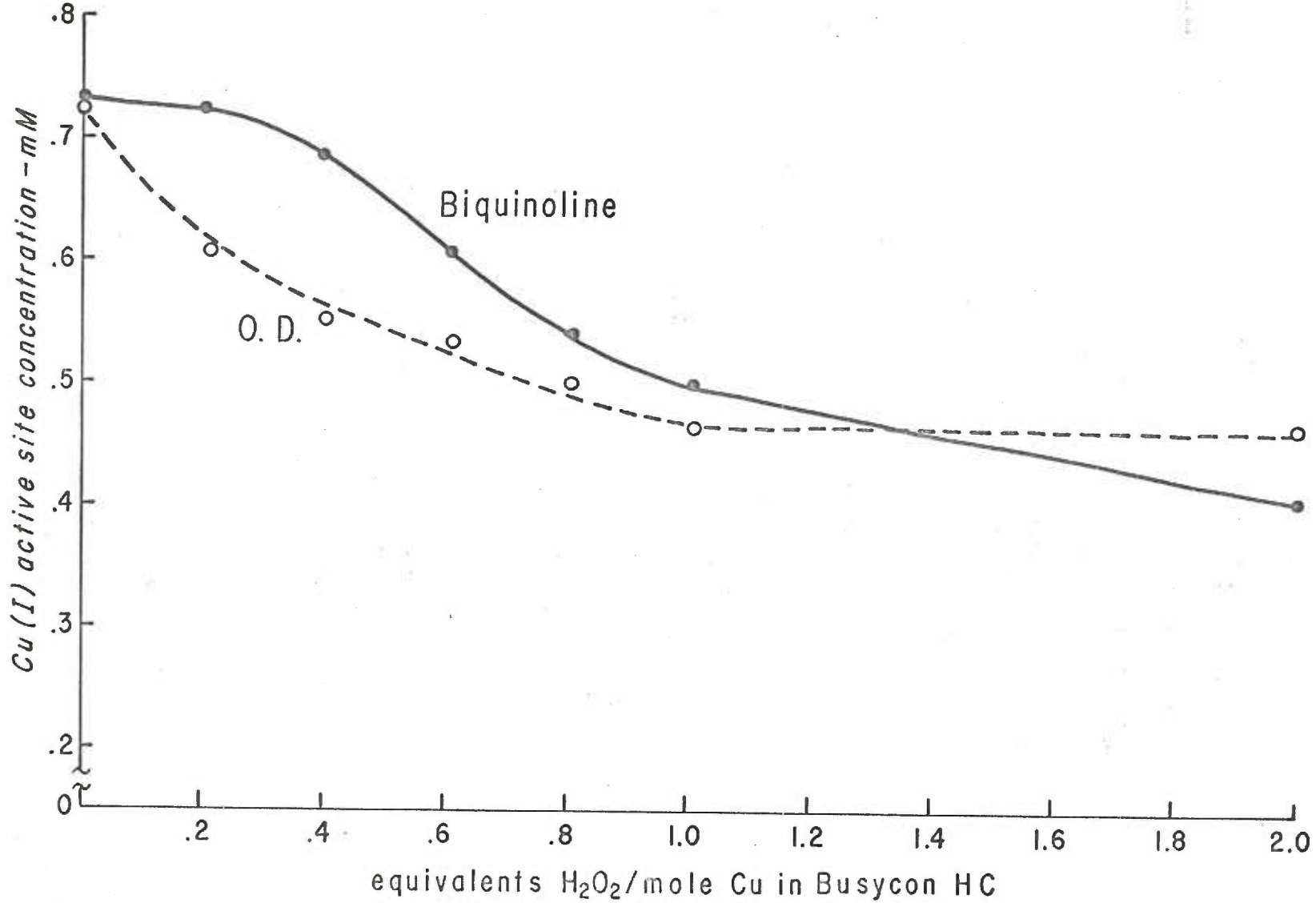
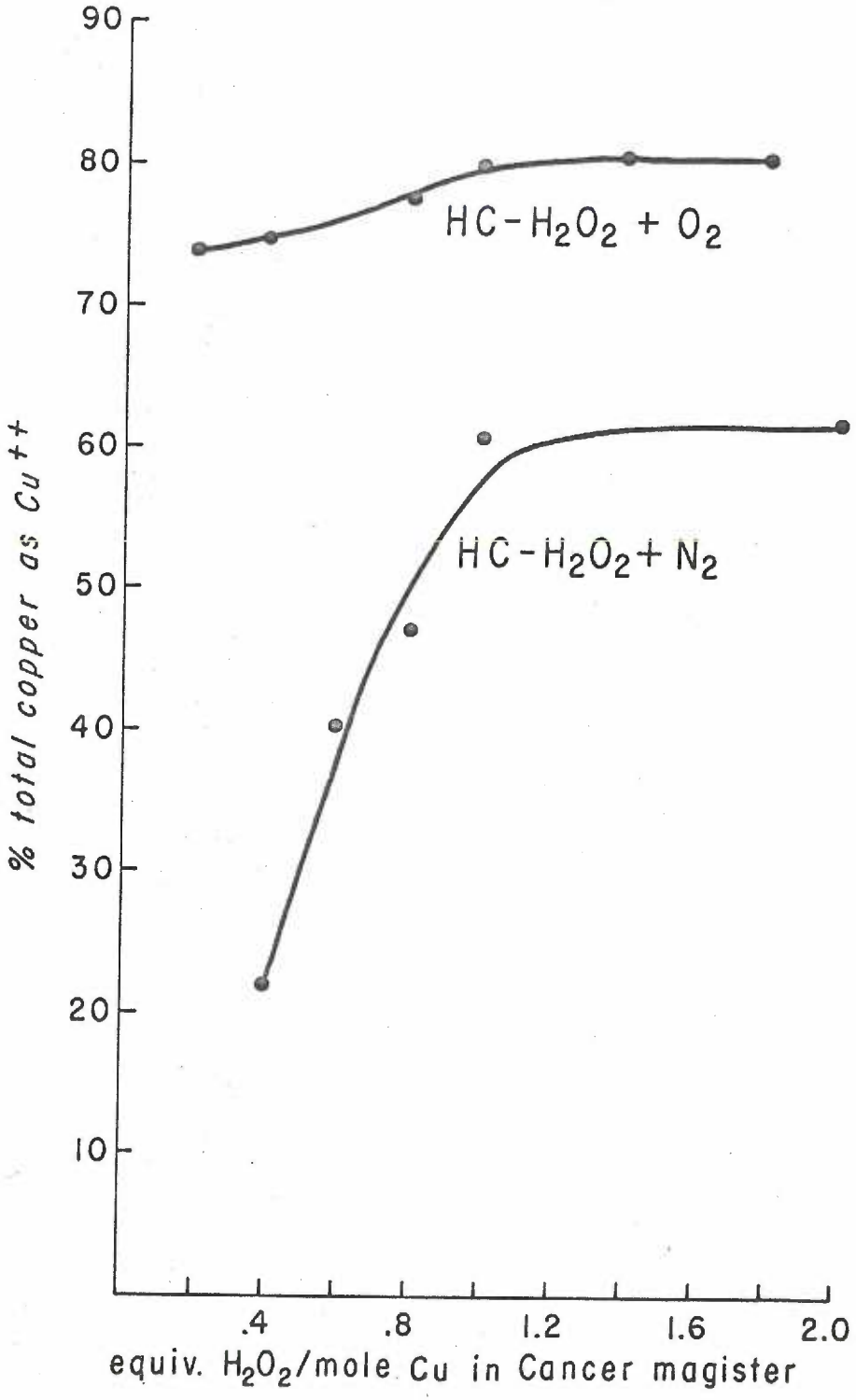

.8
.7
.6
.5
.4
.3
.2
0
Cu (I) active site concentration - mM
Biquinoline
O. D.
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
equivalents H_2O_2/mole Cu in Busycon HC

Figure 13

The titration of Cancer magister deoxyhemocyanin with hydrogen peroxide. Nitrogen bubbled through solutions after addition of peroxide, see text p. 66. Values of Cu(I) in HC-H_2O_2 (N_2) were determined using deoxygenated biquinoline reagents.


90
80
70
60
50
40
30
20
10
% total copper as Cu++
HC-H2O2 + O2
HC-H2O2+ N2
.4
.8
1.2
1.6
2.0
equiv. H2O2/mole Cu in Cancer magister

magister deoxy HC-H_2O_2 mixture in the presence of oxygen reduced the % total copper as Cu(II) to 65% or 15% above that expected in native oxy HC. This corresponds to a loss of at least 7% of the active site. The addition of catalase in the absence of oxygen, however, never decreased the % total copper as Cu(II) below 45%. This indicated that oxidation of more than 7% of functional Cu(I) had occurred.

As in the case of Busycon, no ESR signal could be detected in the "methemocyanin" either in the presence of nitrogen or oxygen. Unlike the case of Busycon, however, there was no loss of OD at 340 mu corresponding to the increase of Cu(II) determined by the biquinoline method. Since both the band position at 370 mu, and the low copper extinction (260 $M^{-1}cm^{-1}$) of the HCH_2O_2 complex under nitrogen, Fig. 12, c., do not correspond with the oxyhemocyanin, spectrum 340 mu and 2,700 $M^{-1}cm^{-1}$, the anomaly between chemical and optical determination of Cu(II) in Cancer magister cannot be attributed to that complex species. The evidence indicates that upon addition of O_2, this species is converted to one, spectrally very similar to oxy HC, but with an increased cupric content, by chemical determination. A reduction of this material and recycling through oxygen and nitrogen was not tried, and should be. Its properties are reminiscent of a blue oxyhemocyanin-like complex which was formed by reduction of a HC-Nitric Oxide complex at pH 5, followed by exposure to oxygen. This

material also had no ESR Cu(II) signal, an optical spectrum similar to oxyhemocyanin and was not reversible to oxygen association. It is fully described in Experimental Section II, D.

In summary, chemical and optical evidence were obtained for the formation of a methemocyanin from Busycon as in 1959 (36) and chemical evidence was obtained for the formation of an oxidized complex of Cancer magister hemocyanin. Neither of these exhibited a significant Cu(II) ESR spectrum. In this way, the Cu(II) species detectable in hemocyanin after reaction with H_2O_2 is similar to that detectable in native oxygenated hemocyanin .

B. Experiments on the reaction of apo-protein with cupric ion.

1. Aim:

A strong cupric ESR signal has been observed in an apo HC-Cu(II) complex (87) prepared by dialysis of a .1 mM protein solution against a .1 mM Cu(II) solution. The following experiments were carried out to determine the specificity of the cupric attachment to the protein, the extent to which native material can be reconstituted from the apo-cupric complex and the nature of the cupric ESR signal.

2. Materials and Methods:

Apo-hemocyanin was prepared by dialysis of an aliquot of fresh concentrate against 2 changes of two liters 0.05M phosphate buffer pH 6.8 or 0.05 M tris buffer pH 6.8 and 0.01

M in CN^- at 4° C. The water and reagents used were the same as described in Experimental Section I, B, 2. All the work to be reported, with the exception of G 75 treated apo-protein was undertaken before .01 M Ca was being routinely added to all buffer solutions. Protein determinations were done by the Biuret method (46) and biquinoline determinations on total copper content were checked against the bathocuprotein method as already described.

All ESR determinations were performed at low temperature in the variable temperature dewar with sample cell, cavity and unit as described in Experimental Section I, B, 2., with the exception of the Field dial control unit and the xy recorder. Field control and strip chart recorder were the same as used in II, A, 2. Determination of Cu(II) concentration from cupric ESR signal was done by double integration of the spectrum of a Cu(II) EDTA standard of known concentration and two or three representative signals of the protein sample recorded at the same modulation. This was necessary since the first derivative of the absorption was recorded, while only the area contained by an actual absorption spectrum is proportional to total concentration of a resonating species.

The determined area and known Cu(II) concentration in the standard allowed calculation of the value of "k" in the expression

$$\iint f'(x)dx^2 = k\ HmN$$

$f'(x)$ is the equation of the first derivative curve, some

function of x, gauss

$\iint f'(x)dx^2$ = value of the area under primary absorption curve

Hm = modulation amplitude

N = molar concentration of cupric

k = constant of proportionality between area and concentration. Its value depends on experimental conditions.

This value of "k" and the area determined for a sample cupric signal, usually having a different shape from that of the standard, can be substituted into the equation to determine "N", cupric concentration in the sample. Since signal height varied linearly with pen gain setting, once the signal heights of spectra from samples have been adjusted to a uniform gain, the Cu(II) in each can be estimated by comparison with the samples in which the relationship between total area or concentration and signal height has been established. The difference between determinations of total copper chemically or by integration of ESR signals from urea denatured material was rarely greater than 7% or ± 2 mM for the concentration ranges studied.

Control samples of native hemocyanin were dialyzed for the same length of time as the apo-samples in the absence of CN^- in the first stage and Cu(II) in the second stage. All protein samples showed an opalescence upon extended dialysis and were centrifuged in the Servall for 20 min at

17,000 rpm before recording ESR and optical spectra and making pertinent protein and copper determinations. Unless otherwise noted reagent grade dithionite was used for reconstitution. Approximately 6 reducing equivalents per mole Cu(II), were added to a deoxygenated apo-protein-Cu(II) solution during continued gentle bubbling of nitrogen through the solution, or swirling of the solution by a strong N_2 stream above it. The mixture was allowed to stand under N_2 from 2-12 hours in the cold before being exposed to oxygen.

3. Results and Discussion:

The data from four separate experiments are collected in Table VI. The entries in item 10 were obtained in the following manner. Total functional copper, in reconstituted material expressed at 575 mu was estimated using the extinction obtained for a parallel native sample. This value of functional copper divided by a calculated total functional copper, item 2, if all the protein present were reconstituted, gave % total protein reconstituted. This value was independent of the amount of total copper present, item 8. In case b, for example, total conversion of the .8 mM Cu into functional Cu would allow a maximum of only 32% total protein present to be reconstituted. With the exception of G 75 treated samples, reconstitution of 80-95% of the total apo-protein could be obtained by the addition of CuCl. It must be colorless and dry, and added in slight excess of equivalents needed, to a deoxygenated

TABLE VI

Chemical and paramagnetic properties of apo-hemocyanin reconstituted by addition of Cu(II) followed by reduction with dithionite. Columns a, b, c, and d refer to separate experiments. The apo-hemocyanin in column d was passed through a Sephadex G 75 column before dialysis against cupric copper.

TABLE VI

1.	Protein in apo-HC (mM)	0.0665[a]	0.097[b]	0.095[c]	0.059[d]
2.	Equivalents in copper (mM)	1.75	2.50	2.25	1.50
3.	Cu(II) in dialysis solution (mM)	2.2	2.2	0.01	0.01
4.	Dialysis time apo-HC vs Cu(II) (hrs)	48	24	48	48
5.	ESR Cu(II) before reduction (mM)	2.58	0.59	1.23	0.338
6.	% total copper as ESR Cu(II) before redn. (%)	90	74	61	66
7.	ESR Cu(II) after reconstitution (mM)	0.35	0.08	0.44	---
8.	Cu total in reconstituted apo-HC (mM)	2.88	0.8	2.16	---
9.	% total chemical copper as Cu(I) in reconstituted apo-HC	86.5	90	77.8	---
10.	% total protein functional at 575 mu	69.0	27.6	67.0	---
11.	ϵ $M^{-1}cm^{-1}$ copper in HC control	584	530	517	---
12.	% total chemical copper functional at 575	43.0	86.5	69.0	---

apo sample after the manner of Lontie (74) or Ghiretti (41). Although the reconstitutions by reduction of the apo-Cu(II) complex were only partial, at best, and unpredictable, several interesting observations were made.

Contrary to the observations of Bayer (8) the hemocyanin protein is not a specific chelate only for Cu(I). In case c., for example, the total concentration of Cu(II) in the dialysis solution was 0.01 mmoles. A 10 ml aliquot of the apo-protein was dialyzed for 48 hrs against 2 liters of this solution, .02 mMoles absolute conc., in an effort to minimize the non specific attachment of Cu(II). Ten ml of a .09 mM protein solution needed approximately .0225 mMoles copper for full reconstitution. Over a period of 48 hrs, the protein concentrated Cu(II) from the solution about 200 fold, while under similar conditions the G75 treated apo-protein, case d., concentrated Cu(II) from the solution about 50 fold.

The second curious observation is that except for case a, in which a substantial amount of excess copper was in the dialysis solution, the amount of total copper by cupric ESR signal before reduction is significantly lower than that which could be determined chemically. This was especially true when absolute concentrations were used in Cu(II) dialyses solutions, as in cases c. and d. It is probable that Cu(II) is attached both non-specifically and specifically to electron donating ligands, e.g. nitrogen and sulfur, which stabilize Cu(I).

The percentages in item 12 were calculated by taking total mM copper functional by OD 575, the same as in item 10, and dividing by total copper present, item 8. This gives an index of specific vs. non specific Cu(II) contributing to the ESR spectra of apo-Cu(II) mixtures before reduction. In case a, for example, a substantial amount of Cu(II) was reduced non specifically, item 9. Only 43% of the total copper present in the apo-Cu(II) complex was destined to become functional. In case b., however, at least 86.5% total chemical copper was destined to become functional. The cupric spectrum from this apo-Cu(II) sample before reduction is shown in Fig. 15. It is unlike the spectrum of the Cu(II) EDTA standard and more like the Cu(II) signal seen in frozen solutions of native ceruloplasmin (81, 82) in both narrowness of the $g_{\perp}$ signal and hyperfine splitting of 60 gauss. A similar signal appears for case c., Fig. 16, c., but without the high field shoulder which can be power saturated, Fig. 15.

Reconstitution of native material from apo-protein which had been treated with Sephadex G75 and then dialyzed against Cu(II) was not possible with 6 excess (xs) equivalents of dithionite, 10xs reducing equivalents of dithiothreitol (20) or 100 xs equivalents of formamidine sulfinic acid (113). The ESR signal of this material showed no evidence of narrow hyperfine, Fig. 16, d. It is possible that treatment of the apo-protein resulted in an alteration

Figure 15

Low temperature ESR spectra of apo-hemocyanin in the presence of Cu(II), before reduction, at four different attenuations. Sample b, of Table VI is shown here.

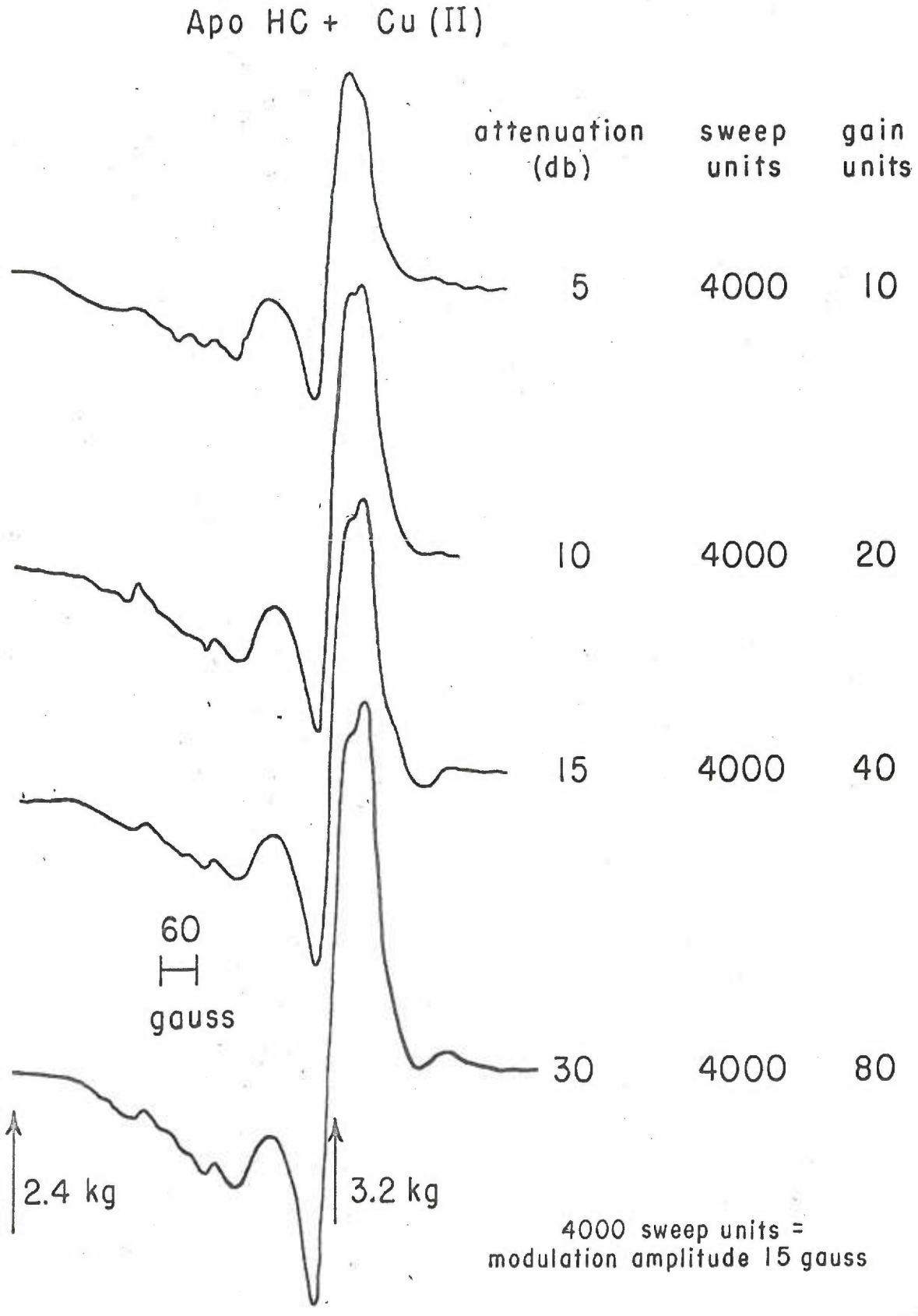
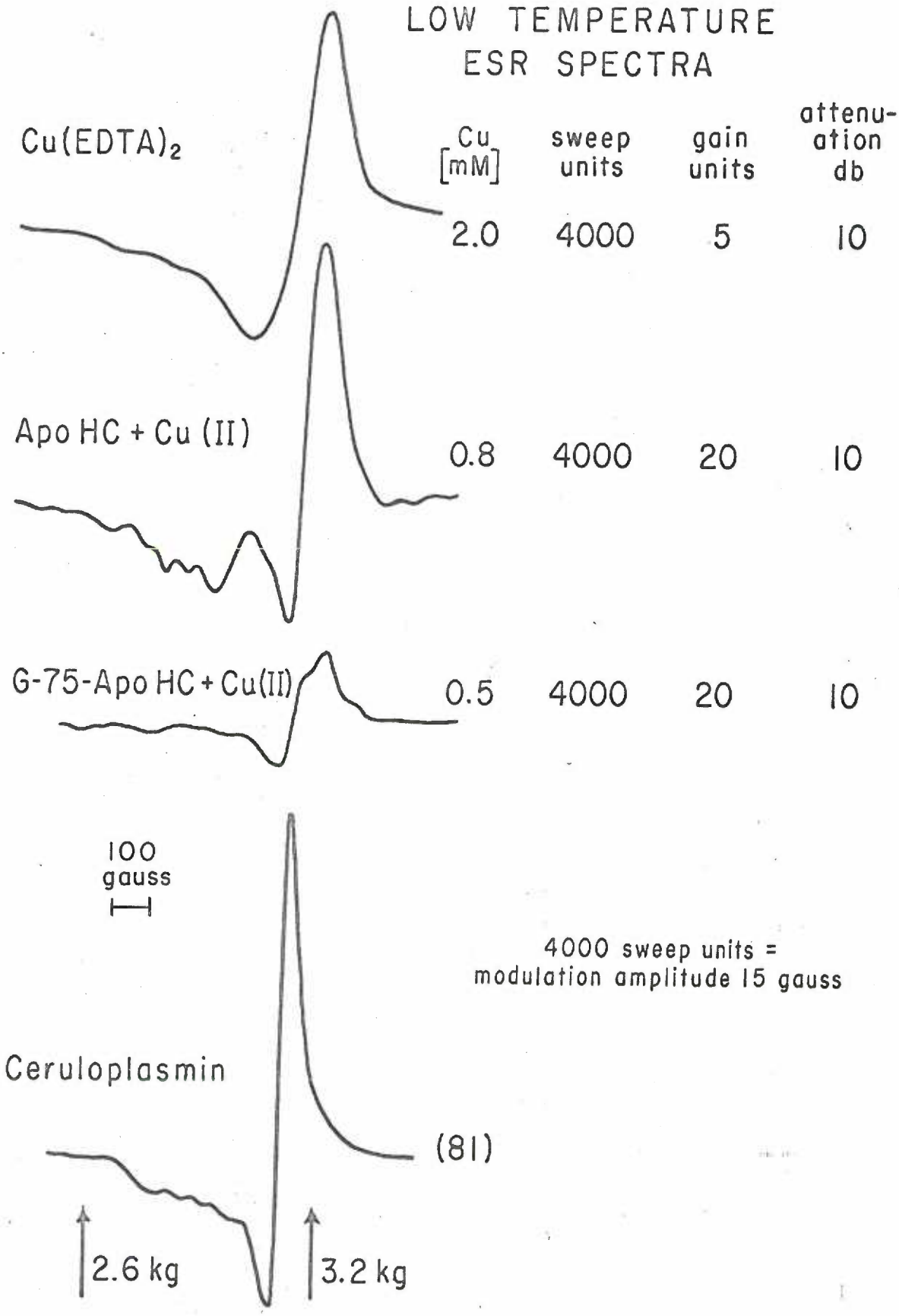

Apo HC + Cu (II)
attenuation (db)
sweep units
gain units
5
4000
10
10
4000
20
15
4000
40
30
4000
80
60
gauss
2.4 kg
3.2 kg
4000 sweep units =
modulation amplitude 15 gauss

Figure 16

Comparison of the low temperature ESR spectrum of apo-hemocyanin-Cu(II) with those of $Cu(EDTA)_2$, ceruloplasmin and a non-reversible apo-hemocyanin-Cu(II).


LOW TEMPERATURE
ESR SPECTRA
Cu(EDTA)2
Cu [mM]
sweep units
gain units
attenu-ation db
2.0
4000
5
10
Apo HC + Cu (II)
0.8
4000
20
10
G-75-Apo HC + Cu(II)
0.5
4000
20
10
100 gauss
4000 sweep units = modulation amplitude 15 gauss
Ceruloplasmin
(81)
2.6 kg
3.2 kg

of the protein, otherwise undetectable. An attempt to reconstitute with CuCl was not made. Dialysis of the apo-Cu(II) complex against fresh buffer in cases a. and d., Table VI had no effect on the total copper content or specificity of attachment. Dialyses against .005 M EDTA solutions to remove non-specific copper, as done in ESR studies on ceruloplasmin but not laccase (82) were not tried in any case.

The Cu(II)-apo signal cannot be described as a true methemocyanin signal. It most probably arises from copper, specifically and non-specifically attached to amino acid residues. This is the first time such a signal with narrow hyperfine has been reported for a protein, other than an untreated copper protein. The narrow hyperfine structure may be somehow related to Cu(I)-Cu(II) interaction through a bridging ligand as in those described for ceruloplasmin (82) since total chemical copper is also not accountable by cupric ESR signal here as in ceruloplasmin. The ESR spectrum of an inorganic model system, cupric acetate, with Cu(II)-Cu(II) interaction has been described (11) and is not similar to the Cu(II)-protein spectra. There has been no similar analysis of an ESR spectrum arising from Cu(I)-Cu(II) interaction.

C. Experiments on photooxidation of the functional copper.

1. <u>Aim</u>:

The binding of oxygen by Cancer magister makes two new transitions possible, one in the region of 575 mu and one in the region of 340 mu. All speculations about the sources of these bands involve an excitation of a Cu(I) electron for the lower energy, 575 mu, band, e.g. transition (138) and a partial transfer of a Cu(I) electron to O_2 for the higher energy, 340 mu, band (83).

In the physiological state, hemocyanin combines with oxygen in the dark. The characteristic blue color of oxyhemocyanin indicates that certain transitions are made possible in the presence of oxygen. The blue color can, of course, only be seen when oxyhemocyanin is exposed to light. That is, it representsan excited state of the oxyhemocyanin. In fact, it is possible that a description of the mechanism of oxygen binding may not necessarily involve a contribution from a Cu(II) species at all. For example, only Cu(I) may be involved, a Cu(I) which is slightly altered in the presence of oxygen to allow d-d and charge transfer transitions to occur in the presence of light. That is, a Cu(I) .. O_2 .. Cu(I) model may be consistent with chemical determinations, performed in the light, and inability to detect an ESR signal when oxyhemocyanin is irradiated only with frequencies close to 9500 mc/sec, in the dark.

The following experiments were undertaken in an attempt to detect any paramagnetic species of the active center, which may form upon irradiation with intense light. The

experiments were designed to accurately measure and make maximum use of the intensity and resolving power of the Bausch and Lomb high intensity monochrometer.

2. Materials and Methods:

Samples of oxy and deoxyhemocyanin, 1.9-2.0 mM Cu, were combined 1:2 with ethylene glycol and 1:1 with glycerol, to form rigid glasses at low temperature as described in Experimental Section I, B, 2. The quartz sample tubes, multipurpose cavity and monochrometer were the same as used in the triplet studies. The variable temperature dewar was used during irradiations above 340 mu and the constant temperature liquid N_2 dewar below 340 mu. The ESR spectrometer and accessories were the same as described in I, B, 2.

The field was centered at 3.1 kg with 1,000 gauss equivalent to a full sweep of the x axis and incident power was 25 mW. The complete cupric signal from a 2 mM Cu-EDTA standard could be recorded at these settings. The monochrometer slits were completely open, entrance/exit ratio 6/6, allowing a 44 mu band to pass with 1.70 mV total light power at 575 mu, 2.6 mV at 340 and 1.65 mV at 290 mu, as measured by the Eppley thermopile. Deoxy and oxyhemocyanin samples were irradiated at each of these three wavelengths and at one setting which gave mixed visible light of 29 mV.

3. Results and Discussion:

Unirradiated glycerol and ethylene glycol hemocyanin mixtures exhibited from 5-15% total copper as Cu(II) at 3.1 kg.

There was no difference in expression of total copper as Cu(II) between the unirradiated oxy and deoxyhemocyanin samples, 1:1 with glycerol. On one occasion the value for both was 6%, on another 13%. The unirradiated ethylene glycol samples, however, showed about 5% total copper as Cu(II) in the deoxy and 10% for the parallel oxy-sample and on another occassion 12% in the deoxy and 14% in the oxy. The different effect of the 1, 2 and 3-carbon saturated alcohols, methanol, ethylene glycol and glycerol is noteworthy. Methanol denatured the protein on contact. In the triplet study, ethylene glycol altered the triplet half life of the native oxygenated material, making it close to, or longer than, $t_{\frac{1}{2}}$ for native deoxy, unlike both water and glycerol analogues. Ethylene glycol also made a small amount of the Cu(I) susceptible to oxidation by O_2. Glycerol, on the other hand, gave half lives comparable to those found in aqueous solutions, and while giving rise to Cu(II) signals greater than the 1% total copper found in aqueous solutions the value was unaffected, as in the native case, by the presence or absence of oxygen. The different effects of these solvents on the aqueous hemocyanin system may be related more to their size than polarity and may depend on efficiency of penetrating the aqueous solvent shell of the protein.

No alteration or increase of the existing cupric signal was seen upon irradiating oxy or deoxyhemocyanin in ethy-

lene glycol or glycerol, up to 10 minutes with light bands of 575 ± 22 mu, or 340 ± 22 mu. Light of 290 ± 22 mu gave rise to a signal at g = 2 (3.25kg) which did not arise from organic solvents or copper interaction with oxygen. In a deoxygenated aqueous solution of apohemocyanin it had a half life of about 70 seconds. This free radical signal evidently arises from the protein portion of the molecule. There is some evidence that unpaired electrons may be stabilized by some structural feature of proteins (22), not necessarily the alpha helix, but some feature easily destroyed upon denaturation of the protein. The oxidation of methemoglobin and metmyoglobin by H_2O_2 has been shown to give rise to a free radical intermediate derived from oxidation of part of the globin molecule (59). Irradiation with light at 290 mu also gives rise to a triplet signal at 1.45 kg, which has been characterized for each sample in Experimental Section I, B.

Irradiation with intense, mixed visible light, 29 mV, gave rise to a free radical signal at 3.247 kg, but not to a triplet signal at 1.45 kg. The free radical signal was similar to that produced by irradiation with 290 mu light. The one produced by 29 mv mixed visible light, however, developed almost twice as fast in deoxy-native samples as it did in oxygenated ones. For example, when the oxy signal height was 4.2 after 10 minutes irradiation, the deoxy signal height at the same settings and gain was 7.8 at 10

minutes. A deoxygenated apo sample was not irradiated with 29 mV light to see if this signal is peculiar to copper-containing samples.

Calculation of electron volts, (V), equivalent to one mole of light at 290, 340 and 575 mus, according to the scheme in Appendix I, gave values of 4.2, 3.6 and 2.12 V, respectively. These values of V associated with a transition at a particular wavelength can also be calculated by dividing cm^{-1} by 8,086 cm^{-1} per electron volt.

The unequivocal result from the attempt to photo-oxidize the copper at the active site is that 2.74×10^{-7}V or 7.64×10^{-9} moles of photons at 340 mu, and 1.8×10^{-7}V or 8.45×10^{-9} moles of photons at 575 mu are not sufficient to cause a detectable valence change in the copper. The fact that even less powerful sources of visible light cause transitions at these wavelengths is consistent with current proposals as to the sources of the transitions. Neither d-d nor charge transfer transitions involve a permanent transfer of an electron out of a metal orbit. It is possible that a more powerful source at 340 mu may cause the average time which the electron remains on the oxygen to be sufficiently increased to make a Cu(II) species detectable by ESR (see General Discussion II, d.).

D. Experiments on the Reaction of Deoxyhemocyanin with Nitric Oxide.

1. Aim:

Introduction of an unpaired spin at the active site by

equilibrating deoxyhemocyanin with nitric oxide (NO) was suggested to us by J.S. Griffith. Three-electron bonds, possible only between identical nuclei or nuclei of nearly identical electronegativities, have been proposed for both O_2 and NO.

The experimentally-observed O-O bond distance for oxygen in ozone is 1.26 ± 0.02 Å, a value inbetween that calculated for a single bond, 1.48 Å and a double bond, 1.10 Å, (67, b., 49). The resonance form of NO

involves a double bond and the observed NO distance is 1.14 Å, which lies between the values calculated for this molecule of 1.18 and 1.06 for a double and triple bond respectively. It exhibits the degree of paramagnetism expected for a substance with one unpaired electron; at room temperature it is approximately 1.8 Bohr magnetons. The electronic structures of some metal nitrosyl complexes have been calculated recently (48) and will be referred to later.

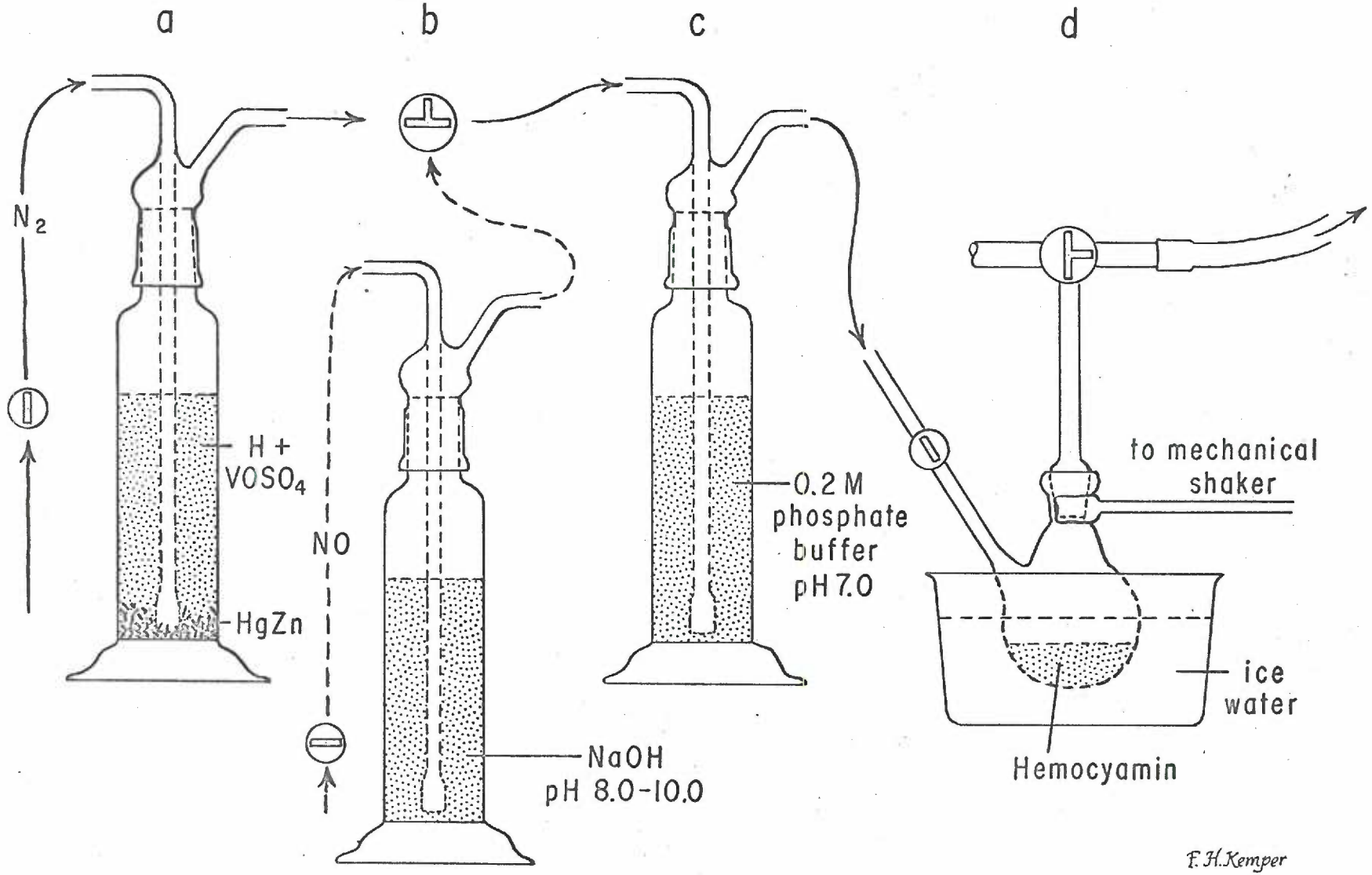
2. Materials and Methods

Nitric oxide, NO, combines rapidly and completely with oxygen at room temperature to form nitrogen dioxide, NO_2, which spontaneously combines with water to form nitric acid, HNO_3. For this reason, every precaution to exclude oxygen, was taken. The equilibration apparatus is shown in Fig. 17.

Figure 17

Schematic diagram of the flow system to remove oxygen and HNO_3 vapors for the nitric oxide experiments.

SCHEMATIC OF N_2 AND NO PATHWAYS THROUGH WASH SOLUTIONS



Prepurified nitrogen, Industrial Air, O_2 content less than 3 ppm, was scrubbed in a vanadyl sulfonate solution over amalgamated zinc (88) and the NO, nitric oxide, Matheson, New Jersey, was scrubbed in a sodium hydroxide solution, pH 8-10, with phenolpthalein as an indicator. Both gases were passed through a 0.2 M phosphate wash buffer, pH 7-7.6, with nitrophenol as indicator, before contact with the protein solution. A five to ten ml sample of 2.0-2.5 mM Cu hemocyanin was gently swirled in an ice bath in a 75 ml round bottom flask, fitted with a sidearm and a two-way stopcock, to allow the insweep of gas to pass directly above the solution.Fig. 1, a. Tygon tubing was used to connect the cylinders with the wash bottles and the wash bottles to the sample vessel. Nitrogen was flushed through all solutions, A, B, and C, Fig. 17, during its two hour equilibration with hemocyanin to form deoxy hemocyanin. Equilibration of deoxy HC with NO was interrupted for sample taking by adjustment of the three-way stopcock to begin flushing the system with nitrogen. Interruptions of gas flow were done quickly and under positive pressure since there is always a slight equilibration with air across tubing.

Samples were always withdrawn under a positive N_2 pressure with a capillary tube attached to a 1 cc syringe flushed with nitrogen immediately before. Samples of about 0.5 ml were placed into quartz ESR tubes of 3 mm bore and

immediately frozen in liquid N_2. This sample volume insured a fully loaded ESR tube for the height of the cavity and therefore eliminated exacting volume measurements at the time of withdrawal.

All ESR spectra of frozen aqueous solutions were recorded at -170 to -180° C in the variable temperature dewar, using the spectrometer with the Field control unit, microwave circuit, cavity and xy recorder as described for the triplet studies, Experimental Section I, B, 2. The incident power was 25 mW unless otherwise noted. The pH of the protein solution, 6.8, was adjusted to pH 5 or 8 by dialysis against two, 2 liter changes of Tris buffer 0.05 M, .01 M in Ca. Dialyses were carried out in a Bellco dialysis vessel. Low temperature optical spectra were recorded using the same apparatus as described in Experimental Section II, A, 2., and Fig. 10, a.

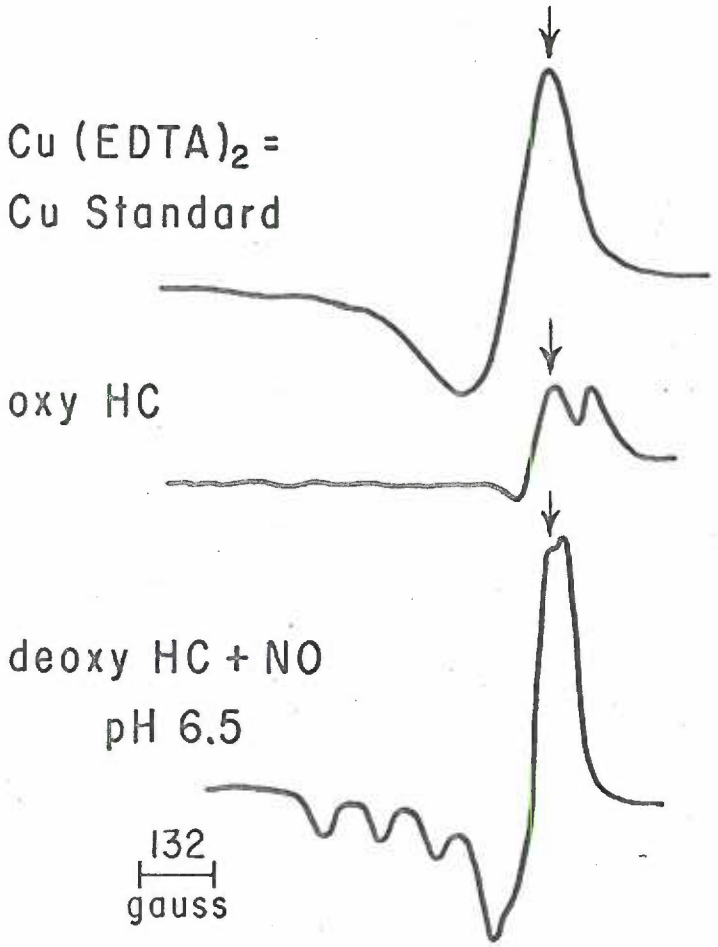
3. Results and Discussion:

a. Description of the ESR signal and the extent of reversibility of HCNO to HCO_2. The low temperature ESR spectra of oxy and deoxyhemocyanin consisted of small signals with a characteristic double peak, Fig. 18, b. Upon double integration and comparison with the Cu $(EDTA)_2$ signal, the concentration standard, Fig. 18, a., the Cu(II) in these signals represented from 1-% of the total copper present. Near pH 5 or 8, the % total copper as Cu(II) by ESR in the native deoxy-protein was always about twice that in the

Figure 18

Low temperature ESR spectra of HCO_2 and HCNO.

LOW TEMPERATURE EPR SPECTRA



	[Cu] mM	Sweep units	Gain units
Cu (EDTA)$_2$ = Cu Standard	1.98	4000	5
oxy HC	2.03	4000	100
deoxy HC + NO pH 6.5	3.0	4000	5

4000 sweep units =
modulation amplitude 15 gauss

oxy: pH 8, .88% oxy and 1.45% deoxy or pH 4.9, 1.76% oxy and 4 % deoxy. The % total copper as Cu(II) by ESR in the deoxy native HC was generally only slightly greater than that in the oxy at neutral pH.

Upon equilibration of the deoxy protein with NO, a green color and an ESR signal, such as seen in Fig. 18, c., with gm 2.074, // 2.299 and A 0.017 were developed. The g-value at maximum absorption (gm) should be close to $g_{\perp}$ (82). The four components of the g// portion of the signal arise from interaction of the unpaired electron with nuclear spin of copper, 3/2. Their existence is evidence of electron density, from the unpaired d electron in this case, at the nucleus. The g// portion or fine structure then gives some indication of sigma or "s" character of the unpaired electron.

A definite increase in "s" character, as compared with Cu $(EDTA)_2$, accompanied the signal at each of the three pHs studied. Double integrations were made of the HC-NO cupric signals in samples taken at regular intervals during 4-6 hr equilibrations of the protein with the gas. Observations from 15 separate experiments, made over two years, are summarized in Fig. 19. Determination of total copper by the biquinoline method was more reproducible than measurement of the Cu(II) ESR signal from a urea denatured protein sample. Equilibration at pH 8 took substantially longer in either buffer. Fig. 20 shows the difference in develop-

Figure 19

The development of the Cu(II) ESR signal in HCNO with time and the dependence of final % total copper as Cu(II) on the buffer used and the pH of reaction.

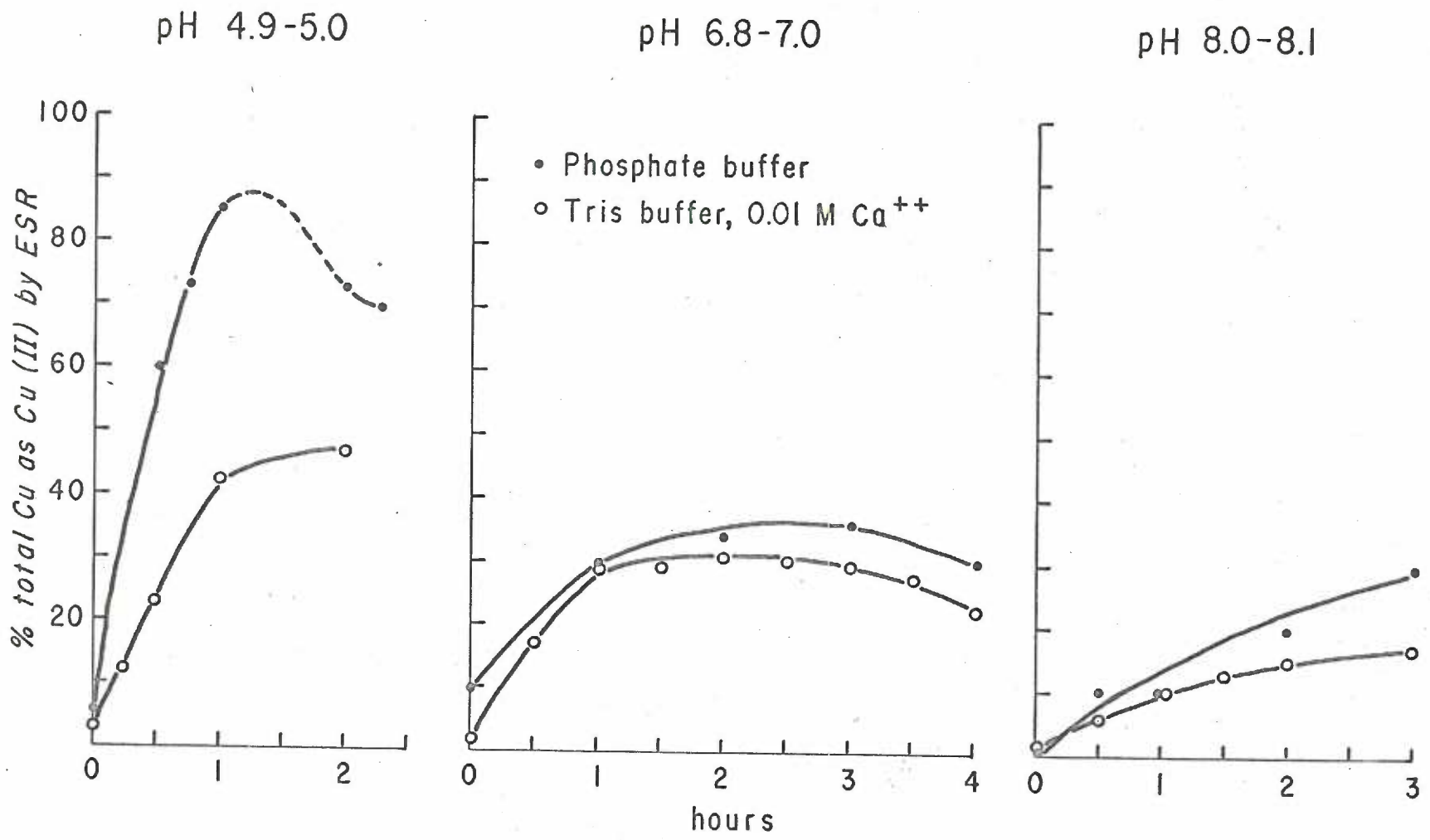
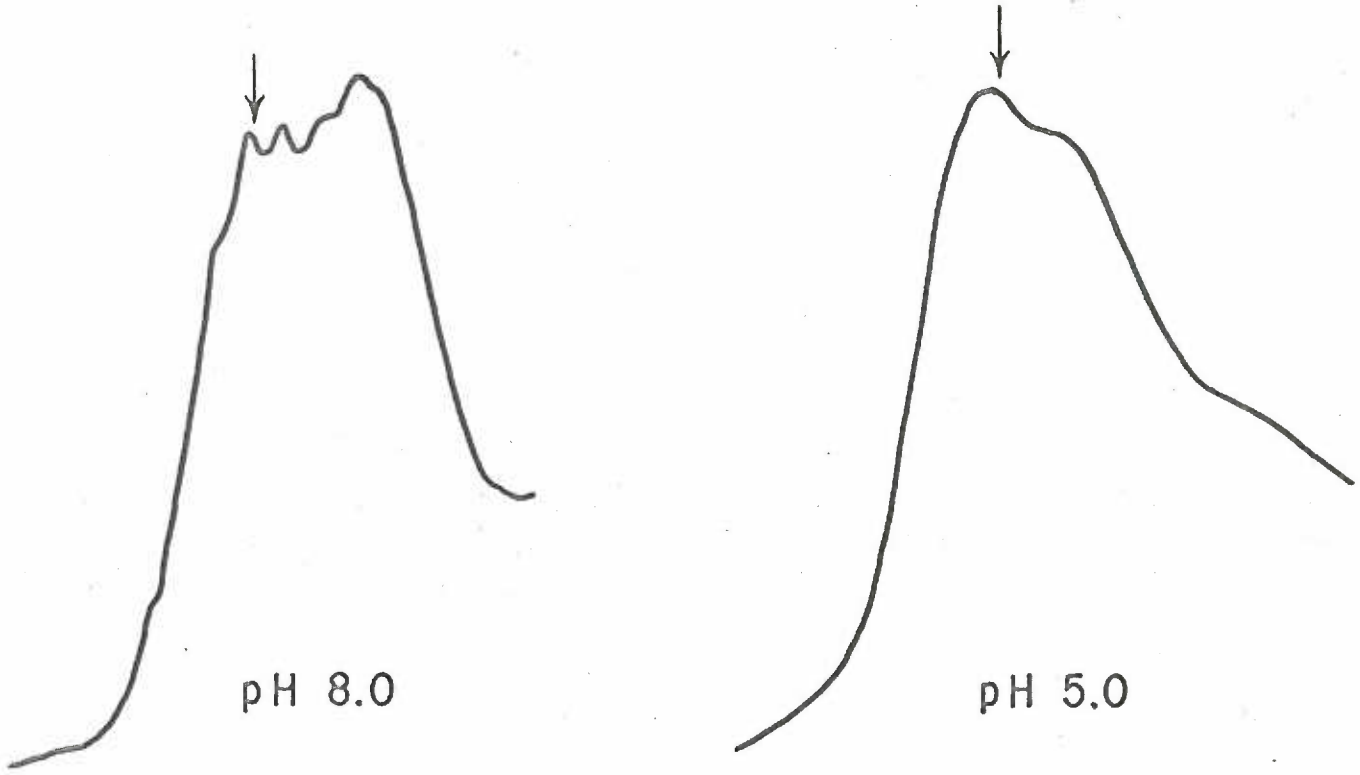

pH 4.9-5.0
pH 6.8-7.0
pH 8.0-8.1
% total Cu as Cu (II) by ESR
100
80
60
40
20
0
1
2
3
4
hours
• Phosphate buffer
o Tris buffer, 0.01 M Ca++

Figure 20

Low temperature ESR spectra after 30 minutes equilibration of deoxy-HC with NO at pH 8 and pH 5.

EPR SPECTRA AFTER 30 MINUTES EQUILIBRATION OF DEOXY HC WITH NO



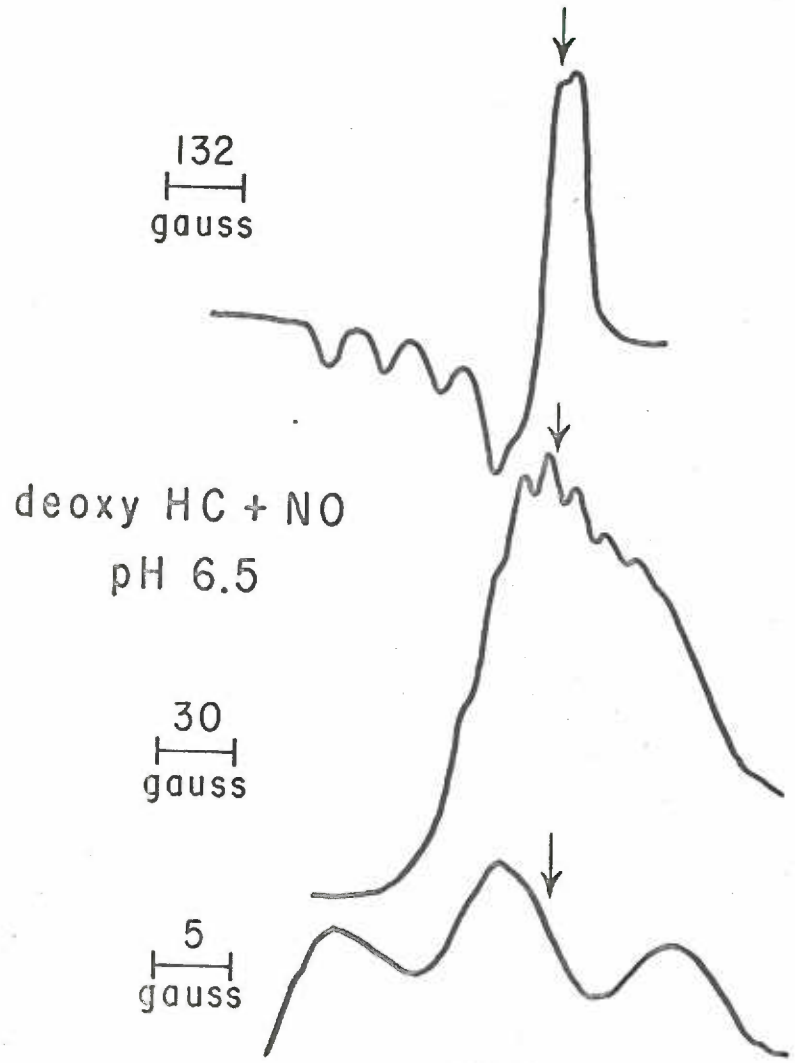
ment of the signal after 30 minutes at pH 5 and 8. The signal at pH 6.8 develops symmetrically compared to these. All signals retained their original symmetries upon continued equilibration. Further resolution of the region around g = 2 revealed an 8, possibly 9, component hyperfine structure, Fig. 21, with splittings from 10-12 gauss. This hyperfine was difficult to resolve in samples which had been passed through Sephadex G75 and almost impossible to resolve at pH 5.0, except in samples taken 10-20 min after equilibration with NO at this pH or with 1 mW incident power in the 2 hr sample.

A series of experiments was carried out to determine whether the main cupric signal and the hyperfine lines at g ⊥ arose from the delocalization of an electron over the same structure. Deoxy hemocyanin was equilibrated at pH 6.9, with NO for 1 hr at 0° C. The signal height of the main signal, between 3.17 and 3.293 kg, and one hyperfine peak, 3.1917-3.20 kg, Fig. 21, were recorded with varying incident power at each of four temperatures, minus 176, 135, 113 and 45 degrees centigrade. Six values including and between 1 and 150 milli Watts incident power gave six recordings of signal height for fine and hyperfine structure at each temperature. The variation of SH with the square root of power, Fig. 22, indicate that the hyperfine lines arise from a different structure and are superimposed on the cupric g⊥ signal. Whether one electron is delocalized

Figure 21

Resolution of hyperfine structure in HCNO.

RESOLUTION OF HYPERFINE STRUCTURE

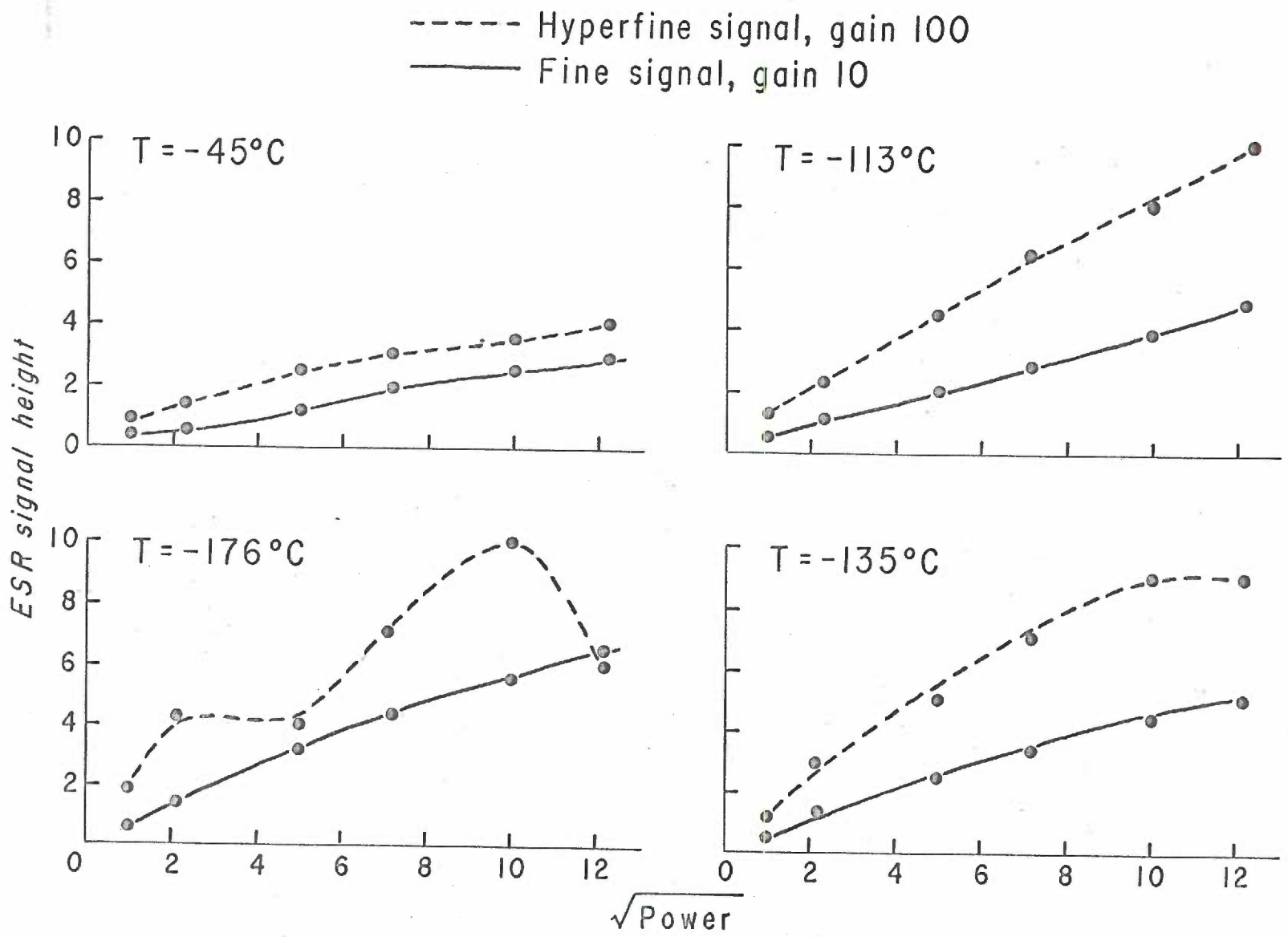


[Cu] mM	Sweep units	Gain units
3.0	4000	5
3.0	200	100
3.0	100	400

4000 sweep units = modulation amplitude 15 gauss

Figure 22

Power saturation behavior of the fine and hyperfine components of HCNO at pH 6.5, after one hour equilibration of deoxy-HC with NO.


Hyperfine signal, gain 100
Fine signal, gain 10
T = -45°C
T = -113°C
T = -176°C
T = -135°C
ESR signal height
√Power
0
2
4
6
8
10
12

over two different types of nuclei or two types of unpaired electrons are involved will be discussed later (Experimental Section II, D, 3, c.).

It was possible to reconstitute native hemocyanin from the HC-NO complex prepared at neutral pH by anaerobic addition of dithionite, 2 mg/ml, following the same procedure as used for reduction of the apo-Cu(II) complex. The recovery of functional hemocyanin was measured by percent recovery of absorption at 575 mu on the basis of that expected according to total chemical copper or protein concentration. Recovery of functional HC was also measured by the disappearance of cupric ESR signal. The latter was estimated by double integration of the Cu(II) signal remaining after reduction. That percentage of the total chemical copper as Cu(II) was subtracted from 100 to give percent total as Cu(I), presumably functional. The correspondence between the total Cu(I) copper by ESR and functional copper at 575 mu was not completely reliable. In three cases 90-95% total copper present as Cu(I) did correspond respectively to 90-95% regeneration of OD at 575 mu. At other times, however, the presence of 70-90% of the total copper as Cu(I) by ESR corresponded to regenerations of as low as 20% OD at 575 mu.

Similar reduction of the HC-NO, prepared at pH 5.0, resulted in the formation of a clear blue solution, just upon standing at 4° C in the presence of dithionite and

nitrogen for four hours. This material had 85% of its total copper as Cu(I) by ESR. On the basis of the extinction of HC-O_2 at pH 5 and 575 mu, restoration of OD in this material was 66%. The color change, however, was not reversible upon flushing with nitrogen. On the basis of total copper present the copper extinction e_{575} M^{-1} cm^{-1} is 270.

b. Magnetic parameters, a discussion. The electron spin resonance parameters, A, the fine structure splitting factor and g// and g$\perp$ have been used to estimate the approximate geometry and degree of covalency in many copper containing organic copper complexes (3, 11, 15, 43, 47, 65, 92, 110) and natural (66, 81, 82) and model (15, 81, 136) biological copper complexes. The molecular orbital approach, to a relationship between the measured resonance parameters and estimated covalency parameters, has been used in many of these studies, (3, 11, 43, 47, 65, 66, 92, 110, 136). More simple approximations, not based on a specific orbital hybridization and evaluation of "S", the overlap integral between copper and ligand orbitals, have been used in some of these studies (15, 81, 82). A brief description of "g", the spectroscopic splitting factor and its relation to α and β, the covalency parameters for in-plane σ and π bonding, should provide sufficient background for the comparison of the HC-NO spectra with spectra from other such studies.

When a free electron in an orbit is exposed to a magnetic field of strength "H", it begins to precess in two ways. It precesses around the original spin direction and its total orbit begins to precess, see Appendix III, a. This is because there is a magnetic moment arising from the spinning of the electron and another magnetic moment arising from the electron traversing an orbital path.

The analogy from electricity is the magnetic moment which arises from a current in a loop and which depends not only on the strength of the current but also the area traversed, or the number of turns.

The angular velocity of precession, ω, is directly proportional to the interaction between the imposed magnetic field with each (either orbital or spin) internal magnetic field and inversely proportional to the orbital or spin angular momentum. The angular velocity of the spin procession of a free electron, ω_s, is approximately twice that of the angular velocity of its orbital precession, ω_o. That is, if a proportionality factor, "k", is unity for ω_o, it is about 2 for ω_s. It is as if the resultant magnetic moment of the spin, μ_s, were twice as long as that expected, Appendix III, b. The constant of proportionality, k, is 2.0023 for a free electron in an external magnetic field. In the most simple case it indicates the proportionality between the orbital and spin angular velocities of precession. It is dimensionless and is also known

as the "g" value and spectroscopic or Landé splitting factor. It is believed that the value may differ from 2.0023 as a result of a contribution to the spin angular velocity of precession, ω_s, from the orbital angular velocity of precession , called spin-orbit coupling. The extent to which this happens is highly dependent on the geometry and occupancy of the orbital. In fact, the g value can be related to a degree of covalency. This will become more clear in the description of g// and $g\perp$.

Since the magnetic moment vectors, u_o and u_s, Appendix III, b., have components parallel and perpendicular to the external magnetic field, H, g// and $g\perp$ may be imagined to be the ratios of the parallel components of $\frac{\omega_s}{\omega_o}$ or perpendicular components of $\frac{\omega_s}{\omega_o}$, respectively. In other words, $g_x = g_y = g\perp$ while $g_z = g//$. Taking Cu(II) in an xy plane of 4 ligands, as an example, Appendix III, b., g// will be greater than $g\perp$ if the hole is in the xy direction, and the structure will resemble an elongated octahedron. The value of $g\perp$ exceeds that of g//, if the hole, or low metal electron density, is in the z direction and the structure will resemble a compressed octahedron (15). For the cubic case $g// = g\perp$. Most Cu(II) complexes have $g// < g\perp$ (65), and in the molecular orbital approximations are considered to be square planar complexes with perturbations along the z axis.

The g values can be directly determined form ESR spectra according to:

$$h\nu = g\beta H$$

where H = field strength in gauss

h = Plank's constant, 2.99×10^{10} erg. sec.

ν = frequency in cm^{-1}

and $\beta = \frac{eh}{4\pi mc}$, 9.27×10^{-21} ergs/gauss

(N.B. $\beta = \mu$ in $\omega = kH\frac{\mu}{p}$

In determination of g//, the field strength, H, is read from the center of the copper nuclear signal, the "fine structure". This is the four line spectrum arising from a copper electron interacting with the nuclear spin of 3/2. The $g_\perp$ or $g_{//}$ value (81) is calculated using the H value at the peak of absorption, zero slope in the original or the point of maximum rate of change in the derivative spectrum. There are five atomic "d" orbitals, three inter-axial and two axial as seen in Appendix IV, a. None has electron density at the nucleus. Since cupric spectra do show nuclear fine structure, however, the "d" electron must have some "s" character. It has been shown, in copper complexes, that the density of ligand orbitals at the Cu nucleus is very small and that the proportion of "s" to "d" character in the Cu atomic orbitals is relatively unchanged by the presence of ligands in most copper complexes (65).

The bonding in transition metal complexes is considered to be electrostatic, ion-ion or ion-dipole and considering the axes in Appendix IV, a., to be inter-atomic axes, it

can be seen that the non axially directed, d xz, d yz, and d xy, orbitals are more stable, Appendix IV, b. Electrons stay away from point charges as much as possible. The energy levels of Cu(II) complexes with bonding and with bonding are given in Appendix V, taken from (65). The g values of a Cu(II) in a square planar complex are approximately related to Δ E xy and ΔE xz by the following equations (15, 81, 65):

$$g// = 2.0023 - \frac{8 \lambda_0 \alpha \beta_1}{\Delta Exy}$$

$$g_\perp = 2.0023 - \frac{2 \lambda_0 \alpha \beta}{\Delta Exz}$$

where

$$\Delta Exy = Exy - E_{x^2-y^2}$$

$$\Delta Exz = Exz - E_{x^2-y^2}$$

λ_0 = Spin orbit coupling constant of the free Cu(II) ion = $-828\ cm^{-1}$

α = Covalency parameter for <u>in-plane</u> σ bonding
(if $\alpha^2 = 1$ the bond is completely ionic
$\alpha^2 = 0.5$ the bond is completely covalent)

β_1 = Covalency parameter for <u>in-plane</u> π bonding
(if $\beta_1^2 = 1$ there are large overlap integrals "S"
$\beta_1^2 = 0.5$ vanishingly small overlap integrals)

β = Covalency parameter for <u>out of plane</u> π bonding

For the case of Cu(II) in a square planar field, B_{1g} represents in-plane σ bonding in group theory notation. B_{2g} represents in-plane π bonding and E_g represents out-of-plane bonding. The sequence of molecular orbitals has not been unambiguously assigned, but "the unpaired 3d electron is almost certainly in the B_{1g} level" (65). In other words, the hole is in the xy direction, the liquid plane, and g// is greater than $g_{\perp}$.

For most Cu complexes, α^2 is in the range of 0.75-0.90. Ceruloplasmin and laccase are exceptions with α^2 values of 0.45 and 0.48, respectively (81). The smaller the value of α^2, the greater the covalent nature of the bond. The trend is in the expected direction with Cu(II) salicylaldehyde-imines and phthalocyanines are more covalent than oxalates (65). The orbital excitation energies ΔE_{xy} and ΔE_{xz}, can often be measured from the visible spectrum. Generally ΔE_{xy} is about 15,000 cm^{-1} while ΔE_{xz} is about 25,000 cm^{-1} (65). They have also been described as the energies needed to raise an electron from the ground state to the first and second excited states, respectively (15, 81). In oxyhemocyanin at room temperature ΔE_{xy} = 17,400 cm^{-1} (575 mu), ΔE_{xz} = 28,600 (340 mu). The magnetic parameters for the HC-NO complex, g_m = 2.074 and g// = 2.299 and A = 0.017 cm^{-1}, characterize the symmetry as elongated octahedral, similar to most Cu(II) complexes.

The α^2 value for HC-NO complex can be estimated using these values in the equation (65, 81):

$$\alpha^2 = -(A/P) + (g//-2) + 3/7(g_\perp - 2) + 0.04$$

with $P = 0.036\ cm^{-1}$ (1). The value is close to 0.49, indicating a high degree of covalency for the in-plane sigma bonding, as was found for both ceruloplasmin and laccase (81).

c. Source of the ESR hyperfine structure and the low temperature optical spectra. For awhile it was considered possible that the hyperfine arose from Cu(II)-Cu(II) spectrum which are distinctly different from the HC-NO spectrum. The cupric acetate ESR spectrum has 1) no $g_\perp$ structure 2) a 7 line fine structure with A = 70 gauss, and 3) it does not follow Curie's law, i.e. the intensity of the signal decreases as the temperature is lowered. This last feature was finally interpreted (11) as a depopulation of an excited triplet state, the only paramagnetic state in this compound. The HC-NO spectrum has 1) a definite $g_\perp$ structure, 2) an 8-9 line hyperfine structure, with A = 10-12 gauss and 3) does follow Curie's law, i.e. the intensity increases as the temperature is lowered. In addition, the power saturation studies at four different temperatures, Figure 22, indicate that the fine and hyperfine structure do not arise from the delocalization of an electron over the same nucleus.

Cobalt 60 gamma irradiation has produced free radicals

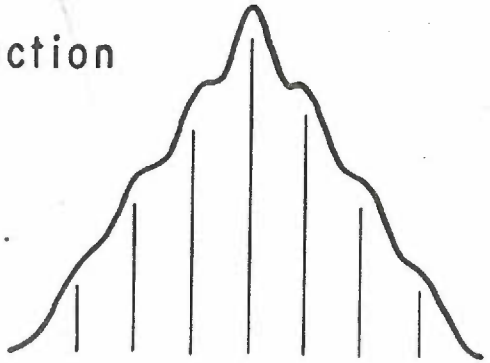
Figure 23

Hyperfine structure models for the low temperature ESR spectrum of HCNO.

HYPERFINE STRUCTURE MODELS

$Cu^{++}-Cu^{++}$ Interaction

100 gauss



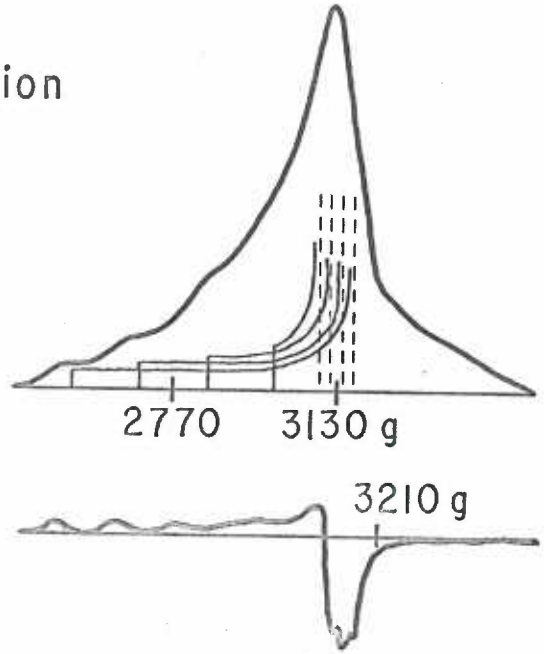
B. Bleaney & K.D. Bowers (1952)

Hyperfine structure from cupric acetate crystal

derivative curve

$Cu^{++}-N$ Interaction

164 gauss



R.H. Sands (1955)

Cu^{++} in silica glass

integrated curve

derivative curve

formed by the loss of the H from the -CHR- group of the polypeptide chain (45). Delocalization of the free electron over hydrogen nuclei gave fine structure in the region of 3.2 kg. Lines with 18-22 gauss separation, with a possible substructure of 2.5 gauss width were observed. Because of the uniform "A" value of 10-12 gauss and the loss of hyperfine structure with increasing proton concentration in the HC-NO spectrum, it is unlikely that proton resonance contributes to the hyperfine of the HC-NO signal. The HC-NO hyperfine structure, on the other hand, corresponds quite closely, in both relative intensity and half width, to that from Cu-N interaction found in Tris complexes of Cu(II) (3) and Cu(II) phthalocyanin (66). The magnetic parameters of Cu(II) 1,10 phenanthroline and 2,2' dipyridine, respectively, were $g_{//} = 2.273\text{-}2.268$ and $g_{\perp} = 2.062\text{-}2.046$ and $A = 0.0161\text{-}0.0166$ cm^{-1} (3). The fine structure of these spectra showed little "s" character or coupling of the unpaired electron to the Cu nucleus. The hyperfine structure of both complexes which contained naturally occurring copper, consisted of 13 components. When samples containing only Cu 63 were prepared and examined, only 9 components were found. This indicated that the unpaired Cu electron was interacting with only four equivalent nitrogens. The four extra lines arose from the slightly staggered superimposition of the same interaction in the less abundant isotope Cu 65. The two Cu isotopes have

slightly different magnetic moments.

If it is the copper electron which gives rise to both fine and hyperfine structure in the HC-NO spectrum, then, considering the probable presence of both Cu isotopes, the 9 component hyperfine would indicate delocalization over only 2 nitrogens. If the electron giving rise to the hyperfine is not the copper electron, then, of course, the 9 component hyperfine would indicate delocalization over four equivalent nitrogens. The fine and hyperfine structure, therefore, may arise from the delocalization of one electron over copper and nitrogen nuclei or two electrons, one in a copper atomic orbital, the other in a nitrogen atomic orbital. The specific possibilities are:

1. 1 electron in 1 CuN molecular orbital (MO)
2. 1 electron in 1 ground state CuN MO
   1 electron in 1 excited state CuN MO
3. 1 electron in 1 Cu atomic orbital (AO)
   1 electron in 1 N AO
4. 1 electron in 1 Cu AO
   1 electron in 1 N AO, with transfer between atomic orbitals.

In the first case, the spin relaxation time is the same for the Cu signal and N signal. That is, the apparent difference in power saturation behaviour may be due to modulation broadening which is noticed in the nitrogen signal first. If this is true, the area (height x ΔH^2) of the Cu signal should have the same dependence on power

as the area of the nitrogen signal. From Fig. 24 this is seen not to be the case. In the second possibility, the saturation properties may truly differ, as we have observed to be the case. The N signal should also increase or decrease relative to the Cu signal as temperature is varied at a single incident power setting. Figure 25 shows this to be true. This automatically excludes the third case in which power saturation properties may differ but the temperature properties should be the same. The fourth case, along with the second could have characteristics consistent with the observations. If there are two different electrons in two different atomic orbitals and some form of transfer or exchange exists between them, it would be possible to see a true difference in both power and temperature saturation. Unfortunately, then, a closer examination of the power saturation data at four different temperatures does not allow a distinction between the two major possibilities. In one possibility the paramagnetism arises from one electron distributed over two nuclei, in the other it arises from two electrons in different atomic orbitals.*

It is also difficult to distinguish between these two possibilities on the basis of the variation of the

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*The interest and suggestions of Dr. Barry Allen are gratefully acknowledged.

Figure 24

Power saturation behavior of the fine and hyperfine components of the ESR ppectrum of HCNO at $77^{\circ}K$. Confirmation of the different behavior of the two components, by consideration of modulation broadening.

height x $(\Delta H \text{gauss})^2$ = area under peak

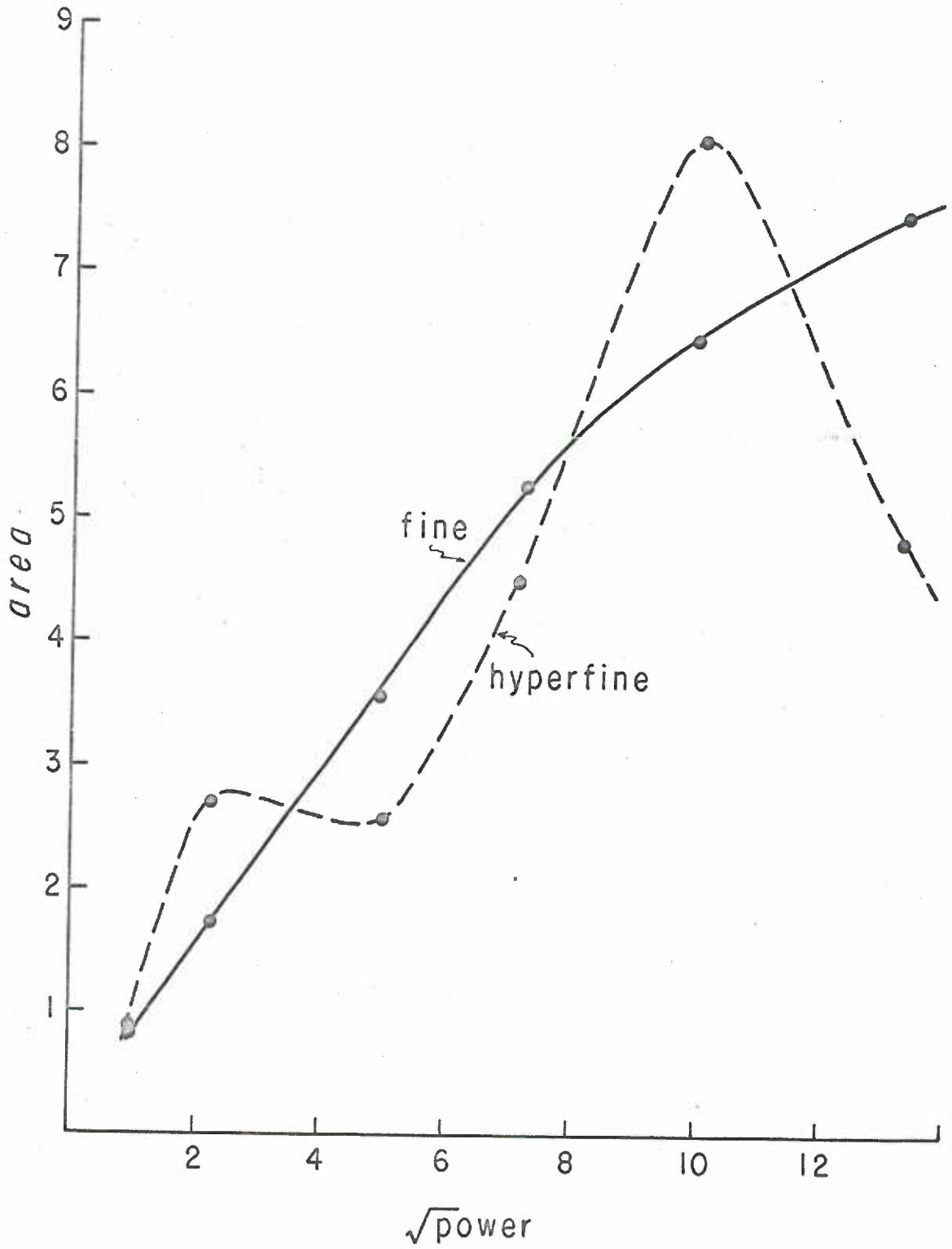
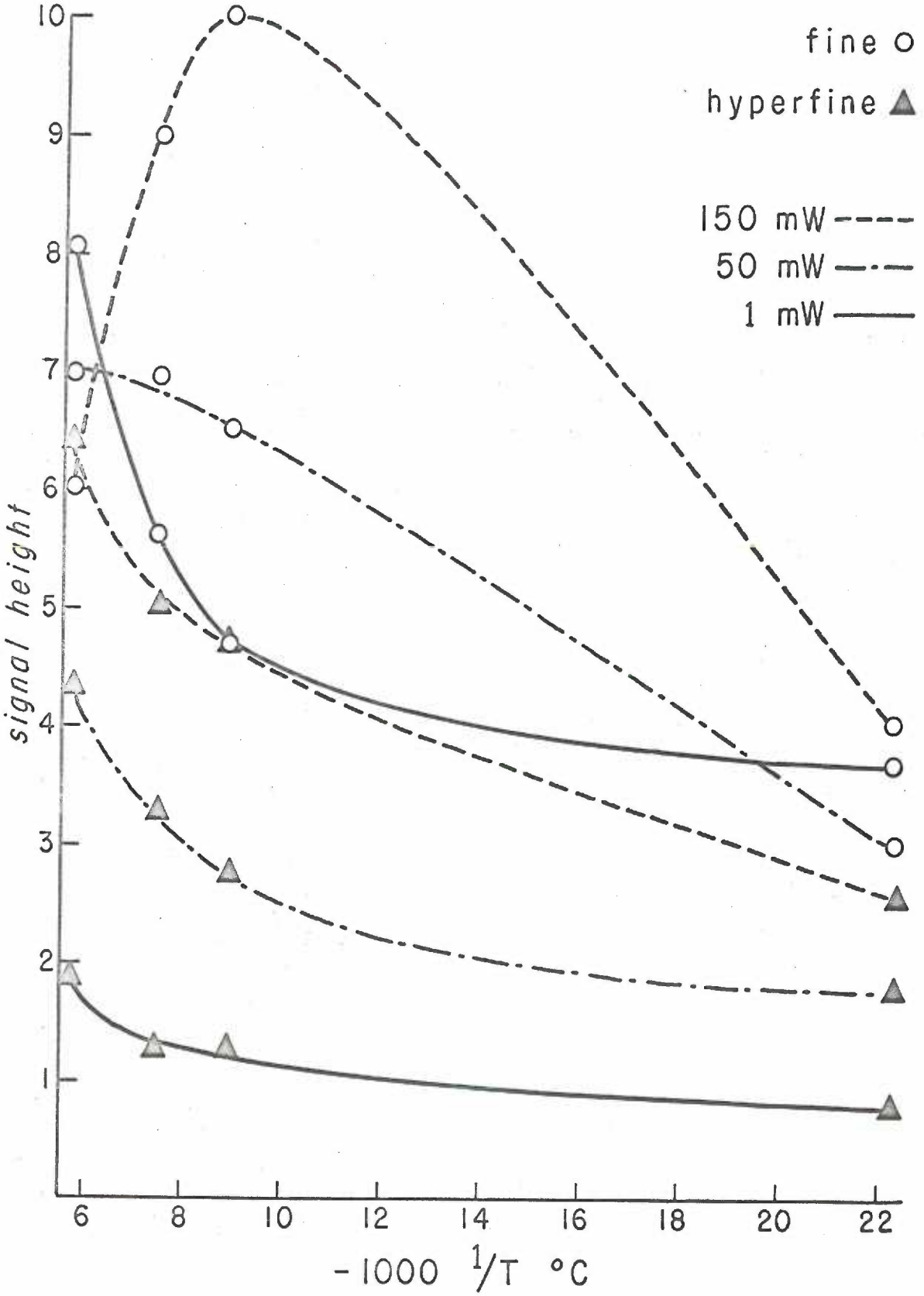


Figure 25

Temperature saturation behavior of the fine and hyperfine components of the HCNO spin resonance signal at three different incident powers; 1 mW, 50 mW and 150 mW.


fine o
hyperfine ▲
150 mW
50 mW
1 mW
signal height
10
9
8
7
6
5
4
3
2
1
6
8
10
12
14
16
18
20
22
-1000 1/T °C

Cu(II) ESR signal with pH. This is because both may be consistent with the variation of hyperfine and total cupric expression with pH. An increased hydrogen ion concentration in either phosphate or Tris buffer was accompanied by a definite increase in total copper expressed as cupric by ESR, Fig. 19, and the virtual disappearance of hyperfine structure, Fig. 20. The amount of delocalization of the same electron over different nuclei could be affected by pH, and could also give rise to different power saturation curves as observed in Fig. 24. If there is a second delocalized electron, not from the copper, the total concentration of paramagnetic species around $g = 2$ may also increase with decreasing pH. The increased hydrogen ion concentration may independently decrease the "s" character of the nitrogen atomic orbitals. In the case of one electron over Cu and N nuclei, the increased paramagnetism at 3.2 kg would arise from the same phenomenon as the decreased hyperfine intensity. In the case of two electrons in two atomic orbitals, the increased paramagnetism and diminished hyperfine structure may arise from different phenomena.

A Cu(II) ESR spectrum develops upon reaction of hemocyanin with NO. This indicates that there has been at least a partial transfer of electrons away from the copper-containing active center, possibly toward the NO molecule. This last idea would be consistent with the belief that the

density of ligand orbitals at the copper nucleus is very small in most Cu(II) complexes (65). It would also be consistent with the proposed accomodation of metal electrons by molecular orbitals, derived from the π electron system of NO, in transition-metal nitrosyl complexes (48). For the purposes of consolidating the observations on HC-NO, an illustrative model will be used. It will be considered unlikely that we are observing any paramagnetism from an electron originally belonging to NO. It should be remembered, however, that the data may be consistent with less conventional models, e.g. Cu^{o}-$NO.^{+}$

The paramagnetism will then be considered to arise either from the unpaired electron remaining at the copper or both this electron and one delocalized over nitrogens. In the former case, the hyperfine structure may arise from only two equivalent nitrogens because of the isotope considerations. If the 8-9 component hyperfine arises from an electron other than the Cu electron, it would indicate delocalization over four equivalent nitrogens. It is not possible to distinguish between a ligand nitrogen and that in NO in these spectra because the nitrogen nucleus in NO may indeed be "equivalent" to ligand nitrogen.

The presence of both copper isotopes has been observed in Cu(II) phthalocyanine (66). The presence of either two copper isotopes or two coppers of slightly different ground state energy in native HC is indicated by both ESR

and low temperature optical spectra of the HC-O_2, Fig. 18 and Fig. 26. The two peaks of the cupric g $\perp$ signal of HC-NO, Fig. 21, do not behave in an exactly parallel manner during power saturation. The low temperature optical spectrum of oxyhemocyanin, Fig. 26, reveals four absorptions, two in the region of ΔE xy and two in the region of ΔE xz. If the 660 (15,200 cm^{-1}) and 420mu (23,800 cm^{-1}) are assumed to belong to one type of copper and the 550-60 (18,050 cm^{-1}) and 360 mu (27,800 cm^{-1}) to a second, the values of ΔExz/ ΔExy are 1.56 and 1.54, respectively. This ratio has been calculated for a number of Cu(II) complexes (65). The values obtained for oxyhemocyanin are similar to those found for denatured laccase (1.51), Cu(II) acetylacetonate (1.54) and Cu(II) phenylporphin (1.5.).

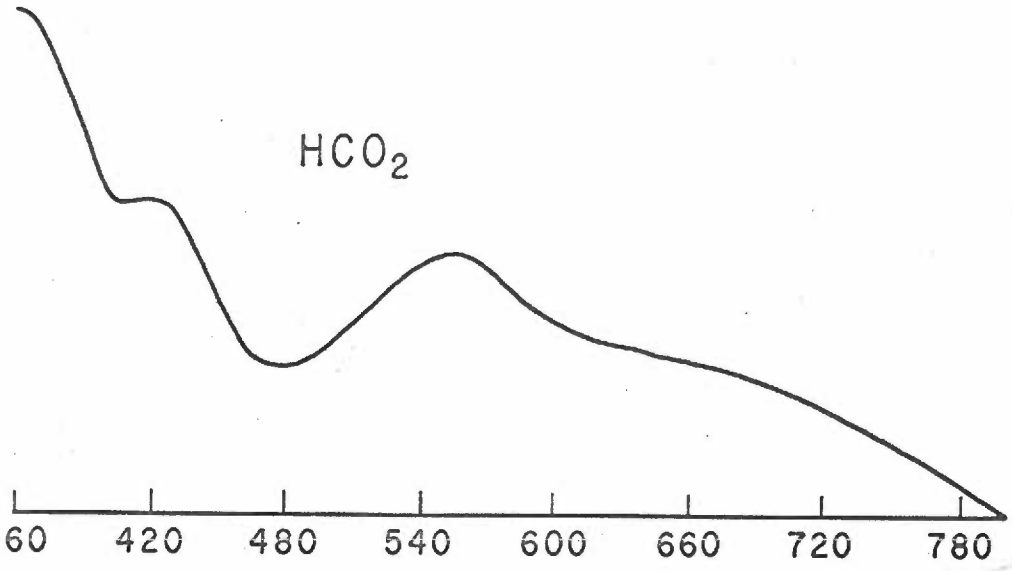
The derivative spectrum of oxyhemocyanin, Fig. 18, b., is very close to that seen in Cu(II) phthalocyanine and ascribed to the presence of the two relatively abundant copper isotopes (66). This phenomenon may contribute slightly to the broader fine structure that was seen in Tris complexes with both a mixture of Cu^{63} and Cu^{65} (3).

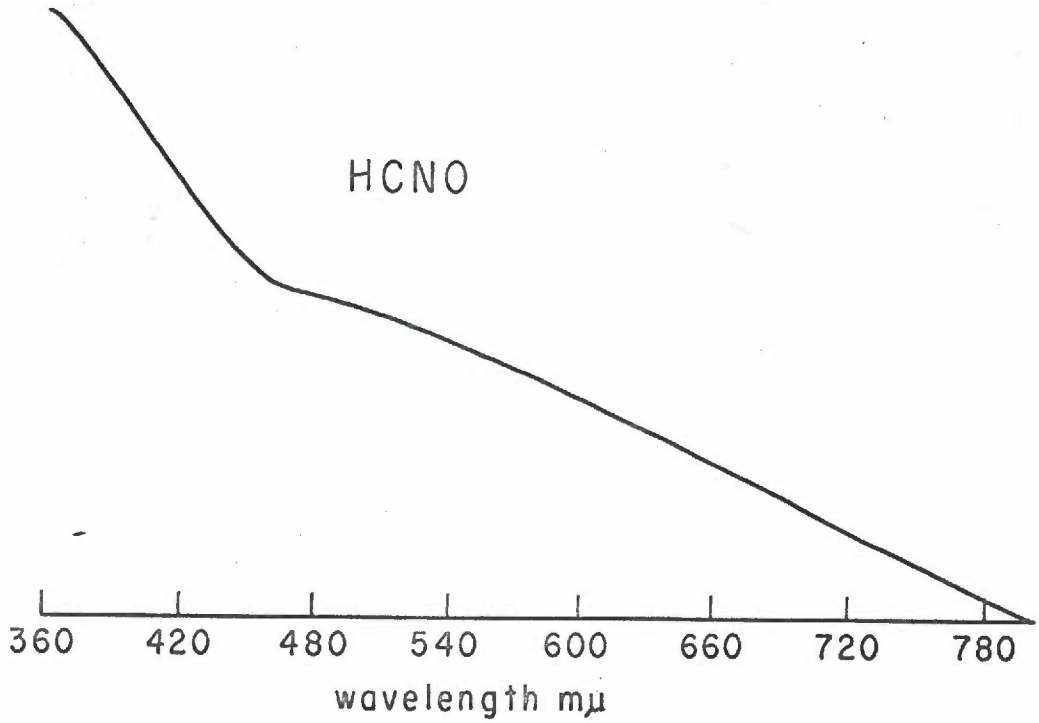
The low temperature spectrum of HC-NO, fig. 26, exhibits two broad absorptions, 560-600 and 350-70 mu. Since the two coppers of slightly different ground state energy, be they in different environments or isotopes of one another, appear to be involved in oxygen binding, it is possible

Figure 25

Low temperature optical spectra of HCO_2 and HCNO.

LOW TEMPERATURE OPTICAL SPECTRA





that they may also be involved in NO binding. In this case it may be reasoned that the degree of oxidation at the active site, produced by NO, is sufficiently greater than that produced by O_2 to obliterate the distinct transitions of each copper. Other interpretations are also possible. We may, for example, be observing the predominance of one type of copper in the NO complex.

In summary, both low temperature optical and ESR spectra indicate the presence of two coppers of slightly different ground state energies, possibly the Cu 63 and Cu 65 isotopes. The hyperfine structure in the ESR spectrum of HC-NO may arise from the delocalization of the cupric electron over two equivalent nitrogens. The possibility that a different electron is delocalized over four equivalent nitrogens, however, has not been ruled out.

GENERAL DISCUSSION

I Ground State Characterization

A. The Number of Coppers at the Active Site

a. By Statistical Ratio. The statistical ratio of 2 Cu/O_2 may be a true representation of the ratio at the active center. It would also be observed if only one copper interacts with O_2 at each active site while a second is either non functional or maintains some structural stability of the protein.

It may be possible to determine whether one or two coppers are functional at the active site by polarographic determination of the ratio in partially denatured hemocyanin. The only unequivocal result from such an experiment would be a decreased Cu/O_2 ratio, indicating that less than two coppers are essential for the binding of an oxygen molecule. The possible results from such an experiment will be considered in the following discussion with reference to Fig. 27. The difficulties in analysing the results of such an experiment arise from not being able to determine whether the partial denaturation occurred in a random or ordered fashion. The extent of denaturation would be equivalent to percent total copper expressed as Cu(II) by ESR. The conditions should be such that this value is close to that which can be obtained from loss of optical activity.

Figure 27

Active site configurations possible in partially denatured hemocyanin.

Possible distributions of Cu^{++} in partially denatured hemocyanin.

a. 25% Cu^{++}

I	II	III	IV
Cu^{++} Cu^{++}	Cu^{++} Cu	Cu^{++} Cu	Cu^{++} Cu
Cu Cu	Cu^{++} Cu	Cu Cu	Cu Cu
Cu Cu	Cu Cu	Cu^{++} Cu	Cu Cu
Cu Cu	Cu Cu	Cu Cu	Cu^{++} Cu

b. 50% Cu^{++}

I	II	III	IV	V
Cu^{++} Cu	Cu^{++} Cu^{++}	Cu^{++} Cu^{++}	Cu^{++} Cu^{++}	Cu^{++} Cu^{++}
Cu^{++} Cu	Cu^{++} Cu^{++}	Cu Cu	Cu Cu	Cu^{++} Cu
Cu^{++} Cu	Cu Cu	Cu^{++} Cu^{++}	Cu Cu	Cu Cu
Cu^{++} Cu	Cu Cu	Cu Cu	Cu^{++} Cu^{++}	Cu^{++} Cu

c. 75% Cu^{++}

I	II	III
Cu^{+} Cu^{+}	Cu^{+} Cu	Cu^{+} Cu^{+}
Cu Cu	Cu^{+} Cu	Cu Cu
Cu Cu	Cu Cu	Cu Cu
Cu Cu	Cu Cu	Cu Cu
		Cu^{+} Cu
		Cu Cu
		Cu^{+} Cu
		Cu Cu

In the case of 25% denaturation one could distinguish a random from an ordered denaturation, but distinguishing between structural and functional coppers is impossible. In Fig. 27, a., if the coppers were lost in pairs, case I, ordered denaturation, the ratio would remain 2 Cu/O_2. The ratio would be higher only if the denaturation occurred in a random fashion, II, III, IV. If the ratio were less than two, i.e. 6 Cu(I)/ $4O_2$, less than two coppers are needed at the active site.

Now consider the oxygen capacity of 50% denatured material, Fig. 27, b. If there is no oxygen binding to the material at all, it is probable that denaturation occurred at each active site in a uniform fashion, b., I. The same result would be obtained if only structural coppers were uniformly oxidized, leaving the reduced active sites intact but non-functional. Structural and functional coppers could not be distinguished if all activity is lost. On the other hand, if the copper present as Cu(I) binds any oxygen at all, the denaturation can be either ordered, II, III, IV or random V. A ratio of 2 Cu(I)/O_2 could be obtained from II, III and IV and as in native material not distinguish structural from functional Cu. If a higher ratio were obtained it would once again be an indication of random denaturation V, discounting the possibility of a non uniform mechanism of denaturation, Fig. 3. However,

if less than two coppers are needed at the active site b., V would give a ratio close to two, making it indistinguishable from II, III and IV. Only a ratio of less than 2 Cu(I)/ O_2 would unequivocally indicate a non correspondence between the statistical ratio and number of coppers at the active center in native material. The same holds true for the 75% denatured material, Fig. 27, c. A ratio of two wouldn't give an indication of type of denaturation since it could arise from II or I, a statistical ratio in a small section of the native material. A ratio higher than two would again indicate random destruction III, indistinguishable from I if less than two coppers are needed at the active site.

These considerations make it clear that knowledge of the Cu(I)/O_2 ratio in partially denatured material may indicate the type of denaturation effected by agents such as urea or chloroform in simple cases. A ratio of less than 2 Cu(I)/O_2 in any case, however, would indicate that less than two coppers are necessary at the active site.

b. From Spectral Information. It is not possible to conclude that two coppers bind one molecule of oxygen at the active site from the statistical ratio, even in conjunction with the optical density information. The ϵ_{575} $M^{-1}cm^{-1}$ copper is 600 if all coppers interact with oxygen. This would be consistent with a favored d-d transition or a low charge transfer transition for each copper, as summarized in the introduction. If, however, only half

the Cu(I) coppers were functioning to bind oxygen, one partially oxidized copper at each active site, the extinction would be doubled to 1,200. A number of cupric complexes are known to have charge transfer bands with λ max in this region and e_{max} $M^{-1}cm^{-1}$ copper, in the order of 10^3 (15), for example, Cu_2 (diazoamino benzene)$_4$ has λ max at 625, with e_{max}, 1850 and Cu (oxalyl dihydrazone of formaldehyde)$_2$ has λ max at 580 with e_{max} of 6700. In addition "blue proteins", with λ max 520-620, have extinctions in the order of 10^3-10^4, and a partially oxidized Cu(I) appears to be involved. In the resting forms of both ceruloplasmin and laccase, the Cu(II) ESR signal accounts only for about 50% of the total copper present in these proteins (82). It has been suggested that Cu(I)-Cu(II) interactions contribute to the intensity of visible absorptions exhibited by these proteins (138). It is most likely that the intense blue of oxyhemocyanin is due to some Cu(I)..O_2 charge transfer complex (82, 87). The interest in whether two coppers are necessary, for this complex, or not arises from the attempt to understand the chemical mechanism of oxygen binding. A decision in this regard is as difficult when considering O_2 interactions with other metal chelate carriers as it is when trying to analyse statistical ratios or optical spectra.

c. Analogy with Known Synthetic Reversible Oxygen Carrying Chelates. All truly reversible Co(II) chelate

carriers contain two metal atoms per O_2 molecule (133). A Mn(II) phthalocyanine reacts with molecular oxygen with the same ratio, but not reversibly. On the other hand, Fe(II) in hemoglobin reversibly associated with a ratio of 1:1 and in dimethylglyoxime, with a possible ratio of one metal to two oxygen molecules (31). Mn(II), Fe(II), Ni(II) and Cu(II) chelates, analogous to the Co(II) chelates, have been prepared but none reversibly absorb oxygen (16). An unstable yellow brown adduct between one Cu(I) and an O_2 molecule has been postulated for a Cu(I)-cysteine complex which adds O_2 reversibly (34). The adduct is not a true oxygen carrier, however, since in the presence of oxygen for a few minutes it irreversibly oxidizes to a violet brown radical complex and finally oxidizes to Cu(II). Recent X-ray analysis (52) indicates that the structure of monopyridine copper (II) acetate contains copper-O_2-carbon bridges.

Fifteen years ago the combining ratio of hemocyanin with oxygen was sufficient to imply "that the prosthetic group contains two atoms of copper in close association" (102). This remains true. Specific functions of atoms at active sites (114) and interactions between active sites have become evident, however, making our knowledge appear less complete.

B. The Nature of Bound Oxygen

On the basis of the $e_{575}\ (O_2)^{-1}$ determined in Experimental Section I, A, an extinction $e_{340}\ (O_2)^{-1}$ can be calculated. The e_{max}/Cu for the 375-340 band of oxyhemocyanin has been reported to be 6,000 (138). In oxyhemocyanin from Cancer magister it is only about 3,700 or approximately six times that found at 575 mu (118). Multiplication of the values found for $e_{575}\ (O_2)^{-1}$, with buffer or deoxyhemocyanin blanks, gives an approximate value of $e_{340}\ (O_2)\ M^{-1}cm^{-1}$ of 4,400 for the difference spectrum and 7,600 when a buffer blank is used. Optical properties of known species of oxygen are summarized in Table VII. The oxygen molar extinctions estimated for oxyhemocyanin are larger than any which have been observed for a particular species of oxygen.

II Excited State Characterization

A. Triplet Quenching; Determination of the Order of Decay

Triplet photoactivation spectra and half lives were observed to vary in the presence and absence of copper or oxygen (Experimental Section I, B.). All decay curves in their initial and major portions were found to be first order; meaning that the primary quenching process was predominantly an intra-molecular one in both the frozen oxygenated and deoxygenated solutions. This not only indicates that all solutions were apparently free of contaminating

TABLE VII

Summary of optical properties of proposed species of oxygen in aqueous solutions.

TABLE VII

Postulated species	max (mu)	$\epsilon, M^{-1}cm^{-1}$ 25° C	Reference
Hydrates	200	30-60	(54)
$(H_2O)_nO_2$	215	4-15	(54)
HO_2	230	1,150	(27)
HO_2 pH < 7	254	2,750 ± 400	(27)
O_2^- pH > 7	240	1,060	(27)
	254	980	(27)
O_2^- pH = 7	250	3,140 ± 470	(27)
O_3	260-270		(55)
O_3	420-440		(55)
Transient forms at pH > 10			
H_2O_3, $O_3^=$ or O_3^-	430-440		(27)
$O_4^=$, $O_2^=$, $HO_5^=$ or $HO_3^=$	240		(27)

quencher species, but that both oxygen and NO complexes of hemocyanin act as single molecules and not loosely associated adducts with respect to triplet activity at this temperature. This may not be true at higher temperatures. Deviation from linearity in the semi-log plots of concentrations were often observed in the last stages of decay at -196° C. The actual quenching mechanism may be either an intra-molecular, first order process or an inter-molecular, second order process. Knowledge of the quenching mechanism, between copper or oxygen and the protein triplet would be valuabe to further characterize the triplet state in the protein.

The two mechanisms might be distinguished by observation of the effect of partial oxygenation on the triplet parameters of hemocyanin at low temperature. If life times decreased with increasing concentrations of bound oxygen, a second order process would be indicated. The absence of such an effect, always experimentally less satisfying, would have to be taken as an indication of a first order process. At this time there is no apparent way in which the intra-molecular process could be independently established for a protein triplet. In other studies of triplet decay processes (94) first order decay rates varied directly with solvent viscosity. Similarly, if half lives of triplets from oxyhemocyanin increased directly with viscosity, an independent measurement of any intra-molecular quenching

would be possible. Such an experiment would be difficult if not impossible at the present time. Amino acid and protein triplets cannot be measured at room temperature (96), as many aromatic hydrocarbons can. Frozen solutions have been used for ESR determinations. Increasing temperature from -196 to -10° C would not provide the necessary viscosity change (94). In addition, any triplet concentration and half life variation with temperature would almost certainly appear to be first order since they would arise only from increased lattice vibrations. Even if fluorescence (singlet) and triplet activation spectra were found to be identical and appropriate non-denaturing solvents, less viscous than glycerol, were in hand, a distinction between triplet quenching in proteins by a first or second order process would not be possible. A decreased excited state concentration with increasing viscosity could be characteristic of either mechanism and singlet life times, 10^{-8}, are too short to measure.

B. Characterization of Excited States in Proteins

With a view toward increasing the information about the excited states in proteins, the determination of a fluorescence (singlet) photoactivation spectrum for apo and native hemocyanin, in the presence and absence of oxygen, would be valuable. Comparison between these and corresponding triplet spectra would reveal energy transfer processes, if they existed. To make the spectra comparable,

a fluorescence spectrum using any uv wavelength for activation, 290 mu, for example, would first have to be recorded to determine the wavelength of maximum activation. A second spectrum, comparable to the triplet activation spectrum could then be recorded with the detector set at that λ max value. The relationship between triplet and singlet quenching has recently been studied (39).

Exposure of insulin or ribonuclease to acid, urea or dodecyl sulfate resulted in enhancement of fluorescence of the tyrosyl residues (25). This indicates that tyrosyl residues are situated in both native proteins so that fluorescenwe is diminished. A similar study, measuring the effect of partial denaturation on both triplet and singlet activation spectra could be carried out for hemocyanins. An agent which unfolds the protein with minimum oxidation of copper would be the most desirable, to minimize quenching by Cu(II). If unfolding by all agents results in significant amounts of Cu(II), the enhancement or change sought after would be easily masked by paramagnetic quenching. The same experiments on well characterized apo-protein solutions would avoid these problems. It is likely that aromatic residues involved in triplet activity are also involved in subunit binding since uv irradiation, alone, can cause dissociation of hemocyanin into subunits (122, 123).

Proteins which reversibly associate with gases or form complexes which can be easily dissociated upon irradiation may be peculiarly suited to initial attempts at characterizing excited states in biological materials. The electron systems involved may be the same as those involved in the biological function and variation among excited state parameters may be directly correlated with an observable alteration in activity. Transitions between energy levels comparable in magnitude to triplet transitions can occur in all protein solutions since they are all sensitive to uv (4, 111) irradiation. The role of the triplet state in biological materials, where these levels are less obviously linked with function, is still unknown. Transfer of the energy of electronic excitation can occur across distances greater than 50 Å by a process which has been called "inductive resonance" and has recently been proposed to contribute to enzyme-coenzyme interactions (115).

C. Nature of Bound Oxygen and the Triplet State

No triplet signal was seen between 950 and 1,950 gauss in unirradiated samples. The ground state of the oxygen molecule in the gas phase is a triplet (49). Attempts to observe the ESR spectrum of either atomic or molecular oxygen in polycrystalline inert materials have been unsuccessful at low frequencies and low fields (60). By using both high magnetic fields and high frequencies,

two resonances, associated with molecular oxygen were recently observed (61). They were at 37 kMc/sec and 71 kMc/sec for oxygen trapped in a single crystal of a β quinol clatherate at liquid helium temperatures.

The failure to observe a triplet signal from oxygen in the hemocyanin experiments was to be expected. These observations are also consistent with recent evidence for the existence of altered forms of oxygen in aqueous solutions at room temperature. Two hydrates of molecular oxygen have been characterized (53, 54). Dissolved oxygen as O_2^- has an absorption maximum at 240 mu while the HO_2 radical absorbs at 230 mu (27) and the O_2 ion at 340 mu, (55). Transient species, HO_2^- or HO_3 and O_3^- or H_2O_3, have been proposed to account for pH dependent absorptions seen at 240 and 430 mu, respectively (27). A mechanism to describe the reversible binding of oxygen to hemocyanin need not account for an undetectable triplet from oxygen.

D. The Energy Required for Photooxidation of Cu(I) in HC, a Speculation.

There is no correlation possible between mV, total power of irradiation and the electrochemical potential, in mV, for the oxidation of a cuprous ion, since the latter is of course measured relative to the standard hydrogen electrode in which the ionization $H_2 \quad 2H \quad 2e^-$ has been set at 0.00 mV (73). If an absolute voltage could be

experimentally determined for this reaction and both the bond dissociation and electron ionization were known in electron volts, a simple proportion between this ratio and the potential for the cuprous cupric couple might give the electron affinity of Cu(I) in electron volts:

$$\frac{\text{mV H couple}}{\text{eV H reaction}} \approx \frac{\text{mV Cu(I) couple}}{\text{eV affinity}}$$

This proportionality between power and energy was assumed to be valid in an attempt to estimate a wavelength of minimum energy required to photooxidize Cu(I) in HC. The reduction potential of the Cu(I)-Cu(II) couple is -0.167V at pH 7 (15). Under standard conditions of unit activity, the negative value of the oxidation potential implies that the oxidized form of the couple is a stronger oxidizing agent than the H^+ ion in solution. This means that Cu(II) has a greater affinity for an electron than does H^+. In other words, it takes more energy to reverse the cuprous couple $Cu(I) \longrightarrow Cu(II) + e^-$, than it does to reverse the hydrogen couple $H_2 \longrightarrow 2H^+ + e^-$. It will be assumed that the experimentally determined energy of dissociation of the hydrogen molecule 4.72 electron volts is the major energy gradient in the hydrogen couple. This is the energy contained by the system in the ionized state and the pressure is even higher in the cuprous-cupric couple since Cu(II) picks up an electron more easily than H^+.

Within the limits of this very approximate estimation, then, one would expect that an energy greater than 4.7 ev would be required to ionize an electron from Cu(I). If it is an energy only slightly greater, for example, any intensity of light with wavelength 250 mu (frequency 40,000 cm^{-1}, 4.9 ev) or shorter, may cause the ionization of an electron from Cu(I), in a simple inorganic system, as the primary photochemical reaction. It is expected that it would be even more difficult to photooxidize Cu(I) in a biological system, not only because of the more negative potential (15) but also because of side reactions of amino acid residues with highly ionizing irradiation (45).

SUMMARY

An average statistical ratio of 1.84 Cu/O_2 was determined for *Cancer magister* hemocyanin using an oxygen electrode. Similar experiments, using partially denatured hemocyanin, may clarify the correspondence between the statistical ratio and the actual number of copper atoms at the active center. Preliminary observations were made on the triplet state in frozen solutions of hemocyanin. Both copper and oxygen decreased the triplet yield over the range of activating wavelengths. Only oxygen consistently decreased the triplet half life.

Four attempts were made to produce an ESR-detectable methemocyanin:

1) Deoxyhemocyanin was combined with excess amounts of H_2O_2. No Cu(II) ESR spectrum could be observed in samples which gave chemical and optical evidence of being methemocyanins. In the presence of excess H_2O_2 *Cancer magister* deoxyhemocyanin had a unique optical spectrum at both room temperature and low temperature.

2) Apo hemocyanin was dialyzed against cupric copper. The Cu(II) ESR signal from this material had several features which have been observed only in ESR spectra of ceruloplasmin and laccase.

3) An attempt was made to photoxidize the copper at the active center using intense light at 340 and 575 mm. Neither a Cu(II) signal nor an increased paramagnetism at 32 kg was observed.

4) Deoxygenated Cancer magister reacted with nitric oxide gas to form a clear green complex which has a Cu(II) ESR signal. The signal accounts for from 16-80% of the total copper present, depending upon conditions of the reaction.

A model of the active center, containing two copper atoms of slightly different ground state energies, was proposed. It is consistent with low temperature visible and ESR spectra observed for both HCO_2 and the HCNO complex.

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APPENDIX I

Calculation of energy in electron volts (V) associated with a particular wavelength:

$$\frac{E}{N} = eV = \frac{hc}{\lambda}$$

where

$e = 1.6 \times 10^{-20}$ emu absolute

$h = 6.5 \times 10^{-27}$ erg sec

$c = 3 \times 10^{10}$ cm/sec

$\lambda = 2.5 \times 10^{3}$ Å

V = electron volts

(1 electron volt = 8,068 cm^{-1} = 23.063 Kcal)

$$V = \frac{hc}{e\lambda} = \frac{6.5 \times 10^{-27} \ (3 \times 10^{10})}{1.6 \times 10^{-20} \ (2.5 \times 10^{3})} = 4.9 \text{ electron volts}$$

4.9 electron volts x 23.063 Kcal/V = 112.55 Kcal/mole photons at 250 mu.

APPENDIX II

Calculation of einsteins from a millivolt reading at a specific wavelength:

1. Thermopile registers 0.6 mV upon illumination at 250 mu with slit ratio, entrance/exit: 6/6.

2. Conditions of illumination during an experiment are simulated in distance of beam from thermopile and flux. Illumination of a low temperature ESR tube, height 2 cm, width 0.3 cm = 0.6 cm^2 flux.

3. $\frac{0.6 \text{ mV}}{0.0592 \frac{\text{mV cm}^2}{\text{mW}}} = 10.1 \frac{\text{mW}}{\text{cm}^2}$; $10.1 \frac{\text{mW}}{\text{cm}^2} \times 0.6 \text{ cm}^2 = 6.06 \text{ mW}$

   $6.06 \text{ mW} = 6.06 \times 10^{-3} \text{W}$

   $6.06 \times 10^{-3} \text{W} \times 24 \times 10^{-4} \frac{\text{Kcal}}{\text{W}} = 14.59 \times 10^{-7}$ Kcal

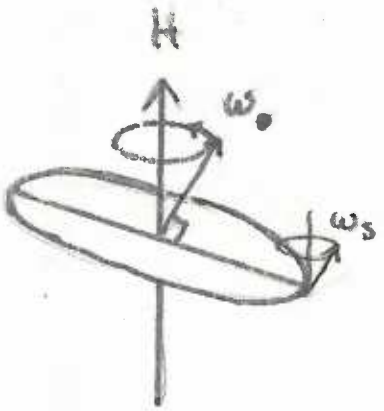
   then

   $\frac{14.59 \times 10^{-7} \text{ Kcal actual}}{112.55 \text{ Kcal/mole photons}} = 1.291 \times 10^{-8}$ moles of photons or Einsteins

4. A 0.36 mv reading was obtained at 250 mu with slit ratio 3.6/2.0. This is equivalent to 7.78×10^{-9} Einsteins. See the entry at 250 mu in Figure 6.

APPENDIX III

a.



$$\omega \text{ orbit} = kH \frac{\mu_o}{P_o}$$

$$\omega \text{ spin} = kH \frac{\mu_s}{P_o}$$

where:

ω = angular velocity of precession

H = strength of external field (gauss)

μ = magnetic moment (ergs/gauss)

P = angular momentum (erg sec)

k = integral number of proportionality between angular momenta

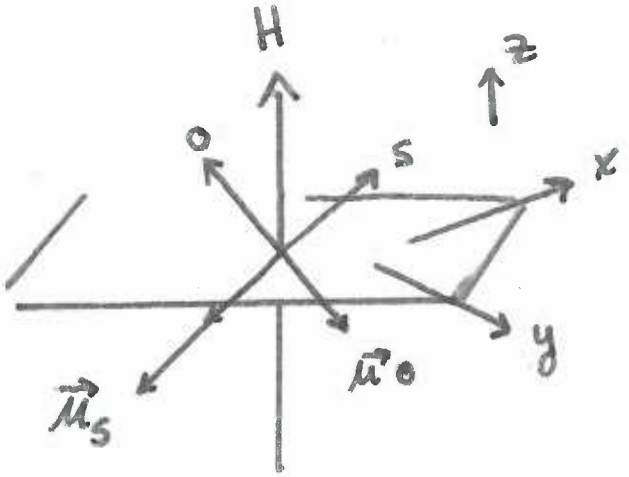
the units of ω are sec^{-1}

since $P = k \frac{h}{2\pi}$ erg sec and $\mu = \frac{eh}{4\pi mc}$ $\frac{\text{ergs}}{\text{gauss}}$

therefore $\omega = \text{gauss} \frac{\text{ergs/gauss}}{\text{erg sec}}$

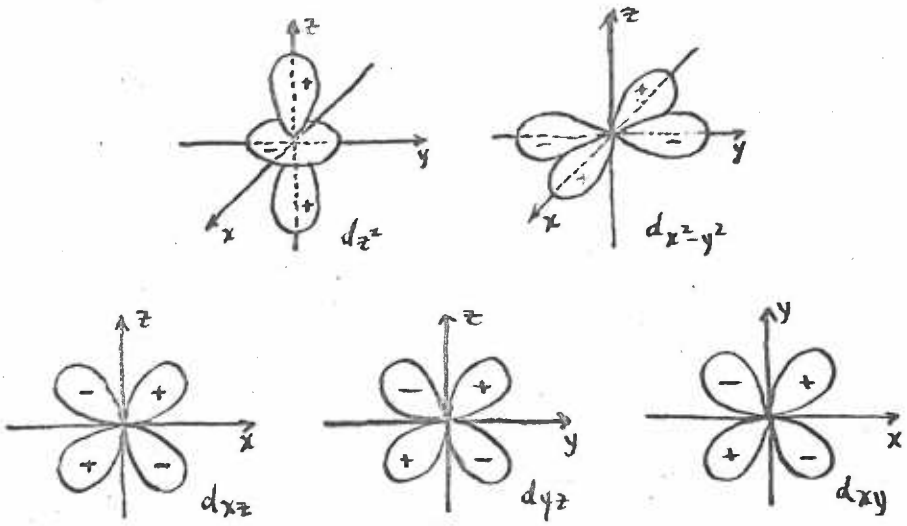
where h = Plank's constant; e = charge of electron;
m = mass of electron

b.

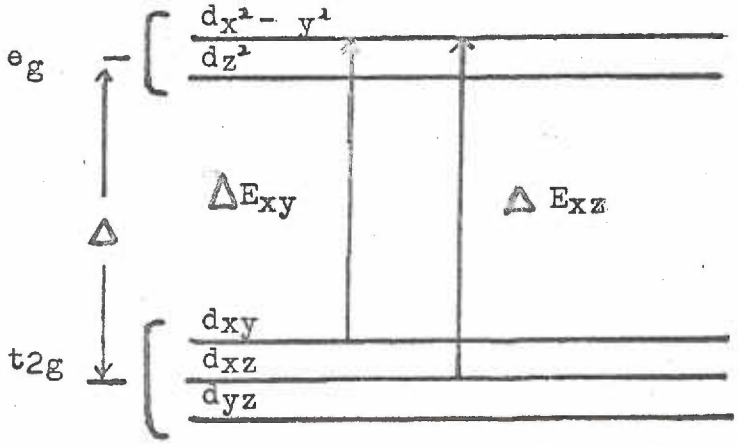


The geometry and energy levels of d-orbitals.

a. e_g and t_{2g} electronic densities (7).



b. Splitting of the metal d-orbitals in an octahedral crystal field (47).



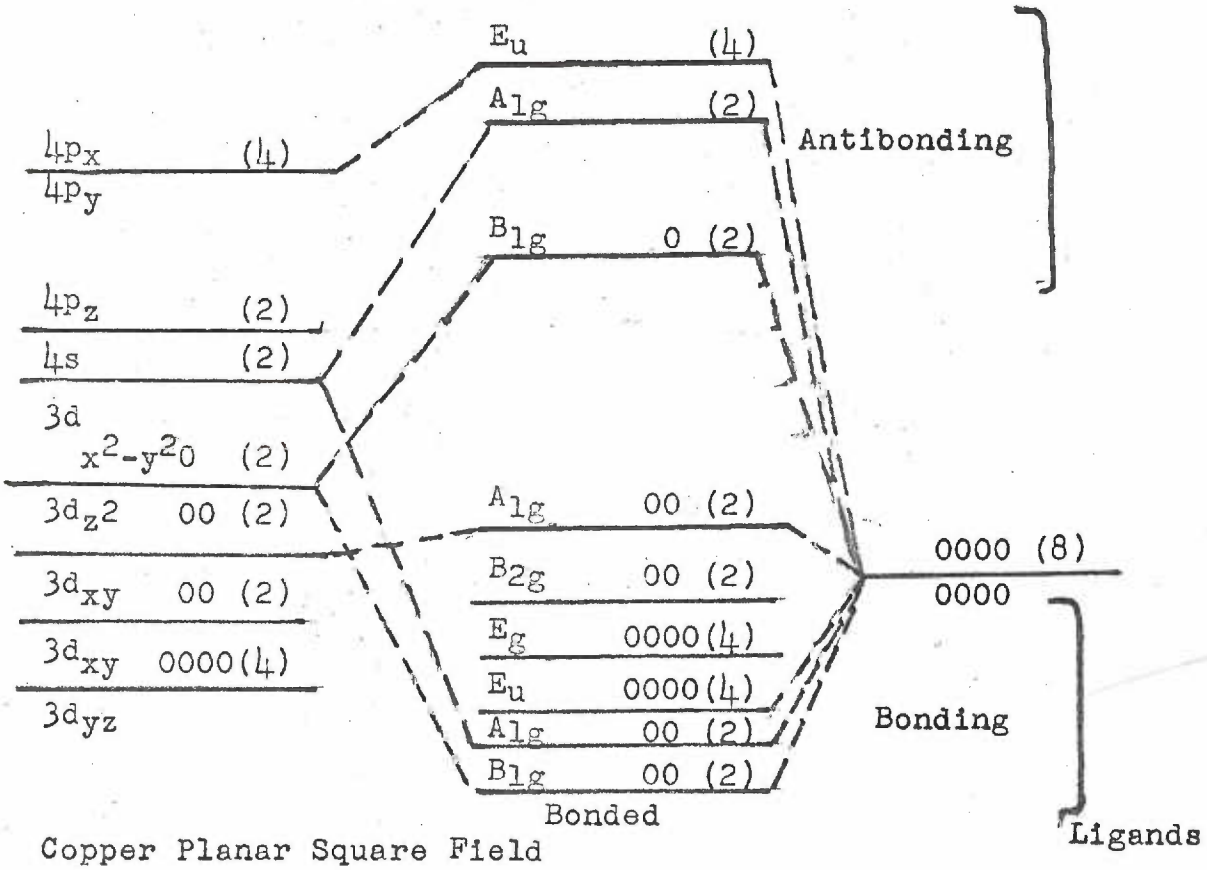
Cu(II) ground state: $(t_{2g})^6(e_g)^3$

excited state: $(t_{2g})^5(e_g)^4$

APPENDIX V

Energy levels for Cu(II) complexes in a square planar field (65).

a. Cu(II) complexes with σ bonding.



Copper Planar Square Field

b. Cu(II) complexes with π bonding.