STUDIES ON SOME PROTEOLYTIC BREAKDOWN PRODUCTS OF CANCER MAGISTER HEMOCYANIN

by

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INTRODUCTION

Part 1

The occurrence of the hemocyanins in the blood of many of the larger invertebrates and their blue color have served to bring these proteins to the attention of investigators relatively early in the history of biochemistry. The blue color of molluscan blood was noted as early as the seventeenth century and the presence of copper in this blood was detected in the mid-nineteenth century. The relationship between the blue color, or pigment, and oxygen transport was discovered by Paul Bert[1]. Fredericq[2,3] made the first study of the properties of hemocyanin and proposed the names hémocyanine and oxy-hémocyanine, these names being analogous to hemoglobin and oxy-hemoglobin, respectively.

Alsberg and Clark[4] studied various chemical and physical properties of Limulus polyphemus (horseshoe crab) hemocyanin and compared their results with previous work on Octopus hemocyanin[5,6]. They found that Limulus hemocyanin differed from Octopus hemocyanin in percentage composition, in its precipitability by dialysis, by full saturation with magnesium sulfate and by half saturation with ammonium sulfate, in not being crystallized, and possibly in being more sensitive to acid. They concluded that these hemocyanins were different. Stedman and Stedman[7] determined the oxygen dissociation curves of the oxy-hemocyanin present in the blood of Maia squinado, Palinurus vulgaris, Cancer pagurus, and

Homaris vulgaris (spider crab, rock lobster, common crab, and common lobster, respectively), found the affinity of the hemocyanins for oxygen to be the same for all four species, and suggested that the hemocyanins present in the species examined were identical. However, upon studying the effect of pH on the oxygen dissociation curves and finding that the shapes of these curves varied with varying pH, these authors reversed their previously held position, expressing the opinion that the pigments of Cancer, Homarus, Maia, and Palinurus, while resembling one another closely in properties, were probably not identical. In the cases of Cancer and Homarus, the two pigments were said to be certainly different[8]. Thus, the hemocyanins are a group of proteins similar in containing copper and in binding oxygen in the labile manner required for respiratory transport, but displaying among themselves distinct, specific differences.

The known distribution of the hemocyanins in the animal kingdom is rather scattered and limited. They are found in the two phyla, the molluscs and the arthropods. Among the former, they occur in the cephalopods and gastropods, but have not been identified in any other classes. Among the latter, they appear to be limited to the decapod crustaceans (crabs and lobsters), the Xiphosura (horseshoe crab) and the scorpions[9]. Copper appears to be the only metal in the hemocyanins. Gatterer and Philippi[10] spectroscopically examined the hemocyanins of Helix pomatia, Busycon canali-

culatum, Homarus americanus, and Limulus polyphemus (edible snail, marine snail, eastern lobster, and horseshoe crab, respectively) for the presence of iron, zinc, and manganese, and found a complete absence of these metals. In general, the hemocyanins are soluble in dilute salt solutions, but if the concentration of electrolyte is sufficiently low, they are insoluble at the isoelectric point. Therefore, they have been classed among the globulins.

The hemocyanins have been shown quite conclusively to exist as aggregates of sub-units. Perhaps the most extensive work on this aspect of the hemocyanins was that of Eriksson-Quensel and Svedberg[11]. These investigators subjected to detailed ultracentrifugal study the blood from 22 species possessing hemocyanin as their respiratory pigment. Sedimentation constants were determined throughout the pH-stability ranges. They observed that the various hemocyanins were perfectly homogeneous with regard to molecular weights both at the isoelectric point and in the regions where reversible dissociation or association occurs. The dissociation reactions were reversible except at extremely low and high pH values. In addition, the stability range for the components of highest molecular weight always comprised the isoelectric point. The molecular weights of the different hemocyanins and their reversible dissociation products were shown to be a relationship of simple multiples. Tiselius and Horsfall[12] demonstrated that electrophoretically distinguishable mixed,

or hybrid, hemocyanins can be produced by dissociating and re-associating two different hemocyanins present in the same solution.

Brohult and Claesson[13] investigated the influence of different types of salts and of a few non-electrolytes in acetate buffer (pH 5.2) and in phosphate buffer (pH 6.0) upon the dissociation of Helix hemocyanin. They obtained, with the ultracentrifuge, well-defined sub-multiples (1/2, 1/8, and 1/16) of the original molecule. Among the non-electrolytes they used were glucose, glycerine, and urea. Glucose and glycerine dissociated hemocyanin into halves only, but urea, while causing this type of dissociation, also caused the production of non-uniform, low-molecular weight compounds. Reversibility was observed in all cases where the dissociation had given only half-molecules. The reaction was not completely reversible when the fragments obtained by the dissociation were smaller than half-molecules. From their results, they conclude that "Certain molecules or groups may have a stronger effect than others, but all types, whether ions or uncharged molecules, influence the dissociation of haemocyanin". Brohult[14] subsequently showed that calcium ion stabilizes hemocyanin with respect to dissociation into sub-units. In addition, he reported that the dissociation was also influenced by temperature, dissociation increasing with increasing temperature.

Burk[15] found, from osmotic pressure measurements in

isoelectric urea solutions (6.66 M), a molecular weight of 142,000 for Limulus hemocyanin, which was smaller than that observed in aqueous isoelectric buffer solutions (565,000). Moreover, this hemocyanin, after treatment with acid at about pH 3, which removed the copper from the protein, was found to have a molecular weight in urea solution of 69,000, or approximately half that of the copper-containing hemocyanin. Further confirmation of the dissociation of hemocyanin was provided by Derrien, et al[16]. These workers investigated the effect of the dissociation of Helix hemocyanin upon its solubility. The modifications in solubility were studied quantitatively and determined in the presence of various salts and at different pH's. Their results confirmed those obtained with the ultracentrifuge.

Polson and Wyckoff[17] have obtained electron micrographs of Busycon hemocyanin. Careful examination of these micrographs indicates that each large particle consists of rod-like sub-units stacked together, apparently in bundles of four. The sub-units are about three times as long as they are thick and have pairs, or all four of their long axes parallel. This gives a cube-like composite particle of constant dimensions. The structure of these clusters of sub-units, according to the authors, is dependent on the chemical environment; in some cases tetrads are formed in addition to the cube-like structures.

Dhéré[18], in 1919, established the general correspondence

between the copper content of various invertebrate bloods and their oxygen-absorbing capacities. Redfield, Coolidge, and Montgomery[19] demonstrated in a series of nine species of animals, that oxygen combines with the hemocyanin in a simple stoichiometrical proportion. In all of these oxy-hemocyanins, one atom of oxygen was combined for each atom of copper in the molecule. This gives, for molecular oxygen, a ratio of copper to oxygen of 2:1. Root[20], using Limulus hemocyanin, has shown that carbon monoxide combines with hemocyanin. The same stoichiometrical relation exists in this derivative as does in the case of oxy-hemocyanin, i.e., one molecule of gas for each two atoms of copper. In addition, it was observed that the carbon monoxide derivative of hemocyanin is less stable than oxy-hemocyanin; the affinity of the gas for Limulus hemocyanin being only about one-twentieth the oxygen affinity. Kubowitz[21] also found that hemocyanin forms a complex with carbon monoxide and that the combining ratio was the same as for oxygen. Rawlinson[22] likewise found a ratio of one oxygen molecule to two copper atoms in the hemocyanin of the Australian crayfish, Jasus lalandii, but was unable to demonstrate the existence of the carbon monoxide derivative.

The average copper content of the various hemocyanins ranges from 0.17 to 0.26 percent[21,23]. From this information, it can be calculated that units which would each contain one atom of copper would possess molecular weights

of 25,000 to 37,000. Since two atoms of copper are required to bind one molecule of oxygen, the minimal molecular weight of the functional unit would be 50,000 to 74,000. As previously described, these units undergo reversible aggregation under the influence of the chemical environment and may exist as exceedingly large molecules having molecular weights ranging from 400,000 to 10,000,000(11). Unlike the hemoglobins of the higher animals, which are contained in erythrocytes, the hemocyanins are non-cellular entities(24). Because the molecular weights of these extracellular pigments are considerable, the osmotic pressure which they exert is extremely small, which seems to correspond to a physiological necessity. In fact, if present in the form of smaller molecules, they would exercise an osmotic pressure incompatible with their physiological participation in the osmotic exchange(24).

In a parallel study of the respiratory capacity of the blood of various animals in which the transporters of oxygen are dissolved in the plasma, Florkin(25) showed that only the respiratory pigments having large molecules can exist in a sufficient quantity to assure oxygenation without disturbing osmotic relationships at the level of the vessels. That 80-97 percent of the oxygen exchange in animals possessing hemocyanin is accounted for by hemocyanin, rather than by some other mechanism, has been shown by Redmond(26). It has also been proposed that the aggregates of hemocyanin may represent the limit of size which has stability without

developing the heterogeneity of structure displayed by true corpuscles and cells[23].

The means by which copper is bound by hemocyanin has been the objective of much of the work on hemocyanin. Early workers, analogizing hemocyanin and hemoglobin, considered the copper to be linked to hemocyanin through a prosthetic group. Philippi[27], after warming the blood of Helix at 40 ° with potassium hydroxide, obtained a dark-green product containing 7 percent copper and giving an intense pyrrole reaction, as judged by the "Fichtenspanreaktion", or spruce splinter reaction. Conant, Dersch, and Mydans[28] treated Limulus serum with potassium hydroxide, warming this reaction mixture to 40-50 ° for 20-30 minutes, and allowing it to stand overnight. A black, copper-containing material resulted and was separated from the mixture. This material, after washing with 50 percent acetic acid and re-precipitating with alkali, was found to contain 14-21 percent copper, which was practically all that was available in the parent protein. In addition to copper, the material was found to contain 3 moles of serine (not demonstrated, only calculated), 1 of leucine, 1 of tyrosine, and a sulfur-containing compound having the empirical formula $C_7H_{15}O_5N_2S_2$. This sulfur compound appeared to be linked through the copper, and not by a peptide linkage to the polypeptide. These authors felt that it may be assumed that the "prosthetic group" of Limulus hemocyanin is a complex copper salt of a polypeptide

and a sulfur-containing compound having the composition described above. They furthur believed that this complex is probably bound to the rest of the protein by some complex salt linkage such as was suggested at that time to be responsible for the binding of iron of the prosthetic group of hemoglobin. Their "prosthetic group", it may be noted, gave no evidence of containing pyrrole.

Schmitz[29] obtained a product containing 41 percent copper by hydrolyzing Octopus hemocyanin with alkali followed by hot alcohol extraction and precipitation after standing in acetic acid. Schmitz considered his derivative as representing the actual prosthetic group of hemocyanin and differing from that of Conant in that it did not possess the sulfur-containing component. He also prepared Conant's derivative and found that it contained 60 percent copper.

By alkaline degradation of snail hemocyanin, Florkin and Toussaint[30] obtained the derivative of Philippi. Upon dissolving this precipitate in 50 percent acetic acid and neutralizing, they obtained the product of Conant. If, on the other hand, the derivative of Philippi was extracted with boiling alcohol followed by acidification of the extract, a precipitate was formed which was the same as the product reported by Schmitz. During the course of observations on these different derivatives, these authors found that the orcinol test for pentoses gave a positive reaction for Conant's derivative and a negative reaction for the derivative

of Schmitz. Moreover, if the derivative of Conant was extracted with boiling alcohol, the extract gave a negative reaction and the residue a positive reaction. The interpretation of these authors was that, if the formula for the sulfur-containing derivative of Conant, the nature of which was unknown, was correct, then it must be responsible for the positive reaction with orcinol. However, they did not definitely establish that the positive reaction was due to the presence of a pentose.

Roche[31] questioned the existence of a prosthetic group in hemocyanin analogous to the heme of hemoglobin. From studies of the isoelectric point, titration curves, and solubilities of hemocyanin and copper-free hemocyanin from Octopus and Limulus, he concluded that the existence of a prosthetic group in hemocyanin was, at best, problematical. Rawlinson[22] has pointed out that the workers mentioned above used whole blood, not pure hemocyanin. He could not repeat their findings by using pure Jasus hemocyanin and suggested that the products obtained by previous workers were artifacts. The reported prosthetic groups were considered by him to be a basic copper sulfide contaminated with protein breakdown products. Rawlinson[32] later suggested that the copper atoms in the hemocyanin molecule do not require a separate prosthetic group to activate them for combination with the protein moiety. He further suggested that "specific orientation of the protein molecule enables the copper atoms

to become activated for their combination with molecular oxygen".

Kubowitz[21] found that copper could be removed from hemocyanin with cyanide without denaturing the protein, the copper salts and excess cyanide being removed by dialysis. The apo-hemocyanin could then be treated with cuprous salts, regenerating hemocyanin. The capacity of hemocyanin to bind oxygen depended upon the presence of copper in the protein, thus leading Kubowitz to conclude that copper is the prosthetic group of hemocyanin, a view which is generally held at the present time.

Zuckerkandl[33], observing that, without exception, denaturation seems to be a necessary condition for the detection of copper by its chemical reactivity, has suggested that in the native state, the copper of the protein may be protected by a "barrier" of a steric nature. In hemocyanin, he continues, only respiratory gases and eventually, if there is not electrostatic incompatibility, small ions such as hydrogen ion and cyanide ion would have access to the copper. Complete denaturation would cause the disappearance of a passage way of attack toward the copper caused by its approach to the molecular surface and a modification of the respective distance of the two copper atoms necessary for the retention of a molecule of oxygen. A liberal interpretation of Zuckerkandl's proposal would be that the copper is contained within the protein matrix, and as such, is accessible only to

small molecules which, for the most part, bear no electrostatic charge. Upon denaturation, the copper is exposed at the protein surface and subject to the action of reagents which are without effect on the copper of the native protein.

Klotz, Faller, and Urquhart[34] have measured the optical absorptions in the region of 400-1000 mμ for the cupric ion complexes of several simple organic acids. The effects of glycine, diglycine, triglycine, tetraglycine, pentaglycine, and hexaglycine on the absorption of cupric ions was also measured. These latter were used as a basis for the interpretation of the observed spectra of copper complexes with serum albumin, serum gamma-globulin, beta-lactoglobulin, and beta-casein, in addition to oxy-hemocyanin. They found that all copper proteins at pH's greater than 7, whether naturally occurring or artificially constructed, absorb in the region of 600 mμ. They interpret these observations to mean that in both types of proteins the cupric ion is tetracoordinated, and that at least two of the bonds are to amine groups in the protein molecule. However, it should be noted that these authors neglect to mention that the absorption at 600 mμ by hemocyanin occurs only with the oxygenated protein, there being no absorption in the absence of oxygen[21,23]. Furthermore, as will be seen shortly, the assumption that the copper of oxy-hemocyanin is in the cupric state is of questionable validity.

As just described, the copper complexes of various pro-

teins as well as oxy-hemocyanin exhibit absorption in the range of 600 mμ. In addition, oxy-hemocyanin also absorbs in the region of 350-375 mμ(23). Klotz, Urquhart, and Fiess(35) have prepared the copper, zinc, cadmium, and lead mercaptides of serum albumin and shown that they absorb in the region of 350-375 mμ and suggest that copper of hemocyanin may also be bound in this manner.

Re-investigating the question of the mode of copper binding by hemocyanin, Lontie(36) expresses doubt concerning the involvement of thiol groups for the binding of copper to hemocyanin. Lontie proposes that the binding of copper by a thiol group alone would seem improbable on the grounds that the copper in Helix hemocyanin is not displaced by silver ions, nor by mercuric chloride. As Klotz, et al(35) have shown, the copper bound by the thiol group of bovine albumin, on the contrary, is readily displaced by these two reagents. Moreover, the treatment of Helix hemocyanin with cyanide, to remove the copper, liberates only a small number of thiol groups with respect to copper. Although Lontie gives no experimental data on this point, this contention would appear to be confirmed by work in this laboratory(37). Further, Lontie reports, after blocking these thiol groups with N-ethylmaleimide, iodoacetamide, or p-chloromercuribenzoate, cuprous copper could still be restored quantitatively. Lontie proposes, from the stability regions and titration curves of hemocyanin and copper-free hemocyanin, that histidine is in-

volved in the binding of copper and suggests that two histidine residues are responsible for the binding of one copper atom.

Another aspect of the chemistry of the hemocyanins is the question regarding the oxidation state of the copper in these respiratory proteins. Conant, Chow, and Schoenbach[38] treated Limulus hemocyanin with the powerful oxidizing agent, potassium molybdicyanide, or alternatively, potassium permanganate, and found that they could oxidize hemocyanin and oxy-hemocyanin to form two new proteins in which the copper was in the cupric state. They determined the oxidation-reduction potential of the cuprous-cupric hemocyanin system and found it to be approximately + 0.550 volt, which, as they remark, is rather high. They further note that with the exception of the complex cyanides of molybdenum and tungsten, and potassium permanganate, the oxidation-reduction potential of their system is among the strongest reversible oxidizing systems known. They conclude that the copper in hemocyanin, as it occurs in nature, is in the cuprous state. However, Rawlinson[32] has cast considerable doubt on these results, proposing that their "oxidized" hemocyanin was contaminated with the original oxidizing agent, and that the high oxidation-reduction potential was due to this contamination. In addition, Rawlinson studied the magnetic susceptibility of hemocyanin and that of hemocyanin "oxidized" by potassium molybdicyanide and found no difference. From

this data, he makes no conclusions regarding the oxidation state of copper in hemocyanin, remarking that the question is still open.

When re-constituting hemocyanin from the apo-protein and copper salts, Kubowitz[21] found that reconstitution would occur only if copper were supplied in the cuprous state. These results have been confirmed by Lontie and Horsman[39]. While Kubowitz was able to restore only 50 percent of the original hemocyanin, Lontie and Horsman demonstrated that they could reconstitute 100 percent of the original hemocyanin, as judged by its ability to reversibly combine with oxygen, rendering the same oxygen dissociation curve and yielding the same extinction coefficient with the copper band at 345 mμ as for the original material. Lontie[36] has interpreted this phenomenon to indicate quite conclusively that the oxygen-binding copper in the hemocyanins is monovalent.

Henze[5] noted that the addition of alkali to Octopus hemocyanin, in the absence of added, extraneous copper, gave the Biuret reaction. Schulman and Wald[40] have found that adding alkali to Limulus hemocyanin also produces the Biuret reaction, but only when the hemocyanin is oxygenated. This result was interpreted to mean that the oxidation state of copper is cupric in oxy-hemocyanin and cuprous in reduced hemocyanin.

Joselow and Dawson[41] carried out some studies on the

dissociability of the copper of Busycon hemocyanin using radioactive copper. When radio-cupric ions were added to a solution of oxy-hemocyanin for periods of time up to 16 hours, it was found that no significant radioactivity was incorporated into the hemocyanin. In addition, no significant exchange occurred while the hemocyanin was undergoing reversible oxygenation-deoxygenation. The argument was presented that if the copper of hemocyanin were cuprous and dissociated in the absence of oxygen, there would have been exchange due to the reduction of some of the radioactive cupric ions to cuprous (by dissociated cuprous ions) and recombination of these radioactive cuprous ions with hemocyanin. These results show that copper is firmly bound by hemocyanin, but give no definitive information about the oxidation state of the copper.

Using 2,2'-biquinoline in glacial acetic acid, which gives a pink color with cuprous ion and no color with cupric ion, Klotz and Klotz(42) have shown that in non-oxygenated Busycon blood, "practically all (87%) of the copper is in the cuprous form". However, they were not able to account for all of the copper (as cuprous) and suggest that this may be due to using whole blood instead of crystalline hemocyanin and to the difficulty of removing the last traces of bound oxygen by a non-chemical method (oxygen-free nitrogen). They also found that oxygenation of hemocyanin converts approximately one-half (61%) of the copper to cupric, the

remainder retaining the cuprous state. Similar results were obtained with the dialyzed bloods of Limulus, Homarus, and Loligo (squid).

From this information and that previously obtained regarding the spectra of mercaptides of serum albumin[35], these authors proposed that the copper in hemocyanin is bound by thiol groups sufficiently close together that two copper atoms may bind, or be "bridged" by, one oxygen molecule. The copper in hemocyanin is cuprous, they suggest, while in oxy-hemocyanin, the copper is present in each of its two valence states, simultaneously. Thus, the oxygenation reaction in hemocyanin, in contrast to that in hemoglobin, also involves a partial oxidation reaction. According to these authors, the oxygen molecule picks up one electron from one of the two cuprous ions and becomes a perhydroxyl ion, or radical, O_2^-. They propose that in hemocyanin some stabilization of this radical may arise from possible resonance of an electron between the two copper ions.

Williams[43] has raised objections to the above hypothesis of the Klotz's, pointing out that they did not test for cupric ions, but merely assumed that the difference between cuprous and total copper was due to cupric copper. Also, he argues, if half of the copper in oxy-hemocyanin is cuprous and upon its removal the oxygen is released, then the remaining half of the copper (as cupric) should revert

to cuprous and thus be accounted for as such.

Several enzymic activities have been reported for the hemocyanins. Bhagvat and Richter[44] have shown that various hemocyanins and other copper-protein complexes can act as, what they term, pseudophenolases, and that the hemocyanins are primarily responsible for the apparent phenolase activity in a number of arthropods and molluscs. They also express the belief that the pseudophenolase activity of the hemocyanins is of very little, if any, physiological significance. Tappel[45] found that copper proteins formed by the binding of copper ions to con-albumin, serum albumin, or caseinate are more effective catalysts for linoleate oxidation than copper alone. Hemocyanin showed a similar catalytic activity. The main product was a conjugated diene linoleate hydroperoxide. The increase in oxidation catalysis of copper proteins as compared to ionic copper was ascribed to the ease of formation and increased stability of the intermediate complex of linoleate peroxide-copper protein.

Ghiretti[46,47] has given evidence for the ability of various hemocyanins to decompose hydrogen peroxide catalytically. The possibility of this catalytic property being due to catalase contamination was quite conclusively ruled out. The decomposition of hydrogen peroxide catalyzed by hemocyanin was shown to be due to the presence of copper. Removal of the metal from the protein caused loss of activity. Ghiretti also found that hemocyanin from different sources

does not have the same activity as calculated per unit of copper. No suggestion of possible physiological implications was presented.

Part II

In as much as the concepts utilized in visualizing the problem investigated in this work and the experimental approach taken toward its elucidation are similar in kind to the researches pertaining to the active centers of enzymes, it seems appropriate to include in this Introduction a brief consideration of the active center concept and some of the methods employed in this type of work.

The concept of active centers in enzymes arose from the discovery of their high substrate specificity. Fischer[48], in 1894, used the analogy of "lock and key" for the enzyme-substrate reaction. Modifications of this concept of varying sophistication are largely accepted at the present time. Earlier workers suggested that enzymes consisted of a specific catalytic molecule attached to a nonspecific colloid. Much of the work on respiratory and other conjugated enzymes tended to favor this hypothesis. However, all attempts to separate activity from the protein fraction were unsuccessful. The necessity for an intact protein structure has been emphasized by the preparation of crystalline homogeneous enzymes such as urease, trypsin, and chymotrypsin, which appear to be entirely protein in character. Thus, the concept that enzymes are proteins or conjugated proteins is

almost universally accepted at the present time.

Although, in general, enzymes are many times the physical size of their substrates, there is considerable evidence that the number of active centers or sites per enzyme molecule is narrowly limited. Conjugated enzymes invariably possess a small fixed number of prosthetic or bound coenzyme groups per molecule, e.g., two DPN groups in liver alcohol dehydrogenase[49]. Myrback[50] showed that invertase is completely inhibited by a concentration of silver ions amounting to 7-8 ions per molecule of enzyme. Unless gross changes of structure occur, not more than 7-8 active centers can exist in the molecule. Urease is completely inhibited when about four silver ions are bound[51]. Many workers have observed reversible inhibition of enzymes by reaction with a small number of thiol groups[52]. The molecules of chymotrypsin and trypsin[53-55] contain only one active center, as is evidenced by their reaction with organo-phosphorus inhibitors. Similarly, acetylcholinesterase can contain only a limited number of active sites[56]. Dialysis equilibrium methods, which estimate the number of molecules of competitive inhibitor[57] or of substrate[58] bound to chymotrypsin, support the hypothesis of a single active center in this enzyme.

A variety of methods and techniques have been employed for the investigation of active centers. Among these methods and techniques are: 1) enzyme kinetics, 2) variation in

activity with pH, 3) chemical modification of specific groups in the enzyme protein, 4) model systems, and 5) in the case of a very limited number of enzymes, it has been possible to isolate and chemically characterize peptides from the active center. Because the first two methods of active center study mentioned above require an enzymic activity in order to be employed, they will not be considered in detail.

Chemical modification of specific groups in the protein can indicate what types of structure are essential for activity, but many conditions must be satisfied. The reagents and conditions must be specific for a limited number of groups, and must not cause denaturation of the protein. Both reagents and the protein groups involved should be estimated quantitatively. Ideally, the inhibition should be capable of reversal. If modification of a particular group causes loss of activity under those conditions, this group may be part of the active center. Alternatively, the substituent may prevent access to the active center by steric hindrance. Another possibility is that the modified group is an essential structural unit in the protein, e.g., a disulphide bridge linking peptide chains. Although there is a considerable literature for this type of experiment[59-61], the conclusions are often ambiguous. Very few reagents show appropriate specificity, and complete balance sheets for the reaction are often not available. In many cases, the conditions used cause inactivation in control solutions.

Model systems which imitate enzyme activity can occasionally clarify the mechanism of enzyme action. Perhaps the most notable success involving the use of model systems for this purpose has been the work of Snell's group[62] in which reactions catalyzed by pyridoxal phosphate plus metal salts have been compared with those catalyzed by enzymes containing this group. Metal-chelate complexes as analogues of enzymes have been considered by Calvin[63].

Model systems for investigating the natural oxygen carriers, hemoglobin and hemocyanin, are conspicuous because of their relative absence. As far as hemocyanin is concerned, there are no known compounds of copper other than the hemocyanins themselves which possess the property of reversibly taking up and releasing molecular oxygen. However, there are several compounds of cobalt which possess this property and which may prove useful as model systems for studying this phenomenon. Several bis-salicylaldehyde imine cobalt derivatives have been prepared and studied in this connection as well as bis-histidino-cobalt[64-69].

The fifth method mentioned above for the investigation of active centers should possibly have been included under method three. But, while falling in the general class of chemical modifications of enzymes, there are certain features which make it desirable that it be considered separately.

The organo-phosphorus compounds, such as diisopropyl-fluorophosphate (DFP), have been found to be highly specific

reagents which react only with certain enzymes having an esteratic action. These reagents do not react with proteins generally, or with any of the constituent amino acids, at any appreciable rate[70]. Since the attachment of the substituted phosphoryl group is irreversible, and it is not removed on denaturation or hydrolysis of the protein, a particularly productive line of work has been the separation and identification of peptides containing it from DFP-inhibited enzymes in order to determine the nature of the groups in the active center with which it combines. This work has been greatly facilitated by the use of DFP labelled with radioactive phosphorus.

The enzymes whose active centers, at least in part, have been isolated and chemically characterized through the use of organo-phosphorus compounds include, at present, chymotrypsin[71-74], trypsin[75-77], cholinesterase[78], ali-esterase[79], and thrombin[80]. The results so far obtained for these enzymes are not consistent in detail, but the main features are clear. It is interesting to note that there is a striking similarity among the peptides thus far obtained. For example, the organo-phosphorus inhibitors all appear to be attached to the hydroxyl group of serine, although there is a certain amount of interchange among the neighboring amino acids. The general sequence of amino acids in the active centers of these enzymes has been found to be asp-ser(PO_4)-gly-glu-ala-val.

It is of considerable interest that the same amino acid sequence has been found to be part of the active center of an enzyme of quite a different type, phosphoglucomutase[81,82]. The serine residue in this sequence acts as the phosphate carrier, transferring the phosphate group of glucose-monophosphate to produce the "co-enzyme" glucose-diphosphate and being re-phosphorylated by the other phosphate group of the "co-enzyme". The mechanism and structure were investigated by the use of P^{32} as a label for the exchangeable phosphate; partial hydrolysis then yielded the peptide pertinent to the active center.

Although the hemocyanins are not, strictly speaking, enzymes, they nevertheless contain "active centers" in the sense that they possess a limited number of binding sites for their natural "substrate", oxygen, and that these binding sites are relatively specific. In the present work, the view has been taken that, instead of introducing artificial "labels", the presence of copper and its functional involvement might provide the means of "labeling" the portion of the protein possessing the "active center", and thus furnish the distinguishing characteristic by which peptides arising from this "active center" by enzymic hydrolysis might be recognized during their attempted isolation.

MATERIALS AND METHODS

Preparation of Hemocyanin

The hemocyanin used in this work was obtained from the crab, *Cancer magister*, supplied by the Bell Buoy Crab Co., Seaside, Oregon. Live crabs were used exclusively. The hemolymph was secured by removal of the pincers and draining the hemolymph into a one liter graduated cylinder. A 1:1 volume of 0.15 M phosphate buffer, pH 7.0, was added immediately to the hemolymph and debris removed by centrifugation for 15 minutes at 10,000 g. All operations were performed in a cold room at 4 °C.

To secure the hemocyanin, the clarified hemolymph was centrifuged at approximately 105,000 g in the Spinco Model L Preparative Ultracentrifuge for three hours. The blue, gelatinous pellets thus obtained were taken up in cold buffer and re-centrifuged twice more, followed by dialysis against deionized water to remove buffer salts. The hemocyanin derived in this manner was essentially ultracentrifugally and electrophoretically homogeneous[37]. This protein exhibited considerable instability upon standing in solution and consequently was lyophilized unless used immediately. The average yield of purified hemocyanin per crab was approximately 500 mg.

Proteolytic Enzyme

The proteolytic enzyme used for the hydrolysis of hemo-

cyanin was a crystalline commercial bacterial proteinase from Bacillus subtilis prepared according to Nagihara[83] and was kindly supplied by Nagase Co., Ltd., Itachibori-minamidori 1-chome, Osaka, Japan. A subsequent method of preparation has been described[84] and the enzyme has been characterized with regard to general properties[85], comparison with trypsin[86], N-terminal amino acid[87], and its reaction with diisopropylfluorophosphate[88]. This enzyme exhibits a wide specificity[89] and is possibly identical with Subtilisin[90], which also possesses a broad specificity[91-93].

Detection of Substances on Paper

Copper was detected using a modification of the procedure described in Block, Durrum, and Zweig[94a] for the detection of the copper salts of amino acids. A mixture of 0.4% w/v of o-tolidine (National Aniline Division, Allied Chemical and Dye Corp., Lot No. 10861) and 1.0% NH_4SCN in re-distilled acetone was mixed with 30% acetic acid (3:1, v/v) immediately before use. This reagent is stable for one day at room temperature and up to one week in the refrigerator at 1 °C. Limits of detection are 0.05 to 0.10 micrograms of copper per cm^2 (as inorganic copper).

Peptides and amino acids were detected on paper with 0.3% ninhydrin (Nutritional Biochemicals Corp., Cleveland, Ohio) in 95% ethanol[94b].

EXPERIMENTAL AND RESULTS

Hydrolysis of Hemocyanin

The hydrolysis of hemocyanin by bacterial proteinase was accomplished according to the following procedure. To a weighed amount of lyophilized hemocyanin was added the proper amount of 0.05 M phosphate buffer, pH 8.0, to give a final concentration of 10 mg/ml of hemocyanin*. This solution was placed in a constant temperature water bath at 37 ± 0.5 °C and allowed to attain thermal equilibrium**. To start the reaction, the enzyme was dissolved in a minimum amount of buffer and immediately added to the hemocyanin solution. The amount of enzyme added was determined by the enzyme/substrate ratio of 1:1000 on a weight basis. During the course of the hydrolysis it was necessary to re-adjust the pH occasionally with 1.0 M NaOH. Although a more concentrated buffer could have been used, it was deemed undesirable because of the subsequent de-salting procedure. The color of the solution changed from pale blue to yellow as the hydrolysis proceeded. At the end of 24 hours, there was no longer any material present which could be precipitated with 20 percent trichloroacetic acid. After completion of

* All buffers and solutions were prepared with deionized water.

** At this time, there remained some undissolved hemocyanin which was subsequently dissolved and hydrolyzed by the enzyme.

hydrolysis (27-30 hours), the reaction mixture was lyophilized and stored at -20 °C until further use.

The Hydrolysis of Hemocyanin by Bacterial Proteinase as Followed with a pH-stat

The time-course of the hydrolysis at pH 8.0 was followed with a pH-stat (Radiometer, 72 Emdrupvej, Copenhagen NV, Denmark). The system consisted of 50 mg of undenatured hemocyanin, 0.05 mg of bacterial proteinase, and 7.0 ml of water. The titrant was 0.2 N NaOH and the temperature was 25 ± 1 °C. The results, corrected for CO_2 uptake, dilution from the calomel electrode, etc., are depicted in Figure 1.

The hydrolysis of hemocyanin catalyzed by bacterial proteinase is a first order reaction as determined from the data obtained using the pH-stat. The first order rate constant, k_1, was determined graphically [$k_1 = -2.303 \times (\text{slope})$] from Figure 2 and from the equation

$$\ln \frac{a}{(a-x)} = k_1 t$$

to be 0.081 / hr for the conditions given (Table 1).

An estimation of the average molecular weight of the peptides formed during the hydrolysis of hemocyanin may be derived from a consideration of the base consumed per mole of hemocyanin hydrolyzed. The total base consumed during the reaction described above was 55 micro-moles (the value asymptotically approached in Figure 1). The reaction vessel contained 50 mg of hemocyanin, corresponding to 5.3×10^{-2}

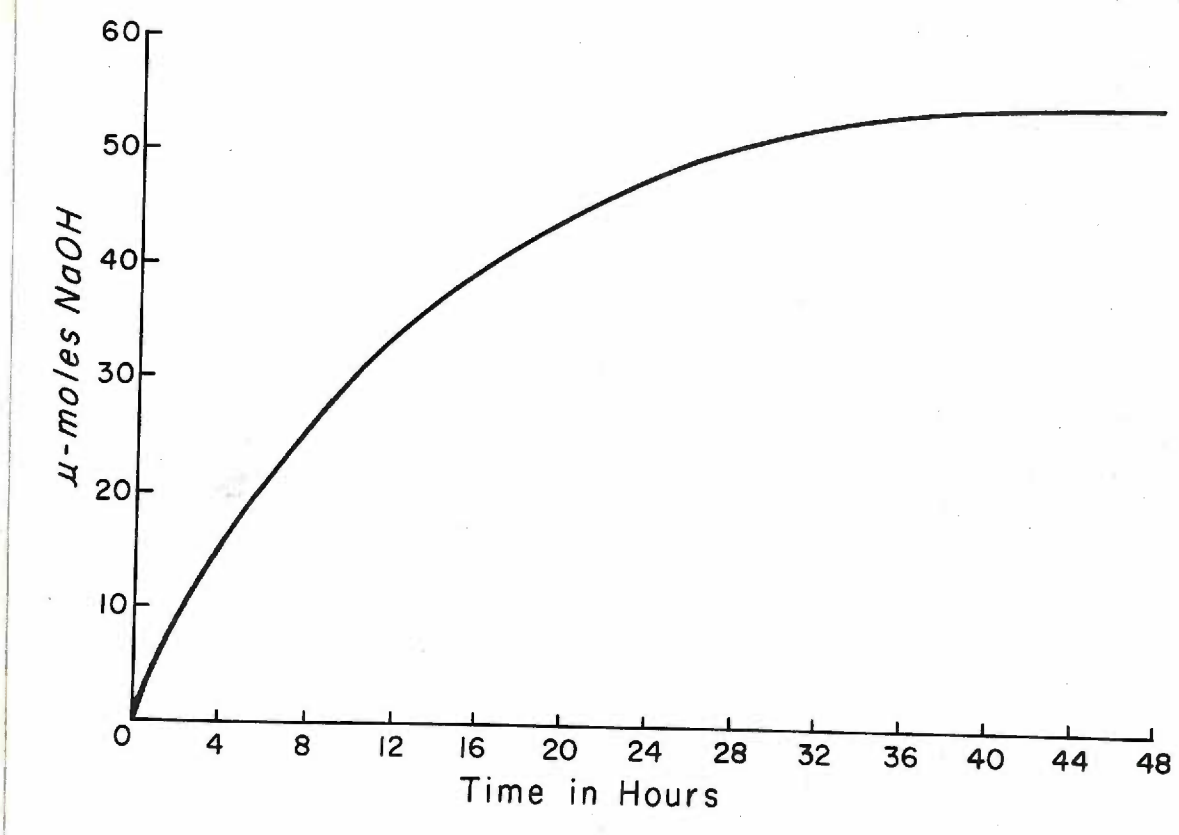

Figure 1. Time curve of the hydrolysis of hemocyanin by bacterial proteinase at 25 ± 1 °C as followed by the pH-stat.

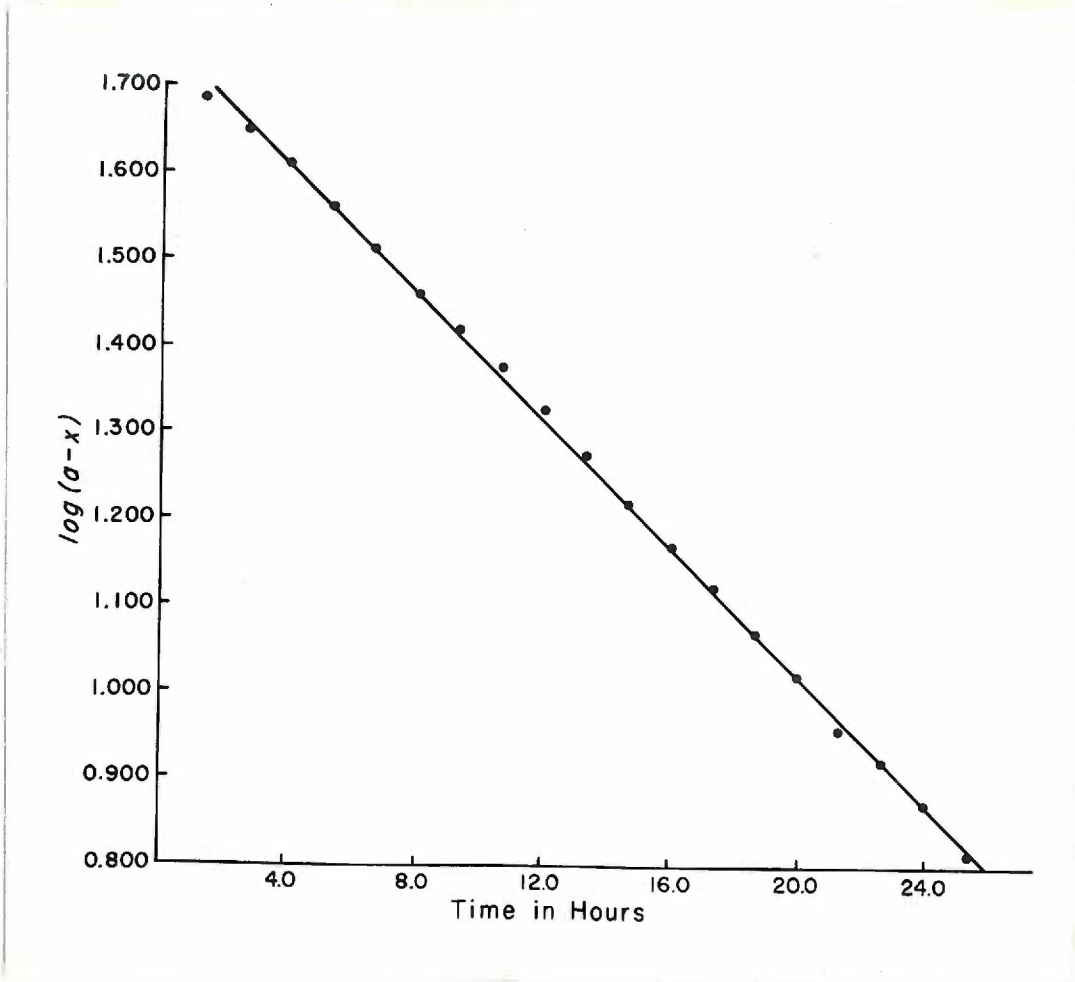

Figure 2. Plot of $\log_{10}(a-x)$ vs t for the hydrolysis of hemocyanin by bacterial proteinase at 25 ± 1 °C.

TABLE 1

Hydrolysis of Hemocyanin at 25 ± 1 °C
Catalyzed by Bacterial Proteinase*

t (hrs)	x (μ-moles NaOH)	k_1 (hrs^{-1})
4	14.4	7.6×10^{-2}
8	26.1	8.0
12	33.8	8.0
16	40.2	8.2
20	44.5	8.3
24	47.5	8.3
		Mean = 8.1×10^{-2}

* a = 55 μ-moles NaOH

μ-moles, the molecular weight of *Cancer magister* hemocyanin being 950,000[37]. Thus, $55 \div 5.3 \times 10^{-2}$, or 1,037 moles of NaOH were consumed per mole of hemocyanin hydrolyzed. Since each mole of NaOH utilized represents the hydrolysis of one mole of peptide bonds, the average molecular weight of the peptides formed is approximately 916, assuming hemocyanin to consist of protein and copper only.

Dialysis of Hydrolyzed Hemocyanin

An experimental evaluation of the above-derived value for the average molecular weight of the peptides resulting from the enzymic hydrolysis of hemocyanin was obtained by dialysis. One milliliter, containing 16.2 mg of hydrolyzed hemocyanin, was dialyzed against three changes of 100 ml each of deionized water for 24 hours each time. The water was stirred continuously, the entire procedure being performed in the cold room at 4 °C. After dialysis, all of the

solutions were lyophilized and analyzed for nitrogen and copper. Nitrogen was determined by the micro-Kjeldahl method and copper according to Peterson and Bollier[95]. Eighty-two percent of the nitrogen and all of the detectable copper were present in the first 100 ml of deionized water. Ultimately, a total of 91 percent of the nitrogen was accounted for outside the dialysis sac. Thus, it would appear that the majority of peptides resulting from the action of bacterial proteinase on hemocyanin are quite small, having molecular weights of approximately 6000 or less[96].

De-salting Procedure for Hydrolyzed Hemocyanin

The lyophilized hydrolyzate was de-salted by extraction with absolute methanol. The amount of methanol required was not critical, being determined by the amount which would result in the formation of a heavy slurry. This slurry was then centrifuged at room temperature, the dark-brown supernatant decanted, and the precipitate re-extracted with additional methanol. Extraction proceeded until the supernatant was colorless. Three extractions usually sufficed. The extracts were pooled and most of the methanol removed under vacuum with a rotory evaporator* (Rinco Instrument Co., Greenville, Illinois). Final traces of methanol were removed by vacuum desiccation over silica gel. The amount of

* Any precipitate of extracted phosphate salts formed during this operation were removed by centrifugation before final drying.

hydrolyzate extractable by this procedure was 88 ± 5 percent while 60 ± 5 percent of the original copper was present in the extract. The extract itself contained 0.12 ± 0.03 percent copper. The copper content of Cancer magister hemocyanin is 0.158 to 0.173 percent[37].

Continuous Flow Paper Electrophoresis of Hydrolyzed Hemocyanin

Continuous flow electrophoresis was used as the initial step for the attempted isolation of a copper-containing peptide. The Spinco Model CP Continuous flow Electrophoresis Cell (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.) was employed for the execution of this procedure, the electrolyte being phosphate buffer, pH 8.0 and 0.02 ionic strength. The extracted hydrolyzate was dissolved in water and diluted to a final concentration of 100 mg/ml*. Instrument settings were: wick feed = 6.8, overflow = 7.2, and sample feed setting = 0.65, which corresponded to a feed rate of 0.32 ml/hr with the 30 mm diameter sample vessel. The sample was applied at the extreme left feed tab (see Figure 3). Conditions of electrophoresis were current = 22 milli-

* The extracted hydrolyzate was dissolved in water rather than buffer because it contained approximately 0.33 percent phosphate by analysis. In general, samples being separated by continuous flow electrophoresis should have a conductivity equal to or less than that of the background electrolyte(94c,97a). Dissolving the material in water resulted in a solution whose electrolyte concentration was about one-third as concentrated as the buffer washing the curtain.

amperes and voltage = 640 volts.

It was noticed during electrophoresis that a yellow streak, or band, was present along the left periphery of the curtain. In addition to being yellow in color, this band was also fluorescent when examined under a long-wave ultraviolet lamp. No other fluorescing region was detected.

At the conclusion of electrophoresis, the curtain was immediately removed and dried in a horizontal position to minimize distortion of the pattern by diffusion and solvent flow effects. After drying, one side of the curtain was sprayed with ninhydrin and the color allowed to develop. Next, the reverse side was sprayed with the o-tolidine reagent. The results of these manipulations are represented in Figures 3 and 4, respectively. The dark band in Figure 3 and the copper band in Figure 4 corresponded to the yellow, fluorescent band just described. The density of the dark band in Figure 3 was enhanced somewhat by the yellow color and should not be construed as being due solely to the reaction with ninhydrin. It may be seen from Figure 3 that with the exception of the dark band along the left periphery, the distribution of material reacting with ninhydrin to give the usual purple color is quite uniform.

The contents of the collecting tubes were analyzed quantitatively for copper by the method of Peterson and Bollier[95]. The distribution of copper among the collecting tubes was obtained by multiplying the amount of copper per

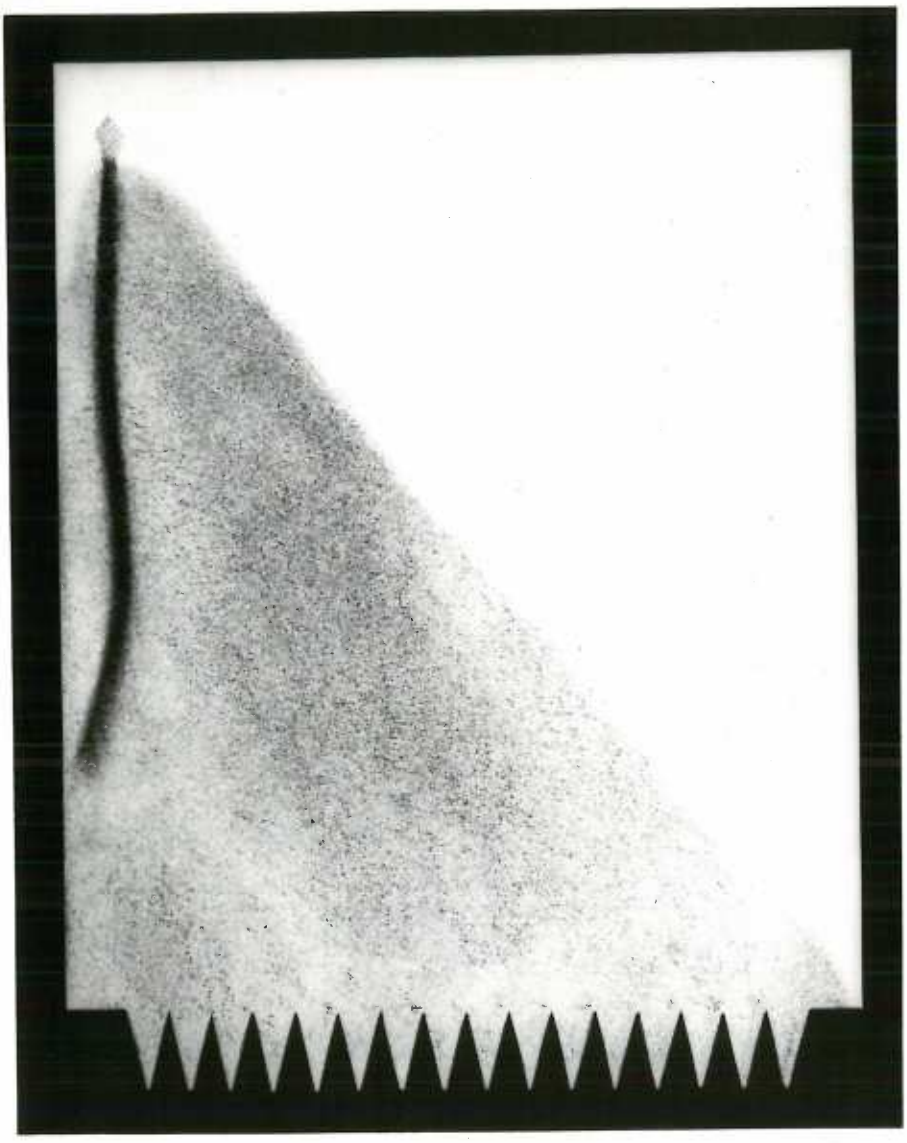

Figure 3. Pattern resulting from electrophoresis of hydrolyzed hemocyanin as revealed by spraying with ninhydrin. This is an artist's drawing of the sprayed curtain. This procedure was necessary because the original curtain did not possess enough contrast to photograph satisfactorily. The cathode is at the left side of the curtain, the anode at the right. See the text for interpretation and discussion.

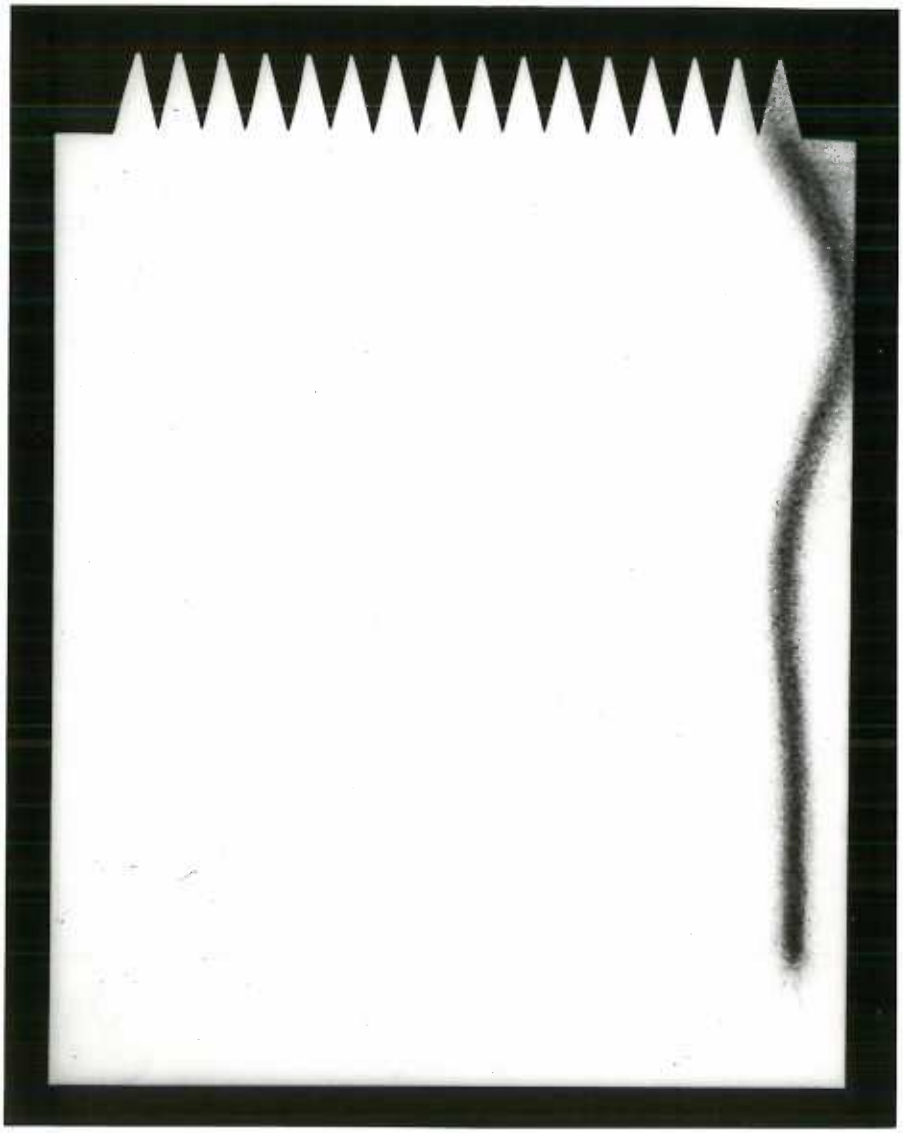

Figure 4. Artist's drawing of the pattern resulting from electrophoresis of hydrolyzed hemocyanin as revealed by spraying with o-tolidine reagent.

ml in each tube by the volume, adding up the amounts in each tube to obtain the total amount of copper, dividing the amount of copper in each tube by the total and multiplying by 100 to derive the percent of the total copper contained in each tube. The distribution of copper is presented in Figure 5. Buffer alone, electrophoresed under identical conditions, contained no copper detectable by this method.

It is not unexpected that tubes 1 and 2 contained a relatively major proportion of the copper since the only copper band in evidence after treatment of the curtain with the o-tolidine reagent was collected in these tubes. The peculiar pattern of the copper band in Figure 4 was caused by the material flowing over to the first two drip points, there being no other means for it to flow off the curtain upon reaching the bottom. The presence of relatively more copper in tube 31 than in the middle tubes could not be suspected from the results of the procedures represented by Figures 3 and 4. There was no more ninhydrin-positive material being collected by this tube than any of the neighboring tubes, as judged by Figure 3. However, this could possibly be due to a relative unreactivity of this material with ninhydrin or it could be interpreted as indicating a material possessing a reactivity toward ninhydrin comparable to the material present on the rest of the curtain, but having a relatively higher copper content. It was also noticed that the contents of tube 31, and tubes 30 and 32 to a lesser

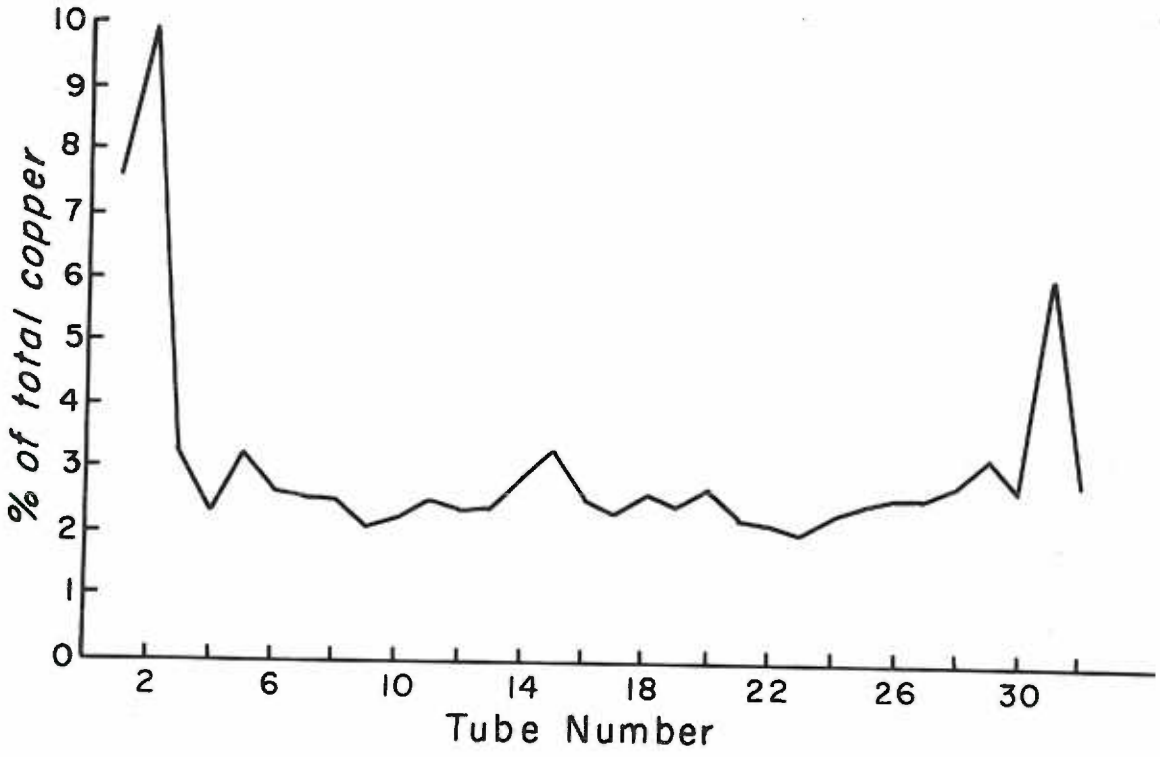

Figure 5. Plot showing the distribution of copper resulting from electrophoresis of hydrolyzed hemocyanin.

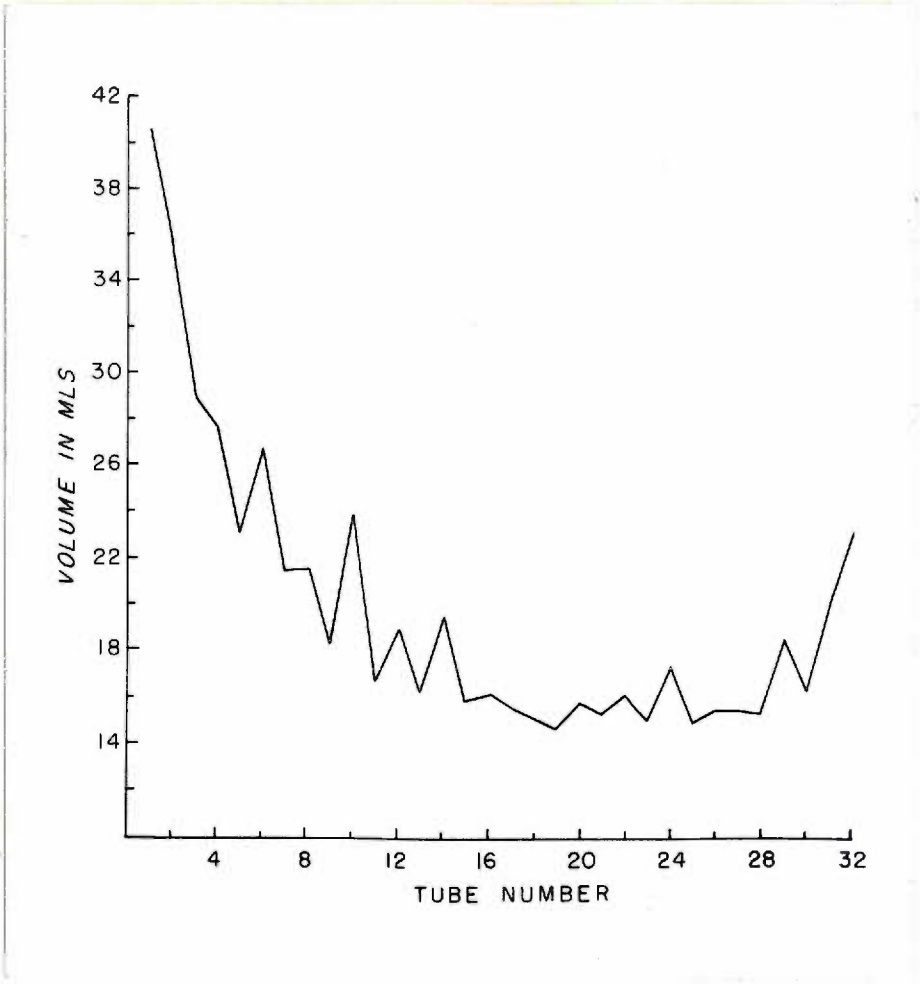

Figure 6. Plot showing the distribution of volumes collected during electrophoresis of hydrolyzed hemocyanin.

extent, were a light yellow or yellowish-brown and non-fluorescent. The distribution of the volumes of the collecting tubes, presented in Figure 6, was also plotted for the purpose of aiding in the decision regarding which fraction or fractions to investigate further. Comparison of Figures 5 and 6 indicates that this maneuver was not particularly successful in this instance, since it cannot be readily deduced whether the distribution of copper is due to an increase in concentration or is merely a reflection of the volumes collected. This decision was made on the basis of the distribution of copper (Figure 5) and the results of spraying the curtain. The combined contents of tubes 1 and 2 were designated Mixture A and those of tubes 30, 31, and 32 as Mixture B.

Copper Content of Mixture A

The contents of collecting tubes 1 and 2 were lyophilized, extracted with absolute methanol, and dried *in vacuo* over silica gel. Before further fractionation, the copper content of this mixture was determined in the following manner. Weighed samples were wet-oxidized using a mixture of concentrated nitric, sulfuric, and perchloric acids. After heating for six hours on a sand bath, the last traces of acid were removed by heating the flasks with Bunsen burners. The residues were dissolved in deionized water and their copper contents determined. Appropriate standards and

reagent blanks were processed simultaneously. This procedure was followed in order to rule out the possibility of competition for the copper by the peptide material and the complexing agent, bis-cyclohexanoneoxalyldihydrazone, used for the colorimetric determination of copper(95), which, if operative, would have resulted in a low value for the copper content. The copper content of Mixture A was found to be 0.094-0.11 percent. In addition, the phosphate content was determined* in order to rule out the possibility of gross contamination resulting from the extraction procedure. The phosphate content was less than 0.37 percent (calculated as Na_2HPO_4), which was too low to affect the results.

A copper content of approximately 0.10 percent does not represent an increase in copper with respect to the starting material (0.12 percent copper). Purification of a peptide of high copper content should involve the removal of extraneous material with a consequent rise in copper content for each procedure resulting in purification.

Continuous Flow Paper Electrophoresis of Mixture A

Mixture A was further fractionated by continuous flow electrophoresis at pH 6.9. The electrolyte was phosphate buffer, 0.02 ionic strength. Wick feed and overflow settings were 7.2. Feed rate of sample (100 mg/ml) was 0.32 ml/hr.

* The author wishes to express his gratitude to Mr. Nicholas Kontaxis for the phosphate analysis of this material and those reported in subsequent experiments.

(sample feed setting = 1.5, 20 mm diameter sample vessel). The sample was applied at the second tab from the right (see Figure 7). Conditions of electrophoresis were current = 30 milliamperes and voltage = 950 volts.

At the conclusion of electrophoresis, the curtain was again removed immediately and dried in a horizontal position. Spraying with ninhydrin and the copper reagent were carried out as previously described. The resulting patterns are represented in Figures 7 and 8, respectively. The interpretation of these Figures as regards the dark band and general ninhydrin-revealed distribution is the same as that of Figures 3 and 4 (vide supra). The copper band illustrated in Figure 8 was fluorescent on the curtain as well as in the collecting tubes. No other fluorescent or copper-positive band could be detected on the curtain. However, upon examination of all the collecting tubes with a long-wave ultraviolet lamp, it was revealed that, in addition to tubes 16-20, tubes 28-31 also exhibited fluorescence.

The distribution of copper in the collecting tubes was determined as before, this distribution being depicted in Figure 9. The presence of two major copper components is readily apparent. Also, comparison of the copper distribution with the volume distribution (Figure 10) indicates that the appearance of the component in tubes 16-19 was due to a concentration effect and not a reflection of the volumes collected. Such a distinction in the case of tubes 28-31

Figure 7. Artist's drawing of the pattern resulting from electrophoresis of Mixture A as revealed by spraying with ninhydrin. Comments regarding Figures 3 and 4 also apply to this Figure and the succeeding Figure.

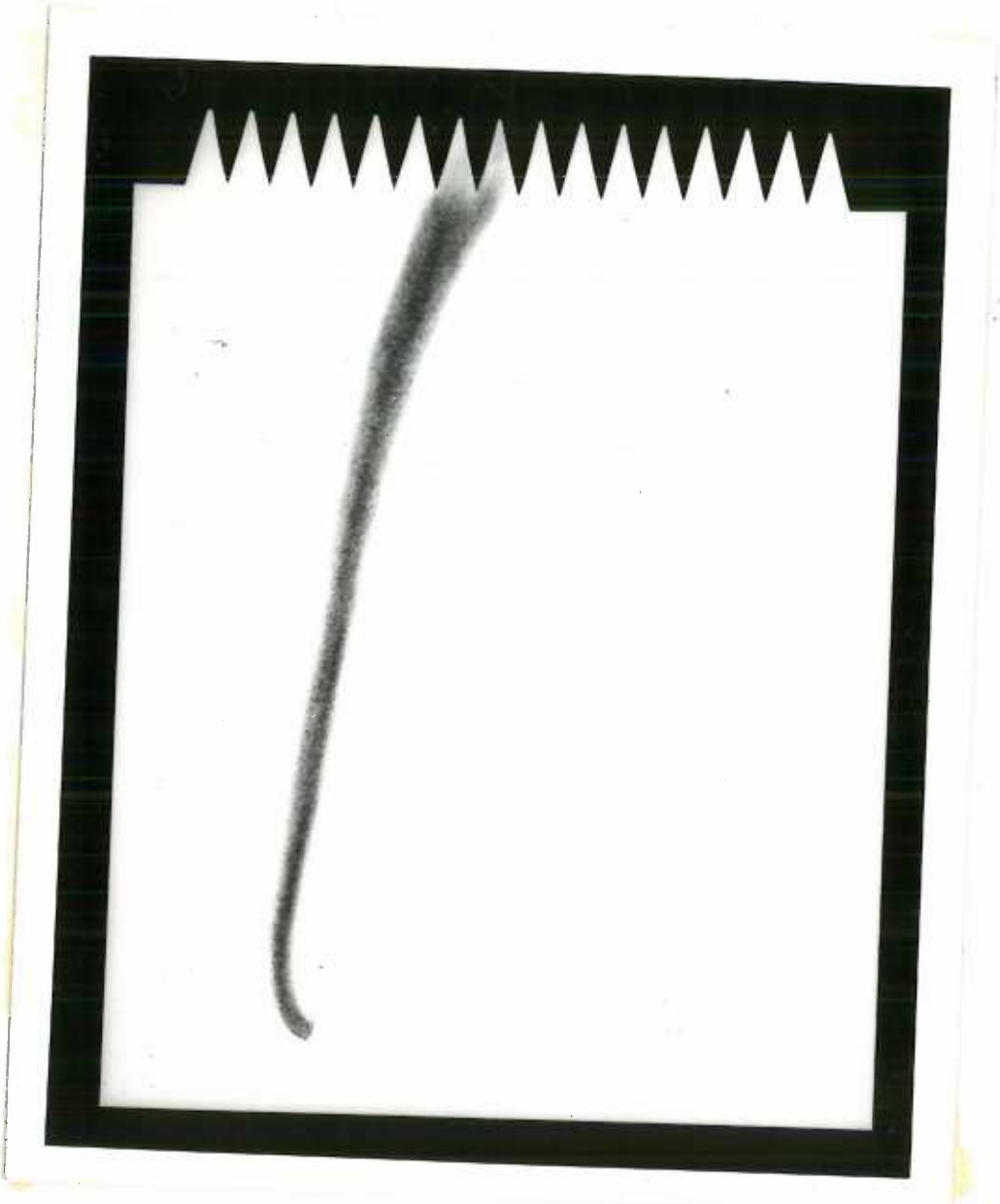

Figure 8. Artist's drawing of the pattern resulting from electrophoresis of Mixture A as revealed by spraying with o-tolidine reagent.

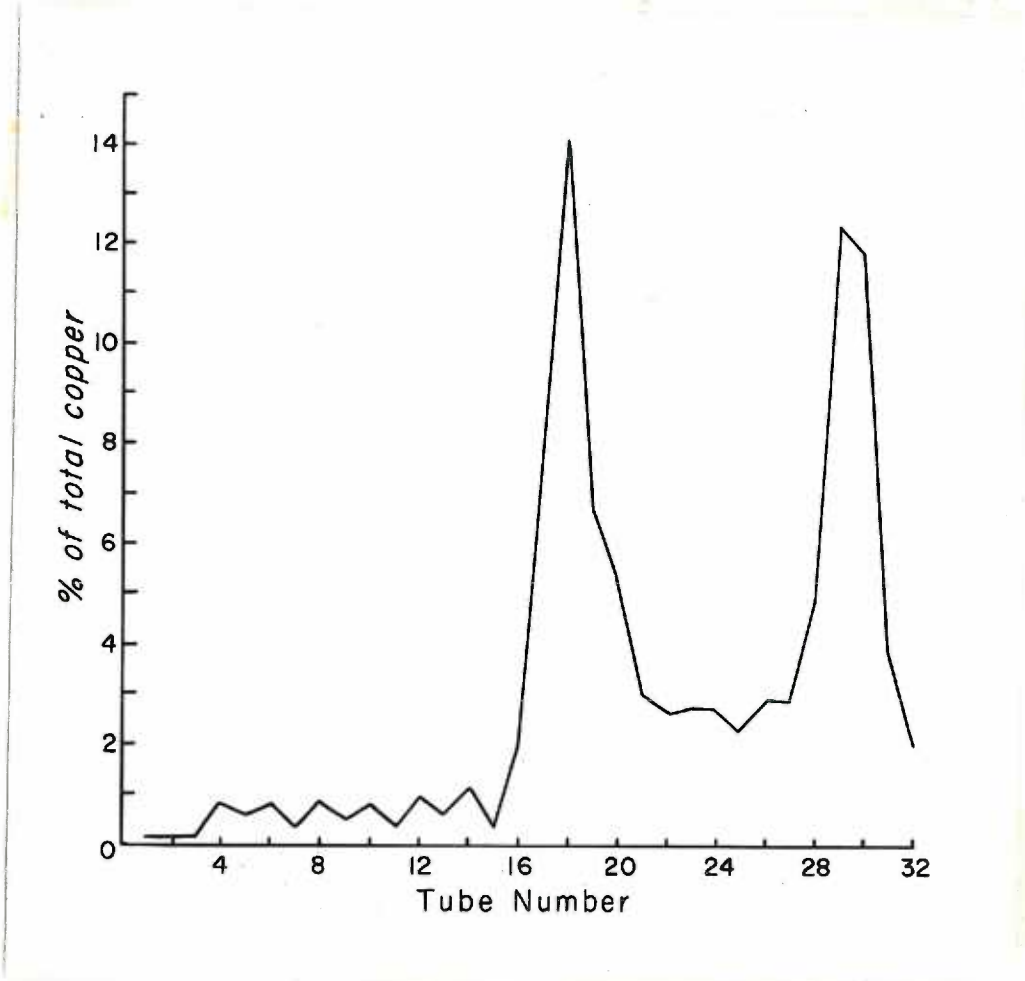

Figure 9. Plot showing the distribution of copper resulting from electrophoresis of Mixture A.

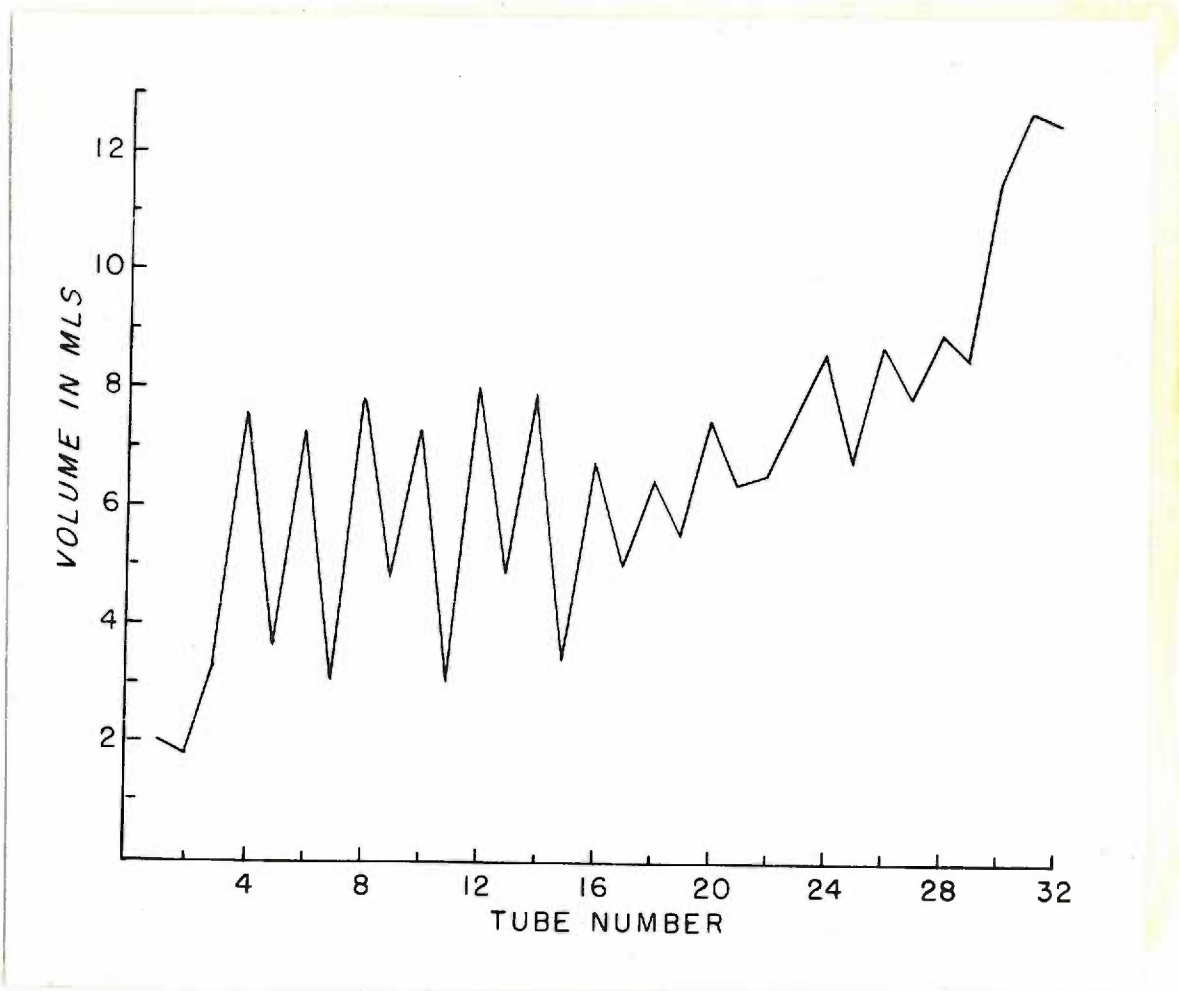

Figure 10. Plot showing the distribution of volumes collected during the electrophoresis of Mixture A.

is not as readily made, however. The significance, if any, of the occurrence of the copper components coinciding with the observed fluorescence is not understood. The contents of tubes 17 and 18, designated Mixture A', and those of tubes 29 and 30, named Mixture A", were lyophilized and extracted with absolute methanol according to the above procedure and dried *in vacuo* over silica gel.

Copper Content of Mixtures A' and A"

Samples of Mixtures A' and A" were weighed into 10 ml Erlenmeyer flasks and ashed in the manner previously described. Copper was quantitatively determined as before. The phosphate contents were also determined quantitatively (see previous footnote), being 11 percent for A' and less than 1.1 percent for A". The copper content of Mixture A' was 0.019 to 0.037 percent and that of Mixture A" 0.14 to 0.17 percent.

A copper content for Mixture A' of approximately 0.03 percent is disconcertingly low considering that this component represents about 25 percent of the total copper recovered from electrophoresis of Mixture A and that the copper content of Mixture A is 3 to 4 times greater than that of Mixture A'. A possible explanation of this result is indicated in the next section.

Paper Chromatography of Mixture A'

Mixture A' was chromatographed on Whatman No. 1 chroma-

tography paper with a solvent system consisting of n-butanol : 95 percent ethanol : water, 1:3:1, (v,v,v). Two separate applications were applied to the paper. After development, the chromatogram was cut in half length-wise, one half being sprayed with ninhydrin and the other with o-tolidine reagent. The results are presented in Figure 11, from which it can be seen that there is present in Mixture A' a considerable amount of material, especially at R_f's 0.42, 0.54, and 0.63, which is not associated with copper. Thus, it appears that although Mixture A' represents a concentration of copper during electrophoresis of Mixture A, it was accompanied by a large proportion of the ninhydrin material as indicated in Figure 7.

The Copper Content of Mixture B

The lyophilized, extracted, and dried contents of collecting tubes 30, 31, and 32 obtained from the initial continuous flow electrophoresis separation at pH 8.0 were analyzed for copper as described above. The copper content of Mixture B was found to be 0.147 to 0.148 percent. Phosphate analyses were not carried out on this material because of the paucity of material and the low phosphate contents previously obtained. This omission, as will be seen from subsequent results, was probably not critical.

Paper Chromatography of Mixture B

Further fractionation of Mixture B was achieved by paper

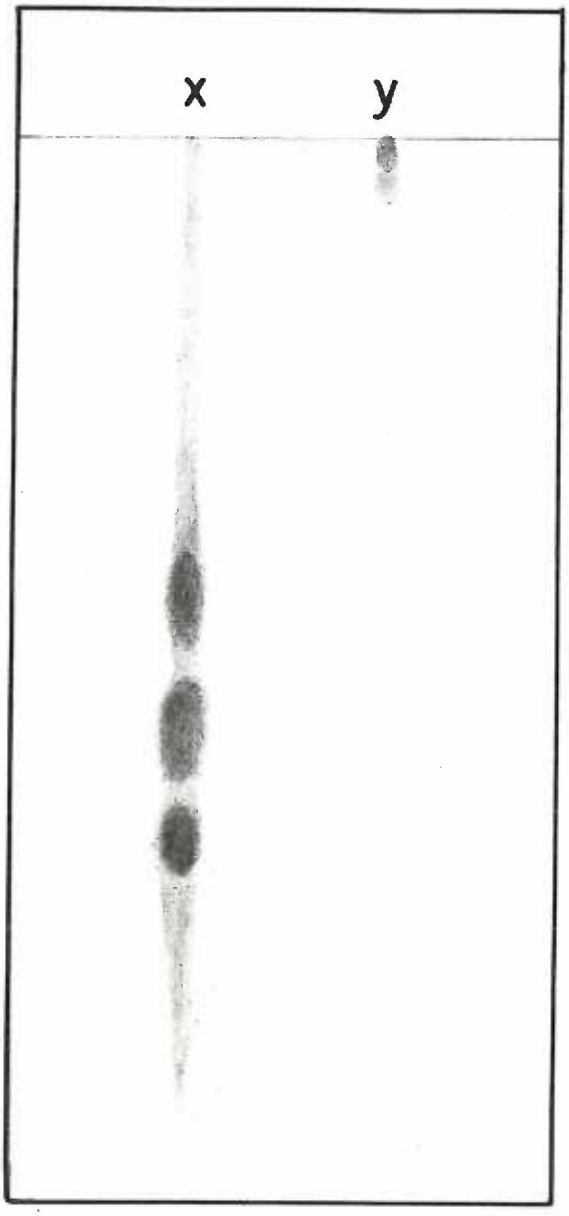

Figure 11. Artist's drawing of chromatograph of Mixture A' in n-butanol : 95 percent ethanol : water, 1:3:1, (v,v,v). x is material responding to ninhydrin and y is copper component.

chromatography with 77 percent ethanol as the solvent. Sheets of Whatman No. 1 chromatography paper, 7 x 22 1/2 inches, were striped with a methanol solution of Mixture B (2.5 mg of material per inch). Descending chromatography was carried out in glass tanks (12 x 24 inches) at room temperature. The solvent was added without any prior equilibration. After 48 hours, the chromatograms were removed from the tanks and air-dried. The positions of two copper-containing components, or bands, were determined by cutting strips from both edges of the chromatograms and spraying with the o-tolidine reagent. These components had migrated from the point of application an average distance of 5 inches and 7 1/2 inches, respectively. Spraying strips of these chromatograms with ninhydrin revealed the presence of 4-5 ninhydrin-reacting components, including those having copper. Additional ninhydrin-responding material may have been present in Mixture B, being undetected because of elution off the end of the paper by the solvent. Occasionally, but not always, a third minor copper component was detected at a distance of about 10 to 11 inches from the origin. This third component, when present, was considered to be a copper peptide possessing an electrophoretic mobility slightly less than that of the other two copper peptides. Its presence or absence was attributed to slight fluctuations in electrophoretic conditions beyond the control of the investigator.

The copper-containing components, designated B' and B"

(slower and faster migrating bands, respectively) were cut from the chromatograms, eluted with deionized water, and dried *in vacuo* over silica gel and potassium hydroxide pellets.

Copper Content of Mixtures B' and B"

Mixtures B' and B" were quantitatively analyzed for copper by the previously described method. Their copper contents were 0.184 to 0.191 percent and 0.157 to 0.160 percent, respectively. The amount of copper that may have been contributed by the chromatography paper (determined by cutting out strips having equal areas as those containing the copper-possessing components and eluting, etc.) was not determined. The copper, if any, contributed by the paper was presumed to be negligible. The consequence, if any, of omitting this procedure would be that the copper content of Mixtures B' and B", as determined, are too high.

The slight increase in copper content after removal of extraneous components indicates that these materials are still heterogeneous and that possibly the copper is associated with a large peptide relative to the peptides not containing copper. Heterogeneity could consist of two types in this instance. On the one hand, heterogeneity could involve the presence of material whose chemical constitution is quite different from that of the component of interest while on the other hand, heterogeneity could consist of material which differs from that containing copper only in

not containing copper. The means by which the latter situation might arise would involve the removal of copper by other peptides in the hydrolyzate*. The amount of copper removed in this manner would depend on the relative binding constants and concentrations of the peptides involved. For additional considerations and details of this proposal, refer to the Discussion.

Re-electrophoresis of Hydrolyzed Hemocyanin Less Mixtures A and B

In order to evaluate the possibility that the initial resolution of hydrolyzed hemocyanin was low because of a fast sample feeding rate, this material, less the fractions previously designated Mixtures A and B, was re-electrophoresed under conditions as nearly identical as possible to those employed for the initial separation (vide supra). The sample feeding rate is the volume of sample to be separated which is fed onto the curtain per unit time. If this rate is greater than the flow of the background electrolyte, a very wide band of sample results with a considerable degree of overlapping, which hinders good resolution[97b]. If this situation prevailed during the initial electrophoresis, which seems unlikely from the narrowness of the dark band in Figure 3, a second electrophoretic separation under the same conditions should yield a copper distribution having

* The converse situation, in which the peptides involved here may represent peptides not associated with the copper-binding site of hemocyanin but arising from the preferential chelation of copper, could also be the case.

the same pattern as previously.

In this experiment, electrophoresis was allowed to continue until most of the material had been washed off the curtain in order to assure sufficient material for analysis. For this reason, the curtain was not dried and sprayed as in the previous experiment. After completion of electrophoresis, the distribution of copper in the collecting tubes was again determined, this distribution being illustrated in Figure 12. Examination of Figure 12 shows that the percent of total copper in tubes 1, 2, 30, 31, and 32 is less than in the initial fractionation (Figure 5). In this instance, 13 percent of the total copper is present in these fractions, whereas 29.5 percent was accounted for in these tubes previously. Comparison of the copper distribution (Figure 12) and the volume distribution (Figure 13) shows a close agreement between these two factors except for the tubes formerly receiving Mixtures A and B. The coincidence between copper and volume distributions of tubes 3 to 29 attained formerly (Figures 5 and 6) was not as close as in this experiment, but the over-all agreement was good. The results of this experiment were interpreted as indicating that the distribution of copper in tubes 3 to 29 is not artifactual, but due to copper-bearing peptides of various mobilities.

It may be concluded that this work did not produce a copper peptide which could be taken as representing the binding site of copper in hemocyanin. Further evaluation of

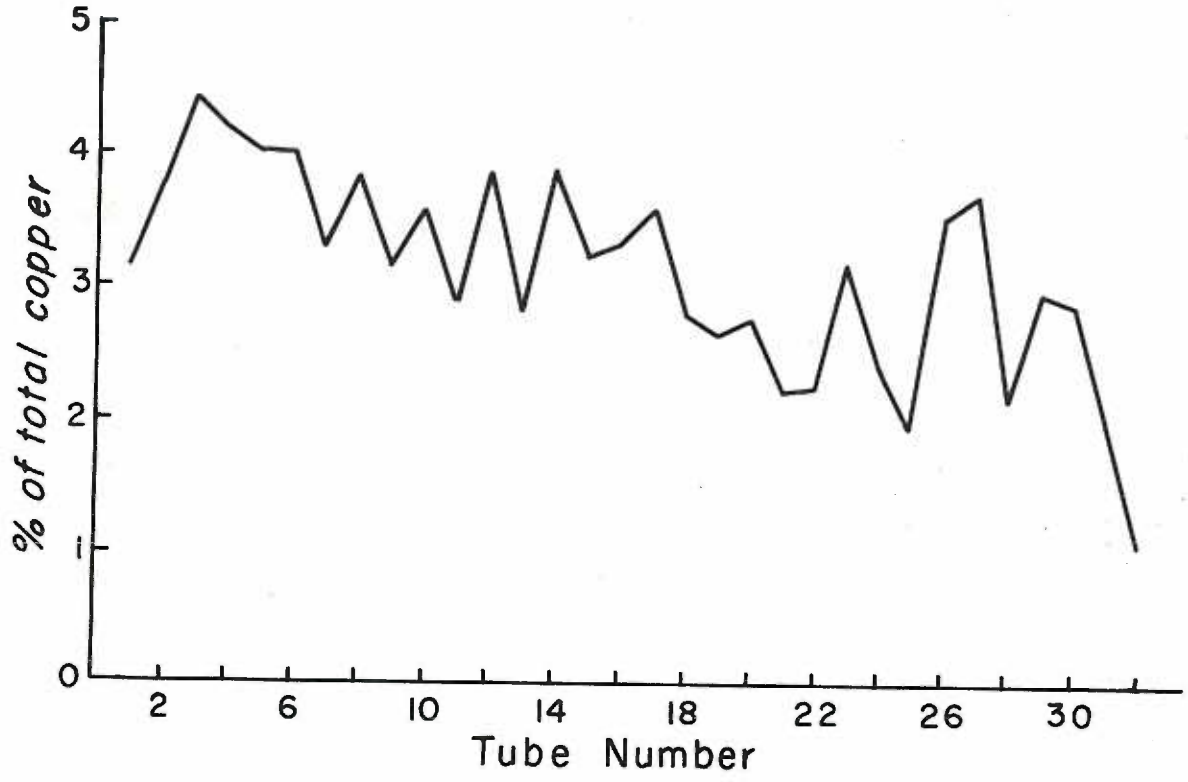

Figure 12. Plot of the copper distribution after electrophoresis of hydrolyzed hemocyanin minus Mixtures A and B.

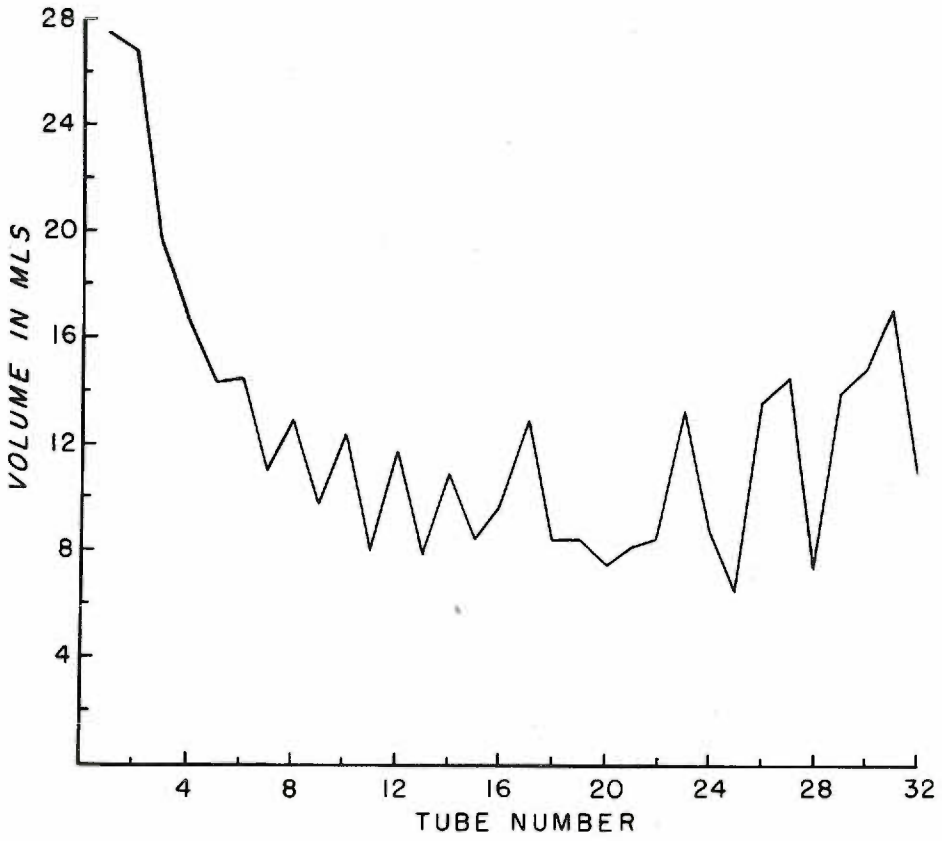

Figure 13. Plot showing the distribution of volumes collected during electrophoresis of hydrolyzed hemocyanin minus Mixtures A and B.

these results and this approach to the question of the binding of copper by hemocyanin appears in the Discussion.

Denaturation Studies with Cancer magister Hemocyanin

As has been indicated in the Introduction, the copper of native, or undenatured, hemocyanin is quite strongly bound by the protein[41]. In addition, reference was made to the decrease in this binding strength upon denaturation of hemocyanin[33]. Thus, it would appear that the binding of copper by hemocyanin is more or less dependent upon the secondary and/or tertiary structures of the protein[98,99], particularly with reference to its ability to bind oxygen. It is well-known that proteolytic hydrolysis of proteins, especially extensive proteolysis, involves denaturation of the protein hydrolyzed[98,99]. Therefore, in light of the experimental results just described, it was deemed desirable to ascertain, in a qualitative way at least, the effect of denaturation upon the binding of copper by the particular hemocyanin employed in these studies. The following sets of experiments were designed to investigate this question.

Dialysis of Denatured Hemocyanin

Several sets of experiments were done in which hemocyanin was dialyzed in the presence of 8 M urea, alone and in the presence of various chelating agents. In addition, experiments were done in which hemocyanin was dialyzed in the presence of these chelating agents, but in the absence

of urea. All solutions were maintained at pH 8.0 with 0.05 M phosphate buffer. These conditions were employed in order to approximate those prevailing in the previous experiments involving the enzymic hydrolysis of hemocyanin. The experimental procedure consisted of placing 24 ml of any particular solution plus 1 ml of hemocyanin solution (in buffer) inside a dialysis sac (5/8 inches diameter) and dialyzing against 25 ml of this solution contained in a 125 ml Erlenmeyer flask. Controls consisting of hemocyanin in buffer at pH 8.0 were always run simultaneously. The flasks were then placed on a mechanical shaker and agitated for 24-28 hours. In a few instances the procedure differed in that only 12.5 ml of solution plus 1 ml of hemocyanin, or 13.5 ml were contained in the sac rather than 25 ml. The theoretical distribution of copper in this case, if it were in a freely diffusible form, is 35 percent inside the dialysis sac and 65 percent outside. The distribution values appear in parentheses in Table 2. All other copper distributions have theoretical distributions of 50 percent outside and 50 percent inside the dialysis sac.

All glassware used in these (and preceding) experiments was thoroughly cleaned and rinsed in deionized water.

After the prescribed time of incubation, the dialysis sacs were removed from the flasks and their contents emptied into other flasks. Copper was then determined for all solutions by the method of Peterson and Bollier[95]. In all

cases, the total amount of copper theoretically present was taken as being equivalent to that found for the control. The results of these experiments have been collected and are presented in Table 2.

Inspection of Table 2 shows that denaturation per se does not cause hemocyanin to release copper (a possible exception to this is the system 6 M urea-saturated guanidine nitrate). Moreover, chelating agents in general (excluding cyanide) do not remove copper from native hemocyanin. These findings are in agreement with the observations of Zuckerkandl[33]. In the case of urea denaturation, temperature in the range usually innocuous to proteins had no effect upon the release of copper. Although these results are inconclusive, they tend to bear out Zuckerkandl's contention that denaturation is a necessary, and in all cases a sufficient, condition for the detection of copper by its chemical reactivity. Detailed considerations of these results are presented in the Discussion.

Sources of Reagents

The sources of reagents used in these experiments were: Urea--Mallinckrodt Chemical Works, New York, "Analytical Reagent" Lot No. 8648; Guanidine nitrate--Matheson, Coleman, and Bell Div., The Matheson Co., Inc., East Rutherford, N. J., Lot No. 5232; Histidine--Matheson, Coleman, and Bell Div., The Matheson Co., Inc., East Rutherford, N. J., Lot No. 5650; Tris [Tris(hydroxymethyl)aminomethane] --Sigma Chemical Co.,

St. Louis, Mo., Lot No. 36-180; Imidazole Acetic Acid Hydrochloride--Source unknown.

TABLE 2

Influence of Denaturation upon the Binding of Copper by Hemocyanin

System[a]	Total Copper (% Theoretical)	% Cu inside sac[b]	% Cu outside sac[b]	Temp. °C	Molar Ratio Chelator : Cu	Remarks
Control	100	100	0	1°, RT, 37°		
75% Ethanol[c]	100	100	0	1°		No buffer present, protein precipitated
75% Ethanol[c]	95.5	100	0	1°		As above
8 M urea[c]	107.3	100	0	1°		No pptn. of protein
8 M urea[c]	103.6	100	0	1°		" " " "
8 M urea	98.5	100	0	37°		" " " "
8 M urea	104.3	100	0	RT[d]		" " " "
8 M urea	105.3	100	0	RT		" " " "
sat. guanidine-NO_3	98.8	100	0	RT		Protein pptd. (sat. guanidine-NO_3 was very nearly 1 M).
sat. guanidine-NO_3	105.3	100	0	RT		
Histidine	82.0[e]	100	0	RT	4.72×10^2	No pptn. of protein
Histidine	76.5[e]	100	0	RT	4.72×10^2	" " " "

TABLE 2, Cont.

System	Total Copper (% Theoretical)	% Cu inside sac	% Cu outside sac	Temp. °C	Molar Ratio Chelator : Cu	Remarks
Tris	97.5	100	0	RT	8.75×10^2	No pptn. of protein
Tris	94.8	100	0	RT	8.75×10^2	" " " "
Imidazole acetic H^+	89.5	100	0	RT	3.59×10^2	" " " "
Imidazole acetic H^+	92.8	100	0	RT	3.59×10^2	" " " "
6 M urea-sat. guanidine nitrate	105.8	60.7	39.3	RT		" " " "
6 M urea-sat. guanidine nitrate	103.7	60.9	39.1	RT		" " " "
6 M urea-sat. guanidine-NO_3-Histidine	101.9	39.0 (35)[f]	61.0 (65)	RT	1.33×10^3	" " " "
6 M urea-sat. guanidine-NO_3-Histidine	98.3	38.9 (35)	61.1 (65)	RT	1.33×10^3	" " " "
8 M urea-Histidine	65.3[e]	47.6	52.4	RT	1.33×10^3	" " " "
8 M urea-Histidine	61.6[e]	64.1	35.9	RT	1.33×10^3	" " " "
8 M urea-Tris	111.6	79.5	20.3	RT	1.33×10^3	" " " "
8 M urea-Tris	111.1	80.1	19.9	RT	1.33×10^3	" " " "

TABLE 2, Cont.

System	Total Copper (% Theoretical)	% Cu inside sac	% Cu outside sac	Temp. °C	Molar Ratio Chelator : Cu	Remarks
8 M urea-Imidazole acetic acid	103.6	41.5 (35)	58.5 (65)	RT	3.59×10^2	No pptn. of protein
8 M urea-Imidazole acetic acid	103.6	41.5 (35)	58.5 (65)	RT	3.59×10^2	" " " "

a-All solutions were 0.05 M, pH 8.0 with respect to phosphate except for 75 percent ethanol.

b-% copper inside and outside sac is % found, not % theoretical.

c-Dialysis time was 18 hours.

d-RT = room temperature (24-28 °C).

e-Total copper found was low because histidine competed with the colorimetric reagent for the copper.

f-Figures in parenthesis represent theoretical distribution of copper.

DISCUSSION

It has been concluded from the experimental findings resulting from this work that no copper peptide was produced which could be taken as representing the binding site of copper in hemocyanin. The tentative interpretation of these results is that, for one reason or another, the copper of hemocyanin has been distributed among various peptides (and possibly amino acids) which were formed during proteolysis. This distribution of copper peptides is as depicted in Figures 5 and 8. The primary factor determining this distribution is considered to be dependent upon the relative binding strengths of the various peptides for copper. However, the insufficiency of information regarding this phenomenon precludes any quantitation of this factor. All of this is not to be taken as indicating that there was no copper peptide, or peptides, existing in the hemocyanin hydrolyzate which may have had their origin in hemocyanin and remained unchanged by proteolysis, but only that if such a peptide or peptides existed, there was no means by which they could be distinguished. It would, of course, be desirable to demonstrate the existence of a large number of copper-bearing peptides by other means. However, the means by which this might be done in a satisfactory manner, as will be discussed later, are rather limited.

The major evidence upon which this interpretation is

based are (1) the low copper contents of the major copper-containing components, (2) the demonstrated reliability of the fractionation procedure (electrophoresis), coupled with the low probability that this procedure, per se, could be responsible for the proposed migration of copper, and (3) the relatively even distribution of the copper among the various fractions. Each of these points shall now be considered in turn. Ancillary evidence derived from the denaturation experiments will be taken up separately (vide infra).

Cancer magister hemocyanin contains 0.158-0.173 percent copper(37). If during the enzymic hydrolysis of hemocyanin, the copper remained bound by a small, discrete number of peptides, any procedure resulting in the removal of extraneous, non-copper-containing material should result in an accompanying increase in the copper content of the material associated with the copper. As was demonstrated, none of the components associated with the majority of the copper possessed a copper content significantly greater than that of the starting material. These results, conceivably, might be attributed to artifacts and the argument presented that the peptide or peptides in question may be associated with the approximately 40 percent of original copper that was not extracted with absolute methanol. An examination of some of the possible reasons for this phenomenon might be instructive at this point. These reasons are that (a) the peptide material associated with this 40

percent copper are insoluble in absolute methanol because of size or other physico-chemical reasons; (b) the insoluble phosphate salts behaved as adsorptive agents analogous to calcium phosphate gels, for example; and (c) that the copper was precipitated as copper phosphate. Regarding the first possibility, the number of large peptides possible would likely be very limited in view of the fact that almost 90 percent of the peptide material was extractable. The result would be that a relatively small number of large peptides would be associated with a relatively large proportion of the copper. That this situation should arise due to the nature of the source material would seem, a priori, improbable. Should the second possibility prevail, it might reasonably be expected that the material extracted represented a more or less random sample of the material remaining behind. The third possibility would seem plausible because of the disproportionality between the amount of ninhydrin material extracted as compared to copper. However, if this possibility were operative and there were originally present in the hydrolysate a small, discrete number of copper-containing peptides, this number should not be changed (or at least not increased) by removal of some of their copper as copper phosphate. A more likely explanation for the observed phenomenon, perhaps, is that there exists an interplay of the three factors mentioned, in addition to factors unknown or unsuspected. In any event, these factors have been considered not to have ap-

preciably altered the situation had they been absent.

There is no apparent reason why electrophoresis, under the conditions of these experiments, should have brought about the release of copper and its subsequent recombination with other peptides. Electrophoresis has been used extensively in the purification of such labile substances as enzymes and proteins, and its success when used for these purposes attests to the generally mild conditions of this technique.

The third major piece of evidence cited for the proposal that no copper peptide representative of the "active center" of hemocyanin has been forthcoming from this work may be considered from several viewpoints. It is believed, for the reasons previously given, that the relatively even distribution of copper among the various fractions represents the actual situation and is not artifactual. However, there are alternative hypotheses regarding the reasons for the demonstrated distribution of copper. Firstly, it might be proposed that the apparently large number of copper-bearing peptides arose from incomplete proteolysis. As pointed out by Tiselius and Eriksson-Quensel(100), there are two possible extremes in the way in which a proteinase can attack a protein. It may split all the possible peptide bonds in each substrate molecule before it goes to the next, or the enzyme may act in such a way that it shuttles from molecule to molecule and breaks, for example, one peptide bond in each before it starts to cleave another bond in each, etc.

In the former instance there would be only substrate and end-products present in the reaction mixture, while in the latter a preponderance of intermediate reaction products would be present. All naturally occurring proteolytic reactions may be considered intermediates between these two extremes(99).

Inspection of Figure 1 shows that, at 25 °C, hydrolysis of hemocyanin was approximately 90 percent complete after 27-30 hours. Hydrolysis at 37 °C, then, may be presumed to be complete after this period of time because of the well-known effect of temperature upon the rate of enzymic reactions. However, if hydrolysis was still incomplete after this time, then the large number of electrophoretically different copper peptides would have to be represented by a very small amount of non-completely hydrolyzed material remaining after the incubation. There is no ad hoc reason why peptides containing copper should be more resistant to hydrolysis by the proteinase than those in which copper is absent. Even though a small proportion of the copper-containing peptides (again assuming a small, discrete number theoretically obtainable) were not completely hydrolyzed when the reaction was stopped, it would seem that the percentage copper appearing with the minor fractions (those not included in Mixtures A and B) should be less than the 70 percent found to be in these fractions (see Figure 5). The result of this manner of reasoning has been to consider

the hypothesis of incomplete hydrolysis as being responsible for the observed distribution of copper to be untenable.

A second proposal regarding the origin of the numerous copper-bearing peptides might be based on the argument that there are in hemocyanin more than one chemically-distinct binding sites for the copper. The maximum number of sites to be expected in this instance would be 24-26, since this is the number of moles of copper per mole of Cancer magister hemocyanin(37). But, considering that two atoms of copper are required to bind one molecule of oxygen and that, although it has not been demonstrated with this particular hemocyanin, hemocyanins in general have been shown to exist as aggregates of sub-units, it would not appear likely that there should exist 24-26 separate, individually characteristic binding sites for copper. Rather, a reasonable assumption to make regarding the number of these sites is that there are one or two, and possibly four(23).

In order to determine as unequivocally as possible that the copper has indeed migrated from its original site, it would be necessary to isolate and determine the amino acid composition and sequence of every copper peptide resulting from the proteolytic hydrolysis. Such an undertaking would involve a considerable amount of time and effort and, in view of the results described herein, would be of questionable value.

As mentioned above, proteolysis involves denaturation of the protein hydrolyzed. The degree of denaturation depends, of course, upon several factors, among which are the extent of hydrolysis and the nature of the protein undergoing attack(99). Denaturation by enzymes and chemical agents have some features in common, such as the breaking of hydrogen bonds. Therefore, it was hoped that studies on the effect of denaturation of hemocyanin by non-enzymic means, with reference to its ability to bind copper, might give an indication regarding the validity of the tentative interpretation presented for the results obtained earlier. It should not be construed, however, that the two types of denaturation have been considered to be strictly analogous.

The copper of native hemocyanin, as far as is known, is not removed by chelating agents (with the previously noted exception of cyanide). No experimental evidence exists by which this phenomenon may be explained, although, as mentioned in the Introduction, Zuckerkandl(33) has proposed that it is due to steric factors. As may be seen from Table 2, the chelating agents histidine, imidazole acetic acid, and Tris [tris(hydroxymethyl)-aminomethane] were ineffectual in removing copper from native hemocyanin. These results are consistent with those obtained using imidazole and di-ethyldithiocarbamate(101,33).

Denaturation, at least denaturation caused by urea,

guanidine, or alcohol, does not bring about a release of copper by hemocyanin. Burk[15] also found that urea denaturation of hemocyanin does not cause labilization of copper. However, it is questionable that urea causes complete denaturation of hemocyanin, in the sense that complete unfolding of the polypeptide chain occurs. Urea has adequately been shown to cause dissociation of hemocyanin into sub-units[13,15] and this phenomenon may be considered to be one type of denaturation[102]. Dervichian, et al,[103], showed that this type of denaturation in the case of hemocyanin is quite dependent upon the concentration of urea used and that the time required for dissociation also depends upon the relative quantities of urea and protein present in the solution. The extent of denaturation in these experiments cannot be ascertained from the observation that urea did not cause precipitation, since urea denaturation does not necessarily cause a decrease in solubility[102].

An interesting point in connection with the denaturation of hemocyanin regards the question of whether this phenomenon causes migration of copper from its original site on the molecule. Zuckerkandl[33] has suggested that it is only after denaturation that the proper chemical nature of the copper bond manifests itself, but evidence supporting this proposition is lacking. There are no indications from the present work either supporting

or denying the validity of this hypothesis.

A possible exception to the conclusion that denaturation does not cause a release of copper by hemocyanin is indicated from the experiment in which hemocyanin was incubated in the presence of 6 M urea-saturated guanidine nitrate. The combination of these reagents would be expected to have a greater ability to denature proteins than either reagent alone(102). The observed labilization of copper could very possibly be due to the denaturing capacity of this system, but alternative mechanisms (e.g., chelation) cannot be conclusively ruled out.

Following denaturation, the chelating agents used in these experiments were able, to some degree at least, to compete with the protein for the copper which it contained. That Tris should exhibit a chelating effect in the case of copper is not particularly surprising since Benesch and Benesch(104) have shown that Tris forms a stable complex with silver analogous to the silver-ammonia complex. In addition, as Corwin(105) has pointed out, since the ordinary coordination number of copper is four and only a few compounds with a higher coordination number are known, copper is large enough to coordinate about itself as many atoms of as large a size as its other properties will permit. A copper-imidazole complex, for example, in which the copper-imidazole ratio is 1:4 has been prepared and studied(106,107). The fact that approximately 80 per-

cent of the copper remained in the dialysis sac after 24-28 hours incubation may reflect slow attainment of equilibrium in the formation of this complex due to steric factors or that the complex, once formed, dialyzes out of the sac at a slow rate. Nevertheless, the results obtained are considered to be real and due to an actual chelation effect.

Histidine and imidazole (as imidazole acetic acid) were of interest because of the presence of these moieties in proteins and peptides. The involvement of histidine in the binding of copper by proteins and peptides has been extensively demonstrated(108,109). The sulfhydryl group of cysteine, the carboxyl group of aspartic acid, and amine groups have also been implicated in the binding of copper by proteins(35,110,34).

Among the elements which undergo complex formation and chelation may be included most of the metals of the periodic system. The transition metals in particular form a large variety of such compounds and have come to be considered as having the strongest tendency for combining with electron donors(111). Copper, being a transition metal, would be expected to form a variety of chelates, and experience bears this out. That copper forms such compounds with proteins and peptides has been known for some time. The ability of peptides to dissolve freshly precipitated copper oxide was noted frequently by Emil Fischer during the course of his researches on the amino acids and proteins(112). Therefore, it does not seem

presumptuous to propose that the occurrence of the large number of copper peptides indicated in this work arose from the random chelation of copper by the peptides resulting from the enzymic hydrolysis of hemocyanin. Thus, after hydrolysis of the protein, a situation prevails in which the copper formerly associated with hemocyanin is distributed, in part at least, among various of the peptides formed; the nature of this distribution would depend on the chemical constitution of the peptides participating, their concentrations, the relative magnitudes of their chelate stability constants, and the concentration of copper.

Let us now consider two alternative approaches to the problem of the nature of the binding of copper by hemocyanin involving the use of proteolytic enzymes; namely, limited proteolysis and the artificial "labeling" of hemocyanin by reagents specifically reacting with various amino acid moieties, followed by hydrolysis.

Limited proteolysis; i.e., proteolysis in which only a few peptide bonds are cleaved, would theoretically result in the formation of relatively few polypeptides, the surviving portion of the protein remaining more or less intact, depending upon the extent to which the protein has been modified(102). The means by which limited proteolysis could be accomplished are either to limit the time of reaction or to employ a proteinase possessing

a narrow specificity toward the type of peptide bond split, or both of these. Restricting the reaction time, although producing relatively few peptides, would not necessarily produce peptides of identical chemical constitution. In order for this to occur, the second extreme for the mechanism of action of proteinases referred to previously would have to prevail. The probability of such a situation existing has already been precluded(99). Regarding the use of enzymes of restricted specificity, it would appear that the enzyme used in these studies is ruled out. Pepsin could also be excluded because of its activity depending on acid media, which would remove the copper of the hemocyanin substrate. The use of trypsin likewise would seem undesirable because of the relatively high arginine content of hemocyanin(47). However, there remain the possibilities of chymotrypsin and various of the plant proteinases, e.g., papain, ficin, and bromelin.

It should be somewhat obvious at this point that the success of the above suggested approach depends largely upon the copper being associated with the peptides formed and not with the bulk of unhydrolyzed protein. For, should the latter situation be the case, which is not unlikely, it then becomes a matter of practical limitations as to whether any more information is forthcoming due to the use of limited proteolysis than could

be obtained from hemocyanin itself.

The artificial "labeling" of various amino acid residues; i.e., the reaction of hemocyanin with certain reagents which would specifically alter the structure of a single class of amino acid such as sulfhydryl, imidazole, etc., followed by enzymic or acid hydrolysis is another approach to the general problem of the means whereby copper is bound to hemocyanin. This approach is, of course, analogous to the work on the active center of chymotrypsin, trypsin, etc. referred to in the Introduction. However, the success to be anticipated from this approach is, at the present time at any rate, quite limited for the following reasons. Firstly, this technique is rendered somewhat undesirable because, as pointed out in the Introduction, there are many conditions which must be satisfied regarding its application. Secondly, the instances in which this technique has been successful have all involved proteins having enzymic activity and that do not possess prosthetic groups, with the possible exception of phosphoglucomutase. Because of their activities, only one amino acid was labeled (the serine of the active center), although there are present in the enzymes additional serine residues, at least in the case of chymotrypsin(113). Moreover, the presence of a prosthetic group, such as the copper of hemocyanin, could quite conceivably complicate matters, especially if more than one type of bond is

responsible for binding copper. Indirect and inconclusive evidence, however, might be obtained by reacting hemocyanin with various reagents under conditions which did not remove the copper and then determining whether any new groupings like the ones modified appear after the removal of copper. A third limitation of this approach is that, at present, there is no clear-cut indication of just which group, or groups, should be investigated. As noted previously, amine, imidazole, and sulfhydryl groups have been suggested as participating in the binding of copper by hemocyanin(34,36,42), but conclusive evidence is lacking.

I would like briefly to point out at this time that many of the procedures and techniques normally employed for peptide isolation were not applicable to this problem. For example, there are many partition chromatography systems available which have been used, and with considerable success, for the isolation of peptides(94,114). However, a large percentage of these systems employ either solvents which are acidic; e.g., n- butanol : acetic acid : water and phenol : water, or the solvents have as constituents various nitrogenous bases, such as ammonia, pyridine, or lutidine. Since most, if not all, copper-peptide linkages are quite labile to acid hydrolysis, and since nitrogenous bases, in general, are good chelators of copper, it was considered inadvisable to use these systems in connection with this work. This same sort of reasoning was applied to the use of ion-exchange resins. The two effects

mentioned here, acid pH and chelation, also limits the number and types of buffers that can be used for maintaining the pH of hydrolysis mixtures or varying the pH in electrophoresis procedures. All of this is not to say that various of these procedures and techniques could not be applied, but that in so doing, a risk is involved in either removing copper from its peptide associations or in creating artifacts.

SUMMARY

The hemocyanin of the crab, Cancer magister, was investigated in this work. The purpose of these researches was to obtain a peptide from hemocyanin which contained copper and which could be further studied in order to secure information regarding the nature of the binding of copper by the parent compound.

A bacterial proteinase was used for the purpose of reducing the protein to the peptide stage. By following the course of the reaction with a pH-stat, it was possible to determine the average molecular weight of the peptides resulting from hydrolysis. This value was calculated to be approximately 916.

Batches of hemocyanin were hydrolyzed at pH 8.0 in 0.05 M phosphate buffer. The temperature was 37 °C. The reaction mixtures were lyophilized after completion of hydrolysis. Absolute methanol was used to extract the peptide material from the buffer salts.

Continuous flow paper electrophoresis was employed as the initial step in the attempted isolation of the peptide sought. Two major copper components resulted from this procedure. The two components contained between them 29.5 percent of the total copper collected, the remainder appearing with an undetermined number of minor constituents. It was demonstrated that this distribution of copper was not artifactual and not due to the buffer used.

Each of the two components just mentioned gave rise to two additional components upon further fractionation. None of these components were shown to be homogeneous. The copper contents of these six fractions were quantitatively determined. In no case was the copper content found to be significantly higher than that of the starting material.

It was concluded that this work did not produce a copper peptide which could be taken as being representative of the binding site of copper in hemocyanin. It was proposed that the wide distribution of copper found, coupled with the low copper contents of the components investigated, arose from a competitive chelation of the copper by the peptides formed during the proteolysis. As a means of qualitatively evaluating this proposal, several experiments were done in which the lability of copper in native and denatured hemocyanin was compared.

These experiments consisted of dialyzing buffered solutions of hemocyanin in the presence of the denaturing agents alcohol, urea, and guanidine nitrate. Hemocyanin was also dialyzed in the presence of the chelating agents histidine, imidazole acetic acid, and Tris [tris(hydroxymethyl)-aminomethane]. Copper was not released by hemocyanin under any of these conditions. However, in the presence of 8 M urea, Tris was effective in removing at least a part of the copper held by hemocyanin, while the other two chelating agents appeared to be quite capable of

combining with the protein-bound copper. These results, although inconclusive, were interpreted as lending credence to the original conclusion.

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