MAGNETIC PROPERTIES OF HEMOCYANIN

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I wish to extend my sincere gratitude to Professor Linus Pauling for suggesting the magnetic investigation of hemocyanin and outlining the nature of the problem. I would further like to express my indebtedness to the system of bond chemistry established by Professor Pauling upon which my theoretical interpretations are based.

I am very grateful to Doctor Charles Coryell for the time he has taken to instruct me in the magnetic technique.

To Doctor George MacGinitie for suggesting the source of hemocyanin and his cooperation in obtaining it I wish to express my appreciation.

Finally I desire to thank the members of the Micro-analytical Laboratory, Mr. Henry Lanz and Mr. Glenn Swinehart, for the analysis of my material.

PREFACE.

The primary purpose of this investigation was to determine the magnetic properties of hemocyanin in order to elucidate the oxygenation reaction. However, a number of other properties were examined as a preliminary basis for this work. These other experiments were also of value in demonstrating the similarity of the hemocyanin used to the other hemocyanins and hence of the applicability of the magnetic work to these other proteins. As the hemocyanins are a class of proteins well adapted to studies from a comparative point of view, these results are of interest in themselves as the animal used was of a group not yet investigated. In order to increase the facility with which these comparative deductions are made I have devoted some space to results on other hemocyanins.

As no review of the hemocyanins has appeared for eight years, and much important work has been done meanwhile, I have felt justified in including some of the results of the recent work that bear on the final interpretation of the magnetic experiments.

The animal chosen for use was the giant key-hole limpet, Megathura crenulata, a Streptoneuran of the class Gasteropoda, found on the rocks in several places along the Pacific coast.

For the convenience of those who are not acquainted with all of the species mentioned I have included a table of common names at the end of the paper.

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INTRODUCTION.

"Le sang du colimacon est d'un blanc bleuatre tres different de celui de l'homme et des grandes animaux qui est rouge fonce." Swammerdam (1737).

The hemocyanins are copper-containing proteins in the blood of various invertebrates and are capable of reversibly combining with oxygen. They are not contained in corpuscles but are dissolved in the plasma. Their labile union with oxygen makes it possible for them to function as respiratory proteins and in a number of cases it has been shown that they do perform a respiratory function in the animal. They differ from hemoglobin, hemerythrin, chlorocruorin and the erythrocruorins in containing copper as an essential part of their molecule. They occur only in the blood of molluscs and arthropods.

The history of hemocyanin goes back to the 18th century where we find that Swammerdam has recorded that the blood of the snail is blue in his final and most important work, the Biblia Naturae. The next mention we find of it is by Baron Cuvier in 1795 in his memoir on the classification of the animals where he generalizes by stating that the blood of molluscs is blue.

During the 19th century we find many men noting the occurrence of this blue color in invertebrate blood (Ermann 1816, Carus 1824, Wharton 1846). Finally in 1847 Harless and von Bibra demonstrated the existence of a protein containing copper and combining reversibly with oxygen. The naming of this substance, however, was left to Fredericq (1878), who combined the Greek words for blood (haimos) and dark blue (kyanos). He

believed that hemocyanin was analogous to hemoglobin and that the copper functioned in the same way as the iron. This copper-protein was immediately assumed to exist wherever a blue color had been seen in the blood (Genth 1852, Haeckel 1857, Witting 1858, Schlossberger 1867, Bert 1867) and this was shown to be true in general when the presence of the protein was shown by its purification from the bloods of many invertebrates: crustacea (Krukenberg 1880a, 1880b, 1882), molluscs (Griffiths 1884, 1891, 1892).

The relation of the blue color of these bloods to oxygen had meanwhile been investigated. Rabuteau and Regnard (1873) and Jolyet and Regnard (1877) confirmed the observations of Harless and von Bibra and found that carbon dioxide would decolorize the blood, driving the oxygen off. Griffiths from 1888 to 1892 had made numerous measurements of the oxygen capacity of various bloods but accurate work was not done along this line until 1909 when Winterstein carefully checked previous values and found them to be incorrect.

Since 1900 there has been a great deal of quantitative work done on hemocyanin; in fact, we can say that our knowledge of hemocyanin is as extensive as that of hemoglobin with one exception — the nature of the prosthetic group. During the last forty years Quagliariello in Italy, Dhere and Roche in France, Svedberg in Sweden, and Conant and Redfield in America have been the most active participants in this field and it is upon their work, as well as upon the work of numerous others, that many of the ideas and deductions of the following investigation are based.

PHYSICAL PROPERTIES.

We can, according to most workers in the field, divide the properties of the hemocyanins into two categories: those based on the protein part of the molecule and those for which the prosthetic group is responsible. The oxygen-combining power and the absorption spectrum are the sole representatives of the latter class. Most of the physical and chemical properties, as well as many of the biological ones, depend on the protein type of structure. They are, however, in some respects different from the "normal" types of protein and it is of interest to compare them with other proteins and also to determine the specific differences which exist between the hemocyanins from various sources.

A. Amphoteric properties.

By amphoteric properties I mean those that depend on the fact that all proteins are built up of amino acids and hence possess a zwitter-ionic type of structure, being capable of combining with either acids or bases and showing a certain charge distribution over the surface, depending on the conditions under which the protein is placed.

1. Iso-electric point.

The iso-electric point is defined as that pH (or range of pH) at which a solution of an amphoteric substance, when placed in a homogeneous electric field, will show no inhomogeneous distribution of this substance after a period of time. It is the pH at which the total positive charge is equal to the total negative charge on the substance in question.

A minimum of solubility for proteins occurs at this point or very near

this point. It would accurately be at this point if proteins did not combine with other ions besides hydrogen-ions and hydroxyl-ions. Thus determinations of the iso-electric point by change of solubility with pH must take the ionic strength of the solution into account (thus the minimum solubility of casein changes from pH 4.8 to pH 4.06 when the ionic strength goes from zero to 0.1). I discuss this point because a number of men have used this method for the determination of the iso-electric point of the hemocyanins. Cataphoresis measurements are in general the only reliable means of determining the true iso-electric point and most of the results in the following table were obtained in this way.

Species	i.e.p.	Method	Author
Mollusca.			
Gasteropoda.			
Helix pomatia	5.05	Cataphoretic	Svedberg (1930b)
	5.05	"	Tiselius (1930)
	5.05	"	Pedersen (1933)
	5.20	Titration	Roche (1936a)
	5.20	Min. Sol.	Roche (1932)
	5.3	Cataphoretic	Stedman and Stedman (1927)
	4.7	Cataphoretic	Quagliariello (1920b)
	5.2	"	Svedberg and Chirnoaga (1928)
	5.20	Min. Sol.	Roche (1936a)
	5.10	Cataphoretic	Roche (1936a)
Helix nemoralis	4.63	Cataphoretic	Pedersen (1933)
Helix hortensis	4.57	Cataphoretic	Pedersen (1933)
Helix arbustorum	5.50	-	Stedman and Stedman (un.)
Paludina vivipara	4.71	Cataphoretic	Pedersen (1933)
	4.71	Cataphoretic	Svedberg (1930)
Paludina contecta	4.63	Cataphoretic	Svedberg (1930)
Littorina littorea	4.34	Cataphoretic	Pedersen (1933)
Buccinum undatum	4.61	Cataphoretic	Pedersen (1933)
Achatina fulva	5.03	Cataphoretic	Pedersen (1933)
Cephalopoda.			
Sepia officinalis	5.05	Titration	Roche (1936a)
	4.80	Cataphoresis	Roche (1936a)
	4.80	Min. Sol.	Roche (1936a)
Octopus vulgaris	4.80	Cataphoretic	Quagliariello (1920b)
	4.90	Cataphoresis	Rpche (1930)
	4.80	Min. Sol.	Roche (1936a)
Eledone moschata	4.80	Min. Sol.	Roche (1936a)

Species	i.e.p.	Method	Author
Eledone moschata	4.70	Cataphoresis	Roche (1936a)
Eledone cirrosa	4.60	Cataphoresis	Pedersen (1933)
Arthropoda.			
Crustacea.			
Homarus vulgaris	4.80	Min. Sol.	Stedman and Stedman (1926)
	4.70	Min. Sol.	Stedman and Stedman (1927)
	4.95	Cataphoresis	Pedersen (1933)
Astacus fluviatilis	4.93	Cataphoresis	Pedersen (1933)
	4.8	Min. Sol.	Redfield (1934)
Dromia vulgaris	4.80	Min. Sol.	Roche (1936a)
Cancer pagurus	4.65	Cataphoresis	Pedersen (1933)
	4.7	Cataphoresis	Stedman and Stedman (1927)
	4.8	Min. Sol.	Stedman and Stedman (1927)
Carcinus maenas	4.70	Min. Sol.	Roche (1936a)
	4.70	Cataphoresis	Roche (1936a)
Maia squinado	4.95	Min. Sol.	Roche (1936a)
	4.70	Cataphoresis	Roche (1936a)
Xiphosura.			
Limulus polyphemus	6.0	Titration	Roche (1930)
	6.30	Min. Sol.	Roche (1936a)
	6.30	Cataphoresis	Roche (1936a)
	6.3	Min. Sol.	Stedman and Stedman (1926a)
	6.4	Cataphoresis	Svedberg (1930b)

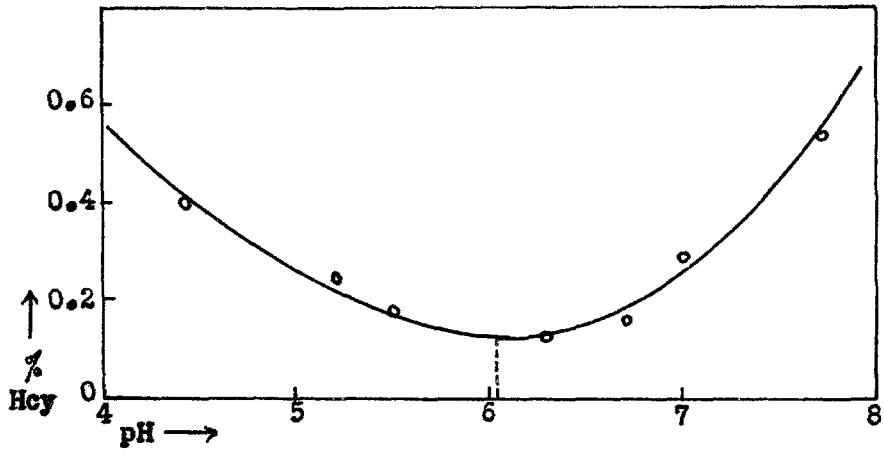
I have investigated the iso-electric point of the hemocyanin of the giant key-hole limpet, Megathura crenulata. Purified hemocyanin (see further) was used in all cases. Three methods were tried: (a) minimum solubility, (b) change of pH on addition of electrolytes and (c) cataphoresis.

Solutions of hemocyanin were made up in 2% NaCl and various amounts of dilute HCl or NaOH were added to give the pHs recorded. A trial run by comparing the tubes visually resulted as follows:

pH	appearance (18 hours)
1.9	clear, no ppt.
4.95	ppt., supernate cloudy
6.69	all pptd., clear supernate
7.11	supernate slightly cloudy
8.15	small ppt., very cloudy
8.85	clear, no ppt.

This indicated that there was a fairly broad range of low solubility and that the minimum was somewhere between pH 6 and pH7. This narrow region was then

further investigated by the same procedure except the supernates were pipetted off and dry-weight determinations made, correcting for the amount of salt present. The results can best be represented on a graph, the lowest point on the curve indicating the approximate iso-electric point.

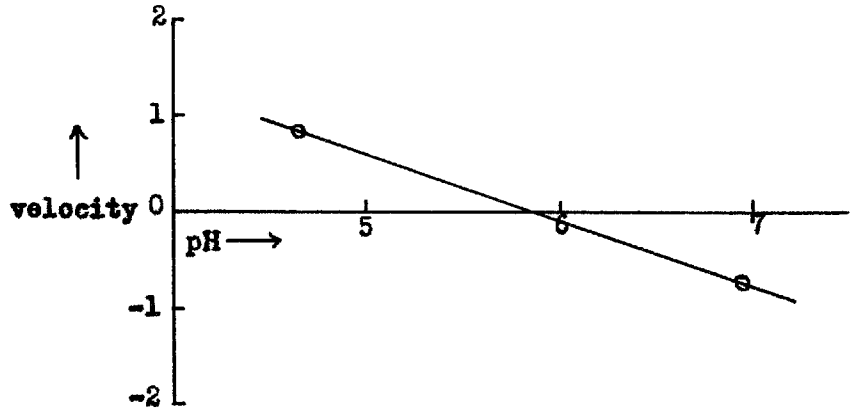


By comparing with other solubility curves we note two facts: (a) the curve is flatter at the minimum than any curve yet recorded and (b) the pH of minimum solubility is higher than any other hemocyanin with the exception of Limulus-hemocyanin.

An attempt was made to determine the iso-electric point by the addition of NaCl to solutions of hemocyanin at different pHs. It is known that the shift in pH on this addition will always be towards the iso-electric point. However, Megathura-hemocyanin has such a wide zone of minimum solubility that it was impossible to approach the iso-electric point close enough at the low ionic strengths necessary for this experiment. Solutions with an original pH of 4 and 8 showed a strong drift when salt was added but the only conclusion that can be made is that the iso-electric point is somewhere between pH 7.5 and pH 4.25 (the pHs to which the solutions drifted).

I was interested in checking the iso-electric point determined by minimum solubility, as this, as mentioned above, sometimes differed from the true

iso-electric point. Dr. James Bonner was kind enough to lend me his micro-cataphoresis apparatus. The hemocyanin was adsorbed onto finely ground quartz particles and the velocity of the particles noted in solutions at various pHs. As the slope of the pH-mobility curve is quite constant for a region of one or two pH units on either side of the iso-electric point, it was sufficient to determine two points on the curve and draw a straight line thru them. Where this intersects the axis of zero velocity we can read off the iso-electric point. The following plot illustrates this and the results of the experiment.



The iso-electric point determined in this manner (5.82) checked quite closely with the pH of minimum solubility (6.05). For the details of the above micro-cataphoretic method see the paper of Brown and Broom (1936) in which is a theoretical treatment of the measurements and a practical routine for making the determinations. Great cleanliness is required: the quartz particles were allowed to stand in distilled water for three days before using and the interior of the micro-cataphoresis cell was cleaned with nitric acid and washed repeatedly with distilled water. The velocity of the particles was determined at a level half-way between top and bottom of the cell and also immediately under the top surface. The formula:

$$V = 0.361\ V_{\bullet} + 0.639\ V_{\frac{1}{2}}$$

was used to calculate the true velocity. It has been shown for numerous

proteins that the adsorption on quartz does not appreciably affect the iso-electric point. The determinations were made in solutions 6% (1.0M) NaCl.

From the broad range of minimum solubility and the small slope of the the pH-mobility curve we can say that Megathura-hemocyanin probably binds a relatively small amount of acid and base per gram of protein. In respect to its iso-electric properties this hemocyanin resembles the hemocyanin from Helix more closely than any other hemocyanin.

For comparative purposes the following table of mobility-slopes is given.

Hemocyanin	du/dpH x 10^{-5}	Author
Helix pomatia	8.1	Pedersen (1933)
Helix nemoralis	11.4	Pedersen (1933)
Helix hortensis	12.1	Pedersen (1933)
Helix arbustorum	7.6	Svedberg (un.)
Paludina vivipara	10.8	Svedberg (un.)
Paludina contecta	11.4	Svedberg (un.)
Littorina littorea	12.8	Svedberg (un.)
Buccinum undatum	13.7	Svedberg (un.)
Achatina fulva	7.8	Svedberg (un.)
Cancer pagurus	16.0	Pedersen (1933)
Homarus vulgaris	18.0	Pedersen (1933)
Astacus fluviatilis	12.1	Pedersen (1933)

As the iso-electric point was the aim in my determination I cannot say accurately from my measurements what du/dpH is for Megathura-hemocyanin, but I estimate it to lie between 7.5 and 9.0.

In conclusion we can say, in agreement with Svedberg, that iso-electric points are a matter of species differences and have no evident relation to phylogeny on a higher level.

2. Solubilities.

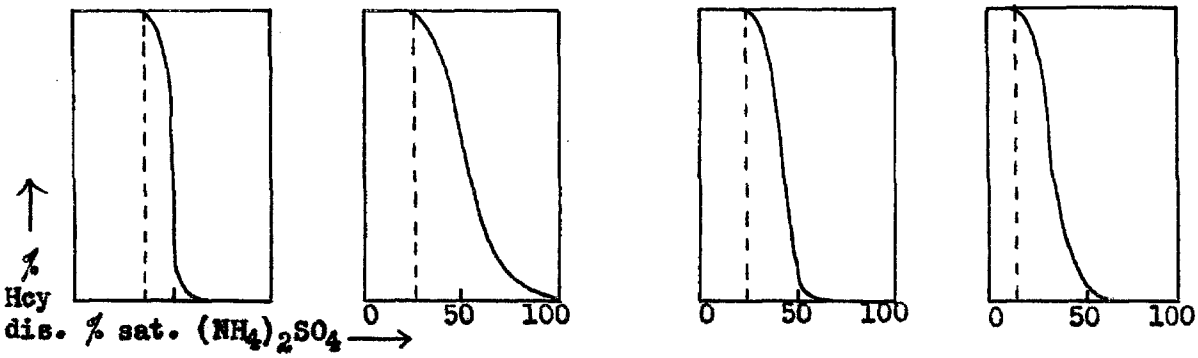
All of the hemocyanins are globulins, i.e. they are insoluble near their iso-electric point in dilute salt solutions, soluble in intermediate salt concentrations over a large range of pHs, and insoluble in high concentra-

tions of electrolytes. However, there are wide differences between the hemocyanins of various species with regard to the quantitative aspects of the problem. It should be remarked that with hemocyanins the solubility is not so definite as with simpler substances: as the medium is changed the size of the particles or aggregates changes in a more or less continuous manner so that we must make some arbitrary definitions of the amount held in solution, e.g. that remaining in the supernate after a certain period of centrifuging at a definite speed.

Hemocyanins differ with respect to their solubility at the iso-electric point: Helix pomatia, Helix hortensis, Helix nemoralis, Limulus polyphemus, Paludina vivipara, Paludina contecta and Achatina fulva hemocyanins are more soluble in dilute salt solutions at the iso-electric point than the hemocyanins of Busycon canaliculatum, Buccinum undatum, Littorina littorea and Astacus fluviatilis. This property is of great importance in the preparation of these hemocyanins because it determines how complete a dialysis must be before the protein is completely precipitated. Megathura-hemocyanin belongs to the latter group and for that reason is easy to obtain by dialysis or dilution of the blood. The high iso-electric point, being near that of carbon-dioxide saturated water, also helps to precipitate it under these conditions.

Hemocyanin can be salted out with strong NaCl solutions but $(NH_4)_2SO_4$ is much more effective. Octopus-hemocyanin is almost quantitatively precipitated at 3.5M (65% saturated) $(NH_4)_2SO_4$ (Henze 1901) although Alsberg and Clark (1910) claim somewhat higher concentrations must be used. Limulus-hemocyanin on the other hand is completely precipitated at concentrations of 40 - 50% saturation. Megathura-hemocyanin is relatively easy to salt out, 40% saturated $(NH_4)_2SO_4$ solutions precipitating the protein almost quantitatively.

The only quantitative work that has yet been done on the salting-out of hemocyanins is that of Roche and Duboulez (1933b) who determined the amount of protein precipitated at various concentrations of $(NH_4)_2SO_4$ and at a pH of 3.8. Their results can be summarized in the following curves.



Helix pomatia	Octopus vulgaris	Carcinus maenas	Cancer pagurus
1/3 - 1/2	1/4 - 2/3	1/4 - 1/2	1/6 - 1/2

The values under the names give the limits of precipitation.

The salting-out of globulins has generally been attributed to a dehydration of the protein brought about by the large hydration of the added electrolyte. The water is "squeezed" out of the protein. Under this assumption we might expect the precipitate to be fairly compact but it is actually the reverse. In precipitating Megathura-hemocyanin by dialysis and salting out (both with NaCl and $(NH_4)_2SO_4$) it has been noted that in the former case the precipitate is compact and adheres together whereas in the latter case it is massive and shows very little internal coherence. In fact it behaves a good deal like the thixotropic gels of the viruses where equilibrium is reached with the protein micelles separated by hundreds of Å., the intervening space being filled with water molecules. This would indicate that under such conditions the micelles possessed electric charges that kept them apart. Although the protein molecules themselves may be dehydrated the micelles are certainly not.

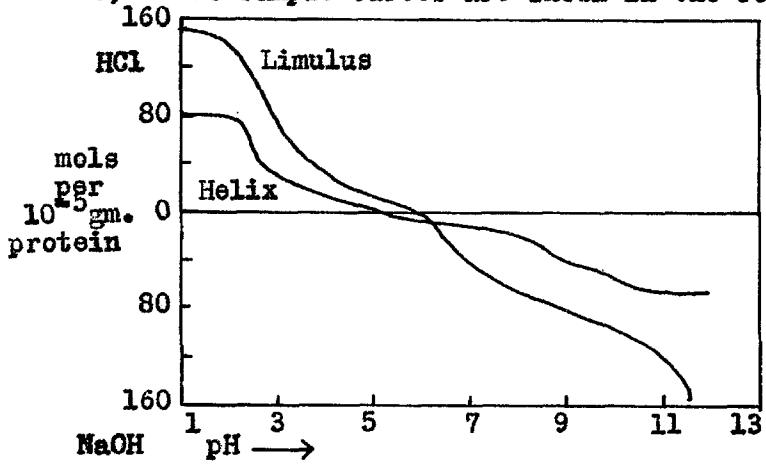
3. Combination with acid and base.

By determining the total amount of acid or base bound by a hemocyanin we can derive some idea of the total number of dissociable (ionizable) groups on the molecule and the relative quantities of diamine and dicarboxy acids present. By titrations we can locate some of the ionizing groups and assign to them a dissociation constant. Later on we shall see that some of these dissociation constants have two values: one for reduced hemocyanin and one for oxygenated hemocyanin. From such measurements we can also make a basis for discussion of the buffering power of hemocyanin in the blood.

The total acid and base bound by various hemocyanins (given in mols of acid or base per 10^5 grams of protein) is shown in the following table.

Species	Acid Bound	Base Bound
Limulus polyphemus	160	140
Carcinus maenas	130	145
Sepia officinalis	108	120
Octopus vulgaris	110	115
Helix pomatia	96	90

The values in the last column are estimated from his curves and are uncertain. (Roche 1936a). Two sample curves are shown in the following graph.



Roche, along with many others, correlatesthe acid-binding power with the diamino acid content. As we shall see later the diamino acid content runs

parallel to the acid bound, but from the values given above we see it also runs parallel to the base-binding power. According to the zwitterion theory, of course, the acid added on the acid side of the iso-electric point is mainly bound to carboxyl groups.

$$^{+}H_3N\text{-}P\text{-}COO^{-} + HCl \longrightarrow {}^{+}H_3N\text{-}P\text{-}COOH + Cl^{-}$$

I believe that interpreting titration curves on the basis of acid or base bound is a highly unsatisfactory method. It would be much more reasonable and practical to follow Brönsted's theory and assume that all the groups were acting as bases. We would then have:

$$H_2N\text{-}P\text{-}COO^{-} + H^{+} \longrightarrow {}^{+}H_3N\text{-}P\text{-}COO^{-}$$

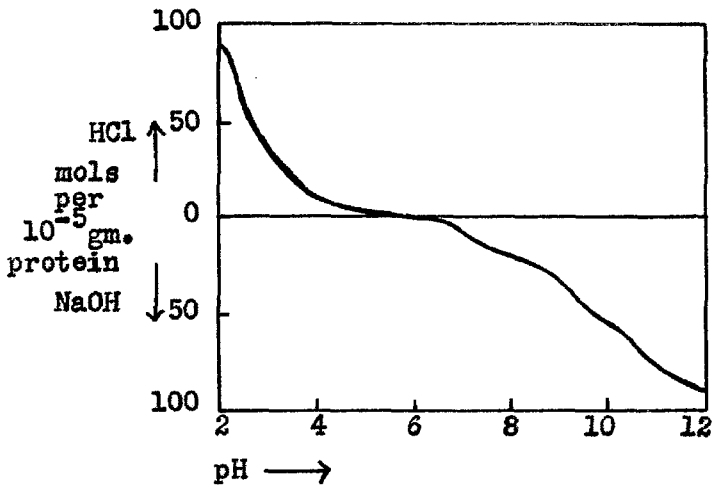
$$^{+}H_3N\text{-}P\text{-}COO^{-} + H^{+} \longrightarrow {}^{+}H_3N\text{-}P\text{-}COOH$$

Looking at it in this manner a titration curve involves a single type of reaction and does not lead to the error that acid and base binding are essentially different as many workers in the field of hemocyanins have thought.

In the acid range hemocyanin behaves as a monovalent base with pKs of 2.3 to 2.9 depending on the hemocyanin used. This is the range of values for the pKs of the carboxyl groups of most of the amino acids. As there is very little deviation in pK from one carboxyl group to another the whole protein acts as a base with a single dissociation constant.

On the alkaline side of the iso-electric point the situation is more complex because the groups dissociating here are more varied in their pKs. Roche (1936a) finds three dissociable groups in Helix-hemocyanin. It is considered in more detail later when we look at the effect of oxygenation on the dissociation constants. The addition of NaCl (0.5M) lowers the pK values of these groups by about 0.5 pH unit in Limulus and Busycon hemocyanins (Redfield 1934, Redfield, Humphreys and Ingalls 1929, Redfield and Mason 1928).

I have made a titration curve for Megathura-hemocyanin for purposes of comparison with other hemocyanins. For this experiment pure hemocyanin was used at a concentration of 0.11 % in a solution 5% in NaCl. NaOH and HCl (0.1M) were added and about five minutes allowed for equilibrium to be attained. They were added carefully and no denaturation was noticed.



Again we find that Megathura-hemocyanin is similar to that of Helix, as we noted above with regard to its iso-electric point and solubility. It binds relatively a small amount of acid (using the terminology of Brönsted's theory) which confirms the deductions made on the basis of the slope of the pH-mobility curve. This means that the buffering power is relatively low and as the concentration of hemocyanin in the blood of Megathura is quite low, we can conclude that the blood is poorly buffered (it has been shown that the hemocyanin is the chief buffer in mollusc blood).

We can detect two dissociating groups in the alkaline range with pKs of 6.9 - 7.2 and 9.5 - 9.8 and perhaps a third around pK 8 but this latter one is not certain. This determination was, of course, made on oxy-hemocyanin. If we compare these values with those obtained by other authors for the oxygenated compound we find that again this hemocyanin resembles that of Helix more closely than others.

Oxyhemocyanin of	pKs	Author
Helix pomatia	7.0	Roche (1932)
	8.1	"
	10.5	"
Carcinus maenas	6.7	Roche (1936a)
Sepia officinalis	6.8	"
Octopus vulgaris	6.4	Roche (1930)
Megathura crenulata	7.1	This work
	9.7	"

The dissociating group with pK around 7.0 is the one that is intimately associated with the oxygenation process as we shall see later.

4. Electrophoresis.

We have discussed the cataphoretic measurements on their relation to iso-electric point and total number of dissociable groups. It remains to see the effect of certain ions and of the ionic strength on the mobility in an electric field.

Since Ca-ions had been noted to exert a stabilizing effect in alkaline solutions, preventing the hemocyanin from dissociating into smaller fractions, cataphoretic experiments were carried out to determine if Ca-ions combined in some specific way with the protein. It was found that the slope of the pH-mobility curve with and without Ca-ions was identical after correction for the effect of the bivalent Ca-ions on the ionic atmosphere of the protein. Thus no specific effect was noticed (Putzeys and van de Walle 1939).

Later experiments (Putzeys and van de Walle 1940) using various ions have shown that the slope of the mobility curve is independent of the type of ion used but does depend on the ionic strength of the solution. The effect is probably due to the depression of the ionization constant with increasing ionic strength.

5. Conclusions.

We can say that the hemocyanin of Megathura crenulata is similar to that

of Helix with respect to the properties depending on its amphoteric nature. That it is similar in the various characteristics discussed above is not surprising as they all depend upon the same factor, the amino acid composition. We may therefore generalize and say that the amino acid make-up of Megathura-hemocyanin is not identical with Helix, but is similar with regard to the amount and ratios of the various types of amino acids. As all proteins (excluding those containing carbohydrate, etc.) differ from each other only in their amino acid composition and the arrangement of the amino acids, the above generalization is of more comparative value than any of the single factors alone.

B. Molecular sizes.

In this section I will discuss one of the most characteristic properties of the hemocyanins: their large size. This is rivalled only by the viruses and the erythrocruorins.

1. Sedimentation constants and molecular weights.

Since it was discovered that the hemocyanins possessed molecular weights of 360,000 to 9,000,000 and that these large molecules were able to break into homogeneous fractions much work has been done in Svedberg's laboratory with them, for they exhibit these interesting properties to a degree not found in other proteins.

The molecular weights and sizes have been determined from osmotic pressure measurements, the ultracentrifuge, ultrafiltration, distribution in a gravitational field, scattering power, diffusion and viscosity measurements.

Since it is known that one molecule of oxygen combines with hemocyanin for every two atoms of copper, one can calculate the minimum molecular weight on the basis of the molecule containing two copper atoms. From the copper content of the hemocyanin of Megathura I have calculated its minimum molecular

weight on the above basis. The copper constituted 0.23% of the molecule and hence the minimum molecular weight is 2 x 63.57/0.0023 which gives a value of 55,300. We can compare this with other values.

Hemocyanin	Minimum molecular weight	Author
Mollusca.		
Octopus vulgaris	50,800	Roche (1934)
Helix pomatia	51,000	Roche (1934)
Sepia officinalis	48,900	Roche (1934)
Crustacea.		
Homarus vulgaris	69,000	Hernler and Philippi (1933)
Carcinus maenas	74,400	Roche (1934)
Xiphosura.		
Limulus polyphemus	73,600	Redfield, Coolidge and Shotts (1928)

These values were calculated from the most accurate copper contents I could obtain. The molluscs have a value of about 50,000 and the crustacea about 72,000 and this is a general rule to which there are no exceptions or intermediate cases yet discovered. The value I obtained for Megathura-hemocyanin falls in well with the molluscs. My value may be slightly high as this hemocyanin could not be made to crystallize, and although the most highly purified preparation was used it is possible that a small amount of impurity was present.

We find on measurements of the actual sizes of the molecules that the above figures are much too low and that each hemocyanin molecule actually contains many copper atoms. A complete collection of all the measurements made on the hemocyanins is given in the following table.

Species	s x 10^{13}	Molec. Wt.	Method	Author
Mollusca.				
Amphineura.				
Tonicella marmorea	61.6		C	Svedberg and Hedenius (1934)
	60.8		C	Svedberg (1933)
Gasteropoda.				
Helix pomatia		1,700,000	OP	Adair and Roche (1934)
		5,100,000	CD	Svedberg and Heyroth (1929)

Species	s x 10^{13}	Mol. Wt.	Method	Author
Helix pomatia (con.)		6,680,000	CE	Eriksson-Quensel and Svedberg (1936)
		797,000	CE	E-K and S (1936)
	12.1	502,000	CD	E-K and S (1936)
	16.0	719,000	CD	E-K and S (1936)
	98.9	6,630,000	CD	E-K and S (1936)
		1,800,000	G	Roche, Roche, Adair and Adair (1935)
	100.7		C	S and Hed. (1934)
		1,500,000	OP	A and R (1934)
		2,400,000	OP	Roche and Roche (1936a)
	99.8		C	S (1933)
	98.0	4,930,000	CD	Svedberg and Chirnoaga (1928)
	63.4		C	S and Ch. (1928)
		5,080,000	CE	S and Ch. (1928)
		814,000	CD	S (1939)
		6,350,000	VD	S (1939)
	98.0	5,000,000	CE	S (1930b)
Helix nemoralis	16.6	799,000	CD	E-K and S (1936)
	99.2		C	S and Hed. (1934)
	99.8		C	S (1933)
Helix arbustorum	94.8		C	S and Hed. (1934)
	99.8		C	S (1933)
Helix hortensis	98.9		C	S and Hed. (1934)
	99.8		C	S (1933)
Limax maximus	97.0		C	S and Hed. (1934)
	99.8		C	S (1933)
Agriolimax agrestis	100.0		C	S and Hed. (1934)
	99.8		C	S (1933)
Paludina vivipara	96.5		C	S and Hed. (1934)
	99.8		C	S (1933)
Paludina contecta	99.6		C	S and Hed. (1934)
	99.8		C	S (1933)
Busycon canaliculatum	13.5	379,600	CD	E-K and S (1936)
	101.7	6,814,000	CD	E-K and S (1936)
	130.4	9,980,000	CD	E-K and S (1936)
Buccinum undatum	101.4		C	S and Hed. (1934)
	131.6		C	S and Hed. (1934)
	99.8		C	S (1933)
Littorina littorea	98.2		C	S and Hed. (1934)
	131.9		C	S and Hed. (1934)
	99.8		C	S (1933)
Neptunea antiqua	107.3		C	S and Hed. (1934)
	99.8		C	S (1933)
Limnea stagnalis	98.4		C	S and Hed. (1934)
	60.2		C	S and Hed. (1934)
	99.8		C	S (1933)
Arion subfusca	64.3		C	S and Hed. (1934)
Arion ater	98.5		C	S and Hed. (1934)
	60.9		C	S and Hed. (1934)

Species	s x 10^{13}	Mol. Wt.	Method	Author
Arion empiricorum	99.8		C	S and Hed. (1934)
	60.8		C	S (1933)
Achatina fulva	95.7		C	S and Hed. (1934)
	63.1		C	S and Hed. (1934)
	15.1		C	S and Hed. (1934)
	99.8		C	S (1933)
Cephalopoda.				
Decapoda.				
Loligo vulgaris	56.4		C	S and Hed. (1934)
	57.1		C	S (1933)
Sepiola oweniana	56.4		C	S and Hed. (1934)
	57.1		C	S (1933)
Rossia macrosoma	56.0		C	S and Hed. (1934)
	57.1		C	S (1933)
Sepia officinalis	55.9		C	S and Hed. (1934)
	57.1		C	S (1933)
Octopoda.				
Octopus vulgaris		2,820,000	VD	S (1939)
		2,780,000	CE	S (1939)
	43.2		C	Svedberg and Eriksson (1932b)
		2,050,000	CD	S and E (1932b)
		700,000	OP	A and R (1934)
	49.5		C	S and Hed. (1934)
	51.1		C	S (1933)
Eledone moschata	48.9		C	S and Hed. (1934)
	51.1		C	S (1933)
		2,791,000	CD	Neurath (1939)
		457,000	CD	Neurath (1939)
	10.6	440,000	CD	S (1939)
	49.1	2,790,000	CD	S (1939)
Eledone cirrosa	46.4		C	S and Hed. (1934)
	51.1		C	S (1933)
Arthropoda.				
Crustacea.				
Pandalus borealis	16.6		C	S and Hed. (1934)
	16.9		C	S (1933)
	17.4	397,000	CE	E-K and S (1936)
Palaemon fabrici	15.6		C	S and Hed. (1934)
	16.9		C	S (1933)
Palinurus vulgaris	16.3		C	S and Hed. (1934)
	16.9		C	S (1933)
	16.4	446,000	CD	E-K and S (1936)
		447,000	CE	S (1939)
Pagurus striatus	16.0		C	S and Hed. (1934)
	16.9		C	S (1933)
Eupagurus bernhardus	17.1		C	S and Hed. (1934)
	16.9		C	S (1933)
Squilla mantis	23.7		C	S and Hed. (1934)
	23.4		C	S (1933)
Nephrops novegicus	24.4		C	S and Hed. (1934)
	23.4		C	S (1933)

Species	s x 10^{13}	Mol. Wt.	Method	Author
Homarus vulgaris	24.4		C	S and Hed. (1934)
	23.4		C	S (1933)
		793,000	VD	S (1939)
		777,000	CD	S (1939)
	22.6	752,000	CD	E-K and S (1936)
Astacus fluviatilis	24.3		C	S and Hed. (1934)
	23.4		C	S (1933)
Hyas araneus	24.2		C	S and Hed. (1934)
	23.4		C	S (1933)
Maia squinado	26.7		C	S and Hed. (1934)
	23.4		C	S (1933)
Cancer pagurus	23.0		C	S and Hed. (1934)
	15.7		C	S and Hed. (1934)
	23.4	360,000	C	S (1933)
Carcinus maenas	24.1		C	S and Hed. (1934)
	16.4		C	S and Hed. (1934)
	23.4		C	S (1933)
Chiridothea entomon	23.0		C	S and Hed. (1934)
	16.1		C	S and Hed. (1934)
Calocaris macandrae	34.0		C	S and Hed. (1934)
	34.1		C	S (1933)
	34.0	1,329,000	CD	E-K and S (1936)
Xiphosura.				
Limulus polyphemus	34.1		C	S and Hed. (1934)
	61.2		C	S and Hed. (1934)
	16.7		C	S and Hed. (1934)
	33.6		C	S and Hed. (1934)
	56.9		C	S and Hed. (1934)
	16.3		C	S and Hed. (1934)
	35.7		C	S (1933)
		360,000	CD	Svedberg and Heyroth (1929)
		1,300,000	CD	Svedberg and Heyroth (1929)
		2,750,000	CD	Svedberg and Heyroth (1929)
		565,000	OP	Burk (1940)
		1,900,000	CD	Svedberg and Heyroth (1929)
	35.7	2,000,000	CE	S (1930b)
Scorpionidea.				
Euscorpius carpaticus	36.6		C	S and Hed. (1934)
	34.1		C	S (1933)

Abbreviations used:

E-K and S	Eriksson-Quensel and Svedberg
S and Hed.	Svedberg and Hedenius
A and R	Adair and Roche
S	Svedberg
S and Ch.	Svedberg and Chirnoaga

-o-

C	Centrifugal sedimentation.
CD	Sedimentation and diffusion.
CE	Sedimentation and equilibrium.
OP	Osmotic pressure.
G	Gravitational field.
VD	Viscosity and diffusion.

I would like to make a few remarks concerning the terms used, the methods employed and their reliability.

Centrifugal sedimentation --- s is the sedimentation constant and is merely the corrected velocity of the molecules in a centrifugal field of unit strength. The higher the sedimentation constant the higher the molecular weight but the relation is not proportional. The approximate relations are:

sedimentation constant	molecular weight
99.8×10^{-13}	5,100,000
57.1	2,750,000
51.1	2,000,000
34.1	1,310,000
23.4	640,000
16.9	360,000

One could calculate accurately the molecular weight from the sedimentation constant if the molecule was spherical, but this is generally not the case.

Sedimentation-diffusion method --- To eliminate this shape factor one can combine sedimentation constants with diffusion constants to calculate the molecular weight. This is a very good method.

Sedimentation-equilibrium method --- This is essentially the same in theory as the above method in that it balances the centrifugal force by the diffusion "force" in the tube after the material has come to equilibrium. It gives good results which check with the other method.

Gravitational method --- This method was used by Roche and Adair (1935) and consists of measuring the change in concentration of hemocyanin in a stationary tube. By relations between the concentration at a certain depth and the osmotic pressure there and the relation between osmotic pressure and molecular weight one can arrive at an approximate value for the molecular weight.

Osmotic pressure method --- The disadvantages of this method are two-

fold: (1) the pressures exerted by substances of such large size is very small and (2) it gives only the average molecular weight and is therefore unsuitable when mixtures of components of varying sizes are present.

Viscosity and diffusion method --- This is a method recently devised by Polson (Svedberg 1939) using an empirical formula for the relation between viscosity and shape factor, the object being again to eliminate the shape factor. The results agree nicely with more standard methods in the three hemocyanins investigated.

It must be understood that in the above table of molecular weights that when values are radically different for the same hemocyanin it does not mean errors in the methods but that the two values belong to different components of the protein having different molecular weights.

On the basis of sedimentation constants Svedberg has divided the hemocyanins (including the components) into classes:

A	133	F	34
B	99	G	24
C	63	H	16
D	56	I	12
E	49	K	5

The values given I have computed by averaging all the determinations for that particular class. This is a good short-hand method for designating the hemocyanins with respect to their molecular weights. For example, he has shown that each phylogenetic group can be represented by the letters above in the following manner:

Mollusca:	Amphineura	C
	Gasteropoda	B (with sometimes A,C and H)
	Decapoda	D
	Octopoda	E
Arthropoda:	Crustacea	F, G and H
	Xiphosura	C, F and H
	Scorpionida	F

One must conclude that the sedimentation constants are characteristic for

classes and orders and reflect biological kinship. More will be said concerning this after a discussion of the dissociation reactions of the hemocyanins.

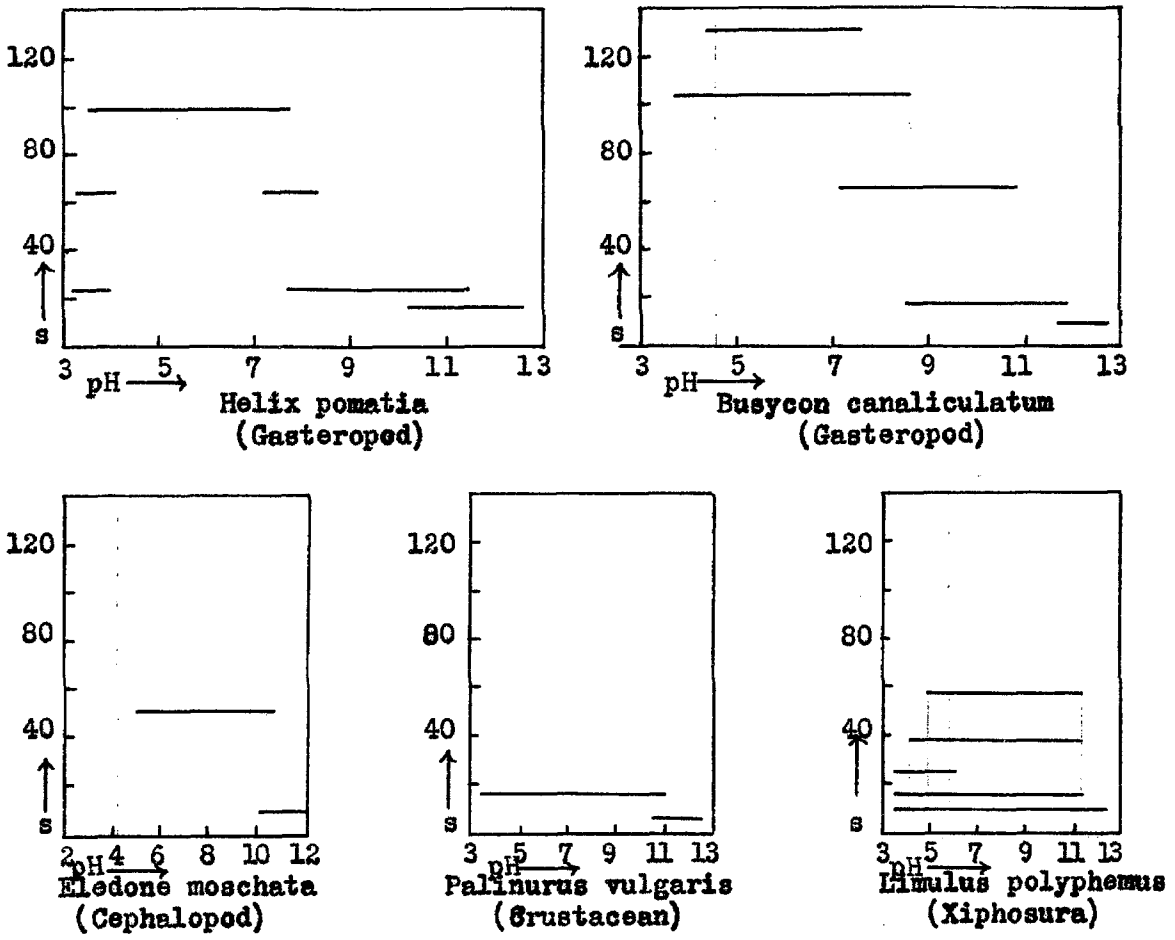
2. Dissociation of hemocyanin molecules.

Svedberg and his collaborators have definitely shown that hemocyanin molecules are true chemical entities by demonstrating that these large molecules are not of random size but occur in a few definite groups of sizes. Furthermore there is an equilibrium between these groups so that by changing the conditions of the medium one form will pass (dissociate or associate) into another, and by bringing the conditions back to the initial state we observe the reverse reaction. This property of sensitive reversible dissociation is exhibited only by the respiratory proteins occurring in solution in the blood.

Factors which bring about dissociation are: changes of pH, salt concentration, temperature, ultra-violet light, ultra-sonic waves, certain organic compound (urea, amino acids, amides, etc.), heavy water, dilution and perhaps oxygenation. One must picture the hemocyanins as made up of some fundamental protein units joined together by bondsmuch weaker than those holding the constituents of the unit together.

Svedberg and Hedenius (1934) determined the sedimentation constants of many hemocyanins as they occur in the blood and also in what manner these constants vary with change in pH. His results can be summarized by so-called "pH-stability diagrams" where the sedimentation constant is plotted as a function of pH. There is in general a region where the high molecular weight molecule, characteristic of the group, is stable; outside of this range dissociation occurs into smaller fragments. To illustrate this phenomenon it will suffice to give such a diagram for a typical

hemocyanin from each major phylogenetic group.



The diagrams were taken from Eriksson-Quensel and Svedberg (1936) and that they can be taken as representative of each group can be seen from the statement of Svedberg: "All species of one and the same order have similar diagrams."

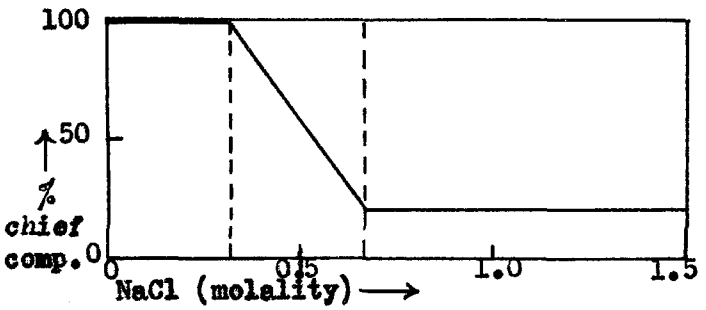
The region of stability seems in general to lie between the iso-electric point and the pH of the blood. The alkaline limit of the stability range lies between pH 8 and 10 for almost all hemocyanins and at the present state of our knowledge I think it can be assumed that this dissociation is due to the titration of the amino groups (which have pKs in this region), the resultant increase in negatively charged groups causing the units to break the weak bonds which hold them together. Although they did not in

most cases investigate the sedimentation constants in solutions on the acid side of the dissociation constant of the carboxyl groups, I am confident that a similar dissociation would be found. This is supported by the appearance of hemocyanin solutions at these pHs. The amount of scattering is dependent on the size of the molecules and it is easily observed that the scattering decreases at these acid pHs in the same way as at the alkaline end of the stability region. In the Helix-hemocyanins this effect was shown by centrifugal analysis to occur around pH 2 - 4.

In view of the above phylogenetic considerations I believe it is safe to make some predictions concerning the size of the hemocyanin of Megathura crenulata. All Gasteropods have as their normal component a molecule of class B (s is 99 x 10^{-13}) having a molecular weight of about 5,000,000. The chief component of Megathura-hemocyanin is, therefore, of molecular weight approximately 5,000,000 - 6,600,000 (it is probably closer to the latter value according to recent determinations of molecular weight on Helix-hemocyanin). This is 16x the "fundamental unit" of 406,000, the lowest molecular weight observed. This dissociates into half-molecules of molecular weight 3,300,000 at pHs near the stability region and finally into eighth-molecules of molecular weight 800,000. Fourth-molecules have never been observed in the dissociation of any hemocyanin. At high and low pHs it is probable that some of the component of molecular weight 406,000 are present. As we shall see later the dissociation into eighth-molecules probably involves a splitting along the long axis of the molecule.

Brohult and Claesson (1939) have recently investigated the effect of electrolytes on dissociation. With 1-1 type of electrolyte (NaCl) we never get dissociation beyond the half-molecules, but with 2-1 type salt the hemocyanin of Helix is split into eighth- and sixteenth-molecules.

When we plot the molecular size against the amount of NaCl added we find a strange phenomenon, as yet unexplained.



(the chief component in this case is 6,500,000)

If we accept an electrostatic theory of dissociation we must say that above 0.7M NaCl has no great effect on the relative charge distribution. These experiments were performed in buffer solutions (0.08M) so the effect cannot be attributed to pH changes.

The above experiments were made at pHs of 5.2 and 6.0 but it had been found by Brosteaux (1937) that at alkaline reactions (pH 7.5 - 9.5) Ca-ions and Mg-ions prevented the dissociation of hemocyanin into smaller fractions. They used higher concentrations of these ions than occur in the blood itself (the calcium in the blood being about 0.001M) and hence no physiological conclusions can be drawn. That the amount of Ca-ions in the blood has very little effect can be seen from Svedberg's sedimentation measurements which were done on the blood of the animals.

Brohult and Claesson (1939) also demonstrated that certain organic substances dissociated the hemocyanin of Helix.

Substance 1.0M	Result
glucose	64% dissociated into half-molecules.
glycerine	32% dissociated into half-molecules.
urea	58% dissociated into smaller fragments, 25% of which were hetero-geneous.

The effect of urea has been investigated in more detail by Burk (1940) who used 6.66M urea solutions. By osmotic pressure measurements he finds that Limulus-hemocyanin under such conditions has a molecular weight of 142,000. When the copper was removed from the molecule with dilute acid the molecular weight dropped to half this value (69,000). He, therefore, assumes a fundamental unit of 142,000, containing four copper atoms.

Heavy water was shown to shift the pH-stability diagram 0.5 pH units to the alkaline side. A corresponding shift is observed in the iso-electric point. This supports the theory of dissociation effects depending on the amphoteric properties of the protein. (Svedberg and Eriksson-Quensel 1936).

Brohult (1937) found that ultra-sonic waves (frequency of 250,000 sec^{-1}) would split Helix-hemocyanin into half- and eighth-molecules in fairly good yields. He claimed that no reversibility was exhibited. The effect of ultra-violet radiation was studied by Svedberg and Brohult (1938) and it was demonstrated that short exposures would dissociate the hemocyanin of Helix into half-molecules. This process was reversible but further exposure resulted in products of lower molecular weight and denaturation and this was irreversible. That the molecular weight of hemocyanin depends on the concentration of hemocyanin itself was shown by Svedberg and Heyroth (1929a) on finding that dilution of the protein past 0.6% resulted in dissociation. Dissociation has also been noted in concentrated solutions but this question has been studied very little.

That the low molecular weight components of two different hemocyanins may recombine on bringing the solution back to the original pH was shown by Tiselius and Horsfall (1939b). This determination was made possible by the hemocyanins possessing different iso-electric points so that mixtures could be recognized by electrophoretic experiments. Two species of Helix were used.

3. Molecular dimensions.

There have been numerous estimates of the diameter of hemocyanin molecules from diffusion, ultra-filtration, sedimentation and comparison with known smaller molecules. However, these estimates were based on the assumption of spherical molecules and it is known that this is not the case. Ultra-filtration has given values of 180 - 290 Å. for the diameter of Helix-hemocyanin (Elford and Ferry 1936, Grabar 1936). From diffusion a value of 240 Å. is obtained (Svedberg and Chirnoaga 1928).

Recently this problem has been somewhat clarified by Neurath (1939) who has determined the dimensions of several hemocyanins on the following basis. (1) From sedimentation and diffusion data we can calculate the dissymetry constant f/f_0 (Svedberg) where f is the measured frictional coefficient and f_0 is the theoretical frictional coefficient for a spherical molecule of the same molecular weight. (2) From the relation between the dissymetry constant and diffusion constant, $f/f_0 = D_0/D$, we can now determine D_0/D. (3) From Perrin's equation relating D_0/D to the axial ratio, a/b, of a prolate ellipsoid we can finally obtain a/b. (4) For absolute values of a and b we can use the formula for the volume of a prolate ellipsoid in terms of the axes. This method is not very accurate but gives quantities of the right order of magnitude. His results are summarized in the following table.

Hemocyanin	f/f_0	b/a	a Å.	b Å.	Mol. Wt.	Cleavage
Palinurus	1.2	4.3	62	268	446,000	
Eledone-M	1.4	7.4	95	715	2,791,000	long axis
D	1.92	18.2	39	707	457,000	
Helix-M	1.2	4.3	153	660	6,630,000	long axis
D_1	1.66	12.4	54	663	814,000	short axis
D_2	1.81	15.7	42	661	502,000	
Busycon-A	1.27	5.4	159	922	9,980,000	short axis
M	1.2	4.3	155	665	6,814,000	both
D	1.4	7.5	57	430	379,000	

A aggregation component M main component D dissociation component

We see that the hemocyanins are far from spherical. Furthermore it is shown that they often split along the long axis. Especially is this noticeable in Helix-hemocyanin where the cleavage into eighth-molecules does not involve a decrease in the length of the molecules. It is as if the units were bound together like a number of parallel rods. I suspect that the larger aggregates formed near the precipitation zone are of even larger axial ratio for they show good streaming double refraction. A solution of Megathura-hemocyanin, when attempts were made to crystallize it, always showed streaming patterns similar to those seen in the tobacco mosaic virus.

C. Optical properties.

1. Absorption spectra.

For almost a hundred years it has been known that oxygenated hemocyanin was blue and the reduced compound colorless but as yet we do not know what group or phenomenon is responsible for this color change. It has always been difficult to separate the effect produced by scattering and by absorption. Sols of particles of similar size to the hemocyanins were known to give bluish suspensions.

The hemocyanins from different species vary in their shade of blue.

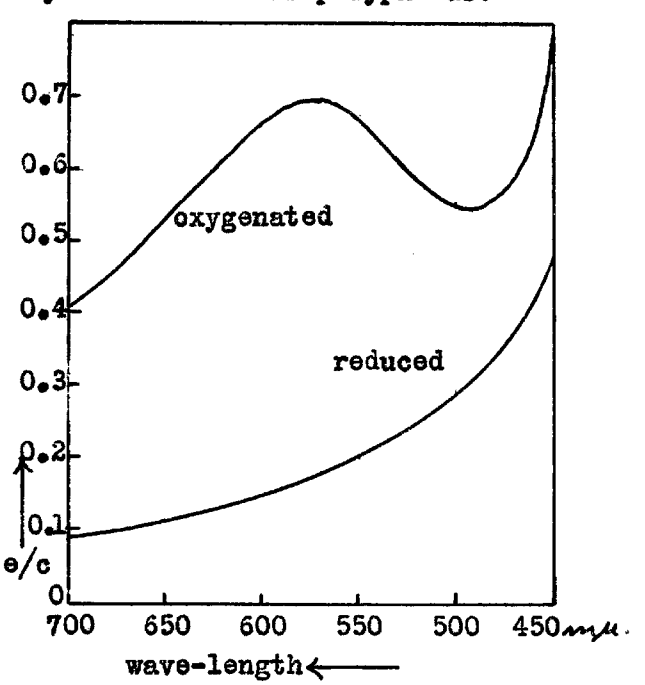
Hemocyanin	Color	Author
Loligo pealei	Clear dark blue with a green cast.	Redfield, Coolidge and Hurd (1926)
Limulus polyphemus	Pure dark blue.	Redfield, Coolidge and Hurd (1926)
	Intense blue with green tint.	Dhere, Baumeler and Schneider (1929)
Cancer irroratus	Dull blue-green, tendency to olive.	Redfield, Coolidge and Hurd (1926)
Busycon canalic.	Blue-violet to purple.	Dhere, Baumeler and Schneider (1929)
Helix pomatia	Violet-blue.	Dhere (1908a)
	Pale gray-blue.	Roche (1934)

Hemocyanin	Color	Author
Helix aspersa	Bluish-white with a purplish tinge.	Mac Munn (1885)
Octopus vulgaris	Blue-gray.	Roche (1934)
	Deep pure blue.	Parsons and Parsons (1923)

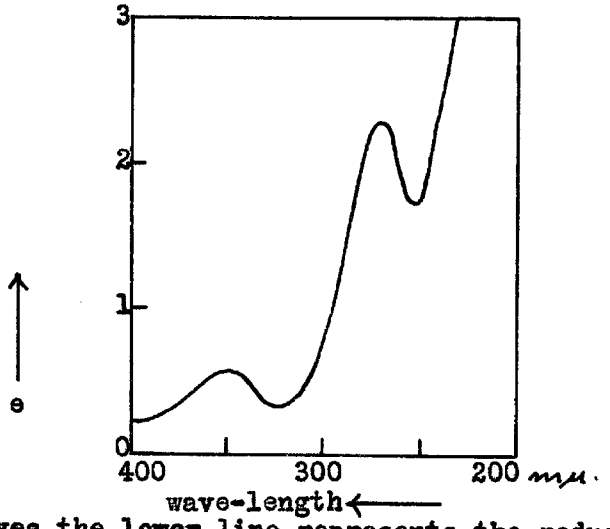
These observations are of no quantitative value because the color of the hemocyanin varies with the conditions under which it is placed. Anything that alters the size of the particles changes the apparent color.

The hemocyanin of Megathura crenulata in a salt solution isotonic with that of the blood of the animal and at a pH of 8.5 is a dark gray-blue by reflected light and a steel-blue with a tint of orange (due to colloidal properties) by transmitted light. The reduced hemocyanin is milky white by reflected light and almost colorless, but with a slight yellow tint, by transmitted light. It actually seems that the scattering of blue light is increased by oxygenation.

True comparisons of the blood, however, can be made by the use of the absorption curves made under similar conditions. The best work has been done by Redfield (1930a) who investigated the spectra of solutions of the pure hemocyanins of Busycon, Homarus, Limulus and Loligo. A typical curve is that of the hemocyanin of Limulus polyphemus.

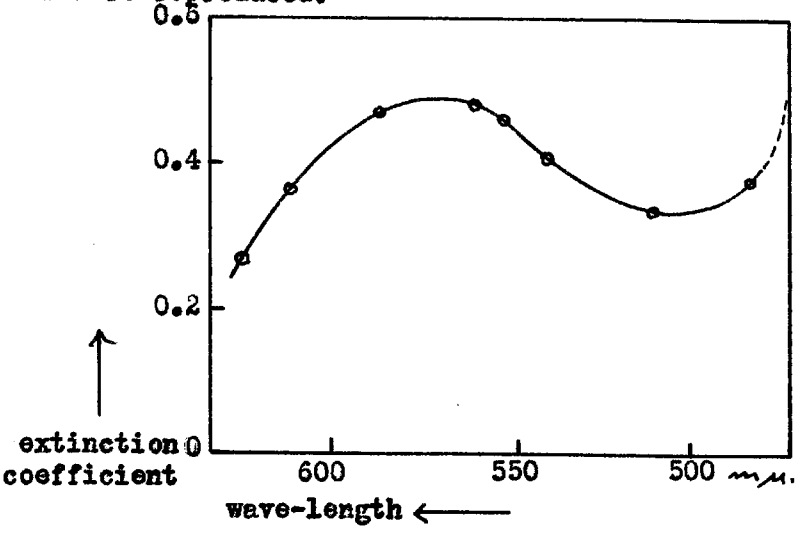


Roche (1936) has investigated the ultra-violet spectra of several hemocyanins and his curve for Helix-hemocyanin is given here.



In both these curves the lower line represents the reduced hemocyanin and the upper line the oxygenated hemocyanin.

I have determined the absorption spectrum of the hemocyanin of Megathura in a solution 4.3% in hemocyanin and 5% in NaCl at a pH of 8.5. A cell of thickness 2.4 cm. was used in a spectrophotometer (visual). The curve obtained is here reproduced.



The absorption maximum was at 5650 - 5680 Å. and the absorption minimum was 4800 - 4840 Å. How this compares with the other hemocyanins can be seen in the table following in which I have collected all the data on this point.

Hemocyanin	1st band.	2nd band.	3rd band.	Author
Limulus polyphemus	570	346		Dhere (1920a), Dhere, Baumeler and Schneider (1929)
	572			Redfield (1930a)
			278	Dhere and Burdel (1919)
	570	345	280	Svedberg and Heyroth (1929a)
Octopus vulgaris	575	345	278	Kubowitz (1938)
		345	278	Roche and Dubouloz (1933b)
	573			Vles (1913)
Busycon canaliculatum		346		Dhere (1920a), Dhere, Baumeler and Schneider (1929)
Helix pomatia		345	278	Roche and Dubouloz (1933b)
			278	Dhere (1920a), Dhere and Burdel (1919)
	555		278	Svedberg and Chirnoaga (1928)
	555			Dhere and Burdel (1913)
Carcinus maenas		345	278	Roche and Dubouloz (1933b)
Eledone moschata	577			Quagliariello (1922)
Sepia officinalis	577			Quagliariello (1922)
Palinurus vulgaris	560			Quagliariello (1922)
Homarus vulgaris	560			Quagliariello (1922)
			278	Dhere (1920a)
Megathura crenulata	567			This work.
Paludina vivipara	600			Svedberg and Hedenius (1933a)

From comparison with reduced hemocyanins it has been shown that the first two bands are connected with the prosthetic group, the third band is common to both (and also to all proteins). The variation in the absorption maxima of the first band is partly responsible for the different colors of the hemocyanins of various species. At various times statements have been made that there is a phylogenetic relation of the absorption maximum, but we see that on the basis of all the measurements made there is no evident relation.

There are specific differences between the hemocyanins, however, and this is probably a reflection of the different environment of the prosthetic groups in the hemocyanins. There is certainly more spread than in the case of hemoglobin where the maximum variation is 575.1 to 578.2 taking into account the hemoglobins (erythrocruorins) of the invertebrates.

The specific absorption of light by the prosthetic group will be taken up in the discussion of the nature of the prosthetic group.

2. Scattering power of hemocyanin.

Redfield (1930a) found that all the light scattered by hemocyanin solutions was polarized when viewed at an angle of 90° to the incident light and furthermore that this scattering was the only factor in the production of the yellow-orange color of the reduced blood. That it was the sole factor was shown by the dependence of the intensity on the reciprocal of the fourth power of the wave-length.

Purified preparations of reduced hemocyanins are, when dissolved in water, practically without scattering power, but the addition of salts, especially Mg and Ca increases the scattering (Dhere and Burdel 1919, Redfield 1930a, Redfield and Ingalls 1932). The curves given in the previous section were made in the blood of the animal and so in the presence of salts.

Dhere's original speculation that the scattering was due to reflection from large particles of protein is incorrect for such scattering does not depend on the reciprocal of the fourth power of the wave-length. The facts can be interpreted satisfactorily on the basis of Rayleigh's theory for particles smaller than the wave-length of light. However, the more recent conception of Raman (1927) that scattering is due to aggregates formed by statistical fluctuations of the molecules gives a somewhat better picture of the effect of electrolytes and change of pH.

Putzeys and Brosteaux (1935) established relations between the scattering power and the size and number of particles of protein. The relation obtained was: $I = Knv^2$ where I is the intensity of the scattered light, n is the number of particles per unit volume and v is their volume. The scattering power is therefore directly proportional to the molecular weights of the proteins. He tested this for several proteins, hemocyanin being among them, and checked this result. Their results showed that hy-

dration and ionization of the protein did not affect the scattering, except by their secondary influence on association and dissociation.

3. Fluorescence.

The strong scattering of hemocyanin bloods does produce an effect that appears like fluorescence so it is no wonder that Lankester (1871), Jolyet and Regnard (1877) and Heim (1892) believed fluorescence to occur. Heim related the phenomenon to the oxygenation of the prosthetic group in Maia hemocyanin. Redfield, Coolidge and Hurd (1926) showed, however, that there was no fluorescence in ultra-violet light of the hemocyanins of Cancer and Limulus. The results on the scattering power (Redfield 1930a) also make the presence of fluorescence very improbable.

Other optical properties are of very little interest for the present investigation. In passing we note that Beer's law has been found to hold for hemocyanin solutions between concentrations of 0.2 and 5.5% (Redfield 1930a) if only the absorption is taken into account, the scattering power , of course changing with the concentration of protein. The index of refraction of hemocyanin solutions has been shown to be similar to other proteins, i.e. an increase of 0.00198 in n for each gram of protein per 100 ml. of solution. This value is independent of salts or pH or source of hemocyanin. The sole observation on the rotatory power of hemocyanin was made by Griffiths (1892b) who showed it to be levo-rotatory (-58.3°).

CHEMICAL PROPERTIES.

Before proceeding to a discussion of the functional properties of the hemocyanins it will be necessary to determine the chemical nature of these proteins. This information will enable us to understand many of their physical properties and the differences in these properties as we go from one species to the next. It will also further substantiate the proposition that there are definite differences between the hemocyanins of phylogenetic groups.

A. Purification and crystallization.

There are two general methods for the purification of hemocyanin: (1) decreasing the electrolyte concentration until the protein precipitates and (2) increasing the electrolyte concentration until the protein precipitates. The first method can be done by dilution with distilled water or by dialysis. Dilution has the disadvantage of producing a very large volume of solution (as some 10 - 20x the volume must be added) and dialysis has the disadvantage of taking a long time. The second method generally involves the use of $(NH_4)_2SO_4$ of $MgSO_4$ and suffers from two disadvantages: (a) the precipitate that is formed in not easy to handle, being massive and containing much solution and (b) the pH of strong solutions of $(NH_4)_2SO_4$ is quite acid and this splits the copper from the protein slowly and produces also some denaturation.

In the present investigation dialysis was the preponderant method. Earlier work with salting-out methods was not satisfactory for the preparation of large quantities of hemocyanin. The general procedure can be summarized in the following scheme.

Preparation of Megathura-hemocyanin:

(1) To obtain the blood from the animal incisions were made around the rim of the mantle. The blood flows immediately and as there is no clotting in these animals most of the blood will drain out. Pressure was applied in most cases to hasten the process. The amount of blood in an individual key-hole limpet depends on the size of the animal and its physiologic state and varies from 40 to 120 ml., the average value being about 90 ml.

(2) The blood is centrifuged at 3500 r.p.m. for half an hour to remove the few cellular elements present and any of the black pigment from the mantle that might have been washed in.

(3) Dialysis is performed in Dupont cellophane tubing against tap-water in the cold room. After one day most of the hemocyanin has precipitated and after two days it is almost complete. The ease of precipitation is due to the fact that the iso-electric point is fairly close to the pH of tap-water.

(4) The hemocyanin is centrifuged out and the supernate thrown away. It is now a compact dark gray-blue paste.

(5) The hemocyanin is dissolved in 5% NaCl solution. There is still some odor present (probably due to amines in the blood) so this solution is dialyzed again in the same way except distilled water was generally used.

(6) The hemocyanin was again centrifuged out and it is found that there is no detectable odor left.

The hemocyanin used in the magnetic work was prepared in this way but that used for analysis was further purified by seven days dialysis to ensure removal of all electrolytes. For the magnetic work it is not necessary

that the hemocyanin be 100% pure but for the analysis it must be reasonably close to this. From the type of procedure it is seen that the only other substances that could be present are other proteins. In the first place in most molluscan blood hemocyanin is the sole protein (Octopus - Henze 1901; Busycon - Montgomery 1930, Menden and Bradley 1906; Limulus - Halliburton 1885a, 1885b, Alsberg 1914). In none of my preparations, using a variety of methods, have I ever found evidence for another protein in the blood of Megathura. In the second place any other protein present would have to be similar in properties to a globulin or else be adsorbed onto the hemocyanin. The two dialyses would have prevented much adsorption. In Svedberg's work no mention has been made of finding other proteins of low molecular weight.

It has been shown by numerous workers that the copper is not split off by dialysis around neutral pHs. There is no denaturation for all of the precipitated hemocyanin will dissolve in the 5% NaCl solution.

Various attempts to obtain good crystals of Megathura-hemocyanin have all failed. The hemocyanins of Limulus and Busycon have never been crystallized although many endeavors have been made. Helix-hemocyanin crystallizes only with difficulty while many hemocyanins will crystallize readily. I utilized five procedures that have been known to give crystalline products with other hemocyanins.

(1) The blood was diluted 10x and 0.006% acetic acid was added very slowly.

(2) A saturated solution of pure hemocyanin was made in concentrated $(NH_4)_2SO_4$ solution at room temperature and this was cooled slowly.

(3) To the same solution as in (2) I added dilute acetic acid slowly.

(4) Slow dialysis, the volume of the surrounding fluid being kept small and changed once a day. It takes almost a week for precipitation

to take place under such conditions.

(5) A saturated solution of hemocyanin was made in dilute (0.01M) Na_2SO_4 and this was warmed slowly and a little HCl was added.

In methods (1), (2), (3) and (5) suspensions were obtained showing a sheen when rotated in a flask. In method (4) only amorphous material resulted. The micro-crystals in the former cases were too small to be seen under a microscope.

The following table shows the type of crystals obtained in the cases where crystallization has been accomplished.

Hemocyanin	Crystal form	Author
Helix pomatia	Rods.	Dhere (1919b)
	Six-rayed stars and octahedra.	Dhere (1908a, 1908b)
	Five- and six-rayed stars and octahedra.	Svedberg and Chirnoaga (1928)
	Hexagonal double-pyramids.	Dhere and Burdel (1914)
Palinurus vulgaris	Hexagonal and regular.	Dhere and Burdel (1914)
	Cubic and rhombohedral.	Redfield (1934)
Eledone moschata	Needles. Hexagonal.	Kobert (1903)
Octopus vulgaris	Prismatic needles.	Henze (1901)
Loligo pealei	Needles.	Montgomery (1930)

It is seen that there is no common crystalline form unless it be the tendency for most hemocyanins to form needles. Naturally various methods will give different crystal forms (at least to outward appearance) as has been demonstrated repeatedly with other proteins.

B. Elementary analyses.

The elementary analysis of most proteins has not been of great use in their study but in the case of the hemocyanins this is not so. I will therefore give a table of the analyses that have been made.

Hemocyanin	C%	H%	N%	S%	Cu%	Author
Mollusca.						
Gasteropoda.						
Helix pomatia	53.0	6.9	15.15	0.76	0.24	Hernler and Philippi (1933)
					0.25	Dhere (1916, 1919a, 1919b)
					0.29	Begemann (1924)
	52.03	6.73	15.22	0.71	0.25	Roche (1934)
	48.59	7.04	14.26	0.71	0.23	Hernler and Philippi (1930)
					0.25	Burdel (1922)
Helix aspersa	53.14	6.78	15.14	0.73	0.24	Roche (1934)
Busycon canaliculatum			15.8		0.24	Montgomery (1930)
	53.5	6.65	15.9	1.23	0.24	Hernler and Philippi (1933)
			14.37			Mendel and Bradley (1906)
Busycon carica					0.23	Montgomery (1930)
Cephalopoda.						
Octopus vulgaris	53.54	6.82	16.09	1.07	0.25	Roche (1934)
					0.25	Kubowitz (1938)
	53.66	7.33	16.09	0.86	0.38	Henze (1901)
	53.4	6.9	15.9	1.04	0.25	Hernler and Philippi (1933)
Eledone moschata	51.53	6.91	15.81	1.13	0.25	Roche (1934)
Sepia officinalis	52.36	6.93	15.81	1.04	0.26	Roche (1934)
					0.34	Griffiths (1892a)
	54.12	7.10	16.26	0.61	0.34	Griffiths (1892b)
Loligo pealei					0.26	Kubowitz (1938)
					0.25	Montgomery (1930)
	52.75	6.8	15.75	1.19	0.26	Hernler and Philippi (1933)
Arthropoda.						
Crustacea.						
Homarus vulgaris					0.34	Griffiths (1892b)
	53.07	6.85	16.78	0.90	0.19	Hernler and Philippi (1933)
	54.17	7.07	16.29	0.67	0.34	Griffiths (1892a)
Maia squinado	52.86	6.56	16.82		0.18	Roche (1934)
Cancer pagurus	54.17	7.11	16.25	0.66	0.32	Griffiths (1892b)
Carcinus maenas	53.15	6.61	16.83	0.95	0.17	Roche (1934)
Dromia vulgaris	52.39	7.07	16.05	1.02	0.17	Roche (1934)
Xiphosura.						
Limulus polyphemus	52.50	6.86	17.05	1.14	0.17	Roche (1934)
			17.5	1.22		Van Slyke (1911)
	53.4	6.9	16.9	1.10	0.17	Hernler and Philippi (1933)
					0.17	Redfield, Coolidge and Shotts (1928)
	48.94	7.10	16.18	1.56	0.28	Alsberg and Clark (1910)

Some of the early values are obviously incorrect (Griffiths 1892a, 1892b, Henze 1901, Alsberg and Clark 1910 for copper determinations). This was due in part to (1) inferior methods of analysis, (2) impure preparations of hemocyanin, (3) change in the hemocyanin by treatment and (4) other factors which no one has been able to determine.

Excluding these values we can summarize the elementary composition of the various hemocyanin groups as follows:

Hemocyanin	C%	H%	N%	S%	Cu%
Gasteropoda -----	52.5	6.9	15.4	0.86	0.24
Cephalopoda -----	52.7	6.9	15.8	1.09	0.25
Crustacea -------	52.9	6.8	16.6	0.92	0.18
Xiphosura -------	52.9	6.9	17.1	1.15	0.17

There are certain phylogenetic conslusions we may draw from this data:

(1) The hemocyanins of the Crustacea contain more N than those of the Mollusca. The difference is definite and we will see we can corellate this with the amino acid content later.

(2) The hemocyanins of the Mollusca contain more Cu than those of the Crustacea.

(3) Limulus-hemocyanin is more closely related to the Crustacea-hemocyanins than to the Mollusca-hemocyanins. This is what one would expect, of course.

If the prosthetic group is the same in all cases we conclude that there are different amounts of protein bound with it in the two phyla.

Are there other metals in the hemocyanin molecule? Mendel and Bradley (1906) found Zn in the hemocyanin of Busycon canaliculatum in a quantity amounting to 0.027% and believed it took the place of Cu. However, Gatterer and Philippi (1933) by spectroscopic methods showed that there were no other metals detectable (less than 0.001%) and I believe we are safe in assuming their absence.

For comparative purposes I have had an analysis made on purified hemocyanin for carbon, hydrogen, nitrogen and sulfur by the Micro-Analytic Department and I have determined the copper in two samples of pure hemocyanin. I have utilized two methods for the determination of copper.

(A) Potentiometric titration. The principle upon which the method is based is the oxidation of I^- by the cupric copper and the potentiometric titration of the I_2 with $Na_2S_2O_3$. The organic material is ashed and dissolved in nitric acid (3 cl). After neutralisation KI solution was added and this was titrated immediately with 0.03N. $Na_2S_2O_3$ solution from a one milliliter syringe graduated in 1/100s of a milliliter (the calibration was checked by weighing Hg-samples taken from it). The titrating vessel was a cup (volume of 2 c.c.) placed in a Beckman pH-meter which had been converted into a potentiometric device by replacing the glass electrode with a Pt-electrode. A stirrer was inserted in the cup. Water and solutions used were boiled to free them of oxygen and the titration was carried out in an atmosphere of nitrogen when particularly accurate results wanted. The readings on the pH-scale were plotted against $Na_2S_2O_3$ added and a good deflection was obtained. To determine the end-point the change of pH per unit addition of thiosulfate was plotted against the thiosulfate added and a sharp peak was obtained, giving the end-point with considerable accuracy. To my knowledge this method has not been used before for copper determinatinns.

(B) Colorimetric method. This method was used when more rapid and not such accurate results were desired. The principle involved is the formation of a Cu-complex with sodium diethyl-dithio-carbamate and the colorimetric determination of this compound in carbon tetrachloride solution with a Klett colorimeter. The procedure was to dilute the unknown copper solution to 25 ml. (after ashing, dissolving and neutralising), add 5 ml. of

40% citric acid, make slightly alkaline with ammonia, add 10 ml. of 0.1% sodium diethyl-dithio-carbamate solution in a separatory funnel, extract with 6 - 7 portions (3 ml. each) of CCl_4 and compare with a standard copper solution treated in the same way. The complex is a deep yellow. Two samples were always run simultaneously and ten readings were made on each sample in the colorimeter. The results of the two analyses always checked within 3%.

With method (A) 100 gamma (micrograms) could be determined quantitatively and with method (B) 5 gamma of copper. I estimate the probable error for the quantities of copper used in the actual work to be less than 1% with method (A) and less than 3% with method (B).

The results of the copper determinations are summarized:

Method (A).

Hemocyanin-sample #1	0.234% Cu
	0.232% Cu
Hemocyanin-sample #2	0.228% Cu
	0.229% Cu

Method (B).

Hemocyanin-sample #1	0.238% Cu
	0.230% Cu
Hemocyanin-sample #2	0.232% Cu
	0.226% Cu
Average	0.231% Cu

The samples were prepared in the same manner (as described above) but were from different lots of blood collected four months apart. The second sample seems somewhat lower in copper.

Hemocyanin for analysis was dried in vacuo for several hours, then at 70°C. in vacuo and finally at 105°C. in vacuo over P_2O_5. It was weighed in the container in which it was ashed. The ashing was done with concentrated H_2SO_4 in a muffle at red heat for twenty minutes.

The results of the elementary analysis on the hemocyanin of the key-hole limpet, Megathura crenulata are:

C	52.08%
H	7.06%
N	16.14%
S	1.00%
Cu	0.231%

These values were obtained by the standard methods of micro-analysis following Pregl.

These values show a typical hemocyanin composition on the whole, but do deviate in some respects to the average analysis of gasteropod hemocyanin. There is somewhat less carbon, more hydrogen and more nitrogen. Of these the nitrogen content is the only deviation that is of significance for comparative purposes. It lies in an intermediate position between the molluscs and the crustaceans and, as can be seen from the table on page 38, it is the highest nitrogen content of any molluscs hitherto reported (with the exception of Griffith's value for Sepia officinalis, which is now considered unreliable). One would at once conclude that it had an intermediate content of diamino acids but its properties do not indicate a content this high. Of course the analysis might mean that it had a higher content in those mono-amino acids having a higher nitrogen content, i.e. a shorter carbon chain. On the other hand the physical properties might mean that some of the amino-groups of diamino acids are shielded or combined with carboxyl-groups. I believe that the latter reason is more probable.

Finally, we must remember that only four species of molluscs have been used for previous determinations and this is not a good statistical series. None of these species are of the same order as Megathura also.

C. Amino acid content.

The amino acids we are particularly concerned about are the diamino acids and the sulfur acids. There have been only three investigations of the individual amino acids themselves and the results are summarized.

Hemocyanin	Cystine	Arginine	Lysine	Histidine	Author
Helix pomatia	2.05	5.27	7.47	5.81	Roche (1936)
Helix aspersa	1.91	5.33	6.91	6.15	"
Octopus vulgaris	1.95	5.32	8.03	5.99	"
Eledone moschata	2.08	5.54	8.47	6.11	"
Sepia officinalis	2.02	5.61	7.90	5.92	"
Carcinus maenas	1.99	6.65	7.84	8.89	"
Dromia vulgaris	1.52	5.72	9.61	7.12	"
Maia squinado	1.88	5.87	-	-	"
Limulus polyphemus	2.26	6.85	8.25	9.08	"
		7.84	7.09	7.83	Van Slyke (1911)
		6.37	8.92	4.52	Mazur (1937)

We see that in general Crustacea-hemocyanin has more dibasic amino acids (diamino acids) than the Mollusca-hemocyanin. This can be correlated with their higher nitrogen content as given by elementary analysis. We cannot directly correlate the diamino acid content with the amphoteric properties until we know the content of dicarboxyl acids, which apparently must increase approximately parallel with the diamino acids since the iso-electric points and acid-base binding properties indicate this.

Compared with other proteins the diamino acid content is high and especially is this true of histidine. Limulus-hemocyanin has more histidine than hemoglobin or, in fact, most histones, and yet it does not have basic properties. We must conclude that hemocyanins probably have a high content of both diamino and dicarboxyl acids and hence have more charged groups per unit area than most proteins. This may be the basic reason for their association-dissociation reactions.

Of the total sulfur in Limulus-hemocyanin Mazur (1937) found cystine to be 47.5%, methionine 49% and an unknown form 3.5%.

PROSTHETIC GROUP.

The prosthetic group of hemocyanin contains copper; this is the extent of our knowledge as far as definite facts go. Several investigations have been carried out on this problem and the results are contradictory and not very reasonable. However, I will first discuss the products obtained by these workers as it indicates the complexity of the problem and gives further support to the theory of individuality of the hemocyanins.

A. Isolation of various products.

Mention must be made of various ideas as to the manner in which the copper is bound to the protein, derived from properties of the hemocyanin itself and not from products isolated from it. Fredericq (1878) believed that the copper was in a prosthetic group like hemoglobin and that acid would split it off. Couvreur (1900) shared this view. Henze (1901) found that tests for copper ions were given only after copper split off by acid and he concluded by analogy that hemocyanin was a compound similar to a Cu-albuminate. This latter view was supported by Kobert (1903) and Alsberg and Clark (1910). Alsberg (1915) stated that spectrum similar to ammoniacal copper solutions and hence was bound perhaps to the amino groups. Quagliariello (1924) took an intermediate position by saying that the copper in hemocyanin was less stable than the iron in hemoglobin but more stable than the copper in copper albuminate. Roche (1930) first believed in the Cu-albuminate idea but later changed (1933) to the theory of a prosthetic group. Dhere (1920b) showed that hemocyanin spectra did not correspond to Cu-albuminate.

1. Prosthetic group of Philippi.

By treating Helix-hemocyanin with strong alkali at 40°C. for several hours he obtained a dark green precipitate, insoluble in water and organic solvents, soluble in concentrated acetic acid or KOH, and giving a strong pine splint test (indicating pyrrols). His conclusion was that the prosthetic group was a copper porphyrin. (Philippi 1914, 1919)

2. Prosthetic group of Schmitz.

By treating Octopus-hemocyanin in the same manner as Philippi he obtained a dark green precipitate soluble in hot water and hot aqueous alcohol. The alcohol solution was wine-red and the substance precipitated when acid added to the solution. He found no evidence of porphyrin structure (high hydrogen content, behavior towards solvents and spectrum) but showed it to be a tetrabasic acid by titration. During the titration the color changed from green in acid solution thru blue at neutral reactions to a purplish tint in alkaline regions and this is roughly similar to the color changes accompaning change of pH on hemocyanin itself. (Schmitz 1930-1-2)

3. Prosthetic group of Conant.

By the same treatment again on Limulus-hemocyanin a greenish-brown precipitate was obtained. This was dissolved in 50% acetic acid and reprecipitated with alkali giving an amorphous black powder. By further treatment with ammonia a colorless amphoteric substance was obtained that was shown to be a polypeptide containing leucine, tyrosine and serine. The other fraction was considered to be an unknown sulfur-containing amino acid. None of the materials gave the test for pyrrols but on fusion with KOH a volatile substance was produced that gave a good reaction with Ehrlich's reagent. Treatment with HI and phosphonium iodide did not give pyrrols and since color gone when copper was split off he concludes porphyrins not present.

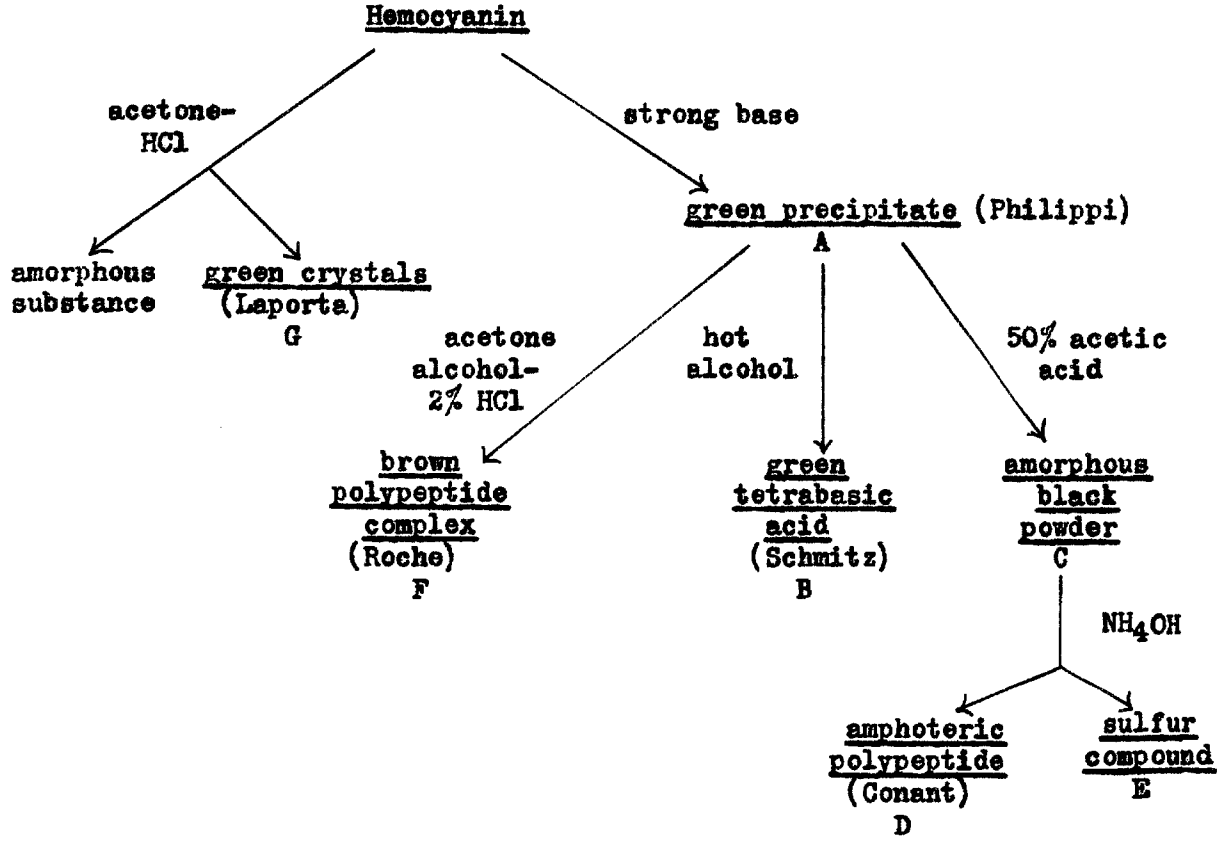
4. Prosthetic group of Laporta.

He precipitated Octopus-hemocyanin with acetone and added HCl which split off a group soluble in the acetone. The acetone was evaporated off leaving an amorphous material and a green crystalline substance. This green substance would crystallize from pyridine and gave the pyrrol test. He considered it to be identical with the "hemocuprin" of Schmitz.

5. Prosthetic group of Roche.

Using the hemocyanins of Cancer and Helix Roche and Dubouloz (1936) combined the method of Philippi with that of Laporta to obtain a substance containing amino acids: tyrosine, arginine and leucine.

To make the relationship between these compounds clear I will attempt to summarize the results in the following diagram.



The chemical analysis of some of these compounds resulted in the following results:

compound A (Philippi) --- Cu 7.0%
Gives pyrrol test.

compound B (Schmitz) --- C 45 %
H 7%
N 12.6% No free amino groups.
Cu 6.27%
S 0
Probably contains 4 COOH-groups.

compound C (Conant) --- C 39.5%
H 5.6%
N 9.2% $C_{31}H_{50}O_{15}N_7S_2Cu_2$
Cu 21.5%
S 8.0%

compound D (Conant) --- C 52.2%
H 6.7% $C_{24}H_{35}O_{10}N_5$
N 13.6%
Contains: 1 leucine, 1 tyrosine, 3 serines.

compound E (Conant) --- $C_7H_{15}O_5N_2S_2$ (not by analysis but by subtraction of D from C)

compound F (Roche) --- Contains: arginine, tyrosine, leucine.

compound G (Laporta) --- Contains Cu.
Free of protein.
Gives strong pyrrol test.

B. Products of Megathura-hemocyanin.

Although I was limited by the quantity of hemocyanin available for this work I have isolated some compounds that may be of help in elucidating the structure of the prosthetic group. Preliminary experiments involving heating of hemocyanin with pyridine in alkaline solution were unsatisfactory as no definite compound could be isolated from the reaction mixture. The red compounds obtained seemed to be associated with protein or protein fragments.

The method of Philippi was, therefore, followed on the assumption that most of the products obtained by other workers were polypeptide complexes of the true prosthetic group and what was necessary was to split this complex in some manner and obtain the non-polypeptide fraction.

Two liters of Megathura blood were dialysed and the precipitated

hemocyanin suspended in water (200 ml.). Enough concentrated KOH solution was added to bring to about 1N. KOH and the mixture was warmed at 45°C. for twenty-four hours. Most of the protein goes into solution and a red color is formed (the Biuret test according to Schmitz after Henze, but it probably is not as trypsin will give it at pH 8.5). After several hours the solution was a dark brown and a precipitate had settled out. After a day it was centrifuged giving a clear yellow supernate and a brown precipitate. This was suspended in water and recentrifuged leaving the supernate colorless. This washing was repeated twice more and a sample was dried at 105°C. in vacuo and analyzed by the Micro-Analytic Department for carbon, hydrogen, nitrogen and sulfur, while I made a copper determination.

Analysis of compound #1.

C	30.0%
H	4.9%
N	4.9% (mean of two: 5.04 & 4.80)
S	5.54%
Cu	19 %
K	6.4%
(O	29.0%)

This analysis showed I had a material high in copper but it was probably quite impure. At the pH of the solution none of the potassium (obtained by the difference in ash and copper) could be combined in salt-form with any carboxyl groups so it was assumed that it was merely adsorbed onto the precipitate. On this assumption we can write an empirical formula for this compound which merely indicates something about the type of substance we are dealing with: $C_{16}H_{32}N_2SO_{11}Cu_2$. From the nitrogen content I assumed that I was not dealing with a pure polypeptide (N 11 - 12%) complex of copper.

This substance did not dissolve in hot alcohol giving a red color as the compound of Schmitz had done. Nor did it give a green color with pyridine as had Conant's material. However, it would dissolve in 50% acetic acid and could be reprecipitated by adding alkali.

It was observed that part of this material would go into solution in 5% HCl so the whole precipitate was so treated, warming to about 50°C. for three hours. When the acid was first added an evolution of some gas was noted and it was not H_2S. Could it be that this prosthetic group can bind oxygen and that HCl forces it off as it is known to do in the complete hemocyanin? The solution was bright yellow and it was estimated that about one-half of the precipitate was dissolved. On slowly adding KOH solution it was found that when neutrality was reached the solution was a very dark wine-red and that on passing into an alkaline pH a red flocculant precipitate separated leaving a clear colorless liquid. This precipitate was centrifuged out and washed a number of times with distilled water and recentrifuged. It was then redissolved in dilute HCl and reprecipitated again with KOH solution and the washings repeated. From carefully observing the precipitation it was seen that there were apparently two substances, one separating out slightly before the other and to which the other (the red substance) was adsorbed. The first appeared a good deal like copper hydroxide in texture and color and it was decided that there was more copper in the solution than could be combined with the prosthetic group. This compound was analyzed by the Micro-Analytical Department and copper determinations were made.

Analysis of compound #2.

C	32.4%
H	4.87%
N	3.41%
O	17.4%
Cu	41.9%
	99.98%

These values are corrected from the original determinations: (1) the compound analyzed was dried at 70°C. in vacuo and it was found later that this did not take all the water off, in fact it contained 7.8% water, determined by drying again at 105°C. in vacuo, and (2) because due to the fact the

precipitation was made from alkaline KOH solution there was KOH in the precipitate and again the K was determined by the difference between the ash and the copper analysis and the total analysis corrected.

It was seen that the compound was even lower in nitrogen than the original brown precipitate and could not possibly be related to a polypeptide or any amino acid structure.

As I had only obtained 12 milligrams of this red substance, and the micro-analysis had consumed 11 milligrams, there was the problem of what was the best way to use this last milligram. I decided to add solid KOH to it in the bottom of a small tube and heat to fusion (360°C.). There were small drops of yellow liquid condensed on the side of the tube and this liquid gave a very strong Ehrlich's reaction as a test for pyrrol-rings. (Note: Ehrlich's reaction I obtained by using a solution of p-dimethyl-amino-benzaldehyde in strong HCl and not by the pine splint test.)

To determine the correct formula for the compound we must now make some assumption regarding the amount of $Cu(OH)_2$ present. The empirical formula for compound #2 is: $Cu_5C_{22}H_{39}N_2O_{12}$. On the basis of the pyrrol reaction, assuming these rings to be present, I think that the most logical method in which to correct for $Cu(OH)_2$ is to assume that there is 1 Cu to every 2N in the compound. This ratio is the one generally obtained in the metal complexes of substituted pyrrols or dipyrryl-methenes. On applying this correction to the above formula we obtain:

$$C_{22}H_{30}N_2O_3Cu$$

for the pure dark red compound. The value of the oxygen is of the most uncertainty because of the large corrections to it and it may be easily 2 or 4. It is seen that the compound is probably unsaturated.

As the only doubtful step in the determination of this empirical formula involves the correction for $Cu(OH)_2$ I wish to discuss the justifications for this. Firstly, from observation it is seen that there are two substances, a red one and a pale blue one, that precipitate together. It is difficult to compare the relative amounts present as the first comes out in small particles and the second is voluminous. The color of this precipitate as it comes down is a rose color of medium intensity. The dried material is almost black. Assuming that the red material does not come down in a condition containing much water it is seen that there must be relatively quite a fair amount of the bluish substance. As the appearance of $Cu(OH)_2$ is altogether characteristic when precipitated in such a way I believe the justification for assuming it to be this substance is well based. As to exactly which precipitates first it is difficult to say. The basis for my statement above that the $Cu(OH)_2$ comes out first is that the initial visible precipitate is of a gelatinous nature, although the color cannot be seen at this time. At the end of the precipitation one can see the last traces of the precipitate formed after most of the red material has come down to be of a bluish cast. As to the assumed ratio of Cu:N, two Ns to one Cu was used because of the reason given above and also that the three other possible ratios are ruled out: (1) no copper present in the red material --- this I believe unlikely due to the solubility behavior (discussed below) but even if this is the case it would not change the empirical formula a great deal with respect to the basic C-H-N ratios ($C_{22}H_{28}N_2O$); (2) one N to one Cu --- no cuprous complexes are known that have this ratio, i.e. where the copper is attached to the nitrogen; (3) one Cu to four Ns --- this would be the case in a porphyrin structure and I think the presence of porphyrin rings can be ruled out because of the

lack of fluorescence in the red compound, the loss of color in strong acid and the fact that cuprous copper would not form such a compound (if a porphyrin were actually present there would be two copper atoms to four nitrogen atoms, which is the ratio assumed).

As this empirical formula is based on there being two nitrogen atoms present any error in the nitrogen determination would affect the values for carbon and hydrogen. From the average error of such a nitrogen determination, as given by Mr. Lanz, I calculate that carbon may vary in the compound from C_{19} to C_{25} and the hydrogen from H_{24} to H_{32}, although the ratio must remain as given, which is the most important factor.

The nature of this compound will be discussed more fully after the magnetic results have been shown, but I would like to make a few general remarks at this point. There are four reasons why I believe this compound to be a pyrrol-complex: (1) Ehrlich reaction --- the KOH fusion showed this definitely (and this is a common method for demonstrating pyrrols in compounds that are substituted in all positions and hence do not give the red color themselves) and the red compound itself gave a brilliant green color with p-dimethyl-amino-benzaldehyde, which is a phenomenon shown by many of the dipyrryl-methenes (Fischer and Orth 1937). (2) Spectral properties --- observation with the spectrophotomer of the red solution immediately before precipitation showed one band which distinguishes it from the porphyrins (which have at least two bands) and indicated its relation to the Cu-dipyrryl-methenes (which have in many cases a similar band (Fischer and Schubert 1924, Fischer and Orth 1937)). (3) Empirical formula --- this indicates unsaturation and, in fact, gives reasonably good agreement with the assumption of a substituted dipyrryl-methene compound. (4) Analogy with hemoglobin and bile-pigment proteins ---

from a comparative point of view it seems to me that when we have a nitrogen-containing, unsaturated compound in a respiratory protein it is probable that it is a pyrrol-type compound.

Naturally these conclusions are not definite but I have attempted to analyze the results obtained on a very limited amount of material to the best of my ability and hope that it may lead to further investigation along these lines.

C. Discussion of previous results.

I would prefer to believe that the same prosthetic group occurs in all the hemocyanins, rather than to attribute my results so some special property of Megathura-hemocyanin. It remains to see what variations in method have given me the results above in comparison to previous work.

The chief difference must be in my elimination of a non-polypeptide compound from parts of the protein that originally remained with the prosthetic group. Conant (Conant and Humphrey 1930, Conant, Dersch and Mydans 1934) split the complex up and analyzed the polypeptide fraction, claiming this to be the prosthetic group. It must be remembered that KOH-fusion of his complete compound gave a good pyrrol test. Roche (Roche and Duboucloz 1936, 1933) has undoubtedly worked with the polypeptide complex as he made no attempt to separate any other fraction and obtained an ultra-violet spectrum of the complex indicative of polypeptide natuse. Laporta (Laporta 1932) alone may have isolated such a non-protein substance for he obtained it in crystalline form, but unfortunately he does not g ve any analysis but states merely that it gives a pyrrol reaction. There is thus no evidence from other workers for the absence of pyrrol compounds.

D. Reactions of hemocyanin bearing on the prosthetic group.

As is the case with hemoglobin, hemocyanin reacts with certain substances by virtue of its prosthetic group: potassium cyanide, carbon monoxide, nitrogen dioxide and hydrogen sulfide.

The first mention of a cyanide complex was made by Kobert (1903) and Cook (1928) showed that this compound did not combine with oxygen. This reaction has since been the means of determining the oxygen content of hemocyanin solutions for the oxygen is driven off in the presence of cyanide. Pearson (1936) in a recent study has found evidence that two cyanide groups react with one copper atom (from dissociation curves of cyanide and mass-action law). However, the problem has been cleared up completely by Kubowitz (1938) who showed that cyanide splits the copper from the hemocyanin molecules. This explains the old observation, many times repeated, that the cyanide reaction was irreversible.

Craifaleanu (1919b) found that carbon monoxide would decolorize hemocyanin and found that the process was reversible when oxygen added again. With Limulus-hemocyanin Root (1934) has found evidence for a CO-compound by comparing the oxygen dissociation curves in the absence and in the presence of carbon monoxide. Although he gives no data he states that they combine in the ratio of two copper-atoms to one carbon monoxide. The hemocyanin has somewhere around twenty times the affinity for oxygen that it does for carbon monoxide. Kubowitz (1938) demonstrated that carbon monoxide did not split the copper from the molecules.

Nitrogen dioxide, NO_2, forms a green compound with hemocyanin as shown by Dhere (1919c) and Dhere and Schneider (1919b, 1920, 1922b) in the case of Helix, Octopus and Eledone, but with difficulty in Astacus and Homarus. Hemocyanin has much more affinity for it than for oxygen.

That hydrogen sulfide and ammonium sulfide decolorized hemocyanin was noted by Krukenberg (1880a) and Craifaleanu (1919b) found that the copper was set free and precipitated as the sulfide.

Some early work of mine in determining oxygen capacities of hemocyanin solutions indicated that cyanide did not react with Megathura-hemocyanin as rapidly as with other hemocyanins. In fact it was not possible under the conditions used to drive off the oxygen sufficiently to give a good determination. I have found that with crayfish blood the addition of dilute cyanide almost immediately decolorizes the hemocyanin, but in the case of Megathura the solution became colorless slowly and the reaction did not seem to be complete until twenty to thirty minutes later.

That cyanide splits off the copper can be demonstrated by adding a little Na-dithio-diethyl-carbamate when we obtain a dark yellow color. When added to pure hemocyanin the solution becomes clear but there is no yellow color. The yellow color was produced when HCl was added as this also splits the copper from the protein.

E. Oxidation of hemocyanin.

Until 1933 there had always been the question of whether there was a "methemocyanin", similar to the methemoglobin, sontaining copper in the oxidized condition. For some reason it had been generally aasumed that the copper was normally in the cuprous state, probably by comparing it with hemoglobin where the iron is in the ferrous state, but there was absolutely no evidence for this. In fact some attempts to oxidize it with reagents capable of oxidizing the iron in hemoglobin had failed. Potassium ferricyanide had been used by Quagliariello (1922) and Cook (1928) without success.

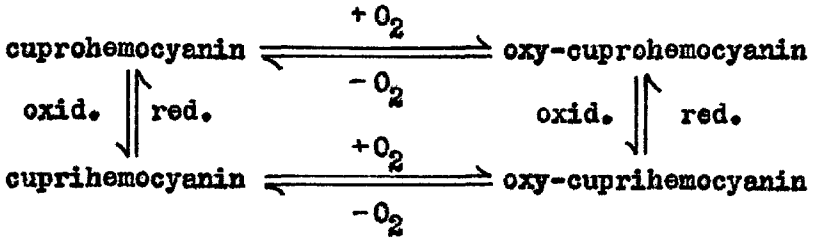
In 1933 Conant, Chow and Schoenbach found that very powerful oxidizing agents would oxidize the copper from the cuprous state to the cupric state in Limulus-hemocyanin. The potential of this system was +0.54 volts. Not only is this the highest potential yet found for any biological substance but it is exceeded by only a few inorganic systems that are reversible. This is the reason for previous failure in the use of ferricyanide and other oxidizing agents with too low potentials. A preliminary observation on the potential of the prosthetic group indicated that it was close to +0.15 volts. This determination was performed on a pyridine solution (alkaline) of the black amorphous substance. In this case, as in most cases where a prosthetic group or active group combines with a carrier protein, the potential is raised. It is to be noted that the potential of the prosthetic group is very close to the normal cuprous-cupric potential (+0.167 volts).

Is it actually the copper that is being oxidized? They showed this by back-titrating with potassium ferrocyanide or 1,4-naphthohydroquinone and found that the amount used was equivalent to the copper present.

One of the most interesting properties of methemocyanin is that it is able to combine with oxygen, thus being unlike hemoglobin in the oxidized state. The reduced compound is colorless and the oxy-compound is green. After reduction of methemocyanin it shows all the reactions of normal hemocyanin showing that the oxidation is reversible.

At this point I would like to indicate the nomenclature I will follow in this paper. The system to be proposed eliminates any possibility of being misunderstood when referring to a certain state of hemocyanin and furthermore brings the nomenclature of hemocyanin into agreement

with the now almost universally accepted nomenclature of hemoglobin and its derivatives (Pauling and Coryell 1936b). It may be summarized in the following diagram.



The prosthetic group has been called hemocuprin and this is generally used now to denote the polypeptide complex. However, I do not feel it is justified yet to change the name, but I suggest that we designate the prosthetic group in the oxidized and reduced forms by "hemocuprin" and "hemocupron".

THE EQUILIBRIUM BETWEEN HEMOCYANIN AND OXYGEN.

Of the three functions of hemocyanin in the animal the transport of oxygen requires the most specialization on the part of the protein. This specialization is brought about by the addition of a metal complex that is capable of combining reversibly with oxygen when it is joined to the protein. This final combination must carry enough oxygen for the needs of the animals under normal and slightly abnormal conditions and must be able to take up oxygen at the partial pressure of oxygen at the surface of the respiratory organ and give it off at the partial pressure of oxygen in the tissues. I will first discuss the quantity of oxygen that the hemocyanin will combine with and then the factors determining the pressures at which oxygen will be taken up and given off.

A. The O_2:Cu ratio.

Dhere (1919a) was the first to determine this important ratio and although his average was close to 1.0 (the ratio will be given as atoms of oxygen per atom of copper in this work) the molluscs fell under and the crustaceans were over this. Such variation has not been confirmed. Begemann (1924) found that Helix and Carcinus hemocyanins gave a ratio of 1.0 but his work was not quantitative enough to permit us to say that the ratio is exactly 1.0.

The first accurate work was done by Redfield, Coolidge and Montgomery (1928) who made several determinations on each of nine different species. The ratios varied from 0.90 to 1.03 showing no specific differences between phylogenetic groups. I have averaged all of his results and find that this

value is 0.934 from 47 determinations. This deviation from unity may be significant when one considers that Peters in 1912 claimed that an error in this direction, due to experimental faults, should not amount to more than 2%

All in all thirteen species have been shown to possess hemocyanins giving this ratio. However, I thought it advisable to determine this ratio with Megathura-hemocyanin because the magnetic work was dependent upon this value. Using a Van Slyke apparatus and utilizing the addition of concentrated HCl to drive off the oxygen I made two determinations. I claim no great accuracy to the results as the exact determination of the oxygen capacity requires a good deal of experience and I felt that it would be sufficient to merely check the ratio of approximately unity against any other integer ratios. The ratio of one was proved for this hemocyanin by obtaining results of 0.89 and 0.94, the first determination being made on a solution of hemocyanin of approximately the concentration occurring in the blood and the second on a solution some five times more concentrated in hemocyanin. Both samples were equilibrated with pure oxygen for half an hour before the determination.

The ratio of unity indicates that the prosthetic group may contain two atoms of oxygen.

B. Oxygen dissociation curves.

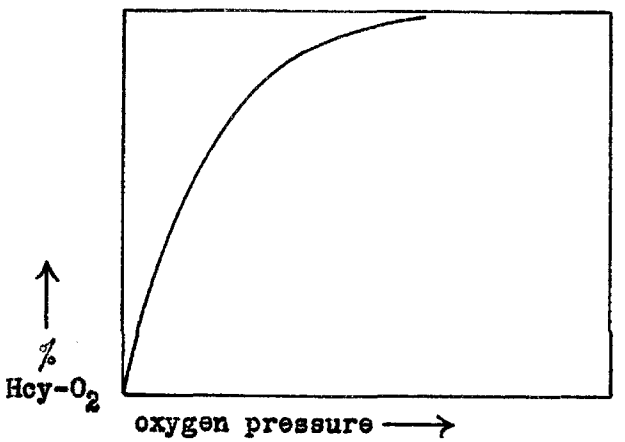
If we equilibrate samples of blood or hemocyanin solutions with gas mixtures of different partial pressures of oxygen and determine the amount of hemocyanin in the oxygenated form we find these values lie on a curve. From the shape of this curve we can derive interesting relationships in the equilibrium between oxygen and hemocyanin. These curves are sensitive

to changes in the medium, such as salt concentration, pH and temperature. We will first consider the curve for solutions of pure hemocyanin only and then discuss the effect of these various factors on changing the position and shape of the curve.

As a great deal of work has been done on a large number of species I have given only generalized curves, typical of all hemocyanins.

1. Dissociation curve of pure hemocyanin solutions.

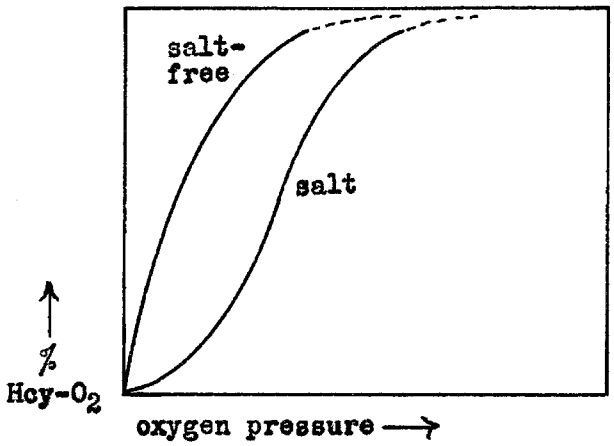
The curves of hemocyanins from all species when pure and present in electrolyte-free solutions are indistinguishable from hyperbolas. Each hemocyanin is characterized by a different affinity for oxygen, i.e. the curves may be shifted along the oxygen-pressure axis, but when this factor is corrected for they all fall on one hyperbolic curve (Redfield 1934). The typical curve for hemocyanin under these conditions is given below.



2. The effect of salt concentration on the dissociation curve.

As the concentration of electrolytes increases the curve departs more and more from an hyperbola and becomes sigmoid in shape up to a certain salt concentration. The salt in the blood is more than sufficient to give this effect and so we find the dissociation curves of blood to be sigmoid. The extent of the departure from a hyperbolic form (the degree of inflection)

is characteristic for the hemocyanin under consideration. The effect can be summarized in the following curves.



The behavior in this respect is similar to hemoglobin solutions.

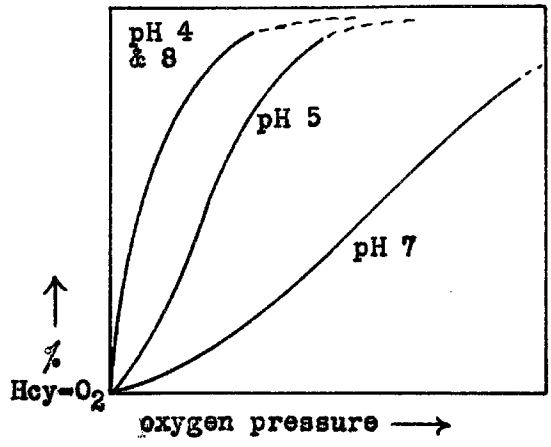
3. The effect of pH on the dissociation curve.

Many workers have claimed that qualitative differences exist between various hemocyanins with respect to their behavior towards oxygenation on change of pH. However, I am certain that there are only quantitative differences and that all the phenomena may be explained on the basis of a general theory (see below).

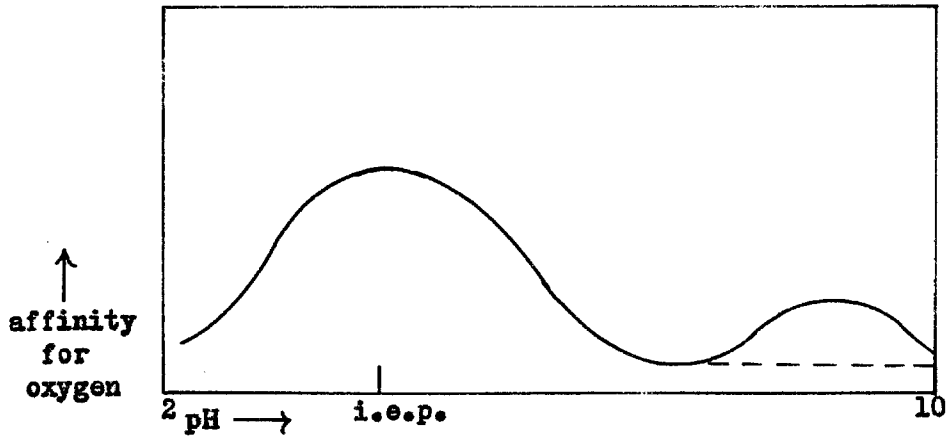
In salt-free solutions the curves are always hyperbolic independent of the pH. The affinity for oxygen may be greatly changed but the shape of the curve is not altered. Thus Redfield (1930b) with Limulus-hemocyanin and Stedman and Stedman (1928) with Helix-hemocyanin found no change in the shape of the curve over a range of pH from 4 to 10.

In solutions containing electrolytes the situation is complicated because of a change in the shape and a change in the affinity as the pH is varied. However, we can make two generalizations: (1) the curves approach the hyperbolic form as we near the limits of the pH range of stability, being distinctly more sigmoid at intermediate pHs and (2) the affinity of hemocyanin for oxygen passes thru a minimum at a certain pH characteristic

of the species, increasing as we leave this pH and often passing thru a maximum at a pH some units removed. The following generalized dissociation curves show this.



I have analyzed every dissociation curve ever published, showing the effect of pH, and I feel I can with confidence give a generalized curve showing the variation in affinity with pH. Such a curve follows.



The presence of the second maximum at alkaline reactions appears to be doubtful in a few hemocyanins.

4. The effect of temperature on the dissociation curve.

This effect is simple and there are no exceptions to the statement that as the temperature is increased the affinity for oxygen is decreased. The oxygenation of hemocyanin is, therefore, an exothermic reaction.

C. Theoretical treatment of dissociation curves.

If we write for the oxygenation of hemocyanin the reaction:

$$Hcy + O_2 \rightleftharpoons Hcy\text{-}O_2$$

where by Hcy we mean that amount containing two copper atoms, and apply the simple mass-action law we obtain for the fraction of hemocyanin in the oxygenated condition:

$$y = \frac{Kp}{1 + Kp}$$

where p is the oxygen pressure and K is the mass-action equilibrium constant:

$$K = \frac{(Hcy\text{-}O_2)}{(Hcy)\ (O_2)}.$$

This simple theory gives an hyperbolic curve, such as we have seen actually occurs in salt-free solutions and at high and low pHs. The physical interpretation is that there is no relation between the various prosthetic groups on the molecule.

To explain the sigmoid curves we must assume that the prosthetic groups are not independent. If we assume a grouping of several copper atoms that is only stable when a certain number, n, of them are in the oxygenated state, we must write:

$$Hcy + nO_2 \rightleftharpoons Hcy\text{-}(O_2)_n$$

where by Hcy we mean the amount of hemocyanin containing these n groups. This results in an expression of the following nature for the fraction of hemocyanin in the oxygenated state:

$$y = \frac{Kp^n}{1 + Kp^n}$$

As the number, n, increases we obtain curves with more and more inflection, i.e. more and more sigmoid character. Experimental curves for hemocyanin, as for hemoglobin, show inflections indicating values of n of two or three (and values intermediate).

These simple theories do not attempt to explain how n may vary as conditions are changed, nor how we obtain non-integral values of n. A great many theories have been put forth to explain the situation in hemoglobin but fortunately most of them are not applicable to hemocyanin. In hemoglobin we have four prosthetic groups per molecules and we can arrange them in certain ways and have them interact with one another and obtain equations that fit the experimental data, but in the case of hemocyanin we have a variable number of prosthetic groups per molecule and these molecules are not stable with respect to size. The only theoretical treatment that was worked out with hemocyanin in mind is that of Redfield and Ingalls (1933) who based their theory on Hill's equation given above but assumed that the blood consisted of two or more components (all hemocyanins of course) each characterized by a different value of n, n being for each component an integer. We therefore obtain the following equation:

$$y = \frac{a_1K_1p}{1+K_1p} + \frac{a_2K_2p^2}{1+K_2p^2} + \frac{a_3K_3p^3}{1+K_3p^3} + \frac{a_4K_4p^4}{1+K_4p^4}$$

where y is the fraction of hemocyanin in the oxygenated condition, the p represents oxygen pressure, a_1, a_2, a_3 and a_4 are the fractions of the total hemocyanin present in the forms characterized by n being equal to 1, 2, 3 and 4, and the Ks are equilibrium constants for each of these components in its reaction with oxygen.

When the conditions of the medium change we find that two factors may vary: (1) the affinity for oxygen or the value K and (2) the relative fractions of the components or the values of a. Since Svedberg has shown that 20 to 80 molecules of oxygen are bound to each molecule of hemocyanin, according to the data of Svedberg we must assume that these changes in the relative amounts of the components are due to changes in the association

of prosthetic groups on the same molecule, resulting from variations in the configuration of the molecule (Redfield and Ingalls 1933).

Redfield and Ingalls determined the oxygen dissociation curves for the hemocyanins of Limulus, Homarus, Busycon and Loligo under a great variety of conditions. From these curves he has shown how the values of a and K vary with pH and temperature. In acid and alkaline solutions there is a relatively greater amount of the component having n=1, while at intermediate reactions components of n=2 and n=4 are present. For none of his curves did he ever have to assume a value of n=3. The Ks vary in a way predictable by the expression derived by Ferry and Green (1929) for the change in the oxygen dissociation constants of hemoglobin. The results in general are those given previously in the generalized curves on pages 60 - 62.

To account for the sigmoid shape of the oxygen dissociation curves any theory must assume some interaction between the prosthetic groups. This word "interaction" does not necessarily mean a direct effect, such as the chemical reaction of two prosthetic groups, but merely means that one group affects the other in some way, perhaps very indirectly. In simple terms it means that when one oxygen molecule has been attached the next one comes on more easily, i.e. the presence of the first oxygen molecule decreases the free energy of oxygenation for the second oxygen.

A theory assuming a generalized interaction to take place between the heme groups in hemoglobin upon oxygenation has been developed by Pauling (1935) and expanded by Coryell, Pauling and Dodson (1939). To explain the effect of pH, interactions were assumed to exist between the hemes and the groups whose ionization constants were affected by oxygenation. The magnitude of the heme-heme interaction and the heme-acid group interaction

is in both cases approximately 6000 calories per mole. The heme-heme interaction can, at the present time, be given no physical interpretation, but recently (Coryell and Pauling 1940) have correlated the heme-acid group interaction with an underlying histidine imidazolium ion and an imino group of another histidine residue lying on the opposite side of the porphyrin ring from the first. Another acid group that is affected by oxygenation may be the iron atom itself which may add an hydroxide ion.

The question here is: can this theory be applied to hemocyanin? I believe it can because the exact nature of the interactions are not specified. Whatever the situation is in hemocyanin I am sure that the curves may be explained on the basis of a change in the free energy of oxygenation. The important problem now is to determine the nature of the interaction.

D. Acid groups and oxygenation.

I wish now to discuss briefly the changes observed in the ionization constants of hemocyanin upon oxygenation.

From the demonstration that the addition of acid decreases the affinity of hemoglobin for oxygen (the Bohr Effect) at pHs corresponding to that of the blood (Bohr, Hasselbach and Krogh 1904) it was shown to be logically necessary by Henderson (1920) that the hemoglobin solution become more acid upon oxygenation. The same reasoning would apply to hemocyanin.

Whether we get the positive Bohr Effect or the reverse Bohr Effect (an increase of affinity for oxygen when acid added) depends on whether we are at a pH on the acid or alkaline side of the oxygen-affinity-minimum (page 62). Some hemocyanins show one effect and some the other, and this is not due to any qualitative difference between the hemocyanins as many workers have thought, but to the fact that their oxygen-affinity-minima are at

different pHs. Shack (1935) has shown that solutions of the hemocyanin of Homarus, giving the positive Bohr Effect, actually do decrease in pH on oxygenation. The phenomenon was described as being similar to ionization of a dibasic group rather than to two monobasic groups.

Roche (1932, 1936) has made comparisons between the titration curves of hemocyanin in the reduced and oxygenated states. He finds that in acid regions the two curves are identical and this is also the case above pH 8.5. However, the group with ionization constant of approximately 7 does change upon oxygenation in the following way.

Hemocyanin	pK(reduced)	pK(oxygenated)
Helix pomatia	7.5	7.0
Carcinus maenas	7.1	6.7
Sepia officinalis	7.2	6.8

In hemoglobin the pKs of the groups affected are about 5.5 and 6.7 (Coryell and Pauling 1940). The shift in the pK value is about the same, however, in both cases.

E. Heat of oxygenation.

From the change in the equilibrium constant of the oxygenation reaction with temperature and the van't Hoff equation:

$$q = \frac{RT_1T_2}{T_1-T_2} \ln \frac{K_1}{K_2}$$

one can calculate the heat of oxygenation in a certain temperature range. This has been done with a number of hemocyanins and under various conditions and the results are summarized in the table on the next page.

Redfield and Ingalls (1933) have, by this method, determined the heat of oxygenation for each component assumed in their theory on the basis of the change in each particular K. These values are strikingly different.

Hemocyanin	Medium	pH	Temp. range	q	Author
Limulus	blood	9.45	5° - 40°	14,800	Brown (1933)
Busycon	"	6.35	10° - 40°	15,600	Brown (1933)
	"	7.95	10° - 40°	17,100	Brown (1933)
	"	10.22	10° - 40°	15,850	Brown (1933)
Cancer	"	7.95	0° - 52°	9,100	Hogben (1926) Hogben and Pinhey (1926)
Maia	"	7.95	0° - 52°	9,100	Hogben (1926) Hogben and Pinhey (1926)
Helix	"		1° - 47°	8,050	Hogben and Pinhey (1926)
Limulus	dialysed	5.7		1,750	Hogben and Pinhey (1927)
	"	7.1		3,150	Hogben and Pinhey (1927)
	"	8.9		900	Hogben and Pinhey (1927)
Maia	"	7.7		5,200	Hogben and Pinhey (1927)

Redfield's results for Limulus-hemocyanin were:

Constant	Temp. range	n	q	q/n
K_1	5° - 40°	1	14,800	14,800
K_2	5° - 44°	2	9,120	4,560
K_4	5° - 15°	4	36,900	9,225
	25° - 44°	4	12,000	3,000

In the first table the q's are given in calories per mole of oxygen combined.

Values for the heat of oxygenation of hemoglobin run from 10,000 to 19,000 calories per mole, probably being closer the first figure. Thus we see that the values are quite comparable.

The most interesting point from these figure is that the heat of oxygenation is much lower in salt-free solutions than in the blood. Another unusual observation is that the heat of oxygenation changes with temperature for the hemocyanin of Limulus in the case of the component with $n = 4$. No explanation has ever been offered for these phenomena.

PRINCIPLES OF MAGNETIC DETERMINATIONS.

As a preliminary introduction to the magnetic work on hemocyanin I shall discuss the theory of magnetism, the equations used in the work, and the relation of magnetic measurements to chemical structure. The last will be briefly illustrated by the work on hemoglobin.

A. Magnetic theory.

A model of the atom based on the theory of Bohr (1913) having the electrons revolving in orbits around a central nucleus is sufficient for our purpose if we include the quantum mechanical spin of the electron (Uhlenbeck and Goudsmit 1925).

On applying a magnetic field to any substance there is an acceleration in the angular velocity of the orbits of the electrons. There is no change in the size of the orbits or of the speed of the electrons in the orbit, but the whole orbit is rotated around the nucleus at a speed proportional to the field applied. This creates a field that is opposed to the applied field and hence the substances is termed "diamagnetic". This diamagnetism is of small magnitude, independent of temperature and is an additive function of the atoms making up a molecule.

On the other hand there are substances that possess a permanent magnetic moment due to the presence of: (1) unpaired electrons and (2) electron orbits that become somewhat polarized. The second factor is often negligible and is always small (except in cases where the electrons are allowed to wander thru several atoms as in poly-benzene ring systems).

We may picture the electron as rotating around its own axis and

thereby producing a magnetic field. The magnetic dipole moment is usually given in Bohr magnetons, this being numerically equal to $\frac{he}{4\pi m_o c}$, and representing the magnetic moment of an electron along any chosen axis. The actual magnetic moment is $\sqrt{3}$ times this value or $\sqrt{3}$ Bohr magnetons.

What is actually measured in a magnetic determination is the so-called magnetic susceptibility. This is classically defined as the ratio of the induced magnetic moment per unit volume to the external field; this is the volume susceptibility. The quantity more often used in practical work is the molal susceptibility, which is given by:

$$\chi_{molal} = \chi_{vol.} \frac{\text{Molec. Wt.}}{\text{density}}$$

Susceptibility is additive for the two types of magnetism, thus:

$$\chi = \chi_{dia.} + \chi_{para.}$$

which, put in more concrete terms, is:

$$\chi_{molal} = N\alpha + \frac{N\mu^2}{3kT}$$

where N is Avogadro's number, α is the molecular diamagnetic susceptibility, k is Boltzmann's constant, μ is the paramagnetic permanent dipole moment and T is the absolute temperature. The first term on the right side of the equation, representing the diamagnetism, is negative in all cases.

We can obtain μ in terms of χ_{molal} and substituting the values for the known constants obtain the useful relation:

$$\mu = \sqrt{3kC/N} = 2.839\sqrt{C} = 2.839\sqrt{\chi T}$$

where C is the Curie constant, $N\mu^2/3k$ and χ is the susceptibility due to paramagnetism.

When there are more than one unpaired electrons in an atom they contribute together to a resultant magnetic moment for the whole atom. This

resultant magnetic moment is given by:

$$\mu = \sqrt{n(n+2)}$$ Bohr magnetons.

when n is the number of unpaired electrons. The theoretical value for the magnetic moment μ calculated from this equation is:

Number of electrons	Magnetic moment
1	1.73
2	2.83
3	3.87
4	4.90
5	5.92

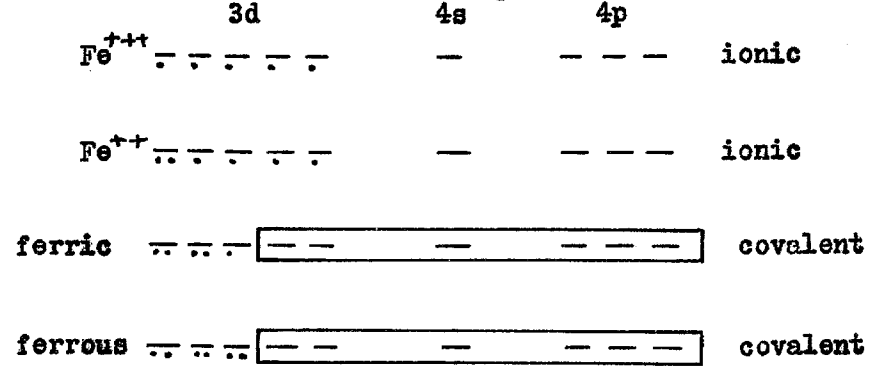
To determine how many unpaired electrons there are in a molecule we determine the molal susceptibility (see later for method) and calculate the magnetic moment, comparing it with the theoretical value given in the table. If we assume no orbital contribution to the magnetic moment and the unpaired electrons do not interact with each other at all, the two values should be coincident.

B. Bonds and paramagnetism.

In most compounds there are only two known types of bonds: the covalent and the electrostatic (or ionic). In the latter the forces holding the atoms together are purely electrostatic in nature and in the former the bond consists of two electrons (for single bonds) with their magnetic spins opposed and hence having no resultant moment.

This is not the place to go into all the rules that determine the way in which one places the electrons in the various orbitals (the word orbital is used to designate a certain definite orbit in which two electrons may be placed, there being a varying number available in the different atomic levels corresponding to the permissible quantum numbers). I will instead give some definite examples of how this is done.

We may write the electronic structure of ionic and covalent ferrous and ferric compounds in the following way:



The symbol at the top (3d, 4s, etc.) indicates the type of orbital, each dash represents one orbital and each dot represents one electron. The enclosure around the outer orbitals in the last two cases means that these orbitals are used in bond formation.

When it is possible electrons have a tendency to spread out over the available orbitals of the same energy, as shown in the ionic iron atoms. This is an experimental fact, shown by spectroscopy and magnetic determinations. In using a group of orbitals to form bonds an atom does not leave orbitals vacant that lie below or at the same level (unless there are not sufficient electrons to fill up the rest of the orbitals at the same level). It is rare when an atom will shift electrons above the orbitals forming the bonds, but it will do this if the energy considerations permit it.

In considering what bonds will be formed under certain conditions we can expect that those bonds will be formed that lead to the greatest stability. Pauling has calculated, with the aid of quantum mechanical considerations and a set of reasonable postulates, the strength of various bonds composed of different orbitals. When we have orbitals available for bond formation from two different energy levels it commonly occurs that

more stable bonds are formed if certain orbitals are combined. We say that the orbitals are then in resonance and each bond possesses somewhat the property of the orbitals of which it is composed. Each bond in such a case is a hybrid of two or more orbitals and we designate this hybridization with symbols indicating the type of orbitals used: e.g. sp^3 means that one s-orbital and three p-orbitals are used to bond four atoms and this is the case in the tetrahedral carbon atom. In the above case of iron the bonds are of the type d^2sp^3 forming six covalent bonds at 90^o angles to each other. We will discuss the nature of the copper bonds in the next section.

What has been learned from the application of these principles to hemoglobin? In a series of papers Pauling and his collaborators (Pauling and Coryell 1936a, Pauling and Coryell 1936b, Coryell, Stitt and Pauling 1937, Taylor and Coryell 1938, Russell and Pauling 1939, Coryell, Pauling and Dodson 1939, Coryell 1939 and Coryell and Pauling 1940) have determined the magnetic properties of a number of substances related to hemoglobin. A summary of their results is given in the following table.

Substance	Unpaired electrons
Ferro-hemoglobin	4
Ferro-Hb-O_2	0
Ferro-Hb-CO	0
Ferro-Hb-CN-C_2H_5	0
Ferro-Hb-NO	0
Ferri-hemoglobin	5
Ferri-Hb-F	5
Ferri-Hb-CN	1
Ferri-Hb-SH	1
Ferri-Hb-OH	3
Ferri-Hb-N_3	1
Ferriheme	5
Ferroheme	4
Hemochromogens	0

We can now say what is the nature of the bonds of iron in hemoglobin and its derivatives. In the cases where we see four or five unpaired electrons it means electrostatic bonds and when we find zero or one unpaired electron it indicates covalent bonds. With ferri-hemoglobin-OH we have an intermediate condition.

Now the oxygen molecule itself has two unpaired electrons (structure to be discussed later) and so when hemoglobin becomes oxygenated the iron and the oxygen both lose their free electrons and this has indicated the true type of bond between oxygen and iron in hemoglobin. Similarly with other complexes as the carbonmonoxy-hemoglobin.

Dissociation constants for these various ferro- and ferri-hemoglobin complexes can be calculated from magnetic data.

Experiments following the reduction of oxy-ferro-hemoglobin to ferro-hemoglobin have shown that there is no magnetic interaction between the hemes nor any magnetic effects brought about by chemical interactions between them.

Recently magnetic data have assisted in the demonstration of a new dissociation constant in hemoglobin that is affected by oxygenation.

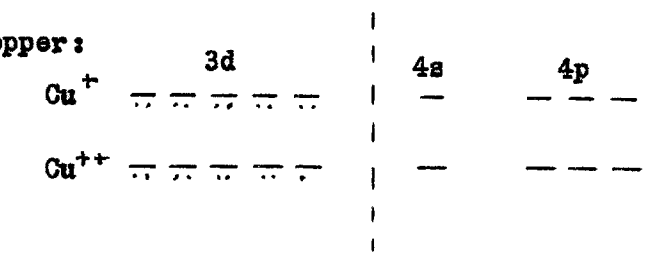
I personally feel that the most important results are yet to come from the magnetic data when contributions from other sources in the future can be correlated with it. I believe that the change from ionic to covalent type bonds will sooner or later be shown to be intimately connected with the interaction phenomenon in oxygen dissociation curves.

MAGNETIC PROPERTIES OF HEMOCYANIN.

When cuprohemocyanin, having no unpaired electrons, reacts with oxygen, having two unpaired electrons, it will be of interest to determine whether the oxygen loses its paramagnetism, as in the case of hemoglobin, or whether it retains it. When cuprihemocyanin, having one unpaired electron per copper atom, reacts with oxygen, having two unpaired electrons, it will be of interest to determine whether the resultant compound is diamagnetic or paramagnetic; if diamagnetic we can say with certainty that one oxygen molecule has reacted with two copper atoms.

A. Magnetic properties of copper.

The following electronic structures can be assigned to cuprous and cupric copper:



We see that all the electrons are paired in the cuprous state and that there is one unpaired electron in the cupric state.

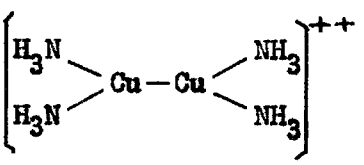
Results of magnetic measurements on cupric compounds are summarized in the following table.

Compound	Calc. moment	Obs. moment
$CuCl_2$	1.73	2.02
$CuSO_4$	1.73	2.01
$Cu(NH_3)_4(NO_3)_2$	1.73	1.82
$Cu(NH_3)_4SO_4 \cdot H_2O$	1.73	1.81

The observed moments are higher than the theoretical ones because of

orbital contribution. This contribution should be less in the square bonded complexes and it is found to be so in all cases (Pauling 1939). This is due to the inhibition imposed by a more unsymmetrical field in the latter case (the tetrahedral atom would be, of course, symmetrical).

What types of bond can we expect copper to form? From the electronic configuration we see that both cuprous and cupric atoms are able to form tetrahedral sp^3 bonds or dsp^2 square bonds. In forming the dsp^2 bonds the shifting of an electron to the third 4p orbital is involved. Ordinarily this would mean a large decrease in stability but in this case it does not. The reason is that in either case there is one of the five orbitals under discussion with an unshared electron and since the interaction energy of a shared pair is the same as an unpaired electron with the copper atom and there is no decrease in energy. Since dsp^2 bonds are of greater strength than sp^3 bonds (in the ratio of 2.7 to 2) this is the deciding factor and we find the square bonds formed in most instances. In the cuprous atom we must shift two electrons up to the third 4p orbital if we are to use dsp^2 bonds and this, I believe, will cause greater instability. Whether this is possible or not depends on whether this factor can be outweighed by the greater strength of the dsp^2 bonds. Complexes of cuprous copper have not been extensively investigated; in most cases sp bonds are formed as in the bi-cyanide complexes. There is a fairly stable cuprous complex with ammonia that can be written:



and it might be assumed that it uses three out of the four sp^3 orbitals as is the case with $B(CH_3)_3$, forming coplanar 120° angle bonds. Whether there is any tendency to add radicals having an unshared pair is not at

present known. It is claimed that oxygen reacts with the copper atoms, splitting the copper-copper bond. Tetrahedral sp^3 bonds have never been found for cuprous copper but are certainly possible.

B. The oxidation of cuprohemocyanin.

One of the greatest difficulties in carrying out the magnetic work was to find a satisfactory method of preparing cuprihemocyanin. A reagent must be used that has the following characteristics: (1) it must possess a potential above 0.6 volts, (2) it must not denature the protein, and (3) it must not have magnetic properties capable of over-shadowing the relatively low magnetic susceptibility of the copper.

I have considered all oxidation systems with potentials greater than 0.6 volts. Positive ions with a valence greater than one will in almost all cases precipitate the protein eliminating such oxidizing agents as ceric-ion and thallic-ion. Many possible reagents must act in solutions too acid for the protein to remain native or for the copper to remain on the protein. Such, I presume, is the case with the chlorates and bromates, which I found to be without effect. Such compounds as permanganate, which was used by Conant to oxidize the cuprohemocyanin, have strong paramagnetic properties.

Electrolytic oxidation was attempted and was found unsuccessful due to the denaturation of the protein at the electrodes, inasmuch as salt solutions had to be used.

The compound finally chosen was potassium molybdicyanide, $K_3Mo(CN)_8$. The potential of this substance is +0.73 (Latimer 1938). The oxidized form is paramagnetic, containing one unpaired electron and the reduced form is diamagnetic. This compound had been shown by Conant to oxidize

cuprohemocyanin. It does not, of course, react specifically with the copper atoms but probably oxidizes some of the other groups on the protein (disulfide groups chiefly). An excess of the substance must, therefore, be added. However, we can be sure that none of it remains in the oxidized state after being in contact with cuprohemocyanin for several hours. Therefore there should be no extraneous paramagnetism present in the cuprihemocyanin solutions.

The molybdicyanide is unstable and so the molybdocyanide was prepared and the oxidized compound made from it at the last moment. The preparation involved the electrolytic reduction of MoO_3 in HCl solution and the addition of the product to hot saturated KCN solution.

The electrolytic vessel consisted of a porous cup (previously put in concentrated HCl for two days) in a large beaker. A coiled lead cathode was placed outside the cup and a thick graphite rod inside for the anode. The solution of MoO_3 in HCl (50 grams of MoO_3 in 500 ml. of 8N HCl) was put outside the cup and a 15% HCl solution inside. A voltage of 2.5 - 3.0 volts was applied for 36 hours. Half again as much MoO_3-HCl solution was added and the whole poured slowly into a saturated solution of NH_4CNS forming a deep red solution. 200 grams of pyridine were added forming a precipitate and the solution was then neutralized with NH_4OH. These steps were done in an atmosphere of CO_2.

This mixture was cooled in an ice-bath and the supernate poured off, leaving a purplish-black tar which was washed several times with distilled water. This tar was added slowly to a hot saturated solution of KCN, dissolving to give a dark brown solution. The solution was boiled until crystals began to separate and on cooling a good supply

of the crystalline material was obtained. These crystals were removed and 95% ethyl alcohol added to the solution; this precipitated the remainder of the potassium molybocyanide.

Purification was obtained in the following manner: the material was dissolved in water and boiled with activated charcoal, filtered, cooled, alcohol added and the procedure repeated. The final crystalline precipitate was washed with absolute alcohol and put in a vacuum desiccator for three weeks. The yield was 80 grams.

In order to establish the absence of paramagnetic material in the potassium molybdocyanide I made some magnetic determinations. It was found that the molybdocyanide was diamagnetic, there being no detectable trace of paramagnetic material. The molybdocyanide was oxidized to molybdicyanide with bromine and magnetic measurements showed that the latter compound was paramagnetic to the extent of one unpaired electron.

That the molybdocyanide is diamagnetic confirms the presence of covalent bonds to the cyanide groups (as otherwise there would be two unpaired electrons), a conclusion reached by Pauling from theoretical considerations (Pauling 1939).

C. Magnetic methods.

The magnetic properties of hemocyanin have been investigated by the Gouy method, which involves the measurement of the force exerted on a tube, containing the substance of interest, placed between the poles of an electromagnet with one end at zero field and the other in a field of approximately 8000 gauss. Magnetic susceptibilities are derived from these force measurements by the use of an equation obtained by integrating

the magnetic field component along the axis of the magnet from one end of the tube to the other. The apparatus is so built that the top of the tube is at zero-field and hence we put the upper limit in the integration equal to zero and obtain:

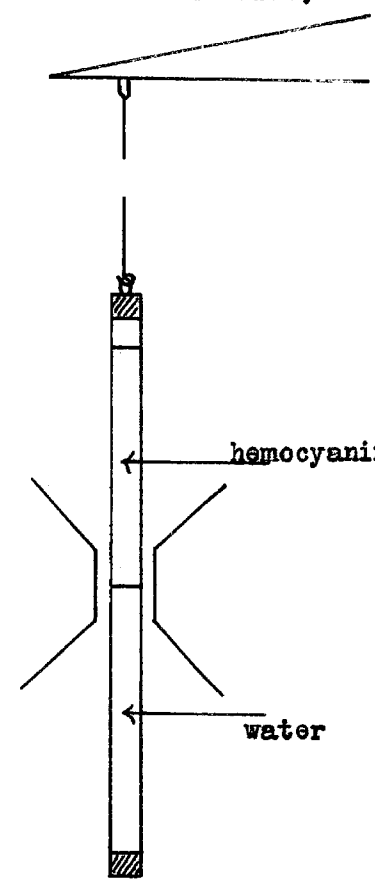
$$F = \tfrac{1}{2} \chi_{vol.} A H^2$$

From the relation between $\chi_{vol.}$ and χ_{molal} and transposing the equation we finally obtain:

$$\chi_{molal} = \frac{(2)(980)(\text{Mol. Wt.})(\Delta w)}{(c)(A)(H^2)}$$

where Δw is the change in weight of the tube hung in the magnetic field from one arm of a balance, in milligrams, c is the concentration of the substance in milligrams $cc.^{-1}$, A is the area of cross-section of the tube, and H is the magnetic field.

A diagram of the general relations in the apparatus is shown at the right. The tubes used were of glass and chosen because of constancy of diameter. The method is comparative, i.e. one determines the magnetic susceptibility of the substance of interest, placed in the top compartment of the tube, with an air-saturated solution of distilled water in the lower compartment of the tube. These two compartments are separated by a glass septum. Measurements were made at two field strengths, using 9 amperes and 12 amperes current, the determinations at the latter value being converted into those of the former by a factor determined experimentally, and these results



are averaged to give the final reading. A paramagnetic substance will increase the weight of the tube and a diamagnetic substance will decrease it. Corrections must be made for the diamagnetism of the substances present. As many measurements are comparative these corrections often cancel out.

As the effects to be expected from hemocyanin were small, involving a change of weight of approximately one milligram, exceptional care had to be exercised in the various procedures. I will mention some of the precautions taken: (1) all preparations were left in the apparatus until they had come to equilibrium, i.e. when there were no changes in weight due to water evaporation or condensation (the tubes are glass-stopped and if care is taken to prevent water from getting in the ground-glass joint there is very little trouble with evaporation). Furthermore, weighings were always made at the beginning and end of each individual measurement. (2) At least two measurements were made on each sample, allowing the tube to stand for an hour or so, in order to make sure that a reaction had completely stopped. (3) All readings must be corrected by the "tube constant", the change in weight of the tube when distilled water is in both compartments. This is due to small nonuniformities in the tube. This value was determined for the tube I used between each experiment and all in all fourteen measurements were made, the average value being taken in making the corrections.

The balance used was a Chainomatic giving readings accurate to 0.05 milligrams. The area of the tube used (determined experimentally by the weighing of water samples from various heights) was 2.16 $cm.^2$.

D. Preparation of hemocyanin samples.

The hemocyanin from five liters of the blood of Megathura crenulata was purified by the method previously given. In order to obtain readings of as great a magnitude as possible the material obtained from centrifugation was used directly in the form of a paste (but which could still be poured). Magnetic measurements were made on the following preparations:

(A) Oxy-cuprohemocyanin --- before centrifugation the suspension of hemocyanin had been equilibrated with pure oxygen for one hour (it has been shown that hemocyanin will take up oxygen in this state) and the centrifuged material was used directly in the magnetic determination. A sample was, of course, removed to determine the copper content. It was observed that there was no perceptible sedimentation after one day standing in such a preparation, the particles being all of approximately equal size, and so I feel justified in stating that a homogeneous distribution of hemocyanin was obtained thruout the measurements.

(B) Cuprohemocyanin --- as evacuation had been found unsatisfactory for the removal of the oxygen, sodium hydrosulfite, $Na_2S_2O_4$, was used in a solution 0.17 grams/cc. and brought to pH 7 by adding base, to obtain the reduced compound. Three milliliters of this solution were added to the hemocyanin in the magnetic tube and it was shaken well and allowed to stand for half an hour before the first measurement was made. The total volume of the solution in the tube is 33.4 ml.

(C) Cuprihemocyanin --- the contents of the tube were removed (the hemocyanin was in solution now due to the salt added) and dialysed in small cellophane tubing against distilled water that had been freed from oxygen by boiling. These 33.4 ml. were dialysed against four portions of water, each of volume two liters. The dialysis took 24 hours. The hemocyanin

was completely precipitated as a white solid. This was centrifuged (the volume having been somewhat increased due to water passing into the cellophane tube) and the solid treated with potassium molybdicyanide, $K_3Mo(CN)_8$. The molybdicyanide was prepared by oxidation with bromine. Potassium molybdicyanide solution (1.19M) was added to the solid material and it soon went into solution. A total of 0.58 ml. of molybdicyanide solution was added over a period of fifteen minutes with vigorous shaking. This represented an excess of molybdicyanide comparable with that found necessary for this substance and potassium permanganate by Conant. The mixture was allowed to stand for five hours at 0°C. before the first measurement.

(D) Oxy-cuprihemocyanin --- the solution from (C) was equilibrated with pure oxygen for three hours and the magnetic measurements made. It was then equilibrated with pure oxygen over-night and measurements again made.

(E) HCl-treated hemocyanin --- the solution from (D) was treated with a small amount of concentrated HCl to bring the pH down to about 1.0 where it is known that the copper is split from the molecule. The solution lost the greenish color produced by the oxy-cuprihemocyanin and became quite blue (this might be due to the formation of some copper complex or salt with the molybdocyanide).

Copper determinations were made on samples (A), (C) and (E), the samples (B) and (D) being calculated from these values.

A small sample of (D) was treated with hydroquinone (potential of +0.7 volts (Oppenheimer and Stern 1939)) to reduce the copper again to form oxy-cuprohemocyanin (Conant had found naphthoquinone useful for this) and the protein precipitated by salting out with Na_2SO_4. This was redissolved in 5% NaCl solution and found to be of a blue tinge. On shaking with pure oxygen the blue color increased and this, I believe, is suf-

ficient to show that oxy-cuprohemocyanin was again obtained after taking the hemocyanin thru a complete cycle.

As it is not convenient to determine the field of the magnet accurately, and calculate the susceptibility by means of the equation on page 80, the best method is to compare the changes in weight of the tube with a standard substance of known susceptibility and concentration. From the equation on page 80 it is seen that the following relation holds:

$$\frac{\chi_{molal}}{\chi'_{molal}} \quad \frac{(\Delta w)\,(\text{Mol. Wt.})\,(\text{conc.})'}{(\Delta w)'(\text{Mol. Wt.})'(\text{conc.})}$$

where the primes refer to the standard substance used. For these experiments I decided to use $FeCl_3$ as a standard. A solution of pure $FeCl_3$ was made up and standardized and found to be 0.0105M in ferric iron. This gave a change of weight (Δw) of 9.62 mg. The molal susceptibility of $FeCl_3$ is given as 1.432×10^{-2} (Pauling 1939). Substituting the appropriate constants in the above equation we obtain:

$$\chi_{molal} = 9.94 \times 10^{-4} \frac{\Delta w}{(\text{conc.})} \qquad \text{(for Cu)}$$

where Δw is given in milligrams and the concentration refers to the concentration of copper in milligrams per cc. This equation was used to calculate the results of the hemocyanin determinations.

The values of Δw obtained by weighing must be corrected by several small factors: (1) the diamagnetism of the protein, (2) the diamagnetism of any substances added, (3) the dissolved oxygen, (4) the tube-constant and (5) the dilution by the solutions added. In the following table I have tabulated all the measurements of Δw and have shown how each correction is made, leading to the final Δw's seen in the last column.

Sample	Substance	Times	Δw_1	Δw_2	Δw_3	Δw_4
A	oxy-cuprohemocyanin		1.33	0.65	0.65	0.81
B	cuprohemocyanin	½ hour	1.82	0.18	0.29	
		1 hour	2.00	-0.02	0.09	
		10 hours	2.27	-0.29	-0.18	0.00
C	cuprihemocyanin		1.11	0.87	0.91	1.09
D	oxy-cuprihemocyanin	3 hours	1.20	0.78	0.82	
		14 hours	1.29	0.69	0.73	0.91
E	acid solution		1.22	0.76	0.77	0.88

Explanation:
Values given are for Δw's after the correction in question was made.
Times --- refers to hours after treatment that measurement made.
Δw_1 --- direct reading from balance.
Δw_2 --- correction for tube-constant.
Δw_3 --- correction for dilution and added substances.
Δw_4 --- correction for diamagnetism of protein.
All the w's are given in milligrams.
Negative values are for diamagnetism.

It was found by calculation and experiment that the dissolved oxygen contributed a negligible susceptibility (difference between oxygen dissolved at 760 mm. and at 153 mm. calculated as the water in the lower compartment of the tube contained oxygen at 153 mm. pressure). The tube-constant was -1.98 (mean of 14 determinations). A correction of 0.11 mg. was applied for the hydrosulfite added and a correction of 0.04 mg. for the molybdocyanide. Corrections for dilution were made in the ratio of the initial and final volumes. It must be noted here that now that we have corrected for dilution in the Δw's we need not correct the copper concentration, but can assume it to be the same in each case. The correction for diamagnetism of the protein was -0.18 mg. and was obtained from the measurements on cuprohemocyanin.

The following table contains the results on the magnetic state of

the copper in the various samples. The last lines contain the theoretical and probable values, with which the others must be compared.

Substance	Δ w	Cu(mg./cc.)	χ molal	μ	Unpaired electrons/Cu
oxy-cuprohemocyanin	0.81	0.654	1.23×10^{-3}	1.71	1
cuprohemocyanin	0.00	0.654	0		0
oxy-cuprihemocyanin	0.91	0.668	1.35×10^{-3}	1.79	1
cuprihemocyanin	1.09	0.668	1.63×10^{-3}	1.96	1
acid-hemocyanin	0.88	0.668	1.31×10^{-3}	1.77	1
theoretical			1.25×10^{-3}	1.73	1
probable			$1.36 - 1.74 \times 10^{-3}$	1.8-2.0	1

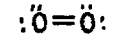
The probable values are derived from experimental data on copper compounds. For copper in solution it is nearer the upper limit and for copper in planar complexes it is nearer the lower.

Before discussing the significance of these results I would like to give some idea of the accuracy of these values. I believe I am justified in claiming an error of less than 5% in the values of the magnetic moment, μ, which would allow for an error of 10% in the Δ w's.

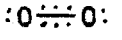
As far as the assignment of unpaired electrons in each case goes there is no question of the result. However, we cannot put much value on the differences between the magnetic moments. The low values for the oxy-compounds may indicate they are not fully saturated with oxygen, as the values given on page 59 show that in most cases it is difficult to completely saturate the protein. The decrease in susceptibility on the oxygenation of cuprihemocyanin I believe is real and I will discuss the possible significance of this in the next section.

E. Discussion of magnetic results.

One might expect the oxygen molecule to posses the following configuration:

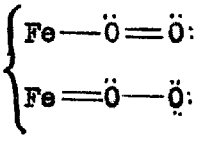


but from spectroscopic and magnetic data it has been shown that two of the electrons are unpaired. The first excited state of the molecule may be represented by this structure but the normal state is better written as:

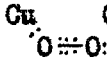
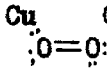
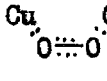
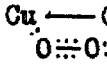
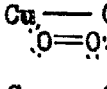
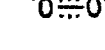


where there is one single bond and two triple-electron bonds joining the atoms (Pauling 1939). The explanation of this phenomenon probably resides in the spin interaction of the unpaired electrons.

In the case of hemoglobin the oxygen loses its paramagnetism when it combines with the iron and can be written:



where there is resonance between these two structures. From the data obtained on hemocyanin we see this is not the case. Let us make a table showing the various possibilities and the results expected.

Structure of complex.	Unpaired electrons per Cu for cupro-	Unpaired electrons per Cu for cupri-
	1	1
	0	1
	1	0
	1	1
	0	1
	1	1

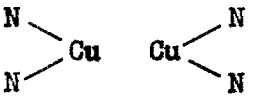
We can eliminate structures #2, #3 and #5 immediately as not being compatible with the data. From the data for the reduced compounds we see that the copper atoms could not be bonded together under these conditions. We have, therefore, two possibilities: (1) assuming structure #1 or (2) that the copper atoms are bonded when the molecule is oxygenated and non-bonded when reduced. In eliminating all possibilities but these two I have assumed that the same structures can be assigned to the cupro- and cupri-compounds with oxygen. Inasmuch as we are ignorant of the other bonds that the copper atoms form and of the oxygenation properties of the cuprihemocyanin this is about all we can do at the present time. However, I do not think the assumption is unreasonable.

Of the two possibilities given above I believe the first is improbable for if this structure is the true one we must assume two different types of copper atoms (different, of course, in the complexes to which they are bound), one capable of combining with oxygen and the other not. From the exact constancy of the $Cu:O_2$ ratio in all hemocyanins it seems that there must be some intimate relation between the copper atoms and this relation is best expressed as a dual functioning in the oxygenation process.

I do not believe it is possible to distinguish between #4 and #6 with the present state of our knowledge. Each copper atom would have the same ability to add an unshared pair and the only reason #6 might not be able to exist would be that there would be too much strain.

A further argument for structures #4 or #6 is the decrease in the magnetic moment on oxygenation of the cuprihemocyanin. This might mean a change from a state in which the copper atoms had the unpaired electrons to a state where the unpaired electrons were on the oxygen. Some decrease in paramagnetism would be predicted if this were the situation.

If we assume for the moment that the work on the prosthetic group signifies the presence of pyrrol-complexes or dipyrryl-methene-complexes, we can write the structures as:



and

or similar structures if the other possibilities mentioned above do occur. We know that cuprous complexes of this nature do occur with the coppers joined by a covalent bond as mentioned before. The strength of the bond, i.e. the stability of such a compound, would depend greatly on the type of N-substance the copper was combined with. One can conceive cases where the Cu-Cu bond is stable with and without oxygen, unstable without the oxygen and stable with it, or unstable under both conditions.

OXYGEN DISSOCIATION THEORY.

Results of the magnetic work, together with some of the physical and chemical properties of hemocyanin related to its oxygenation, have suggested an entirely new conception of the oxygen dissociation curve. The theory is capable of experimental proof or disproof along lines that I will indicate. There are many indications that there are relationships as postulated by the theory and these I will enumerate in part. As the truth of the theory can only be decided by future investigation, and by no amount of argument at the present time, I will limit the discussion to the principles involved and how they bear on some of the phenomenon mentioned in this paper.

There are two fundamental postulates: (A) Oxygen stabilizes a copper to copper bond between prosthetic groups on different fundamental hemocyanin units. (B) Proteins are not rigid spheres or ellipsoids but have a certain degree of plasticity in their molecules.

Instead of attempting to defend these postulates I will allow the results of the theory to speak for themselves.

The nature of the forces existing between Svedberg units in proteins, i.e. between the protein parts of the molecules themselves, are probably of three different types: (1) electrostatic interactions between charged carboxyl and amino groups (including guanidine and imidazole groups), (2) hydrogen bonds and (3) specific groups, especially -S-S- bonds.

This is the first theory to my knowledge that gives a physical interpretation to the interaction between prosthetic groups. The entire theory rests on the interaction involved. I believe that the interaction effects

predicted on the basis of the shape of the oxygen dissociation curve are due to interaction between adjacent fundamental units of the hemocyanin molecule. Assuming that the prosthetic groups occur in a regular pattern over the surface of the hemocyanin molecule, the oxygenation of two groups to form a copper to copper bond would stabilize the union between the units of the protein on which they rest. There are two possibilities: (1) the oxygenation actually produces molecules of higher molecular weight by binding the lower units together and (2) the units are held together weakly in one or two regions and the oxygenation merely brings them closer together.

The proof or disproof of this theory rests on centrifugal analysis. On the basis of this theory I would predict that in the reduced state the hemocyanin molecule will be more easily dissociated. I would also predict that the protein formed by removing the copper (with weak acid or cyanide) would show weaker association properties than the normal hemocyanins.

The effects of changes in the medium on the oxygenation properties would be expected to run parallel with changes in the dissociation reactions. The influence of pH and salts is entirely on the reaction between protein surfaces, affecting the oxygen equilibrium only secondarily.

Before enumerating various indications and correlations that seem to put this theory on a logical basis I would like to make a somewhat more concrete picture of the situation, although this is difficult at present. The oxygenation of one prosthetic group decreases the free energy of oxygenation of the groups adjacent to it. This is due to the fact that to bring parts of the protein together (that were originally not able to come together) it requires energy. The succeeding oxygenations are made

easier and easier to put it in simple manner. Let us write for the total free energy change on oxygenation the following expression:

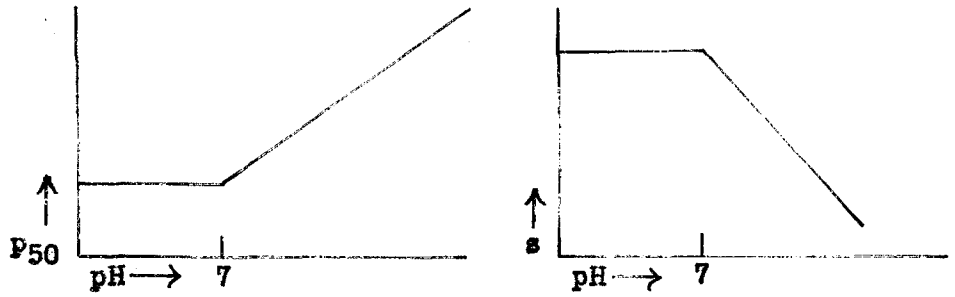
$$\Delta F = \Delta F_c + \Delta F_p$$

where ΔF_c is the energy change for the copper to copper bond and the rest of the reactions that might occur in the prosthetic group and ΔF_p is the energy involved in bringing the protein surfaces closer together (including formation of any specific bond, e.g. hydrogen or sufide bond). The quantity ΔF_c is always negative and the quantity ΔF_p is always positive. The value of ΔF_c is relatively constant and practically independent of the conditions of the medium.

The stabilization interaction energy of Pauling (1935) is to be correlated with the change in ΔF_p as groups become oxygenated. This energy change is very dependent on pH and electrolyte concentration, inasmuch as the interactions involved are chiefly of an electrostatic nature. As the pH changes the number of charged groups and the relative proportion of negative to positive ones will vary. When the repulsion of charged groups becomes too large for the copper to copper bonds (and whatever other bonds there might be) to hold the dissocation fragments together we will get a breakdown of the protein. Until more accurate data are available as to the actual sizes of oxygenated and reduced hemocyanins and the configuration of the molecule it is impossible to state how dissociation would affect the oxygen capacity.

It is not possible to correlate accurately centrifugal data and oxygenation data for in most cases they were obtained on entirely different solutions. However, in a few cases such a comparison is possible, e.g. in Busycon hemocyanin (data of Eriksson-Quensel and Svedberg 1936 and

Redfield and Ingalls, 1932) where we find the following situation:



where p_{50} is the oxygen pressure required to half saturate the hemocyanin and is inversely proportional to the equilibrium constant. There are other correlations like this and some that do not correlate at exactly the right pH region. I believe this may be due to the fact that other bonds (hydrogen bonds, -S-S- bonds, etc.) interfere with the purely electrostatic nature of the phenomenon.

Let us define a term denoting the degree to which oxygenation makes it easier for succeeding oxygenations, the "stabilization". I do not want to use the word "interaction" because of the confusion that might arise with the word used in the sense of repulsion and attraction between the protein surfaces. This stabilization varies from one step to the next, i.e. we define it as:

$$S_1 = \Delta F_p - \Delta F'_p$$

$$S_2 = \Delta F'_p - \Delta F''_p$$

etc.

where ΔF_p is the free energy change for the oxygenation of the first group, $\Delta F'_p$ that for the second group, $\Delta F''_p$ for the third group, etc. The quantity S will be positive when we get stabilization and the greater its value the more inflected the oxygen dissociation curve is.

What are some of the direct experimental supports of this theory?

(1) Burk (1940) has shown that in the presence of urea Limulus-

hemocyanin is dissociated into units of molecular weight 142,000. When the copper was removed from the molecule it was dissociated into fragments of molecular weight 69,000.

(2) Burk (1940) noticed (as did Steinhardt) that urea decolorized solutions of hemocyanin, driving the oxygen off.

(3) The radical change of spectrum upon oxygenation indicates a parallel radical change in the electronic configuration of the complex, such as the change from an ionic to a covalent state (as in hemoglobin). That solutions of hemocyanin are decolorized to some extent in all dissociation reactions makes it probable that the copper to copper bond is important in this respect.

(4) The general observation made by many workers, including myself, that under many conditions solutions of reduced hemocyanin are clearer than those of oxygenated hemocyanin, indicates that the particle size is somewhat smaller in the former case as the scattering of light is less.

(5) The magnetic work of this investigation does not prove the theory but makes it the most probable explanation.

I would like to conclude the discussion of this theory by listing a number of pertinent points, which added up may have a fair amount of weight in supporting the theory.

(1) In this theory it is seen that it is likely that not all the groups can be oxygenated, due to the fact that they cannot all get together in pairs (there will always be some left on the outside) and this is born out by the average O_2:Cu ratio of 0.934 from many determinations on a number of species.

(2) Where conditions are such that there is high repulsion between the protein surfaces we would expect low stabilization values. Where are the conditions for high repulsion?

(a) high and low pHs.

(b) salt-free solutions.

(Redfield measured the scattering power of hemocyanin in various concentrations of electrolytes and showed that the scattering drops to a low value in salt-free solutions, indicating smaller particles or a high degree of repulsion). Where do we find low stabilization values? Under exactly these same conditions. This theory, therefore, explains the oxygenation phenomena seen in salt-free solutions where we get hyperbolic curves.

(3) Data on heats of oxygenation (page 68) show these values to be much lower in salt-free solutions. We can write the following expressions:

$$\Delta H_1 = \Delta F_1 + T\Delta S_1 \qquad \text{(salt-free)}$$

$$\text{and } \Delta H_2 = \Delta F_2 + T\Delta S_2 \qquad \text{(salt)}$$

where the subscripts refer to the conditions at the right. If we can assume the change in entropy is approximately the same in salt-free and salt solutions we can write:

$$\Delta H_2 - \Delta H_1 = \Delta F_2 - \Delta F_1$$

and from the equation on page 92 it follows that:

$$\Delta F_2 - \Delta F_1 = \Delta F_{p_1} - \Delta F_{p_2}$$

since the difference in the F_c's disappears. Now when salt is present ΔF_{p_2} is low and when no salt is present ΔF_{p_1} is high so we find that the quantity $(\Delta H_2 - \Delta H_1)$ must be negative and this is the case (the values of heats of oxygenation are negative as they represent the heat

evolved in oxygenation).

Another interesting point concerning heats of oxygenation is the change in this value with temperature in some cases (Redfield and Ingalls 1933). When the only reaction affected was the oxygenation reaction it was an anomaly, but on the present theory we see that the effect of temperature on the dissociation reaction would be important.

(4) Hemocyanins possessing a large number of acid and basic groups would be expected to show repulsion phenomena to a greater extent (as the pH deviates from around the iso-electric point). Limulus-hemocyanin is one with such a composition and Helix-hemocyanin is of the opposite nature (page 11) and we find that the dissociation curves for oxygen of the former are hyperbolic (or have low n's) under a greater variety of conditions than for the latter.

(5) Molluscan-hemocyanin combines with oxygen at quite low pressures and has a high molecular weight; Crustacean-hemocyanin is half-saturated with oxygen at higher pressures and has a lower molecular weight. Since how easily the molecules may associate into larger groups is correlated with oxygenation in the above theory this result is to be expected.

(6) The recombination of hemocyanins of two kinds (page 26) might indicate that copper to copper bonds were of importance for generally we do not get combination of proteins of different species (because of their different surface patterns) when there is no "active" group.

(7) The alkaline end of the pH stability range generally coincides pretty well with the pH of the blood and thus dissociation phenomena may play a large part in the effect of CO_2 on the transport of oxygen.

(8) The only proteins showing such high molecular weights and such sensitive dissociation reactions are the respiratory proteins, possessing metals

as the active groups, and the viruses, possessing nucleic acids as the active groups. Thus we see a correlation between molecular size and the presence of active groups. This indicates the groups play some part in the binding between units.

One last thing that may be mentioned in favor of this theory is that it has always been impossible to visualize how interaction can take place when the prosthetic groups are lying over the surface of the protein, unless we assume that the prosthetic groups actually react together and are not lying flat on the surface. Such an assumption would mean that at least three groups would be able to react together ($n = 3$), that is actually come into contact with each other. Under any condition it has always been difficult to imagine how changes in pH or salt concentration can change not only the affinity for oxygen but the shape of the dissociation curves. Especially is this true as many of these changes show an amphoteric character, increasing on either side of some pH value.

CONCLUSIONS.

The physiological aspects of oxygen transport are not simple as many investigators would seem to think. For the oxygen to reach the metabolites in the tissues it must diffuse from the water across the gill surfaces, diffuse thru the blood, combine with the hemocyanin, be transported to the tissues, dissociate from the hemocyanin, diffuse thru the vascular walls and intercellular spaces, diffuse thru the cellular walls and finally combine with the respiratory enzymes present in the cell. Yet in such a complex chain of events we often find one of the processes limiting the rate of the total process and we can correlate this step with the various requirements of the animal. In other words, in evolution if a system is evolved to be more efficient in the transport of oxygen, a speeding up of this limiting process is the only thing that will be of any advantage.

We have noted that there are many differences between the hemocyanins of a quantitative nature and that these are generally correlated with the phylogenetic grouping. The differences in oxygenation phenomena have been shown by Redfield (Redfield, Coolidge and Hurd 1926) to be well correlated with the habitats of the animals. Thus those animals that are often exposed to conditions of low oxygen pressure (buried in the sand, shell closed, etc.) have hemocyanins capable of combining with oxygen at a low pressure. There are several correlations of this nature.

The question arises: what are the differences in oxygenation phenomena between the various hemocyanins due to? From comparison with other respiratory proteins, the ideas discussed in this work and lack of demon-

stration to the contrary, we may assume these differences are due to variations in protein structure. We see, therefore, why most of the physical, chemical and physiological properties of hemocyanin fall in with the phylogenetic scale; it is for the same reason that the proteins of animals within a certain group are more similar than those of different groups.

Due to the extreme number of structures possible for a protein using only a few amino acids the proteins must be classed as the group best suited for modification when evolution feels called to change an organism. Thus we see that the respiratory needs of an animal can be fairly easily kept up with as the environment changes for all it requires is a change, perhaps quite small, in the configuration and composition of the respiratory protein involved.

That these differences between one respiratory protein and another are not due to changes immediately underlying the prosthetic group is obvious for there are at most only a few ways that a prosthetic group could be joined to a protein (I suspect in most cases only one) and we cannot imagine such radical changes to occur from the mere change of amino acid configuration in the neighborhood of the prosthetic group, for reactions between the prosthetic group and amino acids to which it is not bound are probably negligible. The differences, instead, are due to general structural variation throughout the entire protein, and this is reflected in differences between hemocyanins in their physical and chemical properties.

I have investigated various properties of the hemocyanin of Megathura crenulata and it is seen that it is a typical hemocyanin. Therefore, I believe, we are justified in applying the results of chemical, magnetic

and theoretical investigation to other hemocyanins. Can we apply them further to substances that are not hemocyanins?

The hemerythrins are respiratory proteins occurring in the blood of certain sipunculoid worms, remarkably similar to hemocyanin, but containing iron instead of copper. No porphyrin rings are evident and the prosthetic group behaves very much like hemocuprin. Their molecular weights are of the same magnitude as the hemocyanins and they show the same tendency towards dissociation. The oxygen dissociation curves are sigmoid and it is very interesting that both the ferrous and ferric states can combine with oxygen. I believe that many of the conclusions above can be applied here.

The hemocyanins are not the only proteins containing copper occurring in nature. The other great group comprises the polyphenol oxidases (potatoe oxidase, phenol oxidase, polyphenolase, laccase, tyrosinase, catechol oxidase, adrenalin oxidase, dopa oxidase, polyamino oxidase, etc. are various names applied to these copper-proteins). These substances probably act in tissue respiration as a link between the pyridine-nucleotides and oxygen with the help of various phenolic compounds. At least they will act in vitro in such a system. Kubowitz (1938) and Keilin and Mann (1938) have prepared crystalline material containing the same amount of copper as hemocyanin does and find it to be colorless or slightly yellow. Instead of binding oxygen reversibly they are oxidized by it. I believe the reason may be in the difference in the N-compound to which the copper is bound. We find a similar situation in the heme compounds: hemoglobin will bind oxygen reversibly and other heme proteins (catalase, cytochrome-c, etc.) will be oxidized to the ferric state. It must be remembered that the other half of the cycle, the oxidation of phenolic compounds by cupric copper, does occur in hemocyanins. Bhagvat and Richter (1938) claimed

that hemocyanin showed "pseudo-phenolase" activity and correlated the activity of invertebrate blood to oxidize various phenols to a protein fraction very closely allied to hemocyanin.

In the future I believe it will be recognized that there are parallel lines of evolution in the iron and copper respiratory proteins. In the simplest situation we have copper ions, some substance they are able to weakly combine with and substrate, and we find the copper alternately oxidized and reduced. In the next stage we have copper ions, protein and substrate and the process is as above. Reversible combination with oxygen is impossible at this stage. The next advance is the development of a simple type of pyrrol-complex for a prosthetic group and we find that both oxidation and oxygenation are possible here. The last stage is the formation of a complicated pyrrol-system, the porphyrin ring, of which the only copper derivative known (turacin) is non-functional, but which condition is fully utilized by the iron series to form the most efficient oxygen transporters and the large group of heme oxidation-reduction enzymes.

SUMMARY.

It has been shown that the hemocyanin of the giant key-hole limpet, Megathura crenulata, is a typical hemocyanin with the aid of the following properties: iso-electric point, absorption spectrum, solubility, mobility, acid and base binding power, elementary analysis, oxygen combining ratio and general behavior.

It is seen that it approaches the Molluscan-type of hemocyanin (particularly Helix-hemocyanin) more closely than the Crustacean-type and thereby conforms to the phylogenetic rule of protein specificity.

Degradation experiments have given products that appear to be related to pyrrol-complexes and this places the hemocyanins in a closer relation to the other respiratory pigments.

Determinations of the magnetic susceptibility of the oxygenated and de-oxygenated states of the oxidized and reduced compounds have shown that the situation is very different from hemoglobin. The most probable interpretation is that a copper-to-copper bond is formed upon oxygenation and that simultaneously there is a radical change in electronic structure.

The part this copper-to-copper bond plays in the oxygenation reaction is discussed. It is shown that the interactions between the protein parts of the molecule can explain the sigmoid shape of the dissociation curve and the changes it undergoes upon change of the external conditions.

COMMON NAMES.

Arion ater	European black slug
Astacus fluviatilis	crayfish
Buccinum undatum	whelk
Busycon canaliculatum	conch
Busycon carica	conch
Callinectes sapidus	blue crab
Cancer pagurus	common crab
Cancer borealis	Jonah crab
Carcinus maenas	common shore crab
Eupagurus bernhardis	hermit crab
Helix aspersa	garden snail
Helix pomatia	edible snail
Homarus vulgaris	common lobster
Libinia emarginata	spider crab
Limax maximus	slug
Limulus polyphemus	horse-shoe crab
Littorina littorea	periwinkle
Loligo pealei	American squid
Loligo vulgaris	European squid
Maia squinado	spider crab
Nephrops norvegicus	Norway lobster
Octopus vulgaris	common octopus
Ovalipes ocellatus	lady crab
Pagurus striatus	purse crab
Paludina contecta	fresh-water periwinkle
Palinurus vulgaris	rock lobster
Pandalus borealis	prawn
Patella vulgata	limpet
Planorbis corneus	fresh-water snail
Portunus depurator	lady crab
Sepia vulgaris	cuttlefish
Squilla mantis	shrimp

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I have included in the bibliography some papers I have not referred to specifically in the text for several reasons: in making many of the generalizations (such as the dissociation curves) I have taken from many papers and not from any one in particular; in reading these papers or reports of them the ideas expressed therein all influenced to a greater or less degree my viewpoint; and lastly, I believe that the complete literature of hemocyanin up to the present time will serve perhaps a useful purpose.

THE PHOTOSYNTHETIC UNIT

The following investigation represents a portion of the work done for my minor; the remaining two-thirds is comprised of one year's work on the growth-substances of pollen and a series of courses in the Chemistry Department.

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RELATION BETWEEN QUANTITY OF CHLOROPHYLL AND CAPACITY FOR PHOTOSYNTHESIS

ROBERT EMERSON, LOWELL GREEN, AND J. LEYDEN WEBB

(WITH ONE FIGURE)

Seeking evidence as to the number of chlorophyll molecules concerned in the reduction of one molecule of carbon dioxide, EMERSON and ARNOLD (3) measured the maximum amount of carbon dioxide which could be reduced by a measured quantity of cells, under conditions designed to make photosynthesis dependent only on the amount of chlorophyll present, and independent of other factors, both internal and external. The limiting effect of chemical processes in the cell under saturating external conditions was avoided by illuminating with extremely short flashes of light (about 10^{-5} seconds) and allowing sufficient dark time (about one-tenth of a second) between flashes so that the chemical processes or Blackman reaction could run to completion between light flashes. The intensity of the flashes was so great that higher intensities resulted in no increase in carbon dioxide assimilation. With light flashes as short as 10^{-5} sec., it was believed impossible for a significant number of individual chlorophyll molecules to act more than once per flash in the reduction of carbon dioxide. Under these conditions it was anticipated that chlorophyll would be the only factor limiting the amount of carbon dioxide reduced per flash. The number of chlorophyll molecules should limit the energy available for carbon dioxide reduction after each flash. The ratio (ρ) of number of chlorophyll molecules present to number of carbon dioxide molecules reduced per flash was found to remain approximately constant, even when the chlorophyll content per unit volume of cells was varied several fold. Unexpectedly high values were obtained for ρ, showing that the cells contained two or three thousand times as much chlorophyll as the maximum amount of carbon dioxide which they could reduce per flash of light.

Calculations made by GAFFRON and WOHL (7) show that essentially the same relationship prevails in continuous light. They compared the maximum rate of carbon dioxide reduction with the chlorophyll content for a variety of material, including leaves as well as suspensions of algal cells. Taking the average time required for the completion of the Blackman reaction in Chlorella from the results of EMERSON and ARNOLD, they found that assimilating cells in general contain over two thousand times as much chlorophyll as seems necessary to account for the highest observed rates of photosynthesis.

The measurements of EMERSON and ARNOLD were made only with *Chlorella pyrenoidosa*, but ARNOLD and KOHN (1) later reported measure-

ments with six species, representing four phyla. Their results, and the calculations of GAFFRON and WOHL, have led to the proposal that chlorophyll exists in the cell in some sort of units, each containing some 2000 chlorophyll molecules, such that when a quantum of light is absorbed by any member of a particular unit, the energy may become available for the reduction of a carbon dioxide molecule associated with the unit (*cf.* ARNOLD and KOHN, **1**; GAFFRON and WOHL, **7**; WOHL, **8, 9, 10**). Some of ARNOLD and KOHN's values for ρ run up to four and five thousand, suggesting that ρ may be subject to some variation. Nevertheless, they regard its relative constancy as evidence for the existence of chlorophyll units. Therefore it seemed to the writers important to find within what limits ρ might vary in a single species, *Chlorella pyrenoidosa*. The experiments we report here show a much greater range in the value of ρ than had been indicated previously, so that hypotheses formulated on the basis of the supposed constancy of ρ may not be tenable. The existence and significance of the chlorophyll unit is now the subject of active discussion (*cf.* WOHL, **8, 9, 10**; also FRANCK and HERZFELD, **5**; FRANCK and TELLER, **6**) on which our results appear to have some bearing.

Experimental

Except for the use of a culture medium saturated with five per cent. carbon dioxide instead of carbonate-bicarbonate mixture as suspending fluid for the cells, our technique was the same as EMERSON and ARNOLD's. In view of the results reported by EMERSON and GREEN (**4**), who compared the behavior of photosynthesis in carbonate mixtures and in phosphate buffers, we thought it possible that the phenomena in flashing light might depend in some respect on the pH or on the method of carbon dioxide provision. In a culture medium saturated with five per cent. carbon dioxide, photosynthesis is unaffected by considerable variations in both pH and carbon dioxide concentration.

The carbon dioxide assimilation during flashing light is of the same order of magnitude as the respiration, so the correction applied for respiration is of the utmost importance. Respiration measurements were made in darkness, between the exposures to flashing light. Separate experiments on respiration indicated that the small amount of assimilation during flashing light was without measurable influence on the subsequent rate of respiration. As in all measurements of photosynthesis, it remains possible that the respiration is higher during illumination so that the use of a subsequent respiration measurement to compute photosynthesis may lead to a value lower than that actually attained by the organism.

The chlorophyll content was determined by extracting aliquot samples of cells with hot methyl alcohol, and measuring the extinction spectrophotometrically for the Neon line 6598 Å. The same method was used by EMER-

SON and ARNOLD, and by ARNOLD and KOHN. It has been standardized with weighed samples of Chlorella chlorophyll (EMERSON and ARNOLD, 3) and with chlorophyll prepared from higher plants (ARNOLD and KOHN, 1) and the values obtained were practically identical.

The accuracy of the method depends on several factors besides the precision of the standardization. Chief among these are the completeness of extraction of the chlorophyll from the cell samples, the freedom of the extract from other substances which absorb appreciably at 6598 Å, and the uniformity of the ratio of the two chlorophyll components. While we cannot state quantitatively how closely these requirements are fulfilled, it seems conservative to say that the error in the analyses is probably less than ten per cent. Since we are concerned here with variations of several fold in the value of ρ, such an error in the chlorophyll determinations would not alter our conclusions.

Most of the photosynthesis measurements were made at 25° C., with a frequency of 20 flashes per second, making the dark time between flashes 0.05 sec. According to EMERSON and ARNOLD, at 25° this dark time is sufficient for the completion of the dark processes after each flash. Some of our experiments suggest that cells from young cultures grown over bright light may require a longer dark period. If this proves to be the case, then the

TABLE I

THE RATIO ρ IS SHOWN FOR CULTURES OF VARIOUS AGES, GROWN OVER THREE DIFFERENT SOURCES OF ILLUMINATION. ALL CULTURES WERE GROWN AT 17° C. ± 1°. MEASUREMENTS OF ρ WERE MADE AT 25° C.

CURVE	LIGHTING CONDITIONS FOR GROWTH OF CULTURES	AGE AT TIME OF HARVESTING	DENSITY AT TIME OF HARVESTING	ρ, MOLES OF CHLOROPHYLL / MOLES CO_2 REDUCED PER FLASH
		days	*mm.³ per ml. of culture*	
A	Four forty-watt incandescent lamps 10 cm. from culture flasks, and Corning "Noviol C" filter, transmitting only wave lengths longer than 480 mμ	2	0.1	3,750
		4	0.5	6,180
		8	1.6	11,120
B	Four forty-watt incandescent lamps 10 cm. from culture flasks, no filter.	4	0.6	3,920
		9	1.6	6,850
		17	2.2	9,720
C	Single forty-watt incandescent lamp, 15 cm. from culture flasks.	15	0.2	6,650
		21	0.5	11,000
		29	0.7	14,500

values of ρ shown in table I may have to be reduced somewhat for the younger cultures.

The ratio ρ was determined for cells from cultures grown for different periods of time and with different conditions of illumination. In all cases it was found to increase with increasing age of the cultures, but the rate of increase and the value at any given age depended on the illumination used for growing the cultures. Much experimental work has been done on the influence of wave length and intensity of light on the changes in chlorophyll content and photosynthetic capacity with age of culture. The action of these factors is obscured by the increasing selective absorption of light by the cells in a growing culture. This is minimal in a young culture which contains so few cells that light passes through the suspension almost unchanged. But as the culture grows older and increases in density, certain wave lengths are absorbed more strongly than others, and the light which has passed through the peripheral parts of the suspension reaches the cells in the interior greatly diminished in intensity and changed in wave length distribution. The cells are circulated by shaking the cultures at regular intervals, and by the slow stream of five per cent. carbon dioxide in air which bubbles through continuously, so that all are exposed from time to time to the entire range of illumination prevailing within the culture. The changing environment inevitably associated with this simple technique renders it difficult to separate and analyze the influence of the factors in question. While it is clear that the age of the culture, the wave length distribution, and light intensity used for growing the culture all have profound effects on the photosynthetic apparatus, a separation of these effects probably requires more elaborate culture technique. Consequently the present paper deals only with variations in ρ, without attempting to trace the influence of culture conditions on the more fundamental internal factors upon which ρ probably depends.

Figure 1 shows three curves for the value of ρ plotted against age of culture for cultures grown under different conditions of illumination. The conditions of illumination for the three curves, as well as the density of each culture at the time of harvest, the age, and the value of ρ, are given in table I. The lighting used for the cultures for curves A and B was identical except for the use of a filter[1] for the "A" cultures which transmitted only wave lengths longer than about 480 mμ. The cultures for curve A were therefore deprived of blue and violet light, while the cultures for curve B were exposed to the full spectrum of the incandescent lamps. Comparison of the densities for equal ages of the A and B series in table I shows that the removal of the blue and violet leaves the rate of growth practically unchanged, although for corresponding densities ρ is always higher for the cells grown without blue and violet light.

[1] Corning "Noviol C" filter.

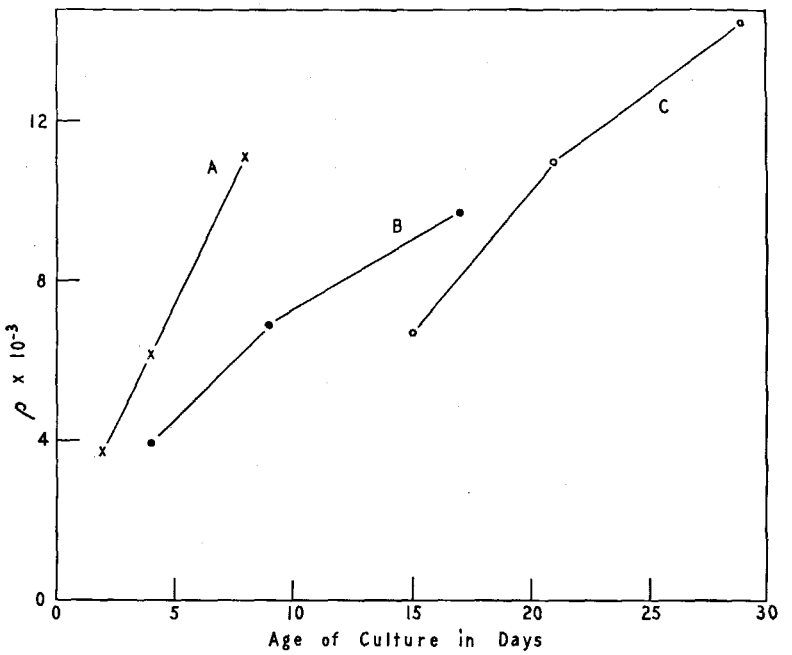

FIG. 1. Three curves showing the influence of age of culture on the value of ρ. The illumination for growing the cultures for curve B was from four forty-watt incandescent lamps spaced close together in a square, and 10 cm. distant from the bottoms of the culture flasks. The same lighting arrangement was used for the cultures for curve A, but in this case the light was passed through a glass color filter transmitting only wave lengths longer than about 480 mμ. The cultures for curve C were grown over a single 40-watt incandescent lamp at a distance of 15 cm.

Curve C, made with a series of cultures grown at a very low light intensity, shows higher values of ρ for corresponding culture densities, or lower values for corresponding ages.

Discussion

Our results indicate that the value of ρ regularly increases with the age of the culture, and is influenced by the lighting conditions used for growth. None of the values reported here are as low as the highest values of EMERSON and ARNOLD (3), but no attempt was made to duplicate their culture conditions. Their primary purpose was to obtain a wide range of chlorophyll concentrations. This they achieved by growing cultures over neon and mercury sign-lighting tubes. The approximate constancy of ρ over a wide range of chlorophyll concentrations seems remarkable, but may be attributable in part to the fact that their cultures were regularly harvested as soon as they had reached a density which provided adequate amounts of material for the measurements, without reference to age.

It might be supposed that the increase in ρ with age of culture indicates an increasing proportion of inactive or moribund cells which still retain their cholorophyll. This would be consistent with the opinion of ARNOLD and KOHN that in all active cells there exists a chlorophyll unit of approximately constant size. There is no reason however, for thinking that our cultures contained increasing quantities of inactive cells. The decline in growth rate with age indicated by the figures for culture density in table I is no more than would be anticipated from the increasing competition for light and nutrients with increasing population density. Differential centrifuging failed to reveal the presence of more and less active fractions among the cell population. We regard it as unlikely that there are appreciable quantities of inactive cells present in cultures during periods of rapid growth, although table I shows that this rapid growth may be accompanied by a sharp increase in the value of ρ.

It seems more probable that a chlorophyll unit within the meaning of ARNOLD and KOHN does not exist, and that the large amount of chlorophyll present in comparison with the capacity to reduce carbon dioxide must be explained in other terms. FRANCK and HERZFELD (5), and FRANCK and TELLER (6) have found it difficult to reconcile the existence of such a unit with modern concepts concerning energy transference between large molecules. EMERSON (2) has suggested that the amount of carbon dioxide which can be reduced per flash of light may not depend upon the amount of chlorophyll, but may be a measure of some other substance taking part in photosynthesis. For example, it is possible that assimilating cells contain a limited amount of some substance with which carbon dioxide must combine before it can undergo reduction. Carbon dioxide saturation in flashing light would then be a measure of the full capacity of this substance for combining with carbon dioxide. The fact that ρ is always large would mean that the amount of chlorophyll always greatly exceeds the amount of this proposed substance. Other catalysts or enzymes present in small amount could also account for the observed phenomena, provided they could set a fixed limit to the yield per flash under saturating conditions. The best obtainable yield per flash appears to be always far short of what might be expected from the amount of chlorophyll present.

Summary

During photosynthesis in flashing light under optimum external conditions, the ratio of moles of chlorophyll present to moles of carbon dioxide reduced per flash is not a constant in *Chlorella pyrenoidosa* cells, but depends on conditions of previous growth, increasing sharply with age of culture and varying with color and intensity of culture illumination. Thus the maximum amount of carbon dioxide reducible per flash is not directly

related to the amount of chlorophyll but depends on some other internal factor.

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