[6] Guilliermond, A., *Protoplasma*, **9**, 1930 (133–174).
[7] Beams, H. W., *Anat. Rec.*, **45**, 1930 (138–162).
[8] Emberger, L., *Arch. Morph. gén. expér.*, **1**, 1921 (1–184).
[9] Mangenot, G., *ibid.*, **9**, 1922 (1–340).
[10] Motte, J., *Ann. Sci. Nat.*, Series 10, **10**, 1928 (298–543).

THE NATURE OF THE PROSTHETIC GROUP IN LIMULUS HEMOCYANIN

(Preliminary Paper)

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At the suggestion of Professor A. C. Redfield we have undertaken a chemical investigation of the hemocyanin of *limulus polyphenus* with special reference to the nature of the prosthetic group. We are now reporting a few preliminary results which we have obtained, since considerable time must elapse before the next large quantity of horseshoe crab blood can be decomposed and the product purified.

The only previous work on the nature of the prosthetic group in hemocyanin is that of Philippi[1] who studied the hemocyanin of the snail. He made the important discovery that if the protein is decomposed with alkali a black insoluble material is formed which contains the copper of the original pigment. This material he believed to be analogous to the hematin formed from hemoglobin by a similar treatment; apparently the quantity of material at his disposal was too small to allow of a detailed examination since the copper content (7%), solubility and a strongly positive pyrrol test are the only facts reported.

We followed Philippi's procedure in the decomposition of the protein but employed the whole blood of the horseshoe crab without any attempt at a separation of the pure hemocyanin. Potassium hydroxide to the amount of 10 per cent was added to the blood and the reaction mixture was warmed to 40°–50° for 20–30 minutes at the end of which time the protein was largely in solution. After standing overnight, a greenish brown precipitate settled which was removed by centrifuging and washed with dilute potassium hydroxide and then water to remove the protein. The precipitate at this point contains, in addition to the copper complex of the prosthetic group, magnesium and calcium hydroxide and probably copper sulfide (and some protein unless the washing has been very complete). In working up one large lot of blood, the material was collected, dried and stored

in this form; the copper content was only 2.6 per cent. From 85 liters of blood 200 grams of such material was obtained; the copper content represented practically all the copper in the original blood (200 x 0.026 = 5.2 grams Cu, compared with 5.5 grams in the original 85 liters).

The copper complex may be separated from the other alkali-insoluble materials by first washing with 10 per cent acetic acid containing sodium chloride (to prevent the formation of a colloidal solution of the copper compound) and then dissolving the material in 50 per cent acetic acid, removing the insoluble copper sulphide and reprecipitating with alkali. A considerable quantity of the crude material referred to above was purified by essentially this procedure and was obtained as an amorphous black powder with the following composition: Cu = 21.5 per cent; N = 9.2 per cent; C = 39.5 per cent; H = 5.6 per cent; S = 8.0 per cent. Unfortunately the crude material appeared to have undergone considerable decomposition after standing six months and only a few grams of purified material were obtained; the dilute acid washings were bright green. In a series of experiments in which the final purification was carried out at once, the yield was about 320 milligrams per liter, and the acetic acid washings were colorless. The copper content of the product was 23 per cent which corresponded to the entire copper content of the whole blood.

The purified material is a black powder, insoluble in all the common neutral organic solvents and in aqueous acid or alkali. When freshly precipitated it dissolves readily in 50 per cent acetic acid and tends to pass into a colloidal solution in dilute acids in the absence of salts; after drying, the material is insoluble in all strengths of acetic acid. Although the copper complex is insoluble in aqueous alkali, it will dissolve in alkaline solutions if ammonia or some amine is present, apparently a soluble complex salt is found. Dark green solutions in aqueous ammonia and pyridine (with the addition of some sodium hydroxide) and a brown solution in dilute hydrazine hydrate are readily prepared with even the dried material. On acidification, the material is precipitated apparently unchanged. The use of ammonia or hydrazine hydrate is the best method of getting the dried material into solution. The green solutions apparently contain cupric copper, the brown, cuprous copper, since the latter may be formed by the addition of a reducing agent; the transformation back and forth by means of sodium hydrosulphite and hydrogen peroxide is rapid but attended by some decomposition. The absorption spectra of the green and brown solutions consist of only one broad band in the blue which extends into the extreme violet.

The copper may be removed from the complex in acid or alkaline solution. If the dark green solution in 50 per cent acetic acid is treated with an equal volume of concentrated hydrochloric acid and warmed, the color disappears, the copper passes into the ionic condition, and hydrogen sulphide

is evolved; at least two organic products are formed, one an amphoteric substance insoluble at the neutral point and the other a material soluble in water which has not been obtained in a crystalline form. The removal of the metal in alkaline solution appears to take place with less drastic decomposition of the whole molecule. If a solution of the pigment in ammonia is allowed to stand in the air with frequent shaking for several days, the color almost vanishes; on cautious acidification with acetic acid, an amphoteric solid is precipitated. The filtrate contains ionic copper but no sulphide; after removal of the copper, as copper sulphide, and evaporation to dryness, a residue is obtained amounting to about one-third of the material while the amphoteric precipitate amounts to about two thirds.

The amphoteric precipitates from both the acid and alkaline decomposition contain nitrogen and sulphur and appear to be similar. The yield from the acid decomposition is very small, however. The amphoteric material is slightly soluble in hot alcohol. The nitroprusside, Molisch, biuret, and ninhydrin tests were negative; the material does not reduce Fehling's solution. Boiling with moderately concentrated hydrochloric acid does not render the material water-soluble in neutral solution, although when the copper complex is decomposed with hydrochloric acid, the bulk of the product is water soluble. However, since the sulphur linkage is also attacked under these conditions (hydrogen sulfide is evolved), it is clear that the decomposition in this case involves more than the mere removal of the copper.

From the evidence now available it would appear that the black copper-containing material is a complex salt of an amino acid which contains sulphur; on the basis of the tests and properties just described it is extremely unlikely that we are dealing with any of the known amino acids.

Unlike Philippi's product from snail hemocyanin, our material did not give any pyrrol test with a pine splint or with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde). These tests were uniformly negative on the crude and purified material and on the products obtained after the removal of copper. However, on fusion with potassium hydroxide, both the copper-containing pigment and the copper-free amphoteric material yielded a volatile substance which gave a strong positive test with the pine splint and Ehrlich's reagent. In the hope of obtaining volatile pyrrols by reduction, the pigment was treated with hydriodic acid and phosphonium iodide under conditions which yield large amounts of volatile pyrrols from hematin; no trace of pyrrols could be detected either from the crude or purified material. Thus, if pyrrol rings are present in our material they must be carboxylated; however, since the tests in question are also given by indole derivatives, and since decomposition with fused potash is very drastic it would be unwarrantable to conclude that the compound contains a pyrrol ring. The absence of an intense color or characteristic spectrum after

the removal of the copper, as well as the negative results obtained in the hydriodic acid reduction prove that the prosthetic group is neither a porphyrin nor a closely related substance such as a bile pigment.

The stability of the black copper complex and its tendency to form soluble, highly colored complexes with amines (and also denatured albumen) indicates that the compound we have in hand is involved in the union of the copper to the protein in hemocyanin. The function of the prosthetic group thus appears to be the same as that of protoporphyrin in hemoglobin, namely, to provide a basis for a very stable metallic complex. Beyond this analogy, however, there seems to be little or no chemical relationship between the prosthetic groups in limulus hemocyanin and hemoglobin. It should be noted that since limulus hemocyanin differs markedly in its copper content from the hemocyanin of other species,[2] the conclusions we have drawn do not necessarily apply to the nature of the prosthetic group in the other hemocyanins.

We are greatly indebted to Mr. I. S. Danielson for his assistance in procuring the initial material and for carrying through a number of the experiments.

[1] E. Philippi, *Zeit. Physiol. Chemie*, **104**, 88 (1919).

[2] Redfield, Coolidge and Shatts, *J. Biol. Chem.* **76**, 185 (1928).

THE PHOTOCHEMICAL DISSOCIATION OF NITROGEN PEROXIDE

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When the substance, nitrogen peroxide, consisting of a mixture of two gases, nitrogen peroxide, NO_2, and nitrogen tetraoxide, N_2O_4, is illuminated with approximately monochromatic light from a quartz mercury arc, an increase in pressure occurs which is greater than that to be expected from the heating effect of the radiation on the gas. The abnormal rise in pressure has been attributed to photochemical decomposition. Norrish[1] almost exclusively studied the equilibrium involved, and in later work[2] investigated the quantum yield so far as to determine the number of oxygen molecules formed in the reaction system per quantum of light absorbed. One molecule of oxygen was produced for every quantum absorbed in the spectral regions 2700–3160 Å and 3660 Å, while at longer wave lengths of 4050 Å and 4360 Å, 0.37 and 0.0 molecules of oxygen, respec-