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THE STRUCTURAL AND FUNCTIONAL RELATIONSHIPS BETWEEN HEME AND CHLOROPHYLL*

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THERE ARE two prominent pigments of living matter on the earth. One is the green pigment, chlorophyll. The other is the red pigment, heme. Both of these pigments are porphyrin compounds. Tonight I should like to analyze in some detail the structure of one, the red pigment, then discuss some of the biochemical and functional relations between the red and the green pigments, and finally, consider the biosynthetic chain of chlorophyll formation as an evolutionary development.

I

Let us first consider the red pigment. The red pigment, heme or iron protoporphyrin, is a constituent of a number of important protein molecules. The heme is the active or prosthetic group of hemoglobin—the molecule that transports O_2 in the blood stream. Heme is also the active group of the enzyme catalase, of the enzyme peroxidase, and of some other heme enzymes.[1, 2, 3] We may use as an example of a heme protein the molecule hemoglobin which has been the most intensively studied protein. In Figure 4 is presented a picture of a hemoglobin molecule. Its structure may best be remembered simply as a four-layer cake made up of a colorless protein globin, and for the frosting there are placed on its sides four red heme molecules. We shall return to the details of this picture later.

Let us examine the structure of these heme molecules more closely. In Figure 1 the chemical structure of iron protoporphyrin or heme is presented. This is a rather complicated molecule whose structure was finally solved only after some 30 years of detailed studies in organic chemistry—studies which have included the Nobel prize work of Willstätter and Hans Fischer. What is the meaning of such a complicated structure? More and more are we becoming aware that just as the anatomy of the body consists of

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various organs which are used for specific functions, so the anatomy of a biological molecule may be considered to consist of various parts that serve specific functions. Let us examine the anatomy of the heme molecule and inquire into the functions of its different parts.

The heme or iron protoporphyrin molecule consists of four pyrrole rings attached to each other through CH methene bridges.

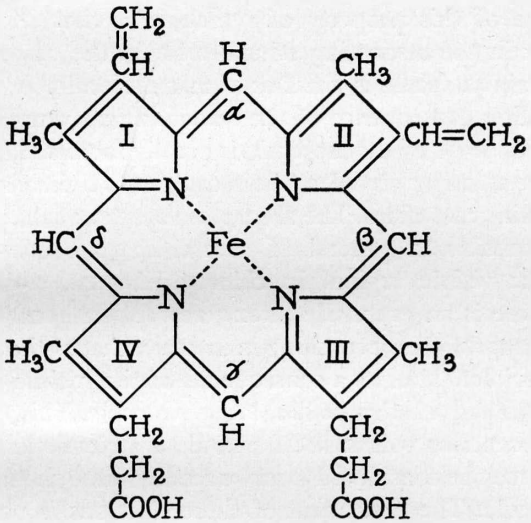

FIG. 1. Structure of iron protoporphyrin 9, or heme.

This attachment forms an inner 16-membered ring of C and N atoms. The formation of such a large ring was proposed by Küster, already in 1914, but was considered implausible because at that time it seemed that it would be a very unstable structure. What may be the significance of such a large ring and how is such a large ring stabilized? In the figure we see an alternating single double bond arrangement. This alternating single double bond structure is a description of the ring in terms of the organic chemist. In terms of the physical chemist the structure is said to resonate. The structure tells us that π electrons of the double bonds are present which move readily from one atom of the ring to the next. In this large ring we have what may be thought of as a circuit for the conduction of these mobile π electrons.[4]

There are a number of properties which derive from such a resonating structure. In the first place the 16-membered ring is greatly stabilized by this resonance. Like benzene, this molecule can be treated with strong acids without being split into fragments; for example, it can be nitrated, sulfonated, or halogenated. The individual pyrrole rings which normally are quite reactive structures by themselves, and which would normally polymerize to resins, also take part in this resonating structure and are stabilized. Another consequence of this property of resonance is that all the atoms taking part in the resonating structure lie in the same plane; so the porphyrin molecule is a flat or planar molecule.

Still another consequence of the resonating structure is that the absorption of light by the molecule is greatly increased, absorption taking place at longer wave lengths in contrast to light absorption by non-resonating molecules. The absorption spectra of the porphyrins therefore consists in general of several sharp bands extending through the visible region. In chlorophyll (i.e., a dihydroporphyrin) there is an especially strong absorption band in the red region, an absorption approaching in intensity that of the Soret band in the ultraviolet. Thus as a consequence of the mobile π electrons we have obtained a rather stable, large molecule. This molecule is flat, and it contains within itself a kind of a circuit in which the π electrons may be considered to move readily throughout the resonating molecule. The movement of these electrons is undoubtedly connected with the functioning of the heme and chlorophyll structures but of this we know very little.

Next let us consider the space in the center of this molecule. The space in the center of the porphyrin is of the right size to accommodate an iron atom. Larger atoms and smaller atoms can be held, but they are in general held less firmly than is iron (or copper). When iron is incorporated into the porphyrin we have the coordination compound, iron porphyrin or heme. The iron is held by six bonds. It can combine with six atoms or atom groups. In the center of the ring it combines with four N atoms, i.e., four bonds link the iron to the four N atoms in the plane of the ring. Now the iron may bind one atom or atom-group below the plane of the ring and one above the plane of the ring.

As mentioned before, iron protoporphyrin or heme is the prosthetic group known to be present in hemoglobin, catalase, peroxidase, and cytochrome b. In a slightly modified form it probably also represents the prosthetic groups of the other cytochromes. In heme proteins we are dealing essentially with the catalytic properties of the iron atom itself. But the properties of the iron atom have been specialized and intensified by being incorporated into the porphyrin and by the iron porphyrin being attached to different special proteins.

Too little is yet known about the coordination link of iron to the protein. We shall not discuss this important problem but rather illustrate by an example the specificity of behaviour brought about by the Fe-protein link. When the bond of the iron below the plane of the porphyrin ring is attached to a certain group in the globin molecule, possibly an imidazole-N, then the bond of the iron above the plane of the ring can attach reversibly to the O_2 molecule and can act as an oxygen transporter. If the iron bond below the plane of the ring attaches to a particular group in peroxidase, then the bond above the plane of the ring can attach to and activate H_2O_2.

Next let us consider the side chains sticking out from the edges of the porphyrin ring. There are three kinds of side chains present, namely, methyl, vinyl and propionic acid. There are 15 possible arrangements of the side chains around the ring. The only protoporphyrin that occurs in nature normally is designated as protoporphyrin 9, and this has the following arrangement of side chains around the ring: methyl, vinyl, methyl, vinyl, methyl, propionic acid, propionic acid, methyl.

In our laboratory we have been interested in studying the functions of these side chains. We were fortunate to have the collaboration of Dr. Helena Gilder in these studies. I should like to tell you what we found out about the function of these side chains.[3, 5, 6]

The technique we have used is essentially a simple one. An organism is selected which lacks the ability to make its own heme. Without heme it cannot respire and cannot grow. Then one adds porphyrins or iron porphyrins that have been modified slightly in their side chains and sees whether growth can occur. An experiment

performed by Lwoff in 1934[7] will illustrate the phenomenon. Lwoff used a flagellated protozoan, Strigomonas, which requires heme for growth. He grew this organism on a medium containing a very low concentration of heme. Its multiplication ceased, its oxygen consumption (as measured in the Warburg manometric apparatus) was 40 per cent of normal; it was barely surviving. Then to these organisms in the Warburg vessel he tipped in excess heme. Within a few minutes the O_2 consumption was observed to increase and after several hours the normal rate was attained. Only after attaining the normal rate of respiration did the organisms begin to multiply. Lwoff calculated that about 500,000 heme molecules were required per new individual. The obvious interpretation of this experiment is that on adding heme, the heme is taken into the cell, it is attached to the correct protein or proteins to form the proper heme enzymes, i.e., the proper oxidation catalysts, and then the metabolism of the cell can proceed normally.

Instead of the protozoan Strigomonas, we used the bacterium Hemophilus for our studies. This bacterium requires heme (iron protoporphyrin) for its growth. It cannot make its own heme. When protoporphyrin was supplied in place of iron protoporphyrin, it was found that the organism would grow and multiply and it could be shown that iron protoporphyrin was present in the organism. It was concluded that oxidative heme catalysts were formed which functioned to permit growth to take place.

Further experiments using porphyrins and iron porphyrins modified in their side chains showed that iron could not be inserted into the porphyrin ring by this organism unless the porphyrin possessed *vinyl groups*. The need for the vinyl groups was demonstrated in the following way. If one feeds iron porphyrins that are modified in the side chains so that they lack vinyl ($-CH=CH_2$) groups, it is found that such iron porphyrins support growth (for example, iron hemato-, iron deutero-, iron meso-porphyrins, in which the vinyl groups are replaced by $-CHOHCH_3$; $-H$; and $-CH_2CH_3$, respectively). However, if the porphyrins themselves are supplied and these porphyrins lack vinyl groups, then such porphyrins cannot support growth. From these experiments it appears that the organism cannot insert iron into porphyrins lack-

Effect of Porphyrins on Growth and Nitrate Reduction of *H. influenzae*

Protoporphyrin						Harderian Gland Protoporphyrin					
Gamma per 5cc. media											
0.05	0.15	0.25	0.25	0.25	0.25	0.05	0.15	0.25	0.25	0.25	0.25
			Hematoporphyrin						Hematoporphyrin		
			0.5	1.5	2.5				0.5	1.5	2.5

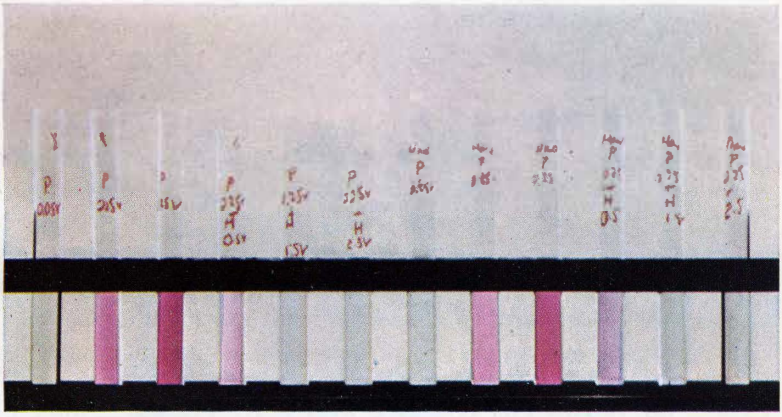

FIG. 2. Example of competitive inhibition in Hemophilus influenza between the growth-promoting porphyrin containing vinyl (— CH = CH_2) groups (protoporphyrin) and the growth-inhibiting porphyrin lacking vinyl groups (hematoporphyrin in which the vinyl groups are replaced by — CHOH — CH_3 groups). The intensity of pink color is proportional to the growth of the organism. (The color actually represents a diazotization coupling, utilizing the nitrite produced by the organism, the nitrite produced being proportional to growth.)

The first three tubes illustrate the increase in growth as the concentration of protoporphyrin is increased. In the next three tubes the concentration of protoporphyrin has been maintained at a constant high level but at the same time hematoporphyrin has been added in increasing concentrations. The growth is seen to be depressed by hematoporphyrin, less than five molecules being required to completely inhibit the growth promoting activity of one protoporphyrin molecule.

As seen in the next six tubes, the porphyrin isolated from the harderian glands of rats behaves in the same manner as does the protoporphyrin prepared from the heme of the red blood cells. This biological test is further evidence that the porphyrin of the harderian gland is protoporphyrin.

ing vinyl groups, thus no iron porphyrins are made and no heme enzymes can be formed. If, however, vinyl groups are present, as in protoporphyrin, then the organism is able to insert iron into this porphyrin, and the resulting iron porphyrin can be used as prosthetic group of heme enzymes.

Not only are the porphyrins which lack vinyl groups incapable of supporting growth, but such porphyrins are actually found to act as competitive inhibitors. Such porphyrins compete with iron porphyrins for the protein enzyme surfaces. A porphyrin lacking iron will become attached to a protein surface and prevent the iron porphyrin from attaching to this spot on the protein. Such a protein will then not function as a catalyst.

The vinyl groups are essential for the insertion of iron. In addition it has been found that the presence of vinyl groups enhances growth either because the heme enzymes which are produced may be more active, or perhaps because certain enzymes may be formed which, although not essential for growth, still definitely increase growth. For example, maximal growth on iron hemato-, iron meso-, or iron deutero-porphyrins, i.e., on iron porphyrins lacking vinyl groups, is only about half of that on iron protoporphyrin. We have also found that a specific enzyme, a nitrate reductase, is produced in the presence of the porphyrin with vinyl groups and not in its absence. Iron porphyrins lacking vinyl groups support growth but the organism cannot reduce nitrate to nitrite. If iron protoporphyrin is supplied to the organism an enzyme is produced which reduces nitrate to nitrite.

Some of these phenomena are shown in Figure 2. Here, the intensity of the pink color is proportional to the growth of the organism. (The pink color is a test for nitrite—the amount of nitrite formed being proportional to the growth of the organism.) When protoporphyrin, which has two vinyl groups is supplied to the medium, the Hemophilus organism can insert iron into the ring, forming iron protoporphyrin. This iron protoporphyrin can act as a prosthetic group, attaching to the proper proteins and forming heme enzymes. These heme enymes are required for the growth of the organism. In the first three tubes we see that the concentration of protoporphyrin has been increased progressively and the

multiplication of the organisms was observed to increase. The next three tubes show the phenomenon of competitive inhibition. Here the *proto*porphyrin concentration has been kept at a level to attain maximum growth, at the same time that increasing concentrations of *hemato*porphyrin have been added. This latter porphyrin lacks vinyl groups and iron cannot be inserted into it by the organism. It is seen that growth decreased as the hematoporphyrin concentration was increased. In this experiment less than five molecules of hematoporphyrin completely inhibited the growth-promoting effect of one molecule of protoporphyrin.

The function of the propionic acid side chains was next investigated. By similar experiments with the Hemophilus organism it was found that the free carboxyl groups are essential for growth. If the propionic acid groups of iron protoporphyrin were esterified, the resulting heme would no longer support growth. If a porphyrin was esterified which was normally a competitive inhibitor—for example, if hematoporphyrin was esterified—such a compound would no longer be inhibitory. It was concluded from such experiments that the propionic acid side chains and particularly the free carboxyl groups are essential for growth.

Support for the idea that the carboxyl groups are necessary for the functioning of iron porphyrins is obtained from the chemical experiments of Warburg and Negelein,[8] and of Theorell.[1] Warburg & Negelein prepared renatured globin by the method of Anson & Mirsky. They then added different iron porphyrins to the globin and tested these artificial hemoglobins for reversible oxygen-carrying capacity. They found that the presence or absence of vinyl groups was not essential for activity. However, iron porphyrins lacking free carboxyl groups could not function as prosthetic groups. In a similar manner Theorell prepared the peroxidase proteins free from heme, and then attempted to combine various iron porphyrins with this protein to see if peroxidase activity resulted. In general he found that for the iron porphyrins to function as prosthetic groups of this protein it was necessary to have free carboxyl groups on the iron porphyrins. Vinyl groups were not essential.

We have seen that free carboxyl groups of heme are necessary

both for the growth of Hemophilus and for the formation of functional hemoglobins and functional peroxidases. Why is it necessary to have free carboxyl groups to obtain activity? At body pH the carboxyl groups are ionized and thus bear negative charges. It seemed reasonable to us to postulate that the two negatively charged carboxyl groups of the heme would serve to anchor the heme to the protein—most probably by attaching to two positively charged groups of the protein surface. To test this idea, mesoporphyrin was used. In mesoporphyrin the two vinyl groups of protoporphyrin are replaced by the ethyl groups, no iron is present, and the two propionic acid groups are in the same relative positions as they occur in iron protoporphyrin. To the mesoporphyrin, renatured globin was added and then the mixture was diluted with buffers of various acidities.[9]

If the porphyrin attached to the globin, the sharp bands of the monomeric mesoporphyrin would be present and the absorption at 502 mμ would be high. If the mesoporphyrin did not combine with the globin the mesoporphyrin would tend to polymerize (i.e., become colloidal), and the absorption at 502 mμ would decrease. (The polymeric form has an absorption spectrum consisting of several low absorption bands with maxima shifted toward the red.) By plotting the intensity of absorption at 502mμ against the pH of the buffered solutions the curve of Figure 3 was obtained. The curve at its height is a plateau region indicating practically complete binding of the mesoporphyrin to the globin. The complex is formed in a region of pH between 5—11, but not at a pH much below 5 or much above 11. What does this result mean? The pK of the acid groups of propionic acid of heme are around 4.8. That is, at a pH of 4.8 half of the carboxyl groups have already taken up protons from the medium and are uncharged. At a pH of 3.8 over 90 per cent of the carboxyl groups are uncharged. From this experiment it may be concluded that when these carboxyl groups are uncharged the porphyrin does not attach to the globin, presumably because only negatively charged groups would attach to the positively charged groups of the globin.

What may be the positively charged groups of globin which attract the negatively charged carboxyl groups of the porphyrins?

From the curve we note that the positively charged groups remain charged up to about pH 11, i.e., the pK of these groups appears to be in the neighborhood of 11-12. In this alkaline region, only

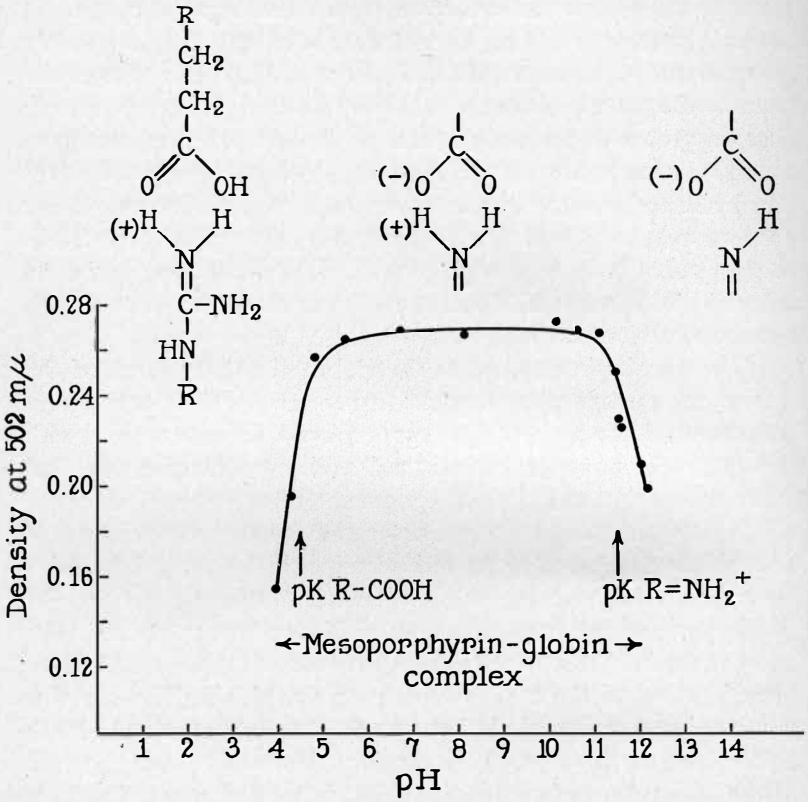

FIG. 3. The pH dependence of attachment of mesoporphyrin to globin. The extinction remains high between pH 5.5-11 indicating formation of the mesoporphyrin-globin complex in this range, but not outside this range.

The experiment was carried out in the following way. To 94 γ mesoporphyrin, in 0.1 cc. of a solution containing 50 per cent ethanol and 0.01 N KOH, was added rapidly 0.5 cc. of aqueous dialyzed renatured globin, pH 8.5, containing 4.50 mg. globin. After 30 seconds 1.00 cc. of buffer solution of 0.1 ionic strength was added. (The pH of the buffer solution was determined with the hydrogen electrode.) After half hour the solution was measured in 1.0 cc. capacity cell of 1 cm. light path in the Beckman spectrophotometer at 502 mμ. Between pH 5.5-11.0 about 94 per cent of the mesoporphyrin was calculated to be attached to the globin in the monomeric form. If mesoporphyrin does not attach to the globin it polymerizes under the experimental conditions—the absorption of the polymer being considerably depressed, with bands shifted toward longer wave lengths.

two kinds of amino acid groups are present with such a high pK, namely, the ε-ammonium groups of lysine with a pK of about 9.4-10.6, and the guanidinium groups of arginine, with a pK of 11.6-12.6. In Figure 3 we have pictured the positively charged group as that of the guanidine group of arginine. According to this interpretation the formation of the mesoporphyrin-globin complex occurs in the region between pH 5-11, a region where the two carboxyl groups bear a negative charge and the two guanidinium groups bear a positive charge. We may infer that the positively charged groups are sufficiently close together on the globin surface so that they can attach to the two propionic acid carboxyls of the hemes. Since hemoglobin bears four hemes, this would mean that eight positively charged groups of the globin are involved in the attachment of the four hemes.

The third kind of side-chain on protoporphyrin is the methyl group. The function of the four methyl groups is not known but it is not unreasonable to assume that they are present to stabilize the heme, that is, to block any side reactions that might occur in those positions if the methyl groups had been absent.

The properties of protoporphyrin may be summarized by considering how they come into play, for example, in the formation of the hemoglobin molecule (assuming that what has been learned of the porphyrin chemistry in Hemophilus may be applied in interpreting the reactions in higher organisms). Figure 4 shows a hemoglobin molecule reconstructed in part from some of the recent x-ray studies of Perutz, Boyes-Watson & Davidson[10, 11] of Cambridge. Hemoglobin has a molecular weight of 68,000. Its shape approximates that of a short cylinder. This short cylinder contains four layers of polypeptides. In each polypeptide layer are present polypeptide chains running the length of the molecule along the x direction. The polypeptide chain is a wrinkled one, containing regularly spaced folds, some 5A apart, the folds being equivalent to a distance of two amino acid residues along the chain. This kind of fold is similar to the one described by Astbury as the contracted or alpha keratin type of polypeptide. According to this interpretation the wrinkled polypeptide chain running along the x direction would have the dimensions of a roughly cylindrical elongated rod with an average cross section diameter of 10.5 A. In one polypeptide

layer, there could fit about five such rods, or the four polypeptide layers could contain a total of 20 such rods. The data of Porter & Sanger[12] reveal only six polypeptide chains suggesting that the

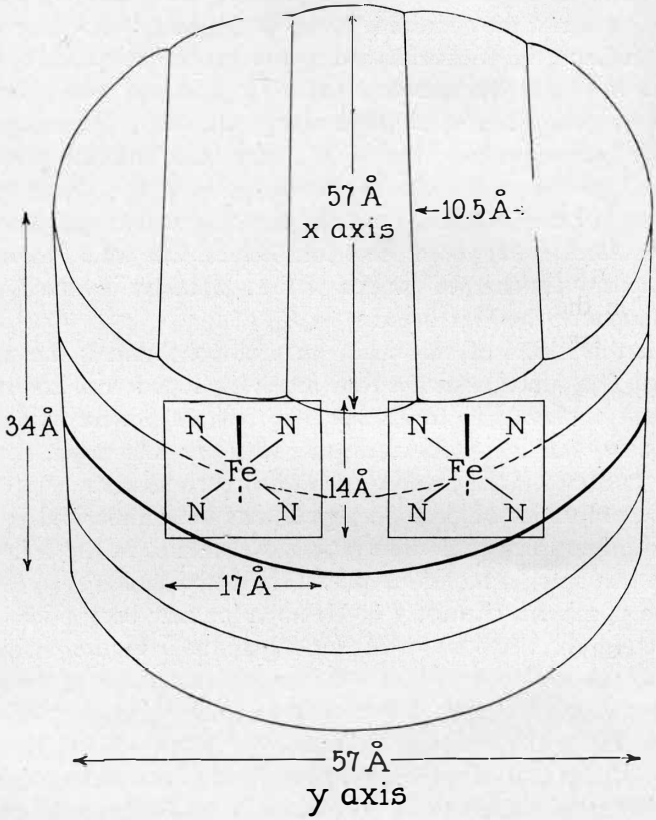

FIG. 4. Molecule of horse hemoglobin as reconstructed from x-ray and biochemical data.

chains might be folded back and forth on themselves either along the x or z direction.

There are four flat heme molecules per globin. The hemes lie parallel to each other and also lie perpendicular to the plane of the polypeptide layers. One interpretation for the disposition of the hemes is presented in Figure 4. Two hemes are shown on the

proximal surface of the globin attached to the upper two polypeptide layers; hidden from view on the distal surface are the other two hemes attached to the two lower polypeptide layers.

One may imagine the following course of events in the synthesis of such a heme protein in an immature red blood cell. The protoporphyrin ring is first formed, and then because of the presence of the vinyl groups, iron can be inserted into the ring forming iron protoporphyrin or heme. This newly completed heme molecule now migrates into the neighborhood of a newly formed globin molecule. Then the negatively charged carboxyl groups of the heme seek out and attach to two positively charged groups of the globin—possibly guanidinium groups. This coulombic attachment now orients the planar heme molecule so that it can fold down onto the globin surface, thus bringing the iron atom into contact with the all-important group of the globin to which the iron coordinates—possibly an imidazole group of histidine.[2] The imidazole link to the iron is a weak one by itself. This iron link would be stabilized by the coulombic attachment of the carboxyls of the heme to the protein and also by the Van der Waals forces between the planar porphyrin ring and the globin surface.

II

After having studied some phases of iron metabolism and then the functions of porphyrin structure, we became interested in finding out how such molecules were put together biologically. There are two places in nature where porphyrin synthesis is going on at a rapid rate. One place is the bone marrow where heme is manufactured as a constituent of hemoglobin and the other place is in the chloroplasts where chlorophyll is synthesized. The problem of obtaining fresh, red bone marrow in sufficient quantity appeared hopeless. We therefore turned to a study of chlorophyll synthesis in plant cells with the hope that some of the steps in the synthesis of chlorophyll might possibly be related to the steps occurring in heme synthesis.

The problem of biological synthesis includes the identification of the intermediate compounds and enzymes along a chain of syntheses as represented by Equation 1. In a general way we may pic-

ture a compound, say 5, being synthesized through a series of intermediate substances, 1, 2, 3, 4, by a number of different enzymes, A. B. C. D:

Equation 1.

$$1 \xrightarrow{A} 2 \xrightarrow{B} 3 \xrightarrow{C} 4 \xrightarrow{D} 5$$

The best example illustrating such a chain process in which not only the substrates but also the enzymes have been identified is the glycolytic scheme of Embden-Meyerhof-Cori; here by a series of well-known steps pyruvic acid molecules can be converted into glycogen. Biologically, this is a well-trodden metabolic road; all the stepwise reactions are reversible and some of the intermediates are present in concentrations of at least several milligrams per 100 g. tissue.

In the biological synthesis of many compounds the intermediates may be present to only a minute extent in the steady state equilibria of the cell. A number of methods have been used to increase the concentration of intermediates into the detectable range. Two general methods are available. One can either arrange conditions so as to trap the intermediate compounds, say 2, 3, or 4, or one can inhibit a particular enzyme, A, B, or C, which would have removed the intermediate by converting it to the next stage.

In the first method, the intermediate may be accumulated by combining it with some specific reagent. For example, in the Neuberg method for the production of glycerol by yeast, the pyruvic acid is trapped by addition of bisulfite, forming the pyruvate-bisulfite addition compound. The method of trapping an intermediate is of limited application since specific reagents must be found, and these reagents must be non-toxic to the cell.

In the second method a specific enzyme is prevented from acting on a particular intermediate, resulting in the accumulation of this particular intermediate. This method has a more general application since more ways are available for interfering with the specific activity of an enzyme. For example, the enzyme may be poisoned by specific reagents (e.g., iodoacetate on triose phosphate dehydrogenase); or its activity may be decreased by adding competitive inhibitor substrates (e.g. malonic acid on succinic dehydrogenase);

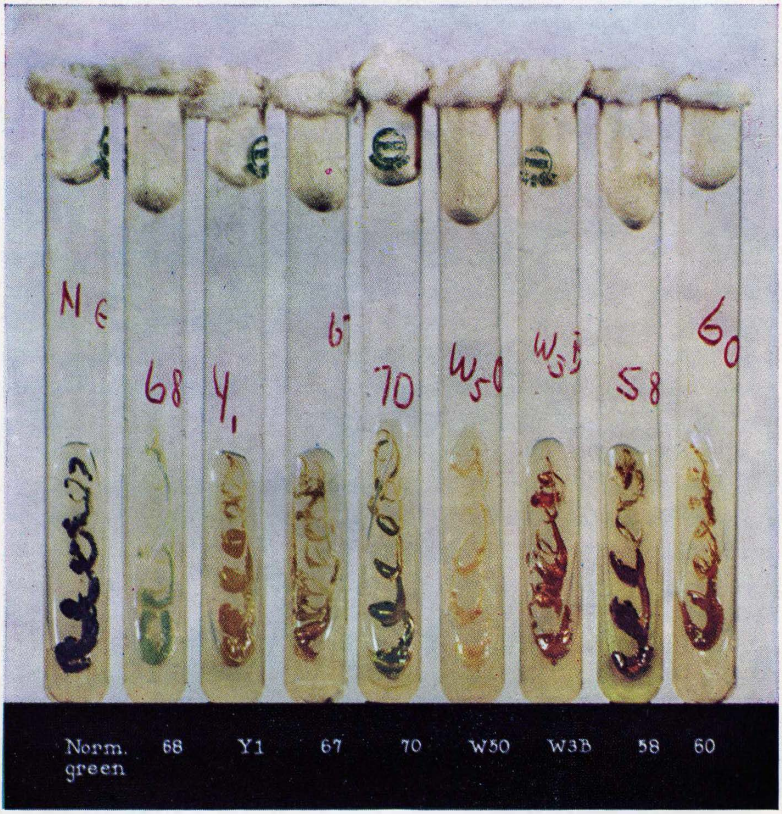

FIG. 5. Examples of chlorella mutants produced by x-radiation. The mutants are grown in the dark on agar-inorganic salt-glucose medium. The normal chlorella (norm. green) grows green in the dark and contains chlorophylls as well as carotenoids. Mutant Y_1, lacks chlorophyll but contains carotenoids and traces of Mg vinyl pheoporphyrin a_5; in the light this mutant produces chlorophyll. Mutant W_3B is brown in color. It lacks the yellow and green pigments. The brown color is due to protoporphyrin 9. By irradiation of W_3B, a mutant W_5O was isolated which lacked protoporphyrin but contained an orange carotenoid. From mutant #60 Mg protoporphyrin was isolated.

or the active enzyme may be diminished in concentration or may be completely prevented from forming in cells, by knocking out certain genes—a technique used with such success on Neurospora by Beadle and Tatum.[13]

By irradiating Chlorella cells with x-rays we have found it possible to bring about the accumulation of certain intermediates along the pathway of chlorophyll synthesis. Mutant cells are formed by this treatment and those mutants are selected which presumably have some derangement in enzymes directly or indirectly concerned with chlorophyll synthesis.

For these studies the single-celled alga *Chlorella vulgaris* was used. This organism is about the size of a red blood cell. It contains a cup-shaped chloroplast consisting of a thin green layer of material embedded in a somewhat thicker layer of cytoplasm appressed to the cellulose wall. This green plant cell, widely used in studies on photosynthesis, will multiply in the presence of an inorganic salt solution and sunlight. If glucose is added to the solution the alga will grow in the complete absence of light. Chlorella differs from the higher plants in that it can produce chlorophyll in the dark. In higher plants chlorophyll is produced only in the light.

The mutants were prepared in the following way: A thick suspension of rapidly growing Chlorella cells was irradiated for 15-20 minutes with x-rays at the rate of 2,000 Roentgens per minute. The cells were then plated out on a solid medium. Colonies arose from the single cells and were examined for variation in color and growth. In this way mutants were isolated which were pale green in color and lacked yellow pigments (i.e., the carotenoids), or which were yellow and lacked green pigments, or were brownish red, or were variously tinged from orange to almost colorless (Fig. 5). All the mutants which have been studied thus far can be grown in the dark on a simple glucose-inorganic salt medium. All of these mutants form starch. One property of the chloroplast is its ability to form starch. The fact that these mutants form starch is considered evidence that the chloroplast or a remnant of it is still present in these mutants.

One of the mutants (W_3B) when grown on solid media turns

deep brown. So much of this brown pigment is developed that it deposits as minute granules in the cells. This pigment was isolated, crystallized and derivatives were made. It proved to be protoporphyrin isomer 9, that is, it was identical with the porphyrin making up the heme of the red blood cells.

The idea that some relationship existed between the two most prominent pigments of living matter—the red blood pigment and the green plant pigment—was merely a satisfying philosophical concept in the early 19th century. The first experimental evidence for such a relationship was provided by Hoppe-Seyler in 1880 who found that treatment of chlorophyll with alkali gave rise to a product with a porphyrin-like spectrum somewhat resembling the porphyrin derived from heme. The studies of Nencki, Piloty, Kuster, and Willstatter gradually revealed the basic pyrrole structures and the porphyrin ring structures of these two pigments. Finally Hans Fischer and Conant and their coworkers determined the basic similarities of their side chains. At one time it was believed that the aetioporphyrin derivatives of the two pigments were identical but this was shown to be incorrect. If one compares the structures of heme and chlorophyll, the similarities are at once apparent but certain differences are also seen. Whether these differences between the two pigments arose early, that is, before the formation of the porphyrin ring, or arose late in the synthesis, could not be determined.

In Chlorella the appearance of relatively large amounts of protoporphyrin 9 in a mutant devoid of green pigments and the finding of Mg protoporphyrin in another mutant lead to the conclusion that protoporphyrin is a normal precursor or intermediate in chlorophyll synthesis. There is one other idea we may infer from this study. When we began this work we had hoped that the pathways for heme synthesis and chlorophyll synthesis might be similar. Since in the red cell which makes heme, and in the chloroplast which makes chlorophyll the same protoporphyrin, i.e., isomer No. 9, is produced, it must be concluded that protoporphyrin is the metabolic precursor of both and that the steps up to the synthesis of this porphyrin must be identical in both. The biochemical relation between the red blood pigment and the green plant pig-

ment is now apparent from these studies. Here is another example of the fundamental biochemical unity of protoplasm.

Up to now two other intermediates have been identified in Chlorella, namely, Mg protoporphyrin and Mg vinyl pheoporphyrin a_5. The proof that these compounds are intermediates rests at

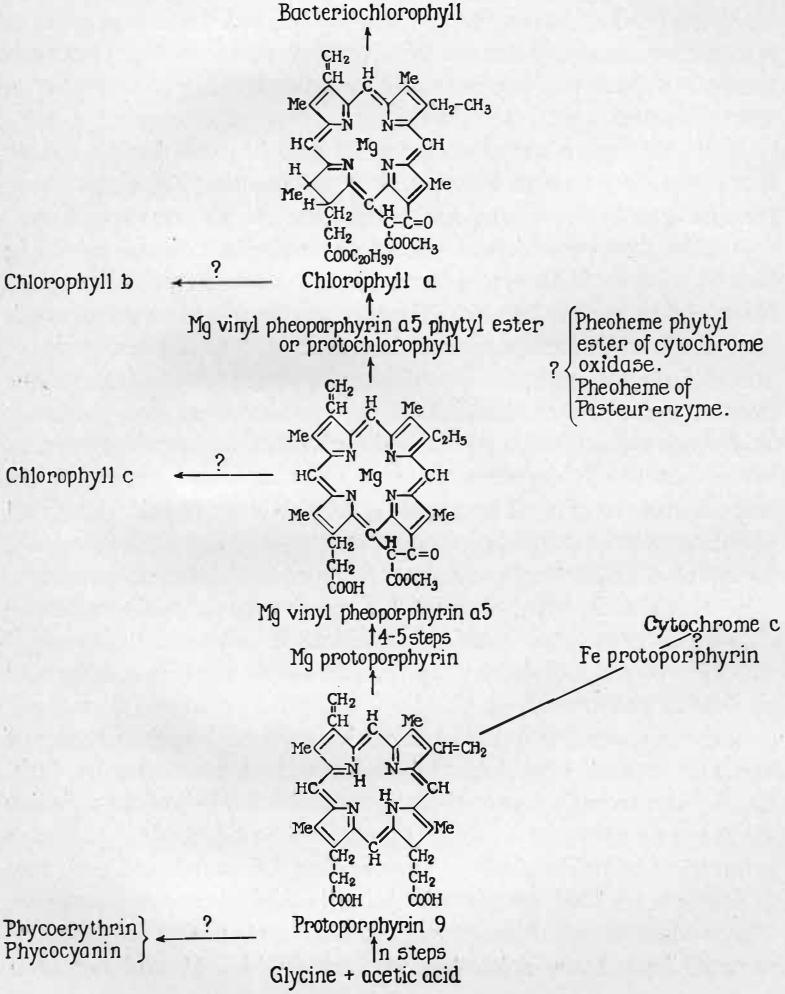

FIG. 6. Scheme of biosynthesis of heme and chlorophyll.

present on the fact that the known intermediates can be fitted into a consistent chemical scheme. An outline of the present status of the biochemical synthesis of chlorophyll is presented in Figure 6. We may start with glycine and acetic acid which have been demonstrated by Shemin and Rittenberg[14] to constitute important building blocks of the pyrroles. Especially elegant was the demonstration by these workers, using tracer techniques, that the N of glycine was incorporated to form the N of the pyrroles in heme. The intermediate steps up to the next known compound, protoporphyrin, are unknown.

From protoporphyrin two branches may be considered to arise, one containing iron as the central atom, and the other containing magnesium. Let us consider the Mg branch. In chlorophyll synthesis the step beyond protoporphyrin is the incorporation of Mg into the ring to form Mg protoporphyrin. This compound has been identified in mutant No. 60. Then follow four or five steps which have not been identified but which must include the reduction of one of the vinyl groups, the oxidation of the β carbon of one of the propionic acids, the esterification with methanol of this propionic acid, and the oxidation to form the cyclopentanone ring between the α C atom of this propionic acid and the γ C of the porphyrin ring. There results then a pale greenish compound, Mg vinyl pheoporphyrin a_5, isolated from mutant No. 31, and a few other mutants. A further step, the esterification of the second propionic acid with the C_{20} alcohol, phytol gives rise to the pigment protochlorophyll, not yet isolated from Chlorella. The protochlorophyll structure was elucidated by the studies of Noack & Kiessling and by Fischer and coworkers.[15]

This compound is found in the pale green seed coats of cucurbits and also in barley seedlings which have been grown in the dark. Smith[16] has recently shown that the protochlorophyll of these barley seedlings on exposure to light is converted to chlorophyll a. In this conversion to chlorophyll a, pyrrole ring IV is reduced, i.e., two H atoms are added. In normal Chlorella, light is not necessary for the production of chlorophyll. However, a number of chlorella mutants have been isolated (for example No. 31 and No. Y_1)

which produce only traces of chlorophyll in the dark, but in the light the normal quantity of chlorophyll is produced as in the higher plants. Evidently the reduction of pyrrole ring IV is brought about in the normal Chlorella by an enzyme system which is lacking or possibly only present in traces in the Chlorella mutants No. Y_1 and No. 31, and in the higher plants.

Bacteriochlorophyll is a further reduction product of chlorophyll a in which two H atoms are added to pyrrole ring No. 2. Whether chlorophyll b can arise directly from chlorophyll a by conversion of the 3-methyl side chain to a -CHO side chain is not known. Chlorophyll c does not contain a reduced pyrrole ring; it is therefore related to Mg vinyl pheoporphyrin rather than to chlorophyll.[17]

Let us next consider briefly the iron porphyrin branch. For the synthesis of iron protoporphyrin, two hypotheses had been suggested. One was that the pyrroles might congregate around an iron atom and couple to form the completed porphyrin ring. The other possibility was that the porphyrin ring would be formed first and the iron would then be inserted. The fact that organisms like Strigomonas and Hemophilus, which do not make their own heme, can utilize protoporphyrin to form heme, supports the second hypothesis. The insertion of iron into the protoporphyrin ring is also compatible with the chemical properties of this compound. The chemical structure assigned to the heme of cytochrome c by Theorell[1] suggests that this heme might arise directly from iron protoporphyrin. The meagre data on the nature of the heme of Warburg's respiratory ferment, a heme which Warburg has recently claimed to contain the phytol alcohol itself,[18] and on the heme of the Pasteur enzyme of Stern & Melnick[19] suggest some resemblances to the structures of the pheoporphyrins. Whether these are to be derived directly from iron protoporphyrin or are to be derived from the magnesium branch of the scheme remains to be seen.

III

I have presented the evidence to indicate that the two major pigments of protoplasm are porphyrins which are related biochemically, arising from a common precursor protoporphyrin. Not only

are these two pigments related biochemically but they are related functionally. This functional relationship becomes apparent when we attempt to express and summarize in the following simple chemical equation the primary energy changes of protoplasm:

$$\textit{Equation 2.}\quad O_2 + 4\,H^+ + 4\,(e) \xrightarrow[\text{Chlorophyll}]{\substack{-\Delta H \\ \text{Fe protoporphyrin}}} 2\,H_2O + nh\,\nu$$

In this equation (e) represents electrons of high potential energy forming the shared electron-pair bonds of organic substrates; the heat produced in oxidation is $-\Delta H$; and the number of light quanta of frequency ν absorbed in photosynthesis is $nh\,\nu$. Why have I expressed the primary energy changes of protoplasm in this form? From the discussion which follows I hope to make this clear. The forward reaction expresses an oxidation. Here the electrons of high potential energy present in various organic combinations, combine with protons and O_2 to form water, where the electrons have a lower potential energy. This oxidative process does not take place spontaneously but is catalyzed by enzymes which have hemes as prosthetic groups. Suppose we take as an example the oxidation of succinic to fumaric acid. In this oxidation two electrons are lost by the succinic acid in going to fumaric. Let us analyze the events in this oxidation. First the succinic acid combines with the enzyme succinic dehydrogenase. In this combination the succinic acid is activated to give up one electron at a time to a heme enzyme, cytochrome c. Cytochrome c then transfers this electron to an activated O_2 molecule, the O_2 molecule having been activated by another heme enzyme, cytochrome oxidase to accept the electron. H_2O_2 which may be produced as an intermediate in these reactions may be activated by another heme enzyme, peroxidase; the activated H_2O_2 then has strong affinity for electrons, i.e. it behaves as a strong oxidizing agent. Or the H_2O_2 may be destroyed by still another heme enzyme, catalase. The forward reaction then expresses an overall oxidation in which energy is

released when the electrons of high potential energy combine with protons and O_2 to form water where the electrons have a lower potential energy. This release of energy is utilized by the protoplasm to make more of itself from the components of its environment. This release of energy is catalyzed by the red pigments—the heme enzymes.

What of the reverse reaction? This reverse reaction is essentially the reaction of photosynthesis. It is an energy-storing reaction, the energy of sunlight being stored primarily in the electronic configuration of organic compounds. The catalyst which makes this reaction possible is also a porphyrin, more precisely a magnesium dihydroporphyrin called chlorophyll.

Studies on suspensions of chloroplasts by a number of investigators have recently established the following basic facts concerning the first stages of photosynthesis. Hill[20] made the discovery that on illuminating a suspension of chloroplasts O_2 would be released if an electron acceptor were present in the suspension. Such an electron acceptor may be a ferric ion or quinone molecule, etc.,[21] that is, a substance which will take up the electrons released in photosynthesis. Next, it was established by Ruben and coworkers[22] that the O_2 released in photosynthesis arose from the water. This was demonstrated by using water, labeled with heavy oxygen; on illumination the O_2 released was found to contain heavy oxygen atoms. More recently Holt and French[23] have shown that protons (hydrogen ions) were released at the time O_2 was produced.

Putting these facts together gives us the reverse reaction of Equation 2. This reaction represents the first stage of photosynthesis occurring in isolated chloroplasts. Water is decomposed in the presence of sunlight to yield O_2, protons and electrons, where (e) represents the electrons taken up by some electron acceptor. This reaction is catalyzed by chlorophyll.

One important fact which still eludes the investigators of photosynthesis is the potential of the electron, that is, the reducing ability of the electron liberated in this process. On the reducing ability of the electron would depend the nature of the primary electron acceptor in the chloroplast. For instance, if photosynthesis required

only four quanta per CH_2O formed, (i.e., 4 $h\nu$), then the electron would have a potential in the neighborhood of the hydrogen electrode.

From these considerations we see that the release of energy in protoplasm is essentially an oxidation with oxygen, water being the end product. The reverse reaction, the reduction of water, is the primary reaction of photosynthesis. This is the manner in which heme and chlorophyll are related: They are the catalysts of the fundamental energy equation of protoplasm.

IV

The scheme of porphyrin synthesis as outlined in figure 6 not only tells us about the pathways of chlorophyll and heme synthesis in the cells. It suggests something more fundamental. It suggests the evolutionary pathway.

A basic hypothesis of biochemical genetics is that the genes, directly or indirectly, determine the formation of specific enzymes. On that hypothesis the evolution of a chain of biosynthesis (e.g., Fig. 6) would be the expression of the evolution of a series of genes developing in ordered sequence. Each new gene would determine the formation of a new enzyme and each new enzyme would act to change a product of the step before it in the sequence. Thus (in equation 1) gene B would give rise to enzyme B and this enzyme would convert product 1 to product 2.

The evolution of a biochemical sequence is perhaps more clearly envisioned at the present time in this scheme of porphyrin synthesis than in any other biochemical synthesis since it leads to two clearly defined end products, heme and chlorophyll, of especial functional significance.

I shall discuss here only one of the corollary implications which follow when we think in terms of a biochemical evolutionary sequence.* In general it is necessary to assume that at some time each

* Here it is not possible to present adequately the other current views of evolution—including the well documented view by Lwoff[27] of an evolution in microorganisms where losses of genes and structures are involved; the view of Oparin[28] that life originated in a complex organic environment; the view of Horowitz[29] that the functional product at the end of the chain was first produced, and that mutations occurred one at a time in a backwards direction from that in which

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one of the intermediate compounds in the sequence was the end product of the sequence. As an end product, such a compound had to be more or less useful to the economy of its protoplasm. For example, on the above assumption, at one time Mg protoporphyrin would have been an end product and would have had some function. When additional steps were added to the sequence, the original function of Mg protoporphyrin might have been lost, but Mg protoporphyrin itself could not be lost because it now functioned as an intermediate. (Here a loose parallel may be seen in the embryology of the mammal, where the gill slits were functional at one stage of phylogeny, and at present represent only an intermediate embryological phase without functional significance.)

From these considerations we are led to ask the question whether any of these intermediates still function in the cell apart from their being intermediates. We are also led to ask whether in certain species some one or other of the intermediates might retain remnants of such functional activity? These are questions which will have to be considered in this and other schemes of biosynthesis of end products.

I should like to present a hypothesis here which might greatly simplify the problem of understanding the function of the intermediates, at least as it concerns the intermediates in the chlorophyll scheme. It does not seem reasonable to consider that after many different evolutionary steps finally giving rise to heme and chlorophyll that suddenly the properties of oxidation and photosynthesis were created. Rather does it seem more reasonable to consider that the functions of oxidation and photosynthesis were so fundamental that they were part of the first protoplasm arising from inorganic origins. The properties of heme are essentially those of the iron atom and may be found in those of the inorganic iron compounds themselves; perhaps the property of chlorophyll might also be represented by some colored inorganic compound. This hypothesis would consider that the two functions of oxidation and photosynthesis

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synthesis to the functional product now proceeds. A brief summary and analysis of these hypotheses is presented in an excellent review by Van Niel.[30] These and other hypotheses besides the one presented here on evolution are not mutually exclusive and may all have to be considered and their significance evaluated.

were established very early in protoplasmic development and the evolutionary process would thenceforth represent the progressive elaboration of mechanisms for carrying out these functions in a more efficient manner.

According to this hypothesis all of the colored compounds arising in the evolutionary sequence leading eventually to chlorophyll would have had the same function as chlorophyll itself, namely that of photosynthesis. Tending to support this hypothesis of the photosynthetic function of intermediates are several incomplete bits of evidences.

1. The reduction of protochlorophyll to chlorophyll a in the plant cell is a photochemical reaction according to the work of Smith[16] and of Frank.[24] Thus protochlorophyll is itself a pigment which, like chlorophyll, can bring about a photoreduction. Whether O_2 is released in this process is not known.

2. In the brown algae there is present besides chlorophyll a another green pigment, first isolated by Strain et al[25] and characterized only by its absorption spectrum to which the name chlorophyll c was given. We have recently investigated this pigment[17] and were able to show that chlorophyll c is related chemically not to chlorophyll a but rather to one of the intermediates, Mg vinyl pheoporphyrin a_5. Chlorophyll c is not on the direct line to chlorophyll a but is a side shoot. Whether it possesses photosynthetic activity is not yet known. If chlorophyll c is found to have photosynthetic activity then it might be considered as an example of a photosynthetic pigment which might have been of importance in the distant past—a kind of biochemical fossil.

3. The phycocyanins and phycoerythrins are found in the red and blue green algae. They are proteins containing open chain tetrapyrroles as the colored prosthetic groups. Blinks[26] has made the important observation that under certain conditions the light absorbed by these pigments but not by chlorophyll a, is active photosynthetically. If these pigments could be shown to function in the cell independently of chlorophyll a then phycoerythrin and phycocyanin might also represent an early evolutionary step in the development of photosynthetic pigments.

Let us then consider not just one chain of biochemical synthesis but the sum total of all the chains of syntheses which constitute protoplasm. Then one might regard the evolution of protoplasm in terms of the development of chains of genes giving rise to these chains of syntheses.

Just as the study of embryology revealed a remarkable sequence of developmental steps of phylogenetic significance, so the steps in biochemical synthesis might reveal to us the evolutionary steps in these syntheses. Just as the embryologist in his contributions to evolution has used as his guiding principle the idea that "Ontogeny recapitulates phylogeny" so the biochemist working on a molecular level might use as his guiding principle of evolution the idea that "Biosynthesis recapitulates biogenesis."

SUMMARY

The two prominent pigments of the protoplasm of the earth, are heme or iron protoporphyrin and chlorophyll. The structure of one of these pigments, heme, has been analyzed in some detail to illustrate how different parts of this structure serve specific functions. The resonating ring of the porphyrin contributes to its stability, also making the molecule colored so that it absorbs light strongly in the visible region of the spectrum. The vinyl side chains of the porphyrin appear to be necessary if iron is to be incorporated into the porphyrin to form the iron protoporphyrin or heme molecule. The propionic acid side chains of the heme serve to attach the heme to the protein by virtue of the ionized carboxyl groups, the attachment of the negatively charged carboxyl groups being to two positively charged groups of the protein.

The two pigments are related biochemically. Studies of chlorella mutants have revealed that protoporphyrin 9 is the precursor of iron protoporphyrin as well as the precursor of chlorophyll. A scheme illustating the probable steps in biosynthesis from protoporphyrin to chlorophyll has been presented. It is suggested that the steps in the biosynthesis of this pigment recapitulate the steps in its evolution.

The two pigments are not only related structurally but they are related functionally as well. The heme pigment is the primary catalyst of biological oxidation. The chlorophyll pigment is the

primary catalyst of photosynthesis. The energy processes of protoplasm are in general limited to the narrow range of 1.2 electron volts, that is, a range between the potential of the hydrogen and the oxygen electrodes, this potential range being the expression of the processes involved in the oxidation and reduction of water. An energy scheme based on the decomposition and formation of H_2O is presented. Heme and chlorophyll are functionally related in being the catalysts of this process of decomposition and formation of water.

It is suggested that this basic plan of oxidation and photosynthesis involving water, might have been a plan of the living substance from its early inorganic beginnings. Further steps in evolution as indicated in the scheme of porphyrin synthesis would represent a progressive elaboration of mechanisms for carrying out these functions of oxidation and photosynthesis in a more efficient manner.

I wish to thank the committee of the Harvey Society for the honor bestowed upon me of addressing the Society. I must share this honor with Dr. Helena Gilder who explored with me the activities of the porphyrins on Hemophilus. But most of all I must share this honor with my good friend and teacher Dr. Leonor Michaelis whose daily example has been my constant inspiration, and of whose insight and advice I have had the great good fortune and privilege to be the recipient.

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