ON HEMOCHROMOGEN.

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I.

The Nature of Hemochromogen.

Introduction.—In a paper (1) concerned mainly with the rôle of the protein, globin, in determining the fitness of hemoglobin as an oxygen carrier, we presented, in a preliminary way, some observations on the nature of hemochromogen. Since then the study of hemochromogen has assumed an entirely new importance. On the one hand, it has been shown by Keilin (10) that substances related to hemochromogen are present in the aerobic tissues of plants and animals generally. On the other hand—as will appear much more clearly in later papers—hemoglobin has proved to be peculiarly suitable material for the study of the denaturation and coagulation of proteins, and when hemoglobin is denatured in alkaline solution in the presence of a reducer, hemochromogen appears. We have accordingly repeated in greater detail and extended our earlier work.

In hemoglobin the simple protein, globin, is combined, in a manner not yet known, with the iron pyrrol complex, $C_{34}H_{32}N_4O_4Fe$ which we call *heme*. The familiar crystalline substance known as hemin is the hydrochloride of heme. The pigment obtained when a reducing agent such as sodium hydrosulfite ($Na_2S_2O_4$) is added to heme we call *reduced heme*. It is necessary to introduce the word heme in order to be able to refer without confusion to a single substance of definite composition.

It was discovered by Stokes (12) that the addition of alkali and a reducing agent to hemoglobin produces a pigment with a well defined two-banded spectrum whose sharpness greatly impressed the noted physicist. Hoppe-Seyler (9) later gave this pigment the name hemo-

chromogen in order to express the *theory*, which has been generally accepted, that the pigment with the two-banded spectrum is identical with the simple iron pyrrol complex, reduced heme.

We define as a *hemochromogen* a substance containing heme whose spectrum has the characteristic pattern described by Stokes. This definition contains no hypothesis about the nature of hemochromogen or about its relation to reduced heme.

It was noticed over 30 years ago by Bertin-Sans and Moitessier (3) that if pure heme is reduced with sodium hydrosulfite, one does not obtain hemochromogen but that hemochromogen is obtained if one adds further to the solution some protein. No precise suggestion was made about the function of the protein. Despite this work the theory of the identity of reduced heme and hemochromogen continued to be accepted. Sodium hydrosulfite was considered to be an "unsuitable" reducing agent. It was well known that hemochromogen could be obtained from pure heme even without any protein by the use of a "suitable" reducing agent such as hydrazine hydrate. Later Dilling[1] (5) and von Zeynek (13) believed pyridine hemochromogen to contain pyridine in addition to reduced heme. Their notion was that pyridine combined with hemochromogen to give a hemochromogen derivative and that pyridine combined with hemochromogen merely because it was a base.

In this paper we propose to show that reduced heme and hemochromogen are different substances with different properties, and that every hemochromogen consists not simply of reduced heme but of reduced heme combined with some nitrogenous substance.

The Preparation and Properties of Reduced Heme and Hemochromogen.

If pure crystalline hemin is dissolved in $\frac{1}{5}$ N NaOH, in which it is readily soluble, and reduced with sodium hydrosulfite ($Na_2S_2O_4$) there results a sparingly soluble pigment, reduced heme, whose spectrum has a diffuse band in the red. When now an excess is added of any of a great variety of substances which *without exception* contain some

[1] "Hemochromogen acts as an acid and it becomes evident that it might combine with several bases, page 61."

"Pyridine as a base has the power of combining with the double hematin or hemochromogen radicle to form an ester-like substance, page 60."

nitrogen group, two striking changes occur—the precipitate formed by reducing the heme goes into solution, and the spectrum now shows in place of the diffuse band in the red the two sharp characteristic hemochromogen bands first described by Stokes. One can use for the synthesis of hemochromogen from reduced heme proteins like globin or egg albumin, potassium cyanide, hydrazine hydrate, amines, pyridine, pipiridine, ammonia, pyrrol itself, nicotine, and so on. The hemochromogens described in the past were all obtained in the presence of one or more of these substances.[2] The "suitable" reducers such as hydrazine hydrate contained nitrogen, the "unsuitable" ones such as sodium hydrosulfite were nitrogen-free. Although all these hemochromogen-forming substances contain some nitrogen group, not all contain an amino group. On the other hand, not all nitrogen-containing substances yield hemochromogen.

Crystallization.—Some, although not all of the hemochromogens can be crystallized. Dilling (5) found that one can readily obtain a permanent preparation of pyridine hemochromogen crystals by dissolving some hemin in a drop of pyridine on a microscope slide, covering the drop with a cover-slip, and then closing it in with Canada balsam and letting the solution reduce "spontaneously."

We repeated this experiment, using in addition however the reducing agent, sodium hydrosulfite, and obtained the crystals pictured by Dilling.

In the best of such preparations practically all the pigment is in the form of distinct crystals. There is always, however, in addition to the intensely colored crystals a faintly colored background of a very fine precipitate. One cannot see whether this precipitate is crystalline. In order to prove that pyridine hemochromogen can be crystallized it is necessary to show—and this Dilling omitted to do—that the large crystals as well as the fine precipitate give the hemochromogen spectrum, that the crystals are not simply heme whose absorption band is weak.

In the first place, both the crystals and the background are of the same color, a peculiar shade of orange. So they both probably consist of the same substance. We found, furthermore, with the aid of an oil

[2] Dilling did not realize that the various bases contained in his hemochromogens were all nitrogenous substances, or that nitrogen groups play any rôle in hemochromogen formation.

immersion lens and a Zeiss microspectroscope, that a background free of any visible crystals gave a hemochromogen spectrum much less intense than that given by a single group of crystals with a practically colorless background, one actually much less colored than the control background. This indicates that pyridine hemochromogen whatever its precise nature, is a definite crystallizable compound.

Differences between Hemochromogens.

The hemochromogens as a class, then, have certain characteristic properties in common which distinguish them from reduced heme. They are soluble in alkali and have a sharply defined two-banded spectrum, while reduced heme is only slightly soluble in alkali and has a diffuse spectrum. But although all the hemochromogens are similar in a general way, the precise properties of any given hemochromogen depend on what nitrogen substance is used in its preparation. To designate a particular hemochromogen we therefore prefix to the class name the name of the particular nitrogen substance used in its preparation, thus globin hemochromogen or ammonia hemochromogen.

Spectra.—The most convenient and precise although not the only way to distinguish the various hemochromogens is by means of the exact position of the sharp α band. With the aid of the Hartridge reversion spectroscope (6), one can detect and measure differences so small as two Ångstrom units. For example the α band of globin hemochromogen prepared by the addition of $\frac{1}{5}$ N NaOH and $Na_2S_2O_4$ to hemoglobin has its maximum absorption at about 27 Å. u. more to the red than the α band of ammonia hemochromogen.

Different preparations of hemochromogen from the same nitrogen substance always show the α band in exactly the same position. Particularly interesting here is the fact that the hemochromogen prepared from hemoglobin by the addition of alkali and a reducer is spectroscopically indistinguishable from the globin hemochromogen prepared by the addition to an alkaline solution of heme of a reducer and globin prepared by Schulz's method (11).

Color.—Since the individual hemochromogens differ in the positions and intensities of their absorption bands they must differ in color too. These differences are not in all cases apparent to the unaided eye. In

some cases the color difference is striking; for instance, that between pyridine hemochromogen which is somewhat orange and ammonia hemochromogen which is somewhat purplish.

Reduced heme and hemochromogen under the same conditions have very different properties and are, therefore, different substances. Since some second substance is needed for the preparation of hemochromogen from reduced heme and since all that the varied compounds which can be used as this second substance have in common is the possession of some nitrogen group, we conclude that in hemochromogen reduced heme is combined in some manner with a nitrogen group. This combination would account for the change in properties of the pigment when the nitrogen-containing substance is added. The differences in the nitrogenous substances would account for the secondary differences in the various hemochromogens.

Hemochromogen Equilibria.

We shall now describe a few experiments designed to bring out the nature of the reaction between reduced heme and nitrogen groups.

It takes a definite minimum concentration of nitrogenous substance to convert reduced heme into hemochromogen, that is, to produce a soluble pigment whose spectrum shows only the two sharp bands of hemochromogen and none of the characteristic absorption of reduced heme. This minimum concentration varies a great deal from one nitrogen substance to another. In no case is the number of nitrogen groups required less than the number of heme molecules. In some cases it is very much greater. For instance a 3 per cent solution of ammonia is not sufficient to convert reduced heme completely into hemochromogen.

If a solution of ammonia hemochromogen is evacuated, gaseous ammonia is pumped off and the spectrum of reduced heme replaces that of hemochromogen.

If 5 cc. of 28 per cent ammonia is added to 0.1 cc. of a 3 per cent solution in $\frac{1}{5}$ N NaOH of globin hemochromogen prepared from hemoglobin, the spectrum of globin hemochromogen is replaced by that of ammonia hemochromogen whose α band is about 27 Å. u. further towards the blue.

In three ways, therefore, the reaction between reduced heme and

nitrogen compounds is analagous to the reaction between hemoglobin and oxygen or carbon monoxide. It requires a definite concentration of free oxygen or carbon monoxide to give oxyhemoglobin or carbon monoxide hemoglobin. Likewise a definite concentration of nitrogen compound is needed to give hemochromogen. When an oxyhemoglobin solution is evacuated reduced hemoglobin appears. Likewise when an ammonia hemochromogen solution is evacuated, reduced heme appears. When carbon monoxide is bubbled through an oxyhemoglobin solution the spectrum of oxyhemoglobin is replaced by that of carbon monoxide hemoglobin. Likewise when ammonia is added to globin hemochromogen the α band is shifted to the position characteristic of ammonia hemochromogen. We conclude from these facts that just as there is the equilibrium:

$$Hb + O_2 \rightleftarrows Hb\ O_2$$

there is also the equilibrium:

$$\text{Reduced heme} + \text{Nitrogenous substance} \rightleftarrows \text{Hemochromogen}$$

and that hemochromogen is always partially dissociated into its two components, reduced heme and a nitrogen substance. In the case of hemoglobin the lower the temperature the more the equilibrium is shifted to the right in favor of oxyhemoglobin. We shall see that the same holds true in the case of the hemochromogen equilibrium.

As has just been described, if enough ammonia is added to globin hemochromogen the spectrum of globin hemochromogen is replaced by that of ammonia hemochromogen. If, however, less ammonia is added then the α band of the resulting solution is intermediate between the α band of globin hemochromogen and that of ammonia hemochromogen. The more ammonia is added, the nearer is the band to that of ammonia hemochromogen. These intermediate spectra can be imitated by means of optical mixtures of the spectra of the two hemochromogens such as are observed by looking at the same time through cells containing separate solutions of the two hemochromogens.

The mere fact that the α band of the solution becomes indistinguishable from that of NH_3 hemochromogen when enough ammonia has been added does not mean that all the globin hemochromogen has

disappeared. A small amount of globin hemochromogen cannot be detected spectroscopically in the presence of sufficient ammonia hemochromogen.

II.

The Reactions between Reduced Heme and Cyanide.

We shall now discuss the reactions of a particular nitrogenous substance, cyanide, with reduced heme. These reactions have a peculiar interest because due to cyanide's great affinity for reduced heme these reactions provide a means of determining the composition of cyan-hemochromogen and because cyanide is able in small concentration to inhibit tissue respiration.

The Two Cyanide Compounds.

Two distinct pigments can be obtained by adding cyanide to reduced heme (1) cyan-hemochromogen which has not hitherto been described and (2) what will be provisionally called the second cyanide compound of reduced heme. This non-commital name is given because the nature of the compound is not yet certain. If the concentration of cyanide is low enough one gets only cyan-hemochromogen, if high enough only the second compound. Intermediate concentrations of cyanide give mixtures of the two pigments. Cyan-hemochromogen has two typical hemochromogen bands designated α and β. The α band is much more intense than the β and has its maximum absorption at about 5535 Å. u. The spectrum of the second compound likewise has two sharp bands, in this case of about equal intensity. The α band is about 150 Å. u. toward the red of the corresponding band of cyan-hemochromogen. In mixtures of the two pigments the two α bands do not fuse. From the relative intensities of these two bands one can, therefore, obtain a rough idea of the relative concentrations of the two components of the mixture.

The Composition of Cyan-Hemochromogen.

The difficulty of isolating hemochromogen free of uncombined reduced heme and nitrogenous compound led to an attempt to determine the composition of hemochromogen by an indirect method. The following experiments are designed to determine how much cyanide

has to be added to reduced heme to convert it into hemochromogen, that is, to cause the complete replacement of the characteristic spectrum of reduced heme by that of hemochromogen. Four solutions of 2×10^{-4} M hemin in 1 per cent Na_2CO_3* are made up to contain .65, .75, 1, and 1.25 molecules of KCN per molecule of heme. After addition of the reducer $Na_2S_2O_4$, layers 1.5 cm. thick are examined spectroscopically. Solution .65 shows the α band of cyan-hemochromogen and marked reduced heme absorption in the yellow; solution .75 more hemochromogen and less reduced heme; and solution 1.25 no reduced heme but in addition to cyan-hemochromogen a small amount of the second cyanide compound. Since one molecule of cyanide is adequate to convert one molecule of heme into hemochromogen, cyan-hemochromogen cannot contain more than one cyanide group per heme.

The following control experiment shows that were 25 per cent of the pigment in solution 1 reduced heme, the reduced heme would be detectable spectroscopically, despite the presence of the hemochromogen. Solution 1 contains 2×10^{-4} M reduced heme in 1 per cent Na_2CO_3 plus 2×10^{-4} M KCN. Another solution is made up which contains $.5 \times 10^{-4}$ M or 25 per cent as much reduced heme and no cyanide. The two solutions, each 1.5 cm. thick, are placed together in front of the spectroscope and observed at the same time. One can distinctly see reduced heme in addition to the hemochromogen.

The Affinity of Reduced Heme for Cyanide.

The experiments which have just been described do not exclude the possibility that cyan-hemochromogen contains less than one cyanide group per heme for we have shown that hemochromogen is always partially dissociated into and in equilibrium with its two components, reduced heme and the nitrogenous substance. In the case of the typical hemochromogen, cyan-hemochromogen, there is the equilibrium mixture.

$$\text{Cyan-hemochromogen} \rightleftarrows \text{Reduced heme} + \text{Cyanide}.$$

*Na_2CO_3 was used instead of NaOH because, as R. Hill (7) has pointed out, the precipitation of reduced heme is slower in Na_2CO_3 than in NaOH. Our original experiments with NaOH, however, gave essentially the same results.

Thus if a solution of cyan-hemochromogen containing just enough cyanide to cause the disappearance of the reduced heme bands is warmed or diluted, the characteristic absorption of reduced heme reappears. In solution 1, therefore, part of the cyanide is combined with reduced heme as cyan-hemochromogen and part is free serving to drive the equilibrium to the left, to prevent much dissociation of the hemochromogen. If the concentration of free cyanide is small compared with 2×10^{-4} M, that is, if the affinity of reduced heme for cyanide is very great, then the free cyanide in solution 1 can be neglected and cyan-hemochromogen contains neither more nor less than one cyanide per heme. If, however, the free cyanide is not negligible, then cyan-hemochromogen contains less than one cyanide per heme.

Let us assume, although there is as yet no experimental basis for this assumption, that the concentration of free cyanide needed to convert a given fraction of the heme into hemochromogen is independent of the total concentration of the pigment. For any given value of the fraction $\frac{\text{Reduced heme}}{\text{Cyan-hemochromogen}}$ the lower the total concentration of pigment, the lower the concentration of cyan-hemochromogen or of bound cyanide. The concentration of free cyanide, however, according to our assumption is always the same. Therefore, the lower the total concentration of pigment the greater is the free cyanide relative to the bound. By lowering the pigment concentration enough one can make the free cyanide practically equal to the total cyanide added. This condition is met in the following experiments.

A solution is made up in 1 per cent Na_2CO_3, 3.75×10^{-6} M in respect to heme and 3.75×10^{-5} M, or 10 times more concentrated, in respect to cyanide. The pigment is reduced with $Na_2S_2O_4$. From the previous experiments the molarity of the bound cyanide is at most equal to the heme with which it is combined, that is at most 3.75×10^{-6} M and, therefore, less than 10 per cent of the total cyanide. The solution is examined spectroscopically through a polarimeter tube 20 cm. long. The intensity of the bands is about the same as that of a more concentrated solution in a narrower vessel. One can see only cyan-hemochromogen—no reduced heme and none of the second cyanide compound. If the cyanide concentration is doubled the

intensity of the α band of the hemochromogen is not visibly increased. If the cyanide concentration is halved, the α band is less intense and one can see reduced heme. To prevent any visible dissociation of cyan-hemochromogen then, when the cyan-hemochromogen concentration is 3.75×10^{-6} N in respect to iron, the concentration of free cyanide must be about 3.75×10^{-5} N.

To discover what differences in cyan-hemochromogen concentration are detectable, three solutions of cyan-hemochromogen are made up in 1 per cent Na_2CO_3. In all three cases the cyanide concentration is $2 \times 3.75 \times 10^{-5}$ N, that is more than adequate to convert reduced heme into hemochromogen. The heme concentrations are 3.75×10^{-6} N, 25 per cent more, and 25 per cent less. In none of the solutions can either reduced heme or the second compound be seen. The differences in the intensities of the cyan-hemochromogen bands, however, are distinct. Thus in the experiments already described, the formation of 25 per cent additional hemochromogen on doubling the cyanide concentration would be detectable.

The assumption has been made that the cyanide concentration needed to give a definite value of the fraction $\frac{\text{Reduced heme}}{\text{Cyan-hemochromogen}}$ is independent of the total pigment concentration. The percentage of reduced heme which is detectable spectroscopically is likewise roughly independent of the pigment concentration for a decrease in the pigment concentration is compensated for by an increase in the thickness of the layer examined. It follows that the concentration of free cyanide needed to prevent visible dissociation of cyan-hemochromogen is the same, namely about 3.75×10^{-5}, when the total heme concentration (reduced heme plus hemochromogen) is 2×10^{-4} M as in the first experiments as it is found experimentally to be when the total heme concentration is only 3.75×10^{-6} M.

When the total heme concentration is 2×10^{-4} M however, in order to prevent visible dissociation of the hemochromogen, the total cyanide concentration must be 2×10^{-4} M. If the free cyanide is only 3.75×10^{-5} or about $\frac{1}{5}$ of the total cyanide then it is within the rather large experimental error and can be neglected. The bound cyanide can be set equal to the total cyanide.

In brief the experiments with the relatively concentrated heme show

that cyan-hemochromogen does not contain more than one cyanide per heme. The experiments with the relatively dilute heme show that reduced heme in alkaline solution has a high affinity for cyanide, and taken together with the other experiments they indicate, although they do not prove conclusively, that cyan-hemochromogen does not contain less than one cyanide per heme.

The Second Cyanide Compound.

The solution containing 1.25 molecules of cyanide per molecule of heme shows in addition to cyan-hemochromogen the second compound. As the cyanide concentration is increased one sees more and more of the second compound and less cyan-hemochromogen. Finally the reduced heme is converted completely into the second compound and the addition of more cyanide causes no further change in the spectrum. Experiments similar to those with cyan-hemochromogen show that the concentration of free cyanide needed to convert reduced heme completely into the second cyanide compound is some 25 times as great as is needed to prevent the dissociation of cyan-hemochromogen.

The factors which determine the composition of the equilibrium mixture:

$$\text{Second compound} \rightleftarrows \text{Cn Hc} \rightleftarrows \text{CN} + \text{Reduced heme}$$

are the cyanide concentration, the total heme concentration, and the temperature, and the hydrogen ion concentration. The importance of this last factor will be discussed in a later paper. Thus in a mixture of the two cyanide derivatives, more of the second compound appears and less cyan-hemochromogen if first, more cyanide is added; second, the absolute amounts of cyanide and heme being kept constant, the concentrations of both are increased by using less water; and third, the solution is cooled.

The method used to determine the composition of cyan-hemochromogen is likewise applicable to the second compound. Much more free cyanide, however, is needed to prevent its dissociation than the dissociation of cyan-hemochromogen. In order that the free cyanide be negligible therefore in comparison with the bound cyanide, the total pigment concentration must be much greater than is the case with cyan-hemochromogen. In the concentrated solution it is difficult to

determine spectroscopically the precise number of cyanide molecules needed to convert a molecule of reduced heme completely into the second compound. We are prepared to state only that the number is a small one.

Our experiments to determine the relations of the second cyanide compound to other hemochromogens and to carbon monoxide hemochromogen will not be described here because they do not seem capable at present of any simple explanation. This applies especially to experiments in which cyanide is added to reduced heme in the presence of various concentrations of other nitrogen substances.

This investigation of the reactions of cyanide with reduced heme in alkaline solution has shown, so far as we know for the first time, that there are two distinct cyanide derivatives of reduced heme; that one is a typical hemochromogen in which cyanide behaves like a typical nitrogen substance; that reduced heme has a great affinity for cyanide; and that cyan-hemochromogen probably contains one cyanide group per heme.

There have been two attempts to determine the composition of pyridine hemochromogen. The first was made by von Zeynek (13) before the nature of hemochromogen was understood. He found solid pyridine hemochromogen to contain 2.2 molecules of pyridine per molecule of heme. Von Zeynek regarded his single experiment as only preliminary and gave no conclusive evidence that the solid analyzed was pure pyridine hemochromogen. Recently R. Hill (7) has concluded that pyridine hemochromogen contains two pyridine groups per heme. This conclusion is hardly justified by the experiments given and the soundness of the experiments we regard as questionable. Hill's work will be discussed in detail in a separate paper.

III.

The Reactions between Reduced Heme and Proteins.[3]

We shall now consider the reactions between reduced heme and the most complicated of the hemochromogen-forming substances, the proteins. The complication consists in the fact that the individual

[3] These experiments were described in the doctoral dissertations of the authors which were submitted to Cambridge University in the spring of 1926.

protein molecule contains a large number of free nitrogen groups. One cannot know in advance which and how many of these groups combine with reduced heme. These protein reactions are of special interest because it is by combination with a particular protein, native globin, to form hemoglobin that heme acquires the variety of properties which permit it to act as an oxygen carrier.

Globin.

The first experiments are an attempt to find out how much heme a given amount of globin can convert into hemochromogen. More and more heme is added to the protein until there appears in addition to the hemochromogen bands, the characteristic absorption of reduced heme.

A stock solution of globin hemochromogen is prepared by adding to 1 cc. of 3 per cent horse hemoglobin, 2 cc. of $\frac{1}{5}$ N HCL (which denatures the protein) and then adding NaOH. From this there is prepared a series of solutions all $\frac{1}{40}$ N in respect to NaOH, all containing .033 per cent of globin hemochromogen from hemoglobin, and finally containing in addition varying amounts of extra hemin. On the assumption that the molecular weight of globin is 16,700, the minimum molecular weight, the solutions contain 1, 2.5, 5, 7.5, 10, and 25 molecules of heme per molecule of globin. The solutions are reduced with $Na_2S_2O_4$ and examined spectroscopically.

The differences in the intensities of the hemochromogen bands are striking. The more heme in the solution, the darker and broader the hemochromogen bands. Only in solution 25 can any reduced heme at all be detected. The following experiments show that such results are not given by optical mixtures of reduced heme and hemochromogen such as are observed by looking through two cells at the same time which contain separate solutions of hemochromogen and reduced heme.

Cell 1 contains globin hemochromogen in the same concentration as in the experiments just described, and cells 2 and 3 contain reduced heme, the concentration in respect to heme being 2.5 times greater in cell 2 and 5 times greater in cell 3 than in the hemochromogen solution. Looking through the hemochromogen solution and cell 2

at the same time one can just distinguish reduced heme. When cell 3 is used instead of cell 2 the reduced heme absorption is marked. These experiments show that in the original experiments where reduced heme is added to globin hemochromogen and not kept in a separate cell there did not result a simple mixture of hemochromogen and reduced hemochromogen. One can detect reduced heme in such mixtures.

The fact that the original solution 10 shows no reduced heme and much more hemochromogen than solution 1 therefore indicates that a single molecule of denatured globin can convert at least 10 molecules of heme into hemochromogen.

On adding extra heme to globin hemochromogen two changes can be noted besides the increase in the intensity of the hemochromogen band. Solutions 10 and 25 are hazy and there is a shift of the α band to the red which in solution 25 amounts to about 10 Å. u. The haziness may be due to a decrease in the solubility of the molecule when the globin is loaded with heme; the shift of the band either to a change in the chemical composition of the pigment or in its dispersion.

Since the individual globin molecule seems to have a considerable number of nitrogen groups which can react with reduced heme to form hemochromogen, there must result theoretically, when denatured globin is added to reduced heme, a complicated equilibrium in which a number of nitrogen groups compete for the heme. Practically, however, this complexity is often of no great consequence. The hemochromogen prepared from hemoglobin is of constant composition and is not detectably dissociated into reduced heme at all, any more than acetic acid in strongly acid solution is dissociated—except to a minute theoretical extent—into acetate ions.

Whether extra heme hemoglobin corresponding to the artifical globin hemochromogens which contain extra heme can be prepared is, in the absence of experiments, an open question. Hill and Holden (8) estimate native globin by its oxyhemoglobin-forming capacity. This procedure depends on the tacit and unproven assumption that the composition of the synthetic hemoglobin is independent of the concentration of heme during the synthesis.

Edestin and Zein.

All proteins have not the same hemochromogen-forming capacity. Thus we have found that it takes much more edestin than globin to convert a given amount of heme into hemochromogen and more zein than edestin. These differences are not surprising. We have already seen from a study of the reactions between reduced heme and the simpler nitrogen compounds that the amount of hemochromogen present at equilibrium—the heme concentration and the temperature being kept constant—depends on the number and the nature of the nitrogen groups. It requires a minimum amount either of cyanide or ammonia to convert a given amount of reduced heme into hemochromogen, but it requires much less cyanide than ammonia. In the case of the different proteins the nature of the nitrogen groups and their number per gram of protein varies a great deal. It is not surprising that accompanying these differences in the number and nature of the nitrogen groups, proteins have differences in hemochromogen-forming capacity. The low hemochromogen-forming capacity of zein may be connected with its low diamino acid content (4).

The Difference between Globin Hemochromogen and Hemoglobin.

Based on the classical theory that hemochromogen and reduced heme are identical is the further theory which, so far as we know, has never been questioned that when hemoglobin is converted into hemochromogen the protein, globin, is split off from the iron pyrrol complex, heme. We have shown, however, that globin hemochromogen like hemoglobin itself is a compound of globin and heme. The question thus arises, as to what the difference is between hemoglobin and the hemochromogen obtained from it. We have already (2) pointed out one difference, namely that hemochromogen is a compound of heme and denatured globin while hemoglobin is a compound of native globin. The evidence given for this was that hemoglobin is a typical coagulable protein, and that the denaturation of hemoglobin in alkaline solution as tested for by solubility always runs parallel with formation of hemochromogen as tested for spectroscopically. There may be still other differences between hemoglobin and globin hemochromogen. We do not know whether these two substances have the same molecular weight or whether heme and globin are combined in the same way

in hemochromogen as in hemoglobin, or in precisely what way they are joined together in either compound.

Hill and Holden (8) have confirmed our view that globin hemochromogen is a denatured protein without seeing fit to mention our paper.

CONCLUSIONS.

1. Every hemochromogen consists of the iron pyrrol complex, reduced heme, combined with some nitrogenous substance.

2. In every hemochromogen there is the equilibrium:

$$\text{Hemochromogen} \rightleftarrows \text{Reduced heme} + \text{Nitrogenous substance.}$$

3. Cyanide can form two distinct compounds with reduced heme, one of which is the typical hemochromogen, cyan-hemochromogen.

4. Reduced heme in alkaline solution has a great affinity for cyanide.

5. Cyan-hemochromogen probably contains one cyanide group per heme.

6. The hemochromogen prepared from hemoglobin is a compound of denatured globin and reduced heme.

7. The individual molecule of denatured globin, of hypothetical molecular weight 16,700, can convert at least 10 molecules of reduced heme into hemochromogen.

8. The hemochromogen-forming capacity of globin is, under given conditions, greater than that of edestin, which in turn, is greater than that of zein.

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