THE REACTIONS OF CYANIDE WITH GLOBIN HEMOCHROMOGEN

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Hemochromogen and the Cyanide Compounds of Reduced Heme

Four pyrrol groups can combine to form a porphyrin. There are many different porphyrins with different side chains (Fischer, 1927). Each porphyrin can combine with iron to form a heme. The familiar hemin crystals are composed of the chloride of the particular iron-porphyrin or heme which is part of hemoglobin. Hemochromogen is defined as a pigment containing heme whose spectrum has the two characteristic bands first described by Stokes (1864).

In previous papers (Anson and Mirsky, 1925, 1928) it was shown that (1) Every hemochromogen consists of reduced heme joined to some nitrogen group; (2) Hemochromogen is always partly dissociated into and in equilibrium with its two components:

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

(3) Ordinarily, nitrogen substances react with reduced heme to form only one hemochromogen-like pigment. Cyanide, however, can react with reduced heme to form two different hemochromogen-like pigments; (4) The first cyanide compound of reduced heme contains one cyanide group per heme.

These results have been confirmed and evidence further given that the second cyanide compound contains two cyanide groups per heme (Hill, 1929).

The properties of cyanide are such as to permit of the estimation of the composition of the two cyanide compounds of reduced heme. But

it has not been clear what the relation is of these two compounds, both of which resemble hemochromogen spectroscopically, to an ordinary hemochromogen such as globin hemochromogen. The nitrogenous substances other than cyanide have the advantage of forming with heme only a single hemochromogen-like pigment which is undoubtedly a typical hemochromogen. But the properties of the other nitrogenous substances, as will be explained later in this paper, are such as to make difficult and uncertain the estimation of the compositions of the hemochromogens which they form from heme. As a result it has not been known whether hemochromogen in general contains one or two nitrogen groups per heme and it has not been known which of the two cyanide compounds is the typical hemochromogen, or why cyanide in particular forms two compounds with reduced heme. It was stated in the previous paper (Anson and Mirsky, 1928) that the relation of the cyanide compounds to ordinary hemochromogen was being studied by an investigation of the reactions of cyanide with heme in the presence of other nitrogenous substances.

The present experiments show that cyanide can react with globin hemochromogen in two entirely different ways. First, cyanide without displacing globin can combine with globin hemochromogen. In this reaction cyanide is *not* competing with globin for a place of attachment to the heme; it is behaving not like a typical nitrogenous hemochromogen-forming substance but probably like carbon monoxide. In addition, however, cyanide can displace globin from its combination with heme just as can ammonia and other nitrogenous substances (Anson and Mirsky, 1925, 1928). In the light of these results, the simplest although not the only explanation of the reactions of cyanide with heme in the absence of globin is that cyanide first combines with heme to form a typical hemochromogen containing one nitrogen group per heme and then combines with this cyan-hemochromogen just as it combines with globin hemochromogen.

The Biological Importance of the Hemochromogens

The biological interest in the chemistry of hemochromogen used to consist solely in its relation to the chemistry of hemoglobin which was the only heme pigment of known physiological importance. It is now clear that hemochromogens as such are of wide distribution and peculiar importance in nature. Pigments related to hemochromogen are present in aerobic tissues of plants and animals

generally in concentrations great enough to permit of direct spectroscopic observation (MacMunn, 1886; Keilin, 1925, 1926; reviewed in Anson and Mirsky, 1930b). In addition the investigations of Warburg and his colleagues show that there is universally present in much smaller amount another substance related to heme and probably to hemochromogen which plays an essential rôle in the catalysis of biological oxidations (reviewed in Warburg and Negelein, 1929, and Anson and Mirsky, 1930b).

The study of the heme pigments in nature has depended entirely on a knowledge of the chemistry of pure known heme compounds *in vitro*. It is therefore important to know as much as possible about the kinds of reactions in which heme pigments can take part. In particular the ability of heme to react with nitrogen groups is significant. Aerobic cells contain a substance which can catalyze oxidations. The catalysis stops when this respiratory enzyme is combined with carbon monoxide and goes on again when the carbon monoxide is dissociated from its compound with the enzyme by light (Warburg, 1926). Similarly heme can catalyze oxidations *in vitro* and it forms with carbon monoxide a catalytically inactive compound which can be dissociated by light. But the catalytic activity of the heme so far tried is much less than that of the cellular enzyme and the sensitivity of the heme's carbon monoxide compound to light is also much less. When heme is combined with some nitrogenous hemochromogen-forming nitrogen group then the catalytic activity of heme and the sensitivity of its carbon monoxide compound to light are very much increased (Krebs, 1928). In other words, combination with a nitrogen group makes heme more like the cellular respiratory enzyme (reviewed in Warburg and Negelein, 1929, and Anson and Mirsky, 1930b).

The Reactions of Globin and Cyanide with Heme

In the present experiments different amounts of cyanide are added to reduced heme in the presence of different concentrations of globin. From the spectra of the resulting solutions it can be decided when the cyanide is competing with globin for a place on the heme molecule and when the cyanide is not competing with globin. Before describing the experiments in detail it is necessary to recall what has already been shown to happen when either globin or cyanide alone is added to reduced heme.

If denatured globin is added gradually to an alkaline solution of reduced heme the spectrum of reduced heme is gradually and finally completely replaced by that of globin hemochromogen. The addition of further globin then causes no further change in the spectrum (Anson and Mirsky, 1928). If cyanide, however, is gradually added to reduced heme there appears first a typical hemochromogen spectrum whose α band is 50 Å to the blue of that of globin hemochromogen.

But then on further addition of cyanide there appears a second two-banded spectrum similar to but definitely different from that of a typical hemochromogen. The α band is 100 Å to the *red* of that of globin hemochromogen and is much weaker than the α band of either globin hemochromogen or the first cyanide compound. This second cyanide compound obtained by adding an excess of cyanide has long been known (see spectrum in Oppenheimer's Handbuch, 1909). The first cyanide compound, which can exist only in a narrow range of low cyanide concentrations, was not observed until recently (Anson and Mirsky, 1928).

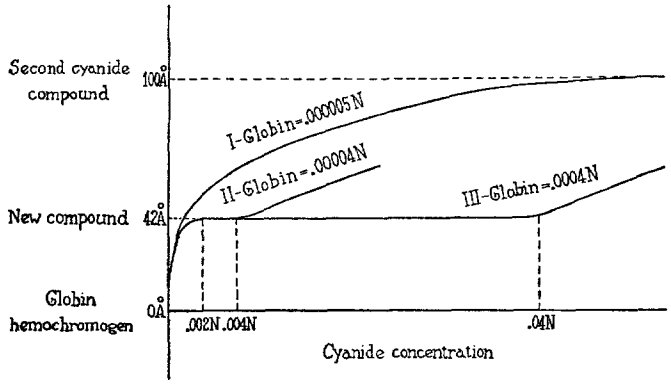


FIG. 1. Positions of α bands of pigments obtained on adding cyanide to globin hemochromogen.

The Reactions of Cyanide with Globin Hemochromogen

The new experiments are these:

1. To a dilute alkaline solution of globin hemochromogen prepared from hemoglobin (0.0084 per cent or 0.000005 N in respect to the iron of hemoglobin) there is added more and more cyanide. The α band is shifted more and more to the red, until after a shift of 100 Å it has reached the position characteristic of the second cyanide compound of reduced heme prepared by adding cyanide to heme in the absence of globin. (See Curve 1. In order to give a simple picture of all the phenomena in one graph, the graph has been made schematic and grossly inaccurate. The actual observations are given in the tables.)

2. More and more cyanide is added to globin hemochromogen in the presence of extra globin, the total globin concentration being 0.00004 N instead of 0.000005 N. The band moves to the red until when the cyanide concentration is 0.002 N, the band is 42 Å to the red of the band of globin hemochromogen. Doubling the cyanide concentration

TABLE I

CN / N	Position of α band — Å to red of 5585 Å		
	Globin = 0.0004 N	Globin = 0.00004 N	Globin = 0.000005 N
0	0	0	0
0.0001	18	18	16
0.0002	27	28	26
0.0004	33	34	33
0.001	39	40	44
0.002	41	42	49
0.004	43	43	
0.01		48	
0.02	42	57	

TABLE II

CN / Globin	Position of α band — Å to red of 5585 Å		
	Globin = 0.00008 N	Globin = 0.00004 N	Globin = 0.00002 N
0	0	0	0
125	45	43	
250	48	48	48
500	57	57	57
1,000	69	66	65
50,000			100
			No globin
			100

(making it 0.004 N) now causes no further change in the spectrum. But the addition of still more cyanide causes the band to move to the red again. (See Curve II.)

3. Finally, more and more cyanide is added to globin hemochromogen in the presence of a concentration of globin ten times greater still, namely 0.0004 N. Until the cyanide concentration is 0.004 N the

results are *precisely* the same as with the solutions containing ten times less globin. But whereas in the more dilute globin solution further addition of cyanide causes the band to shift to the red, in the solution 0.0004 N in respect to globin the cyanide concentration must be increased from 0.004 N to 0.04 N before the band is shifted to the red again. (See Curve III.)

4. Whenever the globin concentration is *greater* than 0.00004 N, the cyanide concentration is *less* than 0.002 N, and the band is accordingly *less* than 42 Å to the red of the band of globin hemochromogen, the position of the band depends solely on the cyanide concentration. Increasing the globin concentration has no effect on the spectrum so long as the cyanide concentration is kept constant.

5. Whenever the cyanide concentration is *greater* than 0.002 N and the band is *more* than 42 Å to the red of the band of globin hemochromogen, the position of the band depends solely on the *ratio* of the concentrations of cyanide and globin. Increasing the cyanide concentration has no effect on the spectrum provided only that the globin concentration is increased to the same extent.

6. The nature of the mixed spectrum given by a solution containing two different pigments depends on how far apart are the bands of the pigments. If the bands are far enough apart they do not fuse completely but can be distinguished separately in the mixed spectrum. For instance, the α bands of the first and second cyanide compounds of reduced heme which are 150 Å apart can both be seen in the spectrum given by a mixture of the two cyanide compounds (Anson and Mirsky, 1928). If the two bands of the two pigments are close enough together, however, they fuse to give a single band whose maximum absorption is at some wave length intermediate between the wave lengths at which the two individual pigments have their maximum absorptions. The more of one of the pigments is present the closer will the position of the intermediate band be to the band of that pigment. Thus the α bands of oxy and carbon monoxide hemoglobins (Hartridge, 1912) and of globin and ammonia hemochromogens (Anson and Mirsky, 1925, 1928) fuse to give intermediate bands whose positions are determined by the compositions of the mixtures. Finally, the bands of two pigments may not be close enough to mix to give a single intermediate band and still not far enough apart for two points of maximum

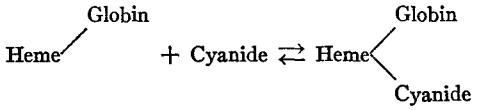
absorption to be distinguished easily in the mixed spectrum. In such cases the mixed spectrum has a band much broader than the band of a single pigment and this band becomes more and more asymmetrical as the relative concentration of one of the pigments in the mixture is increased.

In general the same mixed spectrum which is seen when one looks through a single cell containing a solution of two different pigments is also seen when one looks at the same time through two cells, one containing a solution of one pigment alone, the other containing a solution of the other pigment alone.

If one looks through two cells, one containing a solution of globin hemochromogen, the other a solution of the second cyanide compound of reduced heme, one sees a very broad asymmetrical band. It is impossible by means of optical mixtures of the spectra of globin hemochromogen and the second cyanide compound to imitate the single bands given by solutions of cyanide plus globin hemochromogen. All the spectra actually observed, however, can be imitated by means of optical mixtures of the spectra of globin hemochromogen, the second cyanide compound and the product with the band 42 Å to the red of the band of globin hemochromogen.

Two conclusions are drawn from the new facts:

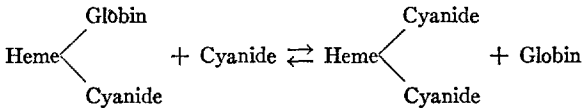
1. Cyanide can combine with globin hemochromogen without competing with or displacing globin to form a pigment with an α band 42 Å to the red of the band of globin hemochromogen.[1] In this reaction it is not behaving as a typical nitrogenous hemochromogen-forming substance but probably like carbon monoxide.



2. Cyanide can further react with cyanide-globin hemochromogen to form the second cyanide compound of reduced heme which contains

[1] Hill (1929) has observed an analogous pigment obtained by adding both nicotine and cyanide to heme. He believed that every hemochromogen contains two nitrogen groups per heme and that the compound of nicotine and cyanide with heme is a typical hemochromogen which happens to contain two different nitrogen groups.

two cyanide groups per heme. In this reaction cyanide is competing with and displacing the typical nitrogenous substance, globin.



From Curve I alone one might suppose that cyanide in reacting with globin hemochromogen simply displaces the globin and forms the second cyanide compound of reduced heme. On this basis any intermediate α band observed must be the result of the fusion of the α bands of globin hemochromogen and the second cyanide compound. This theory is disproved by the fact that one cannot imitate the observed spectra by means of optical mixtures of the spectra of globin hemochromogen and the second cyanide compound. To explain the mere existence of single intermediate bands one must assume the existence of a new compound with a band between the bands of globin hemochromogen and the second cyanide compound. Since the compounds of heme with either globin or cyanide alone do not have such a band, the new compound probably contains both globin and cyanide.

Curve III shows that cyanide first reacts with globin hemochromogen to form a new compound with a band 42 Å to the red of the band of globin hemochromogen and only then reacts further with this new compound. The extent of formation of the new compound is independent of the globin concentration, so globin is not being displaced. The new compound therefore contains globin as well as cyanide, a conclusion already drawn from Curve I.

If the new compound contains globin, and if cyanide reacts with the new compound to form the second cyanide compound which does not contain globin, then this reaction must involve the displacement of globin by cyanide. In confirmation of this the extent to which the second cyanide compound is formed from the new compound of cyanide and globin hemochromogen depends solely on the cyanide to globin ratio, which is precisely what one would expect were the cyanide and globin competing for the heme.

The reason why Curve I deviates from Curves II and III even before the cyanide concentration reaches 0.002 N is now clear. If the globin concentration is low enough some globin is displaced by cyanide even

if the cyanide concentration is not high enough to convert globin hemochromogen completely into cyanide-globin hemochromogen. To separate the two reactions the globin concentration must be above a certain minimum. Theoretically the two reactions can never be separated completely.

The fact that cyanide can displace globin from its combination with heme is most simply explained in the assumption that cyanide can react like a typical nitrogenous substance such as globin. This assumption, however, is not a necessary one. The affinity of hemoglobin for carbon monoxide depends on the hydrogen ion concentration; carbon monoxide and hydrogen ions compete for the hemoglobin and can displace each other. Yet these two groups do not react with hemoglobin in the same way. In all probability, carbon monoxide combines with the iron and the hydrogen ion does not, but combines with a group close to the iron and so influences the affinity of the iron for carbon monoxide.

Experimental Procedure

It remains to describe in detail the manner in which the values put down in the tables were obtained. In all cases the solutions were at O°C. in a cold room, the final concentration of NaOH was 0.5 N, solid sodium hydrosulfite ($Na_2S_2O_4$) was added as a reducer, and the bands read after 5 minutes with a reversion spetroscope (Hartridge, 1912). In some cases the bands were read again after several hours. No change was found. The purpose of the strong alkali was to bring out the hemochromogen-forming capacity of globin (Anson and Mirsky, 1928).

The three solutions of Table I were 0.000005 N, 0.00004 N and 0.0004 N in respect to globin. The 0.000005 N solution was prepared by adding 2.5 ml. of 0.2 N HCl to 1 ml. of 8.5 per cent horse carbon monoxide hemoglobin and then diluting with NaOH. This solution was accordingly 0.000005 N in respect to globin, heme and iron. The 0.00004 N and 0.0004 N solutions contained 0.00001 N globin and heme from hemoglobin and in addition denatured globin prepared by the oxalic acid-acetone method (Anson and Mirsky, 1930a).

The 0.00008 N solution of Table II contained 0.00002 N globin and heme from hemoglobin and in addition 0.00004 N globin prepared by the acetone method. To obtain the 0.00004 N and 0.00002 N solutions, the 0.00008 N solution was diluted with 0.5 N NaOH.

The band 42 Å to the red of the α band of globin hemochromogen is a sharp band intermediate in intensity as well as in position between the α bands of globin hemochromogen and the second cyanide compound of reduced heme.

When the cyanide to globin ratio reaches 1,000 the band begins to be too asymmetrical to be read with the reversion spectroscope.

Alkaline solutions of hydrosulfite attack the cements which were tried, including de Khotinsky cement. The decomposition products of the cements influence the spectra. This difficulty was avoided by using cells made of paraffin blocks and pieces of glass microscope slides. Such cells can be made in a few minutes. From paraffin blocks 1.5 cm. or 3 cm. thick depressions are melted out with a hot iron. These depressions are closed on the sides of the blocks by melting in pieces of glass. To make a double cell to permit looking through two solutions at once, a piece of glass which is to act as a partition is first melted between two blocks of paraffin. If cells only a few millimeters thick are desired, it is better to place slightly softened but not melted paraffin between two pieces of glass which are then pressed together in a vise until they are the desired distance apart. The glass sides of the cell are thus automatically made parallel. Finally some of the paraffin is melted out to form the cell.

The Nature of the Two Cyanide Compounds of Reduced Heme

A priori there are four possibilities in regard to the nature of the two cyanide compounds of reduced heme. Neither of the two cyanide groups may be a typical nitrogenous hemochromogen-forming group; both may be; cyanide may be acting as a typical nitrogenous group in forming only the first; or in forming only the second compound. The present experiments do not decide conclusively which of these four possibilities corresponds to the truth. What has been made clear is that cyanide can combine with heme in two different ways. In one reaction it does not compete with or displace globin; in the other it does. In the light of these results and of the fact that the spectrum of the first compound is similar to that of globin hemochromogen and the spectrum of the second compound is similar to that of cyanide globin hemochromogen, the simplest theory is that the first compound is a typical hemochromogen and the second compound a compound of cyanide with cyanide hemochromogen.

The Composition of Hemochromogen

If the first cyanide compound of reduced heme which has one cyanide group per heme is a typical hemochromogen, then it is probable that hemochromogens in general contain one nitrogen group per heme. It would obviously be desirable to confirm or refute this conclusion by independent investigation of the composition of some hemochromogen whose nitrogenous substance forms only one hemochromogen-like compound with heme. The attempts to do this have not yet led to any conclusive results. Von Zeynek found by analyses of

the precipitates that ammonia hemochromogen (1898) contains one nitrogenous group and pyridine hemochromogen (1910) two nitrogenous groups per heme. But such direct analyses must remain of dubious significance until it is shown that in the preparation of the precipitates no hemochromogen-forming nitrogen groups are split off from the heme and no non-hemochromogen-forming nitrogen groups added to the carboxyl groups.

Two molecules of pyridine must be added to each molecule of heme in order to convert heme into pyridine hemochromogen (R. Hill, 1926, 1929). If all the pyridine added were combined with the heme this would mean that pyridine hemochromogen contains two pyridines per heme. But some of the pyridine added must be free to drive the equilibrium:

$$\text{Pyridine hemochromogen} \rightleftarrows \text{Heme} + \text{Pyridine}$$

to the left. It cannot be decided from Hill's experiments whether or not this free pyridine is negligible. In dilute solution of the pigment the concentration of free pyridine needed to prevent visible dissociation of the hemochromogen may be determined. It is not known whether the same concentration of free pyridine is needed to prevent visible dissociation of the hemochromogen in the concentrated solutions actually used. As has already been pointed out (Mirsky and Anson, 1929) the equilibrium is complicated by the aggregation and precipitation of both pyridine hemochromogen and reduced heme:

$$\begin{array}{ccc} \text{Pyridine hemochromogen} & \rightleftarrows & \text{Reduced heme} + \text{Pyridine} \\ \downarrow & & \downarrow \end{array}$$

Precipitation or aggregation of the hemochromogen would drive the equilibrium to the left and so reduce the concentration of free pyridine needed to prevent detectable dissociation of the hemochromogen. Precipitation of the heme would have the opposite result. It is not known what effect concentration has on the relative velocities and extents of the aggregation of the two pigments.

Dr. Northrop has suggested that it might be possible to estimate the free pyridine in a solution of pyridine hemochromogen by shaking the aqueous solution with a solvent which does not mix with water but in which pyridine is soluble, estimating the total pyridine in this solvent, and then calculating the free pyridine in the water from the distribution constant.

Cyanide and Carbylamine

Warburg, Negelein and Christian (1929) have recently described some reactions of carbylamine with hemoglobin which in some ways resemble the reactions of cyanide with heme. Carbylamine can combine with hemoglobin in two different ways. In one reaction it competes with carbon monoxide, in the other it does not. Native globin in hemoglobin is not behaving like a typical nitrogenous substance such as ammonia or denatured globin. Whether one of the carbylamines is behaving like a typical nitrogenous substance has not yet been determined.

Conclusions

Cyanide can react with globin hemochromogen in two different ways. In the first reaction cyanide combines with globin hemochromogen without displacing or competing with globin. In the second reaction cyanide displaces globin.

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