33. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

2. FORMATION AND PROPERTIES OF CHOLEGLOBIN¹

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(Received 3 March 1941)

In a preliminary publication [Lemberg, Legge & Lockwood, 1938] we have reported that by the coupled oxidation of haemoglobin and ascorbic acid the prosthetic group of haemoglobin is oxidized. Under appropriate conditions a product, still mixed with haemoglobin, can be obtained, in which the new prosthetic group is combined with native globin. Acids easily split off iron from the product of oxidation and form bile pigments. These observations suggest that the compound is a bile pigment-iron-native globin complex, which we have called choleglobin.

In Part 1 of this series [Lemberg, Legge & Lockwood, 1939] we have discussed the initial mechanism of the reaction. In the present paper we describe in detail the preparation and properties of choleglobin, and we compare it with related substances which also contain easily detachable iron and yield bile pigment on acid splitting. Such substances are verdohaemochromogen obtained from pyridine-haemochromogen by coupled oxidation with ascorbic acid [Lemberg, Cortis-Jones & Norrie, 1938], and the 'pseudohaemoglobin' obtained by Barkan & Schales [1937; 1938] by the action of hydrogen peroxide on haemoglobin in the presence of cyanide.

EXPERIMENTAL

Preparation of choleglobin. We have used solutions of crystalline horse oxy-haemoglobin prepared by the method of Heidelberger [1922], and also washed erythrocytes of sheep, ox and horse, laked by freezing and freed from stroma by adjusting the pH to 5.8 and keeping in the refrigerator. All these solutions behaved qualitatively in the same way.

At first the solutions were Seitz-filtered and the experiments carried out under strictly sterile conditions, but this precaution was later found to be unnecessary for experiments up to 2 hr. incubation time. No signs of bacterial contamination were observed and the rate of formation of the choleglobin absorption band was the same as in experiments under sterile conditions.

The haemoglobin concentration varied between 0.3 and 3%, the majority of experiments being carried out with a haemoglobin solution of about 0.7%. Crystalline ascorbic acid (B.D.H.) was used. Its concentration in the reaction mixture was usually 100 mg. per 100 ml. The solutions were buffered with phosphate; phosphate is not necessary for the reaction with ascorbic acid, choleglobin being formed without the addition of buffers, or with borate buffers. The

¹ The work was carried out under a grant from the National Health and Medical Research Council of Australia.

majority of experiments were carried out either at pH 7.2, which was obtained by adding 20 ml. of *M*/20 phosphate buffer pH 6.6 to 30 ml. of a 1.2% haemoglobin solution, or at pH 8.5 obtained by adding 5 ml. of a solution of disodium hydrogen phosphate saturated at room temperature (20°) to 45 ml. of an 0.8% haemoglobin solution. The final haemoglobin concentration was thus 0.72%.

The reaction mixtures were incubated at 37° in flat culture flasks exposed to atmospheric oxygen without shaking in a layer of 4 mm. depth. A few experiments were carried out in Warburg vessels with shaking.

Spectroscopic observations

Choleglobin. Under these conditions the absorption band of reduced choleglobin at 628–630 μ appears a few minutes after the reaction has been started by the addition of ascorbic acid. There was no noticeable formation of reduced haemoglobin and the bands of oxyhaemoglobin remained visible. The band in the orange can be due neither to methaemoglobin nor to methaemalbumin [Fairley, 1938; 1939], since addition of $\text{Na}_2\text{S}_2\text{O}_4$ does not destroy it, but increases its strength. Alkali shifts the band to 618–622 μ , transforming choleglobin into denatured globin-cholehaemochromogen. If pyridine is added to the choleglobin solution until the precipitate of denatured globin-haemochromogens is redissolved and $\text{Na}_2\text{S}_2\text{O}_4$ added at once, there is also only the band of cholehaemochromogen at 618–620 μ beside the bands of protohaemochromogen in the green. $\text{Na}_2\text{S}_2\text{O}_4$ must be added without delay in order to stop the formation of verdohaemochromogen from pyridine protohaemochromogen by coupled oxidation with the excess of ascorbic acid.

During the incubation the absorption band of reduced choleglobin gradually increases in intensity, but later, after about 30 min. of incubation, it is replaced by an absorption band at about 670 μ . On reduction with $\text{Na}_2\text{S}_2\text{O}_4$ this band gives place to the band of reduced choleglobin at 630 μ ; it is therefore due either to ferric choleglobin or to oxycholeglobin. Carbon monoxide also causes the disappearance of the band at 670 μ and the formation of a strong band at 628 μ . The position of this band is very close to that of reduced choleglobin and may lead to the assumption that CO acts only as an inert gas allowing the excess ascorbic acid to reduce the oxidized choleglobin. It can, however, be proved that a CO-choleglobin is formed. Shaking with a mixture of 20% O_2 and 80% CO causes the band at 670 μ to be replaced by that at 628 μ , while shaking with air leaves the band at 670 μ unaltered. CO also reacts with the alkaline solution of cholehaemochromogen shifting the absorption band from 618 μ to 628 μ . CO-choleglobin and CO-cholehaemochromogen thus have their absorption bands in the same position, just as do CO-haemoglobin and CO-protohaemochromogen. In the chole-series, however, the CO-compound has an absorption very similar to that of the reduced choleglobin, while in the proto-series the bands of the CO-compound differ widely from that of reduced haemoglobin.

These observations do not offer sufficient proof that the oxidized form of choleglobin is oxycholeglobin with O_2 reversibly bound to ferrous choleglobin. Ascorbic acid is still present in the solution and CO may not react directly with the oxidized compound, but by combining with ferrous choleglobin it may shift an equilibrium between ferric and ferrous forms, normally much in favour of the ferric form, in favour of the ferrous form, until the formation of CO-choleglobin is practically complete. Experiments carried out in order to decide whether the oxidized form of choleglobin is ferric or contains O_2 reversibly bound, will be described later.

Cholehaemochromogen. After about 45 min. incubation a green precipitate begins to form as a bottom layer and as a thin surface skin. We shall call this precipitate 'green pigment'; it has been previously described by Anderson & Hart [1934] and by Edlbacher & v. Segesser [1937]. After 80 min., more green pigment has been formed which, up to this stage, is almost free from protohaemochromogen. It is insoluble in water, soluble in dilute alkali, 1 % acetic acid or pyridine. These solutions are olive-green. On reduction with $\text{Na}_2\text{S}_2\text{O}_4$ they turn pure green, but seen against artificial light the colour is brown. The absorption spectrum of the reduced alkaline solution shows, beside weak protohaemochromogen bands, the band of ferrous cholehaemochromogen at 616–618 $\text{m}\mu$, which is shifted by CO to 628 $\text{m}\mu$. In pyridine the band lies at 619 $\text{m}\mu$, while in dilute acetic acid or in neutral aqueous suspension the band is found at 628 $\text{m}\mu$. Green pigment consists thus mainly of denatured globin choleparahaematin (ferric cholehaemochromogen). The absorption spectrum of the unreduced ferric compound varies with the conditions. In pyridine there is a broad band with its centre at about 612 $\text{m}\mu$ and a second band at 665 $\text{m}\mu$. This second band is not that of verdohaemochromogen, since it is abolished by $\text{Na}_2\text{S}_2\text{O}_4$ reduction, while the similar absorption band of verdohaemochromogen is not altered by $\text{Na}_2\text{S}_2\text{O}_4$ in neutral pyridine solution. In 1 % Na_2CO_3 there is only an indistinct absorption band at about 595 $\text{m}\mu$, while in $N/15$ NaOH a sharp band at 646.5 $\text{m}\mu$ appears. The solution in dilute acetic acid displays an absorption band at about 610 $\text{m}\mu$.

Although the absorption spectrum of ferrous cholehaemochromogen in neutral suspension or in weakly acid solution does not differ from that of choleglobin, the two compounds differ totally in solubility. Cholehaemochromogen is insoluble at neutral reaction and is quantitatively precipitated from its alkaline or acid solutions by neutralization or by dialysis against distilled water. Choleglobin is soluble at neutral reaction and this solubility is not due to the protective colloid action of admixed haemoglobin. A haemoglobin solution of pH 7.2 or 8.5 does not dissolve green pigment; this can be quantitatively removed by filtration and no choleglobin band is observed in the filtrate. Choleglobin, however, can be redissolved in water after precipitation with ammonium sulphate, and can be absorbed on aluminium hydroxide and eluted from it by a phosphate buffer of pH 8.5.

Denaturation of choleglobin. It is of interest that there is no denaturation of oxyhaemoglobin under the conditions of our experiment, if ascorbic acid is absent. Two explanations of the denaturation leading to green pigment are possible. Either choleglobin is less stable than haemoglobin and undergoes spontaneous denaturation similar to that of free native globin; or the denaturation is due to an oxidation of the globin part of the haemoglobin molecule accompanying the oxidation of the prosthetic group. After the removal of ascorbic acid, choleglobin, though undergoing secondary changes to be described later, does not appear to be very readily denatured. It might be assumed, however, that the replacement of the four groups of haemoglobin by cholehaem groups does not occur simultaneously and that choleglobins with one or two bile pigment-haem groups are stable, while a choleglobin with all four haem groups replaced by cholehaem would undergo spontaneous denaturation. We have recently shown [Lemberg, Norrie & Legge, 1939] that catalase is probably a compound with three protohaematin and one bile pigment-haematin group per molecule. We were indeed, unable to obtain a choleglobin solution with more than 60 % cholehaematin and less than 40 % of protohaematin.

However this may be, the second explanation certainly also holds. Holden and his collaborators [Holden, 1936; 1937; Rawlinson, 1939] have repeatedly

described oxidative denaturations of haemoglobin. Lemberg & Legge [1938] have shown that a part of the oxygen evolved from oxyhaemoglobin by acids is taken up through oxidation of the protein part of the molecule. While up to 80 min. incubation with ascorbic acid there is little denaturation of haemoglobin, after 2 hr. the precipitate contains a considerable proportion of ferric denatured globin-protohaemochromogen (cathaemoglobin), and the longer the reaction proceeds, the more haemoglobin is denatured. There appear to be also further changes in the nature of the protein in the precipitate; its solubility in alkali and pyridine decreases. Finally, as will be described in the fifth paper of this series, the prosthetic groups, both protohaem and cholehaem, are bound to the protein of the precipitate much more firmly than in the choleglobin solution or in the haemochromogens produced from it by alkali denaturation.

These observations enhance the steadily increasing amount of evidence that the products obtained by 'denaturing' one and the same protein by different methods may differ considerably (cf. the discussion of this problem in the *Cold Spring Harbor Symposia on Quantitative Biology* [1938]).

*Products of the later stages of the reaction and of further oxidation
of cholehaemochromogen*

After incubation for several hours there is a great deal of precipitate, now more brown than green. The supernatant solution contains oxidized choleglobin, but the band at $670\text{ m}\mu$ no longer disappears completely on reduction with $\text{Na}_2\text{S}_2\text{O}_4$ or with CO. On addition of pyridine and $\text{Na}_2\text{S}_2\text{O}_4$ an absorption band at $665\text{ m}\mu$, in addition to the cholehaemochromogen band at $618\text{ m}\mu$, appears even if $\text{Na}_2\text{S}_2\text{O}_4$ is added at once to the pyridine solution. The compound which causes the band at $665\text{ m}\mu$ may be verdohaemochromogen, and verdoglobin¹ may be present in the supernatant solution before addition of pyridine. Addition of alkali to the reduced solution decreases the strength of the band in the red, and reduced verdoglobin behaves in a similar way, being transformed into a yellow compound. The strength of the cholehaemochromogen band at $618\text{ m}\mu$ appears, however, to be increased by the addition of alkali; verdoglobin is not transformed into cholehaemochromogen by alkali, and if the increase of the band at $618\text{ m}\mu$ accompanying the decrease of the band in the red is due to a transformation of the compound causing this band into cholehaemochromogen, the compound cannot be verdoglobin.

A substance with a similar absorption band in the red is produced if a pyridine solution of 'green pigment', which contains practically no protohaemochromogen, is left standing in the presence of air and ascorbic acid. Brown solutions are obtained which on reduction with $\text{Na}_2\text{S}_2\text{O}_4$ no longer display the absorption band of cholehaemochromogen, but a band at $657\text{ m}\mu$ and a weaker band at $534\text{ m}\mu$. This compound is not formed if the ascorbic acid is washed away from the 'green pigment' before dissolving it in pyridine, or if the solution is left standing *in vacuo*. There can be no doubt, therefore, that the new compound is an oxidation product of cholehaemochromogen, formed from it by coupled oxidation with ascorbic acid in pyridine. In its absorption spectrum it resembles verdohaemochromogen and also in its behaviour towards $\text{Na}_2\text{S}_2\text{O}_4$ in alkaline solution. Nevertheless its identity with verdohaemochromogen is doubtful. The latter is green and gives rise to yellow compounds only by the action of $\text{Na}_2\text{S}_2\text{O}_4$ in alkali, while the colour of the oxidation product from cholehaemochromogen is brown in the ferric state as well as when reduced in neutral pyridine solution.

¹ Cf. Lemberg & Wyndham [1937, p. 352]. We prefer now to call the compound of verdohaem with native globin verdoglobin in spite of the fact that it does not reversibly unite with oxygen.

Absorption curves

The absorption curves of choleglobin and its derivatives were investigated with a Visible Absorption Spectrophotometer of Adam Hilger, consisting of the Barfit Hilger Constant Deviation Wavelength Spectrometer with prism n_D 1.62 and the Barfit Hilger Nutting Photometer on the Standard Accessory Bar.

Fig. 1 shows the absorption curves in the region from 740 to 600 $m\mu$ of the oxygenated choleglobin solution (full-drawn line), that of the same solution after reduction with $\text{Na}_2\text{S}_2\text{O}_4$ (dotted line) and again after saturation with coal gas (interrupted line). The absorption curves in the region of 600–500 $m\mu$ were mainly those of the accompanying haemoglobin compounds, the maximal

Fig. 1.

Fig. 1. Choleglobin. Curve A (full line), oxygenated. Curve B (dotted line), reduced. Curve C (interrupted line), CO.

Fig. 2.

Fig. 2. Cholehaemochromogen. Curve A (full line), green pigment in 1% Na_2CO_3 . Curve B (dotted line), reduced with $\text{Na}_2\text{S}_2\text{O}_4$. Curve C (interrupted line), reduced with $\text{Na}_2\text{S}_2\text{O}_4$ in coal gas.

density of the CO-haemoglobin at 570 $m\mu$ being 1.63 times as strong as that of the CO-choleglobin at 630 $m\mu$. The absorption band of the oxygenated solution at 670 $m\mu$ is far less marked than when viewed through a pocket spectroscope. Reduced choleglobin and CO-choleglobin both show a strong absorption band at 630 $m\mu$, that of the CO-compound being somewhat sharper. Their absorptions in the wave-length region of 700–655 $m\mu$ are markedly less than that of oxygenated choleglobin.

The absorption curves of some cholehaemochromogen derivatives are given in Fig. 2. (The fully drawn line represents the absorption curve of green pigment dissolved in 1% Na_2CO_3 , the dotted line that of the ferrous cholehaemochromogen obtained by reduction with $\text{Na}_2\text{S}_2\text{O}_4$.) CO without reducer has no effect on the absorption curve of ferric cholehaemochromogen, but in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ the band of the ferrous CO-cholehaemochromogen at 628 $m\mu$ appears which is stronger and sharper than that of the CO-free compound. The opposite

is true with regard to the bands of protohaemochromogen at 559 $m\mu$ and of CO-protohaemochromogen at 570 $m\mu$.

From the appearance of these maxima in the absorption curves of 'green pigment' derivatives it is evident that green pigment contains some protohaemochromogen. The ratio $\epsilon_{630} : \epsilon_{570}$ of its CO-haemochromogen was 1.70. We have obtained other preparations with a higher ratio, the highest being 2.08. The last-mentioned preparation gave only a just noticeable hump at 560 $m\mu$ due to ferrous protohaemochromogen. If we assume as a first approximation: ϵ_{570} of cholehaemochromogen = ϵ_{570} of CO-cholehaemochromogen = ϵ_{557} of cholehaemochromogen = ϵ_{557} of CO-cholehaemochromogen = x , and ϵ_{570} of CO-protohaemochromogen = y , we have two equations with two unknowns:

$$\epsilon_{570}^{\text{CO}} = x + y, \quad \epsilon_{557} = x + 2.14y,$$

the factor 2.14 being the measured ratio ϵ_{557} of protohaemochromogen to ϵ_{570} of CO-protohaemochromogen. If we substitute the values of $\epsilon_{570}^{\text{CO}}$ and ϵ_{557} from the curves in Fig. 2, we obtain $x = 0.99$ and $\epsilon_{630} : \epsilon_{570}$ of CO-cholehaemochromogen = $2.33 : 0.99 = 2.35$. From this figure and the highest figure actually observed, we may safely conclude that 2.22 for this ratio is a good approximation; this figure will be used in Part 3 for establishing the absolute absorption values of choleglobin and cholehaemochromogen.

Does choleglobin combine reversibly with oxygen?

The question whether or not choleglobin unites reversibly with oxygen has an important bearing on the problem of the 'labile iron' in blood which we shall discuss in Part 4. We have therefore studied it in some detail.

A solution of choleglobin, obtained by 90 min. incubation of oxyhaemoglobin with 100 mg. per 100 ml. ascorbic acid at pH 8.5 under the conditions described above, was left at room temperature for 16 hr. in order to complete the oxidation of ascorbic acid, and then filtered. This solution failed to show any oxygen uptake in a Warburg respirometer during 10 min., but still contained small amounts of indophenol-reducing substance in the trichloroacetic acid filtrate (less than 3 mg. ascorbic acid per 100 ml.). Fig. 3 shows the effect of coal gas with acid but without added $\text{Na}_2\text{S}_2\text{O}_4$ on the absorption curve of the choleglobin (oxygenated solution fully drawn line). CO without reducer (interrupted line) still causes the appearance of a distinct absorption maximum at 630 $m\mu$ and a decrease of absorption at 670 $m\mu$, but the effect of CO plus reducer is more marked. Similar results were obtained with a solution of choleglobin dialysed for 16 hr. against distilled water. On exhaustion of a solution of CO-choleglobin in a Thunberg tube, followed by reoxygenation, the band at 630 $m\mu$ disappeared and was again replaced by the band at 670 $m\mu$. These observations lend some support

Fig. 3. Reaction of choleglobin with carbon monoxide. Curve A (full line), oxygenated. Curve B (interrupted lines), in coal gas. Curve C (dotted line), in coal gas with $\text{Na}_2\text{S}_2\text{O}_4$.

to the assumption that the oxygenated form of choleglobin reacts with CO and is, therefore, ferrous oxycholeglobin, with oxygen bound reversibly as in oxyhaemoglobin. The presence of small amounts of reducing substances in the solution does not, however, allow a definite conclusion.

We have attempted to remove the last traces of reducing substances by prolonged dialysis or by precipitation with ammonium sulphate followed by careful washing of the precipitate with saturated ammonium sulphate solution. Solutions were thus obtained which no longer reacted with CO without $\text{Na}_2\text{S}_2\text{O}_4$ (Fig. 4). The oxygenated solution, however, failed now to show the typical absorption band at $670\text{ m}\mu$ of the original oxygenated choleglobin. During the dialysis some methaemoglobin was formed from oxyhaemoglobin, and if oxycholeglobin exists,

Fig. 4.

Fig. 4. Dialysed choleglobin and carbon monoxide. Curve A (full line), oxygenated. Curve B (interrupted line), in coal gas. Curve C (dotted line), in coal gas with $\text{Na}_2\text{S}_2\text{O}_4$.

Fig. 5.

Fig. 5. Effect of ferricyanide on oxygenated choleglobin. Curve A, oxygenated choleglobin. Curve B, the same after addition of ferricyanide.

it may be more easily transformed into a metacholeglobin than oxyhaemoglobin into methaemoglobin and thus lose its property of combining with CO without $\text{Na}_2\text{S}_2\text{O}_4$. This view is supported by the observation that ferricyanide destroyed the band at $670\text{ m}\mu$ and decreased the absorption in this wave-length region (Fig. 5), but we failed to restore the band at $670\text{ m}\mu$ by reduction of the ferricyanide-treated solution with an amount of $\text{Na}_2\text{S}_2\text{O}_4$ just sufficient to reduce the accompanying methaemoglobin to reduced haemoglobin and subsequent oxygenation; oxyhaemoglobin could be thus reconstituted.

Evacuation of a fresh choleglobin solution also caused the absorption band at $670\text{ m}\mu$ to disappear and produced the band of reduced choleglobin, but the reaction proceeded more slowly than the transformation of oxyhaemoglobin into reduced haemoglobin. Reoxygenation yields again the band at $670\text{ m}\mu$. In long-dialysed choleglobin solutions evacuation had no effect.

Another attempt was made starting from ferric choleglobin cyanide, the preparation of which is described below. Cyanide was removed by precipitation with ammonium sulphate from a solution reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and by washing the precipitate with a saturated ammonium sulphate solution containing some phosphate buffer pH 7.6 and some $\text{Na}_2\text{S}_2\text{O}_4$. The precipitate was dissolved in water and the resulting solution reoxygenated. In this way the accompanying cyanmethaemoglobin was transformed into oxyhaemoglobin. There was an absorption band at about $680\text{ m}\mu$ and CO failed to produce the band of CO-choleglobin without addition of $\text{Na}_2\text{S}_2\text{O}_4$. The band at $680\text{ m}\mu$ differed, however, from that of the oxygenated choleglobin solution produced by ascorbic acid by not being removed by $\text{Na}_2\text{S}_2\text{O}_4$; it is, therefore, due to a ferrous haematin compound different from choleglobin, and not to oxygenated choleglobin. Again this throws no light on the nature of the compound responsible for the band at $670\text{ m}\mu$ in oxygenated choleglobin solutions. Since we failed in our attempts to remove the last traces of reducing substances from choleglobin solutions without altering the oxygenated compound, we cannot decide whether this is metacholeglobin or oxycholeglobin; if oxycholeglobin exists, it must be more labile than oxyhaemoglobin.

Action of hydrogen peroxide on haemoglobin in the presence of cyanide

By the action of hydrogen peroxide on oxyhaemoglobin solutions containing potassium cyanide Barkan & Schales [1938] have obtained a green solution showing the following absorption spectrum after reduction with $\text{Na}_2\text{S}_2\text{O}_4$: I, 617–620, II, 564–568, III, 536–540 $\text{m}\mu$. In repeating these experiments we found concentrations of 2% cyanide and 0.05–0.08% hydrogen peroxide optimal for the transformation into the green compound. The authors recognized that the absorption spectrum is due to the presence of two compounds, one causing the absorption band in the orange and the other the two bands in the green. The first substance they call 'pseudohaemoglobin' assuming that it consists of a bile pigment-iron complex bound to native globin. About the nature of the second substance they offer no definite suggestions, but apparently they believe that it is another product of oxidation of haemoglobin, no longer containing protohaem.

We will discuss this compound first, since the understanding of its nature will help us to understand that of the compound with the band at 617–620 $\text{m}\mu$. The two bands in the green are those of ferrous denatured globin-cyan-protohaemochromogen. The bands can be produced without application of hydrogen peroxide by keeping an oxyhaemoglobin solution containing 2% cyanide 24 hr. at room temperature. Before reduction only one band at $542\text{ m}\mu$ is seen which is that of cyancathaemoglobin (denatured globin-ferric protohaemochromogen cyanide). The denaturation of the globin is caused by the alkalinity of the cyanide. In the presence of hydrogen peroxide there is a more rapid oxidative denaturation, complete in 20 min. as shown by the change of the absorption spectrum of oxyhaemoglobin into that of cyancathaemoglobin. On careful neutralization or on dialysis against a neutral buffer complete precipitation occurs. Both the 'pseudohaemoglobin' and the compound with the bands in the green contain, therefore, denatured globin and not native globin.

Barkan & Schales have overlooked the identity of their second compound with ferrous cyanprotohaemochromogen probably because they observed a few spectroscopic properties, which hitherto have not been described for ferrous cyanprotohaemochromogen. In the weakly cyanide-alkaline solution and faster still in a neutral suspension or in a weakly acid solution the bands at 566 and

536 $m\mu$ of ferrous cyanprotohaemochromogen disappear and are replaced by a single rather diffuse band at 558 $m\mu$, while in a more strongly alkaline solution they remain unaltered. NaOH added to the solution with the diffuse band at 558 $m\mu$ restores the original double-banded spectrum. As far as we know, the compound with the single band has so far not been described and its nature is unknown.¹

Under conditions in which the single-banded compound arises from ferrous cyanprotohaemochromogen, CO transforms the latter at once into CO-protohaemochromogen (absorption bands at 570.5 and 540 $m\mu$), while it does not react with it in strongly alkaline solutions. In a weakly cyanide-alkaline solution the position of the bands indicates an equilibrium between CO- and CN-compounds, which depends on the cyanide concentration and on the pH of the solution.

Under all these conditions the absorption bands in the green produced by the action of hydrogen peroxide behave exactly in the same manner as the bands of cyanprotohaemochromogen.

These observations in the protohaematin series allow us to interpret the nature of 'pseudohaemoglobin' and to explain the changes of its absorption studied by Barkan & Schales in a simple and more correct way. Dialysis, which removes the cyanide, transforms the compound into a substance with all the properties of cholehaemochromogen. 'Pseudohaemoglobin' differs from cholehaemochromogen in not reacting with CO in strongly alkaline solution reduced by $\text{Na}_2\text{S}_2\text{O}_4$; while coal gas shifts the band of cholehaemochromogen from 618 to 628 $m\mu$, the band of 'pseudohaemoglobin' at 618 $m\mu$ remains unaltered. The compound is, therefore, a cyanide-compound, a conclusion also reached by Barkan. Evidently the substance is ferrous denatured globin cyancholehaemochromogen. Its behaviour towards CO is very similar to that of the corresponding protohaematin compound which accompanies it; it reacts with it only in acid or in weakly alkaline solution, in the latter somewhat less completely than the protohaematin compound.

Barkan & Schales believe that the dialysis of 'pseudohaemoglobin' causes an essential alteration changing 'pseudohaemoglobin' into 'pseudohaemochromogen'. If these names were taken at their face value, this would mean that the dialysis causes a denaturation of protein, which cannot be correct, the protein in 'pseudohaemoglobin' being already denatured. If we understand the authors correctly, they mean a slight alteration of the prosthetic group. Their evidence is based on their observations that the fresh cyanide-alkaline solution shows a shift of the absorption band with CO towards the infra-red while a dialysed solution to which the same amount of cyanide has been added, fails to show this shift. This is further complicated by another observation that if the order is reversed and the dialysed compound first transformed into the CO compound (band at 628 $m\mu$), cyanide added now fails to shift the band back to 618 $m\mu$. We cannot confirm these rather confusing observations. Provided that pH and cyanide concentration were actually restored, the dialysed solution to which cyanide had been added behaved exactly as the undialysed solution. Barkan's observation may be explained as follows: the fresh solution still contains some excess hydrogen peroxide and its reaction with $\text{Na}_2\text{S}_2\text{O}_4$ tends to acidify the

¹ Dicyanprotohaemochromogen, obtained by dissolving haemin in 10% KCN and adding $\text{Na}_2\text{S}_2\text{O}_4$ (absorption bands I, 572.4, II, 539.8 $m\mu$, intensities II, I), can also be transformed into a similar single-banded compound with an absorption band at 555 $m\mu$, if much $\text{Na}_2\text{S}_2\text{O}_4$ is added to a very dilute solution in water; the effect is due to the acidifying action of $\text{Na}_2\text{S}_2\text{O}_4$, alkali restoring again the two-banded spectrum.

solution, shifting the equilibrium between CO- and CN-compounds in favour of the former. We have failed to observe the final equilibrium to vary with the sequence in which the reagents were added, provided that sufficient time was allowed for the equilibrium to establish itself.

The globin denaturation during the action of hydrogen peroxide can be avoided by neutralizing the cyanide. When 0.05% hydrogen peroxide acts on haemoglobin in the presence of 1% KCN neutralized to litmus by KH_2PO_4 , a compound with an absorption band in the red (at about 680 m μ) is formed which is soluble at pH 7.2. The band lies further towards the infra-red and is more diffuse than that of oxygenated choleglobin. Reduction with $\text{Na}_2\text{S}_2\text{O}_4$ produces reduced choleglobin. The compound does not react with CO without reducer, but yields CO-choleglobin after addition of $\text{Na}_2\text{S}_2\text{O}_4$. It is evidently cyanmetacholeglobin (ferric choleglobin cyanide); a second absorption band in the green is that of cyanmethaemoglobin.

Attempts to isolate choleglobin from haemoglobin

Two different methods were tried, fractional precipitation with ammonium sulphate and differential adsorption on alumina. By the former no separation whatever was obtained. The ratio $\epsilon_{628} : \epsilon_{570}$ of the CO-haemochromogens was the same in the original solution, the filtrate and the solution of the precipitate.

When a solution with the ratio $\epsilon_{628} : \epsilon_{570}$ of the CO-haemochromogens = 0.37 was adsorbed on Willstätter's alumina C, the filtrate had a ratio of 0.36, the eluate from the alumina with 0.1% ammonia a ratio of 0.405. The latter was brought to pH 6 by addition of acetate buffer pH 4.6 and again partially adsorbed on alumina. The ratio in the filtrate was this time 0.30 and in the eluate from the precipitate 0.50, but the eluate was slightly cloudy. Similar results were obtained with Brockmann's aluminium hydroxide (Merck) and with Fasertonerde according to Wislicenus (Merck). Choleglobin appears to be adsorbed preferentially, but the difference is too slight to encourage attempts at complete separation.

SUMMARY

The preparation of choleglobin and cholehaemochromogen by the coupled oxidation of haemoglobin and ascorbic acid and the absorption spectra of these compounds are described. The oxidation of the haem part of the molecule, causing the formation of choleglobin, is soon followed by an oxidative denaturation of the globin part, leading to cholehaemochromogen.

Choleglobin and cholehaemochromogen reversibly combine with CO. Whether or not choleglobin combines reversibly with O_2 , could not be established.

Cholehaematin is distinct from verdohaematin, although verdohaemochromogen is formed in the same type of reaction from pyridine-protohaemochromogen as is choleglobin from haemoglobin.

The 'pseudohaemoglobin' of Barkan & Schales, prepared by action of hydrogen peroxide and potassium cyanide on oxyhaemoglobin followed by reduction with sodium hydrosulphite, is ferrous denatured globin-cyancholehaemochromogen. With neutralized cyanide cyanmetacholeglobin is obtained. The solutions, however, contain by-products.

No effective way of separating choleglobin from haemoglobin was found.

Further oxidation of choleglobin and of cholehaemochromogen leads to compounds which in their absorption spectra resemble verdohaematin compounds, but which differ from them in some properties. Such products are also formed in the course of prolonged coupled oxidation of haemoglobin and ascorbic acid.

The spectrophotometer used in this research was kindly provided by the National Health and Medical Research Council of Australia, to which two of us are also indebted for grants. One of us is working with support from the T. E. Rofe bequest.

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