XCVI. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN. I[1]

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WHEN a solution of pyridine-haemochromogen containing ascorbic acid is shaken in air, both substances undergo a coupled oxidation leading to verdohaemochromogen and dehydroascorbic acid [Karrer *et al.* 1933; Lemberg *et al.* 1938, 1, 2; cf. also Haurowitz,[3] 1937]. When haemoglobin and ascorbic acid are incubated in air at *p*H 7·2 and 38° a slower oxidation takes place giving choleglobin, a bile pigment-haemoglobin compound [Lemberg *et al.* 1938, 3] and finally products in which the protein is denatured [Anderson & Hart, 1934; Edlbacher & v. Segesser, 1937]. The ascorbic acid is broken down to dehydroascorbic acid, and finally to other products. Here we present our investigations of the reaction in its early stages when the main breakdown product of the haemoglobin is choleglobin.

There are two possibilities for the mechanism of this reaction.

(1) *The autoxidation of the ascorbic acid is independent of the haemoglobin.* The hydrogen peroxide formed in this reaction oxidizes the porphyrin ring to the bile pigment prosthetic group of choleglobin. This mechanism has been assumed by Barkan & Schales [1938, 1, 2, 3]. Hydrogen peroxide, indeed, causes the oxidation of the porphyrin nucleus of pyridine-haemochromogen [Lemberg *et al.* 1938, 2].

(2) *Ascorbic acid and oxyhaemoglobin react directly.* Two hydrogen atoms are transferred from ascorbic acid to oxyhaemoglobin, giving rise to an unstable haemoglobin-hydrogen peroxide compound which breaks down with the formation of choleglobin. Such a mechanism was suggested by Lemberg *et al.* [1938, 2], because the first possibility could be excluded in the aerobic experiments with pyridine-haemochromogen, the ascorbic acid not undergoing a sufficiently rapid autoxidation in the pyridine solutions used.

Under the conditions of the reaction with haemoglobin the autoxidation is faster. The phosphate buffers, ranging from *p*H 7·2 to 8·6, were not copper-free, and in the course of autoxidation hydrogen peroxide is formed and can be detected by the luminol test. If it can be shown, however, that the oxidation of haemoglobin proceeds under conditions in which the autoxidation of the ascorbic acid is prevented, then the first theory cannot hold and the second alone explains the facts.

The autoxidation of ascorbic is no true autoxidation, but is catalysed by copper, and we can investigate the reaction by the use of copper-inhibitors.

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[3] The absorption band in the red observed by Haurowitz, p. 142, is not caused by substances related to chlorophylls or chlorins, but by verdohaemochromogen.

Experimental

The reaction was studied spectroscopically and by measurement of the O_2-uptake. Choleglobin may be distinguished from other haemoglobin derivatives with an absorption band in the red by its behaviour with CO after reduction. The following table shows its differences from methaemoglobin, sulphaemoglobin and *pseudo*methaemoglobin [Fairley & Bromfield, 1937].

Compound	Absorption band in mμ	With $Na_2S_2O_4$	$Na_2S_2O_4$ and CO	$Na_2S_2O_4$ and alkali
Methaemoglobin	630–634	Reduced Hb	CO-Hb	Protohaemochromogen
*pseudo*Met-Hb	622–624	Protohaemochromogen	CO-haemochromogen	Protohaemochromogen
Sulph-Hb	618–622	No change	Band at 614	Protohaemochromogen
Choleglobin	628–632	No change	Band at 627–629	Cholehaemochromogen, band at 618–620

The O_2-uptake from a solution of oxyhaemoglobin and ascorbic acid, shaken in air, was measured by the Warburg technique with KOH in the central cup. A solution of crystalline horse haemoglobin was placed in the main chamber of the vessels, while a solution of 1 mg. crystalline B.D.H. ascorbic acid was added from the side-bulb. All the solutions were buffered with $M/15$ phosphate buffer, in the majority of experiments at pH 7·4. The temperature of the bath was 38°.

Inhibition of the autoxidation

Of the copper-inhibitors used by other workers compounds of the diethyldithiocarbamate type proved best for our purpose. We used the above compound as well as piperidine-dithiocarbamate which was found to be very satisfactory. These were put in the main vessel with the buffered haemoglobin solution.

It can be seen from Fig. 1 that the inhibitor is able to prevent the autoxidation of the ascorbic acid at the pH used (curves 1 and 2). Control experiments without ascorbic acid show that the small uptake observed in the presence of the inhibitor is due to the oxidation of the inhibitor itself. The presence of the inhibitor does not affect the haemoglobin breakdown; control experiments showed that addition of the inhibitor does not increase the O_2-uptake of haemoglobin solutions any more than is due to autoxidation of the inhibitor. While the inhibitor supresses the autoxidation of the ascorbic acid, it does not diminish its reaction with haemoglobin. Choleglobin was found to be present at the end of the experiment with inhibitor in no less strength than in experiments without inhibitor. The curves 3 and 4 of Fig. 1 show that the O_2-uptake is not diminished but even slightly increased by the copper-inhibitor. It can also be seen from these curves that dehydroascorbic acid is further oxidized. There is, in the experiment with inhibitor, a distinct diminution of the rate of O_2-uptake after somewhat more than one oxygen atom per mol. of ascorbic acid has been taken up, while the oxidation in all the other experiments proceeds with unaltered rate beyond this point. Experiments in which dehydroascorbic acid was used in

place of ascorbic acid showed that it was not able to form choleglobin from haemoglobin. The dehydroascorbic acid was prepared by oxidizing ascorbic acid with the calculated amount of iodine.

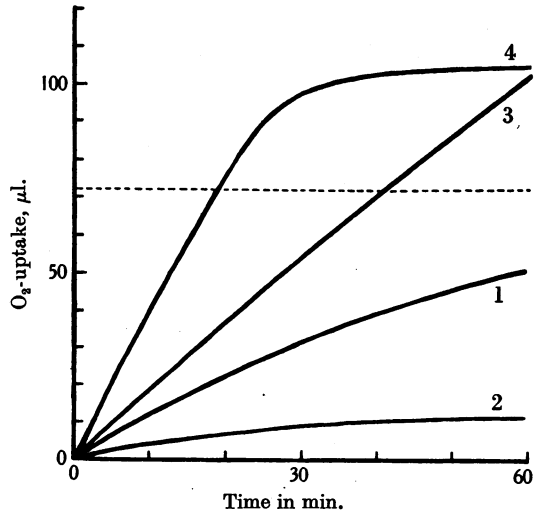

Fig. 1. O_2-uptake in Warburg manometer. The horizontal dotted line represents oxygen required to oxidize ascorbic to dehydroascorbic acid. Curve 1, autoxidation of ascorbic acid in phosphate buffer, *p*H 7·4. Curve 2, the same as 1 with copper-inhibitor (1 mg.). Curve 3, coupled oxidation (0·4 ml. strong haemoglobin solution and acid in phosphate buffer, *p*H 7·4). Curve 4, the same as 3 with copper-inhibitor.

Choleglobin formed with hydrogen peroxide and ascorbic acid

If the haemoglobin and the ascorbic acid are put into a Thunberg tube, and the haemoglobin reduced by evacuation and the use of oxygen-free nitrogen, no reaction takes place. If hydrogen peroxide is now added through the outlet tube, a formation of choleglobin can be observed at 38°. This reaction takes place more rapidly than the choleglobin formation when the same quantities of haemoglobin and ascorbic acid are incubated in air.

Barkan has investigated the formation of choleglobin by the action of hydrogen peroxide on haemoglobin in the presence of cyanide. He concludes that cyanide is necessary for the formation of these bile pigment compounds, and that it cannot be replaced by reducing substances. We find that ascorbic acid can be used instead of cyanide, but we are unable at present to explain satisfactorily the action of cyanide in bile pigment-haemoglobin formation. The mechanism of the two reactions appears to be different. Barkan has withdrawn his earlier explanation that the cyanide only inhibits the catalase that is present; in this we agree.

Carbon monoxide as inhibitor

While we have thus succeeded in blocking the autoxidation without preventing choleglobin formation, we can show that the formation of the choleglobin is prevented by CO without considerably diminishing the rate of the autoxidation.

Barron *et al.* [1936] have shown that CO inhibits the autoxidation of ascorbic acid. The partial pressure of the CO in our experiments was not sufficient to do this to a great extent. Ascorbic acid and haemoglobin were allowed to react in

phosphate buffer at *p*H 7·4 under a gas mixture of 20 % O_2 and 80 % CO. The concentration of the ascorbic acid was measured by precipitating a measured volume of the contents of the vessel *in vacuo* with metaphosphoric acid and titrating the filtrate with 2 : 6-dichlorophenolindophenol [Lemberg & Legge, 1938].

After 2 hr. at 38° nearly all the ascorbic acid had been oxidized, but no trace of choleglobin had been formed. Experiments carried out in air under the same conditions resulted in a copious formation of choleglobin. CO ought, indeed, to prevent the reaction, if the initial step consists in a direct reaction of oxyhaemoglobin with ascorbic acid.

CO does not prevent the formation of choleglobin from haemoglobin by hydrogen peroxide. Washed red cells of sheep were laked by freezing. 1 ml. of this strong oxyhaemoglobin solution was buffered with 6·5 ml. of *M*/15 phosphate buffer *p*H 7·4. Of this solution 2 ml. were placed in the bottom part of a Thunberg tube, the top part of which contained 0·1 ml. of 0·5 % H_2O_2. 2·5 mg. of ascorbic acid were added to the haemoglobin solution and the tube immediately evacuated and filled with oxygen-free CO; 5 min. after the addition of the peroxide a spectroscopic investigation revealed a distinct band at 630 mμ which persisted on reduction by hydrosulphite.

While hydrogen peroxide could be detected in the autoxidation of the ascorbic acid by the luminol test, its application to the reaction catalysed by haemoglobin gave negative results. The test was also made according to the method of Holtz & Triem [1937], whereby the reaction mixture was acidified with H_2SO_4, distilled *in vacuo* at 60°, and the distillate tested with luminol. The test was again negative. This is not, however, definite evidence that hydrogen peroxide is absent, because large amounts of haemoglobin or haematin suppress the chemiluminescence.

Discussion

We have brought forward evidence that the formation of choleglobin from haemoglobin in the coupled oxidation with ascorbic acid is not due to the formation of hydrogen peroxide by autoxidation of the ascorbic acid as Barkan has assumed. This reaction can be prevented without stopping the choleglobin formation. We express the formation of the choleglobin in the following way:

$$HbO_2 + H_2A \rightarrow Hb.H_2O_2 + A \qquad (H_2A = \text{ascorbic acid})$$

$$Hb.H_2O_2 \rightarrow HbOH \text{ (methaemoglobin)}$$
$$\searrow \text{choleglobin}$$

$$2HbOH + H_2A \rightarrow 2Hb + A + 2H_2O$$

$$Hb + O_2 \rightarrow HbO_2.$$

The whole cycle continues until all the reducing agent is oxidized.

This process is very similar to the reaction of pyridine-haemochromogen and ascorbic acid to form verdohaemochromogen [Lemberg *et al.* 1938, 2], yet we can see the specific influence of the globin on the course of the oxidation in the different end-products. The prosthetic group of choleglobin is similar to, but not identical with, verdohaem. We are not able at present to say wherein lies the structural difference between cholehaem and verdohaem.

Barkan uses the collective prefix *pseudo*haem for compounds which may contain either cholehaem or verdohaem and makes no distinction between them. The use of the terms "*pseudo*haemochromogen" for verdohaemochromogen and "*pseudo*haemoglobin" for both choleglobin and cholehaemochromogen makes it appear that the difference between them is only one of the protein moieties of

the compounds, while in fact the main difference lies in the structure of the prosthetic group. Again, the name "*pseudo*haemoglobin" is used by Barkan for the compound with easily detachable iron in the erythrocytes. Its structure is still unknown, but it differs from choleglobin in spectroscopic features [Lemberg *et al.* 1938, 3] and in that carbon monoxide prevents the detachment of iron from it. We cannot, for that reason, conform to Barkan's nomenclature. Fairley has also used the name pseudomethaemoglobin for a quite different class of compounds.

The claim of Barkan & Schales [1938, 3] to have proved the existence of bile pigment-haemoglobin before the experiments of Lemberg *et al.* [1938, 3] cannot be accepted. The fact that iron is easily detached from compounds of this type, which in itself is based on experiments of Lemberg [1935], cannot be considered as sufficient proof of the bile pigment nature of these compounds. Only the isolation of biliverdin from choleglobin has proved that it is a bile pigment-haemoglobin [Lemberg *et al.* 1938, 3]. Full description of the properties of choleglobin will be published later.

Michel [1938] has recently suggested a similar mechanism for the formation of sulphaemoglobin from oxyhaemoglobin and hydrogen sulphide, although here, as in our experiments, hydrogen peroxide can cause the same reaction. In the presence of hydrogen sulphide, however, the product is different from choleglobin, the sulphur atom entering the haemoglobin molecule. In a forthcoming paper we shall confirm and extend Michel's evidence that sulphaemoglobin can be reconverted into protohaemochromogen and thus differs from the bile pigment-haematin compounds, to which Barkan had assumed it to belong. We can, however, confirm Barkan's finding that the iron of sulphaemoglobin can be easily detached. We have here an example of a compound with easily detachable iron which cannot be a bile pigment-haematin compound.

Summary

The coupled oxidation between oxyhaemoglobin and ascorbic acid, leading to a bile pigment-haemoglobin, is not caused by the hydrogen peroxide liberated in the autoxidation of the ascorbic acid. This autoxidation can be prevented by copper-inhibitors without stopping the formation of choleglobin. Oxyhaemoglobin and ascorbic acid react directly to form choleglobin.

While carbon monoxide prevents the formation of choleglobin by the action of atmospheric oxygen on haemoglobin in the presence of ascorbic acid, it does not prevent the formation of choleglobin by hydrogen peroxide.

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