# 34. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

## 3. QUANTITATIVE STUDIES ON CHOLEGLOBIN FORMATION. ESTIMATION OF HAEMOGLOBIN AND ASCORBIC ACID OXIDATIONS[1]

BY R. LEMBERG, J. W. LEGGE AND W. H. LOCKWOOD

*From the Institute of Medical Research, Royal North Shore Hospital, Sydney*

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IN this paper we describe a spectrophotometric method allowing us to measure quantitatively the destruction of haemoglobin and the formation of choleglobin and cholehaemochromogen during the coupled oxidation of haemoglobin with ascorbic acid and with other reducing substances. The influence of various factors, the nature of the reactants, *p*H, temperature and oxygen pressure, on the velocity of the reaction is studied. The oxidation of ascorbic acid during the reactions is measured in the usual way by indicator titration, and the ratio of molecules of ascorbic acid oxidized to molecules of choleglobin formed is thus found. The spectrophotometric determination method enables us, also, quantitatively to correlate the formation of choleglobin with the increase of 'labile iron' and with the yield of bile pigments to be obtained after acid splitting, thus giving us a much more complete insight into the reaction; the results of these studies are described in Parts 4 and 5.

### EXPERIMENTAL

#### *Spectrophotometric determination of choleglobin formation*

*Technique.* A large number of difficulties had to be overcome before a reliable technique could be developed. The solutions derived from the incubation of oxyhaemoglobin and ascorbic acid contain a mixture of oxyhaemoglobin, a little reduced haemoglobin, in some instances methaemoglobin, and reduced and oxygenated choleglobin; in the later stages of the reaction the precipitate of green pigment appears and even in the earlier stages a slight cloudiness occasionally develops. It is evident, therefore, that spectrophotometric study of the absorption of the solutions as such is useless. Means had to be devised to transform the various derivatives of protohaematin and of cholehaematin into one derivative of each and to ensure an optically clear solution. The transformation into the CO-haemochromogens by CO, NaOH and $Na_2S_2O_4$ fulfilled these demands. The CO-free haemochromogens are less suitable, being less stable. Even in the presence of CO we found it necessary to work under strictly controlled conditions in order to get reproducible results. Since $Na_2S_2O_4$ and $O_2$ yield peroxide which produces choleglobin, it is essential to add the reducer only after the solution has been saturated with coal gas and even then to avoid unnecessary exposure to air. We found it also necessary to add the alkali only after the solution had been saturated with coal gas. A CO-haemoglobin solution, which had an extinction coefficient at 570 m$\mu$ of 1·0, gave as CO-haemochromogen

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produced by addition of alkali and $Na_2S_2O_4$ after saturation with coal gas the extinction coefficients at 570 mμ: 1·00; 1·01; 0·98; 0·99. When alkali was added before the saturation with coal gas, the values were smaller and the variation greater: 0·81; 0·91; 0·80; 0·90; 0·91.

The following method was employed. 5 ml. of the solution to be investigated were at once saturated with coal gas for 3 min. The current of gas was as rapid as the frothing of the solution allowed. Only in a few instances did we find it necessary to add a trace of octyl alcohol, which tends to cause some denaturation. 50 mg. of $Na_2S_2O_4$ were then added, the tube once inverted and during further passage of coal gas 0·5 ml. of 20% NaOH was introduced. A vigorous current of coal gas was maintained for 1 min., after which the solution was transferred to the spectrophotometer screw cups. The extinction coefficients at 630 and 570 mμ were then measured without delay, although we found the absorption of the solutions in the cups to change only very slowly. The dilution to the concentration suited for spectrophotometric measurement must be carried out before the addition of $Na_2S_2O_4$ and alkali; the dilution by the alkali has, of course, to be considered. The instrument used was the Hilger Visible Spectrophotometer described in the preceding paper.

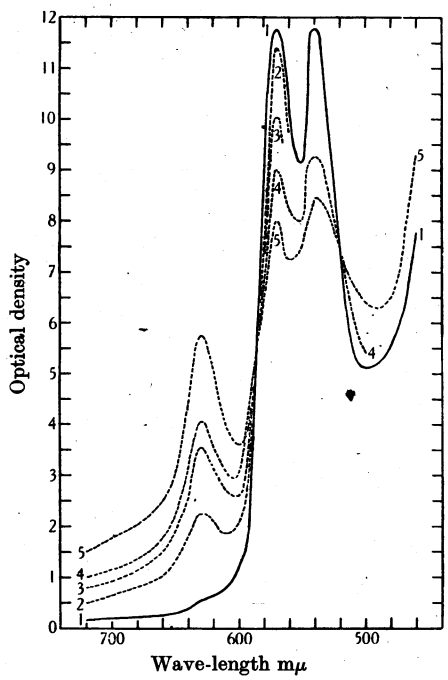



Fig. 1. Formation of choleglobin by coupled oxidation of haemoglobin and ascorbic acid. CO-haemochromogens. Curve 1: from haemoglobin. Curves 2, 3, 4 and 5: after 15, 30, 60 and 120 min. coupled oxidation at 37° respectively.

*Changes of the absorption curves of the CO-haemochromogens during the incubation of oxyhaemoglobin and ascorbic acid*

Fig. 1 shows the changes of the absorption curves of the CO-haemochromogens during incubation of a 1·2% oxyhaemoglobin solution with 100 mg. per 100 ml. of ascorbic acid at 37° and *p*H 8·5 in a culture flask without shaking (cf. Part 2).

The continuous line represents the curve of CO-protohaemochromogen, the interrupted lines those of the CO-haemochromogens obtained after varying times of incubation with ascorbic acid. The absorption of the CO-cholehaemochromogen at 630 mμ rises, while that of the CO-protohaemochromogen at 570 and 540 mμ decreases, the decrease of absorption at 540 mμ being somewhat less than at 570 mμ. Two isosbestic points are observed at 585 and 520 mμ. Owing to the steep course of the CO-protohaemochromogen curve at these wave-lengths the isosbestic point cannot be determined very accurately, but the curves display no evidence of the formation of more than one oxidation product during the duration of this experiment (120 min.).

*Quantitative estimation of choleglobin*

In order to determine the choleglobin concentration in a mixture with haemoglobin from the absorption of the CO-haemochromogens at 630 mμ it is necessary to know the specific extinction coefficients of CO-cholehaemochromogen at this wave-length. Pure CO-cholehaemochromogen has not yet been obtained; we can, however, calculate the specific extinction of CO-cholehaemochromogen from the measured ratio between the decrease of extinction at 570 mμ and the increase at 630 mμ if during the incubation only those compounds are formed which yield CO-cholehaemochromogen or CO-protohaemochromogen with CO, NaOH and $Na_2S_2O_4$. The data reported in the previous section indicate that this is the case, at least in the initial stages of the incubation. It is also necessary to know the ratios of the extinction coefficients at 630 and 570 mμ ($\epsilon_{630} : \epsilon_{570}$) of both CO-cholehaemochromogen and CO-protohaemochromogen. The latter ratio can be determined; the absorption of CO-protohaemochromogen at 630 mμ is small and the ratio $\epsilon_{630} : \epsilon_{570}$ for CO-protohaemochromogen (0·04 as average of a large number of determinations) has only the character of a correction factor. The ratio for CO-cholehaemochromogen cannot be determined correctly, but we arrived at the conclusion that it cannot be far from 2·22 (cf. p. 333, Part 2). If we use these values, the ratio $\epsilon_{630}$ of CO-cholehaemochromogen to $\epsilon_{570}$ of CO-protohaemochromogen, $R$, is obtained by the equation:

$$R = \frac{Q - 0{\cdot}04}{1 - Q/2{\cdot}22},$$

where $Q$ is the observed ratio $\epsilon_{630} : \epsilon_{570}$. $R$ ought to remain constant during the course of the reaction, if our assumption that cholehaematin derivatives are the only reaction products is correct. Table 1 shows that $R$ is approximately 1·0 under widely varying conditions of the reaction. $R$ does not remain absolutely constant during the reaction, but sufficiently so to indicate that at least during the early stages of the reaction choleglobin is practically the only product. Later, lower values of $R$ are found, indicating some formation of by-products.

Table 1. *Values obtained for ratio ($R$) of $\epsilon_{630}$ of CO-cholehaemochromogen to $\epsilon_{570}$ of CO-protohaemochromogen from measurements of absorption decrease at 570 mμ and increase at 630 mμ*

| *p*H 7·2, 37° | | *p*H 8·5, 37° | | *p*H 8·5, 20° | |
|---|---|---|---|---|---|
| Min. of incubation | $R$ | Min. of incubation | $R$ | Hr. of incubation | $R$ |
| 30 | 0·95 | 15 | 1·03 | 19½ | 0·96 |
| 40 | 0·91 | 30 | 1·08 | 31 | 0·84 |
| 80 | 0·83 | 40 | 0·96 | | |
| 120 | 0·84 | 80 | 0·92 | | |
| | | 90 | 0·94 | | |
| | | 120 | 0·81 | | |

For our calculations we assume $R=1$, i.e. equal maximal extinctions of CO-cholehaemochromogen and CO-protohaemochromogen. The specific extinction coefficient (extinction of a 0·1 % solution in 1 cm. layer) of CO-protohaemochromogen was found to be 0·965 at 570 m$\mu$, and the same value is assumed for the specific extinction coefficient of CO-cholehaemochromogen at 630 m$\mu$.

For the specific extinction coefficient at 630 m$\mu$ of the CO-cholehaemochromogen prepared from 'green pigment' we found the value 1·03 when the concentration was measured by the determination of the total iron in green pigment. A lower value (0·76) was obtained when the concentration was measured by weighing the protein denatured by formalin. Green pigment appears thus to contain some protein without strong absorption at 630 m$\mu$ and without iron. The correction for the protohaematin content in green pigment was based on the same data as the calculation of $R$ from the ratio $\epsilon_{630}:\epsilon_{570}$, but the protohaematin content of green pigment is small and the correction therefore of much less importance. The agreement of the values obtained from green pigment with those obtained from choleglobin-haemoglobin solutions can, therefore, be taken as independent confirmation that the values arrived at for $R$ and for the specific extinction coefficient at 630 m$\mu$ of CO-cholehaemochromogen are approximately correct.

We can now determine the content of cholehaematin and protohaematin derivatives in a solution. If $\epsilon^{\circ}_{570}$ is the extinction coefficient at 570 m$\mu$, of the haemoglobin solution before incubation with ascorbic acid, measured as CO-haemochromogen, and $\epsilon_{630}$ and $\epsilon_{570}$ the extinction coefficients at 630 m$\mu$ and 570 m$\mu$ after incubation, we have: choleglobin formed, in % of original haemoglobin $=\frac{102\,(\epsilon_{630}-0\cdot04\,\epsilon_{570})}{\epsilon^{\circ}_{570}}$; and, unaltered haemoglobin $=\frac{102\,(\epsilon_{570}-0\cdot45\,\epsilon_{630})}{\epsilon^{\circ}_{570}}$. If $\epsilon^{\circ}_{570}$ is unknown, the choleglobin percentage of a mixture of choleglobin and haemoglobin is given by the equation: % choleglobin $=\frac{100\,(\epsilon_{630}/\epsilon_{570}-0\cdot04)}{0\cdot96+0\cdot55\,\epsilon_{630}/\epsilon_{570}}$.

*Estimation of protohaematin destruction*

We have established the validity of the spectrophotometric method by estimating the destruction of protohaematin during the incubation of haemoglobin with ascorbic acid in a second way. By treatment of a haemoglobin solution containing ascorbic acid with acetic acid and ether, the protohaematin can be transferred almost quantitatively into the ether and can be determined spectrophotometrically as pyridine-protohaemochromogen in the ammoniacal extract. If this method is applied to choleglobin solutions, the ether contains beside protohaematin only biliverdin which is removed by extraction with 5 % HCl, and a small amount of biliviolin which does not disturb the haematin estimation.

For the determination the following technique was applied: 10 ml. of glacial acetic acid were added to 5 ml. of the choleglobin solution and the pigments were brought into 15 ml. of ether in the manner used for the determination of the bile pigments and described in detail in Part 5. The ether layer was repeatedly extracted with 5 % HCl, washed with water and finally the haematin was extracted with a few ml. of $N/10$ ammonia. Pyridine was added to a concentration of 20 % and after reduction with $Na_2S_2O_4$ the absorption at $\lambda=557$ m$\mu$ (first absorption band of pyridine haemochromogen) was measured spectrophotometrically. The molar extinction coefficient of pyridine-protohaemochromogen at 557 m$\mu$ under these conditions was found to be 31,000.

We have made an interesting observation on the effect of ascorbic acid on the isolation of haematin from solutions of oxyhaemoglobin. 5 ml. of a 0·72 % solution of haemoglobin ought to yield 1·405 mg. haemin. If 5 mg. of ascorbic acid were added to the haemoglobin solution immediately before the acidification with glacial acetic acid, 1·35 mg. of haemin = 96 % were recovered from the ether. Without the addition of ascorbic acid 1·29 mg. = 92 % were recovered, when the ether was added immediately after the addition of the glacial acetic acid; when, however, the acetic acid solution was kept for 15 min. before the addition of ether and the separation of the layers, the aqueous layer contained a great deal of haematin firmly bound to protein and no longer extractable by ether, and the yield of haemin isolated from the ether was only 0·51 mg. or 36 % of the total haemoglobin-haemin. If acid haematin solution is kept in the presence of ascorbic acid, however, the yield of haematin does not diminish. Evidently ascorbic acid protects the globin in the acid solution from an alteration, probably of oxidative nature, which causes the protein-haematin linkage to become stable to acid.

Table 2. *Comparison of the spectrophotometric determination of choleglobin formation with the direct measurement of haemin destruction*

| Conditions | mg. haemin from 5 ml. haemoglobin | % haematin destruction | % choleglobin according to spectrophotometric method |
|---|---|---|---|
| Initial haemoglobin | 1·35 | 0 | 0 |
| *p*H 7·2, 15 min. | 1·215 | 10·0 | 6·9 |
| 30 „ | 1·19 | 11·9 | 14·1 |
| *p*H 8·5, 15 min. | 1·08 | 20·0 | 17·9 |
| 30 „ | 0·905 | 33·0 | 33·3 |

Table 2 shows that the percentage destruction of protohaematin estimated by this method tallies with the percentage formation of choleglobin as determined by the spectrophotometric method. The rather large deviations are due to the fact that the estimation of haematin destruction is less exact than the spectrophotometric estimation of choleglobin formation.

*Measurement of the velocity of choleglobin formation under various conditions*

Table 3 gives the results of measuring the reaction velocity of the choleglobin formation with this method under various conditions of temperature and *p*H. Each of the values given is the average of at least three parallel experiments. The haemoglobin concentration was 0·72 %, that of ascorbic acid 100 mg. per 100 ml. and the incubation was carried out in flat culture flasks as previously described. As is to be expected from the nature of the experiment, variations are fairly large, but they do not affect the conclusions drawn from the experiments in any way. Table 4 shows the variation of a number of experiments carried out under the same conditions.

In the experiments in which a distinct precipitate of green pigment was found, a solution of the precipitate and the filtrate were analysed separately. The results of experiments of longer than 2 hr. can no longer be considered as reliable, since other compounds were formed in addition to choleglobin and cholehaemochromogen (cf. Part 2 and above). There is also occasionally bacterial contamination.

Table 3. *Choleglobin and cholehaemochromogen formation by coupled oxidation of haemoglobin with ascorbic acid*

| Conditions | Duration of incubation | In % of initial haemoglobin: Cholegloblin | Haemoglobin | Cholehaemochromogen | Protohaemochromogen | Total cholehaem | Sum |
|---|---|---|---|---|---|---|---|
| *p*H 7·2, 37° | 15 min. | 6·9 | 92·7 | 0 | 0 | 6·9 | 99·6 |
| | 30 „ | 14·1 | 83·1 | 0 | 0 | 14·1 | 97·2 |
| | 80 „ | 21·6 | 55·1 | 13·0 | 0·8 | 34·6 | 90·5 |
| | 120 „ | 17·2 | 46·2 | 21·5 | 2·7 | 38·7 | 87·6 |
| | 7 hr. | 22·4 | 28·2 | 34·3 | 3·7 | 56·7 | 88·6 |
| | 16 „ | 11·5 | 13·6 | — | — | — | — |
| *p*H 8·5, 37° | 15 min. | 17·9 | 83·2 | 0 | 0 | 17·9 | 101·1 |
| | 30 „ | 33·3 | 69·7 | Trace included in former | | 33·3 | 103·0 |
| | 80 „ | 27·9 | 49·7 | 17·6 | 1·5 | 45·5 | 97·6 |
| | 120 „ | 36·5 | 32·9 | 20·3 | 2·2 | 56·8 | 91·9 |
| | 7 hr. | 20·0 | 26·5 | 21·4 | 6·2 | 41·4 | 74·1 |
| | 16 „ | 13·6 | 14·1 | — | — | — | — |
| *p*H 7·2, 25° | 30 min. | 1·4 | 85·5 | — | — | 1·4 | 86·9 |
| | 60 „ | 6·1 | 83·8 | — | — | 6·1 | 89·9 |
| | 120 „ | 11·8 | 76·9 | — | — | 11·8 | 88·7 |
| *p*H 8·5, 25° | 30 min. | 5·2 | 85·6 | — | — | 5·2 | 90·8 |
| | 60 „ | 16·5 | 82·1 | — | — | 16·5 | 98·6 |
| | 90 „ | 28·3 | 74·4 | — | — | 28·3 | 102·7 |
| | 120 „ | 42·3 | 56·1 | Trace included in former | | 42·3 | 98·4 |

Table 4. *Variations in the yield of choleglobin and cholehaemochromogen*

| 30 min., *p*H 8·5, 37° In % of initial haemoglobin: Cholegloblin | Haemoglobin | Sum | 80 min., *p*H 8·5, 37° In % of initial haemoglobin: Cholegloblin | Haemoglobin | Cholehaemochromogen | Protohaemochromogen | Total cholehaem | Sum |
|---|---|---|---|---|---|---|---|---|
| 22·9 | 80·3 | 103·2 | 30·0 | 49·5 | 16·5 | 4·0 | 46·5 | 100·1 |
| 30·2 | 72·5 | 102·7 | 26·8 | 46·1 | 17·0 | 0·4 | 43·8 | 90·3 |
| 27·6 | 69·1 | 96·7 | 27·0 | 53·6 | 19·3 | 0·2 | 46·3 | 100·0 |
| 35·8 | 69·4 | 105·2 | — | — | — | — | — | — |
| 42·4 | 69·0 | 111·4 | — | — | — | — | — | — |
| 41·8 | 60·9 | 102·7 | — | — | — | — | — | — |
| 32·4 | 67·0 | 99·4 | — | — | — | — | — | — |
| 33·3 ± 2·7 | 69·7 ± 2·0 | 103·1 | 27·9 ± 1·0 | 49·7 ± 2·2 | 17·6 ± 0·8 | 1·5 ± 1·2 | 45·5 ± 0·9 | 96·7 |
| | | | Standard deviation | | | | | |
| 7·3 | 5·4 | — | 1·8 | 3·8 | 1·5 | 2·1 | 1·5 | — |

The reaction velocity of the choleglobin formation at *p*H 8·5 is initially more than double that at *p*H 7·2. The temperature coefficient is rather high ($Q_{10}$ of the initial reaction about 3·5–4·0 between 25° and 37°). The concentration of choleglobin in the solution reaches a maximum, which is higher at 25° than at 37°; after this it remains constant, while more green pigment is formed. At first the latter contains little protohaemochromogen, but later the proportion of the latter increases. Finally the choleglobin concentration begins to decrease. Up to this point there is no indication of by-reactions, the sum of protohaematin and cholehaematin compounds being approximately 100 %. In the later stages, at *p*H 8·5 later than at *p*H 7·2, there is evidently destruction of choleglobin by further oxidation.

*Formation of choleglobin by reducing substances other than ascorbic acid*

Of other reducing substances reducton is of particular interest. Anderson & Hart [1934] describe the formation of green pigment by glucose + glycine + ammonia in alkaline phosphate buffers, and it is possible that reducton, chemically closely related to ascorbic acid, was the active agent in these mixtures. Crystalline reducton was prepared according to v. Euler & Martius [1933]. It is seen from the data in Table 5 that it undergoes coupled oxidation with haemoglobin

Table 5. *Formation of choleglobin by reducing substances other than ascorbic acid*

*p*H 7·2, 37°, haemoglobin 0·7 %, reducing substances in a concentration equivalent to 100 mg. per 100 ml. of ascorbic acid, in the case of thiolacetic acid to 50 mg. per 100 ml. of ascorbic acid.

| | | In % of initial haemoglobin | | | | | |
|---|---|---|---|---|---|---|---|
| Reducing substances | Incubation time | Chole-globin | Haemo-globin | Chole-haemo-chromogen | Proto-haemo-chromogen | Total chole-haem | Sum |
| Ascorbic acid | 90 min. | 25·4 | 61·9 | 18·3 | 0·3 | 43·7 | 105·9 |
| Reducton (crystalline) | 90 min. | 33·8 | 54·6 | 8·4 | 4·6 | 42·2 | 101·4 |
| Thiolacetic acid | 90 min. | 19·5 | 71·5 | 4·2 | 6·6 | 23·7 | 101·8 |
| Glutathione, crystalline | 19 hr. | 24·4 | 65·6 | 5·6 | 4·8 | 30·0 | Assumed 100 |
| Glutathione, purified over Cd-salt | 18½ hr. | 6·5 | — | Small amount insoluble in NaOH | | — | — |

and causes as rapid a formation of choleglobin as ascorbic acid. We were, however, unable to find reducton in the mixture used by Anderson & Hart by the indophenol reduction test. Thiolacetic acid also formed choleglobin only slightly less rapidly than did ascorbic acid. Glutathione caused only a very slow reaction, and even this seems to be brought about by impurities. There was considerably less choleglobin formed when glutathione purified over its cadmium salt was used. With cysteine no formation of choleglobin was observed, although a small amount of green precipitate was formed. Both glutathione and cysteine caused a considerable formation of methaemoglobin (cf. Table 6).

*Formation of choleglobin in the presence of low concentrations of ascorbic acid*

The experiments described hitherto were carried out with ascorbic acid concentrations of 50–100 mg. per 100 ml. In tissue cells the concentration of ascorbic acid is smaller, of the order of 5–50 mg. per 100 ml. (cf. Giroud [1937]). In guinea-pig spleen we found values of 6–15 mg. per 100 ml. with an average of 10·7 mg. per 100 ml.

We have, therefore, carried out some experiments with lower ascorbic acid concentrations. It must be borne in mind that with the comparatively large haemoglobin concentration required for the quantitative study of choleglobin formation, these small amounts of ascorbic acid are rapidly destroyed. In the tissues, however, fresh ascorbic acid can be made available by back-reduction of dehydroascorbic acid with reduced glutathione [Borsook *et al.* 1937; Schultze *et al.* 1937–38; cf. also Genevois & Cayrol, 1939]. Lemberg *et al.* [1938, 1] have shown that reduced glutathione saves ascorbic acid from the oxidation by haemochromogen and does not prevent the oxidation of haemochromogen to verdohaemochromogen. Reduced glutathione accompanies ascorbic acid in the cells of liver and spleen in rather high concentration. In guinea-pig spleen we found 207 mg. reduced glutathione per 100 g. of fresh organ. We have therefore

studied the action of small concentrations of ascorbic acid on oxyhaemoglobin in the absence and in the presence of glutathione on the formation of choleglobin and the results are given in Table 6.

Table 6. *Formation of choleglobin with low concentrations of ascorbic acid in the presence of glutathione*

0·14 g. of haemoglobin in 20 ml. phosphate buffer *p*H 7·2, 37°

| Ascorbic acid mg. | GSH mg. | $\epsilon_{630}$ of CO-haemochromogens after 90 min. incubation | Biliverdin after acid splitting of 5 ml. | Spectroscopic observations after 2½ hr. incubation |
|---|---|---|---|---|
| — | — | 0·27 | None | Oxyhaemoglobin |
| — | 50 | 0·39 | Trace | Oxyhaemoglobin and methaemoglobin; band 634 mμ disappearing on reduction with $Na_2S_2O_4$ |
| 2 | — | 0·62 | Trace | Band at 630 mμ resistant to $Na_2S_2O_4$ |
| 2 | 50 | 1·05 | Considerable amount | Band at 630 mμ resistant to $Na_2S_2O_4$; some green pigment |
| 20 | — | 2·07 | Large amount | Band at 630 mμ resistant to $Na_2S_2O_4$; large amount of green pigment |

Even small concentrations (10 mg. per 100 ml.) of ascorbic acid cause a noticeable formation of choleglobin, much larger than that produced by glutathione in a concentration of 250 mg. per 100 ml. In the presence of both glutathione and ascorbic acid more choleglobin is formed than by ascorbic acid alone, and the increase in absorption is significantly larger than can be explained by additive effect. Under the conditions prevailing in the tissue cell haemoglobin must, therefore, undergo oxidation to choleglobin. Actually the condition in the tissue cells is even more favourable than in our experiment, as it will be seen below that low oxygen pressure increases the rate of choleglobin formation while not affecting the destruction of ascorbic acid.

*Rate of oxidation of ascorbic acid and effect of oxygen partial pressure on the oxidation of haemoglobin and ascorbic acid*

Table 7 gives the percentage losses of ascorbic acid during the incubation of ascorbic acid (100 mg. per 100 ml.) with a 0·7 % haemoglobin solution at 37° and *p*H 7·2 and 8·5; the reaction mixture was kept in flat culture flasks under the conditions previously described.

Table 7. *Ascorbic acid destruction*

| Incubation time in min. | % loss of ascorbic acid | |
|---|---|---|
| | *p*H 7·2 | *p*H 8·5 |
| 30 | 10 | 26 |
| 45 | — | — |
| 60 | 23 | — |
| 90 | 31 | 45 |
| 120 | 40 | — |
| 155 | — | 55 |
| 180 | 51 | — |
| 225 | — | 74 |

By comparison with the rate of choleglobin formation given in Table 3 the oxidation ratio—molecules of ascorbic acid oxidized per molecules of haemo-

globin oxidized to choleglobin—can be found for the time experiments. This ratio is 10·6 after 30 min. and 11·7 after 80 min. incubation at *p*H 8·5; at *p*H 7·2 the values are 9·6 after 30 min. and 14·0 after 120 min. If 1 mol. $O_2$ is required to oxidize haemoglobin to choleglobin and 0·5 mol. to oxidize ascorbic acid to dehydroascorbic acid, about one-sixth of the $O_2$ taken up during the first 30 min. at either *p*H is used for the formation of choleglobin and five-sixths for that of ascorbic acid. Actually somewhat more $O_2$ will be used for the further oxidation of dehydroascorbic acid.

In order to study the effect of the $O_2$ partial pressure on the oxidation of haemoglobin and ascorbic acid and on the oxidation ratio under more defined conditions we have carried out experiments in Warburg flasks in which the reaction mixture was shaken with air and with a nitrogen-air mixture containing 2 % $O_2$. We have shown [Lemberg *et al.* 1938, 2] that in the coupled oxidation of pyridine haemochromogen and ascorbic acid lowering of the $O_2$ concentration decreases the oxidation ratio from 59 in $O_2$ to 32 in air and to 12 in a nitrogen-air mixture containing 4 % $O_2$. The rate of oxidation of pyridine-haemochromogen was little affected, while that of the ascorbic acid oxidation was proportional to the $O_2$ partial pressure. Table 8 shows that in the coupled

Table 8. *Effect of $O_2$ pressure on choleglobin formation and ascorbic acid destruction*

Haemoglobin 0·7 %; ascorbic acid 100 mg. per 100 ml.; *p*H 7·2; 37°.
Total volume of solution: 2·6 ml.; in central cup, 0·4 ml. KOH.

| $O_2$ concentration | Piperidine dithiocarbamate | Incubation time min. | $O_2$ consumption μmol. | Ascorbic acid oxidation μmol. | Choleglobin in % of original haemoglobin | Formation in μmol. | Oxidation ratio | (mol. $O_2$ consumed)/(mol. choleglobin formed) |
|---|---|---|---|---|---|---|---|---|
| 20 % | Absent | 30 | 2·41 | 4·06 | 4·8 | 0·05 | 81 | 48 |
| | | 45 | 2·90 | 4·92 | 9·9 | 0·10 | 49 | 29 |
| Standing in air | Absent | 30 | — | 1·48 | 14·1 | 0·155 | 10 | — |
| 2 % | Absent | 30 | 2·34 | 4·52 | — | — | — | — |
| | | 45 | 3·11 | 5·80 | 38·1 | 0·38 | 15 | 8·5 |
| 20 % | 100 μg. | 30 | 4·7 | — | — | — | — | — |
| | | 45 | 6·5 | — | 23·8 | 0·23 | < 54 | 28 |
| 2 % | 100 μg. | 30 | 3·9 | — | — | — | — | — |
| | | 45 | 5·1 | — | 60 | 0·58 | ~ 16 | 9 |

oxidation of haemoglobin and ascorbic acid the oxidation ratio is also much lower with low $O_2$ concentration. Here, however, the rate of oxidation of ascorbic acid is little affected, while that of the choleglobin formation is greatly increased with low $O_2$ concentrations.

Whatever may be the correct explanation of the reaction mechanism (cf. below), it is clear that the low $O_2$ pressure in the tissue cells favours the oxidation of haemoglobin to choleglobin.

### *The rate of choleglobin formation in laked erythrocytes*

For the experiments described hitherto, solutions of crystalline horse oxyhaemoglobin were used. Table 9 gives the measurements of the velocity of choleglobin formation by coupled oxidation with ascorbic acid under the same conditions as those of Table 3, using, however, laked horse erythrocytes diluted to a haemoglobin concentration of 0·72 %, instead of the solution of oxyhaemoglobin.

Table 9. *Choleglobin formation in laked cells with ascorbic acid*

| Conditions | Incubation time in min. | Choleglobin in % of initial haemoglobin: Laked cells | Choleglobin in % of initial haemoglobin: Laked destromatized cells |
|---|---|---|---|
| *p*H 7·2, 37° | 15 | 1·3 | 5·3 |
| | 30 | 5·1 | 15·0 |
| *p*H 8·5, 37° | 15 | 22 | — |
| | 30 | 35 | — |

A comparison with the data compiled in Table 3 shows that at *p*H 8·5 the reaction velocity is almost the same, when laked horse cells are used instead of a solution of horse oxyhaemoglobin. At *p*H 7·2, however, choleglobin is formed much more slowly from laked red cells. This indicates the presence in the erythrocytes of a factor inhibiting the formation of choleglobin. This factor is contained in the stromata. When the stromata are removed by keeping the laked cells at a *p*H of 5·8 in the refrigerator and centrifuging, the haemoglobin of the destromatized red cells is now attacked by ascorbic acid and $O_2$ as rapidly as that in a solution of crystalline oxyhaemoglobin.

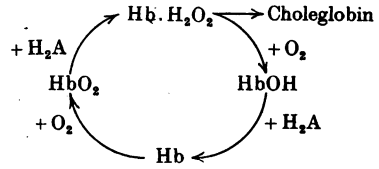
These observations help us to explain why there is no, or at least only a very slow, formation of choleglobin in the red cells. From the figures given in Table 6 it can be calculated that, in the presence of 250 mg. per 100 ml. of glutathione, ascorbic acid in a concentration of 10 mg. per 100 ml. is still able to transform 12 % of 0·7 % haemoglobin into choleglobin in 90 min. The concentration of ascorbic acid in the erythrocytes is still considerably smaller. In rabbit erythrocytes we found only 0·35 mg. per 100 ml., considerably less than the values reported by Stephens & Hawley [1936] for human erythrocytes (0·85–1·2 mg. per 100 ml.); in rabbit's plasma are found higher concentrations than in the cells (0·8–1·9 mg. per 100 ml.), while Stephens & Hawley report 1·1–1·35 mg. per 100 ml. for normal human plasma. From our figures it can be concluded that the membrane of the red cell offers some protection to the haemoglobin contained in it by its very incomplete permeability to ascorbic acid [cf. also Borsook *et al.* 1937]. Even so one would still expect a slow, but substantial haemoglobin destruction in the erythrocytes. This is further decreased, if not prevented, by the inhibiting factor in the stroma, which in the cell is probably still much more effective than in the great dilution in which it is present in our dilute laked cell solutions. Whether these factors are able to prevent the formation of choleglobin or other bile pigment-haemoglobins in the red cell completely, is doubtful, and this problem will be discussed in the succeeding paper.

The protection of the plasma ascorbic acid from oxidation by the red cells [Kassan & Roe, 1940] is probably due to this stroma factor rather than to the glutathione of the red cell, with which the plasma ascorbic acid is not in direct contact; Borsook *et al.* [1937] did not find evidence for the reduction of dehydroascorbic acid added to blood. The rapid destruction of ascorbic acid in the blood following haemolysis is due to the coupled oxidation with haemoglobin.

### *The reaction mechanism*

In Part 1 of this series [Lemberg *et al.* 1939] we have shown that the formation of choleglobin by the coupled oxidation of haemoglobin with ascorbic acid cannot be due to hydrogen peroxide formed by autoxidation of ascorbic acid, since the reaction takes place in the presence of copper inhibitors which prevent the

autoxidation. Nevertheless, hydrogen peroxide acting on haemoglobin in the presence of ascorbic acid forms choleglobin. We have explained these facts by assuming as first step of the reaction hydrogen transfer from ascorbic acid to oxyhaemoglobin with the formation of a ferrous haemoglobin-$H_2O_2$ compound, and have assumed the following cycle in order to explain the destruction of many more ascorbic acid molecules than haemoglobin molecules in the coupled oxidation:




Hb reduced haemoglobin
$HbO_2$ oxyhaemoglobin
HbOH methaemoglobin
$H_2A$ ascorbic acid

We assume the ferrous haemoglobin-hydrogen peroxide compound to undergo an intramolecular peroxidative reaction, the porphyrin ring being oxidized by the $O_2$ of the iron-bound hydrogen peroxide of the same molecule.

The reaction mechanism of the formation of verdohaemochromogen from pyridine-haemochromogen by coupled oxidation with ascorbic acid was explained in a similar way [Lemberg *et al.* 1938, 1, 2]. We have found that hydrogen peroxide acting on pyridine-haemochromogen in the presence of ascorbic acid and in the absence of $O_2$ leads to a precursor of verdohaemochromogen [Lemberg *et al.* 1938, 2]. The green compound obtained by Haurowitz [1937] by reduction of the ferric pyridine haemochromogen-hydrogen peroxide compound by pyrogallol is also verdohaemochromogen.

Hydrogen peroxide forms choleglobin from haemoglobin in the presence of ascorbic acid. We have now found that even in the absence of reducing substances hydrogen peroxide causes a substantial formation of choleglobin from reduced haemoglobin, while much less is formed from oxyhaemoglobin. To 3·5 ml. of an 0·7 % haemoglobin solution, reduced by evacuation under nitrogen, 0·5 ml. of 0·6 % hydrogen peroxide was added under nitrogen. After 2 min. at 20° the excess hydrogen peroxide was destroyed by a drop of catalase solution and the choleglobin content determined spectrophotometrically. It was found that 16 % of the reduced haemoglobin had become transformed into choleglobin. If oxyhaemoglobin reacted with hydrogen peroxide under the same conditions but in air, only 7 % was transformed into choleglobin. Methaemoglobin yielded 6 % choleglobin under nitrogen. Hydrogen peroxide is evidently able to reduce some methaemoglobin to haemoglobin in the same way as catalase is reduced to its ferrous form by hydrogen peroxide [Keilin & Hartree, 1937].

These observations show that the effect of the $O_2$ pressure on the oxidation ratio (cf. above and Table 8) can be understood as autoxidation of the ferrous haemoglobin-$H_2O_2$ compound competing with its transformation to choleglobin. The methaemoglobin-hydrogen peroxide compound is known to be unstable and to give methaemoglobin. The fact that high $O_2$ pressure lowers the oxidation ratio cannot be explained by the assumption that the autoxidation of ascorbic acid competes with its coupled oxidation with haemoglobin, since even at high $O_2$ pressure copper inhibitor does not diminish the oxidation ratio and the effect of $O_2$ pressure in the oxidation ratio in the presence of copper inhibitor is the same as in its absence.

We have ascertained that methaemoglobin can be an intermediate product in the cycle. It reacts with about the same velocity as oxyhaemoglobin, and the effect of $O_2$ pressure on the ratio 'mol. of $O_2$ consumed to mol. of

Table 10. *Choleglobin formation from methaemoglobin*

Methaemoglobin 0·7 %; ascorbic acid 100 mg. per 100 ml.; *p*H 7·2; 37°.
Volume of solution: 2·6 ml.; in central cup, 0·4 ml. KOH.

| $O_2$ concentration | Incubation time min. | $O_2$ uptake in $\mu$mol. | Choleglobin formation: In % of original haemoglobin | Choleglobin formation: In $\mu$mol. | Ratio (mol. $O_2$ consumed)/(mol. choleglobin formed) |
|---|---|---|---|---|---|
| 20 % | 1 | 0·36 | — | — | — |
| | 15 | 1·69 | — | — | — |
| | 30 | 2·59 | — | — | — |
| | 45 | 3·35 | 8·4 | 0·094 | 36 |
| 2 % | 1 | 0·13 | — | — | — |
| | 15 | 1·20 | — | — | — |
| | 30 | 2·00 | — | — | — |
| | 45 | 2·90 | 24·6 | 0·276 | 10·5 |

choleglobin formed' is the same as with oxyhaemoglobin (compare Table 10 with Table 8).

While the effects of the $O_2$ pressure on the oxidation ratios in choleglobin and verdohaemochromogen formation and on the reaction velocities of verdohaemochromogen formation and destruction of ascorbic acid in it can be understood by the notion that the autoxidizable ferrous haem compounds undergo oxidation of their prosthetic groups, it still remains unexplained why the optimum of the rate of choleglobin formation is at low $O_2$ pressure (cf. Table 8), while the rate of verdohaemochromogen formation does not depend on $O_2$. Since the effects of the $O_2$ pressure on the oxidation of ascorbic acid also differ in both cases in an analogous manner (a high $O_2$ pressure favouring the destruction of ascorbic acid in the coupled oxidation with pyridine-haemochromogen, but no influence of $O_2$ pressure on ascorbic acid oxidation with haemoglobin), it is likely that an explanation of the phenomenon must be concerned with the reaction initiating the cycle, the reaction of oxyhaemoglobin with ascorbic acid. There is a remarkable parallel between choleglobin and methaemoglobin formations from oxyhaemoglobin, the rates of both reactions having maxima at low $O_2$ pressures in spite of the fact that the reactions require $O_2$. The range in which this optimum is found, is one in which there is partial dissociation of oxyhaemoglobin. The observations of Brooks [1935] make it likely that the explanation has to be sought in an intermediate between $Hb_4$ and $Hb_4O_8$, e.g. $Hb_4O_2$, being the substance converted into methaemoglobin. The same applies probably to choleglobin formation. Brooks himself rejects this explanation on the basis of calculations involving various constants which hitherto have not been established securely. His own explanation is, however, quite unsatisfactory. Besides assuming an inhibition by $O_2$ of obscure nature, he believes that the rate of methaemoglobin formation is proportional to the concentration of reduced haemoglobin which is decreased by high $O_2$ pressure. Brooks overlooks the fact that the concentration of reduced haemoglobin is low at high $O_2$ pressure, because it reacts more rapidly with $O_2$. There is no reason to assume that this reaction should lead to oxyhaemoglobin exclusively, while another reaction of reduced haemoglobin with $O_2$ would yield methaemoglobin.

## Discussion

In a previous paper [Lemberg *et al.* 1938, 1] we had put forward the hypothesis that dehydroascorbic acid, formed by the coupled oxidation of haemoglobin and ascorbic acid during the bile pigment formation *in vivo*, is reduced

back to ascorbic acid by reduced glutathione, and that oxidized glutathione in turn is reduced by the glucose dehydrogenase system. The latter assumption was based on the experiments of Mann [1932]. Meanwhile Schultze *et al.* [1939] have shown that neither ascorbic acid nor glutathione nor a mixture of the two substances can act as a hydrogen-carrying system between nicotine-haemochromogen and the glucose dehydrogenase system or coenzyme. This result scarcely affects our hypothesis that the ascorbic acid-glutathione system plays an important part in the physiological destruction of haemoglobin, since it is not of importance how the glutathione is restored. What principally matters is that ascorbic acid and haemoglobin undergo coupled oxidation under the conditions found in the cells of those organs in which bile pigment formation takes place; for this we bring forward evidence in the present paper. Secondly, ascorbic acid must be somehow restored, because the daily haemoglobin destruction involves the oxidation of a larger amount of ascorbic acid than is replaced in the food. The fact that dehydroascorbic acid has antiscorbutic activity makes it almost certain that it can be reduced *in vivo*. Several authors (cf. above) have shown that reduced glutathione is able to reduce dehydroascorbic acid in liver cells as well as *in vivo*, but recently Schultze *et al.* [1937–38] have thrown some doubts on the actual occurrence of this reaction *in vivo*. We agree with those authors that their results make it unlikely that the ascorbic acid-glutathione system plays a major role in cell respiration [cf. King, 1939]. A sweeping generalization that the reduction of dehydroascorbic acid by glutathione does not occur in the animal body would, however, not be justified. The experiments of Schultze *et al.* [1937–38, p. 403] were carried out with a proportion of glutathione to ascorbic acid which was much lower than that actually found in liver and spleen, and even so they showed only that the reduction of dehydroascorbic acid by glutathione was not complete.

Other reducing systems may play a role in the physiological oxidation of haemoglobin, but so far the ascorbic acid-glutathione system is the only one known to occur in cells that is able to catalyse the formation of choleglobin under physiological conditions.

## Summary

A spectrophotometric method has been worked out for the determination of the choleglobin content in mixtures of choleglobin and haemoglobin, and thus the measurement of the rate of choleglobin formation.

The spectrophotometric investigation indicates that in the initial stages of the reaction choleglobin is the only product of oxidation of haemoglobin, while later other products are formed in addition to choleglobin and cholehaemochromogen.

The initial reaction velocity of choleglobin formation is about twice as large at *p*H 8·5 as it is at *p*H 7·2. The temperature coefficient is high ($Q_{10}=3{\cdot}5-4$ between 25 and 37°).

Reducton forms choleglobin as fast as ascorbic acid, thiolacetic acid more slowly, glutathione and cysteine very slowly, if at all.

Choleglobin is rapidly formed from haemoglobin under physiological conditions (*p*H 7·2, 37°) and with concentrations of glutathione and ascorbic acid such as occur in tissue cells, glutathione increasing the rate of choleglobin formation more than can be due to a merely additive effect.

At low $O_2$ pressure (15 mm.) the formation of choleglobin proceeds faster than in air, while the oxidation of ascorbic acid is little affected. The same is found in experiments carried out in the presence of copper inhibitor which prevents the

autoxidation of ascorbic acid. The theoretical implications of these observations are discussed. In air, without shaking, about 10 molecules of ascorbic acid are oxidized per molecule of choleglobin formed.

In the red cells haemoglobin is protected from rapid oxidation by the small permeability of the cell membrane to ascorbic acid and by an inhibiting factor in the stromata.

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