# 36. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOGLOBIN

## 5. STUDIES ON THE FORMATION OF BILE PIGMENTS FROM CHOLEGLOBIN AND VERDOHAEMOCHROMOGEN AND ON THEIR ISOLATION FROM ERYTHROCYTES[1]

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IN a preliminary paper [Lemberg, Legge & Lockwood, 1938] we have already reported the isolation of biliverdin (dehydrobilirubin) and of biliviolin from choleglobin solutions. Under remarkably mild conditions haemoglobin is thus transformed into bile pigments; these conditions are such as are found in the cells of organs forming bile pigment, except that acid was used for the removal of the iron. It will be shown in the present paper that the breakdown of choleglobin to bile pigments with the removal of iron probably occurs in the later stages of the reaction with ascorbic acid at a physiological pH.

We describe here in greater detail the bile pigments obtained by acid splitting from choleglobin and verdohaemochromogen. The hope of gaining information on the constitutional differences between cholehaem and verdohaem by the study of the splitting products was not fulfilled owing to secondary reactions by which the primary splitting products are altered and owing to the complicated nature of the mixtures of bile pigments thus formed.

Great difficulties had, therefore, to be overcome in working out quantitative methods enabling us to estimate the yield of bile pigment from the bile pigment-haematin compounds. This was, however, achieved and we can now correlate the yield of bile pigments obtained from choleglobin solutions by splitting with acids with the choleglobin concentration of these solutions.

In the above-mentioned preliminary publication we had also reported the isolation of bile pigments from erythrocytes. In the present paper we describe these experiments in greater detail.

## EXPERIMENTAL

*Preparation of biliverdin and biliviolins[2] from haemoglobin by coupled oxidation with ascorbic acid*

In each of 12 flat-bottomed culture flasks 50 ml. of a 1·5 % solution of crystalline horse oxyhaemoglobin, buffered to pH 8·5 with hydrogen phosphate,

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[2] According to Siedel (*Angew. Chemie*, 52, 38 [1939]) our compounds would be 'bilipurpurins', not 'biliviolins'. Siedel restricts the name 'biliviolins' to a very similar class of substances, probably isomeric with bilirubins, one of which e.g. is formed by the oxidation of mesobilinogen by ferric chloride; for the pigments of an oxidation stage higher than the biliverdins he uses the term 'bilipurpurins'. The extraordinary similarity of the properties of 'biliviolins' and 'bilipurpurins', and the fact that the latter comprise again several pigment classes, makes it appear more practical to maintain the generic name biliviolins, for all these pigments, and to distinguish them by figures and letters, until more is known about their chemical constitution. The term also tallies better with the actual colour of the pigments.

were incubated for 2 hr. with 50 mg. of ascorbic acid at 37°. The supernatant solution was poured off from the green pigment and poured into 2 vol. of glacial acetic acid. After 15 min. at room temperature this solution was poured into 2 vol. of ether (freed from peroxide over sodium) and the protein removed together with the greater part of the acetic acid by repeated washing with dilute sodium acetate solution and finally with water, avoiding shaking in the initial stages.

Biliverdin was extracted from the ethereal solution by repeated extractions with a few ml. of 5 % HCl. After a few hours green flocks of biliverdin hydrochloride settled down; they were collected by filtration and washed on the filter with water. The mother liquor contained a biliviolin. The biliverdin hydrochloride was dried on the filter in a vacuum desiccator and transformed into the dimethyl ester of biliverdin in the usual way. The ester was brought once more into ether and finally recrystallized from methyl alcohol. 20 mg. of blue-green needles. M.P. (on the copper block) 216°, mixed M.P. with an ester prepared from bilirubin (M.P. 212°) was 214°.

After the extraction with 5 % HCl the ether still contained a weakly basic biliviolin and protohaemin. The former was extracted with 20 % HCl. Haemin was then extracted with dilute ammonia and transformed into the pyridine-haemochromogen by pyridine and $Na_2S_2O_4$. Only the absorption bands of proto-haemochromogen could be observed. Cholehaematin must, therefore, have been transformed into the bile pigments, except that part of it which remains unsplit in the protein precipitate. The biliviolin present in the 20 % HCl extract was taken up by chloroform from the diluted solutions. The chloroform solution was washed to neutrality with water. It was then red-violet and showed a rather diffuse absorption band at 562 m$\mu$ and a second weaker band at 520 m$\mu$.

A phenomenon characteristic of the different biliviolins is their hydrochloride formation, when a neutral chloroform phase containing the pigment is shaken with an aqueous phase containing dilute HCl. The absorption spectrum changes from that of the neutral compound to that of the hydrochloride with an absorption band in the orange part of the spectrum, while the colour changes from pink or red-violet to blue-violet or blue, the colour change being more marked in daylight than in artificial light. This change depends on the concentration of the acid in the aqueous phase and also to a remarkable degree on the temperature, a higher temperature favouring salt formation. This can only be explained by the assumption that biliviolins occur in two tautomeric forms, the more strongly basic form being unstable and favoured by higher temperature.

The biliviolin obtained from the 20 % HCl extract behaved in this chloroform-HCl test as indicated by Table 1. We shall call it biliviolin III$a$. Its hydrochloride in aqueous solution was violet, in chloroform or in methyl alcohol in daylight almost blue, in artificial light violet. Its aqueous solution showed a broad absorption band with its centre at 596 m$\mu$, with its maximum, however, somewhat more towards the red at about 604 m$\mu$.

Table 1. *Chloroform-HCl test of biliviolin III a*

| Temperature | 1 % | 5 % | 10 % HCl |
|---|---|---|---|
| 0° | Neutral | Neutral | Partly acid |
| 20° | Neutral | Neutral | Acid |
| 60° | Neutral | Partly acid | Acid |

In chloroform the band was at 622 m$\mu$. The zinc salt in methyl alcohol was blue-green with red fluorescence and a two-banded absorption spectrum (I 646, II 581 m$\mu$; intensities: I, II).

Table 2. *Chloroform-HCl test of biliviolin IIb*

| Temperature | 1% | 5% | 10% HCl |
|---|---|---|---|
| 0° | Neutral | Partly acid | Acid |
| 20° | Neutral | Acid | Acid |
| 60° | Acid | Acid | Acid |

A second biliviolin was isolated in smaller yield from the mother liquor of biliverdin hydrochloride. It showed a different behaviour in the chloroform-HCl test (Table 2). We shall call it biliviolin IIb. The biliviolins can either be derived directly from choleglobin, or they may be products of a secondary oxidation of biliverdin. If about 200 $\mu$g. biliverdin are dissolved in dilute ammonia and added to a 0·7% oxyhaemoglobin solution and this mixture is treated with glacial acetic acid, the biliverdin is transformed into biliviolin, which according to its properties is biliviolin IIb. The same biliviolin had been previously obtained by oxidizing the complex zinc salt of biliverdin with two atoms of iodine per mol. and by exposing the unstable biliviolin IIa, set free from its zinc salt, to atmospheric $O_2$ [Lemberg & Lockwood, 1938, and unpublished experiments]. It is therefore likely that this biliviolin arises by secondary oxidation of biliverdin during the choleglobin splitting with acids.

The choleglobin solutions contain ascorbic acid, and if ascorbic acid in the same concentration (50 mg. per 100 ml.) is added to the mixture of biliverdin and oxyhaemoglobin, there is practically no oxidation of biliverdin to biliviolin. Even in the presence of excess of ascorbic acid or of CO, biliviolin IIIa was always found to accompany biliverdin in the splitting products of choleglobin. Ascorbic acid protects biliverdin from oxidation during the action of acid on oxyhaemoglobin (cf. Table 6), but does not reduce biliviolins to biliverdin. These experiments make it likely that the biliviolin IIIa is a genuine splitting product of choleglobin. It will be shown below that verdohaemochromogen yields also biliviolin besides biliverdin in the presence of ascorbic acid, and that the primary split-product of verdohaemochromogen is apparently oxidized by ferric iron to biliviolin. This biliviolin, however, is biliviolin IIb, the same that arises by oxidation of biliverdin, while the biliviolin IIIa from choleglobin is different.

The same split products were obtained from choleglobin if the method of splitting was varied, e.g. if an acetic acid-ethyl acetate mixture (1:2) was used, or acetic acid containing HCl or if the splitting was carried out at 100°. With acetic acid and hydrazine hydrate or with ferrous acetate in acetic acid-HCl, however, only biliverdin was isolated. In these reactions no trace of the hydroxyporphyrin described below was observed.

### Split products of 'green pigment'

Cholehaemochromogen obtained from choleglobin by alkali denaturation gave the same split products as choleglobin. The 'green pigment', however, which precipitated in the further course of the coupled oxidation of haemoglobin and ascorbic acid behaved somewhat differently in spite of the identity of its absorption properties with those of the first-mentioned cholehaemochromogen. The yield of ether-soluble split products was very small, the major part of the pigment remaining in the denatured protein which precipitated in the aqueous phase on addition of sodium acetate solution. Not only the bile pigments, but also the haemin from the admixed protohaemochromogen were thus largely lost. The kind of linkage of the prosthetic groups with the protein in the 'green pigment' is evidently quite different from that in the native and in the alkali-denatured chromoproteins. This is the reason why earlier observers failed to

isolate bile pigments. The green, amyl alcohol-soluble pigments, obtained by treating 'green pigment' with hot mineral acids [Edlbacher & v. Segesser, 1937] are still compounds of peptide nature, resulting from partial decomposition of the firmly attached protein groups. While their positive zinc acetate-iodine reaction (red fluorescence and absorption band at 636 m$\mu$) proves their close relationship to biliverdin, their solubility properties reveal the presence of large hydrophilic groups. They are insoluble in ether and absolute alcohol, but easily soluble in water.

Nevertheless small amounts of ether-soluble bile pigments were obtained by splitting green pigment with 66 % acetic acid—and these were the same as those isolated from choleglobin. A slightly larger yield of biliverdin was obtained, when 'green pigment' was dissolved in 1 % ascorbic acid (no reduction occurs, the absorption band being that of acid ferric cholehaemochromogen at 610 m$\mu$) and reduction to the ferrous haemochromogen caused by the addition of a minimum amount of $Na_2S_2O_4$ before the addition of acetic acid.

While acetic acid splitting thus yielded the same products as from choleglobin, though in much smaller yield, another split product of different nature was found, when the splitting was carried out with a ferrous acetate-acetic acid-HCl mixture or with glacial acetic acid in the presence of hydrazine hydrate. From the ether solution 0·5 % HCl extracted biliverdin, and 5 % HCl protoporphyrin and small amounts of a compound with a sharp absorption band at 627 m$\mu$. Somewhat larger amounts of this substance were contained in the 20 % HCl extract. The yield of all split products was again small. The new compound was brought back into ether by partial neutralization of the 20 % HCl extract and separated from the last traces of protoporphyrin by repetition of the HCl fractionation. The acid solution was green without fluorescence, while the dirty yellow neutral solution in ether-acetic acid showed red fluorescence. This solution showed four absorption bands: I 684, II 592, III 544, IV 504 m$\mu$ (intensities: I, III, IV, II). The Willstätter number was about 14. The copper complex salt showed the following spectrum: I 628, II 532 m$\mu$ (intensities I, II).

The properties of the substance show that it is different from the hydroxyporphyrin isolated by Lemberg, Cortis-Jones & Norrie [1938] from its haematin which was observed as an intermediate product in the formation of verdohaemochromogen from pyridine haemochromogen with ascorbic acid. It appears, however, to bear a close resemblance to the oxymesoporphyrins of Fischer et al. [1927; 1930], substances of uncertain constitution arising by the action of $H_2O_2$ on mesoporphyrin. Probably it is the corresponding proto compound, the absorption bands lying more towards the infra-red.

The fact that this hydroxyporphyrin cannot be obtained in the same way from choleglobin solutions, makes it unlikely that its haematin compound should be formed as intermediate product in the formation of choleglobin, or that it should be derived from cholehaematin itself. Some, though not all of Fischer's analyses indicate the presence of two oxygen atoms in addition to the four present in the two carboxylic groups of mesoporphyrin. Probably our new compound is a dihydroxyprotoporphyrin, the haematin of which is produced by a side reaction involving the oxidation of a second methene group before the ring is opened at the methene group first oxidized.

*Bile pigments from verdohaemochromogen and verdohaematin*

Verdohaemochromogen was shown by Lemberg [1935] to be transformed into biliverdin by hot methyl alcoholic HCl. Libowitzky & Fischer [1938] have recently claimed that the verdohaemochromogen of the copro series is not split

by a mixture of equal parts of glacial acetic acid and conc. HCl. From this observation they conclude that the 'green haemin' is not a haemochromogen, but a free ferric haematin, probably not of bile pigment-haematin nature. Warburg & Negelein have, however, already shown that the typical three-banded absorption spectrum disappears when the pyridine is washed away from the chloroform solution; it also disappears when the haemochromogen is split by acetic acid or by alkali. We can show also (cf. Table 7 below) that verdohaemochromogen can be split to bile pigments with good yield under much milder conditions than those applied by Fischer. The data collected in Table 7 show that if there is any difference in the reactions of ferric verdohaematin and verdohaemochromogen with acids, the former is split more easily. Libowitzky & Fischer base their assumption also on the observation that hydrazine hydrate destroys the typical absorption spectrum. That hydrazine does not destroy 'green haemins' by reduction, is, however, evident from the fact that it is used in their preparation from haemins. Verdohaemochromogens are unstable in all strongly alkaline solutions which transform them into yellow compounds. We have recently obtained further conclusive evidence [cf. also Holden & Lemberg, 1939] for the formula of verdohaemochromogen as an *iso*biliverdin-haemochromogen with opened ring by condensing it with ammonia and transforming it thus into the haemochromogen of an azaporphyrin with nitrogen-closed ring (unpublished experiments).



Protohaem                        Verdohaem                        Aza-haem
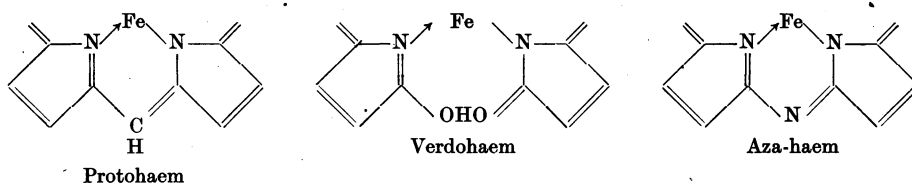
Verdohaemochromogen appears to us best formulated as a resonance structure with a hydrogen linkage between oxygen atoms closing the ring.

We have now subjected verdohaematin, verdohaemochromogen and verdoglobin to the same mild splitting conditions as were used for choleglobin above. Quantitative data will be given later; here we describe the splitting products. Again under all conditions mixtures of biliverdin and biliviolin were obtained (cf. Table 6), whether an alkaline solution of ferric verdohaematin or a solution of the haemochromogen in pyridine was treated with 66 % acetic acids. On keeping at room temperature biliviolins were obtained predominantly, while on short boiling biliverdin became the main product. Mainly biliverdin was formed when the splitting was carried out in the presence of ascorbic acid. Glacial acetic acid plus hydrazine hydrate and ferrous acetate in acetic acid-HCl in the heat produced biliverdin only.

For the isolation of the biliviolins 2 vol. of glacial acetic acid were added to the freshly prepared solution of verdohaemochromogen in 2 % NaOH. After 15 min. at room temperature the solution was diluted with an equal volume of ether. Washing with water removed some unsplit verdohaematin and a little biliverdin. From the red-violet ether solution 2 % HCl extracted two biliviolins, one of which (the main product) passed from 2 % HCl into chloroform, while the second was not extracted by chloroform. In the chloroform-HCl test the first substance behaved as indicated in Table 3.

The acid aqueous solution showed an absorption band at 595 m$\mu$, the acid chloroform solution one at 614 m$\mu$. The neutral chloroform solution had an absorption spectrum with two bands of about equal intensities (I 552, II 512 m$\mu$).

Table 3. *Chloroform-HCl test of biliviolin IIb from verdohaemochromogen*

| Temperature | 1 % | 5 % | 10 % HCl |
|---|---|---|---|
| 0° | Neutral | Neutral | Acid |
| 20° | Neutral | Partly acid | Acid |
| 60° | Partly acid | Acid | Acid |

The zinc salt was blue-green with strong red fluorescence and absorption bands at 640 and 585 m$\mu$, the former being far stronger. In spite of slight differences in the chloroform-HCl tests (cf. Tables 2 and 3) this violin is certainly identical with biliviolin IIb.

The second biliviolin was brought into chloroform by neutralization with sodium acetate. The neutral solution had a somewhat weaker second band than that of biliviolin IIb. 1 % HCl extracted it completely from chloroform. The centre of the absorption band of its hydrochloride was at 592 m$\mu$. This biliviolin, of which only traces are obtained, appears to be identical with that formed as a primary product by dehydrogenation of the biliverdin zinc salt with iodine (biliviolin IIa; unpublished experiments, and Lemberg & Lockwood [1938]).

The original ether solution was further successively extracted with 5, 10 and 20 % HCl. All these extracts contained violin. While the violin in the 5 % HCl extract was also biliviolin IIb, the other fractions contained a third biliviolin of weaker basicity. The absorption bands of the neutral solution in chloroform were at 554 and 514 m$\mu$; those of the acid chloroform solution at 617 m$\mu$. It differs from the biliviolin IIIa isolated from choleglobin by having absorption bands more towards the blue. We shall call it, therefore, biliviolin IIIb. Its behaviour in the chloroform-HCl test (Table 4) does not differ significantly from that of biliviolin IIIa.

Table 4. *Chloroform-HCl test of biliviolin IIIb from verdohaemochromogen*

| Temperature | 1 % | 5 % | 10 % HCl |
|---|---|---|---|
| 0° | Neutral | Neutral | Largely acid |
| 20° | Neutral | Neutral | Acid |
| 60° | Neutral | Partly acid | Acid |

The comparatively strongly basic biliviolin IIb forms the main bulk, while from choleglobin the weakly basic biliviolin IIIa was formed predominantly.

### Methods for the quantitative estimation of biliverdin and biliviolin

The methods for quantitative determination of bile pigments detached from choleglobin or verdohaemochromogen solutions were based in principle on the methods of isolation described above. For an exact determination of the bile pigment yield from these substances the biliviolin cannot be neglected. In the later phases of the coupled oxidation, however, biliverdin prevails. Biliverdin was also obtained as main product from catalase [Lemberg, Norrie & Legge, 1939]. We have therefore worked out a microcolorimetric method for the determination of biliverdin alone, which is simpler and requires less material (method I). For the determination of biliviolin as well as biliverdin spectrophotometric methods were developed. In the first of these (method II) biliverdin and biliviolin are separated by the Willstätter method of fractional HCl extraction from ether and determined separately, while in the second (method III) both are extracted together and their concentrations are calculated from the extinction coefficients at the wave-lengths of the maximal extinctions of biliverdin and biliviolin at 675 and 595 m$\mu$ respectively. Method II is suitable for deter-

mination of the bile pigments from choleglobin solution, since the weakly basic biliviolin $IIIa$ is easily separated from biliverdin, and unsuitable for the determination of the bile pigments from verdohaemochromogen, with which such a separation is difficult owing to the relatively strong basicity of biliviolin $IIb$. Here and for the study of the recovery of biliverdin added to oxyhaemoglobin solutions, in which also biliverdin $IIb$ is formed, method III was applied.

*Method I.* 5 ml. of the choleglobin solution are pipetted into 10 ml. of glacial acetic acid and after 15 min. at room temperature the mixture is poured into 15 ml. of peroxide-free ether in a narrow 50 ml. separating funnel. 5 ml. of saturated sodium acetate solution are added, the tube once inverted and 10 ml. of water added. After removal of the aqueous phase the protein precipitate suspended in the ether is removed together with the main bulk of acetic acid by washing with 10 ml. of dilute sodium acetate solution. Vigorous shaking must be avoided during the initial stages. The ether solution is then washed 2–3 times with water, the last time with shaking. It is essential to have the last trace of protein removed, but on the other hand to leave enough acetic acid in the ether to prevent precipitation of haematin. The first two sodium acetate extracts are overlayered once more with a few ml. of ether and saturated ammonium sulphate solution is added, until the protein comes out in green flocks at the interface and the aqueous layer becomes colourless. The latter is removed and the ether solution is poured off from the protein, washed with dilute sodium acetate solution and added to the main ether extract.

Biliverdin is then extracted by 2 ml. of 2 % HCl, followed by two further extractions with 2 ml. of 5 % HCl each. The united extracts are made up to 10 ml. with alcohol and matched colorimetrically with a biliverdin standard containing 100 $\mu$g. biliverdin in 10 ml. 40 % alcohol with an HCl content of 2·5 %. The standard is prepared freshly each day from a stock solution of 10 mg. pure crystalline biliverdin in 100 ml. alcohol, which is kept in the dark. During 6 weeks the specific extinction coefficient ($\epsilon$ of an 0·1 % solution) of the acidified solution at the maximum of absorption ($\lambda = 675$ m$\mu$) diminished very little, (from 47·1 to 46·5).

*Method II.* Biliviolins cannot be estimated colorimetrically, since their solutions are not stable enough to be used as standards, and other pigments which match the colour exactly are not available. A spectrophotometric method is therefore used. Biliviolins have not yet been obtained crystalline and their specific extinction coefficients are at present unknown. The purification of the biliviolin $IIIa$ from choleglobin in sufficient quantities promised little hope of success. We have therefore used a fresh sample of biliviolin $IIb$. This was isolated from the 5 % HCl fraction obtained after splitting of verdohaemochromogen with acetic acid and extraction with ether. It was brought back into ether with sodium acetate and the acetic acid was removed by extraction with water. The evaporation residue of the ether solution was dried *in vacuo* and weighed. It was then dissolved in 2 ml. of alcohol and diluted to 10 ml. with 20 % HCl. The specific extinction coefficient at the maximum of absorption (at 595 m$\mu$) was found to be 46·0. This value is so close to that of the maximal specific extinction coefficient of biliverdin, that it can be reasonably assumed that the sample of biliviolin was pure, and also that the maximal extinction coefficient of biliviolin $IIIa$ from choleglobin will not be very different from that of biliviolin $IIb$ and biliverdin. The value 46·0 for the specific extinction coefficient of biliviolin as hydrochloride at 595 m$\mu$ and the value 46·5 for that of biliverdin as hydrochloride at 675 m$\mu$ were used to convert spectrophotometric readings into terms of concentration.

For method II 50 ml. of a choleglobin solution obtained from 0·7 % haemo-globin are required. They are worked up as described for method I, the extraction of the bile pigments being carried out, however, in a different manner and with relatively smaller volumes of HCl in order to get sufficiently strong solutions. 10–15 ml. of 5 % HCl are used for the extraction of biliverdin and the same volume of 20 % HCl for the extraction of biliviolin for samples of choleglobin solutions obtained by a short incubation of haemoglobin with ascorbic acid, while for samples of longer incubation time 25–30 ml. of the acids can be used. 5 ml. of alcohol are added and the final volume is measured. In a layer of 4–5 cm. length these bile pigment solutions give extinctions in the optimal range of the spectrophotometer. The instrument was the Hilger Visible Spectrophotometer described in Part 2.

*Method III.* If the extinction coefficients of both biliverdin and biliviolin are known at two wave-lengths at which they differ considerably, the content of a mixture of both substances can be established by measuring the extinctions at these wave-lengths. The wave-lengths of the absorption maxima ($\lambda = 675$ and 595 m$\mu$) are suitable. The specific extinction coefficients of biliverdin hydro-chloride were found to be 46·5 at 675 m$\mu$ and 21·4 at 595 m$\mu$, while those of biliviolin hydrochloride were 46·0 at 595 m$\mu$ and 6·0 at 675 m$\mu$. If $\epsilon_{675}$ and $\epsilon_{595}$ are the extinction coefficients of the solution in 1 cm. depth at the wave-lengths 675 and 595 m$\mu$ the following two equations hold:

$$\mu g. \text{ biliverdin per ml.} = 2\cdot98 \ (7\cdot67 \ \epsilon_{675} - \epsilon_{595})$$
$$\mu g. \text{ biliviolin per ml.} = 10\cdot64 \ (2\cdot17 \ \epsilon_{595} - \epsilon_{675}).$$

For the determination with this method the ethereal solution is extracted twice with 5 ml. each of 5 % HCl and three times with 5 ml. each of 20 % HCl. After the addition of 5 ml. of alcohol to the united extracts the volume is measured and the extinction coefficients at 675 and 595 m$\mu$ are measured.

Table 5 shows that with choleglobin methods II and III give essentially the same results, while the differences are larger when the bile pigments from verdo-haematin or verdohaemochromogen are determined; here method III is pre-ferable.

Table 5. *Determination of bile pigments from verdohaemochromogen and choleglobin*

| | Method II | | | Method III | | |
|---|---|---|---|---|---|---|
| | $\mu g.$ verdin | $\mu g.$ violin | $\mu g.$ bile pigment | $\mu g.$ verdin | $\mu g.$ violin | $\mu g.$ bile pigment |
| Verdohaematin | 52 | 104 | 156 | 33 | 134 | 166 |
| Verdohaemochromogen + ascorbic acid | 167 | 42 | 209 | 165 | 106 | 271 |
| Choleglobin | 950 | 251 | 1201 | 978 | 203 | 1191 |

*Bile pigment yield from verdohaemochromogen*

Experiments on the yield of bile pigments from verdohaematin compounds were carried out in order to examine in what yield bile pigment is isolated from these bile pigment-haematin compounds of established constitution under the conditions used for the splitting of choleglobin.

Verdohaemochromogen was either dissolved directly in 66 % acetic acid (*a*) or it was first dissociated to verdohaematin by dissolving it in 1 ml. of 2 % NaOH (*b*) or dissolved in 1 ml. of 20 % pyridine (*c*) and these solutions added to 2 ml. of glacial acetic acid. Finally a solution of verdohaematin in 1 % Na$_2$CO$_3$

was coupled with native horse globin and 6 ml. of this solution of ferric verdo-globin (*d*) were added to 12 ml. of glacial acetic acid. The bile pigments were isolated as from choleglobin, the unsplit verdohaematin being removed from the ether by repeated washing with water before the extraction of the bile pigments with HCl. Method III was applied to the determination and the results are given in Table 6.

Table 6. *Bile pigment yields after acid splitting of verdohaematin compounds under various conditions*

| Additions to the solution before splitting | Split by | Time min. | Total bile pigment in % of theory | | | | Biliverdin in % of total bile pigment | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *a* Solid verdo-haemo-chromogen | *b* Verdo-haematin in alkali | *c* Verdo-haemo-chromogen in 20% pyridine | *d* Ferric verdo-globin | *a* | *b* | *c* | *d* |
| — | 66% acetic acid | 15 | 13·8 | 25·8 | 6·1 | 14·7 | 16 | 38 | 35 | 19 |
| | | 60 | 18·5 | 32·5 | 13·7 | — | 25 | 44 | 18 | — |
| 5 mg. ascorbic acid | ,, | 15 | — | 25·6 | 6·8 | 10·7 | — | 74 | 62 | 49 |
| | | 60 | 25·3 | 34·3 | 12·2 | — | 58 | 89 | 55 | — |
| 5 mg. ascorbic acid, +CO | 66% acetic acid under CO | 15 | — | 17·0 | — | 12·1 | — | 63 | — | 46 |
| | | 60 | 21·5 | 31·4 | — | — | 54 | 71 | — | — |
| — | 66% acetic acid + 1 ml. conc. HCl | 60 | 19·3 | — | — | — | 28 | — | — | — |
| — | 66% acetic acid, boiling | 2 | 39·5 | 22·6 | 23·7 | 10·4 | 70 | 55 | 87 | 58 |
| — | 5% HCl | 60 | — | 33·0 | — | — | — | 65 | — | — |
| 5 mg. ascorbic acid | ,, | 60 | — | 32·6 | — | — | — | 73 | — | — |

Under the mild conditions used for the splitting of choleglobin the iron from verdohaematin compounds is not detached completely, but already to the extent of 15–35 % at room temperature and up to 40 % by boiling acetic acid. Neither HCl, nor a mixture of acetic and hydrochloric acids increases the yield of bile pigments substantially. The degree of splitting varies a great deal with the conditions. If there is any difference between ferric verdohaematin and ferrous verdohaemochromogen in this regard, ferric verdohaematin is split more readily. The difference is, however, not large. Thus ferrous verdohaemochromogen was observed spectroscopically to persist in the 66 % acetic acid in the presence of pyridine and ascorbic acid or of pyridine, ascorbic acid and CO, while its absorption spectrum rapidly disappeared in the acetic acid in the absence of ascorbic acid; the bile pigment yield was slightly higher in the absence of ascorbic acid. In the absence of excess pyridine CO reacts in the acetic acid with verdo-haem when ascorbic acid is present, yielding a characteristic CO-compound with absorption bands at 611, 541 and 502 m$\mu$ (intensities: I, II, III); when ascorbic acid is absent, CO fails to form this compound. It is, therefore, CO-verdohaem with ferrous iron; pyridine converts it into verdohaemochromogen. Again the yield of bile pigment isolated from the ferrous CO-verdohaem is either the same or slightly less than from the brown ferric verdohaematin solutions obtained by the action of acetic acid on verdohaemochromogen. Verdoglobin is split by 66 % acetic acid with a yield of 10–15 % and neither ascorbic acid nor CO influences the yield significantly. Under all these conditions both biliverdin and biliviolin are obtained, predominantly verdin from ferrous and violin from ferric compounds. Boiling acetic and hydrochloric acids produce less violin from ferric

verdohaemàtin than acetic acid at room temperature. Biliviolin II$b$ contains four hydrogen equivalents less than biliverdin and one would expect, therefore, a yield of 25 % of violin, if oxidation of biliverdin by the ferric haematin iron causes the formation of the biliviolin. Actually much more biliviolin is always found, although the difference in the percentages of biliverdin in total bile pigment from a ferrous and a ferric compound is roughly 25 %. Since biliviolin is not reduced by ascorbic acid, and biliverdin is not oxidized by the small concentrations of ferric acetate set free in the solutions, it is somewhat difficult to explain these results, unless it is assumed that the unstable *iso*biliverdin set free from verdohaematin compounds is more readily oxidized than biliverdin.

### Recovery of biliverdin from mixtures with oxyhaemoglobin

For the determination of the bile pigment yields from choleglobin solutions, which always contain oxyhaemoglobin, it was essential to know to what extent biliverdin can be recovered by our methods from a solution of oxyhaemoglobin. A known amount of biliverdin dissolved in dilute ammonia was added to 50 ml. of an 0·7 % solution of horse oxyhaemoglobin. This amount of oxyhaemoglobin alone gave no detectable amounts of bile pigments. In the majority of the recovery experiments 50 mg. per 100 ml. of ascorbic acid were added, this being about the ascorbic acid concentration of the majority of choleglobin solutions. It has already been mentioned above that ascorbic acid prevents the oxidation of biliverdin to biliviolin during the treatment with acid. The mixture of oxyhaemoglobin, biliverdin and usually ascorbic acid was subjected to the treatment with 66 % acetic acid as described above, and the recovered bile pigment determined by method III.

Table 7 shows that, under the conditions of the determination of the bile pigment yield from choleglobin, between 80 and 90 % (average 84 %) of the added biliverdin can be recovered and that the percentage of recovery does not

Table 7. *Recovery of biliverdin from dilute oxyhaemoglobin solutions*

| $\mu$g. biliverdin added | Ascorbic acid mg. per 100 ml. | In % of added biliverdin | | |
|---|---|---|---|---|
| | | Biliverdin | Biliviolin | Total bile pigment |
| 62·9 | 50 | 79·0 | 2·2 | 81·2 |
| 63·5 | 50 | 79·0 | 0 | 79·0 |
| 95 | 50 | 90·7 | 0 | 90·7 |
| 189 | 50 | 95·9 | 0 | 95·9 |
| 222 | 50 | 83·0 | 0 | 83·0 |
| 222 | 50 | 68·5 | 0·5 | 69·0 |
| 262 | 50 | 90·8 | 0 | 90·8 |
| 408 | 50 | 83·0 | 1·9 | 84·9 |
| 952 | 50 | 75·0 | 0 | 75·0 |
| 62·9 | — | 0 | 85·8 | 85·8 |
| 95 | — | 2·9 | 86·4 | 89·3 |
| | | | Average | 84 % |

depend on the amount of biliverdin in a range between 60 and 400 $\mu$g. per 50 ml., i.e. in the range of bile pigment yields obtained from choleglobin solutions. In the absence of ascorbic acid biliverdin is oxidized to biliviolin II$b$ by the action of acid on oxyhaemoglobin, but the yield of biliviolin is the same as that of biliverdin obtained in the presence of ascorbic acid.

*Bile pigment yield from choleglobin*

We found that splitting at room temperature for 15 min. in 66 % acetic acid gave optimal yields of bile pigment from solutions of choleglobin. Heating, or treatment with the acid for a longer period, or the application of stronger acids, e.g. HCl, decreased the yield. Probably more of the bile pigment-haemoglobin is split, but also more is denatured in a way which prevents the removal of the bile pigment from the protein. In Table 8 the yields of bile pigments obtained from choleglobin solutions after various times of incubation of haemoglobin with ascorbic acid are compared with the amount of choleglobin determined spectrophotometrically. A superficial examination may lead to the assumption that the bile pigment is not derived from choleglobin, since there is evidently no proportionality between the amount of choleglobin and the yield of bile pigment. While the maximum of choleglobin concentration in the solution is reached in about 30 min. at $p$H 8·5 and in about 80 min. at $p$H 7·2, the maximum of the bile pigment formation occurs only after about 7 hr. incubation; for these calculations we have left out of consideration the cholehaemochromogen of the green pigment precipitate, since little bile pigment can be isolated from green pigment, at least in the first stages of its formation. It is assumed that one mole of choleglobin of mol. wt. 16,700 yields 584 g. bile pigment, 584 being the mol. wt. of biliverdin. The mol. wt. of biliviolin is somewhat higher, but the error introduced by this in the calculations, is insignificant. The data in Table 8 show that in the first stages of the reaction only 10–15 % of the bile pigment theoretically expected from the choleglobin content of the solutions is isolated, while later up to 70 % can be recovered. Choleglobin would thus appear not to yield bile pigment by the action of acids, but to be a precursor of bile pigment or of a bile pigment-haemoglobin yielding bile pigment on treatment with acids. While such an assumption would solve the difficult problem: what is the constitutional difference between cholehaem and verdohaem? it would create new vexing problems. If the bile pigments, which are obtained from choleglobin solutions by the action of acids, are not the iron-free prosthetic group of choleglobin, what can this prosthetic group be? Apart from bile pigments and protohaemin no other compounds were isolated from choleglobin solutions treated by acids. Protohaemin is exclusively derived from accompanying haemoglobin, since it decreases exactly in proportion to the haemoglobin concentration (cf. Part 3). We have also shown in Part 4 that the greater part of the choleglobin iron is removed by incubation with $N/10$ HCl for 16 hr.

A closer examination of the data in Table 8 reveals, indeed, sufficient evidence that choleglobin is split to bile pigments with a yield of 11–12 %. Such a yield is found in the initial periods in which according to Table 3 of Part 3 (p. 344) choleglobin is the only product of the reduction. This is evident from Table 9 in which the yield of unknown by-product obtained from Table 3 of Part 3, i.e. initial haemoglobin minus (choleglobin + haemoglobin + cholehaemochromogen + protohaemochromogen), is given in the third column and compared with the percentage yield of bile pigment in the fourth column. The same yield of 11–12 % is obtained from choleglobin produced by coupled oxidation of haemoglobin with glutathione or with thiolacetic acid. With the latter again no formation of by-products was indicated by the balance sheet of the reaction. The apparent bile pigment recovery from haemoglobin is somewhat higher, but still below 20 %, where there is a slight formation of by-product (under 3 % of the initial haemoglobin), e.g. in experiments with ascorbic acid at $p$H 8·5 up to 80 min. and at $p$H 7·2 up to 30 min. incubation time. Only where the balance sheet indicates a

Table 8. *Bile pigment yields from choleglobin solutions*

Haemoglobin 0.72%, ascorbic acid 0.1%, 37°

| pH | Incubation time | Choleglobin in % of initial haemoglobin | μg. biliverdin from 50 ml. | μg. total bile pigment from 50 ml. | Biliverdin in % of theory | Total bile pigment in % of theory | Method | Remarks |
|---|---|---|---|---|---|---|---|---|
| 7.2 | 15 min. | 8.2 | 58 | — | 5.6 | — | I | |
| | | 7.6 | 102 | — | 10.6 } 7.3 | — | I | |
| | | 5.9 | 42 | — | 5.6 | — | I | |
| | | 8.2 | 52 | 112 | 5.1 } 7.8 | 10.9 } 13.5 | II | |
| | | 7.6 | 101 | 170 | 10.5 | 16.1 | II | |
| | 20 min. | 11.2 | 127 | 217 | 9.0 | 15.4 | II | |
| | 30 min. | 18.3 | 123 | — | 5.3 | — | I | |
| | | 14.2 | 173 | — | 9.7 } 8.4 | — | I | |
| | | 10.8 | 138 | — | 10.1 | — | I | |
| | | 13.5 | 121 | 327 | 7.4 | 20.0 } 19.3 | II | |
| | | 13.5 | 138 | 352 | 8.5 } 8.6 | 21.6 | II | |
| | | 18.3 | 230 | 377 | 10.0 | 16.4 | II | |
| | 80 min. | [20] | 360 | 1050 | 14.9 | 43.5 | II | |
| | | — | 393 | 990 | 16.3 } 17.2 | 41.0 } 34.2 | II | |
| | | — | 520 | 753 | 20.6 | 29.8 | II | |
| | | — | 427 | 562 | 16.9 | 22.5 | II | |
| | 2 hr. | [20] | 914 | 1219 | 36.2 | 48.3 | II | Precipitate |
| | 5 hr. | [20] | 1230 | 1500 | 48.7 | 59.5 | II | Precipitate |
| | 6¼ hr. | [20] | 1480 | 1690 | 58.7 | 67.0 | II | Precipitate |
| | 16 hr. | 11.5 | 1080 | — | 71 | — | I | Precipitate |
| 8.5 | 15 min. | 14.8 | 95 | — | 5.1 | — | I | |
| | | 21.7 | 266 | — | 9.7 } 6.5 | — | I | |
| | | 9.9 | 58 | — | 4.7 | — | I | |
| | | 21.7 | 178 | 388 | 6.5 | 14.2 } 10.1 | II | |
| | | 21.0 | 154 | 239 | 5.8 } 5.6 | 9.0 | II | |
| | | 21.0 | 118 | 188 | 4.5 | 7.1 | II | |

Remarks: Precipitate; [ ] = not measured, average value of choleglobin

| pH | Time | | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|
| 8·5 | 30 min. | 32·6 | 482 | — | 11·7 | — | I | |
| | | 32·1 | 465 | — | 11·5 }9·4 | — | I | |
| | | 21·0 | 194 | — | 7·3 | — | I | |
| | | 31·9 | 279 | — | 7·2 | — | I | |
| | | 32 | 242 | 360 | 6·0 | 8·9 | II | Precipitate |
| | | 32 | 322 | 560 | 8·0 | 13·9 | II | |
| | | 32 | 180 | 268 | 4·5 }8·5 | 6·7 }12·4 | II | |
| | | 37 | 465 | 810 | 10·0 | 17·3 | II | |
| | | 24 | 209 | 350 | 7·0 | 11·7 | II | |
| | | 31 | 464 | 616 | 11·9 | 15·8 | II | |
| | 50 min. | 28·7 | 336 | 484 | 9·3 | 13·4 | II | Precipitate |
| | 80 min. | [28] | 595 | 893 | 16·9 | — | I | Precipitate; if filtrate alone split: 468 µg. = 13·3% biliverdin |
| | | 28 | 690 | 553 | 19·5 }14·9 | 25·3 }19·1 | II | |
| | | 28 | 379 | 572 | 10·7 | 15·7 | II | |
| | | 28 | 445 | | 12·6 | 16·2 | II | |
| | 2 hr. | 36·5 | 756 | — | 16·7 }26·6 | — | I | Precipitate |
| | | 21·4 | 990 | — | 36·6 | 32·5 }33·6 | I | |
| | | 21·0 | 681 | 857 | 25·8 }27·2 | 34·7 | II | |
| | | [28] | 1005 | 1225 | 28·5 | — | II | |
| | 3 hr. | [20] | 1060 | — | 42·7 }41·0 | — | I | Precipitate |
| | | — | 990 | — | 39·3 | — | I | |
| | 4 hr. | [20] | 1340 | — | 53 | — | I | Precipitate; if filtrate alone split: 1170 µg. = 43% biliverdin |
| | 5 hr. | [20] | 1505 (a) | — | 60 (a) | — | I | (a) With precipitate. From filtrate alone: 784 µg. = 31%. (b) From filtrate alone |
| | | — | 910 (b) | — | 36 (b) | — | I | |
| | 6½ hr. | [20] | 1030 | — | 41 | — | I | From filtrate alone |
| | 7¼ hr. | [20] | 1060 | 1215 | 42 | 48·3 | II | From filtrate only |
| | | — | 1190 | — | 47 | — | I | |
| | 8¼ hr. | [20] | 1010 | — | 40 | — | I | From filtrate only |
| | 9¼ hr. | [20] | 775 | — | 31 | — | I | From filtrate only |
| | 16 hr. | 13·6 | 620 | — | 36 | — | I | From filtrate only |
| 7·2 | 90 min. | 19·5 | 220 | 307 | Thiolacetic acid 8·5 | 11·9 | II | Ascorbic acid added before acidification |
| 7·2 | 19 hr. | 6·0 | 50 | 86 | Glutathione 6·6 | 11·4 | II | Ascorbic acid added before acidification |

Table 9. *Correlation between by-product formation during coupled oxidation and excess bile pigment not derived from choleglobin*

| pH | Time of incubation min. | By-product formed in % of choleglobin equivalents | Average yield of bile pigment in % of choleglobin equivalents | Average yield of excess of bile pigment over 11·5 % in equivalent % of by-product |
|---|---|---|---|---|
| 8·5 with ascorbic acid | 15 | 0 | 10·1 | — |
| | 30 | 0 | 12·4 | — |
| | 80 | 2·6 | 19·1 | 82 |
| | 120 | 8·1 | 33·6 | 73 |
| | 420 | 25·9 | 48·3 | 28 |
| 7·2 with ascorbic acid | 15 | 0·4 | 13·5 | 38 |
| | 30 | 2·8 | 19·3 | 41 |
| | 80 | 9·5 | 34·2 | 48 |
| | 120 | 12·4 | 48·3 | 59 |
| | 420 | 11·4 | 67·0 | 97 |
| 7·2 with thiol-acetic acid | 90 | 0 | 11·9 | — |

considerable formation of by-product in the later stages of the reaction, much more bile pigment is obtained. It can also be seen from Table 9 that the initial rate of bile pigment formation at $p$H 8·5 is more than double that at $p$H 7·2, exactly as in the case with the rate of choleglobin formation. From these observations we conclude that choleglobin is split by 66 % acetic acid to 11·5 %, i.e. to about the same degree as verdoglobin (cf. Table 6). This also supports the view that the two substances are closely related to each other.

We have attempted to gain information on the question to what degree choleglobin is split by the treatment with 66 % acetic acid in other ways, but have failed to obtain a definite result. The unsplit choleglobin was precipitated from the aqueous solution remaining after ether extraction of bile pigments and protohaematin by sodium acetate and ammonium sulphate. It was then filtered off, washed free from salts and redissolved in $N/10$ NaOH. The absorption maximum at 630 m$\mu$ of the CO-cholehaemochromogen was measured and its strength indicated that about 70 % of the choleglobin (in one experiment 45 %) had been split. The colour of the recovered cholehaemochromogen is, however, rather brown than green, and it is likely that it has become altered and that its absorption has been diminished in intensity. The values found, give therefore, only minimum values of recovery of unsplit choleglobin. In the case of cholehaemochromogen smaller salt concentrations are required for the precipitation of the pigment and the estimation of recovery is more likely to be correct. While incubation with $N/10$ HCl removed 66 % of the iron from green pigment 83 % were recovered unsplit after 15 min. standing at room temperature in 60 % acetic acid.

The later part of the reaction with ascorbic acid is complicated by several factors. First, 'green pigment' precipitates and while little bile pigment is obtained from 'green pigment' precipitated after 80 min. incubation, later on a larger part of the bile pigment may be derived from precipitate (cf. the values of biliverdin obtained from the solution alone (last column) with those obtained from the unfiltered suspension (column 6 of Table 8). This bile pigment from green pigment may either be derived from a cholehaemochromogen, in which the protein has been denatured in such a way that no firm linkage between prosthetic group and protein is established, or, more likely, from an iron-free bile pigment-protein compound. In Part 3 (p. 342) we have indeed reported evidence for the

occurrence of some iron-free protein in the 'green pigment'. On longer incubation ascorbic acid evidently removes iron from cholehaemochromogen. The figures in the last column of Table 8 show, however, that the bile pigment recoveries from the filtrate alone also still far exceed the 11·5 % derived from choleglobin. In the last column of Table 9 the bile pigment derived from choleglobin, assuming a yield of 11·5 %, is subtracted from bile pigment found, and this excess is given in equivalent percentage of the unknown by-product. This percentage cannot be established with great accuracy, but the figures leave no doubt that the by-product yields a much larger amount of bile pigment than choleglobin or verdoglobin. It is therefore likely that prolonged incubation of haemoglobin with ascorbic acid sets free bile pigment from choleglobin, and that this occurs now in the solution still bound to protein, but no longer to iron. This excess bile pigment not derived from choleglobin consists almost entirely of biliverdin. Since biliverdin is reduced by $Na_2S_2O_4$ to a yellow compound with no absorption in the red and green parts of the spectrum, the spectrophotometric determination of the cholehaem and protohaem compounds is not affected by its presence, and in the balances of Table 3, Part 3 the by-product appears simply as deficit.

Additions of further ascorbic acid before the acid splitting of choleglobin or of CO do not alter the total yield of bile pigment; they increase the yield of biliverdin slightly and decrease the yield of biliviolin correspondingly.

### Isolation of bile pigments from red cells

The bile pigment yield from red cells is small and for its estimation rather strong solutions of haemoglobin must be employed. It is to be expected that the recovery of bile pigment from such solutions will be less complete than from dilute haemoglobin solutions. On the other hand the yield of bile pigment obtained from the blood or from strong solutions of haemoglobin can no longer be neglected in the estimation of the recovery of added bile pigment. We therefore measured the amount of bile pigment that can be isolated from red cells in one sample and determined the degree of recovery of added biliverdin in a second sample of the same red cell solution. In order to avoid oxidation of the biliverdin, ascorbic acid (50 mg. per 100 ml.) was added immediately before acidification with acetic acid.

20 ml. of fresh sheep cells, twice washed with saline were diluted to 100 ml. with water and haemolysed. 50 ml. of this solution were treated with ascorbic acid and acetic acid, while to the second lot of 50 ml. a solution of 222 $\mu$g. biliverdin in 1 ml. dilute ammonia was added before they were treated in the same way. Both samples were worked up in the manner described with a few modifications. With the high protein concentrations great care had to be taken to avoid the formation of emulsions. The greater part of the protein was precipitated by the addition of somewhat more ether than was used for the recovery of bile pigment from dilute solution, and filtered off before the addition of sodium acetate solution and water. It was impossible to avoid precipitation of some haemin from the ether before complete removal of the protein; this was filtered off from the ethereal solution, or if there was not so much as to disturb the separation of the layers, from the acid extracts, which had to be filtered in any case. Both samples were worked up in precisely the same manner. 62 $\mu$g. bile pigment (42 $\mu$g. biliverdin and 16 $\mu$g. biliviolin) were isolated from the first sample and 162 $\mu$g. biliverdin from the second. From the added 222 $\mu$g. thus only $162 - 46 = 116$ $\mu$g. $= 52$ % were recovered.

If it is assumed that the same percentage of the bile pigment set free by splitting the bile pigment-haemoglobin of the red cells is recovered as from added biliverdin, and that the bile pigment-haemoglobin of the red cells is split to the same degree as choleglobin or verdoglobin, the amount of bile pigment found has to be multiplied by the factor $\frac{100}{52} \cdot \frac{100}{11 \cdot 5} \cdot \frac{16,700}{584} = 480$ in order to obtain the amount of bile pigment-haemoglobin, from which it was derived. 62 $\mu$g. bile pigment thus correspond to 29·8 mg. bile pigment-haemoglobin. Since the 10 ml. of laked cells contained 3270 mg. haemoglobin, only 0·9 % of the red cell haemoglobin was bile pigment-haemoglobin.

In a second sample 8·75 ml. of sheep erythrocytes containing 2·8 g. haemoglobin yielded 66·2 $\mu$g. bile pigment (45·7 $\mu$g. biliverdin and 20·5 $\mu$g. biliviolin) without the addition of ascorbic acid, while with ascorbic acid only 46 $\mu$g. (23 $\mu$g. biliviolin and 23 $\mu$g. biliverdin) were obtained. These values correspond to bile pigment contents of 1·1 and 0·8 %.

Horse erythrocytes were not studied quantitatively, but qualitatively they gave the same results. Similar yields to those from sheep red cells were obtained from solutions of crystalline horse oxyhaemoglobin. 50 ml. of a 5·7 % solution gave 76 $\mu$g. bile pigment (39 $\mu$g. verdin and 37 $\mu$g. violin), corresponding to a bile pigment-haemoglobin content of 1·3 %, when CO-haemoglobin was split in the presence of 50 mg. ascorbic acid, while the splitting of the oxyhaemoglobin without ascorbic acid gave here a smaller yield of 35 $\mu$g. (of which 30 $\mu$g. were violin) corresponding to 0·6 % bile pigment-haemoglobin.

These estimations are not very accurate, but while proving the presence of some bile pigment-haemoglobin in red cells, they make it unlikely that bile pigment-haemoglobin constitutes more than a part of the 'labile iron' of the blood. Since splitting in the presence of ascorbic acid and of CO decreases the yield of bile pigment little, if at all, the latter cannot be derived to a large extent from oxidative processes occurring during the acidification of oxyhaemoglobin solutions, but must be derived, at least partly, from bile pigment-haemoglobin preformed in the red cells. The biliviolin obtained appeared to be of the weakly basic type III, but not sufficient material was available to identify it with the biliviolin III$a$ obtained from choleglobin.

## SUMMARY

On splitting with acetic acid choleglobin yields biliverdin and a weakly basic biliviolin (biliviolin III$\bar{a}$). Cholehaemochromogen ('green pigment') gives small amounts of the same products, but the greater part of the prosthetic group has become firmly attached to the denatured protein. On splitting with ferrous acetate-acetic acid-hydrochloric acid green pigment yields small amounts of a hydroxyporphyrin. Verdohaemochromogen is split by acids to biliverdin and a biliviolin of medium basicity (biliviolin II$b$).

Quantitative methods are worked out allowing the isolation of bile pigments from solutions that contain oxyhaemoglobin, and for their spectrophotometric and colorimetric determination. An average recovery of 85 % is obtained. Biliverdin is oxidized during the action of acids on oxyhaemoglobin to biliviolin II$b$, and this oxidation is prevented by ascorbic acid.

Verdohaematin is split to bile pigments by 66 % acetic acid to an extent of 25–35 %, verdoglobin to 10–15 %. The bile pigment yield from choleglobin is 11–12 % in the initial phases of the coupled oxidation of haemoglobin and ascorbic acid, in which no by-product is formed. Later considerably more bile pigment is obtained. This bile pigment, not derived from choleglobin, is biliverdin

and the by-product is probably an iron-free biliverdin-protein compound. Prolonged action of ascorbic acid on choleglobin is thus able to remove the iron held in complex combination in choleglobin and to set free biliverdin at a physiological $pH$.

Small amounts of bile pigments (biliverdin and biliviolin) were obtained by treatment with acetic acid from laked red cells of sheep and horse as well as from crystalline horse oxyhaemoglobin. Provided that the bile pigment haemoglobin, from which these bile pigments are derived, is split to the same degree as choleglobin, and that the percentage of recovery of these bile pigments is the same as that of added biliverdin (52 %), only about 1 % of the red cell iron is bile pigment-haemoglobin iron.

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