CLXI. TRANSFORMATION OF HAEMINS INTO BILE PIGMENTS.

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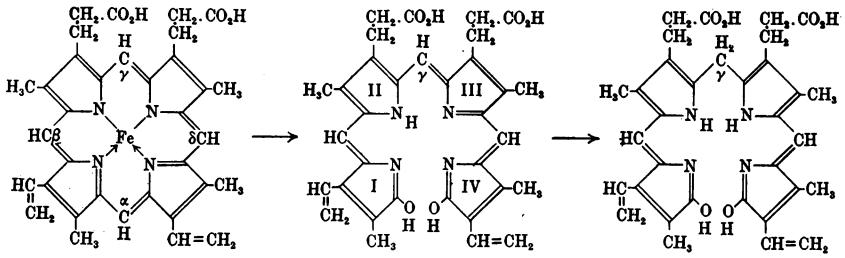
THAT bile pigment arises in the body by decomposition of haemoglobin is generally accepted. The work of Fischer and collaborators has confirmed the close chemical relationship between bilirubin and the colour component of haemoglobin. Hitherto nothing was known about the chemical mechanism of this transformation, nor has the formation of a bile pigment from haemoglobin or haemin *in vitro* been demonstrated with certainty. Mesobilinogen[1] has been transformed into mesoporphyrin [Fischer and Lindner, 1926, 2]; the reverse reaction however has not been accomplished. From a chemical point of view the formation of bile pigments from haemin would mean their total synthesis, since haemin has been synthesised by Fischer and Zeile [1929]. Several mesobilirubins (with a symmetrical arrangement of the side-chains) are now obtainable by direct synthesis, but the natural bile pigments which have unsymmetrically arranged and unsaturated side-chains, like bilirubin and biliverdin, cannot be synthesised in this way. A glance at the formula now established for bilirubin (III) by the investigations of Fischer since 1931 shows that it must arise from the porphyrin nucleus of haemoglobin by oxidative scission. The α CH-group is removed and replaced by two hydroxyl groups, and the porphyrin ring is thus opened. If this reaction leads to bilirubin (I → III), the γ CH-group is reduced



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to CH_2. Although an oxidative-reductive process is not impossible, another alternative suggests itself, *viz.* that the reaction leads at first to biliverdin [dehydrobilirubin, *cf.* Lemberg, 1934, 1] which is subsequently reduced. It has been reported by Barry and Levine [1924] that the liver reduces biliverdin to bilirubin. Moreover the bile of a number of animals (*e.g.* of the frog) contains biliverdin and not bilirubin[2].

[1] The writer proposes to use the shorter term "mesobilinogen" instead of "mesobilirubinogen". The first name is also more logical, since mesobilinogen is the leuco-compound of mesobiliverdin *etc.* as it is of mesobilirubin.

[2] Unlike the product of autoxidation of bilirubin in alkaline solution and like uteroverdin the biliverdin of frog's bile is free from products of secondary alteration. This disproves Fischer's view that there is a genuine difference between biliverdin and dehydrobilirubin.

Several references occur in the literature to the formation of green pigments from haemoglobin or from haemin. In fact, the bile pigment nature of some of these compounds has been claimed, although the evidence has been very scanty. With the exception of Warburg and Negelein [1930] no investigator has obtained a pure substance. Moreover, the work of these authors pointed in a different direction, since they obtained substances containing iron ("green haemin").

A closer study of the literature reveals that the processes observed by the different authors leading to the formation of green pigment from haemin or haemoglobin have more in common than appears at a first glance.

From the description given by Lewin [1901] of the dichroic green-brown substance (or more likely mixture of substances), obtained from the blood of animals poisoned with phenylhydrazine and termed haemoverdin, it remains doubtful whether it belongs to the series of pigments discussed in this paper. This does not seem improbable in view of the existence of oxidative systems produced in erythrocytes by phenylhydrazine [Warburg *et al.*, 1931, 1, 2] and in consideration of the power of this compound to produce jaundice.

Parisot [1911] prepared a green pigment by incubation of haemolysed blood with adrenaline and ammonium sulphide or sodium hydrosulphite. He considered the substance to be biliverdin, since it gave the Gmelin test. This test cannot however be considered as sufficient evidence for the bile pigment nature of a substance, unless the typical absorption bands appearing at some stages of the reaction are observed. Brugsch and Pollack [1924] contested the bile pigment nature of Parisot's substance on the grounds that it did not give the diazo-test. Since biliverdin in contrast to bilirubin does not give this test, the failure of Brugsch and Pollack to obtain it is no evidence against Parisot's assumption.

Fischer and Lindner [1926, 1] observed that (proto-)haemin and also other haemins, dissolved in pyridine, were transformed at 40–50° into green pigments in the combined presence of oxygen and of reducing substances (*e.g.* polyhydric phenols, SH-compounds). Instead of these reductants yeast (which was used in the first experiments by these authors), minced plants or animal tissue (particularly liver) could be used. The authors mention the possibility that the amorphous green pigment might be a bile pigment, but their results do not harmonise in several points with this assumption. They found the green product to contain iron and to show distinct absorption bands different from those of any known bile pigment. From the description of the bands there can be no doubt that they were of the type of those given by the "green haemin" of Warburg and Negelein [1930].

By the action of molecular oxygen at 50° on pyridine-haemochromogen in the presence of hydrazine as reductant, Warburg and Negelein [1930] obtained a "green haemin" showing typical absorption bands, Warburg [1932] considered such "green haemins" as somewhat related to the green haemins of phaeophorbides and brought forward the hypothesis that the "Atmungsferment"-haemin (a green-red haemin) might stand between the red (porphyrin-)haemins and the green (chlorophyll-)haemins, the latter being oxidation products, the former reduction products of the Atmungsferment-haemin, from which they arose in the course of development. The results of the present paper show that the "green haemins" obtainable by oxidation have nothing to do with chlorophyll haemins. Warburg and Negelein did not obtain the "green haemin" itself in a pure state, but they were able to transform it by methyl alcoholic hydrochloric acid into a crystalline ester $C_{36}H_{40}O_6N_4FeCl_4$ which contained chlorine in contrast to the green haemin itself.

Schottmüller [1903] recognised that streptococci (*S. viridans*, *Pneumococcus*) produce a green pigment from haemoglobin when grown aerobically on the surface of blood agar plates or in blood broth. This process has been investigated in detail by Hart and Anderson [1933] and earlier literature on the subject has been reviewed in their paper. The pigment (which in fact is a mixture) shows in addition to other absorption bands a band in the red part of the spectrum resembling that of Warburg's green haemin. With the pigment produced by bacteria from haemoglobin Rich and Bumstead [1925] observed an atypical Gmelin reaction only. A pigment of the same properties as that produced by bacteria was prepared by Anderson and Hart [1934] by the action of non-bacterial reducing systems on haemoglobin in the presence of oxygen, and a similar mechanism was assumed to work in the formation of the pigment by bacteria.

The essential similarity of all the processes described above is that the simultaneous action of a reducing system and of oxygen on either haemochromogen or haemoglobin is necessary for the green formation.

Recently Schreuss and Carrié [1934] reported that a green pigment showing the properties of a biliverdin (it was soluble in ether and passed from it into hydrochloric acid) can be obtained in very small amounts if haemoglobin or haematin is incubated with liver "brei" or liver extract at 70° and p_H 7–8. The authors do not give any details of their method and the pigment was not isolated in the pure state. The formation of biliverdin was regarded by them as an oxidation of bilirubin during the extraction process.

It appeared particularly important to me to make a closer study of the "green haemin ester" of Warburg and Negelein, not only because it was the only pure product obtained but also for important structural implications. This ester contained four chlorine atoms and one iron atom. The results of Warburg and Negelein made it improbable that the substance was a haemin ester of a chloroporphyrin, since unlike the known tetrachloroporphyrins it could not be reduced to a normal red porphyrin. Biliverdins however form ferrichlorides, as has been shown by Fischer *et al.* [1932] and by Lemberg [1932], and it seemed not impossible that the substance in question was such a ferrichloride of a biliverdin.

Experimental.

For the preparation of the "green haemin" the procedure of Warburg and Negelein was used with slight alterations. It is essential to complete the transformation of haemin into green haemin as fast as possible to avoid formation of brown by-products. This could be attained more easily if only small amounts of haemin were used in each experiment; the products of several experiments were then worked up together.

In a 500 ml. wide-necked Erlenmeyer flask with almost vertical sides 0·4 g. of haemin was dissolved in a mixture of 100 ml. pyridine and 250 ml. water. This solution was kept at 50° in a water-bath and was aerated with a violent current of oxygen accompanied by vigorous mechanical stirring. A solution of 1·3 g. hydrazine sulphate in 32 ml. $N/2$ NaOH was then added. The colour changed soon to green and after 3–4 minutes the first band of haemochromogen at 557 $m\mu$ had disappeared and the spectrum of the green haemin (*cf.* below) was seen. At this point the reaction was stopped immediately and the solution cooled with ice. The filtered solutions of several successive experiments were combined and extracted without delay with ether and chloroform as described by Warburg. The black precipitate which appeared during the extraction of the brown by-products with ether included a considerable part of the "green haemin". This

was extracted by an aqueous pyridine solution containing some hydrazine hydrate and some acetic acid. This part of the product however was kept separate; it yielded a considerable amount of "green haemin" but did not give crystalline ester. The main bulk of the chloroform solution was concentrated *in vacuo* at 40° to a few ml. and precipitated with light petroleum (B.P. 50–60°).

The precipitate was transformed into the "green ester" by boiling in methyl alcoholic hydrochloric acid. From 2 g. haemin 50 mg. pure crystalline ester were obtained with the following crystal properties.

Elongated platelets pointed at the ends. Extinction slightly oblique. Pleochroism: light blue-green, if long axis of platelets parallel to plane of polarisation, dark green in perpendicular orientation. Blue and green colours between crossed nicols. The substance did not melt. The solution of the compound does not show distinct absorption bands but a general absorption of the red part of the spectrum.

A few mg. of the ester were dissolved in chloroform. The dark green solution turned blue when shaken with water (colour of neutral biliverdin). The aqueous solution was found to contain ferric iron as indicated by the thiocyanate reaction. This experiment is proof that iron is not present in complex combination in the ester, but as a ferric chloride double salt.

One mg. of the ester was dissolved in 1 ml. methyl alcohol and this solution was poured into a mixture of ether and sodium tartrate solution. Biliverdin ester passed into the ether layer colouring it blue, whereas iron was found in the tartrate solution. With 1 % hydrochloric acid the pigment passed into the acid aqueous solution with green colour.

With zinc acetate and iodine biliverdins like bilirubins [Auché, 1908] are transformed into the complex zinc salts of biliviolins. Biliviolins contain two hydrogen atoms less than biliverdins [Lemberg, 1934, 2]. Their zinc complex salts are characterised by typical absorption bands and extremely strong red fluorescence. The "green ester" reacted immediately with zinc acetate to give a yellow-green solution with an absorption band in the extreme red part of the spectrum (biliverdin zinc salt); on addition of a drop of iodine the solution turned green-blue and displayed a striking red fluorescence and absorption bands at 635·0 $m\mu$ and (less strong) 582·0 $m\mu$. These bands are the same within experimental error as those of biliviolin ester zinc salt, prepared in the same way from biliverdin (634·1 and 580·8 $m\mu$) and distinctly different from those of the corresponding mesobiliviolin compound (*cf.* below). This experiment confirmed the finding that the iron is not bound in complex combination in the green ester, and made its identity with the ferrichloride of biliverdin ester very probable.

The formula of *biliverdin dimethyl ester ferrichloride* is $C_{35}H_{38}O_6N_4.HCl.FeCl_3$. (Found: C, 51·86; H, 4·80; Fe, 6·68; OCH_3, 7·60, 7·47 %. $C_{35}H_{39}O_6N_4FeCl_4$ requires C, 51·91; H, 4·86; Fe, 6·90; OCH_3, 7·67 %.) The analyses reported by Warburg and Negelein are in better agreement with this formula than with the formula $C_{36}H_{40}O_6N_4FeCl_4$ which the authors ascribed to the substance. (Found: C, 51·92, 51·79; H, 4·90, 4·82; N, 6·47, 6·46; Fe, 6·85, 7·13, 6·78, 6·64; Cl, 17·93, 17·66; OCH_3, 8·29, 7·64, 7·83 %. $C_{35}H_{39}O_6N_4FeCl_4$ requires C, 51·91; H, 4·86; N, 6·93; Fe, 6·90; Cl, 17·53; OCH_3, 7·67 %.)

Since the compound has no melting-point it was transformed into *biliverdin dimethyl ester* for identification. 40 mg. were dissolved in methyl alcohol, containing some sodium acetate, and this solution was poured into a mixture of chloroform with a dilute solution of sodium carbonate and sodium tartrate. The blue chloroform solution was washed with water, filtered, dried with Na_2SO_4 and evaporated *in vacuo*. The residue was recrystallised from methyl alcohol. It was

free from iron and chlorine and melted at 208° (uncorr.). Mixing with purest biliverdin ester (M.P. 215°) gave no depression of the melting-point.

From the mother-liquor of the crude "green ester" a second small yield of crystals of the same properties was obtained. The mother-liquor of these however contained a different biliverdin which in the violin reaction with zinc acetate and iodine gave absorption bands of mesobiliviolin zinc salt (623·6, 572·2 $m\mu$). It is not however suggested that the compound in the mother-liquor was mesobiliverdin ferrichloride. The neutral iron-free ester obtained from it was easily soluble in methyl alcohol and did not crystallise completely. The following observations indicate that it might be haematobiliverdin (with two $CH(OH).CH_3$ groups).

21·39 mg. of this biliverdin were heated for 1·5 hours over P_2O_5 in a flask at 150° in a high vacuum (of the mercury pump). The substance lost 0·91 mg. (calculated loss of two mols. of water, corresponding to a transformation of two hydroxyethyl side-chains into vinyl groups: 1·19 mg.). The product had however been altered more fundamentally; it was no longer soluble in methyl alcohol, but soluble in chloroform and did not give the violin reaction. Hence a second sample was heated at 105° in the vacuum of the mercury pump. Under these conditions haematoporphyrin is transformed into protoporphyrin. After half an hour 14·54 mg. had lost no more than 0·12 mg. The heating was continued for three more hours; now the substance gave a violin reaction like biliverdin (protobiliverdin): absorption bands of the zinc salt at 632·3 and 578·7 $m\mu$. Whereas biliverdin dissolved in concentrated sulphuric acid is destroyed by heating for a quarter of an hour on a steam-bath and mesobiliverdin is not attacked at all and remains blue-green, the biliverdin of the mother liquor is changed into a blackish blue-green.

This partial alteration of the side-chains occurs in the preparation of the "green haemin" and not in the succeeding esterification, since biliverdin ferrichloride is completely stable under the conditions of the esterification, and since the amount of by-product formed varies according to the purity of the green haemin.

Preparation of mesobiliverdin ester from mesohaemin.

Since the corresponding mesobiliverdin compounds are well-characterised substances, and since it was interesting to investigate whether or not the reaction mechanism of the formation of "green haemin" is bound up with the vinyl side-chains I subjected mesohaemin to the same process as haemin above. Like haemin, mesohaemin was transformed into a green haemin and the latter into a green ester, containing iron and chlorine. The oxidation time extended for 6 minutes. From 0·8 g. mesohaemin 60 mg. of well crystallised green ester were obtained. The substance decomposed at 261° (uncorr.) under the following conditions. The paraffin-bath was brought to 100°, before the thermometer with the substance was inserted, and then the temperature slowly raised. Under the same conditions mesobiliverdin dimethyl ester ferrichloride ("ferrobilin ester" of Fischer) decomposed at 265° (uncorr.). With more rapid heating higher decomposition points are found. (Found: C, 51·44, 51·97; H, 4·99, 5·25; Fe, 6·77, 6·10; Cl, 18·42 %. $C_{35}H_{43}O_6N_4FeCl_4$ requires C, 51·66; H, 5·33; Fe, 6·87; Cl, 17·45 %.)

With zinc acetate and iodine the solution of the green ester turned blue exhibiting a striking red fluorescence and absorption bands at 623·2 and 572·3 $m\mu$ characteristic for mesobiliviolin zinc salt. Mesobiliverdin ester prepared from

bilirubin showed absorption bands at 623·7 and 573·3 $m\mu$, if treated with zinc acetate and iodine[1].

40 mg. of the ferrichloride were dissolved in chloroform and freed from iron and HCl by washing with dilute sodium carbonate solution containing some tartrate. The residue of the dried evaporated chloroform solution was crystallised from methyl alcohol; 20 mg. of characteristic well-formed prisms crystallised out; M.P. 218–219° (uncorr.), no depression with mesobiliverdin dimethyl ester (M.P. 220° uncorr.). (Found: C, 67·39; H, 6·85 %. $C_{35}H_{42}O_6N_4$ requires C, 68·34; H, 6·89 %.)

From the mother-liquor of the ferrichloride 25 mg. of a biliverdin ester (according to the violin reaction of the meso-type) were prepared which did not crystallise as well, were more easily soluble in methyl alcohol and had a lower M.P. (179–180°). The ferrichloride of this ester was easily soluble in methyl alcohol in contrast to that of mesobiliverdin ester. From these observations it can be concluded that the product is not impure mesobiliverdin ester, but consists of a different biliverdin ester.

Verdohaemochromogen ("green haemin") and mesoverdohaemochromogen.

In contradistinction to the green esters which have now been recognised as ferrichlorides of biliverdins, the green haemins contain the iron still in complex combination. With zinc acetate and iodine they do not give the violin reaction. The iron cannot be removed by washing with water or with alkali. From the results of the investigation to be described below it will be seen that they are pyridine-haemochromogens. I propose to name the "green haemin" of Warburg verdohaemochromogen and the corresponding compound from mesohaemin mesoverdohaemochromogen.

The two compounds are very similar, but they can be distinguished from one another by the position of their absorption bands, those of the meso-compound lying nearer to the ultra-violet. This difference in the situation of the bands of proto- and meso-compounds is also true for the porphyrins, haemochromogens and biliviolins. Verdohaemochromogen dissolved in pyridine-water shows a sharp absorption band in the red with its centre at 662$m\mu$ and two weaker bands in the green (533·1 and 500·8$m\mu$). Mesoverdohaemochromogen under the same conditions shows the bands 644·2, 525·4 and 494·4$m\mu$. In chloroform (containing pyridine) the first two bands lie more towards the infra-red, whereas the third has the same position as in water (verdohaemochromogen 663·5, 535·7 and 500·8$m\mu$, mesoverdohaemochromogen 650·7, 527·8 and 495·1$m\mu$).

If verdohaemochromogen in an aqueous solution containing much pyridine is shaken with air the type of the spectrum is little changed. The bands become less sharp, particularly that in the red, which becomes broader and is shifted towards the infra-red. Mesoverdohaemochromogen behaves in the same way. These aerated solutions contain parahaematins and have a bluish green colour rather than the yellowish green colour of verdohaemochromogen solutions. Parahaematins are also obtained if the verdohaemochromogens precipitated from the chloroform solution by light petroleum have stood some time exposed to the air. The fresh precipitate however consists, at least to a large extent, of

[1] The situation of the absorption bands depends somewhat on the purity of the violin, admixture of much verdin causing a shift of the bands towards the infra-red. It depends also on the p_H of the mixture, alkali shifting the bands towards infra-red. The difference in the position of the biliviolin and mesobiliviolin bands is however so great that compounds of the proto and of the meso series can be clearly distinguished in this way.

the haemochromogen, as will be seen below. Verdohaemochromogens are evidently more stable towards atmospheric oxygen than haemochromogens.

If the verdohaemochromogens are shaken with air in a solution containing no great excess of pyridine, the absorption bands disappear and are replaced by diffuse absorption at both ends of the spectrum. On addition of sodium hydrosulphite the olive solution turns green and displays the bands of the haemochromogens (in the case of verdohaemochromogen it is essential to use only small amounts of reductant). We can conclude from this experiment that the affinity of verdohaematin for pyridine is much less than that of verdohaem. Pyridine-verdoparahaematin which is formed by the oxidation of the verdohaemochromogen decomposes in water into verdohaematin and pyridine, unless a great excess of pyridine is present. By $Na_2S_2O_4$ verdohaematin is reduced to verdohaem, and the latter, owing to its greater affinity for pyridine, recombines with it. This is a complete parallel with the behaviour of haematin and haem towards pyridine.

If sodium hydrosulphite is added to a solution of "green haemin" in pyridine-water the solution turns yellow. The two absorption bands in the green disappear and are replaced by general absorption in the blue part of the spectrum, whereas the band in the red remains but is shifted towards the ultra-violet (652 $m\mu$). On shaking with air the solution turns green again, the bands in the green reappear and that in the red returns to its previous place. A ready explanation for this reversible reduction with sodium hydrosulphite would be that the green colour and the bands in the green were properties of a parahaematin compound which is reduced by sodium hydrosulphite to the yellow haemochromogen. The "green haemin" would then be a parahaematin and not a haemochromogen. This is, however, not in accordance with the experiments described above and it can be proved that such an explanation is incorrect. The reaction with hydrosulphite is given by verdohaemochromogen only, mesoverdohaemochromogen remains green with hydrosulphite and the absorption bands in the green do not disappear. This shows that the unsaturated side-chains of the proto-compound are somehow involved in this reaction.

The behaviour of the "green haemin" towards alkali also gives evidence that the absorption bands in the green belong to the haemochromogen. If a solution of sodium carbonate is added to a solution of "green haemin" in pyridine-water the absorption bands disappear slowly and the olive-green solution obtained shows diffuse absorption in the red and blue parts of the spectrum. If now a small amount of hydrosulphite is added the solution turns green and displays the spectrum with the three bands of the green haemin; if more hydrosulphite is added the solution becomes yellow and the bands in the green disappear. This experiment can only be explained in the following way. Pyridine-verdoparahaematin, which arises by oxidation of verdohaemochromogen in atmospheric oxygen, is unstable in alkali: thus it dissociates into verdohaematin and pyridine. By the reduction verdohaem is formed which unites with pyridine to give verdohaemochromogen, which is stable towards alkali. Since we obtain the absorption bands in the green in this experiment by reduction with hydrosulphite these bands must be those of the haemochromogen, and the further reversible reduction must be explained in a different way. Here again we find the verdohaem compounds behaving like normal haem compounds: haemochromogens are stable towards alkali, parahaematins are not [Keilin, 1926].

It can be shown in this way that the green haemin precipitated from the concentrated chloroform solution with light petroleum still contains verdohaemochromogen. A few mg. of the freshly precipitated "green haemin" were

dissolved in 2 ml. pyridine-water and put into a Thunberg tube the stopper of which contained 1 ml. 10 % sodium carbonate solution. The tube was at once repeatedly evacuated and filled with nitrogen. The alkali was then allowed to run into the solution. The colour turned slightly more olive, indicating that a part of the haemochromogen had been oxidised and split, but the three bands were still strongly visible. The pyridine used in this experiment did not reduce haemin to haemochromogen (as impure pyridine does). When the experiment was repeated with a preparation of "green haemin" which had been kept for some hours in contact with the air, the bands disappeared after the addition of the alkali.

The pyridine-free *verdohaematin* was obtained in the following way. A few mg. of green haemin were ground with water in a mortar and left in suspension for one hour. By the filtration of this suspension a black powder was obtained which was washed several times with water. Its solution in methyl alcohol showed only one diffuse absorption band at 700–680 $m\mu$, that of mesoverdohaematin a band at 690–655 $m\mu$ (in a Zeiss pocket spectroscope). Verdohaematin was dissolved in phosphate buffer p_H 7·5 giving an olive solution without distinct absorption bands. With hydrosulphite the solution became olive-green, but no distinct bands were seen (verdohaem). If CO were passed into this solution, it changed to olive-brown and displayed distinct bands at 665 and 610 $m\mu$ verdohaem-CO). Experiments with pyridine confirmed the results obtained above. A small amount of pyridine did not produce any alteration before reduction, a great excess of pyridine gave rise to bluish green parahaematin solutions with a rather diffuse band in the red and more distinct bands in the green. Unexpected however, was the behaviour towards ammonia. After addition of ammonia $Na_2S_2O_4$ did not give ammonia-verdohaemochromogen, as had been expected, but a brown ammonia-haemochromogen with a completely different spectrum, showing one absorption band at 567 $m\mu$. The same compound was obtained by direct action of ammonia on verdohaemochromogen. Pyridine now produced a dirty yellow solution with a reddish tint in artificial light and a sharp absorption band at 565·7 $m\mu$. A closer study of this interesting reaction has yet to be made. With the exception of its behaviour towards hydrosulphite mesoverdohaemochromogen behaved similarly in every respect.

These experiments prove that the "green haemins" are haemochromogens, containing bivalent iron in complex combination and pyridine bound to the iron atom. It is only by treatment with acid that the iron is removed from this combination. Since haemochromogens are more easily split by acids into the iron-free compounds and iron salt than the compounds with tervalent iron like haemin, the easy removal of the iron from the green haemins is understandable. Unlike haemins, however, verdohaematins are also unstable towards acids and apparently even more liable to alterations than the verdohaemochromogens. Verdohaematin is easily transformed by dilute hydrochloric acid in methyl alcoholic solution into a mixture of biliverdin and some biliviolin.

Verdohaem compounds as bile pigment complex salts.

The evidence thus far brought forward has not yet excluded with certainty the possibility that in the "green haemins" the porphyrin ring was still closed, and that the scission occurred only during the preparation of the "green esters". It was not quite impossible, although improbable, that the ring of the green haemins was closed by a CO-group instead of a CH-group, and that the CO-group was replaced by two OH-groups under the hydrolytic action of the acid.

The reduction of haemins by sodium amalgam leads to porphyrinogens which on reoxidation by atmospheric oxygen yield porphyrins. The reduction of verdohaematin, however, leads to mesobilinogen which is oxidised by atmospheric oxygen to a urobilinoid pigment. 50 mg. of verdohaematin were dissolved in 1 % sodium carbonate solution and shaken with sodium amalgam for 2 hours. The slightly yellow solution was separated from the mercury and extracted with ether containing glacial acetic acid. The ethereal solution gave a strong reaction with Ehrlich's aldehyde with absorption bands at 562 and 495 $m\mu$. With zinc acetate and iodine a strong "urobilin" reaction was obtained. On standing the colourless ethereal solution gradually turned yellow, containing now a typical urobilinoid pigment. The absorption band of the zinc salt was situated at 508·6 $m\mu$, that of the acid aqueous solution of the pigment itself at 494·5 $m\mu$. This is exactly the behaviour of mesobilinogen. I have recently shown [1934, 3] that mesobilinogen, unlike urobilinogen, is oxidised by ferric chloride to a mixture containing biliverdins and biliviolins. The bilinogen obtained from green haemin behaves in this reaction like mesobilinogen and yields a biliviolin which according to the spectrum of its zinc salt is mesobiliviolin. There can be little doubt that the substance in question is mesobilinogen. When haemin was treated in the same way, the ether solution of the chromogen gave only a very feeble Ehrlich reaction. After some standing the spectrum of protoporphyrin appeared. In the aqueous solution a small amount of a yellow substance was present which gave a green fluorescence with zinc acetate, the typical shift of the absorption band towards the red, characteristic for urobilinoid pigments, was not however observed. Like verdohaematin "green haemin" itself was reduced to mesobilinogen by sodium amalgam.

This experiment proves the presence of an open bile pigment chain in the "green haemins" which is maintained in ring form by the central iron atom. The following experiments give additional evidence that the iron-free part of the green haemin is a bile pigment closely related to biliverdin.

When copper acetate was added to a solution of mesoverdohaemochromogen in a mixture of methyl alcohol and pyridine the solution turned at first yellow-green. The two absorption bands in the green had disappeared and that in the red had been shifted towards infra-red (to about 680 $m\mu$). On standing the band in the red gets weaker and is replaced by two bands at 632 and at 578 $m\mu$. The latter spectrum is that of a copper complex salt of mesobiliviolin, the former that of mesobiliverdin-copper which is oxidised by bivalent copper to the latter [*cf.* Lemberg, 1934, 1]. Mesoverdohaematin treated with copper acetate yields the same products.

It has been said above that the verdohaemochromogens do not react with zinc acetate. When however the solution is kept exposed to the air after the addition of the zinc salt, the red fluorescence and the spectrum of the biliviolin zinc salt appear slowly. Even the replacement of iron by another metal is not necessary to form complex salts of biliviolins from green haemins. When a solution of mesoverdohaemochromogen in methyl alcohol containing a little ammonia was shaken with air an absorption band at 633·7 $m\mu$ was observed.

Since iron complex salts of biliverdins and biliviolins have not yet been described[1], I investigated the action of ferrous sulphate on mesobiliverdin in ammoniacal solution. The filtrate was olive-green and showed diffuse absorption in the red and blue. After addition of pyridine and sodium hydrosulphite the green solution displayed absorption bands at 627·2 and 576·4 $m\mu$. This behaviour does not correspond with that of mesoverdohaemochromogen but with that of

[1] The "ferrobilins" are not complex salts in this sense, but ferrichlorides.

a haemochromogen of mesobiliviolin. Shaking the solution with air produced a series of changes which have not yet been investigated in detail. Since bivalent copper oxidises the copper complex salt of mesobiliverdin to that of mesobiliviolin, it was easily understandable that the tervalent iron formed in the presence of oxygen would oxidise the mesobiliverdin iron salt to that of mesobiliviolin as well. For this reason I tried to introduce iron into mesobiliverdin by means of ferrous sulphate in ammoniacal solution in the absence of oxygen.

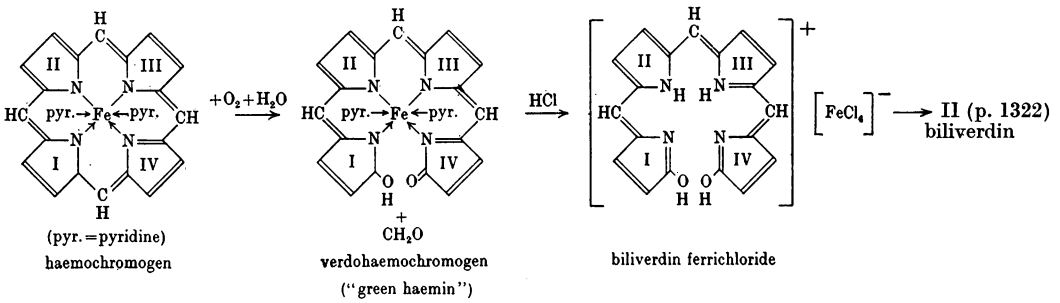
For this purpose an apparatus was used consisting of a tube containing a filter plate of porous glass with a tap below it; a small dropping funnel was fitted into the top of the tube, and the bottom was inserted into a small receiver with a tap in the side arm. Each of the three vessels was connected by means of three-way taps with the vacuum pump and with a nitrogen apparatus. Nitrogen, freed from oxygen by passage over heated copper, passed at first from the receiver, containing a little pyridine with some hydrazine hydrate as reductant, through the tap and the porous glass plate into the lumen of the tube containing mesobiliverdin in ammoniacal solution. To this solution ferrous sulphate solution, freed from oxygen by evacuation and saturation with nitrogen, was added from the dropping funnel, and after a few minutes the reaction mixture was sucked through the filter into the receiver. The two taps were then closed and the spectrum of the solution investigated. Under these conditions no complex salt is formed. The blue-green solution showed the general absorption of the red part of the spectrum characteristic of mesobiliverdin. Under the conditions of the experiment any complex iron salt formed would yield haemochromogen, the sharp absorption bands of which would not escape notice. As long as the solution was kept in nitrogen, no change was observed. When however air was allowed to enter the flask, the spectrum of biliviolin complex salt described above appeared. When hydrochloric acid was now added to this solution, mesobiliverdin ferrichloride was precipitated, leaving a blue-violet solution of mesobiliviolin (spectrum in acid solution: absorption bands with centres at 592·6 and 551·5 $m\mu$, rather diffuse and connected by a shadow; main absorption band of the zinc salt: 624·9 $m\mu$). Mesobiliverdin itself apparently does not form haem compounds.

From these experiments it must be concluded that the "green haemins" are haemochromogens of bile pigments with an open ring. The iron-free compound is probably not identical with biliverdin, but unstable and easily transformed into biliverdin.

Discussion.

The reaction leading from haemins (or more exactly from pyridine-haemochromogens) to "green haemins" and from these to biliverdins can now be formulated as follows:

(The side-chains have been omitted.)



The first step leads from pyridine-haemochromogen by an oxidative process to verdohaemochromogen. The central part of the molecule remains unaltered, but the porphyrin ring is split open and transformed into a bile pigment nucleus. The usual way of writing the bile pigment formula in a straight chain is only a conventional mode of expression. Since there is free rotation around the single bonds combining pyrrole nuclei with carbon atoms the molecules of bile pigments may assume different shapes in space, *e.g.* that of a plane opened ring.

The identification of the "green esters" with derivatives of natural bilirubin (bilirubin IXα according to Fischer's nomenclature) proves that the scission occurs in the same place (α CH-group) as in the transformation of haemoglobin into bilirubin. It must be conceded that the yield of well-crystallised biliverdin ester is rather small and that isomerides with different position of the side-chains, resulting from scission of the ring at other CH-groups, might be present in the mother-liquor, but this applies also to natural bilirubin, a small fraction of which only is obtainable completely pure.

The ring having been opened at one point the stability of the complex salt becomes remarkably decreased. The central iron in its complex combination still holds the ring together, particularly as long as its six coordination places are filled, as they are in the haemochromogens. In contrast with normal haematins the verdohaematins are very unstable compounds.

The formula given above characterises the verdohaem compounds as iron salts of *iso*biliverdins. The latter are unstable and, if set free, are transformed into biliverdins (*cf.* formula II, p. 1322) by a migration of one hydrogen atom from the nitrogen of the pyrrole nucleus III to the α CO-group of ring IV.

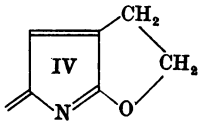
The reaction of verdohaemochromogen with hydrochloric acid consists of the following stages. The pyridine is removed and the verdohaem oxidised to verdohaemin, this is now split into ferric chloride and *iso*biliverdin which rearranges to give biliverdin. This isomerisation transforms the nitrogen in ring III into a basic tertiary nitrogen which adds a proton. There is in addition esterification of the two carboxyl groups in the side-chains by the methyl alcohol. $FeCl_4^-$ ions are always present in solutions of ferric chloride and since the ferrichloride is the least soluble salt, it crystallises out.

The fact that isomerisation of biliverdin is necessary for the formation of "green haemin" explains why this cannot be accomplished from biliverdin with ferrous salt. The zinc and copper compounds of biliverdin are probably complex salts of biliverdin itself and not of *iso*biliverdin, since unlike the iron salt they are obtained from biliverdin, and since they show an absorption spectrum very different from that of verdohaemochromogen. Both types of complex salts can however be transformed by oxidation into complex salts of biliviolins[1].

The transformation of haemin into biliverdin is also of interest for the question of the nature of the side-chains of bilirubin. It suggests that bilirubin and biliverdin contain two vinyl groups like haemin. Fischer has often discussed this question and has repeatedly altered his views. Quite recently Fischer and Haberland [1935] have come to the conclusion that bilirubin contains one vinyl group, whereas the second unsaturated side-chain is present in the form of a dihydrofuran nucleus (*cf.* formula on p. 1333). Although it is not quite impossible

[1] H. Fischer who had previously confused the blue colour of the neutral biliverdins with the similar colour (although very different spectrum) of the acid biliviolins and assumed that the latter were isomerides of the former, has now [Fischer and Haberland, 1935] recognised his mistake and has arrived at the formula for biliviolins which I proposed in my paper before the Biochemical Society on November 16th, 1934, and which has been published in a short note [1934, 2]. Fischer has overlooked this note.

that such a ring is formed during the reaction leading from haemin to biliverdin by closing a ring between the vinyl group in position β and the hydroxyl group in position α at ring IV, it does not seem probable.



Moreover, the evidence brought forward by Fischer for the existence of this ring is unconvincing. It is based (1) on the fact that the behaviour of the unsaturated groups in bilirubin towards reagents is different from that of these groups in haemin; (2) on the isolation of a substance assumed to be a pyrrofuran compound, obtained in small yield by the action of nitrous acid on bilirubin; and (3) on the isolation of a dihydrobilirubin with one vinyl group and one ethyl group by catalytic hydrogenation of bilirubin.

As regards the first point, these differences concern both unsaturated groups in the same way; since only one can be present in form of a dihydrofuran ring (on account of the position of the unsaturated side-chain in ring I relative to the hydroxyl group), the observed differences of the bilirubin side-chains from those of haemin must be explained in a different way.

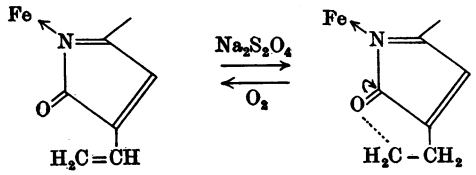
The existence of the pyrrofuran compound is very doubtful. In later experiments Fischer [1915] tried in vain to reproduce this substance, nor was Dr Mühlbauer (unpublished observation) able in my laboratory to obtain a substance of the properties described in the first paper by Fischer and Hahn [1914]. In a short note Fischer and Hess [1931] mentioned that the substance in question reacted with phenylhydrazine and possibly was methylhydroxyvinylmaleinimide (although this substance would yield analytical values quite different from those obtained by Fischer and Hahn). In spite of this uncertainty the substance is now again used as an argument in favour of the existence of a dihydrofuran ring. Previously Fischer had assumed a furan ring, although this was in evident disagreement with the amount of hydrogen necessary for the transformation of bilirubin into mesobilirubin and mesobilinogen.

The third argument of Fischer is the fact that one of the unsaturated groups of bilirubin is more easily reduced to an ethyl group by catalytic hydrogenation. The partially hydrogenated substance has an ethyl group in ring IV and a vinyl group in ring I. Provided that Fischer's hypothesis is correct, it would be necessary to assume that by catalytic hydrogenation the dihydrofuran ring is split open to ethyl and hydroxyl more readily than the vinyl group is saturated. This is very improbable, and Fischer's results are more easily explained by the simple assumption that the vinyl group in the neighbourhood of the α-hydroxyl reacts more rapidly. For these reasons it is suggested that bilirubin contains two vinyl groups like haemin.

The following observation may throw light upon the question why the vinyl group in the neighbourhood of the hydroxyl group[1] reacts more rapidly with catalytically activated hydrogen. Verdohaemochromogen is reversibly reduced by $Na_2S_2O_4$; since mesoverdohaemochromogen is not reduced, the unsaturated side-chain must play a rôle in this reversible reduction, which cannot involve the iron, since this is already in the bivalent form before the reduction. The

[1] Of the lactim form (—C(OH)=N—) or the CO-group of the lactam form (—CO—NH—) of bilirubin, which are probably in equilibrium.

yellow haemochromogen, obtained by reduction from verdohaemochromogen and reoxidisable to it, is probably a monohydroverdohaemochromogen in which one hydrogen atom is added to the vinyl group in ring IV. This radical is stabilised by the proximity of the α CO-group.



(It is hardly necessary to mention that the cyclic structure suggested for the radical has nothing in common with a dihydrofuran ring, the linkage of the side-chain with the oxygen being a single electron.) This double oxidation-reduction system of verdohaemochromogen (in respect of the iron atom and the unsaturated-side chain) might be of interest with regard to the possible catalytic activities of the substance.

The mechanism of the reaction leading from haemin to verdohaemochromogen remains still to be investigated. Undoubtedly it is the haemochromogen which is oxidised, but whether oxygen performs this directly, or the tervalent iron of haematin, or whether oxygen and haematin are necessary for the reaction cannot yet be decided.

The action of H_2O_2 may be taken into consideration. McLeod and Gordon [1922] and Hart and Anderson [1933] have arrived at different conclusions with regard to the rôle played by H_2O_2 in the formation of the "green pigment" from haemoglobin. In this respect it is important to mention that other greenish haematins exist, and that they are present in the "green pigment" obtained from haemoglobin[1]. No definite conclusions as to the reaction mechanism of the formation of verdohaemochromogen can thus be drawn from the investigations of the different authors on the formation of "green pigment" from haemoglobin. Verdohaemochromogens are easily destroyed by H_2O_2 even in very dilute solution. The possibility that H_2O_2 in extremely small concentrations is the active agent, cannot be quite excluded, but it seems more probable that the formation of an unstable iron-peroxide of the haemochromogen itself may be the first step followed by transference of the oxygen to the porphyrin nucleus; or the reaction may be of a more complicated nature and require the presence of haematin compound with tervalent iron together with haemochromogen; the former may either oxidise haemochromogen directly or by the way of a chain reaction. However that may be, it is certain that the reaction is essentially a process in which the catalytic activity of the haem compound is applied to its own molecule.

It is suggested that the formation of bile pigment in the body may be caused by a similar process. The system of Warburg and Negelein studied above is of

[1] I am indebted to Anderson and Hart for sending me samples of green pigment produced by pneumococcus and by non-bacterial reducing systems from haemoglobin. In confirmation of the spectroscopic investigation published in their paper I found that both pigments give the same absorption spectrum in pyridine solution with hydrosulphite. The spectrum is that of a mixture of haemochromogens, *i.e.* of a good deal of unaltered protohaemochromogen, of a haemochromogen with an absorption band at 620 $m\mu$, and of a rather small amount of verdohaemochromogen. The second haemochromogen belongs to a haematin type which can be produced by autoxidation of haematin in alkaline solution (without reductant) and which is also of greenish colour ("cryptohaemin" of Negelein).

course only a model of the system responsible for bile pigment formation in the body. It differs in that the compound oxidised in the body is haemoglobin and not pyridine-haemochromogen, so that the resulting verdohaem compound would be either verdohaemoglobin or globin-verdohaemochromogen. Instead of hydrazine we have reducing systems. It is unnecessary to assume the action of a special enzyme for the oxidation of haemoglobin, the haem of the molecule itself or of cytochrome working as catalyst. Enzymes may play a rôle, however, in the process in so far as they provide the reducing systems [*cf.* Hart and Anderson, 1933; Anderson and Hart, 1934]. The transformation of the verdohaem compound into biliverdin cannot be brought about in the animal body by any of the reactions described above; here further research is necessary. It is evident however from the ease with which this transformation proceeds that its occurrence under physiological conditions is likely.

Apart from these differences the essential process is probably the same. Hitherto only teleological explanations have been offered for the formation of bile pigments from haemoglobin, such as the unconvincing hypothesis of Asher [1924] that the decomposition of haemoglobin is necessary for providing the cells with Atmungsferment-iron. In consideration of the results brought forward in this paper the formation of bile pigments in the animal body is the natural consequence of the catalytic activity of the haem iron working on its own molecule. A similar explanation probably holds for the complexity of the cytochrome spectrum [Keilin, 1926].

From the physiological point of view a great deal remains to be done, but it is believed that the model now worked out will provide a good guide to further research.

Summary.

"Green haemin" is prepared according to Warburg and Negelein by action of oxygen on pyridine-haemochromogen in presence of hydrazine. A corresponding compound is obtained from mesohaemin.

The "green haemins" are characterised as pyridine-haemochromogens of *iso*biliverdins. The name "verdohaemochromogens" is proposed. Their formation is due to oxidative scission of the porphyrin nucleus of the haemochromogen, catalysed by haem iron.

The "green ester", obtained by Warburg and Negelein by action of methyl alcoholic hydrochloric acid on green haemin, is identified with the ferrichloride of biliverdin ester. From mesohaemin the ferrichloride of mesobiliverdin is obtained in the same way.

The ferrichlorides can be transformed into the biliverdin esters and these can be identified with products prepared from bilirubin of gall stones. Since haemin has been synthesised, its transformation into biliverdin means the total synthesis of the latter.

It is suggested that the formation of bile pigments in the animal body proceeds in a similar way yielding biliverdin (dehydrobilirubin) which is afterwards reduced to bilirubin.

The nature of the unsaturated side-chains of bilirubin and biliverdin is discussed and it is suggested that they are vinyl groups like those of haemin.

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