CXXXVII. BILE PIGMENTS. VI. BILIVERDIN, UTEROVERDIN AND OOCYAN.

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By dehydrogenation of bilirubin with ferric chloride a green pigment, dehydrobilirubin, has been prepared. The same pigment is found in nature as oocyan in the egg-shells of gulls and other birds [Lemberg, 1931] and as uteroverdin in the dog's placenta [Lemberg and Barcroft, 1932]. Dehydromesobilirubin, which differs from dehydrobilirubin merely in having saturated side-chains, was prepared by Fischer *et al.* [1932] and called glaucobilin.

Analysis of the dimethyl esters of these pigments showed that these compounds possess two hydrogen atoms less than bilirubin and mesobilirubin, but the same number of oxygen atoms, *i.e.* six. The first analytical values of the oocyan ester showed some divergence, but later analyses [Lemberg, 1932] gave the same results for oocyan and dehydrobilirubin esters. The constitution of dehydrobilirubin is established in the present paper by analyses of dehydrobilirubin (free acid). The first analyses of this substance favoured a formula with eight oxygen atoms, while the results of Fischer on glaucobilin (free acid) corresponded to a formula with seven oxygen atoms. Analyses of carefully purified dehydrobilirubin establish the formula $C_{33}H_{34}O_6N_4$. The analyses of the free acid and the esters show furthermore that only two hydrogen atoms have been removed by the dehydrogenation of bilirubin.

A second point which required further investigation was the relation of dehydrobilirubin and dehydromesobilirubin to biliverdin and mesobiliverdin, pigments showing similar colours and absorption spectra, which are obtained by autoxidation of bilirubin and mesobilirubin in alkaline solution. It is now shown that "mesobiliverdin" is actually a mixture consisting largely of dehydromesobilirubin together with certain by-products which cannot be obtained in a pure state, but give the impression of being resinified substances. The yield of pure dehydrobilirubin from "biliverdin" is lower. This is to be expected as the unsaturated side-chains form a point of attack for alkali and oxygen. It seems preferable now to apply the convenient name "biliverdin" to pure crystalline dehydrobilirubin, instead of to a mixture of the latter with products of secondary alteration. The name mesobiliverdin rather than dehydromesobilirubin or glaucobilin will in future be used for the product with saturated side-chains.

A further simplification is rendered possible by the evidence now brought forward that the differences in optical properties between crystals of uteroverdin ester and oocyan ester, observed by Bernal in 1931 [Lemberg and Barcroft, 1932], are due merely to differences in growth and formation of the crystals, and probably do not indicate structural difference. Further investigation has confirmed the existence of two different crystal forms of biliverdin ester differing only with regard to pleochroism and extinction. One of these forms is obtained from biliverdin synthesised from pure bilirubin, the other (usually mixed with

the first) from biliverdin obtained from bilirubin mother-liquors and from the natural biliverdins, particularly uteroverdin.

This matter has been carefully investigated, as the existence of slight differences between isomeric bile pigments might have an important and interesting significance. According to recent investigations of Fischer and his collaborators bilirubin is represented by a formula in which four pyrrole nuclei are linked together by three carbon atoms to form an open chain. Bilirubin arises from protoporphyrin by oxidative opening of the porphyrin nucleus; one of the four CH-groups of the ring (α in the formula) is removed and replaced by two hydroxyl groups:

$M = .CH_3$
$V = .CH : CH_2$
$S = .CH_2.CH_2.CO_2H$
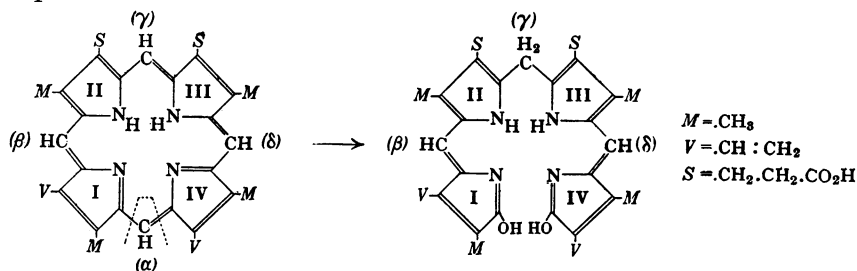
Fischer and Hess [1931] had assumed a symmetrical position of the methyl and vinyl groups in the rings I and IV of bilirubin (CH_3 next to OH in ring IV, instead of vinyl as in the formula above). This would have meant that the formation of bilirubin from protoporphyrin implies decomposition of the latter and resynthesis, as the position of the side-chains in ring IV could not be changed in any other way. Later, however, Siedel and Fischer [1933] proved that the side-chains of bilirubin are unsymmetrically arranged exactly like those in haemin and protoporphyrin. The ring is opened between the rings I and IV and the α-CH-group is removed.

Now three other bilirubins may arise from protoporphyrin if the ring could be opened at one of the other CH-groups (β, γ, δ) instead of at α. Thus isomeric bilirubins would originate differing from each other in the position of the side-chains attached to the rings. Such isomerides are often extremely similar and may even give no depression of the melting-point if mixed. Examples are found in the paper of Siedel and Fischer [1933] and previous ones of Fischer, e.g. the three mesobilirubins (IIIα, IXα, XIIIα) differing in the position of the side-chains attached to the rings I and IV. The difference of the two forms of biliverdin ester, if actually due to a structural difference, could only be caused by a difference in the position of the side-chains. The absence of a melting-point depression, the analytical data, the identity of the absorption curves and of other properties (such as basicity and solubility) leave no doubt that the nuclear system of the four pyrrole rings and the arrangement of the double bonds are the same in both forms.

The biliverdin ester of the form predominantly present in uteroverdin (which is different from the form obtained from pure bilirubin) can be obtained from mother-liquors of bilirubin and from gallstones. If thus structurally different types of biliverdin exist, then it is almost certain that there are two corresponding structurally different bilirubins. Uteroverdin, moreover, is formed in haemorrhages in placental tissue and is definitely of extrahepatic origin; thus the different biological origin is possibly connected with the chemical difference.

In the crystallographic investigations of the esters and their hydrochlorides I had the valuable help of Mr Rawlins of the Mineralogical Laboratory at Cambridge. These investigations show that the assumption of a structural difference between the two different crystalline forms is unnecessary, and that uteroverdin, oocyan and biliverdin may be considered identical. These investigations do not necessarily exclude the above explanation of the observed differences, but they give no evidence for it. Two different forms of protoporphyrin crystals have been observed by Richter [1930], one of which is caused by impurities according to Fischer *et al.* [1931] and Hamsik [1931][1].

The microanalytical part of the work has been done by Dr Roth, Heidelberg.

EXPERIMENTAL.

Mesobiliverdin by autoxidation of mesobilirubin.

The autoxidation of mesobilirubin to mesobiliverdin in alkaline solution was studied under different conditions and the product of the reaction was isolated. The effect of piperidine was tried, as this substance accelerates the autoxidation of dihydropolyenes [Kuhn and Drumm, 1932].

Five small crystallising dishes were filled as follows: I, 1·7 ml. 0·22 N NaOH. II, 3 ml. of the NaOH and 1 ml. water. III, IV and V like II but with the addition of 0·1 ml. $M/10$ ferrous sulphate solution to III, 0·01 ml. of the same solution to IV and one drop of piperidine to V. In each of them 0·1 g. of mesobilirubin was dissolved requiring theoretically 1·54 ml. of the NaOH solution (2 mol.). I contained 2·2, II–V 3·9 mols. of alkali. The dishes were placed in a water-filled desiccator and kept in it at 30°. After 12 hours only the solution in IV had turned green, the other ones were olive-brown. After 4 days' standing I and IV were green; II, III and V olive-green. Therefore the reaction proceeds best if a great excess of alkali is avoided. The reaction is accelerated by traces of iron, but not by piperidine.

The solutions I and IV were mixed and acidified with dilute acetic acid. The pigment (from 0·2 g. mesobilirubin) was extracted with ether, and after evaporation of the ether, the residue was extracted with methanol, which left some unchanged mesobilirubin undissolved. The mesobiliverdin was then esterified by passing in hydrogen chloride. After keeping overnight, esterification was completed by boiling the solution, diluted previously with half its volume of absolute methanol, for 15 minutes. It was then evaporated *in vacuo*. The residue was dissolved in a small amount of methanol, sodium acetate solution was added and the ester was extracted with ether. The extract was washed with 1 % sodium carbonate solution and water. The ether was evaporated and the product recrystallised from methanol. 31 mg. of pure mesobiliverdin dimethyl ester crystallised out. M.P. 221° (corr.). It was identical in every respect with glaucobilin ester [Fischer *et al.*, 1932].

The same substance was obtained from II, III and V, but the yield was smaller.

Biliverdin by autoxidation of bilirubin.

0·58 g. of bilirubin was dissolved in 10·5 ml. of 0·22 N NaOH and 0·07 ml. of $M/10$ ferrous sulphate solution was added. After 6 days' standing at 30° some precipitated bilirubin (10 mg.) was filtered off and the biliverdin taken up in ether and precipitated therefrom as described below. 150 mg. of crude biliverdin were obtained, which however gave only 10 mg. of well crystallised pure biliverdin

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[1] Lindenfeld [1931; 1933] considers the different crystalline forms of haemin and porphyrin esters to be polymorphous modifications.

dimethyl ester. M.P. 220–221° (corr.). The crystals were those termed form A below. No depression of the melting-point was obtained with dehydrobilirubin dimethyl ester [Lemberg, 1932].

The considerable ether-insoluble part of the green pigment was almost insoluble in methanol. From its solution in pyridine it was precipitated by hot methanol in very fine amorphous flocks of almost black colour and resinous character. Dilute methyl alcoholic hydrochloric acid dissolved it with an olive-brown colour due to included bilirubin; more concentrated methyl alcoholic hydrochloric acid again precipitated black flocks.

Mesobiliverdin from the products of the Gmelin test for mesobilirubin.

0·1 g. mesobilirubin was dissolved in 100 ml. of warm chloroform. A few ml. of chloroform were shaken in a test-tube with one drop of fuming nitric acid, and 1 ml. of this added to the mesobilirubin solution. The colour changed slowly to olive-green. After 6 minutes the colour had become green with a yellow tinge at the borders of the liquid. The reaction was stopped at this time by shaking with water containing a little sodium acetate. The chloroform was evaporated and the residue taken up with absolute methanol which left 30 mg. of unchanged mesobilirubin undissolved. The solution was blackish-blue in neutral, blue in acid solution. The acid solution showed, besides the absorption in the red due to mesobiliverdin, a weak band at 590 $m\mu$. On addition of zinc acetate the neutral solution turned green with weak red fluorescence and showed three bands, the strongest at 685 $m\mu$, a weaker one at 630 $m\mu$ and a very weak one at 578 $m\mu$. The violet pigment which causes the band 590 $m\mu$ in acid solution and the bands at 630 and 578 $m\mu$ and the red fluorescence of the zinc salt will be discussed in another paper.

The pigment in the methyl alcoholic solution was esterified and the ester brought into ethereal solution as described above. From the ethereal solution mesobiliverdin ester was extracted with 1 % hydrochloric acid which showed now the characteristic blue-green colour and absorption only in the far red. The by-products, among them the weakly basic violet pigment, remained in the ether. The mesobiliverdin ester was shaken out with fresh ether after addition of dilute sodium carbonate solution. It was recrystallised from methanol. 25 mg. of steel-blue prisms of mesobiliverdin dimethyl ester were obtained. M.P. 220° (uncorr.).

If the Gmelin reaction were allowed to proceed until the blue phase was reached the yield of mesobiliverdin became very small.

Mesobiliverdin by oxidation of mesobilirubinogen with ferric chloride.

0·3 g. mesobilirubinogen (the chromogen of mesobilirubin) was boiled for 1·5 hours with a solution of 1·5 g. FeCl$_3$ in 9 ml. 25 % hydrochloric acid and 50 ml. methanol. On cooling well formed rectangular blue leaflets with straight ends crystallised out. Yield 0·2 g.

Although the crystals looked perfectly uniform, they were mixed crystals of two different substances. Their blue solution in acid alcohol showed, besides strong absorption in the red, a band at 595 $m\mu$ and one at 555 $m\mu$ with a shading between the two. In the neutralised solution on addition of zinc acetate a strong red fluorescence appeared and the blue-green solution showed bands at 685, 631 and 578 $m\mu$. If the crystals were recrystallised twice from methanol containing some HCl flat blue-green needles with straight ends were obtained, whereas the mother-liquor was blue. The solution of the crystals now showed absorption only in the red; the zinc compound was green and without fluorescence. The

mother-liquors gave a blue zinc compound with strong red fluorescence and the bands 631 and 578 $m\mu$; the acid solution showed the bands at 595 and 555 $m\mu$. These bands are caused by mesobiliviolin. The crystals are the ferric chloride double salt of mesobiliverdin dimethyl ester (the "ferrobilin ester" of Fischer et al. [1932]). M.P. 276° (not corr.). (Found: C, 52·04; H, 5·34; Cl, 16·81; Fe, 5·72; OCH_3, 7·52 %. $C_{35}H_{42}O_6N_4$·HCl·$FeCl_3$ requires C, 51·66; H, 5·33; Cl, 17·45; Fe, 6·87; $2OCH_3$, 7·63 %.) The low value for iron is explained by the fact that $FeCl_3$ is volatile and iron was assayed as combustion residue.

Optical properties of the crystals: pleochroism: blue, if long axis of platelets parallel to plane of polarisation (NS), blue-green in perpendicular orientation (EW)[1]. Extinction almost straight.

By mild treatment with alkali they were transformed into mesobiliverdin ester as described by Fischer et al. [1932].

Similar mixed crystals to those mentioned above, but richer in mesobiliviolin chloride, result from shorter treatment of mesobilirubinogen with ferric chloride. They have been obtained previously and considered to be pure mesobilicyanin chloride [Lemberg, 1933]. It will be shown in another paper that "bilicyanins" are mixtures of pigments, which are coloured blue-violet in acid, red-violet in neutral solution, with biliverdins. For this reason the term violin is used, although the "mesobiliviolin" of Fischer and Niemann [1924] is a mixture of still more complicated nature.

Preparation and analysis of biliverdin.

Free biliverdin (dehydrobilirubin) is rather insoluble in ether. The yield could be greatly increased and much ether saved by using for extraction ether rich in methyl alcohol.

The crude ferric chloride double salt of biliverdin chloride was prepared from bilirubin by oxidation with ferric chloride in glacial acetic acid as described by Lemberg [1932]. 0·5 g. of this material is dissolved in 30 ml. of $N/5$ NaOH and filtered from ferric hydroxide; 100 ml. of methanol are added, the solution is acidified with dilute acetic acid and immediately poured into 500 ml. ether and shaken. It is then extracted several times with ether, some more methanol being added after each extraction. The solution must be kept acid with acetic acid. The extraction is continued until ether no longer extracts an appreciable amount of the pigment, even if some more methanol has been added. 1·5 l. of ether have been found necessary. The remaining watery solution contains a fine precipitate of ether-insoluble "biliverdin" which is filtered off and dried (80 mg. of this by-product).

The ether solution is washed with water. The first washings remove with the methanol a part of the pigment. This is precipitated by addition of some more sodium acetate (the reaction must remain slightly acid) and filtered off or taken back into ether. The greater part of the biliverdin precipitates from the ether solution when the methanol is washed away with water. If the ethereal filtrate is concentrated to 50 ml. and washed again, a second portion of the pigment is obtained. The ether remains violet and contains some biliviolin.

The green precipitates of the ether-soluble part are collected, washed carefully and dried in the desiccator. They are dissolved by boiling with 200 ml. of methanol. The solution is filtered and concentrated to 30 ml.; 240 mg. of

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[1] The phrases North-South (NS) and East-West (EW) refer to the position in the field of view of the polarising microscope.

biliverdin crystallise out. This yield, however, can be obtained from the first crystallisation of the ferric chloride double salt only. A much smaller yield is obtained and more of the amorphous ether-insoluble by-product, if that part of the ferric chloride compound is used which comes out of the glacial acetic acid mother-liquors. Thus the total yield of crystalline product is no more than 30 % of bilirubin.

The substance was dried for analysis at 80° and 0·1 mm. over phosphorus pentoxide. (Found: C, 64·63, 64·89; H, 6·00, 5·95; N, 9·06, 8·91, 9·07 %. $C_{33}H_{34}O_6N_4$ requires: C, 68·01; H, 5·89; N, 9·62. $C_{33}H_{38}O_8N_4$ requires: C, 64·04; H, 6·19; N, 9·06 %.)

The analysis of the dimethyl ester of this preparation showed that it contains six oxygen atoms like that of Lemberg [1932]. The starting material, the crude ferric chloride double salt also contains six oxygen atoms only. (Found: C, 50·73; H, 4·59; N, 6·75, 6·67, 7·04; Cl, 17·54, 17·34; Fe, 6·42, 7·11 %. $C_{33}H_{34}O_6N_4.HCl.FeCl_3$ requires: C, 50·71; H, 4·52; N, 7·17; Cl, 18·18; Fe, 7·16 %.) The first iron value was calculated from the weight of the combustion residue, the second obtained by direct precipitation of ferric hydroxide with ammonia. After the biliverdin had been recrystallised twice from methanol the analysis now confirmed the formula with six oxygen atoms for the free acid as well. (Found: C, 67·87; H, 5·79 %. $C_{33}H_{34}O_6N_4$ requires: C, 68·01; H, 5·89 %.) The analytical values for H show that biliverdin has no more than two hydrogen atoms less than bilirubin. (For the free acid have been found: H, 6·00, 5·95, 5·79 %. $C_{33}H_{34}O_6N_4$ requires: H, 5·89 %; $C_{33}H_{32}O_6N_4$: H, 5·56 %. Dimethyl ester, found: H, 6·42, 6·50, 5·96 % (uteroverdin ester); 6·26 % (oocyan ester); 6·39, 6·44 % (biliverdin ester). $C_{35}H_{38}O_6N_4$ requires: H, 6·28 %; $C_{35}H_{36}O_6N_4$: 5·96 %.)

The ether-insoluble by-product still contains some biliverdin. From its methyl alcoholic solution, methyl alcoholic HCl gives a fine blackish precipitate. If this is filtered off, a mixture of amorphous and crystalline chlorides is obtained by addition of hot 2N HCl.

Chlorides of biliverdins.

Mesobiliverdin chloride. 50 mg. of mesobiliverdin are dissolved in 15 ml. of boiling methanol and 40 ml. of hot 2N aqueous hydrochloric acid are added. After a short time an amorphous precipitate appears; this is filtered off. The chloride crystallises from the filtrate in fine green needles.

The substance is dried for analysis *in vacuo* at 20° over P_2O_5 to avoid dissociation and loss of HCl. (Found: N, 8·78, 8·98; Cl, 4·46, 4·21 %. $C_{33}H_{38}O_6N_4.HCl$ requires: N, 8·98; Cl, 5·70 %.)

Mesobiliverdin dimethyl ester chloride is obtained in the same way. It crystallises also, on standing, from its solutions in 1 % hydrochloric acid obtained by extracting its ether solution with the former: M.P. 175–176°; no complete melting, but sintering together.

Biliverdin chloride. Fine green needles. Extinction slightly oblique. Pleochroism: (NS) blue-green (darker), (EW) yellowish-green (lighter). (Found: C, 63·38, 63·36; H, 5·52, 5·75; N, 7·02, 6·84; Cl, 4·81, 4·71 %. $C_{33}H_{34}O_6N_4.HCl$ requires: C, 63·99; H, 5·55; N, 9·05; Cl, 5·74 %.) The nitrogen is 2 % too low. On drying at 0·1 mm. and 80° over phosphorus pentoxide for several hours, 9·615 mg. substance lost 0·363 mg., but there was no considerable increase in nitrogen percentage. (Found: N, 7·09 %.) There can be no doubt however about the existence of a monochloride.

Biliverdin dimethylester chloride. Fine green needles with no definite M.P. The crystal properties are given below.

Complex salts of biliverdins.

For these experiments mesobiliverdin ester was used.

Zinc salt. A few ml. of a solution of the ester in methanol were filled into a Thunberg tube, in which was placed a small test-tube containing zinc acetate solution. The tube was evacuated twice and filled with nitrogen free from oxygen. When the solutions were mixed the colour turned immediately from blue to green. The general absorption in the red part of the spectrum gave place to a sharper band, with its centre at 685 $m\mu$. This is the band of the complex zinc salt. There is however no fluorescence.

On addition of water and ether, the zinc compound passed from methanol into the ethereal layer. When this was shaken with $N/10$ HCl, the zinc compound was decomposed; the liberated mesobiliverdin ester passed into the acid, showing the typical green colour.

On standing exposed to the air the zinc compound was slowly changed, much faster if ammonia is added. The red fluorescence and the absorption band at 630 $m\mu$ characteristic of biliviolins appeared.

Copper salt. The formation of the copper salt was carried out in nitrogen as in the case of the Zn salt. The solution turned olive-yellow when the copper acetate solution was added. No distinct band could be seen, but general absorption in the red, blue and violet. When the copper compound was brought into ether and this shaken with $N/10$ HCl, no mesobiliverdin was extracted. The ethereal solution was now green and showed a strong band at 650 $m\mu$. When now $2N$ HCl was added, a play of colours developed. Immediately under the green ethereal layer the acid aqueous layer showed a blue zone with a red-violet one below. Gradually the acid solution turned blue-violet, but after some time green flocks of mesobiliverdin chloride precipitated and left the solution red-violet, showing absorption bands at 600 and 550 $m\mu$.

There must exist a copper compound, or otherwise the mesobiliverdin would have been extracted by the dilute acid from ether. The copper complex salts of pyrrole compounds are more stable towards acid than those of zinc. The stronger HCl however splits the compound and sets free the bile pigment which has been changed partly into a biliviolin. Whether this more rapid change of the copper salt is due to an oxidation by bivalent copper or occurs as an effect of the stronger acid on the copper complex is not clear.

If ammonia were added the olive copper compound gave a blue solution showing the typical bands of complex salts of the biliviolin type at 632 and 578 $m\mu$.

Biliverdin, uteroverdin and oocyan dimethyl esters.

In the paper of Lemberg and Barcroft [1932] Bernal reported that oocyan and uteroverdin esters differed in the optical properties of the crystals. I found later a similar difference between the crystals of uteroverdin ester and biliverdin ester obtained from bilirubin with ferric chloride.

Different preparations of natural and synthetic biliverdin esters have been examined. All crystallise in one of the two forms which may be called A and B or they may contain a mixture of both. Form A is that observed by Bernal for oocyan ester, form B that found by him for uteroverdin ester. In Bernal's report the orientation of the pleochroism of both forms was given inversely to that observed now, which was probably due to a mistake.

Pure A was obtained if pure bilirubin was dehydrogenated whether with ferric chloride or with atmospheric oxygen in alkaline solution. Even impure samples of the ester crystallised then in the A-form only. The purest was one

obtained by esterification of pure crystalline biliverdin; its M.P. was 223° (corr.). But samples melting as low as 195° crystallised as A.

Very small amounts of B were obtained from pure bilirubin twice. Once bili rubin was extracted with boiling glacial acetic acid. A very small amount went into solution and was simultaneously dehydrogenated to biliverdin which gave B on esterification. Again a very small sample of B was got from the ethereal mother-liquor of biliverdin, after the chief part of the biliverdin had separated.

B was predominant in uteroverdin ester, some samples of which were almost free from A. The ester of oocyan contained more A and less B. Mixtures of A and B resulted also from biliverdin extracted from ox gallstones by extraction with boiling glacial acetic acid or by dehydrogenation of bilirubin, which had been prepared from the mother-liquors in chloroform by precipitation with alcohol. Here again the melting-point varied from 196° up to 209° (for utero-verdin ester) and to 215° for the product of the glacial acetic acid extract of gallstones. There was no melting-point depression on mixing the different A or B esters or A with B ester.

No change could be observed from B to A or reverse, on recrystallisation. If a mixture of A and B were fractionated by crystallisation from methanol, A crystallised first, B from the mother-liquors.

The mesobiliverdin synthesised from analytical neoxanthobilirubic acid [Fischer et al. 1932] is according to Siedel and Fischer [1933] a mixture of the natural (unsymmetrical) mesobiliverdin with two other symmetrical meso-biliverdins with different position in the side-chains. The crystals of the synthetic mesobiliverdin ester were compared with crystals of that obtained from bilirubin ("natural ester").

Natural ester: oblique extinction; pleochroism extraordinarily strong (NS), violet-grey (EW), blue; at 45°, red-violet.

Synthetic ester: Extinction changing from almost straight to oblique. Pleochroism different in different crystals, partly similar to the "natural" pro duct, partly much less distinct.

Although these facts seem to favour the assumption that A and B forms are structurally different, the exact investigation of the optical properties of the crystals showed that the differences can be explained satisfactorily as due to growth and orientation of the crystals; a particular impurity may cause the substance to crystallise in the B form.

If the crystals are monoclinic, then A may be a form in which the crystals are tabular parallel to (110) or another vertical face elongated along c. The extinction would then be oblique. Tilting the crystal along its long axis into the direction in which the light is perpendicular to (100) should give straight extinction, turning it in the opposite direction should increase the angle of extinction up to the value it shows for its (010) plane. That is what actually happens if the A-crystals are tilted on a hemisphere.

The B-crystal may be one showing its (100) or (001) plane. The different pleochroism can be explained by the fact that it is elongated along b. Thus on turning the extinction remains straight.

Further evidence against the assumption of a genuine difference between A and B arises from the investigation of the hydrochloride crystals. No difference could be found between the chlorides of pure A-ester and of uteroverdin ester. Both show the following properties: flat needle-shaped green prisms; complete extinction almost straight (deviation less than 2°). Pleochroism: (NS) blue-green (dark in sodium light), (EW) green (lighter in sodium light), slow wave parallel to long axis. The partial birefringence can be roughly estimated to be 0·02.

DISCUSSION.

Two formulae have been discussed for biliverdin [Fischer and Adler, 1931; Lemberg, 1932; Siedel and Fischer, 1933; Lemberg and Bader, 1933] which differ in the position of the double bonds in the rings I and II:
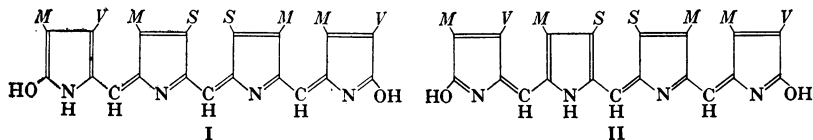
I had preferred formula I in 1932, as biliverdin was then thought to be incapable of forming complex salts, and this formula accounted better for their non-existence. But as complex salts have now been proved to exist, there is no longer any reason for preferring it. Furthermore a substance of formula I should form dichlorides (as the porphyrins do) on its two middle basic nitrogen atoms, whereas in formula II a monochloride, which has been found, is understandable; the two nitrogen atoms of the rings I and IV are less basic being lactim-nitrogen (—N=C(OH)—).

Mesobiliverdin was considered by Siedel and Fischer [1933] to be the blue stage of the Gmelin test. It is shown in this paper that this pigment is the chief product in the green phase of the Gmelin test, whereas it is hardly present when the blue phase is reached. The Gmelin test was carried out at high dilution which favours the successive formation of the single reaction phases. Nevertheless mixtures were obtained. They were separated by use of the Willstätter HCl-number method, which is as applicable to certain types of bile pigments as to porphyrins or chlorophyll derivatives.

Mesobiliverdin has been obtained in the form of the ferric chloride double salt of its ester chloride by oxidation of mesobilirubinogen with ferric chloride. The bearing of this observation on the relation between the green and violet bile pigments (mesobiliviolin and the phycobilins of red algae) and on the urobilin problem will be discussed in a forthcoming paper. A short report has been already given [Lemberg, 1934].

SUMMARY.

The preparation is described of dehydromesobilirubin dimethyl ester from crude "mesobiliverdin" and of dehydrobilirubin ester from "biliverdin." It is proposed that the names biliverdin and mesobiliverdin should be used for the green dehydrobilirubins.

Mesobiliverdin forms the first (green) stage of the Gmelin reaction, from which it could be isolated in a yield of 36 % of the mesobilirubin which entered the reaction.

Mesobiliverdin is obtained from mesobilirubinogen by oxidation with ferric chloride in the form of the ferric chloride double salt of its dimethyl ester chloride ($C_{35}H_{42}O_6N_4.HCl.FeCl_3$).

The method of preparation of biliverdin is improved and its formula $C_{33}H_{34}O_6N_4$ (with two hydrogen atoms less than bilirubin) is confirmed.

Biliverdin dimethyl ester can crystallise in two different forms. The first form is obtained from pure bilirubin. Natural biliverdins (uteroverdin and oocyan) give a second form mixed with more or less of the first; so does biliverdin from the mother-liquors of bilirubin. The two types can be explained by differences of crystal growth and the crystallographic investigation gives no evidence

of the existence of two structurally different substances. Biliverdin, uteroverdin and oocyan may be considered as identical.

Crystalline monochlorides are obtained from biliverdin, mesobiliverdin and their esters. The biliverdins form complex salts with Zn and Cu, but the Zn salt lacks the typical fluorescence of pyrrole pigment zinc complexes. These observations allow of a decision between the two formulae which have been proposed for biliverdin.

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