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Formation of Biliverdin from Legcholeglobin, the Green Pigment in Leguminous Root Nodules

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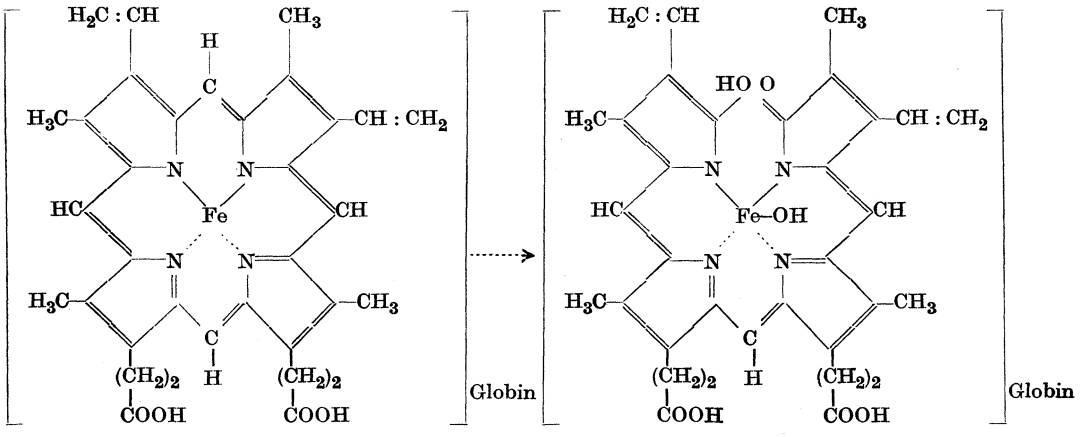
Virtanen and his collaborators [1–4] have shown that the leghemoglobin present in the leguminous root nodules changes to a »green pigment» when the symbiotic nitrogen fixation ceases. The pigment was isolated from the nodules, purified in a high degree by repeated precipitation with ammonium sulphate, and characterized as a precursor of bile pigments. This green pigment to which we preliminarily propose the name *legcholeglobin* until its constitution is fully explained, was noted to resemble in the first place the choleglobin of Lemberg [5] prepared from hemoglobin by oxidation with oxygen in the presence of ascorbic acid. The legcholeglobin was assumed to arise in the nodules from leghemoglobin through a similar oxidation either with hydrogen peroxide or oxygen. The initial and final products of the reaction were assumed to be the following:



Leghemoglobin Legmetcholeglobin

It has not yet been sufficiently explained whether the green pigment contains both bi- and trivalent iron or only either of them and at what stage the possible oxidation of iron to the ferric form takes place.

An important step in the investigation of the structure of legcholeglobin is the finding to be reported in this paper that a fully characterized bile pigment, biliverdin, is formed from the prosthetic group of legcholeglobin. Lemberg, Lockwood and Legge [6] have earlier shown that bile pigments, biliverdin and biliviolins, can be split from choleglobin by a weak acid (acetic acid). Liebecq [7] has shown that biliverdin and biliviolins can also be split from the pseudohemoglobin of Barkan and Schales [8] obtained from hemoglobin by oxidation with hydrogen peroxide in the presence of cyanide, provided that the pseudohemoglobin is not fully denatured during the procedure.

We have employed in our experiments pea plants grown in sterile cultures, inoculated with an effective *Rhizobium* strain. After six weeks growth the culture flasks with plants were transferred into the dark whereby the reddish nodules turned green in three days.

Samples of 4 g (fresh weight) were taken from the separated green nodules, crushed in a mortar with 8 ml glacial acetic acid to a homogeneous suspension, and centrifuged. 15 min after crushing 20 ml ether were added in a separatory funnel to the grass-green acetic acid solution. Legglobin and the main part of acetic acid were washed with small amounts of water from the ether phase, whereupon biliverdin was extracted with small amounts of 5 % hydrochloric acid. The HCl-extracts (2, 3 ml) were joined, filtered clear, made up to exactly 3 ml with water, and the absorption spectrum was determined with Beckman Quartz Spectrophotometer (1 cm cell). The spectrum obtained is given in Fig. 1, curve II. The maxima lie at about 665 mμ and at about 355 mμ. The curve is very similar to the spectrum of a pure biliverdin-hydrochloride measured in glacial acetic acid solution (Fig. 1, curve I). A somewhat differing location of the maxima in these biliverdin preparations may be due to some impurities, *e. g.*, small amounts of other bile pigments. For comparison, a typical spectrum is also given (Fig. 1, curve III) of 5 % HCl-extract from choleglobin prepared according to Lemberg *et al.* [6] at pH 7.4 with 3 h incubation from the cow oxyhemoglobin. It appears from the curve that this preparation contains abundantly biliviolins.

From the choleglobin preparations of Lemberg [6] and pseudohemoglobin preparations of Barkan [7] biliviolins are extractable with 10 and 20 % hydrochloric acid. In the corresponding extracts of legcholeglobin no biliviolins could be detected. Besides, the oxidation products obtained by Lemberg and Barkan from the hemoglobin of blood leave deeply brown pigments in the ether layer after HCl-extraction, whereas the ether solution obtained from

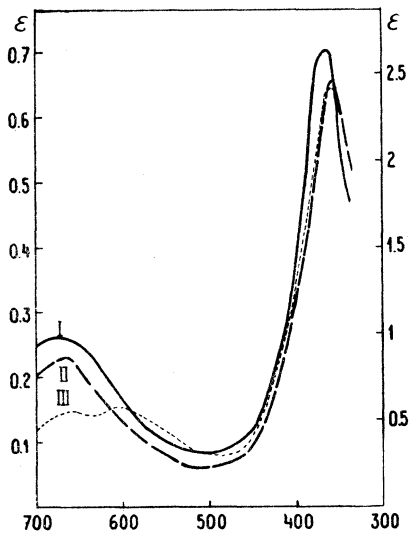


Fig. 1. Spectra of different biliverdin preparations. The ε values on the right refer to curve I, those on the left to curves II and III.

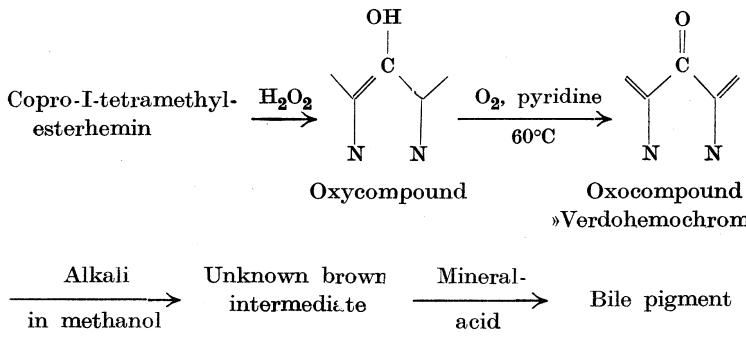
I. Pure biliverdin.
II. Biliverdin from legcholeglobin.
III. Biliverdin with high percentage of biliviolins from choleglobin preparation according to Lemberg.

legcholeglobin is only slightly yellowish after the removal of biliverdin. These observations indicate that legcholeglobin is an appreciably more homogeneous and purer substance than the artificial preparations of choleglobin and pseudohemoglobin.

In the soybean nodules the transfer of the plants into the dark does not catalyze the oxidation of leghemoglobin to green pigment as effectively as in pea [2]. The change of colour requires weeks. We have noted in the soybean grown out-of-doors that with the formation of green pigment the globin part is simultaneously denatured, whereby the prosthetic group and the denatured protein are linked together in some other way than in the native legcholeglobin. Then the prosthetic group can no longer be split by acid treatment. In the nodules of pea, where the change of colour takes rapidly place in the dark, the amount of the denatured green pigment is small compared with that of the undenatured one.

Regarding the structure of legcholeglobin the following facts must be considered. The absorption spectrum of legcholeglobin does not show any marked maxima between 500 and 600 mμ, so it does not seem probable, that the porphin ring is unbroken in the molecule. The easy formation of biliverdin from the legcholeglobin supports this view. Thus the opinion that the porphin ring has opened at the formation of legcholeglobin is well founded on the basis of the observations so far. Barkan and Schales, Lemberg and collaborators, and Liebecq consider the case to be such when pigments of pseudohemoglobin-choleglobin-type are formed.

Hans Fischer *et al.*[9] have found that the porphin ring has not been broken in the »green hemins»[10], another type of precursors of bile pigments, also called »verdohemochromogens» by Lemberg[11]. By oxidation of synthetic copro-I-tetramethylester-hemin they prepared the corresponding »oxo-compound» which has an oxo-group in one of the ms-positions. It has the properties of »verdohemochromogens» but can be reduced to the initial porphyrin.



Thus, the porphin ring of the »green hemins» must still be intact, contrary to the opinion of Lemberg[11]. In the light of the above findings, an intact porphin ring is, however, not probable in legcholeglobin.

The »green pigment», legcholeglobin, isolated in this laboratory in 1945 from the root nodules of pea, is as far as we know the first precursor of the bile pigments, formed in nature which has been isolated in high degree of purity. It is noteworthy, that this compound is found in the plant kingdom, in the root nodules of legumes — the only place where hemoglobins have hitherto been met in the vegetable kingdom. The research on the formation and breakdown of leghemoglobin in the nodules will possibly give further explanation also to the formation of the hemoglobin of blood and to its transformation into bile pigments.

SUMMARY

The green pigment which is formed in the leguminous root nodules as a transformation product of the red pigment, leghemoglobin, at the ceasing of nitrogen fixation is a precursor of the bile pigments. It yields biliverdin by the action of acids. The observations so far are in accordance with the idea that the porphin ring is open in the green pigment. The pigment is called legcholeglobin.

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