Studies on Verdohemochrome*

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ABSTRACT: The properties of verdohemochrome obtained by coupled oxidation of dipyridine iron(II) protoporphyrin IX (protohemochrome) and ascorbic acid have been reinvestigated in order to compare this model reaction to the recently reported enzymatic conversion of protohemochrome to a green precursor of bile pigment. Thin layer chromatography shows that verdohemochrome is heterogeneous, and purification has been obtained on silica gel columns. Elemental analysis of the chromatographed material indicates the presence of two oxygen atoms more than have been previously assigned to the structure, an empirical formula of $C_{43}H_{42}FeN_6O_8$, and an average minimum molecular weight of 827, which is in good agreement with a value of 818 measured by vapor pressure osmometry. Two exchangeable pyridine moieties are present, indicating that verdohemochrome is a dipyridine hemochrome; therefore, the additional molecule of oxygen cannot be present as a ligand to Fe, as has been suggested for the enzymatic reaction product. The visible absorption spectrum of chromatographically purified verdohemochrome has an extinction value for the maximum at 663 mμ which is 50% higher than those previously reported for this compound and for the enzymatic reaction product. The infrared spectrum does not suggest the presence of the terminal formyl group noted in the latter compound, nor does verdohemochrome form derivatives with carbonyl reagents. A carbon monoxide complex of verdohemochrome in deuteriochloroform gives a measurable though weak and broad nuclear magnetic resonance signal, indicating low-spin iron(II). The observations are consistent with a tetrapyrrole ring in verdohemochrome which is closed by an oxygen bridge. The two unassigned oxygen atoms and the failure of verdohemochrome to yield a stoichiometric amount of bile pigment on acid hydrolysis can be explained by assuming that the chromatographed material is still impure, and contains some structures hydroxylated on methene bridge carbon atoms.

The green compound verdohemochrome, formed by oxidation of dipyridine iron(II) protoporphyrin IX (protohemochrome) in the presence of various reducing agents (Warburg and Negelein, 1930), yields bile pigments on acid treatment. This oxidative cleavage of the iron porphyrin ring has been considered to be a model of *in vivo* hemoglobin catabolism (Lemberg, 1935). Recently, a green bile pigment precursor, spectrally similar to verdohemochrome, has been isolated from the *in vitro* oxidation of protohemochrome by a liver enzyme (Nakajima *et al.*, 1963). However, the structures suggested for verdohemochrome (Figure II) (Lemberg and Legge, 1949), and for the enzymatic reaction product (Figure III) (Nakajima, 1963), differ in the nature of the Fe ligands and the level of oxidation of the methene bridge, leaving unsettled the validity of the chemical oxidation as a model of the physiologic process. As an initial step in comparing the two ring-opening reactions, the properties of verdohemochrome have been reinvestigated, using a preparation purified by chromatography, and this preparation clearly distinguished from the structure proposed for the enzymatic reaction product. These studies indicate that verdohemochrome is a dipyridine hemochrome of mol wt 827 and an empirical formula $C_{43}H_{42}FeN_6O_8$.

Materials and Methods

Crystalline bovine hemin, not recrystallized, was obtained from Koch-Light Laboratories, and three-times-crystallized bovine hemin from Nutritional Biochemicals and Eastman Organic Chemicals. Silica gels were obtained from E. Merck AG., and Celite (No. 545, acid washed) from Johns-Manville. Tritiated pyridine obtained from Nuclear-Chicago had a constant specific activity of 48 mc/mmole on redistillation at 116°. Pyridine was redistilled from KOH. Petroleum ether (bp 30–60°) was purified on alumina to remove peroxides (Dasler and Bauer, 1946). Glass-distilled water was used.

Protohemochrome was converted to verdohemochrome with ascorbic acid in 400 ml of a pyridine–water mixture (1:3, v/v) containing the following components (in millimoles): sodium barbital buffer, pH 8.3, 50; hemin, 0.0375; and ascorbic acid, 2. The reaction mixture had an apparent pH of 9.2. The solution was shaken vigorously in an open vessel at 37° for 15 min, cooled in ice, and acidified with 160 ml of acetic acid. After 5 min, the mixture was saturated with sodium acetate and the verdohemochrome was extracted into 200 ml of chloroform (Nakajima, 1963).

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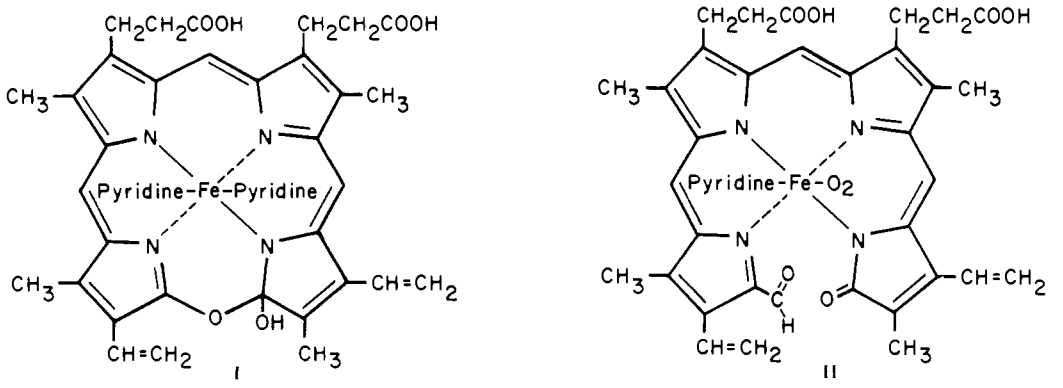

FIGURE 1: Suggested structure of verdohemochrome (I) (Lemberg and Legge, 1949) and of the intermediate in the enzymatic degradation of protohemochrome (II) (Nakajima, 1963).

The chloroform extract was washed five times with water, once with 0.01 M HCl, and again three times with water. The solution was reduced under vacuum to a few milliliters and verdohemochrome was precipitated with petroleum ether (Warburg and Negelein, 1930). This preparation, which had the typical visible absorption maxima described by Lemberg (1935), is referred to as crude verdohemochrome.

Ascending thin layer chromatography was carried out on silica gel G using a mixture of pyridine–chloroform–water (6:4:1, v/v) as the solvent. Preparative columns for verdohemochrome, using the same chromatography solvent, were constructed with 240 g of a mixture of silica gel (less than 0.08 mm) and Celite. The weight ratio of the silica gel (acting as the adsorbent) and the Celite (acting as an inert spacer) varied from 1:1 to 1:3, depending on the batch of silica gel being used, and was determined on a small-scale column to give good separations with a reasonably rapid flow rate. The pooled crude verdohemochrome derived from 16 reaction mixtures (394 mg of hemin) was dissolved in 20–30 ml of chromatography solvent and loaded on a 68 × 3.7 cm column bed. Under a pressure of 2 cm applied to the solvent reservoir, columns ran at about 1.0 ml/min and were completed in 24 hr. The absorption spectra of fractions collected over 10-min intervals were analyzed in the range 350–750 mμ. The selected column fractions were combined and washed three times with 0.01 N HCL and four times with water, additional chloroform being added if necessary. Dark green acid washes were sometimes reextracted with chloroform and the extracts were combined. The chloroform solution was dried with anhydrous sodium sulfate, filtered, concentrated *in vacuo*, and precipitated with petroleum ether as before. The precipitate was washed once with petroleum ether and dried for 40 hr at 22° in a vacuum of 0.02 mm, conditions found to remove residual solvent pyridine and give a constant weight.

Iron was determined by the method of Drabkin (1941) after oxidation of the organic material with HNO_3 and H_2O_2. Carbon, hydrogen, and nitrogen analyses were carried out by Mr. Joseph Walter of The Johns Hopkins University and by Dr. S. M. Nagy of The Massachusetts Institute of Technology, and oxygen analyses by Schwarzkopf Microanalytical Laboratory. Visible absorption spectra were measured in a Cary 14 recording spectrophotometer, calibrated to within 4 A with a mercury arc. Tritiated pyridine was counted in a Packard Tri-Carb liquid scintillation spectrometer, using 15 ml of toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2′-(5′-phenyloxazolyl)benzene, and corrected for quenching from a standard curve. The volumes of pyridine counted were kept below 20 μl, so that all corrections were less than 20%.

Results

Thin Layer Chromatography. In the initial experiments, verdohemochrome was prepared from three-times-crystallized bovine hemin, described by the supplier as having a minimum purity of 92% and a minimum iron content of 8%. However, the iron content of preparations of crude verdohemochrome made from different batches of this hemin was 5.7%, much lower than is reasonable for any type of an iron tetrapyrrole structure derived from hemin. This observation raised the suspicion that these verdohemochrome preparations were heterogeneous, perhaps containing bile pigment or other iron-free breakdown products of hemin. No significant purification of verdohemochrome was obtained by chromatography on silicic acid (Nakajima, 1963) or by partition chromatography on Celite (Caughey and York, 1962), but thin layer chromatography on silica gel fractionated verdohemochrome into a series of colored and fluorescent spots. Although not all of these spots could be completely separated on a single 15-cm long thin layer run, subsequent thin layer chromatography of fractions obtained from preparative column chromatograms revealed at least 10 different components, listed in Table I.[1] Verdohemochrome prepared with ascorbate is evidently a complex mixture, although the proportions of the various colored products are not measured by the chromato-

TABLE I: Thin Layer Chromatographic Fractions of Crude Verdohemochrome.

Spot	Color	R_F Value
1	Green-yellow	1.00
2	Red-brown	0.86
3	Blue	0.82
4	Brown	0.73
5	Green	0.64
6	Red	0.58
7[a]	—	0.43
8	Brown	0.38
9	Green	0.30
10	Blue	0–0.30 (streak)

[a] Fluorescent under ultraviolet light.

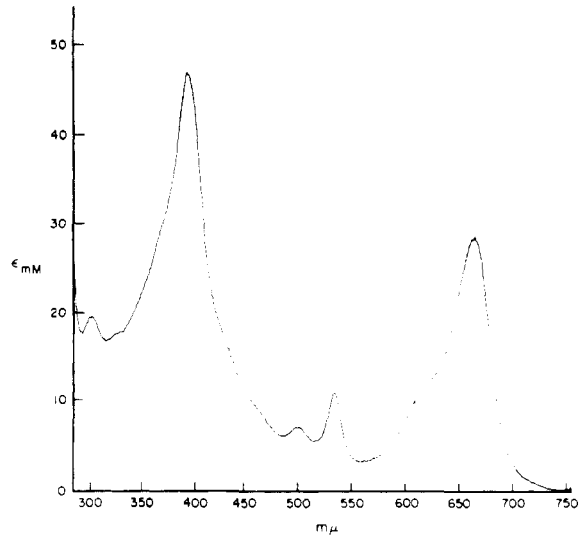

FIGURE 2: Visible absorption spectrum of purified verdohemochrome in 1% pyridine–chloroform (v/v).

graphic method. A similar mixture of colored products has been noted on chromatography of verdohemochrome derived from coupled oxidation of hemin with hydrazine hydrate, supporting the impression that verdohemochrome prepared with different reducing substances is the same crude product (Lemberg and Legge, 1949).

Column Chromatography. The observed fractionation of crude verdohemochrome on thin layer plates suggested the use of the silica gel column procedure for purification on a larger scale. Crude preparations of verdohemochrome fractionated on the silica gel–Celite columns in the same way as on thin layer plates, except that a blue material spectrally resembling biliverdin preceded the green band. After elution of the blue substance from the column, a series of fractions was eluted with a nearly constant spectrum in which the optical densities at 398, 533, 565, and 663 mμ were in the ratio 4.5:1.0:0.3:2.4. Collection of these fractions was continued and spectra monitored until the peak at 663 mμ began to broaden, as measured by an increase in width at half-height beyond 55 mμ. This broadening was caused by the appearance in the eluate of material with a verdohemochromelike spectrum but with a peak at 680 mμ, and subsequent fractions were discarded. Usually 30–40 mg of dried material was recovered from the selected fractions. This substance is referred to as purified verdohemochrome.

The low yield, about 8%, resulted in part from the discarding of many green fractions with spectra indicating an admixture of other colored products. There was also some decomposition of verdohemochrome to an insoluble black material, possibly verdohemin or an oxidation product, which deposited near the top of the column. Aside from this decomposition, the material in the selected fractions was homogeneous on repeated chromatography in the same system.

Absorption Spectrum. The visible absorption spectrum of purified verdohemochrome dissolved in pyridine, or in chloroform containing 1% pyridine (v/v), is shown in Figure 2. This spectrum is very similar to that reported for the enzymatic reaction product in chloroform (Nakajima, 1963), and some of the observed differences between the two are even less apparent if crude verdohemochrome is used for the comparison. Table II compares the position of the absorption maxima for purified verdohemochrome in chloroform containing 1% pyridine with those reported for the enzymatic reaction product in chloroform. The latter material shows a relatively much greater absorption at 398 mμ, but presence of as little as 15% of a compound having a Soret band of ϵ 200 could raise the extinction to this level. The visible spectra, therefore, do not provide unequivocal evidence that the two preparations are different.

The ϵ of the 398-mμ band of verdohemochrome is 49.6, very close to that of biliverdin–HCl at 375 mμ (47.4, methanol) (Gray *et al.*, 1961) and much less than the Soret band of protohemochrome (166) (Gallagher and Elliott, 1965). This may be an indication that

[1] Several batches of the three-times-crystallized hemin preparations (Eastman, Nutritional) could be separated on thin layer into three components with protohemochromelike spectra. These three components were the precursors of the green spots 1, 5, and 9, respectively (Table I), each of which had a verdohemochromelike spectrum. Possibly these components of hemin were artifacts produced by the recrystallization, because once-crystallized hemin preparations (Koch-Light) were homogeneous in this system, and gave spot 9 as the only green component of the verdohemochrome product, although with the other colored fractions unchanged. The nonrecrystallized hemin was therefore used for large-scale preparations of verdohemochrome in order to decrease the number of side fractions. There is no evidence that recrystallization purifies hemin crystals (Falk, 1964), and in fact recrystallization has been found to produce flattened-out minima and maxima in the absorption spectrum (Paul *et al.*, 1953).

TABLE II: Visible Absorption Spectrum of Purified Verdohemochrome and of the Reported Enzymatic Reaction Product.

Prepn	Solvent	Absorption Max (mμ)	Ratio of Absorbancies at These Wavelengths	ε (mμ)
Enzymatic reaction product	Chloroform	657	2.0	20.3[a] (657)
		530	1.0	
		498	0.9	
		397	7.5	
Purified verdohemochrome	1% pyridine in chloroform or in pyridine	663[c]	2.5	29.0[b] (663)
		534	1.0	
		500	0.7	
		397	4.3	
		308	1.8	

[a] Calculated from the published spectrum (Nakajima, 1963) and based on mol wt 760. [b] Calculated for mol wt 827. [c] Minima at 559, 515, 487, 320, and 298 mμ have EmM 3.4, 5.75, 6.4, 17.1, and 18.0, respectively.

when the conjugation is interrupted around the porphyrin ring, it is not maintained through the metal, an explanation that has been offered for the presence of a Soretlike band in the Zn complexes of some bile pigments and in vitamin B_{12} (Falk, 1964).

The verdohemochrome preparations studied by Lemberg and Legge (1949) had ϵ_{657} 19.6 in 20% pyridine in chloroform (v/v), about the same as for the enzymatic product (Table II). Thus the chromatographic purification leads to a 50% increase in the ε of the peak in the red, but the over-all purification must be greater than 50%, since spectrophotometric examination of colored column fractions showed the presence of several other substances which absorb at 663 mμ.

Infrared Spectrum. Biliverdin derived from hemin by the chemical oxidation method is a mixture of isomers, formed by the loss of different methene bridges when the ring is opened (Petryka *et al.*, 1962). Its precursor verdohemochrome, which also has lost one carbon atom (Ludwig *et al.*, 1957), must be a similar mixture, and so might not be expected to give a sharp infrared spectrum. Despite this difficulty, and the low solubility of the compound, it was possible to measure a spectrum in the infrared by using ordinate scale expansion. Figure 3 shows the infrared tracings obtained for verdohemochrome and protohemochrome in pyridine, measured in the Perkin-Elmer Model 521 infrared spectrophotometer. The verdohemochrome spectrum does not closely resemble that reported for the enzymatic reaction product in Nujol (Nakajima, 1963), and no peak in the region of 6 μ is sufficiently sharp and intense as to indicate the presence of the noncarboxylic carbonyl group ascribed to the enzymatic product (Figure 1II). In comparison with the spectrum of protohemochrome, the infrared spectrum of verdohemochrome shows several new bands in the regions 1500–1700 cm^{-1} and at 1635 cm^{-1}. Attempts to assign these new bands to specific functional groups in verdohemochrome are in progress.

Nuclear Magnetic Resonance Spectrum. The nmr[2] spectrum of verdohemochrome was examined in a Varian Associates H A-100 nmr spectrometer, using an internal tetramethylsilane reference. No signal could be observed with 3×10^{-2} M verdohemochrome in deuteriopyridine, and the compound was therefore studied as the carbon monoxide complex, presumably a ferrous diamagnetic species (Lemberg and Legge, 1949), which was formed by dissolving the purified preparation in CO-saturated deuteriochloroform. After filtration to remove undissolved verdohemin, the deep blue CO complex showed absorption maxima at 625, 544, 506, 408, and 353 mμ, having relative heights of 2.4:1.0:0.73:3.60:3.37. A typical verdohemochrome spectrum like that in Figure 2 appeared in about 40% of the expected yield when pyridine was added and the CO removed in a stream of nitrogen; nmr data for the CO complex are shown in Table III. The broadening of the bands may be an indication of very small amounts of ferric impurities, but may also reflect the presence of structural isomers oxidized at different methene bridges. No structural information about the methene bridges can be deduced from this poorly resolved spectrum, but the existence of a carbon monoxide complex which gives an nmr signal indicates low-spin iron(II). The dipyridine verdohemochrome must be the low-spin Fe(II) complex also, as was suggested previously (Lemberg and Legge, 1949). The observed shift of the peaks to higher field, as compared with the corresponding signals in iron porphyrin compounds (Caughey *et al.*, 1965), might be explained in terms of a partial

[2] Abbreviation used: nmr, nuclear magnetic resonance.

TABLE III: Nmr Spectrum of CO-Verdohemochrome.

Band Position (δ)	Description of Band	Probable Source	Width at Half-Height (cps)
2.98	Broad	Methyls	35
2.45	Broad	Methyls	43
7.21	Sharp	Pyridine	5
9.22	Weak	Methenes	11

interruption of the resonance of the porphyrin macrocycle like that suggested in Figure 1I, resulting in a decrease in the ring-current field effect (Caughey and Koski, 1962).

Elemental Analysis. Elemental analysis of verdohemochrome has not been reported previously, although it was noted by Lemberg and Legge (1949) that the carbon content was always much lower than the 65% required by the assumed structure (Figure 1I). Part of this discrepancy may have been due to the heterogeneity of these earlier preparations, but as shown in Table IV, chromatographically purified material still

TABLE IV: Elemental Per Cent Composition of Purified Verdohemochrome.

Element	Prepn I	Prepn II	Mean	Calcd for $C_{43}H_{42}FeN_6O_8$
C	62.59	62.84	62.72	62.47
H	5.12	4.84	4.98	5.12
Fe	6.59	6.81	6.70	6.76
N	10.58	9.64	10.11	10.17
O	16.24	13.77	15.01	15.48
Cl	0.76 (0.17 atom)	—	—	0

gives carbon values of only 62–63%. The complete analysis indicates an elemental composition of $C_{43}H_{42}FeN_6O_8$, with an expected minimum molecular weight of 827. This elemental composition is consistent with the loss of a one-carbon unit from protohemochrome, but it suggests the presence of an average of eight oxygen atoms per molecule, rather than the expected six (Figure 1I). Oxygen content of different preparations ranged from 13.77 (7.12 atoms) to 17.66% (9.13 atoms). No chlorine is present in the compound, indicating that there is no verdohemin or bile pigment complex salt present; and also that there has been no inadvertent

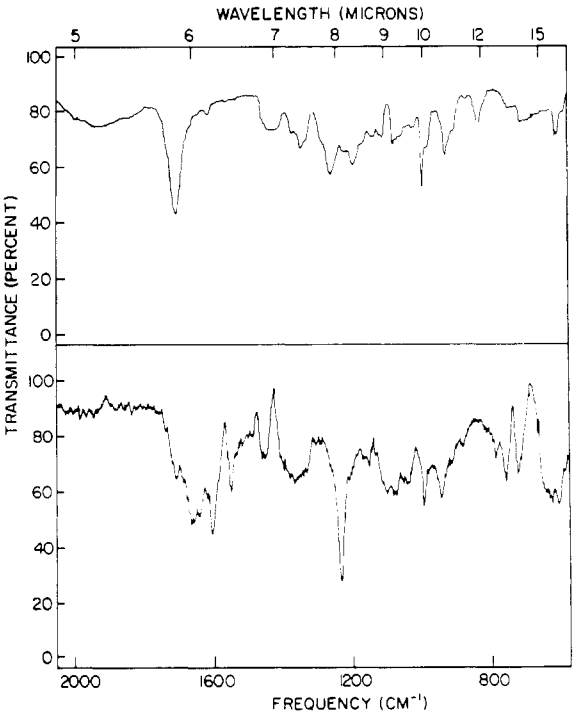

FIGURE 3: Infrared spectrum of 1.3×10^{-2} M verdohemochrome in pyridine, at an ordinate scale expansion of about four times (lower tracing); and of 6×10^{-2} M protohemochrome in pyridine (upper tracing).

mesochlorination, which can occur with chlorins partitioned between peroxide-containing ethers and HCl (Woodward and Skarić, 1961).

Molecular Weight Determination. The molecular weight of a purified verdohemochrome preparation was measured in pyridine solution at 65° on a Mechrolab Model 301 vapor pressure osmometer. The method is limited in accuracy because verdohemochrome is not soluble in pyridine at concentrations greater than about 24 mg/ml (0.029 M). However, five determinations in the concentration range 8–24 mg/ml gave a mean value of 818, within 1% of the molecular weight expected from the elemental composition data. This supports the empirical formula given above, and also establishes that verdohemochrome, like hemin (Shack and Clark, 1947), exists in pyridine solution as a monomer.

Pyridine Content. Lemberg and Legge (1949) concluded that verdohemochrome is a dipyridine hemochrome, because the nitrogen to iron ratio of their preparations was 6:1. Although the iron content of this crude material is less than would be expected for one atom per molecule, nevertheless the assignment of two pyridines/mole of verdoheme is supported by N:Fe ratios of 6:1 obtained on the purified material. In contrast, the product of the enzymatic hemin-degrading reaction contains much less of both carbon and nitrogen than does the suggested structure (Figure 1I) for verdohemochrome, and Nakajima (1963) has therefore

postulated the presence of an oxygen molecule in place of one of the pyridines (Figure 1II). Iron porphyrin compounds with one pyridine and one O_2 ligand have been reported (Caughey *et al.*, 1965) and a structure of this type was suggested for verdohemochrome by Rackow (1957). The molecular weight and elemental composition of purified verdohemochrome are not consistent with structures containing one pyridine and one oxygen molecule as ligands, but it seemed desirable to confirm that two of the nitrogen atoms of the compound are constituents of intact pyridine ligands by a direct method of measuring the pyridine content.

From 3 to 5 mg of purified verdohemochrome was dissolved in 1 ml of diluted tritiopyridine and incubated at 22°. After various times, the verdohemochrome was precipitated with 9 volumes of petroleum ether, collected by centrifugation, washed once with petroleum ether, and dried *in vacuo* for 40 hr. The dried material was redissolved in unlabeled pyridine and incubated for 1 hr at 22°. The amount of verdohemochrome recovered was measured spectrophotometrically at 663 mμ, the pyridine distilled over, and an aliquot of the clear distillate counted. Specific activity of the distillate did not change on redistillation. Table V demonstrates that regardless of the time of incubation, 2 μmoles of radioactive pyridine was incorporated into each 827 μg of verdohemochrome. Recovery of incorporated tritiopyridine was similarly independent of the time of incubation in unlabeled pyridine. The data indicated that 2 moles of pyridine is present as ligands in purified verdohemochrome, and is very rapidly exchangeable with solvent pyridine. Verdohemochrome therefore cannot include an oxygen molecule bound to the iron, as was suggested for the enzymatic reaction product (Nakajima, 1963). Moreover, the close fit of the data to the expected value indicates that the verdohemochrome preparation does not contain any significant amount of bile pigment, and in addition is supporting evidence for a molecular weight of 827.

TABLE V: Incorporation of Tritiopyridine into Verdohemochrome.

Incubn in Tritiopyridine (hr)	Dpm/μmole of Pyridine	Dpm/μmole[a] of Verdohemochrome	μmoles of Pyridine/μmole of Verdohemochrome
0.01	5.24×10^4	9.60×10^4	1.83
0.5	3.06×10^5	6.04×10^5	1.97
1 0	3.06×10^5	6.24×10^5	2.04
2.0	3.06×10^5	5.89×10^5	1.92
4.0	5.24×10^4	9.76×10^4	1.86
27.0	5.75×10^4	1.20×10^5	2.09
		Mean	1.95

[a] Calculated for mol wt 827.

The ease with which pyridine in verdohemochrome exchanges with solvent pyridine is in sharp contrast to the difficulty of directly abstracting this ligand. When heated 3–4 hr at 118° *in vacuo*, verdohemochrome loses only one-half the weight calculated for its pyridine content, even when the measured weight loss is corrected for a measured increase in oxygen content. In one experiment using verdohemochrome binding tritiated pyridine, 30% of the radioactive ligand was not removed by this heating procedure, and about the same fraction of the OD 663 mμ in pyridine was recovered. However, removal of the pyridine by evaporation of solvent from the verdohemochrome band on a thin layer plate in air at 22° rapidly destroys the spectral properties, and this can be prevented by saturating the atmosphere with pyridine. Probably verdohemochrome is labile to oxidation only under conditions where the pyridine ligands can be replaced by molecular oxygen. In pyridine solution, or in pyridine–chloroform (1:100, v/v), the verdohemochrome spectrum is almost unchanged after 24 hr at 22°. Perhaps carbon monoxide is more easily displaced by O_2 than is pyridine, explaining why after brief replacement of pyridine by CO only 40% of the repyridinated molecule was recovered.

Discussion

Earlier studies of verdohemochrome assumed that the material was homogeneous, but recently chromatographic heterogeneity has been observed on silicic acid (Nakajima, 1963). Moreover, biliverdin prepared *via* coupled oxidation of pyridine hemochrome and hydrazine hydrate is a mixture of structural isomers (Petryka *et al.*, 1962) suggesting that verdohemochrome is also such a mixture. Chromatography of verdohemochrome on silica gel does not separate these presumed isomers, but it removes a number of colored side products to give a purification of at least 50% based on ϵ_{663}. Analysis of this chromatographically purified verdohemochrome indicates the presence in the molecule of two more oxygen atoms than had previously been recognized. The electronic and nuclear magnetic resonance spectra are consistent with a structure in which the resonance properties of the porphyrin macrocycle have been destroyed at least in part (Lemberg and Legge, 1949). There is no assurance that the chromatographed material studied in this reinvestigation is pure, but on the other hand the removal of contaminants does not bring the composition much more in line with the proposed structure (Figure 1I).

With crude verdohemochrome, the total yield of bile pigment on acid hydrolysis is usually less than 50%, even when estimated by nonspecific spectrophotometric methods (Lemberg *et al.*, 1941), and the colored side products present in these preparations, which include blue compounds spectrally resembling biliverdin, could have been the source of some of this chromophore. When the product of verdohemochrome hydroly-

sis was crystallized and clearly characterized as biliverdin dimethyl ester ferrichloride, the yield from hemin was less than 2% (Lemberg, 1935). By the spectrophotometric criteria, chromatographically purified verdohemochrome likewise does not give a quantitative yield of bile pigment, possibly because some of the material is degraded to other products in acid-dependent side reactions. In fact, it is difficult to understand how any significant amount of verdohemochrome containing eight oxygen atoms, none of which are present as Fe-bound ligands, can be converted by acid to biliverdin containing only six. Perhaps some of the excess oxygen is accounted for in the bilipurpurins always found accompanying the product biliverdin (Lemberg *et al.*, 1941); or other oxygenated derivatives, such as hematobiliverdin, may be present.

A more satisfactory explanation is that "purified" verdohemochrome actually contains some components more highly oxygenated than the structure in Figure 1I. An intermediate with absorption maximum at 639 mμ observed during verdohemochrome formation has been interpreted as a hemin hydroxylated on a methene bridge (Lemberg *et al.*, 1938). A compound of this type was isolated during the oxidation of coprohemin ester by H_2O_2 (Libowitzky and Fischer, 1938; Libowitzky, 1940). Further oxidation of the hydroxylated bridge carbon would then open the porphyrin ring (Lemberg *et al.*, 1938). Since during the coupled oxidation ring scission can occur at two or more bridge positions (Petryka *et al.*, 1962), the initial hydroxylation may sometimes proceed on more than one *meso* carbon before cleavage occurs at one of them. Purified verdohemochrome would then be not only a mixture of isomers with structures like that proposed by Lemberg and Legge, with different methene bridge carbons removed, but also with some similar structure hydroxylated at one, two, or three of the remaining bridges. A mixture of such hydroxylated compounds in verdohemochrome could account for the two unassigned oxygen atoms found on elemental analysis of purified verdohemochrome, for the variations in oxygen content between different preparations, and for the pronounced shoulder observed in the visible spectrum of verdohemochrome between 590 and 640 mμ. The nonhydroxylated form could be the precursor of the biliverdin formed on acid hydrolysis, the monohydroxylated form the bilipurpurin, and the higher hydroxylated forms the colorless products which account for the low yield of total bile pigment as measured spectrophotometrically. It is possible that verdohemochromelike compounds with different numbers of bridge positions hydroxylated show very similar electronic spectra, as did the *meso*-substituted mono-, di-, and trinitro-octaethylporphyrins (Bonnet and Stephenson, 1965).

It is clear that verdohemochrome prepared with ascorbic acid is different in several respects from the formula suggested for the enzymatic product. Crude or purified verdohemochrome preparations do not yield a crystallizable 2,4-dinitrophenylhydrazone, or release formaldehyde on acid degradation, as does the enzymatic product (Nakajima, 1963), and no absorption band ascribable to a carbonyl can be detected in the infrared spectrum. A terminal formyl group would not be expected in verdohemochrome, because the chemical oxidation is associated with CO evolution, indicating loss of the oxidized methene bridge (Ludwig *et al.*, 1957). The analytical data on the enzymatic product have been interpreted to indicate one pyridine and one oxygen as ligands to the iron, while verdohemochrome certainly has two pyridine moieties as ligands. Finally, if the enzymatic product is pure, there are differences between the electronic spectra. If the enzymatic and model oxidations truly give structurally different stable products, a comparison of the mechanisms of the two reactions should be interesting.

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Studies on the Biosynthesis of the Erythromycins. I. Isolation and Structure of an Intermediate Glycoside, 3-α-L-Mycarosylerythronolide B*

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ABSTRACT: A neutral glycoside, elaborated in large quantities, has been isolated from the fermentation beers of a blocked mutant of *Streptomyces erythreus*. Recovery and purification of the substance can be easily effected by solvent extraction and crystallization. Cofermentation and feeding of the glycoside to a second blocked mutant established that the compound is an intermediate in the erythromycin biosynthetic pathway. The structure of the material has been determined to be 3-α-L-mycarosylerythronolide B.

The isolation and chemistry of the erythromycins, antibiotic glycosides produced by fermentation of *Streptomyces erythreus*, has been well documented (Wiley *et al.*, 1957, and previous papers in this series by investigators of Eli Lilly and Co.). Although some progress has been made toward elucidating the biosynthetic pathway, most of the process is still unknown. Various investigators have reported that *S. erythreus* incorporates ^{14}C-labeled propionate without randomization into the macrocyclic lactone portion of the antibiotic (Kaneda *et al.*, 1962; Corcoran *et al.*, 1960). Studies by Corcoran (1964) suggest that the intact carbon chain of D-glucose is the precursor of the deoxy sugars, desosamine and cladinose, found in the erythromycins. Corcoran (1961) also reported that methionine was involved in the *C*- and *O*-methylation of cladinose. Tardrew and Nyman (1964) isolated erythronolide B, and Hung *et al.* (1965) established that the compound, the aglycone of erythromycin B, was an intermediate in the biosynthesis of erythromycins A–C. In this communication we wish to report the isolation and structure of an erythromycin biosynthetic intermediate, 3-α-L-mycarosylerythronolide B.

Experimental and Results Section[1]

Fermentation Organism. The strain employed in this investigation was *S. erythreus* (Abbott 8EI57). This variant was derived by treatment of a high erythromycin-yielding strain with ethylenimine followed by ultraviolet irradiation. The mutant has a complete block in the erythromycin biosynthetic pathway and accumulates large quantities of the intermediate glycoside in fermentation beers. The compound is devoid of antibiotic activity against *Bacillus subtilis*.

Fermentation Procedures. Seed cultures of variant 8EI57 were prepared in a medium consisting of (in grams per liter) glucose monohydrate (Cerelose), 15.0; soybean meal, 15.0; and $CaCO_3$, 1.0. The cultures were incubated at 32° for 72 hr on a rotary shaker. The seed was added at a level of 3–5% (v/v) into 500-ml erlenmyer flasks containing 50 ml of a chemically defined fermentation medium consisting of the following components (in grams per liter): glucose monohydrate (Cerelose), 10.0; corn starch, 40.0; glycine, 7.5; L-tyrosine, 0.9; triolein, 2.5; NaCl, 2.0; K_2HPO_4, 1.56; KH_2PO_4, 0.78; $MgSO_4 \cdot 7H_2O$, 0.50; $CoCl_2 \cdot 6H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.02; $MnCl_2 \cdot 4H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.05; and $CaCO_3$, 3.0. The potassium phosphate salts were sterilized and added separately. The fermentation flasks were incubated at 32° on a rotary shaker (280 rpm) for 168 hr.

Recovery from Fermentation Beer. Fermentation beer (2650 ml) was centrifuged to sediment the mycelium and the supernatant was recovered. To the supernatant was added with stirring an equal volume of an aqueous

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[1] Nuclear magnetic resonance spectra were determined as 10% solutions in deuteriochloroform with a Varian A-60 spectrometer. Infrared spectra were determined as chloroform solutions.