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# Alteration of the Porphyrin Nucleus of Cytochrome *P*-450 Caused in the Liver by Treatment with Allyl-Containing Drugs

## IS THE MODIFIED PORPHYRIN *N*-SUBSTITUTED?

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A spectral study was carried out of the green pigments produced by allyl-containing drugs and a comparison made with *N*-methylated octaethylporphyrin and 2,4-diformyldeuteroporphyrin. The green pigments resemble the former (and markedly differ from the latter) in the intensity of the bathochromic shifts, titration curves with trifluoroacetic acid and rate of incorporation of metal ions *in vitro*.

Drugs containing allyl substituents cause loss of hepatic cytochrome *P*-450 and conversion of its haem moiety into modified porphyrins, henceforth called green pigments (De Matteis, 1970, 1971; Levin *et al.*, 1973). These abnormal pigments, originally discovered by Schwartz & Ikeda (1955), are characterized by an aetio-type spectrum with a bathochromic shift of all absorption maxima, compared with the absorption maxima of the native porphyrin of cytochrome *P*-450, protoporphyrin (Unseld & De Matteis, 1976; McDonagh *et al.*, 1976). [In the aetio-type spectrum the four bands of the visible spectrum of a porphyrin free base decrease in intensity going from shorter to longer wavelengths (i.e. IV > III > II > I). A bathochromic shift is an increase in the wavelength of an absorption maximum.] It has been suggested that reactive derivatives produced during the metabolism of these drugs by cytochrome *P*-450 (for example epoxides) may attack its haem moiety, giving rise to the green pigments (De Matteis, 1970, 1973; Levin *et al.*, 1973). This view is supported by the finding (Ortiz de Montellano *et al.*, 1978*a*) that, after treatment with one of these drugs, 2-allyl-2-isopropylacetamide, radioactivity from the drug is recovered irreversibly bound to the green pigments. The nature of the reactive derivative responsible, the site within the porphyrin nucleus where the attack takes place and the chemical reaction involved are not known, but a better understanding of the structure of the green pigments should help to clarify these points.

In the present paper we describe spectral studies of the green pigments and a comparison with other modified porphyrins of known chemical structure that also exhibit a bathochromic shift in their absorption maxima as compared with the absorption maxima of their respective parent porphyrins. We now find that the green pigments incorporate metal ions *in vitro*, chemically, much more readily than does their parent porphyrin, protoporphyrin. They are also much more basic than protoporphyrin, and on titration with a strong acid give rise readily to a porphyrin monocation, which then requires relatively large amounts of acid for conversion into the porphyrin dication. In all these respects and also in the intensity of their bathochromic shifts the green pigments closely resemble authentic *N*-alkylated porphyrins and markedly differ from porphyrins bearing electron-withdrawing substituents at the $\beta$-positions of the pyrrole rings.

### Materials and Methods

Male rats (160–180g) of the Porton strain were given phenobarbitone sodium (at a concentration of 1mg/ml) in their drinking water for a week and were starved overnight before further treatment. Green pigments were produced *in vivo* by administering a single subcutaneous dose of 2-allyl-2-isopropylacetamide (400mg/kg body wt.) or secobarbitone [5-allyl-5-(1-methylbutyl)barbiturate] (100mg of the sodium salt/kg) and killing the rats 1.5h later. The methylated pigments were obtained from the liver and purified by two successive t.l.c. runs, as described by Unseld & De Matteis (1976). The major band was eluted from the silica plate with chloroform/methanol (1:1, v/v), evaporated to dryness and stored in the dark under $N_2$ at −20°C until required.

Protoporphyrin IX dimethyl ester was purchased from Sigma (London) Chemical Co. (Poole, Dorset BH17 7NH, U.K.). 2,4-Diformyldeuteroporphyrin methyl ester was a gift from Professor G. H. Elder (Welsh National School of Medicine, Cardiff,

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Wales, U.K.). Octaethylporphyrin and its mono-*N*-methyl derivative were a gift from Professor A. H. Jackson (University College, Cardiff, Wales, U.K.).

For spectral studies the green pigments and the other porphyrins were dissolved in freshly washed chloroform that had been dried over anhydrous $Na_2SO_4$ and their concentrations were adjusted to give an absorption of 1–1.5 at their Soret maximum. Their absorption spectra were obtained with either the Perkin–Elmer model 356 or the Unicam SP. 1800 recording spectrophotometer. Titration with acid was carried out in Teflon-stoppered spectrophotometric cells by adding increasing amounts of a suitable dilution of trifluoroacetic acid in chloroform and scanning the spectrum immediately after each addition. Chemical incorporation of metal ions was studied at room temperature (16°C) by adding to the chloroform solutions of the various porphyrins a methanolic solution of either cobaltous acetate or zinc acetate. The appearance of the two-banded spectrum of the metalloporphyrin was followed in time by repeated scanning. For a more precise measurement of the rate of incorporation the disappearance of band IV was followed in a Perkin–

Table 1. *Absorption spectra of the green pigments obtained after treatment with either 2-allyl-2-isopropylacetamide or secobarbitone [5-allyl-5-(1-methylbutyl)barbituric acid]: comparison with protoporphyrin and with other model porphyrins*

All porphyrins listed here exhibit an aetio-type spectrum; the absorption maxima of the Soret and of the visible bands are given below together with the bathochromic shifts (from the absorption maxima of the parent porphyrins) observed in the green pigments and in three other classes of modified porphyrins. All spectra were determined in chloroform, except those of aetioporphyrin and of its *N*-methyl derivative, where ether was the solvent. The spectra of other porphyrins substituted at one *meso*-carbon position or bearing electron-withdrawing side chains are given by Smith (1975).

| | Absorption maxima or bathochromic shifts (nm) | | | | | | |
|---|---|---|---|---|---|---|---|
| Porphyrin | Soret | IV | III | II | Ia | I | Reference |
| (*A*) Protoporphyrin IX methyl ester | 407 | 505 | 541 | 575 | 603 | 630 | Falk (1964) |
| (*B*) Green pigment methyl ester (2-allyl-2-isopropylacetamide) | 417 | 512 | 544 | 593 | 623 | 651 | Present work |
| Bathochromic shifts (*B*−*A*) ... | 10 | 7 | 3 | 18 | 20 | 21 | |
| (*C*) Green pigment methyl ester (secobarbitone) | 416 | 512 | 543 | 594.5 | | 652 | Present work |
| Bathochromic shifts (*C*−*A*) ... | 9 | 7 | 2 | 19.5 | | 22 | |
| (*D*) Aetioporphyrin | | 495 | 527.5 | 567.5 | | 624.5 | Ellingson & Corwin (1946)* |
| (*E*) *N*-Methylated aetioporphyrin | | 503 | 533 | 587 | | 645 | Ellingson & Corwin (1946)* |
| Bathochromic shifts (*E*−*D*) ... | | 8 | 5.5 | 19.5 | | 20.5 | |
| (*F*) Octaethylporphyrin | 400 | 498.5 | 534 | 566.5 | 594 | 620 | Present work† |
| (*G*) *N*-Methylated octaethylporphyrin | 412 | 508 | 539 | 586 | 616 | 642 | Present work‡ |
| Bathochromic shifts (*G*−*F*) ... | 12 | 9.5 | 5 | 19.5 | 22 | 22 | |
| (*H*) α-Formylated octaethylporphyrin (*meso*-substituted) | 408 | 507 | 541 | 577 | | 625 | Inhoffen *et al.* (1966) |
| Bathochromic shifts (*H*−*F*) ... | 8 | 8.5 | 7 | 10.5 | | 5 | |
| (*I*) 2,4-Diformyldeuteroporphyrin | 435 | 526 | 562.5 | 595 | | 651 | Lemberg & Parker (1952) |
| Bathochromic shifts (*I*−*A*) ... | 28 | 21 | 21.5 | 20 | | 21 | |

* These authors give the range of wavelengths comprising each band; the absorption maximum is assumed to be exactly in the middle of each band.

† Similar absorption maxima have been reported by Bonnett & Stephenson (1965).

‡ Similar absorption maxima have been reported by Grigg *et al.* (1972).

Elmer dual-wavelength spectrophotometer: the difference in absorption between the wavelength of band IV maximum and a reference wavelength 20nm further towards the red (and corresponding to the trough between bands IV and III) was then recorded. Final concentrations of cobaltous acetate and methanol were either 80$\mu$M and 0.1M respectively for green pigments and *N*-methylated octaethylporphyrin or 3.6mM and 2.25M for the other porphyrins studied, since these required much higher concentration of metal ions to obtain a measurable rate of incorporation.

## Results

### *Absorption spectra of green pigments and other modified porphyrins*

Three different types of chemical modification of the porphyrin nucleus have been described that lead to a bathochromic shift in the absorption maxima (as compared with those of the parent porphyrins) while retaining the aetio-type spectrum. These are (1) substitution at the pyrrole nitrogen atom, (2) substitution at the bridge *meso*-carbon atom and (3) presence of electron-withdrawing substituents at the $\beta$-positions of the pyrrole rings. Table 1 shows the absorption maxima of several porphyrins with an aetio-type spectrum and also the bathochromic shifts from the absorption maxima of the parent porphyrins seen in the green pigments and in modified porphyrins of known structure belonging to the three classes mentioned above. It will be noticed that the bathochromic shifts observed with the green pigments closely resemble those exhibited by *N*-alkylated porphyrins, not only in order of intensity (I > II > Soret > IV > III), but also in absolute values: they differ from those of the other two classes of modified porphyrins, where all absorption maxima tend to be shifted towards longer wavelengths to a similar extent.

### *Titration of green pigments and other porphyrins with acid*

On addition of a strong acid the green pigments were found to give rise very readily to the porphyrin monocation, and this could then be converted into the porphyrin dication by further addition of acid. Similar findings have been reported for *N*-alkylated porphyrins (Neuberger & Scott, 1952; Jackson & Dearden, 1973).

Since band IV decreased on conversion of the free base into the monocation and band III when the monocation was converted into the dication, the progressive protonation of the pyrrole nitrogen atoms was followed by measuring the absorption at the wavelength maxima of these two bands. The

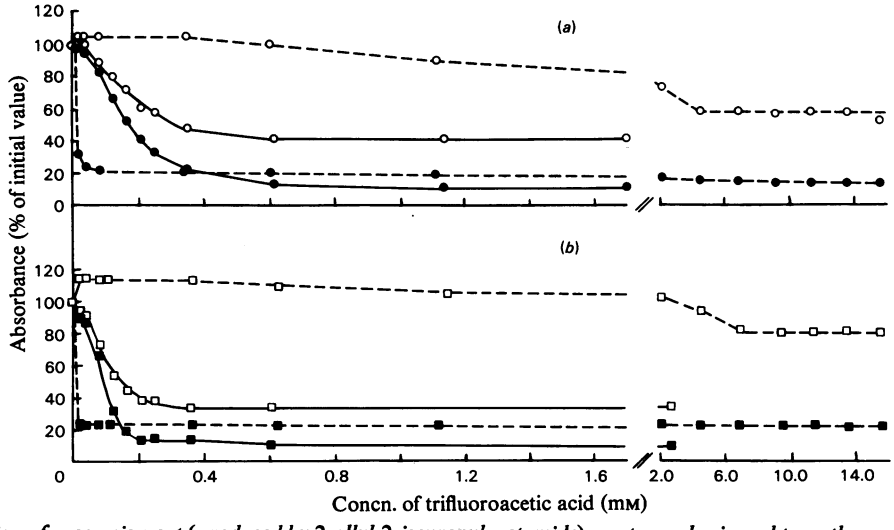


Fig. 1. *Titration of green pigment (produced by 2-allyl-2-isopropylacetamide), protoporphyrin and two other model porphyrins with trifluoroacetic acid*

The absorption at the wavelengths of band IV and band III maxima was followed for each porphyrin at progressively increasing acid concentrations and is expressed as a percentage of the initial value of the free base. (*a*) Protoporphyrin dimethyl ester: ●—●, band IV; ○—○, band III; green pigment methyl ester: ●--●, band IV; ○--○, band III. (*b*) Octaethylporphyrin: ■—■, band IV; □—□, band III; *N*-methylated octaethylporphyrin: ■--■, band IV; □--□, band III. Green pigments produced by secobarbitone [5-allyl-5-(1-methylbutyl)barbituric acid] behaved on titration similarly to those obtained after 2-allyl-2-isopropylacetamide.

titration curves obtained for green pigments and for protoporphyrin (Fig. 1*a*) were compared with those obtained with *N*-methylated octaethylporphyrin and octaethylporphyrin (Fig. 1*b*). With both parent porphyrins bands IV and III decreased together, as the dication was directly formed and no intermediary monocation could be demonstrated: this is probably because the $pK_a$ values of the conjugated acids (estimated to be 7.2 and 4.2 for the unsubstituted porphyrins; Neuberger & Scott, 1952) are relatively close to one another. In clear contrast, with both the *N*-methylated porphyrin and the green pigment band IV decreased rapidly at very low concentration of acid, whereas band III first showed a slight increase and decreased slowly only when relatively large amounts of acid were added. We can therefore conclude that, like *N*-methylated porphyrins (Neuberger & Scott, 1952; Jackson & Dearden, 1973), the green pigments are more basic than their parent porphyrin, and that the $pK_a$ values of their conjugated acids are sufficiently apart to allow the existence of the monocation even after addition of a strong acid. From the similarity of the titration curves one can in fact suggest that the $pK_a$ values of the green pigments must be fairly close to those of *N*-methylated porphyrins.

The same titration experiment was performed with 2,4-diformyldeuteroporphyrin, but this porphyrin was found to be less basic than protoporphyrin, requiring concentrations of trifluoroacetic acid between 4 and 5mM for approx. 50% decrease in band IV absorption. This is to be expected in view of the base-weakening effect of electron-withdrawing side chains (Falk, 1964).

*Incorporation of metal ions into green pigments and other porphyrins*

McEwen (1946) and Jackson & Dearden (1973) have reported that *N*-methylated porphyrins can form complexes with $Zn^{2+}$ and other metal ions. We now find that the rate of formation of $Zn^{2+}$- and $Co^{2+}$-metalloporphyrin *in vitro* is much more rapid with *N*-methylated octaethylporphyrin than with its parent unsubstituted porphyrin. Also in this respect the green pigments resemble the *N*-alkylated porphyrin and differ from 2,4-diformyldeuteroporphyrin. Incorporation of $Co^{2+}$ was measured by dual-wavelength spectrophotometry, and the times required for complete disappearance of band IV were calculated from the initial rate and are given below (average ±S.D. of three determinations): *N*-methylated octaethylporphyrin, 0.6±0.02min; green pigment (2-allyl-2-isopropylacetamide), 10.2±2.4min; and, with a 45-fold greater concentration of $Co^{2+}$, octaethylporphyrin, 83.6±15.3min; protoporphyrin, 370±64min; 2,4-diformyldeuteroporphyrin, 1020±179min. In general the rate of incorporation of metal ion was found to increase with the basicity of the various porphyrins, in line with a displacement-type mechanism for the incorporation reaction, as discussed by Falk (1964). The increased ability of the green pigments to incorporate metal ions probably accounts for the appearance during their purification of a derivative with a two-banded visible spectrum (Unseld & De Matteis, 1976), later identified as their $Zn^{2+}$ chelate (Ortiz de Montellano *et al.*, 1978*a*).

## Discussion

Several authors have considered the possibility that the green pigments may arise from haem by modification of the vinyl side chains into substituents with greater electron-withdrawing character (De Matteis *et al.*, 1977; Ortiz de Montellano *et al.*, 1978*b*). However, any modification of the side chains responsible for such a pronounced bathochromic shift will probably result in decreased basicity of the pyrrole nitrogen atoms and in decreased incorporation of metal ions [see the results obtained here with 2,4-diformyldeuteroporphyrin, and Falk (1964)], whereas we find that the green pigments are more basic and incorporate metal ions at a greater rate than does protoporphyrin. In addition, if only one vinyl group were to be modified, as proposed by Ortiz de Montellano *et al.* (1978*b*), then a marked bathochromic shift would probably be accompanied by a loss of the aetio-type spectrum, as is the case with their model compound, 4-(3-oxopropenyl)-2-vinyldeuteroporphyrin, which in fact exhibits an oxorhodo-type spectrum (Nichol, 1970). This is because of the so-called rhodofying effect, which has been discussed by Falk (1964): strong electron-withdrawing substituents at certain β-positions of the porphyrin ring will increase the intensity of band III, changing the visible spectrum of a porphyrin free base from the aetio-type (IV > III > II > I) to either the rhodo-type (III > IV > II > I) or to the oxorhodo-type (III > II > IV > I).

The present work has shown a number of similarities between green pigments and *N*-alkylated porphyrins. Taking into account the report that radioactivity from 2-allyl-2-isopropyl[2-$^{14}$C]acetamide can be recovered bound to the green pigments in a 1:1 stoichiometry (Ortiz de Montellano *et al.*, 1978*a*), the most likely interpretation for our findings is that a reactive derivative of the drug acts as an electrophilic reagent and alkylates one of the pyrrole nitrogen atoms of cytochrome *P*-450. Substitution at one of the *meso*-carbon positions is less probable, but cannot yet be completely ruled out. On exposure to acids the modified haem will lose iron and give rise to the corresponding free porphyrins much more readily than protohaem (De Matteis & Unseld, 1976). This is also compatible with *N*-alkylation, as the presence

of a bulky substituent in the centre of the molecule may weaken the co-ordination of the metal ion. A contributing factor may be the increased basicity of the modified porphyrin, which will facilitate the protonation of the pyrrole nitrogen atom necessary for elimination of the metal ion.

In a theoretical paper Schoental (1976) has suggested that reactive derivatives of several hepatotoxins, especially carcinogens, may alkylate the pyrrole nitrogen atom of free porphyrins, and these would then accumulate, simulating porphyria. With allyl-containing drugs, however, haem (not free porphyrins) is the target of drug action and most of the accumulating porphyrins are normal metabolites formed in excess.

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## References

Bonnett, R. & Stephenson, G. H. (1965) *J. Org. Chem.* **30**, 2791–2798

De Matteis, F. (1970) *FEBS Lett.* **6**, 343–345

De Matteis, F. (1971) *Biochem. J.* **124**, 767–777

De Matteis, F. (1973) *Drug Metab. Dispos.* **1**, 267–272

De Matteis, F. & Unseld, A. (1976) *Biochem. Soc. Trans.* **4**, 205–209

De Matteis, F., Gibbs, A. H. & Unseld, A. (1977) *Biochem. J.* **168**, 417–422

Ellingson, R. C. & Corwin, A. H. (1946) *J. Am. Chem. Soc.* **68**, 1112–1115

Falk, J. E. (1964) *BBA Libr.* **2**, 27, 38, 78, 232

Grigg, R., Shelton, G., Sweeney, A. & Johnson, A. W. (1972) *J. Chem. Soc. Perkin Trans. I* 1789–1799

Inhoffen, H. H., Fuhrhop, J., Voigt, H. & Brockmann, H. (1966) *Justus Liebigs Ann. Chem.* **695**, 133–143

Jackson, A. H. & Dearden, G. R. (1973) *Ann. N.Y. Acad. Sci.* **206**, 151–174

Lemberg, R. & Parker, J. (1952) *Aust. J. Exp. Biol. Med. Sci.* **30**, 163–175

Levin, W., Jacobson, M., Sernatinger, E. & Kuntzman, R. (1973) *Drug Metab. Dispos.* **1**, 275–284

McDonagh, A. F., Pospisil, R. & Meyer, U. A. (1976) *Biochem. Soc. Trans.* **4**, 297–298

McEwen, W. K. (1946) *J. Am. Chem. Soc.* **68**, 711–713

Neuberger, A. & Scott, J. J. (1952) *Proc. R. Soc. London Ser. A* **213**, 307–326

Nichol, A. W. (1970) *J. Chem. Soc. C* 903–910

Ortiz de Montellano, P. R., Mico, B. A. & Yost, G. S. (1978*a*) *Biochem. Biophys. Res. Commun.* **83**, 132–137

Ortiz de Montellano, P. R., Mico, B. A., Yost, G. S. & Correia, M. A. (1978*b*) in *Enzyme-Activated Irreversible Inhibitors* (Seiler, M., Jung, M. J. & Koch-Weser, J., eds.), pp. 337–352, Elsevier/North-Holland, Amsterdam

Schoental, R. (1976) *FEBS Lett.* **61**, 111–114

Schwartz, S. & Ikeda, K. (1955) *Porphyrin Biosynth. Metab. (Ciba Found. Symp.)* 209–226

Smith, K. M. (1975) in *Porphyrins and Metalloporphyrins* (Smith, K. M., ed.), pp. 873, 878, Elsevier, Amsterdam

Unseld, A. & De Matteis, F. (1976) *Proc. Int. Porphyrin Meeting, Freiburg, 1975*, pp. 71–75, S. Karger, Basel