colonies to establish themselves, 7, 10 and 21 days respectively, the bacteria were centrifuged and the culture medium was decanted. Part of each sediment of bacterial cells (0.5–1.0 ml.) was ground with fine sand and stirred with 2–3 ml. of 0.1N-HCl. After 1 hr. the supernatant liquid and the decanted culture medium were examined by paper chromatography. No canavanine was detected in either the cell extracts or the culture media.

SUMMARY

1. Canavanine has been detected in the seeds of 11 species of leguminous plants.
2. The sensitivity of the method for the colorimetric determination of canavanine has been increased by using a spectrophotometer at 522 m μ .
3. Canavanine stored in seeds of *Medicago sativa* is metabolized by the seedlings during germination and growth.

4. The biosynthesis of canavanine by *Medicago sativa* and *Trifolium pratense* is not dependent upon the presence of nitrogen-fixing bacteria in the root nodules of the plants.

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Spectral-Absorption Coefficients of some Porphyrins in the Soret-Band Region

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All porphyrins exhibit intense fluorescence when illuminated by light of appropriate wavelength and this property has been much used in the past for their quantitative determination. The intensity of the fluorescence varies with pH (Fink & Hoerburger, 1933, 1935; Rimington, 1943; Jope & O'Brien, 1945), however, and is also much affected by impurities and even by the presence of inorganic ions. Notwithstanding the use of a reference standard for each determination, errors may be large and unpredictable.

With the advent of modern photoelectric spectrophotometers, it has become possible to exploit for quantitative measurement another characteristic physical property of porphyrins, namely their intense spectral absorption at about 400 m μ . This band, known as the Soret band, is intense and narrow and is most well developed in acid solution; its position and magnitude differ for each porphyrin. Both parameters are influenced by pH (cf. Jope & O'Brien, 1945) but conditions for measurement are easily standardized and non-absorbing impurities are without effect. For measurement in the presence of substances which absorb light, a correction formula has been devised

by Rimington & Sveinsson (1950). This is generally applicable so long as the absorption of the impurities remains approximately linear over a narrow spectral range on either side of the Soret maximum of the porphyrin in question.

The porphyrins in acid solutions obey Beer's law up to an extinction (optical density) of about 1.0, and spectrophotometry is thus the method of choice for their determination. It is necessary, however, to know the molecular extinction coefficients (ϵ) of the pure materials under the conditions which are to be applied for their measurement. Unfortunately the preparation of porphyrins or their methyl esters in the pure state is difficult; melting points are unsatisfactory criteria of purity and it is thus not surprising that widely differing values for ϵ or $E_{1\%}^{1\text{cm}}$ are to be found in the literature. The situation has been reviewed briefly by With (1955). The purpose of the present paper is to present the values found by examination of a number of carefully purified porphyrins and so to provide data for accurate spectrophotometry. Measurements have been made upon porphyrin and aetioporphylin I for comparison with the naturally occurring porphyrins.

MATERIALS AND METHODS

Measurements of extinction were made in a Unicam model SP. 500 photoelectric spectrophotometer. Purification of the porphyrin methyl esters was effected by repeated crystallization. A weighed quantity was then dissolved in freshly washed and dried chloroform or other solvent as specified in Table 1 and the Soret band was measured. For examination of the free porphyrins, another weighed quantity was left in contact with 7N-HCl for 48 hr., after which the solution was diluted to appropriate concentration of acid and porphyrin for spectrophotometric measurement. With aetioporphyrin I a measured quantity of a concentrated solution in dioxan was diluted appropriately with HCl so that the final acid concentration was 1.37N. N.P.L. grade A glassware was used throughout.

Porphin. Synthetic material (Krol, 1959; Rimington, Mason & Kennard, 1958) was used.

Aetioporphyrin I. This was synthetic material from Hans Fischer's collection.

Uroporphyrin III. This was synthetic octamethyl ester supplied by Dr S. F. MacDonald. Natural material was prepared from turacin (Nicholas & Rimington, 1951).

Coproporphyrin III. This was prepared from residues obtained in production of toxoid from cultures of *Corynebacterium diphtheriae* (Gray & Holt, 1948).

Deuteroporphyrin IX. This was prepared synthetically from haemin (Fischer & Orth, 1937).

Protoporphyrin IX. This was prepared by the method of Grinstein (1947) from haemin.

Phylloerythrin. This was isolated from sheep's faeces (Rimington & Quin, 1934).

RESULTS

Calculated values for ϵ and $E_{1\text{cm}}^{1\%}$ obtained in this investigation are presented in Tables 1 and 2, together with values from the literature.

DISCUSSION

There is reason to believe that the molecular extinction coefficients of porphyrin isomers do not differ significantly from one another. Thus uroporphyrin esters I and III afford substantially the same figure (Rimington & Sveinsson, 1950), and the bands of coproporphyrin esters I and II in the visible region are practically identical (Stern & Wenderlein, 1934), as are the bands of coproporphyrins I, II, III and IV in 3N-hydrochloric acid (Stern & Wenderlein, 1934). Jope & O'Brien (1945) found the same molecular extinction coefficient for coproporphyrins I and III in 0.1N-hydrochloric acid.

Examination of Tables 1 and 2 reveals generally good agreement between the figures here reported and those to be found in the literature which are based upon direct measurement. The somewhat higher figure found for protoporphyrin in acid solution by Grinstein & Wintrobe (1948) is probably preferable to that given by the specimen used in this work. A real difficulty arises, however, when the values for coproporphyrin are considered. Jope & O'Brien (1945) in their study of the I and III isomers used photographic apparatus and reported ϵ values of 5.31×10^5 and 5.30×10^5 respectively for the Soret band in 0.1N-hydrochloric acid. This figure has never been attained by any subsequent investigator; in fact all careful measurements lie around $4.8\text{--}4.9 \times 10^5$. It seems probable therefore that Jope & O'Brien's figure is erroneous. It is important to have an agreed extinction value for use in calculating coproporphyrin concentrations and the adoption of $\epsilon \ 4.89 \times 10^5$ for the Soret band in 0.1N-hydrochloric acid as solvent is recommended as a result of this study. This figure is almost identical with that found by Zondag & Van Kampen (1956).

Absorption of energy manifested in the Soret band is attributable to a single transition affecting the resonating system of the porphyrin ring structure as a whole (Rabinowitch, 1944). Features such as the vinyl groups of protoporphyrin and the isocyclic ring of phylloerythrin would be expected therefore to influence considerably the intensity of this absorption band. The effect of alkyl or carboxyalkyl substituents in the β positions may be elucidated by consideration of the data in Tables 1 and 2. Table 1 records absorption of porphin, aetioporphyrin I and porphyrin esters in neutral solvents and Table 2 gives data for the free porphyrins in acid, arrangement being in order of decreasing ϵ values. The individuals are placed in the same order in Table 1.

Effect of alkyl substitution. Porphin, with all eight β positions unsubstituted, has the highest ϵ of all the porphyrins examined. Substitution with symmetrically arranged electron-repelling methyl and ethyl groups, as in aetioporphyrin I, leads to a profound depression of the molar extinction coefficient whereas substitution with carboxyalkyl groups has less effect. The ϵ of uroporphyrin octamethyl ester lies approximately midway between the values for porphin and aetioporphyrin I; the values for coproporphyrin tetramethyl ester and the dicarboxylic porphyrin esters are considerably lower and approach that for aetioporphyrin I.

Effect of acid medium and of carboxyl groups. All molar extinctions are enhanced when the free porphyrin is dissolved in acid. Thus ϵ of porphin rises from 2.64×10^5 to 3.48×10^5 (1.32 times) but the effect is even greater with the alkyl- and

carboxyalkyl-substituted porphyrins. In these ϵ increases from 2.4 to 2.7 times, except in the special cases of phylloerythrin (1.42 times) and protoporphyrin (1.54 times). The enhanced intensity of absorption in an acid medium is clearly due to the symmetry introduced by protonation of the pyrrolic nitrogen atoms.

Values of the constant for use in the correction formula

In 1950, Rimington & Sveinsson (1950) deduced an expression

$$P_{\max} = \frac{2 D_{\max} - (D_{430} + D_{380})}{k}$$

Table 1. *Extinction coefficients of porphyrin methyl esters, porphin and aetioporphyrin I*

Solvent: chloroform, unless otherwise indicated.

Material	Soret max. (m μ)	$10^{-3} E_{1\text{cm}}^{1\%}$	$10^{-5} \epsilon$	$10^{-5} \epsilon$ values from literature
Uroporphyrin III octamethyl ester (syn.)	405.6	2.30	2.17	—
Uroporphyrin I octamethyl ester (nat.)	406	—	—	2.14,* 1.95†
Coproporphyrin III tetramethyl ester	399.5	2.53	1.80	1.80‡
Aetioporphyrin I	399.5	3.35	1.60	—
Deuteroporphyrin dimethyl ester	399	3.25	1.75	—
Haematoporphyrin dimethyl ester	402	—	—	1.75§
Porphin¶	396.5	8.52	2.64	—
Phylloerythrin methyl ester	415	3.57	1.95	—
Protoporphyrin dimethyl ester	407.5	2.89	1.71	—

* S. Granick (personal communication) on uroporphyrin I octamethyl ester. One specimen supplied by Rimington, one by With.

† Rimington & Sveinsson (1950).

‡ Calculated from data of Brugsch & Kubowitz (1953) for coproporphyrin I tetramethyl ester in dioxan.

§ Granick, Bogorad & Jaffe (1953) for the diester in pyridine.

|| In dioxan.

¶ In benzene.

Table 2. *Extinction coefficients of porphyrins in acid solution*

Material	Concn. of HCl (N)	Soret max. (m μ)	$10^{-3} E_{1\text{cm}}^{1\%}$	$10^{-5} \epsilon$	$10^{-5} \epsilon$ values from literature
Uroporphyrin III (syn.)	0.5	405.5	6.36	5.28	—
Uroporphyrin I (nat.)	0.5	—	—	—	5.41*
Coproporphyrin III	0.1	399.5	7.47	4.89	4.78†
	0.1	—	—	—	4.85‡
	0.1	—	—	—	5.30§
Deuteroporphyrin	0.1	398	8.48	4.33	—
Haematoporphyrin	0.5	401	—	—	4.23
Porphin	1.37	391	11.23	3.48	—
Phylloerythrin	3.0	421	5.08	2.78	—
Protoporphyrin	1.37	408	4.67	2.62	2.75¶

* Rimington & Sveinsson (1950).

† Calculated from data of Brugsch & Kubowitz (1953) for coproporphyrin I.

‡ Zondag & Van Kampen (1956). A second determination gave 4.87×10^{-5} . (Personal communication from Dr Van Kampen).

§ Jope & O'Brien (1945).

|| Legrand (1955). Solvent 0.5N-H₂SO₄.

¶ Grinstein & Wintrobe (1948).

Table 3. *Recommended data for determination of porphyrins by measurement of the Soret band*

Data are included for correction for absorbing impurities according to Rimington & Sveinsson (1950).

Porphyrin	Concn. of HCl (N)	$10^{-5} \epsilon$	$10^{-3} E_{1\text{cm}}^{1\%}$	Extremes for measurement of <i>D</i> in formula (m μ)	<i>k</i>
Uroporphyrin	0.50	5.41	6.52	380, 430	1.844
Coproporphyrin	0.10	4.89	7.47	380, 430	1.835
Deuteroporphyrin	0.10	4.33	8.48	375, 425	1.868
Protoporphyrin	1.37	2.75	4.89	380, 430	1.668
Phylloerythrin	3.00	2.78	5.08	395, 445	1.753

which could be used to eliminate the effect of impurities in measurements made on porphyrins in acid solutions containing absorbing impurities. The expression is generally applicable provided that the increase or decrease of absorption due to the impurities remains approximately linear over the range 380–430 $m\mu$. D_{380} , D_{430} and $D_{\max}$ are the optical densities observed at the wavelengths 380, 430 $m\mu$ and the peak of the Soret band respectively; $P_{\max}$ is the Soret band absorption actually due to the porphyrin, and k is a factor empirically determined from measurements upon the pure porphyrin (Rimington & Sveinsson, 1950; Zondag & Van Kampen, 1956; With, 1955). This expression was originally worked out for uroporphyrin, the Soret maximum of which in 0.5N-hydrochloric acid lies at 405 $m\mu$ and the wavelengths 430 and 380 $m\mu$ were adopted as being equidistant from the maximum and near the foot of the band on either side of the peak. For absolute precision, the lateral values should always be equidistant from the peak, thus for example at 425 and 375 $m\mu$ for correction of coproporphyrin solutions (Soret maximum approx. 400 $m\mu$), but in practice the error introduced by using 430 and 380 $m\mu$ for all the commoner porphyrins, except deuteroporphyrin, is negligible and a standard procedure is much more convenient. In phylloerythrin, the Soret maximum is too far removed from 405 $m\mu$ and here also a new expression has to be applied (see Table 3). In all cases, the value of k must be empirically determined and there has been some discussion about the most acceptable values (see With, 1955). In Table 3 the recommended data are summarized for those porphyrins for which they are available.

SUMMARY

1. The spectral absorption in the Soret region of some common porphyrins has been examined.

2. Values are reported for ϵ at the Soret band, of the esters in a neutral solvent and of the porphyrins dissolved in acid of suitable concentration.

3. Recommended values are tabulated for use with the formula of Rimington & Sveinsson (1950), which permits correction for light-absorbing contaminants of porphyrins in impure solutions.

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