

THE *IN VIVO* LOCALIZATION OF PORPHYRINS

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SUMMARY.—A series of porphyrins with varying side chains was synthesized and tested in the rat for hard and soft tissue binding and photosensitization. It was found that for combination with either type of tissue there was a requirement for groups capable of ionic or hydrogen bonding at more than one edge of the tetrapyrrole nucleus. Thus binding to keratin, collagen and growing bone occurred with purified haematoporphyrin, commercial “haematoporphyrin” and haematoporphyrin “derivative”. Little or no binding with these tissues occurred with deuteroporphyrin, deuteroporphyrin dimethyl ester disulphonic acid, haematoporphyrin dimethylether and unexpectedly, porphyrin *c* and *N,N'*-diacetyl porphyrin *c*. Incorporation of iron into the last two named is thought to account for their apparent lack of binding. In the case of growing bone, the same pattern was apparent with the exception that porphyrin *c* and *N,N'*-diacetyl porphyrin *c* were very strong labels. Combination to bone was with the organic rather than with the inorganic component. All soluble porphyrins tested induced a potential photosensitivity on intraperitoneal injection. Using intravenous colloidal carbon as an indicator of an increase in vascular permeability, the photoresponse following excitation with sunlight, mercury vapour lamp or carbon arc was found to be biphasic, with the delayed response altered by dosage and binding ability of porphyrins, and the amount of light absorbed.

An abnormally high concentration of porphyrin in tissues *in vivo* is usually due to a lesion in the pathway of biosynthesis of protoporphyrin and may be accompanied by some degree of photosensitivity (Neuberger, 1968; Magnus, 1968). Somewhat similar effects are observed following intraperitoneal and intravenous injection of haematoporphyrin (Vannotti, 1954). The red fluorescence of porphyrins in UV light enables their detection in very small amounts (*ca.* 1 in 10⁸), and this technique has been used recently in an attempt to follow the uptake of injected haematoporphyrin by hard tissue in bone growth studies (Prescott, Mitchell and Fahmy, 1968). Also, the fluorescence of administered porphyrin, selectively taken up by malignant tissues, has been used for the localization of tumours (Lipson, Baldes and Olsen, 1961; Gregorie, Horger, Ward, Green, Richards, Robertson and Stevenson, 1968). Little detailed information is available, however, on the types of tissues to which various porphyrins may be bonded or the requirements for binding. For example, the binding of porphyrin to bone and teeth has generally been considered to be by linkage to the calcium of these structures (Vannotti, 1954), and the possibility of linkage to the organic component of the matrix does not appear to have been investigated.

The present study was designed to investigate the combination of various

porphyrins with rat tissues *in vivo*. For this purpose a number of porphyrins with varying peripheral groups and solubilities were synthesized. By suitable masking or rearrangement of particular groups, their importance in the attachment to tissues was investigated. The correlation between tissue combination and the photosensitization which followed the introduction of the porphyrins was also investigated. A preliminary report of this work has appeared elsewhere (Barker, Henderson and Storey, 1970).

MATERIALS AND METHODS

Porphyrins

Protohaemin was used as the starting material for the preparation of the various porphyrins tested (Fig. 1). It was prepared from fresh ox-blood by the glacial acetic acid method (Fischer, 1955).

Protoporphyrin.—Protoporphyrin was prepared from protohaemin by the de-ironing procedure of Morell and Stewart (1956).

Esterification.—Porphyrin dimethyl esters were usually prepared by treatment of the porphyrin free acids in methanol containing 5 per cent (v/v) sulphuric acid for 48 hr at 20° in the dark. For the preparation of small quantities of the esters, diazomethane was used (Falk, 1964; Vogel, 1962).

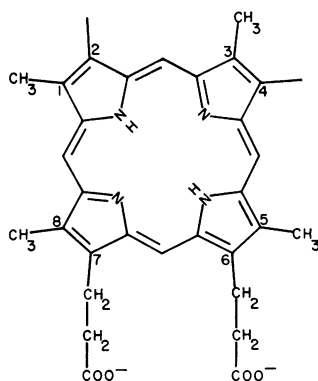


Fig. 1.—Porphyrin structure (Type III, free acid form). Substituent groups of the porphyrins tested

Porphyrin	Substituent on positions 2 and 4	Porphyrin	Substituent on positions 2 and 4
Haematoporphyrin	-CH-CH ₃ OH	Protoporphyrin	-CH=CH ₂ +NH ₃
Haemato-dimethylether	-CH-CH ₃ O-CH ₃	Porphyrin c	-CH-S-CH ₂ -CH CH ₃ COO ⁻
Deuteroporphyrin	-H	N,N'-diacetyl-porphyrin c	HN-C-CH ₃ O -CH-S-CH ₂ -CH CH ₃ COO ⁻
Deuteroporphyrin-dimethyl ester disulphonic acid (DDME)	-SO ₃ ⁻		

In the dimethyl esters, the carboxyl groups on positions 6 and 7 are esterified.

Thin-layer chromatography.—In order to check the purity of the porphyrins they were chromatographed as the dimethyl esters on layers of activated Silica gel G (0.25 mm. thick) using benzene : methanol, 100 : 7.5 (v/v) as the developing solvent (Henderson and Morton, 1967).

Hydrolysis of porphyrin esters.—Hydrolysis, except in the case of haematoporphyrin dimethyl ester (see below), was carried out in 25 per cent (w/v) hydrochloric acid for 48 hr at 20° in the dark.

Deuteroporphyrin.—Deuterohaemin was prepared from protohaemin by the resorcinol method of Schumm (1928), iron was then removed by the formic acid method of Ramsey (1953) and the dimethyl ester of the crude deuteroporphyrin prepared. Purification of the ester was carried out by chromatography on an alumina column using chloroform : methanol, 100 : 1 (v/v) as the eluting solvent.

Deuteroporphyrin dimethyl ester disulphonic acid (DDME).—This was prepared by sulphonation of deuteroporphyrin dimethyl ester with N-pyridinium sulphonic acid essentially according to the method of Walter (1952); the preparations showed pH dependent spectra closely similar to those reported by Neuberger and Scott (1952).

Haematoporphyrin.—This was prepared by treatment of protohaemin with hydrogen bromide in glacial acetic acid followed by hydrolysis of the hydrogen bromide adduct which is formed (Falk, 1964; Fischer and Orth, 1937). The crude product was esterified and separated from impurities on an alumina column using chloroform : methanol, 100 : 1 (v/v) as the eluting solvent. Haematoporphyrin free acid was prepared from its dimethyl ester by hydrolysis in aqueous-alcoholic potassium hydroxide as it has been found (Henderson, Barker and Pardee, unpublished) that acid hydrolysis introduces a number of modified porphyrins. A commercial sample of haematoporphyrin dihydrochloride (Nutritional Biochemicals Corporation, Batch Nos. 2500, 4481) was also used. This has been shown to contain approximately 30 per cent impurities (Henderson, Barker and Pardee, unpublished) and is referred to as commercial "haematoporphyrin".

Haematoporphyrin "derivative".—This derivative was prepared from commercial "haematoporphyrin" by treatment with glacial acetic acid : conc. sulphuric acid, 19 : 1 according to the method of Lipson *et al.*, (1961).

Haematoporphyrin dimethylether.—The hydrogen bromide adduct of protoporphyrin was formed from protohaemin as above, this was then taken to dryness *in vacuo* after which the residue was subjected to methanolysis by the method of Fischer and Orth (1937). The dimethyl ether was then recovered by evaporation of the excess methanol *in vacuo*.

Porphyrin c.—This was prepared from the hydrogen bromide adduct of protoporphyrin by reaction with L-cysteine hydrochloride according to the method of Neilands and Tuppy (1960). The crude product was then purified by resin-column treatment (Morton and Henderson, unpublished).

N,N'-diacetyl porphyrin c.—This porphyrin was prepared by acetylation of resin-column purified porphyrin *c* in 70 per cent (v/v) acetic acid with acetic anhydride (Morton and Henderson, unpublished).

Spectrophotometry.—Where required, spectra were determined using a Cary model 14 automatic recording spectrophotometer.

Porphyrin introduction and detection

Porphyrins were dissolved in Krebs-Ringer-phosphate buffer pH 7.4, or in 0.1 N sodium hydroxide then brought to pH 7.4 with 0.1 N hydrochloric acid. One ml. amounts of these solutions were injected i.p. into 100–140 g. male albino rats at a dose of 50 mg./kg. body weight unless otherwise stated. For i.d. localization, 0.5 mg. in 0.1 ml. was used. Experiments were repeated until consistent results were obtained; at least 2 rats were used to test each variable.

Porphyrin fluorescence of the various tissues was examined by means of an UV light (HBO 200 mercury vapour light source with two 2 mm. UG1 entry filters).

Since porphyrin fluorescence is lost during paraffin embedding procedures, soft tissues were sectioned at 5–7 μ on a freezing microtome and sections mounted unstained in gelatine and viewed under UV light. Confirmation of staining and fluorescence in skeletal tissue was obtained by similarly examining hard ground undecalcified sections.

Photosensitivity

The photosensitivity reaction of the animals was tested by exposing the ears either to sunlight or to one of two artificial light sources. For most experiments a high energy carbon arc from a Leitz microprojector (Model XI C) was used. This incorporated a heat filter which limited the temperature rise of the irradiated tissue to less than 5° in the 10 min. exposure time. UV light was excluded by a plain glass filter, which allowed full utilization of energy in the 400–700 nm. range. Alternatively an HBO 200 mercury vapour lamp light source was used for exposure periods of 2 or 5 min. with entry filters excluding wavelengths below 380 nm. (Leitz U.V.R.) or 420 nm. (Leitz CS 3-73) and greater than 500 nm. (BG12) or 700 nm. (BG38).

In the photosensitivity reaction, erythema was noted as being present or absent, but no attempt was made to measure the degree of redness. Increased vascular permeability was shown by the techniques of blueing following i.v. injection of 1 per cent trypan blue, or of localization in vessel walls of carbon particles following their i.v. introduction in a colloidal solution (Pelikan ink) at a dose of 0.2 ml./100 g. (Cotran and Majno, 1964). The degree of blueing was not measured, but the carbon labelling was assessed by killing the animals 1 hr after injection and clearing the tissues in cedarwood oil. Location in either capillaries or venules was noted and depending on the number of vessels involved and the amount of labelling, it was graded 0, ±, +, ++, +++, +++++. Subsequently the labelling was confirmed by histological examination and any other tissue changes noted.

RESULTS

Tissue localization following i.p. injection

Porphyrins soluble at pH 7.4

Haematoporphyrin was absorbed through the peritoneum and fluorescence was present in serum for up to 3 hr and in the urine for up to 24 hr. There was some fluorescence of faeces at this stage, but none subsequently. Examination at 48 hr showed weak fluorescence of the skin around the nose, and in the accral parts, but little trace was found in the contents and lining of the peritoneum. There was fluorescence of that part of the bony skeleton which had formed during the previous 2 days. Decalcification with 50 per cent formic acid/20 per cent sodium citrate removed the fluorescence and the solution became reddish-brown and fluoresced strongly. However, decalcification in 10 per cent EDTA for 7 days failed to remove this fluorescence, and the solution remained colourless. Commercial "haematoporphyrin" produced different results on i.p. introduction. The serum fluoresced 5 hr subsequent to injection, and excretion in urine and faeces was still evident at 48 hr. At this stage there was a strong red fluorescence of the ears, nose, footpads and accral parts; on dissection, the peritoneal lining and contents were observed to fluoresce red, particularly the fibrous tissue of the sternal cartilage. The forming bone fluoresced more strongly than it did after the use of purified haematoporphyrin. Haematoporphyrin "derivative" produced a similar fluorescence pattern to commercial "haematoporphyrin" and although quantitation of fluorescence was difficult, the haematoporphyrin "derivative" appeared to produce the most fluorescence. In each case, detailed study showed that in the first 24 hr most bone margins fluoresced red, but by 4 days, a red fluorescent line was present within bone underlying areas of subsequent growth. At 14 days there was still fluorescence of that part of the bony skeleton which was forming at the time of injection.

Haematoporphyrin dimethylether.—At 2 and 4 days after introduction there was no evidence of insoluble granules in peritoneal fascia and it appeared that absorption had been complete. However, there was no fluorescence of skin, fibrous

connective tissue, cartilage or bone, but there was slight fluorescence of perinephric and mesenteric fat.

Deuteroporphyrin.—Absorption was complete at 2 days but only very weak fluorescence was apparent in both hard and soft tissues at 2 and 4 days, with slight fluorescence in the adipose tissue of the peritoneum.

Deuteroporphyrin dimethyl ester disulphonic acid.—This was eliminated rapidly from the animal and no trace could be found after 30 min. by fluorescence in either the serum or any hard or soft tissue of the animal.

Porphyrin c and N,N'-diacetyl porphyrin c.—The results with these porphyrins differed in some aspects from the others tested. Excretion in urine occurred in the first 24 hr, with most occurring in the initial 4 hr period. The fluorescence of urine was much stronger with porphyrin *c* than with the diacetyl derivative. Excretion of both porphyrins from the snout and in the tears was much more noticeable even than with commercial "haematoporphyrin", and was still evident at 24 hr. Internal examination at this stage showed that like skin, there was still slight pink fluorescence of the gut contents, but there was no fluorescence of cartilage in the animals which had had *N,N'*diacetyl porphyrin *c*, and only slight fluorescence in the porphyrin *c* injected animals. However, there was strong evidence of uptake into growing bone, the fluorescence being very intense with porphyrin *c* and slightly less with the diacetyl derivative. At 4 days, with each porphyrin, there was no fluorescence of any tissues of the peritoneum but bone still fluoresced strongly.

Porphyrins with low solubility at pH 7.4

Protoporphyrin could not be introduced at neutral pH in a soluble form and was introduced as a suspension. At 48 hr it was found in granular form well localized in peritoneal fascia. No fluorescence could be observed in any of the tissues examined.

Porphyrin esters.—The dimethyl esters of haemato-, deuter-, and protoporphyrin were introduced as suspensions. After 48 hr they were found in granular form well localized in peritoneal fascia. Apart from slight fluorescence of mesenteric fat in the case of haematoporphyrin dimethyl ester, no tissue fluorescence was apparent with any of the esters.

Tissue localization following i.d. injection

Following intradermal administration in previously shaved dorsal skin the area around the site of injection was stained red and fluoresced brilliantly under UV light. However, the tissue stain disappeared at different rates with different porphyrins. With porphyrin *c* and *N,N'*diacetyl porphyrin *c* fluorescence disappeared within 24 hr; with haematoporphyrin, deuteroporphyrin, protoporphyrin and haematoporphyrin dimethylether it remained 2-3 days, while with commercial "haematoporphyrin" and haematoporphyrin "derivative" it remained for at least 4-5 days after introduction. At 2 days, in the case of the retained porphyrins, examination of frozen sections showed some fluorescence of the collagenous fibres of the dermis, some larger vessels were distinctly red, and peri-vascular cells in locations common to mast cells fluoresced strongly. In the epithelium, the basal and Malpighian layers were weakly stained, but the keratin layer was brilliantly fluorescent, as was the keratin of forming hair in the follicles.

The fully mature hair did not fluoresce, but the epithelial cells of the hair follicle appeared to take up porphyrin very strongly. Even when the fluorescence of other structures had faded, the keratin continued to retain this property, this being particularly marked with the commercial "haematoporphyrin" and haematoporphyrin "derivative". Poorly soluble porphyrins were retained in granular form in the tissue, and adjacent structures failed to show any change from their natural fluorescence. The above results are presented in tabular form in the Table.

TABLE.—*Tissue Binding, Photosensitization, and Comparative Solubility of Porphyrins*

	Relative tissue binding*				Comparative‡ solubility at pH 7.4
	Bone	Collagen: keratin	Fat	Photo- sensitivity†	
Haematoporphyrin	+	+	—	+	+
Commercial "haematoporphyrin"	++	++	—	++	++
Haematoporphyrin "derivative"	++	+++	—	++	+++
Haematoporphyrin dimethyl ether	—	—	±	++	++
Haematoporphyrin dimethyl ester	—	—	±	±	—
Deuteroporphyrin	—	—	±	+	+
Deuteroporphyrin dimethyl ester disulphonic acid	—	—	—	+	+++
Protoporphyrin	—	—	—	i.v. only	—
Porphyrin c	+++	—	—	+	+++
N,N'-diacetyl porphyrin c	++	—	—	+	++

* Amount of porphyrin bound was determined by intensity of fluorescence of tissue in UV light.

† Photosensitivity determined by erythematous response of ear of rat following irradiation.

‡ Comparative solubilities are only approximate.

Photosensitivity

Commercial "haematoporphyrin".—Using sunlight, mercury vapour lamp or carbon arc excitation, photosensitivity was consistently demonstrated. At any period from 15 min. to 4–5 days after i.p. injection at the usual dose level of 50 mg./kg., exposure to any of the light sources produced an initial erythema which began as early as 2 min. from the beginning of the exposure period, and which sometimes faded after a few minutes and sometimes persisted. In some cases after initially fading it reappeared at about 2 hr and persisted for 20 hr. When the animals were conscious, this initial erythema was accompanied by scratching; oedema was also noted. During the 15 min. period immediately after exposure there was increased vascular permeability as shown by trypan blue leakage and by carbon labelling of venules. This vascular labelling could be inhibited by the antihistamine "Anthisan" (2.5 mg./kg.) and there was microscopic evidence of mast cell degranulation. Nevertheless some erythema persisted in the presence of "Anthisan". During the subsequent 2 hr period, there was no evidence of increased vascular permeability by carbon labelling although some leucocytic emigration was initiated in this period. After 2 hr, carbon labelling could again be demonstrated both in venules and capillaries, and this "delayed" phase persisted for some hours, the period varying with the type of light source and the duration of exposure. With a 10 min. exposure to the carbon arc, the "delayed" phase lasted only 4–6 hr, whereas after exposure to sunlight or the mercury vapour lamp it lasted at least 24 hr (Fig. 2). Occasionally when erythema

persisted, injection of trypan blue or carbon showed that there were areas of ischemia, and these areas often became necrotic. It was found that this severe reaction was more readily produced with sunshine and the mercury vapour lamp, and with doses of commercial "haematoporphyrin" of 100 and 150 mg./kg.

The possibility that the response following exposure to sunlight was at least partially due to UV light radiation was tested by (a) exposure for the same time of control animals without introduced porphyrin, and (b) exposure of animals with introduced porphyrin for the same period as before but with light filtered through an UV light exclusion filter ($\lambda > 380$ nm.). In the case of test (a) no erythema was seen and there was no evidence of increased vascular permeability using the carbon labelling technique. In test (b) the results were the same as those obtained by irradiation with unfiltered light.

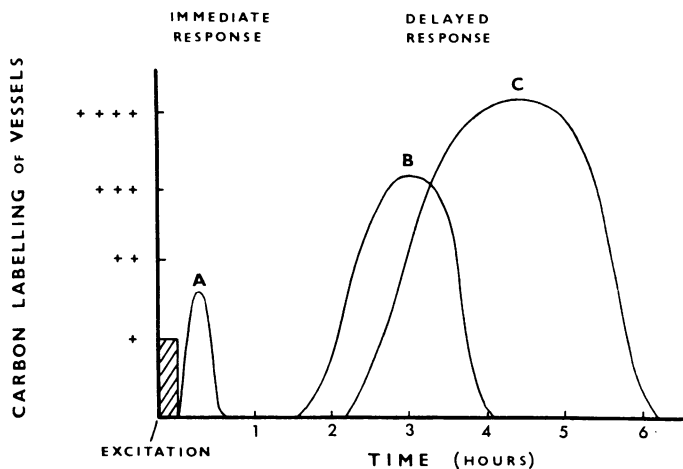


FIG. 2.—Vascular permeability changes in the photo-response (as measured by carbon labelling of vessels in the rat ear following irradiation by light). A. Immediate response, limited to venules, and inhibited by a histamine antagonist. B. Less severe delayed response, involving both venules and capillaries, produced with carbon arc excitation and strongly binding porphyrins, or in the case of poorly binding porphyrins, with sunlight or the mercury vapour lamp. C. More severe delayed response, which occurred with strongly binding porphyrins irradiated by sunlight or the mercury vapour lamp.

The exclusion of light of wavelength greater than 700 nm. (filter BG38) with sunlight or the mercury vapour lamp did not apparently influence the reaction. Although light of wavelength range 380–500 nm. was commonly used with the mercury vapour lamp, a similar photosensitivity reaction could be obtained using a range of 420–700 nm. with the same exposure time.

Purified haematoporphyrin.—At a dose of 50 mg./kg. purified haematoporphyrin produced a less severe reaction than did commercial "haematoporphyrin". Animals showed less distress after exposure to the mercury vapour lamp and although there was little difference in the degree of erythema, the length of the "delayed" phase of increased vascular permeability was reduced, as were the number of vessels showing carbon localization. The time of onset of the "delayed" phase following exposure to either sunlight or the mercury vapour lamp, was

slightly shorter in animals given purified haematoporphyrin than in those given commercial "haematoporphyrin" (Fig. 2).

Other porphyrins.—Deuteroporphyrin, DDME, haematoporphyrin dimethylether, protoporphyrin, porphyrin *c* and its diacetyl derivative, all produced a photosensitivity reaction, as judged by an erythematous response, in animals following their exposure to sunlight. However, with protoporphyrin, an erythema occurred only with i.v. and not with i.p. injection (Table). A similar pattern of vascular permeability change using trypan blue and colloidal carbon was obtained with haematoporphyrin dimethylether, porphyrin *c* and DDME following exposure to sunlight, but no increased permeability was observed with deuteroporphyrin and protoporphyrin. However, using the carbon arc to produce excitation, none of the above porphyrins produced a detectable "delayed" phase of vascular permeability in the period at which such a response was found to occur with commercial "haematoporphyrin".

DISCUSSION

The ability to induce a state of photosensitivity following intraperitoneal or intravenous introduction in rats, appeared to be a property of all porphyrins tested which were sufficiently soluble to be detected in the blood plasma by fluorescence in UV light immediately following injection. The photosensitization accompanying certain porphyrias in humans is due to the presence in cutaneous tissues of either or both uro- and coproporphyrins (Vannotti, 1954). It would thus appear that photosensitization is a property of the basic tetrapyrrole structure (Fig. 1), and is only superficially altered by change in the peripheral substitution of the molecule.

The results with DDME showed that binding to tissue is not an obligatory requirement for a photo-response. Nevertheless, porphyrin bound to skin appears to be capable of eliciting a photo-response. In the case of commercial "haematoporphyrin" when there is little or no detectable porphyrin in the blood plasma, the photo-response is close to the same as that obtained at an early stage (4 hr), when porphyrin is present in the blood plasma in relatively high concentration.

The commencement of decline of the photo-response following intraperitoneal injection (after 4–5 days) correlates well in the case of the firmly bound commercial "haematoporphyrin", with the stage at which it begins to disappear from soft tissues. In the case of DDME, the lack of photo-response after 30 min., correlates with its absence, both from blood plasma and other tissues.

The severity of the photo-response is related to the porphyrin dosage, the degree of tissue binding, and the amount of light absorbed by the porphyrin. The effect of dosage was shown by the increased frequency of tissue necrosis when commercial "haematoporphyrin" was administered at 100–150 mg./kg., instead of 50 mg./kg. The reaction to sunlight was unchanged by excluding any UV light present. It was different from that caused by UV light (Logan and Wilhelm, 1966) in that the "delayed" phase, as shown by carbon labelling occurred much earlier. The shorter length of the "delayed" phase of vascular permeability obtained with the carbon arc compared with that obtained with sunlight or the mercury vapour lamp, if not due to a difference in the amount of irradiation, may be due to the absence of longer wavelength light, as there is some evidence (Runge and Watson, 1962) that infra-red light potentiates the effect of irradiation with light of 400 nm.

wavelength. The results obtained with a mercury vapour light source in conjunction with exclusion filters show that excitation with wavelengths above 420 nm. can still excite a photo-response. This confirms the findings of Rimington, Magnus, Ryan and Cripps (1967) and Magnus (1969).

Of the criteria used, erythema was the most sensitive indicator of a photo-response. Following sunlight irradiation of animals given protoporphyrin (i.v.) or deuteroporphyrin (i.p.), no carbon labelling or enhanced blueing was apparent whereas an erythema was observed in each case. The presence of carbon labelling in the absence of erythema, and *vice-versa*, during various stages of the photosensitivity reaction indicated that these signs reflect different vascular changes. This has also been noted following chemical injury (Steele and Wilhelm, 1966). Using the degree of carbon labelling as an indicator of vascular damage, it appears that the "delayed" phase is prolonged and its onset postponed in the more severe reaction (Fig. 2, type C). Although conclusive evidence has not yet been obtained, the results suggest, where identical dose levels, type and degree of irradiation were used with deuteroporphyrin, purified haematoporphyrin and commercial "haematoporphyrin", that there is a correlation between the tissue binding ability of these porphyrins and the amount of vascular damage which occurs on irradiation (Table and *vide infra*).

As regards the component of bone to which porphyrin binds, it is well known that uroporphyrin can be removed by acid under conditions which also bring about complete or partial decalcification (With, 1955), and we obtained similar results with haematoporphyrin (purified and commercial), haematoporphyrin "derivative", porphyrin *c* and N,N'-diacetyl porphyrin *c*. This does not necessarily mean, however, that the porphyrin is bound to calcium, in fact our experiments indicate that in the case of haematoporphyrin, porphyrin *c* and N,N'-diacetyl porphyrin *c* the binding is to the organic component of bone, as treatment of the stained bone with EDTA until it had completely lost its rigidity, failed to remove the pigment in each case. This points to combination of porphyrins with a protein complex both in bone and in soft tissue. It has thus been shown that porphyrin *c* and N,N'-diacetyl porphyrin *c* may be used selectively and haematoporphyrin or its derivatives less selectively, as markers of forming bone in skeletal growth and remodelling studies.

For the staining of either hard or soft tissue by porphyrin, the first requirement is that it should have adequate solubility in tissue fluids. This criterion was not met by protoporphyrin, which like most of the dimethyl esters tested, remained in the peritoneum at the site of injection. In a number of cases, however, where solubility was practically complete, the plasma fluoresced following i.p. introduction of the porphyrin and a strong photo-response could be obtained, but there was little or no staining of bone or collagenous tissue. This applied to deuteroporphyrin; the introduction, however, of hydroxy ethyl groups at the 2,4 positions to form haematoporphyrin (Fig. 1) resulted in binding to both hard and soft tissues. Then, when the hydroxyl groups were masked by the formation of the haematoporphyrin 2,4-dimethylether, binding was again inhibited. Further, DDME, which is similar to deuteroporphyrin in that two negative charges are present on only one edge of the porphyrin nucleus, did not combine with either hard or soft tissue. Also, the octacarboxylic acid, uroporphyrin, is well known to bind to bone and to soft tissues, although more strongly to the former (Vannotti, 1954). These results indicate that binding to both hard and soft tissues requires peripheral

groups to be present at more than one edge of the planar square formed by the tetrapyrrole nucleus of the porphyrin and that such groups be capable of forming either hydrogen or ionic bonds with a protein complex of the stained tissue.

The results with porphyrin *c* and N,N'-diacetyl porphyrin *c* are in agreement with the above hypothesis as far as bone is concerned and it was surprising that they were apparently taken up by soft tissue so weakly. Porphyrin *c* is, after the insertion of an iron atom to the tetrapyrrole nucleus, the non-fluorescent prosthetic group of the wide-spread pigment cytochrome *c*. Neilands (1961) has shown by *in vitro* experiments, that non-enzymic incorporation of iron by porphyrin *c* takes place readily at 23° and pH 7.5. The possibility exists therefore that porphyrin *c* and N,N'-diacetyl porphyrin *c* are both taken up by soft tissue, but then incorporation of iron takes place either non-enzymically or enzymically, with the loss of fluorescence.

The hydrophobic tetrapyrrole nucleus is, of course, common to all porphyrins and it is very likely that, in balance with the hydrophilic forces present, it plays a part in tissue staining. Such a balance has been shown to be of importance in the affinity of dyestuffs for textile fibres (Alexander and Hudson, 1963) and also in the uptake by serum albumin of aromatic sulphonic acids (Flanagan and Ainsworth, 1968) and of cholanic acids (Rudman and Kendall, 1957). In this regard, it is interesting that the most generally intense porphyrin stain for both hard and soft tissues of those tested, was the so-called haematoporphyrin "derivative" resulting from the treatment of haematoporphyrin with glacial acetic and sulphuric acids (Lipson *et al.*, 1961). Thin-layer chromatographic examination of the product (Henderson, Barker and Pardee, unpublished) showed one of the main components to be a porphyrin with an R_f value intermediate between the R_f values of protoporphyrin and haematoporphyrin. The conditions of the preparation are conducive to dehydration, and it is likely that the porphyrin in question is the partially dehydrated derivative of haematoporphyrin, where one of the hydroxyl groups has been converted to a vinyl group. This is presumably not readily metabolized or converted to the non-fluorescent iron complex and it could be expected to be held to tissues more firmly than haematoporphyrin, as it retains the binding group pattern found necessary but has an increase in its hydrophobic nature. The presence in commercial "haematoporphyrin" of porphyrins with similar R_f values to the above, suggests that the increased staining of both hard and soft tissues of this preparation, over that of purified haematoporphyrin, may be due to the presence of one or more isomers of partially dehydrated haematoporphyrin.

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REFERENCES

- ALEXANDER, P. AND HUDSON, R. F.—(1963) 'Wool: Its Chemistry and Physics', 2 ed. London (Chapman and Hall).
BARKER, D. S., HENDERSON, R. W. AND STOREY, E.—(1970) *J. dent. Res.*, **49**, 659.
COTRAN, R. S. AND MAJNO, G.—(1964) *Am. J. Path.*, **45**, 261.
FALK, J. E.—(1964) 'Porphyrins and Metalloporphyrins'. Amsterdam (Elsevier).
FISCHER, H.—(1955) *Org. Synth.*, **3**, 442.

- FISCHER, H. AND ORTH, H.—(1937) 'Die Chemie des Pyrrols'. Leipzig (Akad. Verlagsgesellschaft).
- FLANAGAN, M. T. AND AINSWORTH, S.—(1968) *Biochim. biophys. Acta (Amst.)*, **168**, 16.
- GREGORIE, H. B., HORGER, E. O., WARD, J. L., GREEN, J. F., RICHARDS, T., ROBERTSON, H. C. AND STEVENSON, T. B.—(1968) *Ann. Surg.*, **167**, 820.
- HENDERSON, R. W. AND MORTON, T. C.—(1967) *J. Chromatog.*, **27**, 180.
- LIPSON, R. L., BALDES, E. J. AND OLSEN, A. M.—(1961) *J. nat. Cancer Inst.*, **26**, 1.
- LOGAN, G. AND WILHELM, D. L.—(1966) *Br. J. exp. Path.*, **47**, 286.
- MAGNUS, I. A.—(1968) *Proc. roy. Soc. Med.*, **61**, 196.—(1969) *J. invest. Derm.*, **53**, 400.
- MORELL, D. B. AND STEWART, M.—(1956) *Aust. J. exp. Biol. med. Sci.*, **34**, 211.
- NEILANDS, J. B.—(1961) in 'Haematin Enzymes', eds. Falk, J. E., Lemberg, R. and Morton, R. K. Oxford (Pergamon).
- NEILANDS, J. B. AND TUPPY, H.—(1960) *Biochim. biophys. Acta (Amst.)*, **38**, 351.
- NEUBERGER, A.—(1968) *Proc. roy. Soc. Med.*, **61**, 191.
- NEUBERGER, A. AND SCOTT, J. J.—(1952) *Proc. roy. Soc. A.*, **213**, 307.
- PRESCOTT, G. H., MITCHELL, D. F. AND FAHMY, H.—(1968) *Amer. J. Phys. Anthropol.*, **29**, 219.
- RAMSEY, V. G.—(1953) *Biochem. Prepn.*, **3**, 39.
- RIMINGTON, C., MAGNUS, I. A., RYAN, E. A. AND CRIPPS, D. J.—(1967) *Quart. J. Med.*, **36**, 29.
- RUDMAN, D. AND KENDALL, F. E.—(1957) *J. clin. Invest.*, **36**, 538.
- RUNGE, W. AND WATSON, C. J.—(1962) *Proc. Soc. exp. Biol. (N.Y.)*, **109**, 809.
- SCHUMM, O.—(1928) *Z. Physiol. Chem.*, **178**, 1.
- STEELE, R. H. AND WILHELM, D. L.—(1966) *Br. J. exp. Path.*, **47**, 612.
- VANNOTTI, A.—(1954) 'Porphyrins'. London (Hilger and Watts).
- VOGEL, A.—(1962) 'Practical Organic Chemistry'. London (Longmans Green).
- WALTER, R. I.—(1952) *J. biol. Chem.*, **196**, 151.
- WITH, T. K.—(1955) *Biochem. J.*, **60**, 703.
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