Formation of metal complexes of tumor-localizing porphyrins

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Whereas the tumor localizer and photosensitizer hematoporphyrin derivative (Hpd) has its fluorescence emission maximum at 610–630 nm, several authors have reported that in aqueous solutions of hematoporphyrin (Hp) and Hpd, or in tumors after an injection of Hpd, a compound is formed which has its fluorescence emission maximum at 570–590 nm. This work (HPLC and fluorescence analysis) indicates that this peak is due to the formation of Zn-porphyrins either in vitro or in vivo. Cu- and Co-porphyrins may be formed as well, from traces of these metallic ions. In contrast to free porphyrins and Zn-porphyrins the latter complexes are non-fluorescent and do not act as photosensitizers.

Metalloporphyrin *Tumor localizer* *Photosensitizer* *Photochemotherapy*
Fluorescence *Chromatography*

1. INTRODUCTION

Hematoporphyrin derivative (Hpd) is a photosensitizing tumor localizer that is being tested in several clinical trials of photoradiation therapy of cancer [1,2]. Furthermore, because of its characteristic red fluorescence and its preferential localization in tumor tissue, it may be used as an aid in cancer diagnosis [3,4]. Hpd is a mixture of several porphyrins and it seems that the least polar of these are the most potent tumor localizers [5,6].

When bound to serum proteins or to cells and tissues, the porphyrins in Hpd have fluorescence emission maxima at 620–635 and 680–700 nm [7–9]. Recently, a different fluorescence emission spectrum was observed in hematoporphyrin and Hpd solutions [10,11]. This spectrum had peaks at 575–580 and 630–640 nm. A corresponding spectrum was observed in mouse 3T3 cells labelled with Hpd [12] and in mouse mammary tumors after injection of hematoporphyrin [9]. Several explanations of this phenomenon have been proposed: porphyrin monomers bound to polymeric structures of porphyrins [10], porphyrins bound to dead cells, time-dependent changes in the binding of components of the Hpd solutions, biochemical modification of Hpd, and changes in the state of aggregation of the porphyrins [12].

Here we show that this new fluorescence spectrum is probably due to the formation of Zn-porphyrins. This may take place in vivo, as well as in vitro if, under suitable conditions, trace amounts of Zn^{2+} are present. The formation of metalloporphyrins is of great importance for the diagnostic and therapeutic use of porphyrins since incorporation of metals frequently changes the tumor-localizing property of a porphyrin [13] and/or reduces or even eliminates its fluorescence and/or its photosensitizing effect [14].

2. MATERIALS AND METHODS

Hpd was prepared as in [3]. Hematoporphyrin (Hp), hydroxyethylvinyl deuteroporphyrin (Hvd) and protoporphyrin (Pp) were obtained from Porphyrin Products (Logan, UT). The porphyrins were dissolved as in [15]. Other chemicals used were of the highest purity commercially available.

Reversed-phase high-performance liquid chro-

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matography (HPLC) was carried out as in [15]. Optical absorbance and porphyrin fluorescence were recorded simultaneously during the chromatographic runs. Absorption and fluorescence spectroscopy were carried out by means of a Cary 118 spectrophotometer and a Hitachi Perkin Elmer MPF-2a spectrofluorimeter, respectively.

Cells of the human cell line NHIK3025 (derived from a carcinoma in situ) were cultured in Pucks medium E2a with 30% serum as in [16]. The cells were incubated with porphyrins in E2a with 3% serum for 18 h at 37°C. Porphyrins were extracted from the cells as in [17]. Photodynamic inactivation of the cells was assayed as in [15].

Lewis lung carcinomas were grown in B6D2 mice as in [18]. Hpd (12.5 mg/kg) was given by intraperitoneal injection; 24 h after the injection the mice were killed and tumors and several other tissues were removed. Porphyrins were extracted from the tumors and tissues as in [6]. Relative yields of photoinduced singlet oxygen were measured as in [19].

3. RESULTS

Hp (10 μM in Dulbecco's phosphate-buffered saline, PBS) was incubated at 37°C with different concentrations of the individual metal ions Zn^{2+}, Cu^{2+} and Co^{2+}. It was found that after 18 h incubation in the presence of 100 μM metal, the transformation of Hp into its metal complex was complete. The resulting absorption spectra, together with that of Hp are shown in fig.1. These 3 metal complexes of Hp have characteristic chromatographic retention times as shown in fig.2 and may easily be separated from each other and from Hp itself. The corresponding metal complex of Hvd and Hpd were also analysed. Incorporation of the metal changed the absorption and fluorescence spectra as shown for Hp in fig.1,3. The complexes of protoporphyrin were not stable in the mobile chromatographic phase (methanol/water) and were not studied further. The Co and Cu complexes were non-florescent while the Zn complexes were fluorescent, as were the free porphyrins. Under the present conditions Mg^{2+} and Fe^{2+} did not form complexes with Hp. The fluorescence emission maxima of ZnHp are located at 578 and 630 nm, those of Hp at 612 and 675 nm (fig.3). The mentioned fluorescence peaks are all slightly shifted towards the red by porphyrin binding to serum proteins or cells (not shown). When NHIK3025 cells were incubated with Hpd or ZnHpd under similar conditions (18 h at 37°C and 12.5 μg/ml or porphyrin in E2a with 3% serum) they accumulated 10 ± 2-times more Hpd than ZnHpd. Their photosensitivity was also determined, and the fluence needed to reduce the surviving fraction to 50% was 10–15-times larger for ZnHpd than for Hpd. Cells incubated with Hpd alone for 18 h and then removed from the tissue culture flasks by a cell scraper showed strong fluorescence of Hpd but never any trace of fluorescence of ZnHpd. Neither was any ZnHpd fluorescence seen in cells that were first incubated with Hp and then with Zn^{2+} (1 mM) for a further 4 h, indicating that Zn^{2+} did not penetrate to the site(s) of Hpd in the cells during this time.

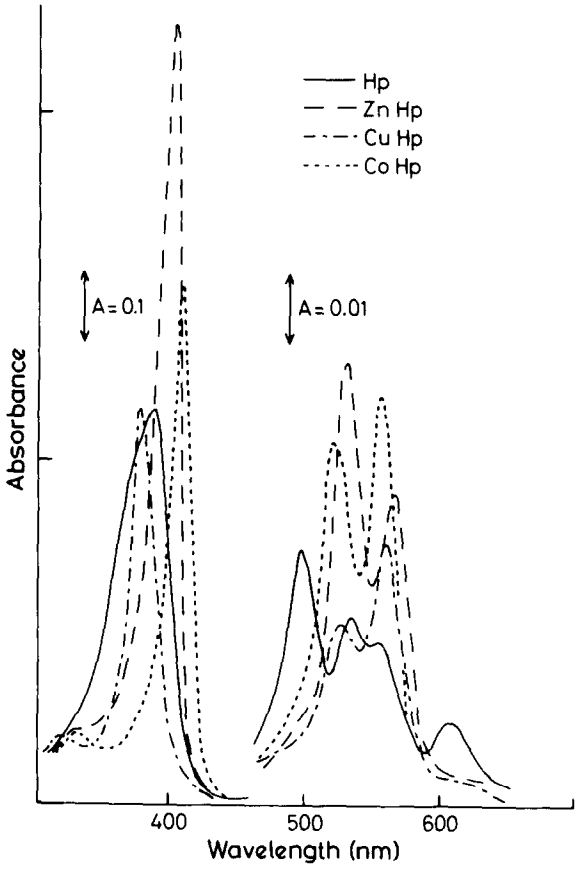

Fig.1. Absorption spectra of the following porphyrins in PBS: (——) Hp; (– – –) ZnHp; (— · —) CuHp; (-----) CoHp.

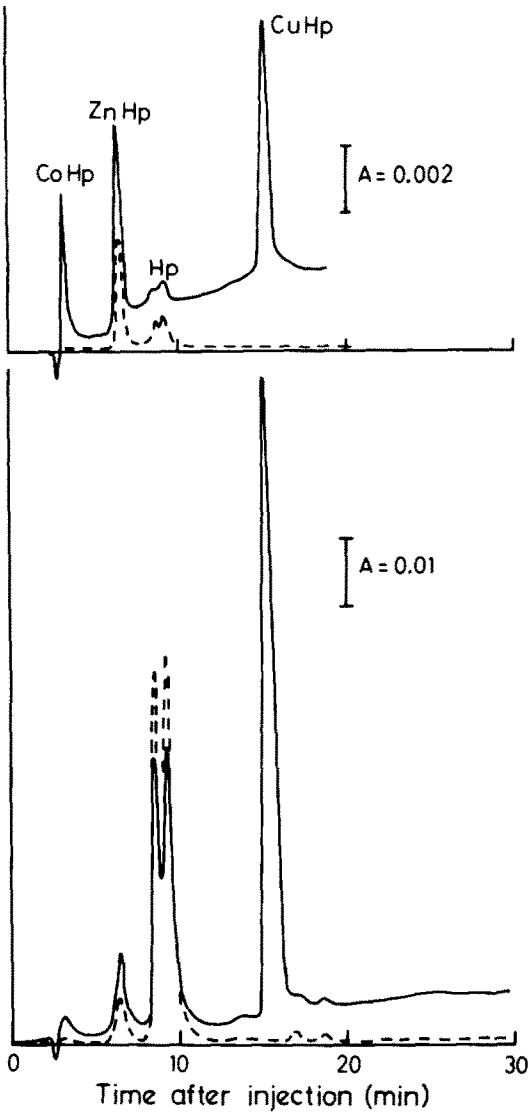

Fig.2. Reversed-phase HPLC. (——) Absorbance at 392 nm, (---) fluorescence excited in the region of the Soret band. The upper part of the figure shows the chromatogram of a mixture of CoHp, ZnHp, Hp and CuHp. The peaks were identified by separate runs of the pure compounds. The lower part of the figure shows the chromatogram of 10 μM Hp after storage for 24 h in PBS at 37°C in a disposable glass tube.

Lewis lung tumors in B6D2 mice which had been injected with Hpd 24 h earlier were analysed by both HPLC and fluorescence spectroscopy. Neither extracts nor crude suspensions of sonicated tumor tissue showed any fluorescence at 570–590 nm. Only the ordinary free porphyrin fluorescence was

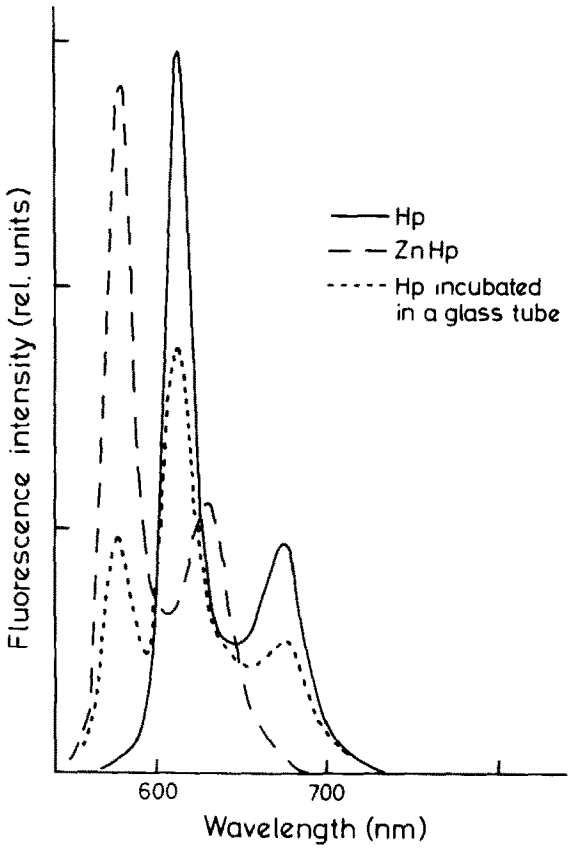

Fig.3. Fluorescence emission spectra of 10 μM Hp (——), ZnHp (— —) and Hp stored for 24 h in PBS at 37°C in a disposable glass tube (---).

seen. When samples of such extracts were kept in glass tubes washed with 2 M nitric acid, HPLC chromatograms showed no sign of metalloporphyrins. However, when disposable glass tubes were used in the extraction procedure, significant amounts of metalloporphyrins were apparent. This is exemplified in fig.2,3. It can be seen that incubation of 10 μM Hp in such disposable glass tubes for 24 h at 37°C resulted in the formation of significant amounts of metalloporphyrins, notably of Cu-porphyrins. Metalloporphyrins were formed whether or not serum or SDS (an agent that efficiently favours monomerization of porphyrins) were present. Thus, protein binding and state of aggregation of the porphyrins are not of crucial importance for the formation of metalloporphyrins under the conditions of the experiment. When the glass tubes were washed with 2 M nitric acid before use, practically no metalloporphyrins were

formed. Several types of disposable plastic tubes were also significantly less contaminated with metals than were the disposable glass tubes.

The fluorescence and $^1O_2$-quantum yields were estimated, and found to be 70 and 50%, respectively, lower for ZnHp than for Hp.

4. DISCUSSION

This work clearly indicates that the fluorescence peak at 570–590 nm observed by several authors [9–11] in what they call 'aged' solutions of porphyrins is due to the formation of Zn complexes of the porphyrin. We have shown that disposable laboratory equipment of glass and plastic is contaminated with several metals that may give rise to the formation of metalloporphyrins when in contact with porphyrin solutions. This may result in either quenching of the porphyrin fluorescence (i.e., formation of Cu^{2+} and Co^{2+} complexes) or a shift of the fluorescence and absorption spectra due to the formation of Zn^{1+} complexes. Fe^{2+} complexes are also non-fluorescent but the rate of their formation would be relatively slow under the present conditions [20].

In Lewis lung carcinomas in B6D2 mice no metalloporphyrins seem to be formed under the present conditions. However, in other tumor systems, such as in [9], at least Zn^{2+} complexes of hematoporphyrin seem to have been formed, notably after injection of small amounts of Hp. The fluorescence maxima reported in [9] are located at slightly lower wavelengths than found by us and other workers. However, the difference is not larger (~10 nm) than may be due to experimental factors. In [9], the fluorescence spectra were recorded in vivo and, therefore, no external contamination seems to have taken place. Several metals are present in the body in high enough concentrations to transform a significant fraction of clinically used Hpd doses into metalloporphyrins. The formation of ZnPp in red cells, in which it represents up to 90% of the total non-heme protoporphyrin, is well known ([21] and references therein), and Zn plays a vital role as an essential constituent of many enzymes.

The apparent polarity of the metal complexes of Hp decreased in the order Co > Zn > Cu (fig.2). This behaviour may be explained by the structure of their chelate systems. The d^{10} Zn^{2+} chelates have no unpaired electrons. They normally have a square-planar structure with the ability to add one extra ligand, e.g., an aquo ion, perpendicular to the plane. Co^{2+} forms low-spin chelates with one unpaired electron and may add 2 extra ligands to form somewhat distorted octahedra. The Cu^{2+} chelates, on the other hand, are of the high-spin type, the lone electron occupying a $d_{x^2-y^2}$ orbital along the axis pointing to the 4 N atoms. Reluctance to add further ligands is due to the repulsive effect of the filled anti-bonding orbital perpendicular to the plane. Thus 'polarity' in the HPLC system in a series of metal chelates of a porphyrin would be dependent upon ease of extra ligand binding of aquo ions such that Co > Zn > Cu. One may expect that the rate of passive cellular uptake of a substance decreases with increasing polarity of the substance. This was found to be true for ZnHp, Hp and CuHp, which were tested here.

The photosensitizing efficiency of ZnHp seems to be about 50% lower than that of Hp both with respect to photoinactivation of cells and formation of singlet oxygen. On the other hand, the Cu^{2+}, Co^{2+} and Fe^{2+} complexes are inefficient since practically no singlet oxygen is formed when they are exposed to light [22].

The facility of free porphyrins to react with metal ions is greatly influenced by the environment [23].

Such differing situations may be expected to be encountered within the living cell and are almost certainly exploited in enzymatic reactions, e.g., by ferrochelatase which catalyzes the incorporation not only of iron, but also of Co^{2+} into protoporphyrin [24].

In is not surprising that porphyrin species taken up into a cell may be further changed, as by chelation with metal ions, and this may affect their phototherapeutic activity – probably adversely should they form Cu^{2+} or Fe^{2+} conjugates. The biological implications of such reactions still await investigation. It is also important to realize that such conjugates will not be included in any quantitative analytical technique for porphyrin content based on fluorescence spectrophotometry.

To avoid errors, the importance of using metal-free reagents and experimental apparatus and of exercising caution in the interpretation of the observed findings cannot be overemphasized.

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