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INHIBITION OF DNA DEPENDENT RNA SYNTHESIS BY PORPHYRIN PHOTOSENSITIZERS

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SUMMARY

Select porphyrin photosensitizers were studied to determine their effects on DNA-dependent RNA synthesis in the presence and absence of visible light. All of the porphyrins were found to inhibit wheat germ polymerase II to some degree in the dark. In the presence of light, the inhibitory effects of the porphyrins was found to result from both inactivation of the enzyme and impairment of the ability of DNA to serve as a template.

INTRODUCTION

Natural and synthetic porphyrin photosensitizers are receiving considerable attention as tumor-localizers (1-3) and photochemotherapeutic agents (4). Their photosensitizing effects on cultured cells has been demonstrated by various investigators (5-8). The mechanism of their photocytoxic action is unknown, although, Kessel has recently presented data which supports the hypothesis that the cell membrane is a primary target (9). No relationship has been established, however, between Kessel's results and the complex events involved in the cell and tissue destruction observed during photochemotherapy. Moreover, a "membrane effect" does not explain the ability of at least one porphyrin, hematoporphyrin, to induce skin tumors in the mice exposed to sunlight (10). Appropriately, several investigators have examined other substrates for the photosensitizing effects of porphyrins, particularly, nucleic acids (11,12).

The present work deals with a select series of porphyrins that includes hematoporphyrin derivative (HPD), the photosensitizer used in clinical trials of photochemotherapy (4). This, and three related acidic

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porphyrins were compared to mesotetra(4-N-methylpyridyl)porphine (T4MPyP), a photosensitizer that has been shown to intercalate into DNA (13,14). The effects of these porphyrins on the enzyme activity of wheat germ DNA-dependent RNA polymerase and on the template function of DNA, were studied in illuminated and dark reactions.

Experimental.

ATP, GTP, and CTP were obtained from Boehringer Mannheim, [^{3}H] -UTP (sp. act. 20 Ci/m mole) was obtained from New England Nuclear, and wheat germ DNA dependent RNA polymerase II (EC 2.7.7.6) was prepared as described by Jendrisak and Burgess (15).

Hematoporphyrin IX (HEM IX) was purchased from Porphyrin Products. Mesotetra(4-sulfonatophenyl)porphine (TSPP) and meso-tetra(4-carboxylphenyl) porphine (TCPP) were obtained from Dr. N. Datta-Gupta, South Carolina State College. HPD was provided by Dr. T. Dougherty of this Institute. The tetraiodide salt of T4MPyP was purchased from Strem Chemicals. TCPP, TSPP, HEM IX, and HPD were dissolved in 0.02M Tris, 0.15M NaCl, pH 7.9, and T4MPyP was dissolved in 0.01M Tris, pH 8.0.

Two light sources were used for illumination. The enzyme was illuminated for 30 minutes in an ice bath with a Lafayette high intensity lamp (equipped with a GE 1133 bulb). The power output was approximately 80 mWatts/cm^2 at a distance of 20 cm. DNA was illuminated with a mercury vapor lamp through appropriate filters to cut-off wavelengths below 365 nm. The power output was approximately 100 mWatts/cm^2 at a distance of 35 cm.

RNA polymerase assays were carried out in duplicate using a reaction mixture of 0.25 ml containing 0.5 mM ATP, CTP, GTP and 0.02 mM [^{3}H] -UTP (253 cpm/p mole), 1.6 mM $MnCl_2$, 3.0 mM DTT, 100 mM $(NH_4)_2SO_4$, 40 mM Tris-HCl, pH 7.9, 0.016 mM EDTA, 25 µg heat denatured calf thymus DNA and enzyme as indicated. After incubation at 25°C for 15 minutes, the samples were processed as previously described (16) and counted in a Beckman LS-100 liquid scintillation counter at 57% efficiency. Porphyrin quenching was determined by internal standardization.

Electrophoresis was carried out with BioRad 4% and 7.5% acrylamide gels using Biophore SDS buffer. The gels were stained with 0.05% Coomassie Blue R 250, and diffusion-destained in 7% acetic acid. Molecular weights of the bands were estimated by comparison with BioRad MW markers. The bands were identified according to the results of Jendrisak and Burgess (17).

RESULTS AND DISCUSSION

As shown in Figure 1, all of the porphyrins inhibit RNA polymerase in the absence of light. TSPP, TCPP, HPD and T4MPyP begin to inhibit at a concentration of approximately 1 x 10^{-5} M, and reach 100% inhibition at 1 x 10^{-4} M. HEM IX appears to be somewhat less effective and has a narrower range of inhibition beginning at 1 x 10^{-4} M.

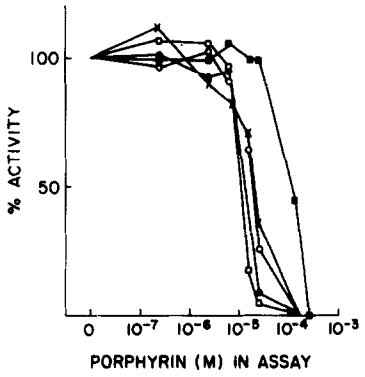


Figure 1: Effect of increasing porphyrin concentration on DNA-dependent RNA synthesis. Increasing amounts of porphyrin were added to assay mixtures containing 20 µl enzyme (approximately 20 µg protein) at 0 time followed by incubation for 15 minutes at 25°C. ●=TSPP, O=TCPP, ■=HEM IX, □=HPD, x=T4MPyP.

Some insight into the mechanism of this inhibition can be obtained by preincubating the enzyme for 15 minutes with all the assay components except UTP. This allows the formation of an initiation complex, but prohibits chain elongation (18). After the pre-incubation, UTP and porphyrin are added, and the reaction allowed to proceed. As shown in Table I the same degree of inhibition is obtained with and without the pre-incubation period. This indicates that the porphyrin inhibits synthesis by blocking chain elongation. If the inhibition obtained in the pre-incubated series were significantly less than in the series in which pre-incubation was not used, it would suggest that blocking of the initiation step was a factor in the inhibition. This, however, assumes that porphyrin added after pre-incubation cannot interfere with the initiation complex.

Besides their ability to inhibit RNA polymerase in the absence of light, the photosensitizing effects of the porphyrins on both the enzyme and the DNA template activity were investigated. Photoinactivation of DNA-dependent RNA polymerase (19) and RNA-dependent DNA polymerase (20) has already been noted. The mechanism of the photoinactivation is not known; however, it is apparent from these studies (19,20) that the enzyme is more susceptible than the template to the action of the photosensitizer plus

Table I

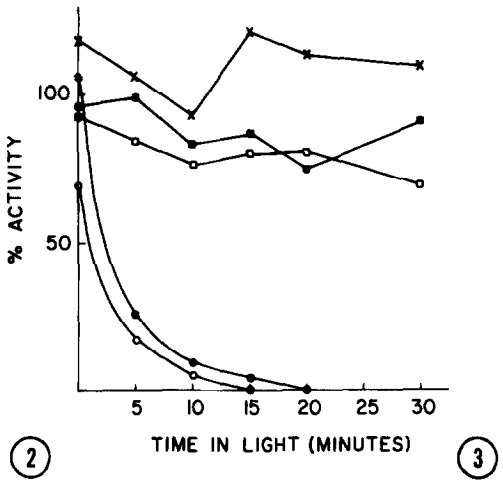
	CPM	% Inhibition
Control	31,968 ± 3,342	
TSPP	3,257 ± 214	89.8
TSPP (PI)	3,909 ± 286	87.8
TCPP	4,989 ± 1,534	84.4
TCPP (PI)	4,241 ± 562	86.4
HPD	661 ± 128	97.9
HPD (PI)	2,532 ± 251	92.1
T4MPyP	12,105 ± 3	62.1
T4MPyP (PI)	8,361 ± 2	73.9

Table I: Effect of porphyrins on chain elongation of RNA. All assay ingredients, except UTP and porphyrin, were preincubated for 15 min. at 25°C. At the end of the pre-incubation, UTP and porphyrin (final concentration = 2 x 10^{-5}M) were added at 0 time and RNA synthesis allowed to proceed for an additional 15 min. at 25°C. The values are acid precipitable counts per minute from which blank values have been subtracted. Parallel experiments were also done in which porphyrin was added at 0 time. (PI) = pre-incubated.

light, and that dithiothreitol provides effective protection against inactivation. This protective effect suggests that thiol groups of the enzyme are oxidized by the photodynamic effect of the porphyrin. It is not known, however, whether photo-oxidation of thiol groups is correlated with enzyme inactivation.

Other photodynamic modifications of proteins are known which may be responsible for enzyme inhibition. The most prominent of these is cross-linking. This was first demonstrated by DeGoeij and van Steveninick in the photoirradiation of red blood cell ghosts and intact cells in the presence of protoporphyrin (21).

The present investigation determined the ability of four different porphyrins to photo-induce cross-linking of the subunits of RNA polymerase. The relative effectiveness of the porphyrins to photoinactivate the polymerase is shown in Figure 2. TCPP and TSPP are very active photosensitizers providing 100% inhibition at 15 and 20 minutes respectively. HPD and HEM IX have moderate activity and T4MPyP appears inactive under these conditions.



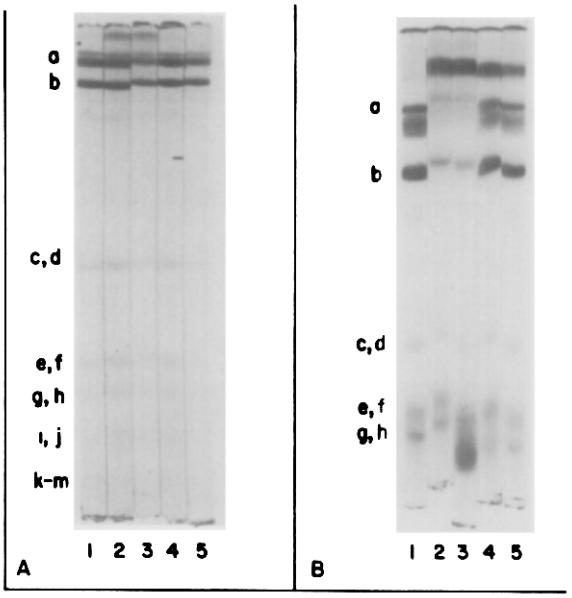


Figure 2: Effect of porphyrins and light on DNA-dependent RNA synthesis. 90 µl enzyme was mixed with 5 µl porphyrin (1.9 x 10^{-4} M) and illuminated as described. At designated times, 5 µl aliquots were removed and assayed. The controls contained buffer, but no porphyrin. The % activity is relative to that of controls at the same time period.
●=TSPP, O=TCPP, ■=HEM IX, □=HPD, x=T4MPyP.

Figure 3: SDS electrophoresis of photoirradiated enzyme. 45 µl enzyme was mixed with 2.5 µl porphyrin and illuminated as described. Following illumination, 100 µl of solubilization buffer (0.04 M Tris pH 6.6, 0.001 M EDTA, 1% SDS, and 0.1 M B-mercaptoethanol) was added followed by incubation for 18 hours at 37°C in the dark. Electrophoresis was done at 15 v/gel toward the anode (bottom). (a) 7.5% gels (porphyrin = 1 x 10^{-5} M), (b) 4% gels (porphyrin = 1 x 10^{-4} M). Gel 1, control; Gel 2, TSPP; Gel 3, TCPP; Gel 4, HPD; Gel 5, T4MPyP.

The results shown in the inactivation curves correlate with those of electrophoresis experiments, which indicate photo-induced cross-linking of the enzyme subunits. This is shown in Figures 3A and 3B. SDS electrophoresis was carried out on the enzyme after photoirradiation in the presence of 1 x 10^{-5} M porphyrin in Figure 3A. For TCPP and TSPP (gels 2 and 3) the gels show the presence of a high molecular weight (cross-linked) component near the top of the gel. The HPD treated enzyme appears to have a trace of the same high molecular weight component. None can be

Table II

	Pre-Column (CPM)	% Inhibition	Post-Column (CPM)	% Inhibition
Control	34,093 ± 93		30,518 ± 2,372	
TSPP	20,781 ± 860	39.1	32,681 ± 1,371	0
TCPP	16,316 ± 393	52.2	31,213 ± 1,433	0
HPD	11,002 ± 1,513	66.9	32,764 ± 2,077	0
T4MPyP	25,373 ± 1,148	24.1	21,363 ± 372	32.6

Table II: Effect of porphyrins on the DNA template in the absence of light. One ml mixtures containing 450 µg HD DNA and buffer or porphyrin at a final concentration of 4 x 10^{-5}M (TCPP and TSPP) or 8 x 10^{-5}M (HPD and T4MPyP) was incubated for 24 hours in the dark. Two 50 µl aliquots were removed and assayed as described. The remainder was chromatographed on a Sephadex G-25 column fashioned from a pasteur pipette. 0.5 ml fractions were collected and monitered for fluorescence or absorbance at 260 nm. Two 50 µl aliquots were removed from the peak fraction and used for assay. Values are acid precipitable counts per minute from which blank values have been subtracted.

detected, however, in gel 5, in which the enzyme was treated with T4MPyP. HEM IX was not included in this series.

At a porphyrin concentration of 1 x 10^{-4} M, as shown in Figure 3B, cross-linking is much more apparent. Moreover, resolution of the high molecular weight component is improved when the photoirradiated enzyme is analyzed in a less concentrated gel. Under these conditions, photoirradiation in the presence of TSPP and TCPP, gels 2 and 3 respectively, produces a high concentration of the high molecular weight-component. Furthermore, this component appears to be formed from subunits a and b, both of which show a decrease in staining intensity. HPD and T4MPyP also show an effect at this higher concentration, as indicated by the presence of a high molecular weight-component in gels 4 and 5 respectively. No comparable effects were noted if porphyrin and enzyme were incubated in the absence of light.

Besides the effect of porphyrins on the enzyme, interaction with DNA and subsequent modification of its template function may result in inhibition of the polymerase activity. Table II lists the degree of inhibition

Table III

	Pre-Column (CPM)	% Inhibition	Post-Column (CPM)	% Inhibition
Control	32,671 ± 1,246		28,751 ± 1,138	
TSPP	7,690 ± 25	76.7	7,683 ± 43	73.3
TCPP	3,565 ± 176	89.1	3,662 ± 99	87.4
Control	82,893		78,596 ± 2,564	
HPD	15,364 ± 126	81.5	16,392 ± 154	79.2
T4MPyP	2,453 ± 188	97.1	2,398 ± 134	97.0

Table III: Effect of porphyrins and light on the DNA template. The same experimental protocol as described in Table II was used except the samples were illuminated for 24 hours prior to assay.

obtained when the porphyrin is pre-incubated with the DNA template in the dark. All of the porphyrins were found to inhibit the polymerase activity. A portion of each incubation mixture containing porphyrin and the DNA template, was chromatographed on Sephadex G-25 to remove unbound porphyrin, and the peak fraction used as the template to assay enzyme activity. As indicated in Table II, only T4MPyP retains its inhibitory capacity due, apparently, to its ability to bind to DNA (13,14).

A similar series of experiments, shown in Table III, were carried out with illumination of porphyrin treated DNA followed by removal of any unbound porphyrin using Sephadex G-25. The results of these experiments indicate that photosensitized damage of the DNA template is induced by each porphyrin, and that this is responsible for the subsequent inhibition of the polymerase reaction.

In conclusion, it is apparent that critical enzymes as well as the cell genome may be substrates for the photosensitizing and dark reactions of this series of porphyrins. Furthermore, inhibition of the polymerase activity can occur as a result of photosensitized or dark reactions of the porphyrin with the enzyme and/or the DNA template. These observations must be considered in regard to the mechanism of porphyrin photosensitized cytotoxcity, mutagensis and carcinogenesis.

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