

Porphyrin-sensitised Photodecomposition of Thymine in Aqueous Triton X-100 Solution

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Hematoporphyrin (Hp) and protoporphyrin (disodium salt) sensitise the photodecomposition of thymine, in aqueous solutions of Triton X-100, under CW excitation ($\lambda = 320\text{--}490\text{ nm}$). A number of products have been detected by HPLC. The effect of addition of ascorbate ion (AA), tryptophan (TRP) and sodium azide (NaN_3) on the yields of the most prominent photoproduct has been studied. From this, the involvement of type-I mechanism in the observed photodecomposition of thymine, i.e. electron transfer from thymine to the excited sensitisers has been suggested.

The photoradiation therapy of malignant tumours, based on the photosensitised destruction of transformed cells, is of great interest at present¹⁻³. It is well known that the therapeutic effect is due to comparatively selective accumulation of the sensitizer hematoporphyrin derivative (HpD) in tumours. As a rule the activation of HpD in cells is achieved by coherent or incoherent CW red light which can penetrate rather well into tissues and be absorbed by HpD molecules.

The mechanism of toxic action of the excited molecules of HpD on the cells is still not clear. It is known³⁻⁶ that the CW excitation of hematoporphyrin (Hp) and HpD in solutions produces a series of reactive intermediates ($^1\text{O}_2$, $\text{O}_2^{\bullet-}$, $\text{OH}^\bullet$). The role of these species in the cytotoxic action has not yet been clearly defined¹⁻³.

The role of light intensity in the decomposition of thymine sensitised by Hp and HpD has been studied earlier⁷ by analysing the thymine photoproducts by high performance liquid chromatography (HPLC) technique. It has been shown⁷ that with $I \geq 10^4\text{ W cm}^{-2}$ (337.1 nm), Hp and HpD were excited by two quantum mechanism, resulting in thymine products whose quantum yields were proportional to I^2 . However, such dependence could not be observed at CW excitation of the sensitisers. In the latter case, the main products of thymine were somewhat dependent on the scavengers of the intermediates ($\text{H}^\bullet$, $\text{OH}^\bullet$,

$^1\text{O}_2$). However, for both CW and high-power pulsed excitation of Hp and HpD the resulting products of thymine were almost the same⁷.

The purpose of the present investigation was to study the effect of different quenchers on the efficiency of formation of thymine photoproducts in order to understand the mechanism of sensitised decomposition of thymine by Hp and HpD under CW excitation. Thymine, being a constituent of nucleic acids, is a potential "target" which can be acted upon by the excited molecules of HpD in the cells, leading to the destruction of the former^{8,9}. The available aqueous solution of HpD ("Photofrin" medicine) is a composite mixture of porphyrins and so it would be rather difficult to interpret the experimental results. In our experiments, therefore, pure Hp and sodium salt of protoporphyrin-IX ($\text{Na}_2\text{-PP}$) were used.

Materials and Methods

Hematoporphyrin (Hp) was synthesised and purified at the Physical Institute, USSR Academy of Sciences (Minsk). Protoporphyrin-IX sodium salt (Sigma), sodium azide (Reachim, USSR) and tryptophan (Reachim, USSR) were certified pure compounds and were used as such.

Fresh porphyrin solutions were prepared as follows. In a 2% solution of Triton X-100 (TX-100) in phosphate buffer (0.1 mol dm^{-3} , pH 7.4; 100 ml), thymine was dissolved to a concentration of $10^{-2}\text{ mol dm}^{-3}$. Porphyrins were added to an aliquot (4 ml) of this solution to get a concentration of $8 \times 10^{-5}\text{ mol dm}^{-3}$ as determined spectropho-

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tometrically. All the solutions were prepared in triply distilled water. Before irradiation the solutions were filtered through 0.2 micron membrane filters. The samples were irradiated in quartz test tubes of 1.5 cm internal diameter or in quartz cells of 0.2 cm path length. The test tubes containing the sample (4 ml) were irradiated in a Rayonet photochemical reactor (Southern New England Ultra Violet Company, USA), fitted with 16 "black phosphor" coated low pressure Hg lamps (output range 320-380 nm approximately, with a peak at 350 nm) in a cylindrical geometry, giving about 1×10^{16} photons $\text{cm}^{-2}\text{s}^{-1}$. The cells with 0.25 ml of sample were irradiated by light in the wavelength range 330-490 nm using a mercury quartz lamp and a set of appropriate glass filters.

High pressure liquid chromatography (HPLC) was employed to separate thymine photoproducts. Preliminary qualitative results were obtained with a Laboratory Data Control Chromatograph (USA) equipped with a C-6 column (length 250 mm, i.d. 4.6 mm, spherisorb C-6, 5 μm). The elution rate of triply distilled water was 0.75 ml min^{-1} . The quantitative measurements were carried out using a Spectra Physics Chromatograph (USA) (SP 8700 solvent delivery system, SP 8750 organizer, SP 8400 UV/vis detector and SP 4100 computing integrator) with a RP-18 column (C-18, 10 μm Lichrosorb, 250 mm length, 4.6 mm i.d.). The rate of elution by 3% methanol in triply distilled water was $\sim 1 \text{ ml min}^{-1}$. In both cases the detection of thymine products was performed at $\lambda = 206 \text{ nm}$, but other wavelengths were used whenever necessary.

Results and Discussion

In the present experiments, the aim was to study the effect of different quenchers on the efficiency of formation of thymine photoproducts in order to understand the mechanism of the photochemical reactions between excited porphyrins and thymine.

Figure 1 shows the absorption spectrum of thymine-Hp solution, showing that in experiments reported below, excitation light was exclusively absorbed by the porphyrin. Thus direct excitation of thymine to its excited state or energy transfer from the excited porphyrin to thymine are improbable. Any photosensitisation leading to thymine photoproducts must therefore involve an indirect mechanism.

Figure 2 shows a HPLC chromatogram of the Hp-thymine mixture irradiated by 330-490 nm light. The first and last intense peaks correspond

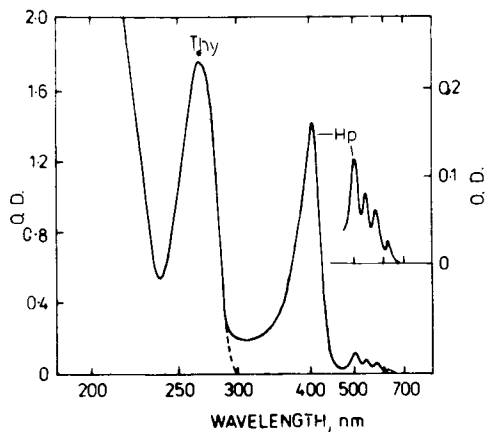


Fig. 1—Absorption spectrum of mixture of thymine ($2.1 \times 10^{-3} \text{ mol dm}^{-3}$) and Hp ($1.1 \times 10^{-4} \text{ mol dm}^{-3}$) in 2% TX-100 aqueous solution [The dashed line corresponds to the absorption tail of thymine in the absence of Hp]

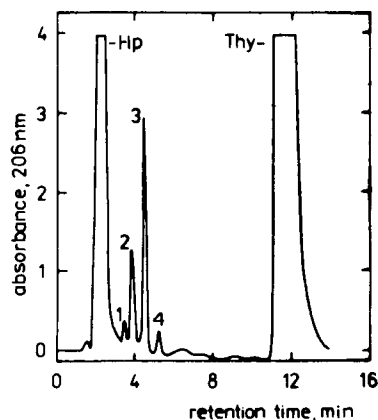


Fig. 2—HPLC of the mixture of thymine and Hp after irradiation with 330-490 nm CW light [The sample volume is 0.25 ml, the absorbed light dose is 5 J. Peaks 1,2,3 correspond to the products of sensitized decomposition of thymine]

to Hp and thymine respectively. Although the peaks 1-3 (Fig.2) correspond to the decomposition products of thymine photosensitized by Hp, no attempt was made to unambiguously identify these peaks¹⁰. As is evident from Fig.2, peak 3 is the most intense at 206 nm. The integrated area of peak 3 is higher than those of peaks 1 and 2 by about 24 and 2 times respectively. Peak 4 is attributed to an admixture of thymine with 5-hydroxymethyluracil.

The products obtained in the photo-sensitized oxidation of thymine by 2-methyl-1,4-naphthoquinone (menadione) in aqueous oxygenated solutions have been analysed earlier¹⁰. Although all the photoproducts were not identified unambiguously, it was observed that the photoproducts

were more or less similar to those produced by the gamma radiolysis of oxygenated solutions of thymine. Furthermore, employing ^{14}C -labelled thymine in the photo-sensitisation experiments, it was shown that the prominent peaks seen in HPLC with UV absorbance detector set at 229 nm correlated well with the major photoproducts of sensitised oxidation of thymine as estimated by the tracer technique¹⁰.

In our experiments, the products were also monitored at several other wavelengths including 229nm¹⁰ at which the extinction coefficients of both saturated and unsaturated pyrimidines would have comparable values. In these analyses also, the peak 3 was found to be the most intense in HPLC showing that this peak corresponded to a prominent product from the photo-sensitised damage of thymine. However, since the sensitivity for detection at 206nm was better than that at 229nm, the chromatograms were routinely monitored at 206nm.

The yields of products, determined from the corresponding integrated absorption peaks, increased linearly with absorbed light energy at least upto 20 J, when the sample volume was 0.25 ml. Similar products with almost the same yield were formed when thymine was sensitised by $\text{Na}_2\text{-PP}$.

When Hp is excited to the first singlet excited state (S_1), intersystem crossing to the first long-lived triplet states (T_1) occurs¹¹ with a high quantum efficiency of 0.92. So it may be assumed that the triplet state of Hp takes part in the sensitised photodecomposition of thymine. However, it is known^{3,12} that the triplet state energy of many porphyrins, including Hp and $\text{Na}_2\text{-PP}$, is effectively transferred to molecular oxygen ($^3\text{O}_2$) forming chemically active singlet $^1\text{O}_2$. The reaction rate constant of $^1\text{O}_2$ with thymine is rather low¹³, e.g. $k \leq 1 \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. If $^1\text{O}_2$ was to participate in the modification of thymine, the quenchers of $^1\text{O}_2$ must affect the yield of thymine products. Therefore, ascorbate ion (AA), tryptophan (TRP) and sodium azide (NaN_3) were used as quenchers of $^1\text{O}_2$.

The results of measurements of the relative efficiency of product 3 formation are given in Figs. 3 and 4. It can be seen that for both Hp and $\text{Na}_2\text{-PP}$, the yield of the product 3 is most sensitive to the addition of AA. The relative yield decreases to half when AA concentration is $1 \times 10^{-4} \text{ mol dm}^{-3}$. In presence of $1 \times 10^{-3} \text{ mol dm}^{-3}$ AA, the yield of product 3 is almost zero, but the yield of the product 1 increases by 2.5-3 times and the in-

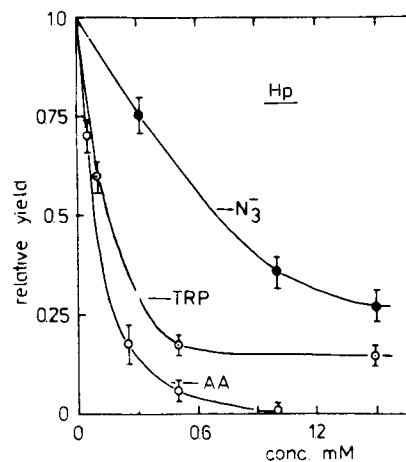


Fig. 3—Effect of addition of AA, TRP and N_3^- on the yield of thymine product 3 sensitised by Hp [The sample volume is 0.25 ml and the absorbed light dose is 5 J]

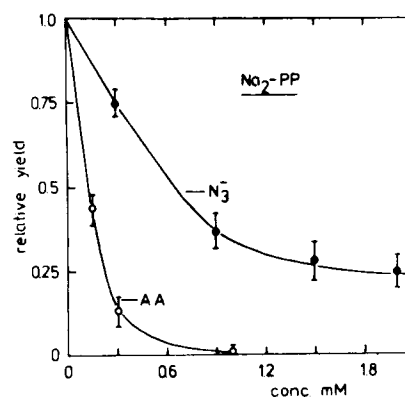


Fig. 4—Effect of AA and N_3^- on the yield of thymine product 3 sensitized by $\text{Na}_2\text{-PP}$ [The sample volume is 0.25 ml and the absorbed light dose is 5 J]

tegrated area under peak 2 decreases by $\sim 35\%$ due to its narrowing. This means that peak 2 corresponds to at least two thymine products. When the concentration of tryptophan is upto $5 \times 10^{-4} \text{ mol dm}^{-3}$, it is an effective quencher of the sensitised decomposition of thymine. At higher concentrations (upto $1.5 \times 10^{-3} \text{ mol dm}^{-3}$) thymine product yield remains practically constant (Fig.3). Tryptophan has qualitatively the same effect on the yield of all thymine products.

Sodium azide was found to be the least effective quencher of thymine photoproducts formation (Figs. 3 and 4). For example, at a concentration of $5.7 \times 10^{-4} \text{ mol dm}^{-3}$ sodium azide, the relative yield of product 3 slowly decreases and reaches a value of 0.25 when the azide concentration is $2 \times 10^{-3} \text{ mol dm}^{-3}$. The yields of the other thymine products decrease likewise.

Product 3, the integral absorption of which at 206 nm is 60-70% of all the thymine decomposition products, cannot be explained by the participation of $^1\text{O}_2$. As far as the quenching efficiency of product 3 formation is concerned, the quencher molecules follow the order: $\text{AA} > \text{TRP} > \text{NaN}_3$. But this result does not agree with the rates of $^1\text{O}_2$ quenching by these molecules¹³ ($k = 8.3 \times 10^6$, 2.5×10^8 and $2.3 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ for AA, TRP and NaN_3 respectively) since NaN_3 and AA are the most and least effective quenchers of $^1\text{O}_2$ respectively.

It may be assumed that the sensitised formation of thymine product 3 in the absence of AA occurs in agreement with type-I mechanism which includes an electron transfer from thymine to the excited triplet state of Hp. With addition of AA the yield of thymine product 3 decreases due to the competition between thymine and AA as electron donors (AA is a very good electron donor¹⁴) for the triplet state of the porphyrins.

The assumption qualitatively agrees with other results^{15,16} on the relative role of type-I and type-II photoreactions sensitised by Hp and HpD. For example, it has been shown¹⁵ that at Hp concentration of $5 \times 10^{-5} \text{ mol dm}^{-3}$, photo-oxidation of TRP in the presence and in the absence of sodium dodecyl sulphate micelles occurs in accordance with the type-I mechanism. In this experiment the electron, however, is assumed to be transferred from the triplet state of Hp to tryptophan.

In the lysis of phosphatidylcholine liposomes sensitised¹⁶ by Hp and HpD, it was found that for small Hp concentrations ($\leq 1 \times 10^{-6} \text{ mol dm}^{-3}$) the lysis of liposomes could be explained by the participation of singlet oxygen (type-II mechanism), whereas, at higher concentrations ($15 \times 10^{-5} \text{ mol dm}^{-3}$) the destruction of liposomes by type-I mechanism prevailed. Methylene blue¹⁷ and 2-methyl-1,4-naphthoquinone¹⁰ also sensitise photodecomposition of thymine by an electron transfer to the excited triplet states of the sensitisers.

The results of the present experiments show that in aqueous solutions containing TX-100 micelles the role of $^1\text{O}_2$ in the decomposition of thymine is not important. This is in agreement with an observation¹⁸ that guanine is the only base of nucleic acids which can be modified to a great extent by $^1\text{O}_2$. Under modified conditions, however (thymine adsorbed on the surface of silica gel)¹⁹, thymine can be affected by $^1\text{O}_2$.

In the presence of *n*-butanol, an effective scavenger of $\text{H}^\bullet$ and $\text{OH}^\bullet$ radicals, peak 1 decreased by half when *n*-butanol concentration was

$6 \times 10^{-2} \text{ mol dm}^{-3}$ and could not be observed when *n*-butanol concentration was 0.18 mol dm^{-3} . Thus $\text{OH}^\bullet$ radicals are probably involved in the formation of the product 1. In a concentration range of upto 0.18 mol dm^{-3} the integral absorption under peak 2 remained constant and that under peak 3 increased by ~ 1.5 times.

On the addition of $^1\text{O}_2$ quenchers, not only there was a decrease in the yield of the main thymine product 3 but also an increase in the yield of some products; e.g. ascorbic acid favoured the formation of product 1.

Thus the effect of quenchers on the yield of thymine photoproducts 1,2 does not allow us to make unambiguous conclusions about the mechanism of their formation.

The experimental results obtained here enable us to conclude that: (i) Hp and $\text{Na}_2\text{-PP}$ under CW excitation sensitise the decomposition of thymine in aqueous solution of TX-100 leading to formation of products 3-4; (ii) product 3 which is dominant at 206 nm is apparently formed by type-I mechanism with an electron being transferred from thymine to the excited triplet state of the sensitisers; and (iii) the role of $^1\text{O}_2$ in the thymine modification at the porphyrin concentration of $8 \times 10^{-5} \text{ mol dm}^{-3}$ is negligible.

Acknowledgement

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