Investigation of the Quantum Yield of the Dye-Sensitized Photoinactivation of Ribonuclease

Quantum yields for the photodynamic inactivation of trypsin as a function of several kinetic variables have been reported earlier[1]. The present paper describes the preliminary results of a similar study using bovine pancreatic ribonuclease A. Although the photodynamic inactivation of ribonuclease has been studied rather extensively[2–8], no quantum yield data for the process have been published.

Materials and methods. Three dyes were used as sensitizers: methylene blue (MEB), eosin Y (EOY), and flavin mononucleotide (FMN). 2 ml of a reaction mixture containing ribonuclease, phosphate buffer, and sensitizing dye, were illuminated in a temperature-controlled plexiglas cell by a 500-watt slide projector provided with Baird-Atomic multi-layer interference filters: 6750 Å with MEB, 5170 Å with EOY, and 4370 Å with FMN. The optical densities of the reaction mixtures at the wavelengths of irradiation were 1.3 for MEB, 1.9 for EOY, and 3.0 for FMN, as measured with a Cary Model 14 spectrophotometer using a silica cell with a 1 cm path length. The light energy absorbed by the system was measured with a thermopile-millimicrovoltmeter combination calibrated with a standard lamp. The light intensity was adjusted to deliver equal photon fluxes at the 3 wavelengths used. Standard reaction conditions of 36.6 μM ribonuclease, 0.125 M phosphate buffer at pH 8, $3\times10^{-5}M$ MEB and EOY, $3\times10^{-4}M$ FMN, 15 °C, 8.34×10^{15} photons/cm²-sec, carried out in air with stirring, were used except as noted below. Ribonuclease activity was assayed after 0, 5, 10, 15, and 20 min of illumination using cytidine-2′, 3′-cyclic phosphate as substrate. The inactivation process followed first-order kinetics. Control experiments were: (1) irradiation of the reaction mixture minus the dye, and (2) maintaining the reaction mixture containing the dye in the dark for the appropriate length of time. Quantum yields, defined as the number of ribonuclease molecules inactivated per number of photons absorbed by the dye, were calculated as before[1] from the initial rate of loss of enzymic activity divided by the initial rate of absorption of photons by the system.

Results and discussion. No inactivation of the enzyme was observed in either the light or the dark control experiments.

The quantum yield was independent of light intensity over the range 4.17×10^{15} to 11.3×10^{15} photons/cm²-sec with all 3 dyes used as sensitizers. Similarly, the concentration of phosphate buffer did not markedly affect the quantum yield over the range 0.0625 M to 0.250 M. In contrast, as the concentration of ribonuclease was increased from 18.3 to 73.2 μM, the quantum yields nearly tripled with all 3 dyes.

Quantum yields of inactivation of ribonuclease as a function of various other reaction conditions are shown in Figures 1–4. As can be seen in Figure 1, MEB and EOY were most efficient as sensitizers at a concentration of $3\times10^{-5}M$, whereas FMN sensitized most efficiently at 10 times this concentration. As is typical in these investigations the EOY- and FMN-sensitized systems had quantum yields of nearly the same magnitude, while the values for the MEB-sensitized system were lower. With all 3 dyes used as sensitizers, the quantum yield increased rapidly with increasing oxygen concentration, then leveled off above 10–15% oxygen, as shown in Figure 2.

With increasing pH, the quantum yields with all 3 dyes remained quite low until pH 6, and increased sharply from pH 6 to 8. Above pH 8 the quantum yields increased with MEB or EOY as sensitizers, but decreased for the FMN-sensitized system. The pH-dependence of the quantum yield indicates that the inactivation of ribonuclease might be due primarily to a destruction of

[1] B. W. Glad and J. D. Spikes, Radiat. Res. *27*, 237 (1966).
[2] D. Shugar, Bull. Soc. Chim. biol. *33*, 710 (1951).
[3] L. Weil and T. S. Seibles, Arch. Biochem. Biophys. *54*, 368 (1955).
[4] B. R. DasGupta and D. A. Boroff, Biochim. biophys. Acta *97*, 159 (1965).
[5] K. Waku and Y. Nakazawa, J. Biochem., Tokyo *57*, 578 (1965).
[6] U. W. Kenkare and F. M. Richards, J. biol. Chem. *241*, 3197 (1966).
[7] G. Jori, G. Galiazzo, A. Marzotto and E. Scoffone, Biochim. biophys. Acta *154*, 1 (1968).
[8] F. Sawada, J. Biochem., Tokyo *65*, 767 (1969).

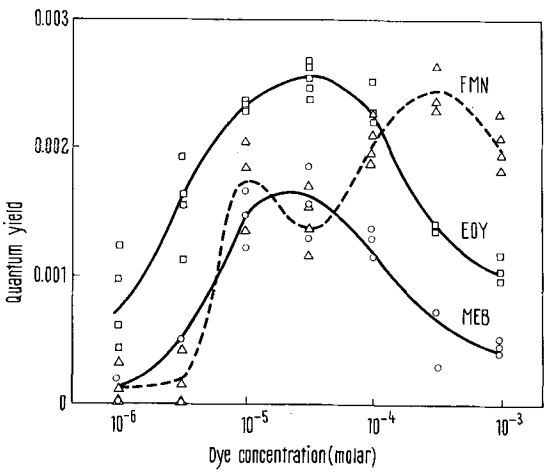

Fig. 1. Effect of dye concentration on the quantum yield of the photodynamic inactivation of ribonuclease A. Standard reaction conditions, except dye concentration, as described in the text were used. The sensitizing dyes used were methylene blue (MEB), eosin Y EOY) and flavin mononucleotide (FMN).

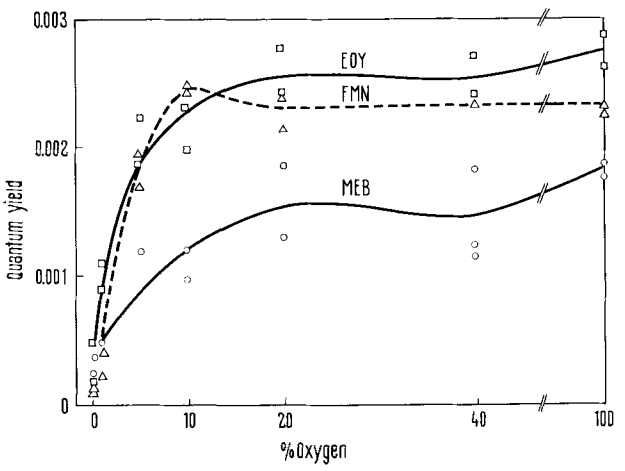

Fig. 2. Effects of oxygen concentration on the quantum yield of the photodynamic inactivation of ribonuclease A. Standard reaction conditions, except oxygen concentration, were used. Mixtures of various percentages of oxygen in nitrogen were bubbled through the reaction mixture during illumination.

histidyl residues, for the photodynamic destruction of histidine shows a very similar pH-dependence[9-10].

The quantum yield values increased gradually with increasing temperature until approximately 50 °C, above which the values increased markedly with all 3 sensitizing dyes, as shown in Figure 4. This increase in yield probably results from the thermal unfolding of ribonuclease[11] which presumably exposes more residues susceptible to photodynamic oxidation. At temperatures greater than 65 °C the inactivation of the enzyme in the dark, presumably due to thermal denaturation, became appreciable. Therefore quantum yield values could not be determined accurately at these higher temperatures.

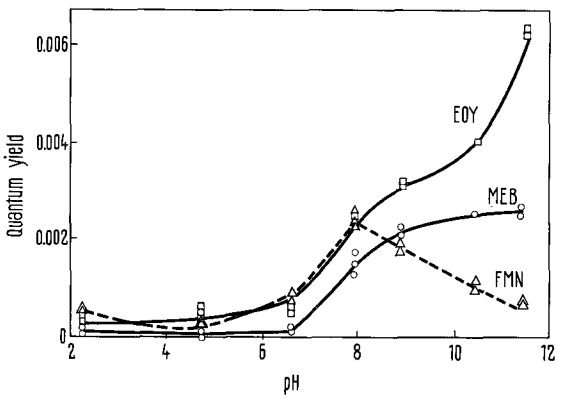

Fig. 3. Effect of pH on the quantum yield of the photodynamic inactivation of ribonuclease A. Standard reaction conditions, except pH, were used.

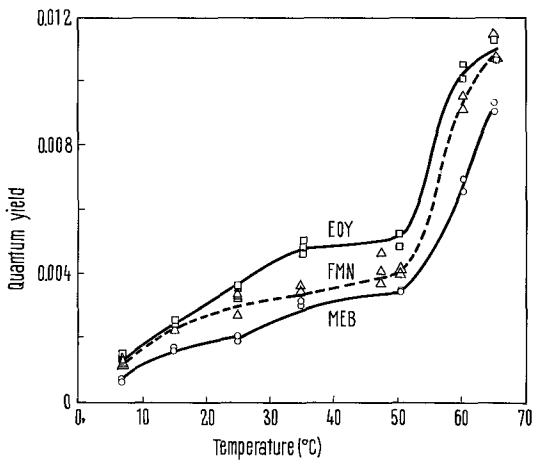

Fig. 4. Effect of temperature on the quantum yield of photodynamic inactivation of ribonuclease A. Standard reaction conditions, except temperature, were used.

Arrhenius plots of these data yielded approximate energies of activation of 9.3, 9.1, and 3.9 kcal/mole for the MEB-, EOY-, and FMN-sensitized systems, respectively, for temperatures below 50 °C. Above 50 °C the corresponding values were 15.0, 14.7, and 15.3 kcal/mole.

In general, for all the conditions studied, the MEB-sensitized system responded in much the same way as the EOY-sensitized system, although the magnitude of the quantum yield values was less with MEB. The FMN-sensitized system, however, behaved in a somewhat different manner in response to the reaction conditions examined, indicating that perhaps FMN sensitizes the inactivation by way of a different mechanism from that of the other two dyes[12].

Résumé. L'efficacité quantique de la photooxydation de la ribonucléase A sensibilisée par le bleu methylène, l'éosine Y et par FMN a été étudié sous plusieurs conditions de réaction. On a varié le pH, la température, l'intensité de la lumière et les concentrations de la ribonucléase, du tampon phosphate, de l'oxygène et des colorants.

MARTHA L. MACKNIGHT and J. D. SPIKES

Department of Biology, University of Utah, Salt Lake City (Utah 84112, USA), 27 August 1969.

[9] L. WEIL, W. G. GORDON and A. R. BUCHERT, Arch. Biochem. Biophys. *33*, 90 (1951).
[10] L. WEIL, Arch. Biochem. Biophys. *110*, 57 (1965).
[11] W. F. HARRINGTON and J. A. SCHELLMAN, C. r. Trav. Lab. Carlsberg *30*, 21 (1956).
[12] This work was supported by National Science Foundation Graduate Fellowship stipends to M.L.M. and by the U.S. Atomic Energy Commission under Contract No. AT(11-1)-875.

Protoporphyrines Erythropoiétiques et Griséofulvine

L'action de la griséofulvine sur le métabolisme des protoporphyrines a été étudiée au cours de ces dernières années. Administrée chez le rat à la dose journalière de 2 à 4 g elle provoque un tableau biologique de porphyrie à la fois hépatique et érythropoiétique: élimination urinaire importante d'acide deltaaminolevulinique et de porphobilinogène, excrétion fécale massive de proto et coproporphyrines érythrocytaires (DE MATTEIS et RIMINGTON[1]). Chez l'homme atteint de porphyrie aiguë la griséofulvine peut être responsable d'une poussée sévère, parfois mortelle (EALES[2], REDECKER[3]). Chez le sujet normal, l'action de la griséofulvine a été étudiée par RIMINGTON[4], WATSON[5] et ZIPRKOWSKI[6], plus particulièrement sur les porphyrines fécales.

Nous avons étudié les protoporphyrines érythropoiétiques (PPE) chez 14 malades traités pour des trichophyties par la griséofulvine à la dose journalière de 1 à 1,5 g, depuis 2 mois au maximum. L'âge de ces 14 malades, 12 hommes et 2 femmes, s'échelonnait de 15 à 69 ans.

Le dosage des protoporphyrines érythropoiétiques a été effectué selon la méthode de Wranne. Après précipitation par un mélange, acétate d'éthyle – acide acétique, puis extractions successives par l'acide chlorhydrique 5*N*,

[1] F. DE MATTEIS et C. RIMINGTON, Br. J. Derm. *75*, 91 (1963).
[2] L. EALES, S. Afr. J. Lab. clin. Med. *9*, 304 (1963).
[3] A. REDECKER, R. STERLING et R. BRONOW, J. Am. med. Ass. *188*, 466 (1964).
[4] C. RIMINGTON, P. MORGAN, K. NICHOLLS, J. EVERALL et R. DAVIES, Lancet *2*, 318 (1963).
[5] C. WATSON, F. LYNCH, I. BOSSENMAIER et R. CARDINAL, Arch. Derm. *98*, 451 (1968).
[6] L. ZIPRKOWSKI, M. CRISPIN et A. SZEINBERG, J. Isr. med. Ass. *70*, 415 (1964).