The Inhibition of the Dye-Sensitized Photoinactivation of Trypsin Using Tyrosine and Thyronine Analogues

We reported earlier that certain tyrosine and thyronine analogues exhibited protective effects on the dye-sensitized photoinactivation of trypsin[1]. Results using additional analogues and an additional photosensitizing dye, eosin Y, are described in the present paper. The protective compounds used were potassium iodide, L-tyrosine (TYR), 3-monoiodo-L-tyrosine (MIT), 3, 5-diiodo-L-tyrosine (DIT), DL-thyronine (THYR), 3, 5-diiodo-L-thyronine (T_2), 3, 3', 5-triiodo-L-thyronine (T_3), and L-thyroxine (T_4).

Quantum yields for the photodynamic inactivation of trypsin were determined over the range of 0–1000 μM inhibitor concentration. The reaction mixtures were 42 μM in trypsin, 0.125 M in sodium phosphate buffer at pH 8, with 150 μM riboflavin-5'-phosphate (FMN), 25 μM eosin Y or 12.5 μM methylene blue. The reaction mixture was illuminated at 15 °C while being stirred. Illumination was provided either by a 500 W slide projector (for the methylene blue system at 6750 Å and the eosin Y system at 5170 Å) or by a General Electric A-H6 mercury arc lamp for the FMN system at 4370 Å. The wave-lengths desired were obtained using multilayer interference filters, and the light energy absorbed by the reaction system was measured either by a vacuum thermocouple-galvanometer combination, or by a thermopile-millimicrovoltmeter combination, each previously calibrated with a standard lamp. Tryptic activity was determined after 0, 5, 10, 15 and 20 min of illumination using benzoyl-L-arginine ethyl ester (BAEE) as substrate; inactivation was first-order in all cases. Quantum yields for the inactivation are defined as $(dS/dt)_0/(dQ/dt)_0$, where the numerator is the rate of loss of enzymatic activity and the denominator is the rate of loss of absorption of photons, both at zero time. These experimental methods have been described in detail elsewhere[2].

The quantum yields for the dye-sensitized photoinactivation of trypsin as influenced by the protective compounds listed above are shown in Figures 1–3. All 8 compounds have very similar protective activities as a function of concentration when FMN is used as the photosensitizer, even though they differ markedly on a chemical basis (Figure 1). Potassium iodide is thought to quench long-lived excited states of FMN[3–6], whereas tyrosine and

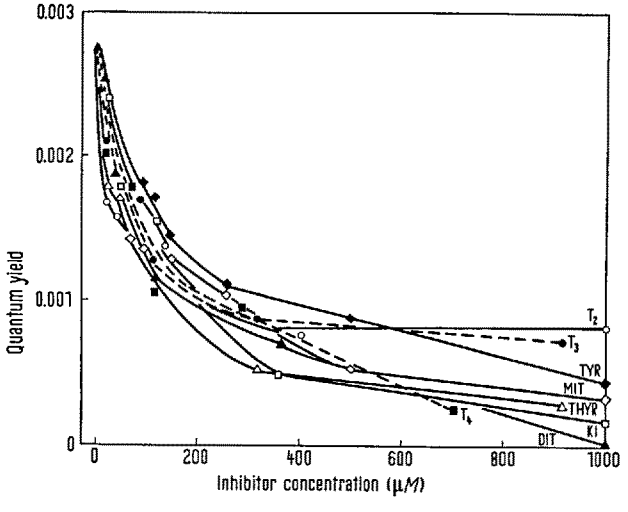

Fig. 1. Effects of potassium iodide and tyrosine and thyronine analogues on the photodynamic inactivation of trypsin in the presence of 150 μM FMN. The reaction mixture was 42 μM in trypsin and 0.125 M in sodium phosphate buffer at pH 8 and 15 °C. The following inhibitors were used in the concentrations indicated: potassium iodide (KI), L-tyrosine (TYR), 3-monoiodo-L-tyrosine (MIT), 3,5-diiodo-L-tyrosine (DIT), DL-thyronine (THYR), 3,5-diiodo-L-thyronine (T_2), 3,3',5-triiodo-L-thyronine (T_3), and L-thyroxine (T_4).

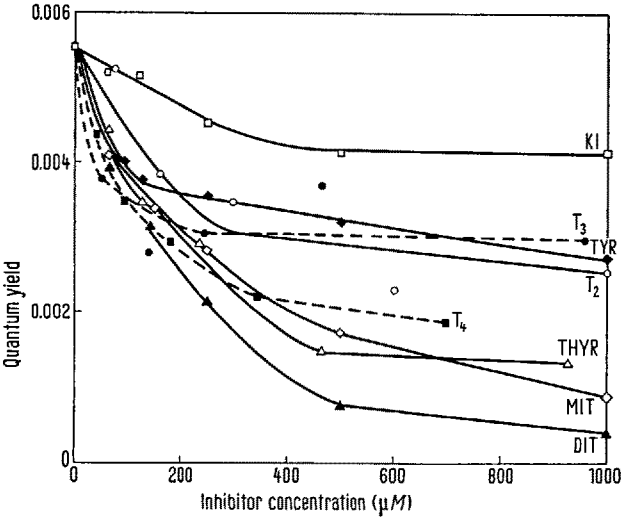

Fig. 2. Effects of potassium iodide and tyrosine and thyronine analogues on the photodynamic inactivation of trypsin in the presence of 25 μM eosin Y. The reaction mixture and protective agents used were the same as given for Figure 1.

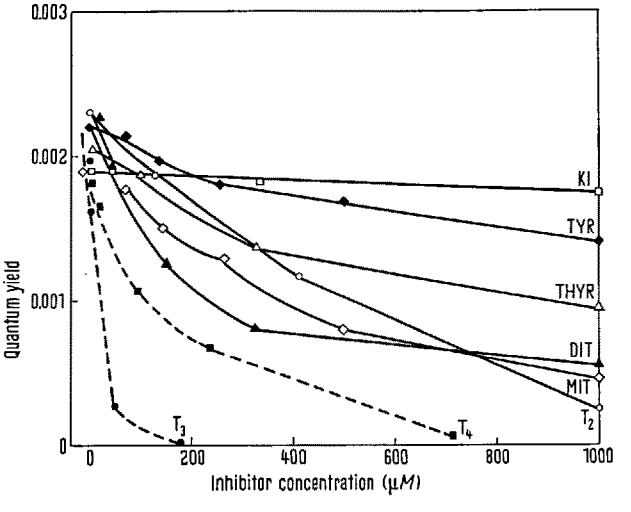

Fig. 3. Effects of potassium iodide and tyrosine and thyronine analogues on the photodynamic inactivation of trypsin in the presence of 12.5 μM methylene blue. The reaction mixture and protective agents used were the same as given for Figure 1.

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thyronine probably act as 'competitive' inhibitors[7-9]. Studies with FMN are complicated by the fact that the iodinated forms of tyrosine and thyronine are deiodinated upon illumination in the presence of this sensitizer[10-13]. If photochemical deiodination occurred rapidly in the present experiments, some of the protective compounds present could change significantly during the period of illumination. This might account for the very similar results obtained with the different protective agents when FMN is used as the sensitizer.

The protective efficiencies of the different compounds varied widely with eosin Y as sensitizer as shown in Figure 2; for example, DIT was a good protector while potassium iodide showed relatively little protective effect. Eosin sensitizes the photochemical deiodination of T_4[14], although its efficiency in this respect as compared to FMN is not known. The sensitivities of the other protective compounds in our series to photochemical deiodination with eosin have not been determined.

Finally, the results with methylene blue as sensitizer are described in Figure 3. As may be seen, the protective efficiencies of the different compounds vary enormously. In contrast to FMN and eosin, methylene blue does not sensitize the photochemical deiodination of T_4[14]. The methylene blue system is interesting because the degree of inhibition demonstrated by these protective compounds is similar, in general to their known biological metabolic activity[15]. This system, then, may merit further study as a potentially simple photochemical system for the in vitro assay of thyroid compounds and congeners in terms of their likely pharmacological activity[16].

Zusammenfassung. Es wurde Tyrosin- und Thyroninwirkung auf die farbensensibilisierende Photoinaktivierung des Trypsins untersucht. Sensibilisatoren waren Methylenblau, Flavinmononukleotid und Eosin Y. Die Analoge von Tyrosin und Thyronin haben sehr ähnliche konzentrationsabhängige Schirmwirkung bei Verwendung von Flavinmononukleotid als Photosensibilisator. Die Abschirmungseffekte der Sensibilisatoren werden besprochen.

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Formation of Hydroxyapatite Nuclei Induced by Dehydration of Calcifying Solutions

Of fundamental importance to our understanding of the process of biological calcification is knowledge of the mechanism(s) which induce the formation of nuclei of crystallization of hydroxyapatite from calcifying media. Nuclei formation is a phase transition and may be described as the emergence from a metastable solution of the first microaggregates (nuclei) of a new solid phase. Based on theoretical studies of energy requirements[1], nuclei formation is a distinct process and should be considered separately from the subsequent step of growth of these nuclei into larger aggregates of crystals of hydroxyapatite. Although our knowledge of the kinetics of nuclei formation is meager, empirical data[2] would indicate that nucleation of calcium phosphate salts is favored by an elevation of the activity product of calcium and phosphate ions. In a biological calcifying system in dynamic equilibrium, an increase in the activity product of calcium and phosphate ions could be simply accomplished by the addition of a quantity of one or both ions. Alternatively, removal of water from the system would result in a relative increase in calcium and phosphorus ion product without necessitating an absolute increase. It is known that the first case is true[3], but the alternative, although appearing obvious is not fully documented by direct experimentation. Thus in the foregoing experiment, we examined this premise that nuclei of crystallization of hydroxyapatite could be induced to form in a nuclei-free metastable calcifying solution by the simple technique of partially removing water from the system.

Materials and methods. The barbiturate-buffered calcifying solution of Fleisch and Neuman[4] was used in this experiment. In our test system, we utilized the simple expedient of slow freezing to remove water from the calcifying solutions. 100 ml of barbiturate-buffered calcifying solutions (nuclei-free), pH 7.4 $\pm$ 0.05, with Ca $\times$ P products ranging from 25–60 were placed in 125 ml glass bottles. The lower third of the bottles were immersed in an ice-salt bath at $-$ 10 °C. After an interval of approximately $^1/_2$ h, ice crystal began to form in the bottom of the bottles and rapidly spread toward the top. When the ice crystals occupied approximately 50% of the total volume of calcifying solution, the bottles were rapidly withdrawn from the ice bath and the concentrated liquid supernatant phase quickly decanted and saved. Controls were of 2 types: (a) untreated calcifying solutions and (b) calcifying solutions which were partially frozen as above and then

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