Radioprotective action of haematoporphyrin on X-irradiated trypsin

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Summary. Dose-response curves were obtained for trypsin X-irradiated in aqueous solution (4.6×10^{-6}M), alone or after incubation with haematoporphyrin (Hp). When trypsin was irradiated alone (exposure doses ranging from 10 to 25 kR) the exponential dose-response curve showed a D_{37} equal to 17200 R. When trypsin was incubated with Hp before irradiation a D_{37} equal to 61900 R was obtained, with a dose reduction factor of 3.6. Therefore in our experimental conditions Hp shows a highly significant radioprotective action.

Trypsin inactivation by ionizing radiations is oxygen-dependent and affected by the pH and the nature of the anions present in the trypsin solution[2]; hydrogen transfer mechanisms seem to play an important role in the repair of trypsin damage by ionizing radiations[3].

Haematoporphyrin (Hp) forms reversible complexes with many types of biomolecules by weak chemical bonds[4]. While much is known about the in vivo effects of Hp administered before whole-body irradiation few data are available from in vitro experiments: Hp induces the oxidative photoinactivation of lysozyme, but protects it against radioinactivation[5]; Hp shows a radioprotective action on aldolase[6].

In this paper we report the effects of Hp on the radiosensitivity of trypsin irradiated in water solution.

Materials and methods. Crystalline trypsin was purchased from Biochemia SRL (Milano, Italy); haematoporphyrin disodium salt, chromatographically pure, was purchased from Zilliken (Genova, Italy); DL-PABA (benzoyl DL-arginine p-nitroanilide hydrocloride) was purchased from Sigma Chemical Company. Direct and indirect absorption spectra were preliminarily evaluated.

Indirect spectrum. Trypsin and Hp were dissolved in water at a molarity ratio Hp versus trypsin 10:1 (Hp=2×10^{-5}M, trypsin 2×10^{-6}M); 5-ml fractions of the solution were incubated at room temperature for 4 h.

Direct spectrum. Trypsin was dissolved in water at the final concentration of 2×10^{-6}M and incubated at room temperature as above. The pH of trypsin solution in water was 5, while the pH of trypsin plus Hp solution in the same solvent was about 6. In some experiments trypsin was dissolved in Tris buffer, pH 7.8, so to ascertain the effects of the pH on the behaviour of the absorption spectra.

Spectra were read in the wavelength range from 300 to 500 nm: indirect spectrum was read against a blank of 2×10^{-5}M Hp in water, direct spectrum against water.

Irradiation. 2 series of experiments were set up: in the 1st trypsin alone, 4.2×10^{-6}M in water, was irradiated in 1-ml fractions; in the 2nd, trypsin was preincubated with Hp at room temperature for 4 h in a molarity ratio Hp versus trypsin 10:1 in water solution (Hp=4.2×10^{-5} M, trypsin 4.2×10^{-6} M) and afterwards irradiated in 1-ml fractions. 3 non-irradiated controls were prepared in both series of experiments. Irradiation was performed in plexiglass containers through a bottom 5-mm-thick: exposure doses ranged from 10 to 25 kR (X-rays: 200 kV_p; HVL 0.45 mm Cu; 843 R/min).

Enzyme assay. Trypsin activity was assayed using benzoyl DL-arginine p-nitroanilide hydrocloride (DL-BAPA) as substrate by the method of Erlanger et al.[7] that measures the increase in optical density at 410 nm as nitroaniline is released in the reaction. All the assays were performed in 1 h from irradiation and repeated in 6 independent experiments.

43.5 mg DL-BAPA was dissolved in 1 ml dimethylsulfoxide and the solution was brought to 100 ml with 0.05 M Tris buffer pH 8.2 containing 0.02 M $CaCl_2$: this substrate stock solution 10^{-3} M was stored at 25 °C.

Trypsin was dissolved in water at the concentration of 100 µg/ml and immediately used. At the moment of enzyme assay water (0.9 ml) was added to 5 ml of substrate stock solution and the mixture was allowed to equilibrate in a thermostatically controlled bath at 25 °C for 5 min. 0.1 ml of trypsin solution freshly prepared was added to the substrate at 25 °C and the reaction was allowed to run for 600 sec. Reaction was stopped by adding 1 ml of 30% acetic acid and the amount of released p-nitroaniline was estimated spectrophotometrically at 410 nm.

Statistical analysis of the results was performed by Student's t-test.

Results. Remarkable differences were observed between the direct and indirect absorption spectra of trypsin: we could detect in the indirect spectrum 2 peaks in the wavelength range between 370 and 400 nm which were

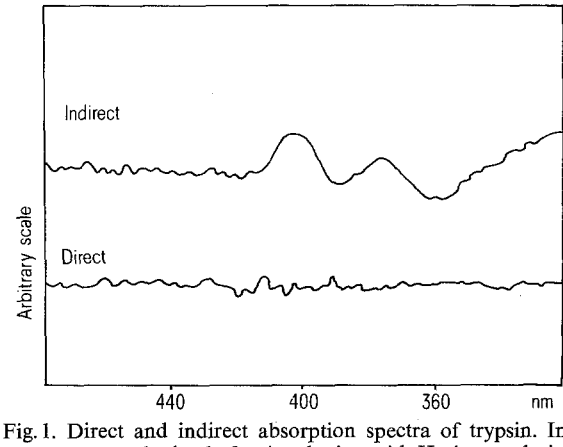

Fig. 1. Direct and indirect absorption spectra of trypsin. Indirect spectrum was obtained after incubation with Hp in a molarity ratio Hp vs trypsin 10:1 using as blank an equimolar Hp solution. Direct spectrum was read against water. Solution were incubated in full dark for 4 h at room temperature.

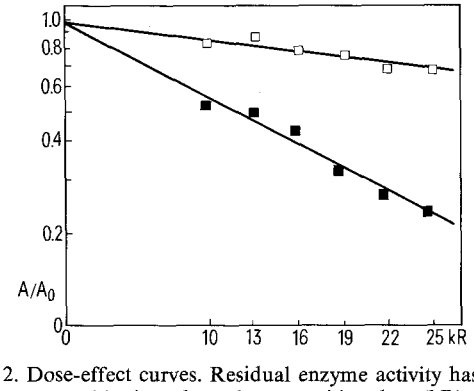

Fig. 2. Dose-effect curves. Residual enzyme activity has been plotted on a logarithmic scale against exposition dose (kR). ■, Trypsin alone; □, trypsin plus Hp.

absent in direct spectrum (figure 1). These peaks are probably the result of the binding of the porphyrin ring to the protein.

The pH of the solution had no effect on the behaviour of the absorption spectra. In the analysis of results account was taken of trypsin inactivation per se induced by Hp: the mean loss of trypsin activity in presence of Hp was 27%. Dose-response curves are shown in figure 2, where residual enzyme activity is plotted on a logarithmic scale against radiation doses (kR) in the 2 series of experiments: both curves are exponential with a D_{37} of 17,200 R in absence and 61,900 R in presence of Hp: the G values are respectively 0.19 and 0.05. Hp shows therefore a highly significant radioprotective action on trypsin irradiated in water solution ($p < 0.005$), with a dose reduction factor (DRF) of 3.6.

Discussion. This radioprotective action of Hp may depend partly on a radical scavenging mechanism by free Hp in solution, partly on a direct interference of the porphyrin ring in the dissipation of excitation energy of the protein following the binding of trypsin to Hp.

The true role of these mechanisms will be better defined by experiments performed using different molarity ratios of Hp versus trypsin and irradiating the solutions under vacuum or in pure oxygen.

The evidence of this radioprotective action suggests, as previously proposed by Cittadini[8], that the inconstant radiosensitizing effect of Hp observed in whole-body irradiated mice might be ascribed to an overlap of a Hp-induced radiodynamic disease. Finally, it is remarkable that the DRF obtained in our experiments is quite comparable to that known for the best radioprotective compounds.

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The effect of dietary administration of aspartic acid on thymus weight in C57 black mice

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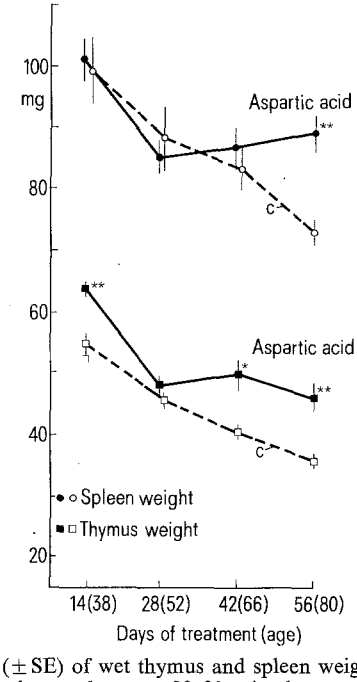
Summary. D,L aspartic acid incorporated at a level of 0.5% w/w into the diets of C57B1/10 male mice for 2–8 weeks increased significantly the thymus weight. Similar effects were observed after a 10 days' treatment of the mice with K- and Mg-salts of D,L aspartate (1%) in drinking water.

Aspartic acid deserves attention for its broad physiological and pharmacological properties. Of particular interest is its role in central nervous transmission[1] and its participation in metabolic pathways of citric acid cycle[2]. K- and Mg-salts of aspartic acid ameliorate some metabolic disturbances resulting from local as well as generalized tissue hypoxia[3]. Favourable effects of aspartates on hemopoietic cell renewal systems perturbed by radiation have been demonstrated[4]. In this communication the enhancing effect of dietary administration of aspartic acid on the weight of the thymus in mice is described.

Materials and methods. Male mice of the C57B1/10 strain were used. At the weaning age of 24 days the mice were matched according to body weight and caged in groups of 10. The experimental mice were fed with basal standard diet (20% protein) supplemented with D,L aspartic acid, which was incorporated into the pellets by food-drug trituration at a level of 5 mg/g of food (0.5%). The average daily ingestion of aspartic acid thus varied between 10 and 25 mg per mouse. The aspartic acid-containing diet, the

Mean values (± SE) of wet thymus and spleen weights in aspartate treated and control mice in the various experiments. 10–30 animals per group were used. Statistical significance as compared with controls: * $p < 0.01$

		Thymus weight (mg)	Spleen weight (mg)
Experiment 1	Aspartate	43.7 ± 1.6*	84.9 ± 2.2
	Control	37.5 ± 1.3	82.9 ± 2.7
Experiment 2	Aspartate	37.2 ± 1.9*	96.4 ± 6.5
	Control	29.5 ± 1.5	88.8 ± 5.4
Experiment 3	Aspartate	40.2 ± 1.4*	94.0 ± 2.9
	Control	29.0 ± 1.1	94.7 ± 4.3
Experiment 4	Aspartate	38.4 ± 0.6*	81.4 ± 4.1
	Control	31.7 ± 1.2	79.1 ± 3.2



Mean values (± SE) of wet thymus and spleen weights in aspartic acid treated and control groups. 20–30 animals per point were used. Statistical significance as compared with controls: ** $p < 0.001$, * $p < 0.01$.