PHOTOINACTIVATION OF L-GLUTAMATE DEHYDROGENASE IN A SPECTROPHOTOFLUORIMETER

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The inactivation of enzymes by ultraviolet radiation has been studied (Setlow and Doyle, 1957; Mee, 1964), but knowledge of which amino acid residues are affected is still incomplete. We have recently noted that beef liver L-glutamate dehydrogenase (GDH) lost enzyme activity as well as protein fluorescence under certain conditions of spectrophotofluorimetric assay. Since the fluorescence of proteins is due almost wholly to tryptophan residues (Teale, 1960), these observations seem relevant to the mechanism of photoinactivation of enzymes. This communication documents these findings and also discusses the significance of photodecomposition in fluorimetric analysis.

Materials and Methods

Crystalline beef liver L-glutamate dehydrogenase (ammonium sulfate suspension) and DPN^+ were purchased from the Sigma Chemical Company, St. Louis. DPNH was prepared by reduction of DPN^+ with $Na_2S_2O_4$ and purified by chromatography on DEAE-cellulose as previously described (Chen and Plaut, 1963). Crystalline ovalbumin was a product of Armour Labs., Chicago. Enzyme assay: The rate of oxidation of DPNH after addition of enzyme was followed spectrophotometrically by the change in A_{340}. The final reaction mixture in a 1.0 cm path length cell was at pH 7.8 and contained 3.3×10^{-2} M Tris-Cl^- buffer; 8.3×10^{-3} M α-ketoglutarate; 0.17 M NH_4Cl; 1×10^{-5} M EDTA; 8.3×10^{-5} M DPNH;

water and enzyme in a final volume of 3.0 ml. Fluorescence was measured with an Aminco-Bowman spectrophotofluorimeter fitted with a standard Osram 150 watt high pressure xenon arc lamp. The design of this instrument originated in this laboratory (Bowman et al, 1955). Photodecomposition studies were done with a quartz microcuvette having a capacity of 0.2 ml (see Figure 3). The monochromators were calibrated with a mercury arc lamp.

Results and Discussion

Figure 1 shows the following: a) When GDH was exposed to 290 mμ light in a microcuvette, the observed protein fluorescence intensity fell rapidly, so that 40% of the initial value had been lost in about 7 minutes. b) Since only part of the solution was in the light path, blockage of the activating beam for a short time resulted in an apparent "recovery" of fluorescence due to diffusion of molecules to and from other parts of the cell. c) Photodecomposition could be prevented by attenuating the activating beam with a piece of ordinary glass. d) Ovalbumin fluorescence was diminished under exposure to intense

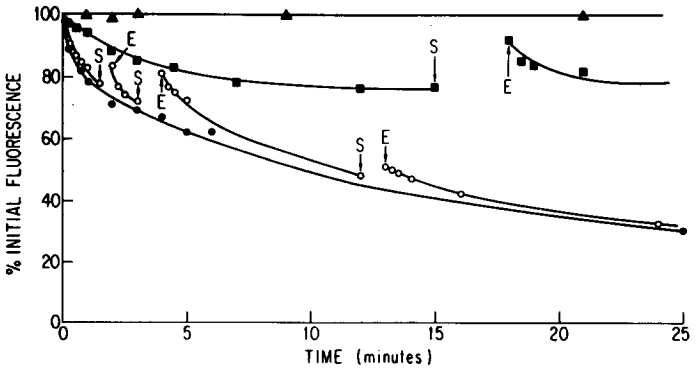

Fig. 1. Observed protein fluorescence as a function of time. Temperature, 8.0°. With the activating beam blocked, each cuvette containing 0.14 ml of solution was kept in the cell holder for 5 minutes to allow for temperature equilibration before exposure to radiation. Excitation, 290 mμ. Nominal bandwidth, 20 mμ. Emission observed at 350 mμ. The protein was dissolved in 0.10 M Tris-Cl⁻ buffer, pH 7.8. ●—● , 0.5 mg/ml GDH. ○—○ , 0.5 mg/ml GDH, with intermittent shuttering (S) and re-exposure (E) of the cell as indicated. ■—■ 0.5 mg/ml ovalbumin. ▲—▲ , 0.5 mg/ml GDH excited through a microscope slide (300-fold attenuation).

ultraviolet radiation, although more slowly than GDH. The difference in observed rates may be partly due to more rapid diffusion of the smaller ovalbumin molecules in and out of the light path, but this would not explain why the rates for photodecomposition of tryptophan (Fig. 2) and ovalbumin are similar. Instead, it seems probable that the specific environments of the tryptophans in the two proteins differ and influence the rate of decomposition.

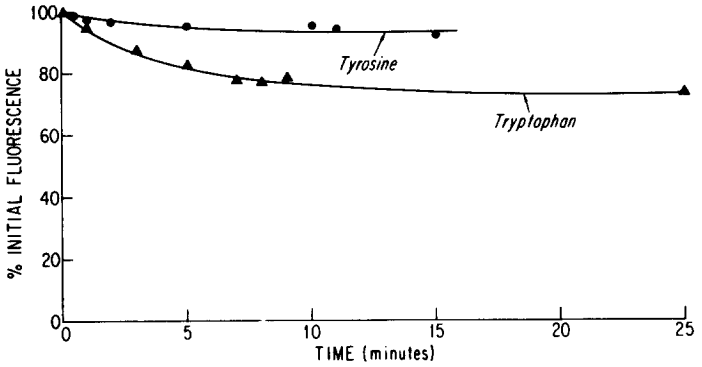

Fig. 2. Observed amino acid fluorescence as a function of time. Buffer and temperature the same as in Fig. 1. ▲—▲, Tryptophan, 3.3 x 10^{-5} M. Activation, 290 mμ; emission, 350 mμ. ●—●, Tyrosine, 3.3 x 10^{-5} M. Activation and emission were at 278 and 303 mμ, respectively, both wavelengths being apparent maxima.

TABLE I

EFFECT OF IRRADIATION ON ACTIVITY

Fluorescence (% of initial)	Activity* (% of initial)
100	100
87	83
67	60
53	33
34	20
31	12

*In this experiment, 100% activity represents a turnover number of 87,000 min^{-1} in terms of moles of DPNH oxidized per 10^6 gm protein in the standard assay. The cell contents were mixed before each fluorescence reading. A 2 μl sample was used in each activity assay.

As seen in Table I, loss in enzyme activity paralleled closely the loss of protein fluorescence. In other experiments it was found that partial inactivation (70%) involved no change in Michaelis constants (K_m) for either α-ketoglutarate or DPNH. These studies show that the mechanism of inactivation of GDH by 290 mμ light must differ from that due to X-irradiation (Adelstein and Mee, 1960), which is attended by little change in protein fluorescence and by an increased K_m for substrate. While tyrosine also decomposes if exposed to 278 mμ light (Figure 2), we have been unable to detect photosensitivity to 290 mμ light under these conditions. Thus, destruction of tryptophan rather than tyrosine appears to underlie the observed photoinactivation of GDH.

It should be noted that a microcuvette rather than the usual 1 x 1 cm cross-section cell was used in showing photodecomposition. The dimensions of these cells are compared in cross-section in Fig. 3. It is obvious that with 1/16" slits, the volume of irradiated solution

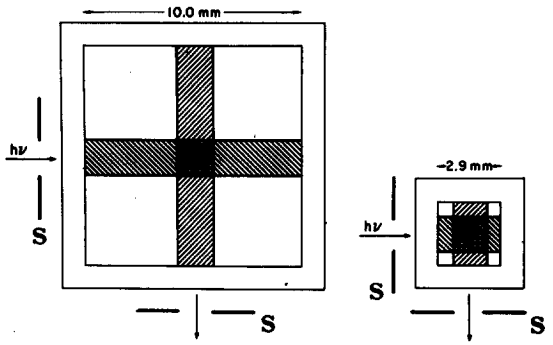

Fig. 3. Cross-sections of regular and micro cuvettes. , Solution exposed to activating light. , Solution observed by detector. Slits (S) are 1/16" wide, walls are 1.0 mm thick.

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which is observed (doubly cross-hatched area) is the same in the two cells. The chief advantage of the microcell is its very short light path, so that inner filter effects due to absorption of incident or emitted light are minimized. Photodecomposition is more evident in the microcell, since a relatively larger portion of the total volume is in the light path. With

concentrated solutions in the large cells, photodecomposition may be evidenced only by fluctuations in the reading due to destruction of solute in various parts of the exciting beam both in front of, and within the observed irradiated area. When the spectrophotofluorimeter was originally developed in this laboratory (Bowman et al., 1955), a high light intensity was deliberately achieved by focusing the arc of the xenon lamp in the center of the cell, an arrangement normally advantageous for routine analytical work. Photodecomposition must be looked for, however; and it may be easily prevented by attenuating the activating beam (Fig. 1). Compensation for the lower fluorescence then can be made by increasing the amplification of the photomultiplier tube response (see p. 104ff. of Udenfriend, 1960).

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