Inactivation of Enzymes by Visible Light in the Presence of Riboflavin

Arthur W. Galston and Rosamond S. Baker

Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena

During a study of the mechanism of light action on plant growth (*2*, *3*) it was found that riboflavin can sensitize the photo-oxidation of various indole-containing compounds, including the plant growth hormone indolacetic acid and the amino acid tryptophane. Such photo-oxidations proceed rapidly at low concentrations of riboflavin (0.1–10 γ/cc) and at moderate light intensities. Because tryptophane is normally present as a constituent of proteins, it seemed desirable to investigate the possibility that riboflavin could have a similar effect upon the tryptophane of protein molecules.

For convenience in determining the effectiveness of our photoinactivation system we decided to use various enzyme preparations as experimental proteins. In such experiments we have invariably found that enzymes suspended in riboflavin solutions are rapidly inactivated by visible light. Thus urease is completely inactivated in 2½ hr, α-amylase in 6 hr and tyrosinase in 17 hr. Since no enzyme which we have investigated has proved insensitive to this photoinactivation procedure, the phenomenon seems to be quite general. In this paper, we shall report in some detail the results of our experiments with the α-amylase of malt diastase.

The α-amylase investigated was present in Merck diastase of malt. Preliminary investigations demonstrated that the enzyme reacted most rapidly in the pH range 5.9–6.6. M/15 pH 6.1 phosphate buffer was therefore employed as standard throughout these experiments.

The malt diastase was made up into a 1% stock solution which was stored under toluene in the refrigerator. Such stock solutions were found to retain full activity for at least 1 week. Immediately prior to use, the enzyme was diluted tenfold. For the study of the reaction, 1 cc of diluted enzyme was mixed with 4 cc of buffer and then with 5 cc of previously boiled 1% soluble starch. At desired time intervals, 1-cc aliquots of the reaction mixture were removed and added with vigorous stirring to 10 cc of $KI.I_2$ solution. The stock solution of this reagent was 2% KI, with enough resublimed iodine crystals added to give a distinct yellow color. The stock was diluted 1:100 immediately prior to use.

The blue color resulting from mixing the 1-cc aliquot of the reaction mixture with 10 cc of the 1:100 $KI.I_2$ was found to be quite stable and convenient to measure. All determinations of color intensity were made in a Klett-Summerson photoelectric colorimeter, using a red filter. Colorimeter readings at zero time were approximately 230; these readings fell linearly to zero in approximately 30 min at room temperature (23° C).

In order to compare the activities of control and experimental enzyme preparations, it was necessary to adopt some arbitrary unit of activity. For this purpose, we determined the time t necessary for complete disappearance of the starch color, and then expressed enzyme activity as $1/t$. In almost all cases, t was directly determined, but where partially inactivated enzymes were reacting very slowly, t was approximated by extrapolation.

To determine whether riboflavin could sensitize the photoinactivation of α-amylase, 1 cc of the diluted enzyme was mixed with 1 cc of pH 6.1 phosphate buffer either lacking (control) or containing (experimental) 100 γ of the riboflavin (Rbf). Tubes were either stored in the dark or were illuminated by 400 ft candles of fluorescent white light for 1 hr. At the end of this time, 3 cc of buffer and 5 cc of 1% soluble starch were added to each tube and aliquots removed for colorimetric analysis every 10 min. In order to insure that differences in activity would be due to the pretreatment of the enzyme and not to riboflavin effects on starch hydrolysis itself, riboflavin was incorporated into the buffer added to enzymes not previously so treated. The results of such an experiment are shown in Table 1. It is clear that visible light or riboflavin applied separately have but slight effect on the rate of enzyme action, but that illumination of enzyme solutions containing riboflavin result in a marked reduction of enzyme activity.

TABLE 1

EFFECT OF VISIBLE LIGHT ON ACTIVITY OF AN ENZYME SENSITIZED BY RIBOFLAVIN*

Contents during pretreatment	Illumination during pretreatment	Starch—$KI.I_2$ color after 10 min	20 min	30 min
1 cc enzyme 1 cc buffer	dark	118	64	9
1 cc enzyme 1 cc buffer containing 100 γ rbf.	"	134	71	12
1 cc enzyme 1 cc buffer	400 ft candle fluorescent white light	127	79	16
1 cc enzyme 1 cc buffer containing 100 γ rbf.	"	157	135	112

* Temp = 23° C, pretreatment period = 1 hr. After pretreatment, all tubes were made up to contain 1 cc enzyme, 3 cc buffer, 1 cc buffer-riboflavin and 5 cc 1% starch solution.

We next endeavored to arrive at some understanding of the nature and kinetics of the inactivation reaction. For this purpose a series of tubes was prepared in the dark room, necessary illumination for the operations being supplied by a 7½ W ruby-red bulb. Into each tube was placed 1 cc of enzyme and 1 cc of buffer containing 100 γ of riboflavin. The tubes were then carried into the laboratory and exposed to the light. At various intervals, tubes were removed from the light rack and returned to the dark room. At the end of 17 hr, all exposed tubes were tested for residual enzyme activity. To each was added 3 cc of buffer and 5 cc of 1% starch solution. Aliquots were then removed at frequent intervals, and the time, t, necessary for complete disappearance of the starch color was determined. Data are presented in Table 2. A plot of the log of relative en-

zyme activity against duration of preillumination yielded a straight line (Fig. 1). This indicates that the inactivation follows the time course expected for a first-order reaction and probably involves a "one-hit" mechanism.

TABLE 2

EFFECT OF DURATION OF ILLUMINATION ON ACTIVITY OF α-AMYLASE SENSITIZED BY RIBOFLAVIN

Duration of exposure to light	Time (t) in min to complete disappearance of starch	$\frac{1}{t}$	Relative activity
0	34	.0294	100
10 min	39	.0256	87
30 min	48	.0208	71
1 hr	66	.0152	52
2 hr	114	.0088	30
3 hr	220	.0045	15
4 hr	360	.0028	10
5 hr	582	.0017	4
17 hr	∞	0	0

Subsequent experiments with antibodies and with bacteriophage have demonstrated that these types of proteins are also rapidly photoinactivated in the presence of riboflavin.

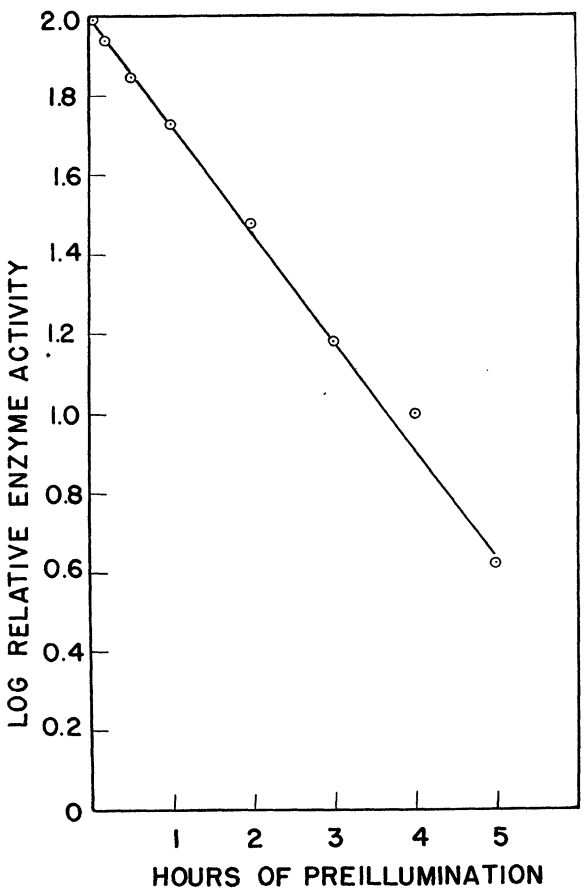

FIG. 1. Effect of duration of preillumination upon the inactivation of α-amylase.

As early as 1879 (*1*) it was reported that sunlight, especially the blue-violet wavelengths, could cause the aerobic photoinactivation of a crude enzyme preparation. Von Tappeiner (*6*) (see also other references in the review by Schomer [*5*]) later reported that the addition of fluorescent dyes such as eosin greatly accelerated such photoinactivation. It is perhaps not surprising, therefore, to find that riboflavin, which is strongly fluorescent in blue light, produces a similar effect.

Our investigations (unpublished) on the relation of light intensity and riboflavin concentration to the rate of photoinactivation strongly indicate that this type of reaction could proceed *in vivo*, at least in green plants. This reaction may therefore be of some importance in one or more of the light-growth reactions of plants such as phototropism, photoperiodism, and the light inhibition of internode growth.

It is also interesting to note that numerous investigators (*4*) have attributed the effects of ultraviolet irradiation of enzymes to a specific absorption by tryptophane. Since riboflavin is known (*3*) to cause the photoinactivation of tryptophane, this reaction may provide a mechanism whereby visible light produces the same sort of effect on enzymes as ultraviolet light.

References

1. DOWNES, A., and BLUNT, T. P. *Proc. roy. Soc. Lond.*, 1879, **28**, 199.
2. GALSTON, A. W., and HAND, M. E. *Amer. J. Bot.*, 1949, **36**, 85.
3. GALSTON, A. W. *Proc. Nat. Acad. Sci.*, 1949, **35**, 10.
4. GORBACH, G., and LERCH, K. *Biochem. Z.*, 1930, **219**, 122.
5. SCHOMER, H. A. *Biological effects of radiation*, edited by B. M. Duggar. New York: McGraw-Hill, 1936, Chap. 37.
6. VON TAPPEINER, H. *Ber. Deut. Chem. Ges.*, 1903, **36**, 3035.

A New Method for Isolation and Purification of Mammalian Striated Myofibrils[1]

Armin F. Schick and George M. Hass

The Rush Department of Pathology, Presbyterian Hospital, Chicago

The following method has been devised for isolating large numbers of mammalian skeletal and cardiac myofibrils in highly purified form without modifying their microscopic structure or reactivity to adenosine triphosphate.

The method with slight variations is suitable for isolation of myofibrils of man, rabbit, dog, and guinea pig. The present discussion is concerned principally with the isolation of skeletal myofibrils of rabbits and properties of the segregated fibrils.

A block (25 × 25 × 10 mm) of living white muscle from the anterior thigh was rapidly frozen and cut with a cold

[1] This research was aided by a grant from the Otho S. A. Sprague Memorial Fund.