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RIBOFLAVIN-SENSITIZED PHOTOÖXIDATION OF INDOLEACETIC ACID AND RELATED COMPOUNDS

BY ARTHUR W. GALSTON*

KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY

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Indoleacetic acid (IAA) is known to exert profound effects upon growth and morphogenesis in higher plants (cf., the review by Went and Thimann[17]). Since its natural occurrence in plants has been directly demonstrated by chemical isolation (Haagen-Smit, *et al.*,[7, 8]) its status as a plant hormone seems well established. Obviously, any information concerning the genesis and disappearance of this hormone is of fundamental importance to plant physiology.

During a series of experiments on the physiology of light action in etiolated peas (Galston and Hand[5]), we noticed that the addition of small amounts of riboflavin (Rbf) to the growth medium resulted in a marked growth inhibition if the tissue were exposed to light. No such inhibition occurred in the absence of light. Since this inhibition could be partially reversed by the addition of relatively large quantities of IAA to the medium, it appeared possible that riboflavin in some way caused the photo-inactivation of indoleacetic acid. This interpretation was completely confirmed by *in vitro* experiments, as described below.

Methods.—The test solutions were put into Erlenmeyer flasks of such capacity that a layer 1–2 cm. deep was formed. Duplicate series were prepared, one being mixed and stored in a dark room, the other being exposed to about 200 foot-candles of light from "Daylight" fluorescent bulbs. Such light as was necessary in the "darkroom" was supplied by a $7^1/_2$-watt ruby-red bulb. In the determination of IAA, a 1-cc. aliquot of the reaction mixture was removed to a test tube, mixed with 4 cc. of Salkowski reagent (see Tang and Bonner[14]), and allowed to stand for 30 minutes. The intensity of the resultant pink color was then measured in a Klett-Summerson photoelectric colorimeter, using a green filter, and the concentration of IAA in the aliquot determined by reference to a previously prepared standard curve.

The riboflavin employed was the Merck product, made up into a stock solution of 100 γ/cc. and stored in a red bottle in the refrigerator to retard decomposition. The IAA utilized was made up into a stock solution of 50 γ/cc., adjusted to pH 7.0 and similarly stored in the refrigerator until use.

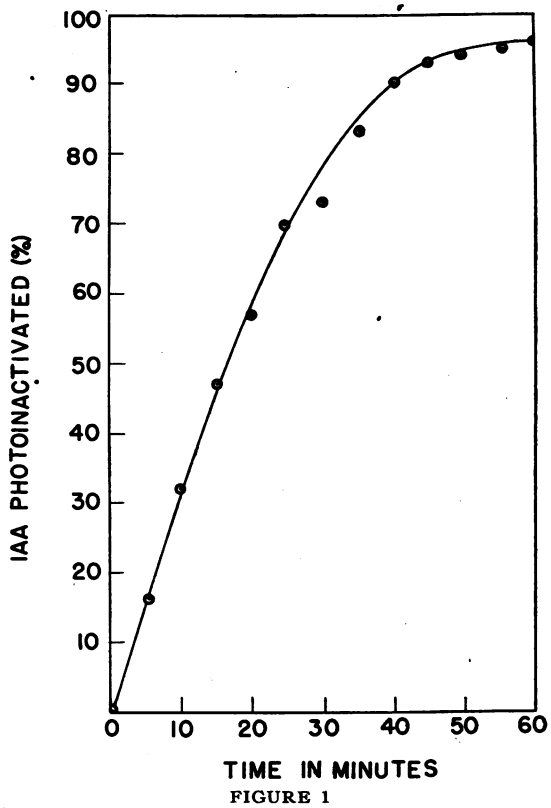

FIGURE 1

The time course of photoinactivation of indoleacetic acid. Solution contained 25 γ/cc. IAA and 10 γ/cc. Rbf.

Experiments.—Although solutions of indoleacetic acid are quite stable both in light and in the dark, the addition of as little as 0.01 γ/cc. of riboflavin renders the IAA susceptible to rapid light inactivation. Thus, in a solution containing 25 γ/cc. IAA and 1 γ/cc. riboflavin, practically all of the indoleacetic acid is inactivated within 1 hour. Typical data are presented in table 1.

To determine the time course and rate of the reaction, 25 γ/cc. IAA and 10 γ/cc. of riboflavin were incubated at 23°C. under the fluorescent lights.

Every five minutes, a 1-cc. aliquot was removed from the solution and subjected to colorimetric analysis. The data (Fig. 1) show that the reaction is 50% complete in about 16 minutes, 75% complete in about 31 minutes, and virtually complete at the end of one hour.

TABLE 1

THE EFFECT OF RIBOFLAVIN AND OF LIGHT ON THE DISAPPEARANCE FROM SOLUTION OF IAA. DURATION OF EXPERIMENT, 1 HOUR; TEMPERATURE, 23°C.

SOLUTION		% DISAPPEARANCE OF IAA	
γ/CC. IAA	γ/CC. RIBOFLAVIN	DARK	LIGHT
25	0	0	0
25	0.01	0	16.0
25	0.1	0	22.4
25	1	0	98.8
25	10	0	98.4

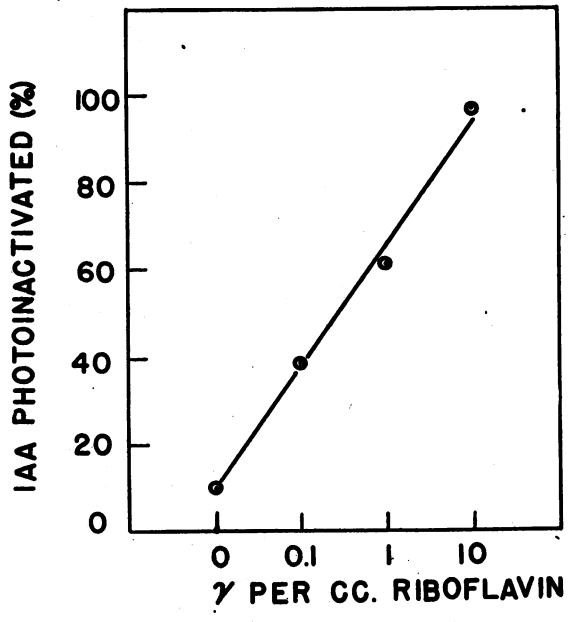

FIGURE 2

The effect of riboflavin concentration on the rate of photoinactivation of IAA. Solution contained 25 γ/cc. IAA; duration of reaction was 40 minutes.

If the log of the residual concentration of IAA is plotted against time, a straight line is obtained, indicating that the reaction is first-order. The reaction rate constant, K, as determined by multiplying the slope of this line by 2.303, was 1.2×10^{-4} reciprocal seconds. This value checked closely with other determinations of K made by means of the formula

$$K = \frac{2.303}{t} \log \frac{C_o}{C}$$

where C_o = original concentration and C = concentration at time t.

To determine more precisely the relation between rate of the reaction and concentration of riboflavin, a series was set up similar to that in table 1, but aliquots for colorimetric analysis were taken after 40 minutes. As shown in figure 2, there is approximately a straight line relationship between the log of the riboflavin concentration and the rate of the reaction. This indicates that the reaction will be strictly first-order with respect to IAA only in the presence of an excess of riboflavin.

The effect of temperature and of pH on the reaction rate are shown in tables 2 and 3. Raising the temperature from 3°C. to 23°C. results in a 50% increase in the rate of the reaction, and further elevation of the temperature to 48°C. has a proportionately smaller effect. The fact that the reaction proceeds best at the lower pH values would indicate that the undissociated molecule reacts more rapidly than does the indoleacetate ion. The pK of indoleacetic acid is 4.75 (D. Bonner[1]).

TABLE 2

THE EFFECT OF TEMPERATURE ON THE RATE OF PHOTO-INACTIVATION OF IAA. ALL SOLUTIONS HAVE 25 γ/CC. IAA AND 1 γ/CC. RIBOFLAVIN

TIME AFTER START OF EXPERIMENT, MIN.	% OF IAA PHOTO-INACTIVATED		
	3°C.	23°C.	48°C.
15	18.0	28.8	32.0
30	26.8	38.9	51.9
45	38.4	55.3	60.0
60	46.8	65.6	70.4

TABLE 3

THE EFFECT OF PH ON THE RATE OF PHOTO-INACTIVATION OF IAA. ALL SOLUTIONS HAVE 25 γ/CC. IAA, 1 γ/CC. RIBOFLAVIN, AND 0.01 *M* KH_2PO_4-Na_2HPO_4 BUFFER

PH	% OF IAA PHOTO-INACTIVATED AFTER 40 MINUTES
4.5	62.4
5.6	60.7
6.5	56.8
7.0	50.4
7.4	44.7
8.0	40.0
9.2	32.0

To determine whether the photo-inactivation requires oxygen, duplicate flasks containing 25 γ/cc. IAA and 1 γ/cc. riboflavin were prepared in the dark room. Through one flask a swift stream of argon gas was bubbled for 30 minutes and the flask was then sealed. The other flask was kept as an aerobic control. At zero time, both flasks were brought into the light.

It was noted that the riboflavin in the anaerobic flask became decolorized in about 20 minutes, whereas that in the aerobic flask remained bright yellow. At the end of 1 hour, aliquots were removed from each of the flasks and assayed for residual IAA as before. Whereas 95.5% of the IAA had been photo-inactivated in the aerobic flask, only 11.6% had been photo-inactivated in the anaerobic flask. Admission of oxygen to the latter flask resulted in the rapid disappearance of IAA. This indicates that the reaction may be represented as follows:

$$\text{Rbf} + h\nu \rightarrow \text{Rbf}^*$$
$$\text{IAA} + \text{Rbf}^* \rightarrow \text{oxidation product} + \text{Rbf}\cdot\text{H}_2$$
$$\text{Rbf}\cdot\text{H}_2 + {}^1/_2\text{O}_2 \rightarrow \text{Rbf} + \text{H}_2\text{O}$$

The activated riboflavin (Rbf*) therefore acts as a hydrogen carrier between IAA and oxygen.

In order to elucidate the mechanism of the reaction and the nature of the products formed, experiments were conducted in a Warburg apparatus equipped with a battery of 60-watt bulbs seated under the reaction vessels. Into control cups were placed 1 cc. of either IAA (500 γ/cc.) or riboflavin (20 γ/cc.); experimental cups contained both the IAA and riboflavin mixed after the cocks were closed. Vessels with and without KOH in the center well were employed to determine both O_2 absorbed and possible CO_2 evolved in the reaction. At the conclusion of the experiment, a 1-cc. aliquot of the reaction mixture was removed for the determination of residual IAA, and therefore of the quantity of IAA photoöxidized. Typical data are presented in table 4. They seem clearly to indicate the release of 1 molecule

TABLE 4

GAS EXCHANGE DURING PHOTOÖXIDATION OF IAA

EXPT. NO.	μM IAA OXIDIZED	μM O_2 CONSUMED — TOTAL	μM O_2 CONSUMED — PER μM IAA OXIDIZED	μM CO_2 EVOLVED — TOTAL	μM CO_2 EVOLVED — PER μM IAA OXIDIZED
P-308	2.76	3.16	1.15	..	..
	2.47	2.91	1.18	..	..
P-314	2.73	2.58	0.95	2.78	1.02
P-315	2.77	3.23	1.17	2.94	1.06
	2.77	3.24	1.17	3.03	1.09

of CO_2 per molecule IAA oxidized, and the absorption of about 1 molecule of O_2 per molecule oxidized, though oxygen absorption values averaged about 12% higher than this value. A balanced over-all reaction may therefore be written:

$$\text{IAA} + \text{O}_2 \xrightarrow[\text{light}]{\text{riboflavin}} \text{oxidation product} + \text{CO}_2$$

The nature of the oxidation product is still under investigation and will be reported separately.

Specificity of the Reactants.—It became of interest to determine whether compounds related to riboflavin and to indoleacetic acid could react in the same way. Samples of lumichrome and lumiflavin (originally obtained from Dr. J. W. Foster) were made available to the author through the kindness of Dr. H. K. Mitchell. As shown by the data of table 5, lumiflavin

TABLE 5

COMPARATIVE EFFECTIVENESS OF RIBOFLAVIN, LUMICHROME AND LUMIFLAVIN IN SENSITIZING THE PHOTOOXIDATION OF IAA. TEMPERATURE, 21°C.

SOLUTION	% OF IAA DESTROYED AFTER 1 HR.	2 HRS.	17 HRS.
25 γ/cc. IAA	0	0	0
25 γ/cc. IAA + 1 γ/cc. riboflavin	70	92	96
25 γ/cc. IAA + 1 γ/cc. lumichrome	12	16	90
25 γ/cc. IAA + 1 γ/cc. lumiflavin	0	0	0

was completely incapable of sensitizing the photooxidation of IAA, whereas lumichrome reacted at a much slower rate than did riboflavin.

To determine whether other compounds containing an indole ring could be photooxidized in the presence of riboflavin, 10^{-4} *M* solutions of such compounds were made up and sensitized by the addition of 10 γ/cc. riboflavin. At zero time, a Hopkins-Cole color was obtained by mixing 3 cc. of the solution with 1 cc. glyoxylic acid reagent, 0.2 cc. 0.01 *M* $CuSO_4$ and 5 cc. concentrated H_2SO_4. At intervals, aliquots were removed to determine whether the intensity of the Hopkins-Cole color had decreased. Such decrease with respect to the zero time color for each compound was taken as evidence of its photochemical alteration. All indole compounds tested were found to react in the same way as does IAA, although at considerably lower rates. A list of such compounds is found in table 6.

TABLE 6

COMPOUNDS CAPABLE OF BEING PHOTOCHEMICALLY ALTERED IN THE PRESENCE OF 10 γ/CC. RIBOFLAVIN. ALL SOLUTIONS TESTED WERE 10^{-4} *M*

Indole	Indole aldehyde
Skatole	Tryptamine
Indoleacetic acid	Tryptophane
Indolebutyric acid	5-Methyl tryptophane

Possible Biological Significance of the Reaction.—It has been reported by several investigators (van Overbeek,[16] Burkholder and Johnston,[3] Koningsberger and Verkaaik,[9] Stewart and Went,[13] Oppenoorth,[11] Gustafson[6]) that tissue exposed to strong light has a lower auxin content than does unilluminated tissue. If this auxin is indoleacetic acid, then the reaction described in this paper may proceed, destroying either IAA, its precursor, tryptophane (Wildman, *et al.*[18]), or both. Whereas it has been reported (van Overbeek[15]) that only auxin *a* and *b* may produce differential growth

effects in light and dark, this reaction provides a mechanism whereby indoleacetic acid may also yield such differential effects, as previously reported by Galston and Hand.[5]

Since the absorption maximum of riboflavin in the visible portion of the spectrum (Ca 460 mμ) is quite close to the most effective wave-length in the action spectrum for phototropism, it is also possible that riboflavin is a receptor pigment in the phototropic response of plants. Recent demonstrations (Galston,[4] Bonner and Thurlow,[2] Leopold and Thimann[10]) that there may be a relation between auxins and photoperiodic response of plants may also be interpreted by means of such a reaction, although the reported action spectrum for photoperiodism (Parker, *et al.*[12]) seems to preclude the participation of riboflavin. These and related questions are at present under investigation in this laboratory.

Summary.—In *in vitro* studies, it has been found that riboflavin can sensitize the photoöxidation of various indole-containing compounds, including the plant growth hormone indoleacetic acid. Under the conditions studied, the reaction goes to completion in 1 hour. Kinetic studies indicate that it is first-order.

The reaction requires oxygen. Warburg manometric studies indicate that approximately 1 mol. O_2 is absorbed and 1 mol. CO_2 is liberated per mol. indoleacetic acid oxidized. The nature of the oxidation product is as yet unknown.

The reaction proceeds best at the lower pH values tested, suggesting that the undissociated IAA molecule reacts more quickly than the indoleacetate ion. The reaction rate is somewhat accelerated by an increase in temperature. Riboflavin analogs work more slowly than riboflavin, or not at all. All indole-containing compounds tested reacted.

It is suggested that riboflavin-sensitized photo-inactivation of auxin may be an important phenomenon in the normal physiology of the plant.

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*AGGREGATION PHENOMENA IN EGG ALBUMIN SOLUTIONS AS DETERMINED BY LIGHT SCATTERING MEASUREMENTS**

BY M. BIER AND F. F. NORD

DEPARTMENT OF ORGANIC CHEMISTRY,** FORDHAM UNIVERSITY, NEW YORK 58, N. Y.

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The various ultracentrifugal measurements reported for the molecular weight of egg albumin are not in complete agreement, as values between 34,500 and 46,000 have been obtained.[1,2] Investigating the behavior of this protein, Sjögren and Svedberg[3] have reported also that, within the pH range 4 to 9, egg albumin is stable and monodisperse.

During the course of extensive studies on the effect of freezing on various proteins and enzymes, it was found, however, that freezing causes, solely because of its physical influence, a change in the state of aggregation of egg albumin, inducing an aggregation at higher concentrations and a disaggregation at lower concentrations of the protein particles in solution.[4] These results were recently confirmed by Neduzhii[5] and others. Furthermore, experiments of Putzeys and Brosteaux,[6] and of Heller and Klevens,[7] extended these results by noting that egg albumin also undergoes aggregation at normal temperatures within the above mentioned pH range of alleged stability.

The causes influencing the state of aggregation of this protein under the above conditions were not yet investigated, and hence we studied the factors governing the aggregation occurring at normal temperature. To study the influence of concentration and time, we adopted the light-scattering method, which is very suitable for the study of aggregation phenomena,[7] the rapidity of the single measurements offering a notable advantage over the determination of particle weights by ultracentrifugation. This is to our knowledge the first attempt of a study of the mechanism of protein aggregation and denaturation by a continuous direct measurement of changes of particle weights.

Regarding the kinetics of aggregation, Oster[8] has pointed out that, in a system of polymerizing or aggregating particles, the light scattering increases with the progress of the reaction. Namely according to Rayleigh,[9]