Inhibition of Cell Growth by Photooxidation Products of Indole-3-acetic Acid*

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Indole-3-acetic acid is well known as an inducer as well as an inhibitor of several growth and differentiative processes in plants, lower metazoan, and microorganisms. The principle of unity in biology suggests that these diverse effects of indole-3-acetic acid may all be consequences of interference with a common reaction or series of reactions; therefore, the study of the action of indole-3-acetic acid in the simplest of these species should have relevance to its action in the other species. The simplest organism previously known to have been reproducibly affected by small amounts of indole-3-acetic acid is Schizosaccharomyces pombe (1). Its growth is markedly inhibited by indole-3-acetic acid. Accordingly, this organism was the biological material of choice for the initiation of studies on the biochemistry of indole-3-acetic acid action. Preliminary studies revealed that a photooxidation product of indole-3-acetic acid rather than the acid itself was the active material. This observation could be extended to an even simpler, better known organism, Escherichia coli, with which most of the work in this report has been performed.

A brief preliminary description of some of these observations has been presented (2).

EXPERIMENTAL PROCEDURE

Organisms—The microorganisms employed in this study were Schizosaccharomyces pombe (ATTC 2478) and Escherichia coli W. The yeast strain was grown in Wickerham's complete synthetic medium (Difco's Bacto-yeast nitrogen base) at 30° with aeration. E. coli W was grown with aeration at 37° in a glucose-salts mixture (3) modified by the omission of CaCl2. For the growth in the dark of both organisms, the culture vessels were wrapped in foil. Turbidity of the cultures was measured in a Klett-Summerson colorimeter with a No. 42 or 62 filter. Growth is expressed as milligrams of dry weight of cells per ml of culture based on a calibration relating the turbidity of the culture to its dry weight. Pea seeds were allowed to germinate in the dark at 25°. For each experimental or control value, 30 peas were placed in foil-wrapped Petri dishes containing substances under test and distilled water to a volume of 30 ml. After 72 hours, the six longest radicals were excised, blotted dry, and weighed. The elongation of tomato root tips was estimated by the method of White (4), with the use of five tips for each control and experimental value.

Photooxidation of IA—Aqueous mixtures of IA and riboflavin were irradiated by exposure to standard fluorescent lamps (Cool-White) at ambient temperatures from a distance of 5 cm. These lamps have several narrow emission bands clustered around 550 mλ and a single band at 445 mλ. For large scale production of the photooxidation products, IA and riboflavin concentrations were 175 and 7 mλ per liter, respectively. Irradiation of the mixture in 35- to 40-ml batches was continued until the IA was completely oxidized. The crude irradiated mixture was clarified by centrifugation at 3000 × g for 20 minutes and extracted with ether for 15 hours. The ether extract was evaporated to dryness, and the resulting residue was dissolved in small quantities of water.

Chromatography of Photooxidation Products—Paper chromatograms of the ether extract were developed by the descending technique in the cold. Whatman No. 3MM paper was used throughout. The principle solvent employed was Solvent A, isopropanol-water (5:95). Solvent B consisted of saturated (NH4)2SO4-0.1 m phosphate, pH 7.0-water-isopropanol (50:38:10). Solvent C had the composition chloroform-methanol-water (80:80:48, upper phase). Solvent D was 1-butanol-benzene-methanol-water (20:30:40:10).

The chromatograms were routinely examined for ultraviolet-absorbing materials and for inhibitory substances. The inhibitory substances were detected by layering the developed chromatograms on glucose-mineral salts-agar plates seeded with E. coli W and noting zones of inhibition after incubation at 37° for 15 hours. The ultraviolet-absorbing spots associated with inhibitory activity were cluted with water.

In experiments with IA-α-14C (8.5 mc per mg), 50-ml portions were irradiated and the radioactive products were removed by extracting five times with 2 volumes of ether. Chromatograms were assayed for radioactivity with a Nuclear-Chicago Actigraph 11 strip counter.

Analysis—Disappearance of IA during photooxidation was measured with the Salkowski reagent according to the method of Tang and Bonner (5). Ultraviolet absorption spectra of the compounds were determined with a Beckman model DU spectrophotometer or with a Cary recording spectrophotometer. Infrared spectra of chloroform solutions were obtained with a Perkin-Elmer model 21 infrared spectrophotometer.

Chemicals—IA-α-14C and oxindole were commercial prepara-

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† The abbreviation used is: IA, indole-3-acetic acid.
tions. 3-Methyloxindole and 3-bromo-oxindole-3-acetic acid were generous gifts of Dr. R. L. Hinman. When dissolved in water, the bromo derivative is rapidly converted to 3-methyl-oxindole. 3-Methyloxindole was kindly provided by Dr. K. V. Thimann.

RESULTS

Effect of Growth Media on IA Toxicity—Initial experiments on the effect of IA on S. pombe were carried out in Wickerham's complete synthetic medium. As reported by Kennell (1), IA markedly inhibited the growth of S. pombe (Fig. 1A). In the complete medium, the growth rate of this fission yeast was 0.29 generation per hour. IA at 10 μg per ml reduced the growth rate to 0.25 generation per hour and then, after one generation, caused complete cessation of growth. No effect on the lag period was observed.

To re-examine the phenomenon of IA toxicity, a minimal synthetic medium for the growth of S. pombe was employed. Single eliminations of the organic compounds present in the complete medium led to the minimal medium shown in Table I. This medium differs from the complete medium by the omission of the amino acids and the following vitamins: p-aminobenzoic acid, pyridoxine, riboflavin, and thiamine. The growth rate of S. pombe in the minimal medium was 0.25 generation per hour.

In contrast to its effect on the growth of S. pombe in the complete medium, 10 μg per ml of IA was essentially inactive on the yeast growing in the minimal medium (Fig. 1B). There was only a slight, transitory inhibition of growth. A 10-fold increase in IA concentration did not significantly alter this growth response.

Requirement for Riboflavin and Light for IA Toxicity—It was apparent that a compound present in the complete medium was required for the expression of IA toxicity. Single additions to the minimal medium of the amino acids and vitamins omitted from the complete medium indicated that the compound responsible for the divergent response of S. pombe to IA in minimal and complete media was riboflavin. The addition of 0.20 μg per ml of this vitamin restored full sensitivity to IA.

Galston's (6) report on the riboflavin-catalyzed photooxidation of IA suggested a possible role of this vitamin in the expression of IA toxicity for S. pombe. If added riboflavin does in fact catalyze the photooxidation of IA under the growth conditions employed, it would be reasonable to expect that the toxic effect of IA should be light-dependent. This expectation was fulfilled. The toxic effect of IA could be observed only when the cultures were incubated in the light; cultures of S. pombe grown in the dark were insensitive to IA as well as to the combinations of IA and riboflavin (Table II). Analysis of the culture grown in the presence of both light and riboflavin for IA indicated that 85% of the auxin had disappeared. No significant loss of IA was detected in similar cultures grown in the dark. In the absence of riboflavin, but in the presence of light, 14% of the IA disappeared, possibly because of limited photooxidation of the auxin catalyzed by flavins produced by S. pombe. This is reflected by the slight inhibition of growth observed in the absence of added riboflavin.

Growth Inhibition by Photooxidation Products of IA—These observations strongly suggested that a photooxidation product, rather than IA itself, was the cause of growth inhibition. To test this possibility, mixtures containing 100 μg of IA and 2 μg of riboflavin per ml were irradiated by exposure to standard fluorescent lamps for a period of 10 minutes and then added to darkened cultures of S. pombe as well as to cultures of E. coli W. The results of such an experiment are shown in Table III. The amount of irradiated mixture added to the cultures of S. pombe and E. coli W was equivalent to the addition of 10 and 20 μg per ml, respectively, of IA. Mixtures of IA and riboflavin had essentially no effect on the growth in the dark of S. pombe or of E. coli W. If, however, the mixtures were irradiated prior to their addition to the darkened cultures, inhibition of growth was observed with both organisms. Preirradiation of IA by itself or of riboflavin by itself did not produce an inhibitor. The effect of the duration of irradiation on the formation of toxic products paralleled the disappearance of IA.

The photooxidation products also had inhibitory effects on higher plants. The average fresh weight of the radicals of germinating pea seeds at 72 hours was reduced from 0.06 g to 0.02 g, and to 0.03 g, and unaffected by photooxidation products...
TABLE II
Light requirement for indole-3-acetic acid toxicity

The inoculum for S. pombe (2478) in minimal synthetic medium was prepared as described in Fig. 1.

<table>
<thead>
<tr>
<th>Additions to minimal medium</th>
<th>Growth*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg dry wt/ml</td>
</tr>
<tr>
<td></td>
<td>Light</td>
</tr>
<tr>
<td>None</td>
<td>1.38</td>
</tr>
<tr>
<td>IA, 20</td>
<td>1.18</td>
</tr>
<tr>
<td>IA, 20 + riboflavin, 2</td>
<td>0.28</td>
</tr>
</tbody>
</table>

* After 24 hours of incubation.

TABLE III
Inhibition of cell growth by irradiated solutions of indole-3-acetic acid plus riboflavin

Aqueous solutions of IA (100 μg per ml), riboflavin (2 μg per ml), and IA plus riboflavin were irradiated for 10 minutes. Cultures of S. pombe (2478) and E. coli W were grown in the dark for 24 and 3 hours, respectively, with the indicated additions.

<table>
<thead>
<tr>
<th>Additions to growth media*</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. pombe</td>
</tr>
<tr>
<td></td>
<td>mg dry wt/ml</td>
</tr>
<tr>
<td>None</td>
<td>1.40</td>
</tr>
<tr>
<td>IA + riboflavin</td>
<td>1.58</td>
</tr>
<tr>
<td>Irradiated solutions</td>
<td></td>
</tr>
<tr>
<td>IA + riboflavin</td>
<td>0.04</td>
</tr>
<tr>
<td>IA</td>
<td>1.42</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1.40</td>
</tr>
</tbody>
</table>

* IA and riboflavin concentrations per ml were 10 and 2 μg, respectively, for cultures of S. pombe and 20 and 2 μg, respectively, for cultures of E. coli W.

equivalent to $6.7 \times 10^{-4}$ M, $6.7 \times 10^{-3}$ M, and $6.7 \times 10^{-2}$ M IA, respectively. The elongation of tomato root tips in culture was more sensitive: photooxidation products equivalent to $3.4 \times 10^{-5}$ M, $3.4 \times 10^{-4}$ M, and $3.4 \times 10^{-3}$ M IA reduced tip elongation during the second 30-hour interval of incubation from 11 mm to 2, 5, and 10 mm, respectively.

Photooxidation of IA in the absence of added riboflavin can be achieved by the use of ultraviolet light (7). The oxidation product produced by the action of ultraviolet light from a General Electric AH-5, 250-watt mercury vapor lamp from a distance of 7 cm for 80 minutes also proved to be toxic for E. coli W.

Isolation and Properties of Toxic Photooxidation Products—Concentration of the photooxidation products from the irradiated mixture was achieved by extraction into ether and evaporation of the ether extract to dryness. The residue was dissolved in small quantities of solvents. A precipitate formed slowly in highly concentrated aqueous solutions, probably because of the formation of polymeric substances. Polymerization was prevented by dissolving the ether-extracted residue in small quantities of chloroform, ether, or ethanol.

The ultraviolet absorption spectrum of the products extracted by ether exhibits a maximum at 248 μm. When treated with alkali, this peak was rapidly lost and was not recovered on neutralization. Treatment with alkali also destroyed the biological activity of the photooxidation product. The spectrum of the ether-extracted material closely resembles those reported by Ray and Thimann (8) and Ray (9) for the oxidation product produced by the action of a fungal (Omphalia flavida) enzyme on IA and closely approximates the spectrum exhibited by a synthetic mixture of 3-methyloxindole (B) and of oxindole (C) was 10 mg per ml.

Infrared analysis of the ether-extracted material provided further evidence for the oxindolic nature of the photooxidation product. The infrared spectrum of the toxic material is compared with the spectra of oxindole and 3-methyloxindole in Fig. 2. In general, the spectrum bears a close resemblance to the oxindoles in showing strong absorbance at 1095 cm$^{-1}$, which represents the carbonyl group at position 2. The photooxidation product is further characterized by a peak at 2900 cm$^{-1}$, which is not found in the spectrum of either oxindole or 3-methyl-oxindole. This peak, however, is evident in the spectrum reported by Ray and Thimann (8) for the products of the fungal IA oxidase reaction. Examination of the similarities in the ultraviolet and infrared spectra observed between the photooxidation and fungal oxidase products suggests that the products are similar if not identical in chemical composition.

Paper chromatography of the toxic material revealed the

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*Personal communication from Dr. R. L. Hinman, Union Carbide Research Institute.

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![Infrared absorption spectrum of the photooxidation products of indole-3-acetic acid, 3-methyloxindole, and oxindole.](https://example.com/infrared-spectrum.png)
presence of two ultraviolet-absorbing compounds. Zones of inhibition, revealed by bioautography, and peaks of radioactivity coincided with the two ultraviolet-absorbing compounds (Fig. 3). Compound I ($R_F$, 0.72), present in much larger amounts than Compound II ($R_F$, 0.42), produced correspondingly larger zones of inhibition. The $^1$C label was originally present in the methylene group of IA; therefore, the methylene carbon must be retained in both inhibitors. The areas of the chromatograms corresponding to Compound I and Compound II contain 58 and 14%, respectively, of the total radioactivity. The radioactivity at the origin is associated with fluorescent, probably polymeric substances which are nontoxic. Chromatograms developed with Solvent B gave similar results. Two ultraviolet-absorbing compounds with $R_F$ values of 0.61 and 0.29 were detected which were associated with zones of inhibition and with peaks of radioactivity. The faster moving compound in Solvent B was again associated with the largest radioactive peak.

**Chemical Nature of Compound I and Compound II**—The ultraviolet absorption spectrum of Compound I eluted from paper chromatograms (Fig. 4) closely approximates the spectrum obtained with the unfraccionated ether extract and also resembles the spectrum of 3-methyloxindole. However, the latter compound migrates in Solvent A with an $R_F$ of 0.65 as compared with an $R_F$ of 0.72 for Compound I and is nontoxic for *E. coli* W. Next, the possibility was considered that 3-methyldioxindole is a product of the photooxidation. This compound has been suggested by Ray and Thimann (8) as the product of the fungal IA oxidase reaction. However, comparison of 3-methyldioxindole with Compound I showed several important differences. 3-Methyldioxindole was more polar than Compound I, with an $R_F$ of 0.81 in Solvent A, as compared to an $R_F$ of 0.72 for Compound I. In addition, 3-methyldioxindole was nontoxic for *E. coli* W and had an ultraviolet absorption maximum at 254 mp. The hypothetical structure suggested for Compound I is 3-hydroxymethyloxindole. This structure would retain the methylene group and would satisfy the stoichiometry of the photooxidation reaction which indicates oxidative decarboxylation (6).

Compound II has been characterized as 3-methyleneoxindole. The ultraviolet absorption spectra of Compound II eluted from paper chromatograms and 3-methyleneoxindole are shown in Fig. 5. The inhibitor exhibits a double maxima, 248 and 254 mp, which is characteristic of 3-methyleneoxindole, a compound identified by Hinman, Bauman, and Lang (10) as a product obtained from the oxidation of IA catalyzed by horseradish peroxidase. Further confirmation of the identity of Compound II and 3-methyleneoxindole was based on paper chromatography and biological activity. Compound II had the same $R_F$ as synthetic 3-methyleneoxindole with Solvents A and B. In addition, 3-methyleneoxindole at $10^{-4}$ M caused complete bacteriostasis of *E. coli* W.
incubation, the cells were removed by centrifugation. The cul-

Fig. 6. The conversion of Compound I to 3-methyloxindole by
Escherichia coli W. Compound I, isolated from IA-α-14C photo-

oxidation mixtures, was added to exponentially growing cells of

E. coli W. After 10 hours of incubation, culture filtrates were

prepared and extracted with ether. The ether extracts were

examined chromatographically with Solvent A (isopropanol-

water, 5:95). Compound I without cells served as the control

(A). The experimental system contained 0.17 mg (dry weight)

cells per ml of culture medium (B).

Biological Conversion of Compound I and Compound II to 3-

Methyloxindole—The addition of Compound I to heavy suspen-

sions of E. coli W in exponential growth did not cause permanent

bacteriostasis; after a short interval, exponential growth resumed

at a rate somewhat lower than the control. The fate of Com-

pound I in cultures which had recovered in this manner is shown

in Fig. 6. Compound I prepared from IA-α-14C was added to

exponentially growing cells of E. coli W, and after 60 minutes of

incubation, the cells were removed by centrifugation. The culture

filtrates were extracted with ether and the extracts examined

chromatographically. Compound I in the growth medium

without cells served as the control. With Solvent A, two addi-
tional peaks of radioactivity were found in the culture filtrates;
a major peak with an Rp of 0.65 and a minor peak with an Rp

of 0.42. On elution from the paper chromatogram, the faster

moving compound had an ultraviolet spectrum identical with

that for synthetic 3-methyloxindole and migrated with the syn-

thetic compound in Solvent Systems A, B, C, and D. The

minor peak was present in insufficient amounts for analysis.

Its Rp value, however, was similar to that for 3-methyl-
exindole. It is conceivable that the latter compound might be

an intermediate in the conversion of Compound I to 3-methyl-
exindole.

Since the conversion of 3-methyloxindole to 3-methyl-
exindole would substantiate the possibility that the former is an

intermediate in the conversion of Compound I to 3-methyl-
exindole, similar growth experiments with 3-methyloxindole as

the inhibitor were performed. On examination of the culture

filtrates, it was found that 3-methyloxindole had been quan-
titatively converted to 3-methyloxindole. These observations,

therefore, suggest that the minor peak (Fig. 6) observed in

filtrates of a culture recovering from the bacteriostatic effects of

Compound I is 3-methyloxindole and that this compound is an

intermediate in the formation of 3-methyloxindole from Com-
pound I.

Nonenzymatic Formation of 3-Methyleneoxindole from Com-
pound I—If the riboflavin-catalyzed photooxidation of IA was

carried out in 0.2 M acetate buffer at pH 5.0, conditions employed

by Hinman, Bauman, and Lang (10) for the oxidation of IA

catalyzed by horseradish peroxidase, the ultraviolet absorption

spectrum of the irradiated solution showed the characteristic
double maxima, 248 and 254 μm, of 3-methyleneoxindole, in

contrast to the single maximum at 248 μm characteristic of

Compound I observed when the irradiation was carried out in

aqueous solutions at uncontrolled pH. It is possible that in

both conditions of irradiation, the primary photooxidation

product is Compound I, but that in the presence of acetate buffer

it is converted to 3-methyleneoxindole. This possibility is

strongly supported by the spectral change observed when Com-
pound I was placed in acetate buffer (Fig. 7). The ultraviolet

spectrum of Compound I in 0.2 M acetate buffer, pH 5.0, under-
went a progressive change to the characteristic double maxi-

ma of 3-methyleneoxindole. The 254-μm peak appeared within

90 minutes and reached a maximum in 12 hours. An increase in

optical density in the 275- to 295-μm region became noticeable at

4 hours and reached a maximum in 12 hours. These progres-

sive changes in acetate buffer are similar to those reported by

Hinman and Frost (11) for the changes which took place after 1

hour when IA was treated with horseradish peroxidase under the

same conditions.

DISCUSSION

The fact that IA is inactive by itself as an inhibitor of S.
pombe and E. coli W, but that its riboflavin-catalyzed photo-
oxidation yields two products, 3-methyleneoxindole and a com-
pound tentatively identified as 3-hydroxymethyleneoxindole, both

potent inhibitors of these organisms, may well be responsible for

the many variable and even conflicting reports cited by Kennell

(1) of the sensitivity of microorganisms to inhibition by IA. In

these investigations, neither the riboflavin content of the medium

nor the exposure of cultures to light was taken into account.

Therefore, it is reasonable to assume that the experiments were

performed under conditions in which the concentrations of the

inhibitors varied widely in an uncontrolled manner. In addi-
tion to inhibiting the growth of microorganisms, the photooxida-

tion products were found to be inhibitors of the growth of tomato

root tips and of the germination of pea seeds. These effects on
growth and differentiation in plant tissue invite speculation about a possible role of one or both of these products in phototrophic responses. Unequal illumination of living plant tissue rich in both IA and flavins would result in the accumulation of the inhibitors at the part nearest the light source which would cause a differential rate of growth, and thus a bending toward the light.

The evidence for the characterization of one of the inhibitory photooxidation products as 3-methyloxindole includes an ultraviolet absorption spectrum identical with that of an authentic sample, $R_f$ values identical with those of the authentic sample in two solvent systems, and similar potency as an inhibitor of E. coli. On the other hand, the proposal that Compound I is 3-hydroxyoxindole is dependent in part on less direct evidence. Compound I has ultraviolet and infrared spectra characteristic of oxindoles. Compound I must retain the methylene group of IA as shown by tracer studies. Although its spectrum closely resembles that of 3-methyloxindole, Compound I cannot be identical with 3-methyloxindole: (a) 3-methyloxindole is not sufficiently oxidized to satisfy the stoichiometry of the riboflavin-catalyzed photooxidation; (b) 3-methyloxindole appears to be less polar than Compound I as it migrates less rapidly in several solvents; and (c) 3-methyloxindole is not an inhibitor of E. coli. 3-Methyloxindole, which neither satisfies the stoichiometry of the reaction and is more polar, was suggested by Ray and Thimann (8) to be a product of fungal IA oxidase. However, a synthetic sample of this compound proved to be different in three respects: it has a different ultraviolet absorption spectrum from that of Compound I; it has no biological activity; and it migrates slightly faster than compound I in paper chromatography. Another possibility for the chemical nature of Compound I is 3-hydroxyoxindole. This compound is at the proper state of oxidation and has the appropriate carbon content for the stoichiometry of the photooxidation reaction. The hydroxyl group would account for a greater polarity than 3-methyloxindole, yet would have relatively minor effects on the ultraviolet absorption spectrum. This proposed structure retains the methylene carbon. Furthermore, this structure is readily compatible with the observed conversion of Compound I to 3-methyloxindole via 3-methyleneoxindole.

The binding of an IA metabolite to RNA in plant tissue has been reported by Keppord, Kaur-Sawhney, and Galston (12). This metabolite is produced under conditions (oxidation of IA by horseradish peroxidase at pH 7.0) which might easily result in the formation of one or both of the bacteriostatic products described here. Thus, it will be of interest to determine whether the bacteriostasis caused by these inhibitors is a result of RNA metabolism. On the other hand, the observation that both Compound I and 3-methyloxindole can be reduced to 3-methyleneoxindole, a nontoxic substance, not only accounts for the transitory inhibition caused by these compounds, but also suggests that the enzyme system responsible for the conversion should be sought out and examined as another possible site for the action of Compound I and 3-methyleneoxindole.

### SUMMARY

The inhibitory effect of indole-3-acetic acid on Schizosaccharomyces pombe is indirect. Riboflavin-catalyzed photooxidation products of indole-3-acetic acid rather than the acid itself are the active agents. These products not only affect S. pombe but also Escherichia coli and other bacteria. They also inhibit the growth of tomato root tips and the germination of pea seeds. One of these products has been tentatively characterized as 3-hydroxyoxindole (Compound I) and the other has been identified as 3-methyloxindole (Compound II). Both Compound I and Compound II are converted to 3-methyleneoxindole, a nontoxic substance, thus, accounting for the transient bacteriostasis by these inhibitors.

A possible role of Compound I and Compound II in phototrophic responses and their possible sites of action are discussed.

### REFERENCES

Inhibition of Cell Growth by Photooxidation Products of Indole-3-acetic Acid

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