

# STUDIES ON THE MECHANISM OF PHYTOHORMONE DAMAGE BY IONIZING RADIATION. I. THE RADIOSENSITIVITY OF INDOLEACETIC ACID<sup>1</sup>

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The phytohormone auxin is widely recognized as a regulator of growth and morphogenesis. Since retarded growth and altered developmental patterns in plants are known to follow exposure to ionizing radiation, Skoog (26) investigated the inactivation of auxin by x- and γ-rays. His data indicated that both native auxin preparations and pure 3-indoleacetic acid (IAA) in solution were highly labile when irradiated. A marked reduction in auxin levels also occurred when plant tissues were given moderate doses of x-radiation. Auxin thus appeared to be radiosensitive *in vitro* and *in vivo*. Skoog pointed out the parallel effects of decreased auxin levels and low doses of radiation in growth and correlation phenomena. His results were used subsequently by other workers to interpret various radiation responses on the basis of auxin radiosensitivity (10, 16, 27).

However, certain considerations discussed below cast doubt on the implied direct sensitivity of the auxin molecule, and prompted our reinvestigation of auxin radiosensitivity. Concomitantly, a more precise evaluation of IAA inactivation kinetics was obtained. The present work deals with the destruction of auxin in aqueous solution by ionizing radiation. Auxin sensitivity in the plant, and the biochemical mechanisms whereby auxin levels are lowered *in vivo*, will be treated in subsequent papers of this series.

## PRELIMINARY CONSIDERATIONS

The loss of auxin in the irradiated plant was attributed wholly or in part to direct inactivation (26). A direct auxin photolysis seems remarkable in view of the low auxin concentrations relative to other, potentially protective, cellular components in plant tissues. It is likewise striking that irradiation of both *in vivo* and *in vitro* systems with comparable doses resulted in similar percent inactivations. In order to approximate the kinetics and efficiencies of auxin inactivation *in vitro*, we combined the data from Skoog's (26) tables 2 and 4, and figure 1. In the range of 0 to 4,000 roentgens, these data could be characterized satisfactorily by the relationship

$$C_r = A_1 e^{-b_1 r} + A_2 e^{-b_2 r} \quad (1)$$

where  $C_r$  is the auxin concentration in  $\mu$  moles/ml at any roentgen dose,  $r$ .  $A_1$ ,  $A_2$ ,  $b_1$ ,  $b_2$  are constants  $> 0$ , and  $A_1 + A_2 =$  the initial auxin concentration,  $C_0$ . With  $C_0 = 0.015 \mu$  moles/ml, graphical fit yielded the following values of the constants:  $A_1 = 0.005$ ,  $b_1 = 0.041$ ,  $A_2 = 0.01$ ,  $b_2 = 0.00011$ . For later comparison, the fitted curve of  $C_r$  versus dosage is given in figure 1, curve A.

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The chemical effects of ionizing radiation on dilute aqueous solutions are attributed chiefly to free radicals formed through excitation and ionization of water (6, 17). For a known dose of a given radiation energy, the number of primary ion pairs formed per unit weight of water can be calculated, and the efficiency of solute inactivation conveniently expressed as ionic yield ( $M/N$ ), i.e., the number of solute molecules inactivated ( $M$ ) per ion pair ( $N$ ). Under the conditions of continuous uniform irradiation and nonlimiting solute concentrations, with no competition for the radicals by solute inactivation products, and with back reactions at a steady-state, the concentration of solute decreases linearly with the dose (17). The ionic yield would thus remain constant. Where varying rates of back reactions, competition, or a chain inactivation occurs, residual solute concentration will diminish either exponentially or at rates which can be described empirically by sums of exponentials. In these instances  $M/N$  will vary with increasing dosage. Under such circumstances an estimate of the intrinsic radiosensitivity of the solute could be obtained by determining the inactivation efficiency at *initiation* of irradiation, i.e., as the dose approaches zero. This initial ionic yield with exponential inactivations may be estimated by calculating the  $M/N$  for the various experimental values and either approximating graphically the ionic yield at zero dose or evaluating empirically the relationship  $\lim_{r \rightarrow 0} M/N = (f)r$ .

$$r \rightarrow 0$$

A convenient and more accurate approximation may be made of the initial ionic yield when  $M/N$  vs  $r$  is nonlinear. Ionic yield for any roentgen dose may be expressed as

$$(M/N)_r = \left[ \frac{C_0 - C_r}{r} \right] K \quad (2)$$

where  $K$  is the quotient of Avogadro's number divided by the number of ion pairs formed per  $r$  per unit volume, and  $C_0$ , the initial solute concentration, and  $C_r$ , the concentration remaining at dose  $r$ , are in moles per unit volume.

Let

$$C_r = \sum_{i=1}^n A_i e^{-b_i r} \quad (3)$$

where the  $A_i$ 's and  $b_i$ 's are constants  $> 0$ , and

$$\sum_{i=1}^n A_i = C_0.$$

Then

$$(M/N)_r = \frac{\left[ C_0 - \sum_{i=1}^n A_i e^{-b_i r} \right] K}{r} \quad (4)$$

Expanding the exponential terms, and recalling

$$\sum_{i=1}^n A_i = C_0,$$

$$(M/N)_r = K \sum A_i b_i \left[ 1 - \frac{b_i r}{2!} + \frac{b_i^2 r^2}{3!} - \dots \right]. \quad (5)$$

Hence

$$(M/N)_{r \rightarrow 0} = K \sum_{i=1}^n A_i b_i \quad (6)$$

For  $n = 1$ ,

$$(M/N)_{r \rightarrow 0} = K A_1 b_1$$

For  $n = 2$ ,

$$(M/N)_{r \rightarrow 0} = K (A_1 b_1 + A_2 b_2) \text{ etc.}$$

If we assume an effective wavelength of 0.02 Å in Skoog's experiments (heavily filtered 900 kvp x-rays), and  $1.8 \times 10^{12}$  ionizations per ml solution for this wavelength (17),  $K$  becomes  $2.4 \times 10^{11}$ . By inserting the constants of equation (1) in equation (6),  $n = 2$ , an initial ionic yield of ca 70 is obtained. An even higher intrinsic sensitivity is shown in the inactivation of auxin by gamma radiation. A fit of the data obtained by irradiating auxin solutions with a radium source (26, p. 236) gave residual concentrations the values of

$$C_r = 0.0027 e^{-0.35r} + 0.012 e^{-0.0009r} \mu \text{ moles/ml} \quad (7)$$

From equation (6) an initial ionic yield of 325 results; the curve obtained by exponential fit of the ionic yields calculated for each experimental point yielded an initial inactivation rate of 350 molecules inactivated per ion pair.

These ionic yields are unusually high. Many biological substances are inactivated by ionizing radiation with efficiencies on the order of a few hundredths to somewhat above 1 molecule per ion pair (17). Ionic yields somewhat greater than 1 have been interpreted as resulting from the production of several active radicals after the primary splitting of water to H and OH (4, 14). However, yields of 70 and 325 indicate that a chain or autocatalytic sequence of auxin inactivation had been induced, similar to the radiation induced auto-oxidation of unsaturated fatty acids (19, 22) or the decomposition of indole with very low dosages (Allsopp in (6)).

The difference in the initial inactivation efficiency of very hard x- and  $\gamma$ -irradiation is difficult to interpret. The number of ionizations produced per ml solution per  $r$ , as well as the linear ion density in this instance, are similar for both radiations. One would expect the radiochemical effects to be essentially the same, as they have been found to be with even wider ranges in energies (17, 21). Therefore, we first at-

tempted to duplicate the above radiochemical efficiencies for auxin inactivation.

#### EXPERIMENTAL METHODS

Stock solutions of IAA (Eastman) in purified water (13), generally 0.25 mM, were made up monthly and stored at 3 to 5° C. Lower concentrations were freshly prepared from stock solution. To obtain higher concentrations, the more soluble Na salt (recrystallized from the acid) was utilized. Unless otherwise noted, the solutions were in equilibrium with air and irradiated at a pH of 6 to 7. Solutions with low oxygen concentration were prepared by the evacuation apparatus and method described by Hart (13). The same apparatus was used to saturate solutions with oxygen (15).

IAA was usually assayed colorimetrically with iron-perchloric acid reagent (11). In testing the protective action of other solutes, IAA calibration curves were determined in the presence of the protective agent in amounts equivalent to those experimentally used. When bioassay was used to measure auxin concentration, diluted aliquots of a solution were mixed with an equal quantity of 3% liquid agar and assayed by the standard Avena curvature method using sand-grown plants. Simultaneously, several known concentrations of IAA were run in order to convert experimental curvatures directly to IAA mole equivalents.

Native auxin of the kidney bean plant was extracted with ether and purified by paper chromatography. Several kg of 3-week-old plants, cut at the soil level, were frozen in liquid  $N_2$  and pulverized. The material was covered with diethyl ether (distilled from aqueous  $Ca(OH)_2-FeSO_4$ ) which was allowed to remain at 0 to 3° C for ½ hour and then decanted. This extraction was twice repeated. The combined extracts were reduced in volume to ca 100 ml and partitioned 3 times with 20 ml of 1%  $NaHCO_3$  in water. The combined aqueous phases were washed once with ether, adjusted to pH 3.0 with HCl, and repartitioned with ether (3 × 2 vols). After removal of the ether from the acid fraction by distillation, the residue was taken up in 0.5 ml of purified 95% ethanol (distilled from Zn-KOH). The alcohol was then quantitatively transferred and evaporated in a nitrogen stream as the starting line of a chromatogram (6.25 cm strip Whatman No. 3 MM). Following equilibration in the chromatograph chamber, the strip was developed by descending isopropanol:28% ammonia:water (10:1:1) at 25° C for 16 hours.

The following radiation sources were employed:

1. For hard x-rays,
  - a. Westinghouse 200 kvp, filtration 0.75 mm Cu plus 3 mm Bakelite, half-value layer 1.07 mm Cu,  $\lambda$  effective 0.15 Å.
  - b. G. E. Maximar 250 kvp, filtration 0.25 mm Cu plus 1.5 mm Bakelite, hvl 1 mm Cu,  $\lambda$  effective 0.15 Å.
2. For soft x-rays, Picker 50 kvp, no added filtration, hvl 1.07 mm Be or 0.07 mm Al,  $\lambda$  effective approximated as 1.8 Å.

3. For  $\gamma$ -rays,
  - a. 100 mg radium needle in equilibrium with its decay products, whose  $\lambda$  effective was considered as 0.015 Å on the basis of the average total photon energy (5).
  - b. The 25 curie  $\text{Co}^{60}$  source described in (13).

Unless otherwise noted, the dose rates were between 50 and 250r/min for x-rays, 12.2r/min with the Ra source, and 440r/min with the  $\text{Co}^{60}$  source. (As will be shown below, there is no appreciable effect of dose rate on the ionic yield in this range.)

In a large fraction of the experiments with hard x-rays, the solutions were in equilibrium with air and irradiated in 50- or 100-ml silica glass cylinders under a vertical beam. Since the heights of the solutions were 2.5 to 5 cm, the filtered hard x-rays were considered as monochromatic and as decreasing exponentially through the water. Victoreen thimble chamber measurements of dose rate were made at the aqueous surface of a phantom and converted to integral dose by Mayneord's approximation (18, equation (7)), using 0.181 as the absorption coefficient for water at  $\lambda$  0.15 Å (7). In a typical vertical irradiation of a cylindrical volume of solution by the 200 kv x-ray beam, target to surface distance 25 cm, the Mayneord approximation gave an average dose rate of 85r per minute. A depth-dose plot (8) for the volume yielded 83r, while a determination by ferrous ion oxidation, under identical irradiation conditions, gave a value of 73r. Radiation dose was determined in all other experiments by ferrous sulfate oxidation, using the value of 19.7  $\mu$  moles of  $\text{Fe}^{++}$  oxidized per liter per kiloröntgen to convert the iron yield to r (20, 21). The experimental values given for radiation inactivation are corrected for similarly treated but unirradiated controls.

### RESULTS

A 15.5  $\mu\text{M}$  solution of cryst. IAA in air-saturated water was irradiated with hard x-rays. The changes in residual auxin concentration were followed colorimetrically, and are shown in figure 1, curve B. The concentration decreased exponentially,

$$C_r = .0155 e^{-0.000158r} \mu \text{ moles/ml} \quad (8)$$

from which, using equation (6), an initial ionic yield of 0.87 is obtained. These results may be contrasted with those in (26) as differing both in rate order of inactivation and in direct molecular radiosensitivity.

**METHOD OF AUXIN ASSAY:** Two changes from the procedures used in (26) were made in the above experiment. First, the concentration of auxin involved permitted use of the more rapid and convenient colorimetric assay instead of biological assay as a measure of changes in IAA concentration. If we assume that the products arising from IAA on irradiation are of reduced activity or are relatively inactive as auxins when measured by coleoptile curvature—certainly a reasonable assumption in view of the postulated structural requirements for biological activity—it is still possible that such products could react with the color

reagent. Colorimetrically determined values might, therefore, be spuriously high. This possibility is ruled out by the curves later shown in figure 7. Concomitant analyses by both assay methods of aliquots taken from a solution of IAA during irradiation yielded essentially the same values.

**RADIATION ENERGY:** Second, since we used "hard" (not "very hard") x-rays, it was possible that the kinetics of auxin inactivation were uniquely dependent on photon energy in this frequency range. Though the probability of such a dependence was low, as indicated above, the inactivation efficiencies of gamma and hard and soft x-rays were examined. Figure 2 shows the IAA concentrations during irradiation of 53.6  $\mu\text{M}$  solution by radium and by 200-kv and 50-kv x-rays. It is evident that the inactivation curves are similar for the three radiations, the initial ionic yields being about 1. In an analogous experiment, an 860  $\mu\text{M}$  solution was irradiated with the  $\text{Co}^{60}$  source. An initial ionic yield of about 1.2 was obtained.

**AUXIN CONCENTRATION:** A series of indoleacetate concentrations was then irradiated by hard x-rays. The solutions were unbuffered (pH 6 to 7), and ranged in concentration from 15 to 1200  $\mu\text{M}$ . Residual IAA concentrations at the various doses for seven typical experiments are shown in figure 3. The exponential form is apparently followed irrespective of initial concentration or dose. With the three lowest concentrations, the exponential relation holds till approximately 90% of the auxin originally present was inactivated.

The residual concentration curves in figure 3 can be generalized as

$$C_r = C_o e^{-br}$$

with the initial condition  $C_r = C_o$  when  $r = 0$ . It is evident that the slopes of the linear relationships  $\log C_r$  versus dosage are not equal but vary inversely with  $C_o$ . Therefore, let

$$b = \frac{k}{C_o}, \text{ whence}$$

$$C_r = C_o e^{-kr/C_o} \quad (9)$$

$$C_o - C_r = C_o (1 - e^{-kr/C_o}) \quad (10)$$

The experimental data permitted a test of the preceding generalizations. A log-log plot of  $b$  versus  $C_o$  for nineteen irradiation experiments, in which each slope was approximated by least squares, is given in figure 4. A least squares fit yields the value for  $b$  of  $3.7 \times 10^{-6} C_o^{-0.90 \pm 0.034}$ , with  $C_o$  expressed in  $\mu$  moles/ml. Assuming that the exponent of  $C_o$  is  $-1$ , the intercept value of  $k$  for equation (9) is  $2.8 \times 10^{-6}$  (fig 4). If the initial concentrations below 0.05  $\mu$  moles/ml, where the yield tends to fall, are not considered,  $b = 3.1 \times 10^{-6} C_o^{-1.03}$ . Equations (9) and (10) may therefore be considered as reasonably correct formulations of auxin inactivation by hard x-rays within the concentration and dose range studied.

We have no specific knowledge of the number, identity, concentrations, or reactivities of the auxin inactivation products formed during the irradiation.

However, the concentration of such products may be expressed in terms of the IAA inactivated,  $C_0 - C_r$ , having the average radical reaction affinity relative to IAA of  $\beta$ . Per unit volume the increment of IAA molecules inactivated on any dose increment will depend on the ratio of IAA to the total number of reactive molecules and their reaction affinities. If we assume that the only solutes present in pure solution are auxin and its inactivation products, and that solute concentrations are sufficiently high so that we may neglect the probability of radical elimination by activated water interactions, then:

$$dC_r = -kC_r dr [C_r + \beta(C_0 - C_r)]^{-1}. \quad (11)$$

This approximation may be integrated to

$$C_r(1 - \beta) + \beta C_0 \ln C_r = -kr + A \quad (12)$$

For  $\beta = 1$ ,

$$\ln C_r = -kr/C_0 + A/C_0$$

$$C_r = A'e^{-kr/C_0} \quad (13)$$

For the initial conditions  $C_r = C_0 = A'$  when  $r = 0$ , equations (9) and (13) are identical. We may therefore infer that the kinetics of IAA destruction in pure solution are defined in part by the competitive action of the inactivation products, and that such products have an average reaction affinity for the activated water about equal to that of IAA. A like reaction order was found in the x-ray inactivation of carboxypeptidase by Dale et al (3). There, similarly, the proportion of active enzyme concentration remaining at various doses was a function of the ratio of the dose to initial enzyme concentration over two orders of magnitude of  $C_0$ .

Finally, the initial ionic yields were calculated for each of the above irradiations with various auxin concentrations. They are shown in figure 5, curve A. Within two orders of concentration magnitude, the ionic yields are essentially in the range of unity. There is a trend toward lower initial ionic yields at higher dilutions. This is consistent with previous observations (17), in which falling radiochemical yields at high solute dilutions are attributed to an increased probability of radical recombination before reaction with the solute. The fall in yield at higher dilutions, as well as the relatively constant absolute amount of auxin inactivated for a given dose over the major portion of the concentration range, is approximated by equation (10).

**OXYGEN:** The presence of dissolved oxygen generally enhances the oxidation of a solute by x- or  $\gamma$ -rays. We therefore considered the possibility that the efficiency of auxin inactivation might be critically dependent upon oxygen concentration in the solutions. Various concentrations of auxin in water were either evacuated or saturated with oxygen and then exposed to hard x-rays. The initial ionic yields calculated for each auxin concentration are given in figure 5. In comparison with the ionic yields of about 1 for auxin solutions in equilibrium with air, the yields dropped to between 0.5 and 0.3 in the absence of air and were

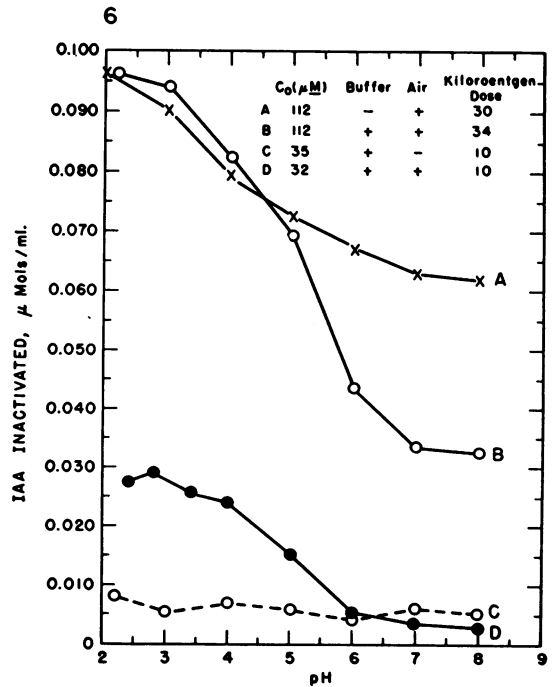
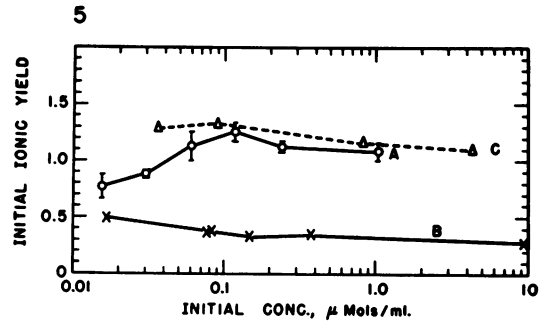
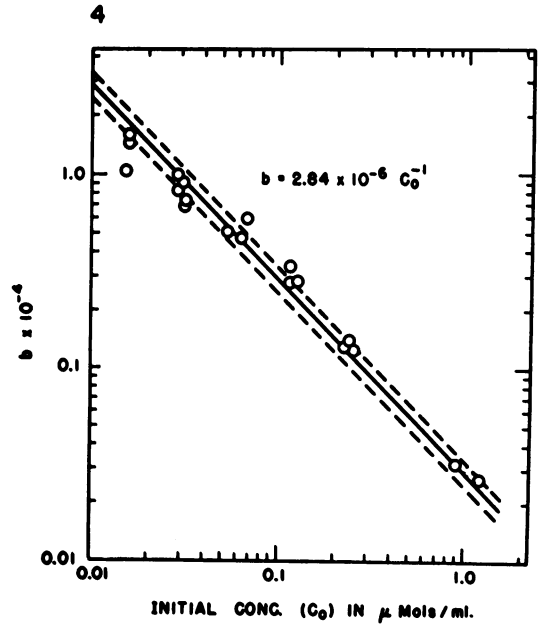
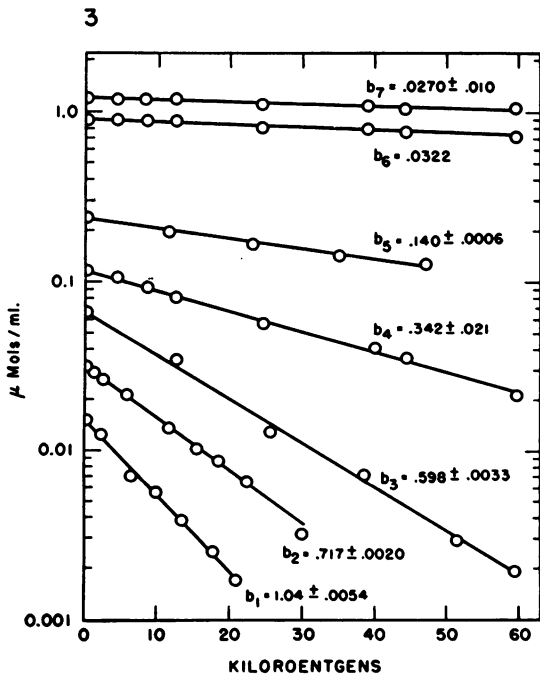
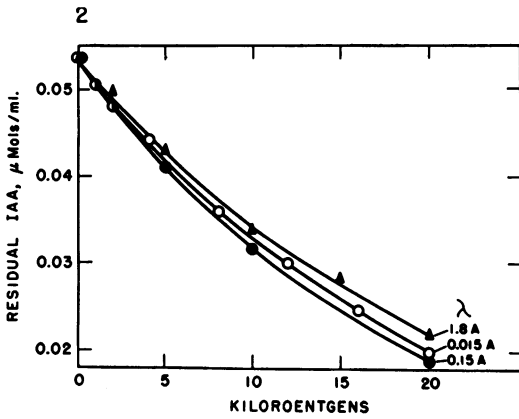
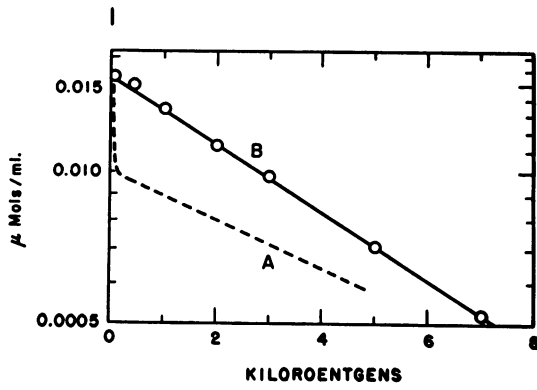
slightly above 1 with oxygen saturation. In no instance was there any indication of initiation of a chain sequence of auxin inactivation or highly accelerated inactivation rates.

**pH:** The statement that the extent of auxin inactivation in aqueous solution (in presence of air) is at least as great in the pH range well above the pK of the acid as in the range below it (26), is not substantiated by our observations. When IAA solutions adjusted to various pH's by HCl or NaOH were irradiated, the extent of inactivation increased with increasing acidity (fig 6, curve A). The difference in auxin sensitivities at neutral and acid pH's was enhanced when the medium was buffered (fig 6, curves B and D). Indeed, the inactivation curves in buffer containing dissolved air bear a striking resemblance to the dissociation curve of IAA, which has a pK 4.75. This resemblance suggested that the radiosensitivity was a function of the concentration of associated molecules, a possibility considered by Skoog in examining the pH effect. However, when evacuated solutions were irradiated, no significant difference was found in the amount of inactivation between pH 2 and pH 8 (fig. 6, curve C). Since IAA dissociation equilibria presumably are unaffected by evacuation, the apparent higher sensitivity of the associated form of IAA may be related to the particular radical species produced by irradiating aerated water. Alternatively, oxidant concentration may increase with  $H^+$  concentration when water containing dissolved  $O_2$  is irradiated.

It seems unlikely that photolysis of the HCl used for acidification caused the pH effect in solutions without added buffer (fig 6, curve A); a similar difference in the amount of IAA inactivated was observed for solutions adjusted to pH 5 and pH 2 with  $H_2SO_4$  before irradiation. Competition for radicals by buffer citrate may have caused the decreased IAA inactivation near neutrality (fig 6, curves B, C, and D). Yet, IAA inactivation increased at the more acid pH's at which citrate concentrations were also greater. Though the mechanism of yield dependencies on pH is not clear (Amphlett in (6)), it is pertinent that  $H_2O_2$  levels in irradiated water were found to increase with increasing acidity (1).

As an index of inactivation efficiency in acid medium, IAA solutions at pH 2.9 were then x-irradiated. The typical exponential decrease in concentration was observed, and, as indicated in table I, no greatly enhanced inactivation efficiency resulted at the more acid pH. The initial ionic yields are roughly double those observed on irradiation of neutral solutions.

**PROTECTIVE EFFECTS:** With ionizing radiation, one would anticipate that the ionic yield for auxin inactivation would be decreased by the presence of other solute molecules able to react with activated water. Such protective effects have been frequently observed and investigated. Nevertheless, it is possible that enhanced inactivation might be caused by impurities present in an indoleacetate preparation or by certain cosolutes. Therefore, the inactivation of IAA by hard x-rays was determined after IAA recrystallization, in



the presence of single added substances, and in the presence of extracts of the *Avena* coleoptile and cocklebur plant.

Commercial IAA was recrystallized four times (as the Na salt from alcohol, again as the salt from water-alcohol, as the acid from water and from ethylene dichloride). The inactivation curves, given in figure 7, indicate no significant effect of recrystallization upon IAA sensitivity, the initial ionic yields being approximately one.

Inactivation curves were then determined for IAA in the presence of added solutes at several mole ratios. Irradiation and periodic sampling were continued (with the single solute added as well as with the plant extracts) till 10 to 20% of the IAA initially present was inactivated. Initial inactivation efficiencies were calculated, and are given in table II, column 4, in order of increasing protective action. No great enhancement of IAA inactivation was observed with any of the substances listed. Glutathione, cysteine, asparagine, glucose, and ascorbate, in equimolar ratios, showed no protective effect under these experimental conditions. Significantly reduced IAA inactivation rates resulted with the last two compounds when present in 10 mole excess, and in the presence of ethanol, ferricyanide, and malic acid. It should be noted, as a potential model for *in vivo* auxin sensitivity, that protein protected the IAA with relatively high efficiency. The presence of the albumin in 0.1 molar ratio reduced initial IAA inactivation efficiency by ca 2/3, whereas the leaf protein, at a molar ratio of  $10^{-2}$ , lowered the ionic yield 100 fold.

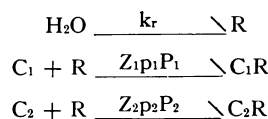
Lea (17) has formulated the number of solute molecules undergoing chemical change by radical interaction, in a unit volume, as  $n CZpP$ , where  $n$  is the number of active radicals,  $C$  is the solute concentration,  $Z$  the solute-radical collision frequency for unit solute concentration,  $p$  the probability of radical elimination on collision with the solute, and  $P$  the proportion of those radical eliminations which results in solute change. By a procedure analogous to that followed by Lea in evaluating the data of Dale (2), in which the concentrations of various cosolutes required for 50% protection of flavin-adenine-dinucleotide against a single x-ray dose were listed, the relative radical deactivation probabilities on solute collision may be deduced for the experiments reported here. Let us consider the cosolute ( $C_2$ ) as competing with

TABLE I  
IONIC YIELDS BY HARD X-IRRADIATION OF INDOLEACETIC ACID AT pH 2.9, McILVAINE'S CITRATE-PHOSPHATE BUFFERS

	$C_0$	$b$	$(M/N)_{r \rightarrow 0}$
	$Moles \times 10^{-6}/ml$	$\times 10^{-4}$	
Buffered . . . . .	0.126	0.57	2.5
Unbuffered . . . .	0.111	0.53	2.1
Buffered . . . . .	0.032	1.95	2.2

$$C_r = C_0 \cdot e^{-br}; (M/N)_{r \rightarrow 0} = C_0 \cdot b \cdot K; K = 3.52 \times 10^{11} \text{ (see text)}$$

IAA ( $C_1$ ) for radicals produced from the water, and assume that a steady state of free radical concentration is rapidly established (13) under a constant dose rate. Using the above symbols, radical (R) production and radical and solute deactivation may be characterized by the following rate constants:



In the steady state

$$\frac{dR}{dt} = 0$$

$$R = \frac{k_r}{C_1 Z_1 p_1 + C_2 Z_2 p_2}$$

$$-\frac{dC_1}{dt} = \frac{Z_1 p_1 P_1 C_1 k_r}{C_1 Z_1 p_1 + C_2 Z_2 p_2} \quad (14)$$

If  $C_2 = 0$

$$-\frac{dC_1}{dt} = P_1 k_r \quad (15)$$

Let  $\alpha = \frac{dC_1 \text{ in presence of } C_2}{dC_1 \text{ in pure solution}}$ , where  $0 < \alpha < 1$ .

Then  $\frac{Z_1 p_1 P_1 C_1 k_r}{C_1 Z_1 p_1 + C_2 Z_2 p_2} = \alpha P_1 k_r$ , whence the relative probability of radical deactivation

$$\frac{p_2}{p_1} = \frac{Z_1 C_1 (1 - \alpha)}{\alpha Z_2 C_2} \quad (16)$$

[Equation (14) may be considered as a more explicit

FIG. 1. Residual concentrations of IAA in water after various dosages of x-rays. Initial concentrations 15  $\mu$ M. Curve A. Calculated from data of Skoog, 900 kvp, 50r/min. Curve B. This work, 200 kvp, 50r/min.

FIG. 2. The inactivation of auxin by radium  $\gamma$ , 200-kvp and 50-kvp x-rays. The initial ionic yields are, respectively, 0.89, 0.97, and 0.84.

FIG. 3. Residual concentrations of auxin as a function of hard x-ray dose at various initial IAA concentrations. The fitted slopes are listed and all are  $\times 10^{-4}$ .

FIG. 4. Test of equations (9) and (10), with slopes of residual concentration curves as a function of hard x-ray dose plotted against initial auxin concentration. Broken lines indicate 99% confidence limits from fit using  $C_0^{-1}$ .

FIG. 5. Initial ionic yields obtained by hard x-irradiation of various concentrations of indoleacetate in water. Solutions unbuffered, pH 6-7. Curve A. Solutions in equilibrium with air. Vertical brackets indicate the standard error. Curve B. Evacuated. Curve C. Oxygen saturated.

FIG. 6. The effect of pH on IAA inactivation by hard x-rays. Buffer: McIlvaine's standard citrate-phosphate.

TABLE II  
EFFECT OF ADDED SOLUTES ON INITIAL IONIC YIELDS FOR IAA INACTIVATION BY HARD X-RAYS

1 SOLUTES	2 INITIAL IAA CONC	3 MOL RATIO ADDEND/IAA	4 (M/N) <sub>r→o</sub>	5 p <sub>2</sub> /p <sub>1</sub> †	6 Z <sub>p</sub> /MOL WT ††	7 Z <sub>p</sub> ‡	8 Z <sub>2</sub> p <sub>2</sub> /Z <sub>1</sub> p <sub>1</sub>
	<i>μM</i>						
Glutathione .....	59	1	1.0	....	....	....	....
Glutathione .....	63	10	2.3	....	....	....	....
Cysteine .....	65	1	1.3	....	....	....	....
Cysteine .....	61	10	1.6	....	....	....	....
Asparagine .....	63	1	1.2	....	....	....	....
Asparagine .....	62	10	1.2	....	....	....	....
Glucose .....	65	1	1.2	....	....	....	....
Glucose .....	62	10	0.57	0.11	2.1 × 10 <sup>8</sup>	3.7 × 10 <sup>10</sup>	0.11
Ascorbic acid .....	62	1	1.2	....	....	....	....
Ascorbic acid .....	62	10	0.41	0.19	3.8 × 10 <sup>8</sup>	6.6 × 10 <sup>10</sup>	0.19
Ethanol .....	62	1	0.98	0.33	1.6 × 10 <sup>9</sup>	7.3 × 10 <sup>10</sup>	0.21
Ethanol .....	62	10	0.46	0.25	1.2 × 10 <sup>9</sup>	5.5 × 10 <sup>10</sup>	0.16
K <sub>3</sub> Fe(CN) <sub>6</sub> .....	60	1	0.66	0.59	8.5 × 10 <sup>8</sup>	2.8 × 10 <sup>11</sup>	0.82
Malic acid .....	62	1	0.51	1.5	3.5 × 10 <sup>9</sup>	4.8 × 10 <sup>11</sup>	1.4
Bovine serum albumin * ...	62	0.1	0.37	0.81	1.1 × 10 <sup>8</sup>	7.6 × 10 <sup>12</sup>	22
Leaf protein ** "Fraction I"	61	0.01	0.011	110	6.3 × 10 <sup>9</sup>	3.8 × 10 <sup>15</sup>	11,000
IAA alone .....	62	....	1.2	1	1.9 × 10 <sup>9</sup>	3.4 × 10 <sup>11</sup>	1
IAA alone .....	21	....	0.88	....	....	....	....
IAA in 1/2 strength coleoptile juice .....	21	....	0.016	....	....	....	....

\* Armour cryst., molecular wt 70,000.

\*\* Prepared from spinach leaves according to (28), incorporating two cycles of differential centrifugation, and presumed to be over 95% pure, molecular wt 6 × 10<sup>6</sup>.

† Relative probability of radical elimination on single collision with a solute molecule.

†† Relative radical affinity of solutions containing 1 gm/l.

‡ Relative radical affinity of solutions containing 1 gm mole/l.

Z represents the number of collisions per sec per ml between solute molecules and an active radical calculated by the relationship  $Z_{AB} = n_A n_B \delta_{AB}^2 [8\pi RT(1/m_A + 1/m_B)]^{1/2}$ , where n = the number of molecules per ml, m the molecular wt,  $\delta_{AB}$  the mean molecular diam in cm, and R the gas constant in ergs per mole per degree (9). The molecular diameters have been approximated as  $1.33 \times 10^{-8}$  m<sup>3/8</sup> cm (17).

form of equation (11) in the case when C<sub>2</sub> represents not a cosolute added to the IAA solution, but the product(s) of IAA inactivation, whose concentration was defined as (C<sub>0</sub> - C<sub>1</sub>). Thus, when the relative radical reaction affinity of the product is 1, i.e., Z<sub>1</sub>p<sub>1</sub> = Z<sub>2</sub>p<sub>2</sub>, equation (9) likewise follows.]

The relative probabilities of radical elimination with a number of the cosolutes used are given in table II, column 5. It may be seen that the probability of radical deactivation with a single collision is less for low molecular weight cosolute molecules (except for malic acid) than for IAA. The probability for the serum albumin molecule is, surprisingly, likewise less, though the spinach leaf protein has an effectiveness of from 10<sup>2</sup> to 10<sup>3</sup> greater than the other substances evaluated. Since the collision frequency rises with increase in solute molecule dimension, the proteins have, on an equimolar basis, an appreciably greater net radical affinity than has IAA; thus they exert a strong protective effect. Columns 7 and 8 indicate an apparent radical distribution ratio between IAA and protein of 1:20 for the serum albumin and 1:11,000 for the leaf protein.

Protective action rather than enhanced inactiva-

tion was likewise observed when IAA was irradiated in the presence of plant extracts. Avena coleoptile sheaths and Xanthium leaves and buds were ground, in the cold, for 2 minutes in a mortar with quartz sand. The dispersions were centrifuged at 25,000 × g for 1/2 hour at 2° C and the supernatants diluted with water. IAA was added to each diluted extract and the mixtures were transferred to cylinders imbedded in cracked ice. A fifty-fold decrease in IAA sensitivity to x-rays was provided by the presence of 1/2 strength coleoptile juice (table II, col 4); 1/2 strength Xanthium extract yielded roughly a twenty-fold decrease in initial sensitivity (fig 8). It can be observed from the figure that IAA sensitivity increases as the extract becomes more dilute, and that the curve tends to extrapolate, at high dilutions, to the sensitivity of pure IAA in solution, i.e., initial ionic yields in the region of unity.

DOSE RATE: The tendency for initial ionic yields to fall when initial IAA concentrations were below 0.06 μ moles/ml (fig 5, curve A) was interpreted as reflecting the increased probability of radical recombination and consequent lessening of radical-solute interactions. Extending the time in which equivalent energies were

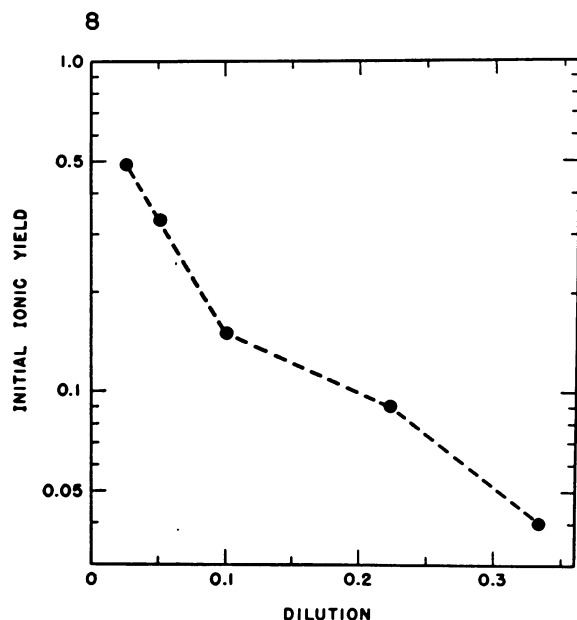
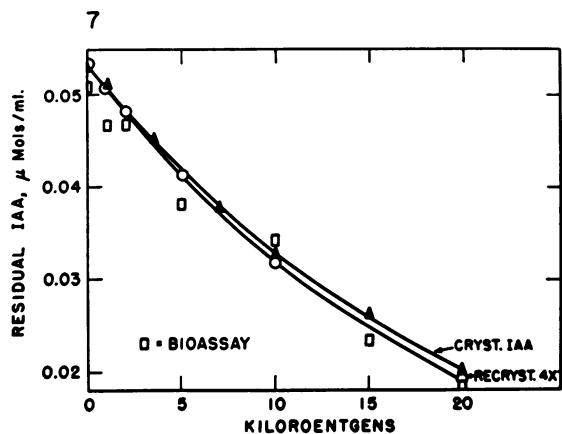


FIG. 7. The effect of recrystallization on IAA inactivation. Initial concentration  $53.5 \mu\text{M}$ .  $(M/N)_{r \rightarrow 0}$  for cryst. IAA, 0.93; for recryst. IAA, 0.99. The bioassay points represent residual auxin concentrations as determined by the Avena curvature test in aliquots taken from the "cryst" IAA solution at the indicated radiation doses.

FIG. 8. Protective effects of Xanthium extract at various dilutions. Initial ionic yields for IAA inactivation are plotted vs dilution of extract (tissue weight as a fraction of final mixture). Initial IAA concentrations ca  $0.1 \mu\text{moles/ml}$ .

absorbed under continuous irradiation should result in an increased separation of the photon tracks and thereby of the radicals formed. Thus, enhanced radical-solute reaction rates might occur at low solute concentrations when the dose rate is lowered. Therefore, IAA solutions in equilibrium with air were irradiated with hard x-rays at dose rates ranging from 1.4 to 840 r/min, the intensity range being obtained by

varying the target distance and tube current. Sampling was continued until 20 to 30% inactivation occurred. The effect of dose rate on initial ionic yield, together with the rate constants of IAA disappearance, are given in figure 9. It can be seen that inactivation efficiencies are virtually independent of dose rate above 20 r/min. At dose rates below 10 r/min the inactivation rate rises. The ionic yields can be extrapolated to a value near 4 at the relatively low rates of energy absorption. The figure also indicates that the dose rates ordinarily used here, as well as in (26), produce ionic yields of about unity.

**SENSITIVITY OF AUXIN OBTAINED FROM THE PLANT:** The possibility that auxin isolated from the plant might possess a radiosensitivity different from that of synthetic auxin was investigated. The plant auxin was obtained by chromatographic resolution of the ether-soluble acidic fraction of kidney bean plants (see METHODS). Preliminary experiments with pure IAA showed that this auxin could be delimited on a chromatogram either by examination under UV light or by spraying with iron-perchloric acid reagent. By either method the  $R_f$  was 0.29 to 0.30. Moreover, 90 to 95% recoveries of the IAA originally applied could

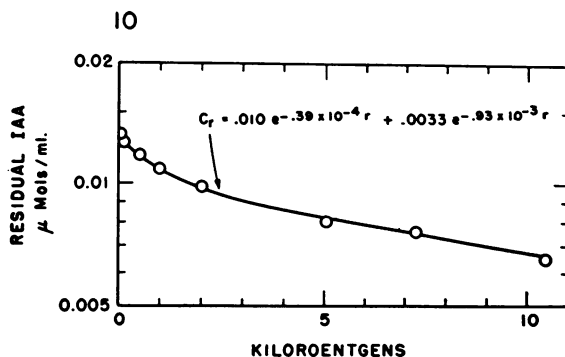
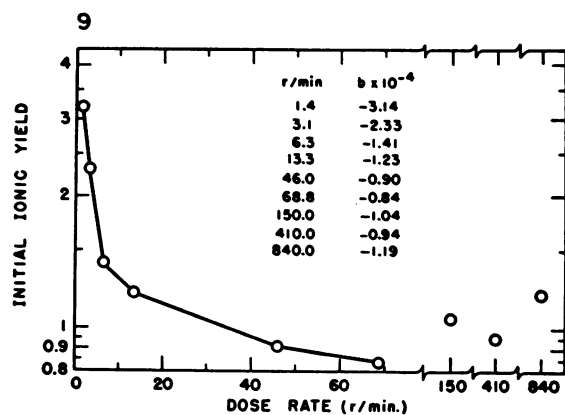


FIG. 9. The effect of x-ray dose rate on initial ionic yield. Initial IAA concentration,  $28.6 \mu\text{M}$ . Tabulated are the rate constants of concentration change at each dose rate.

FIG. 10. Inactivation of auxin obtained from bean plants. The points are mean experimental values, the curve is the calculated fit.



be obtained on elution: the UV outlined spot on a developed strip was cut out and immersed in water, adjusted to pH 3, and both paper and water extracted with ether.

The developed chromatogram of the acid fraction of the plant extract showed several distinct components when surveyed under UV light ( $R_f = 0.08, 0.20, 0.29, 0.61, 0.90$ , and frontal). However, a parallel guide strip of an aliquot of the plant extract, showing corresponding components under UV, yielded the crimson IAA color only at  $R_f 0.30$  when sprayed with the iron-acid reagent. Segments of a similar strip analyzed by *Avena* curvature bioassay showed significant activity only at about  $R_f 0.30$ . Therefore, this region was cut from the unsprayed chromatogram, eluted as described in the previous paragraph, and taken up finally in 10 ml water. Aliquots of this solution assayed  $0.013 \mu$  moles IAA per ml.

The remainder of the solution, in air equilibrium, was irradiated as usual with hard x-rays till approximately 50 % of the original concentration remained. Figure 10 shows the effect of irradiation on this auxin preparation. Here the decrease in concentration as a function of dose departs from the exponential form, unlike the behavior of synthetic IAA (cf., in particular, the lowest curve in figure 3). The concentration-dose relationship for auxin extracted from the plant could be reasonably characterized by two exponentials, and the constants derived for this relation are given in figure 10. Again no unusual auxin radiosensitivity is indicated. Initial inactivation efficiency determined by substitution of the constants in equation (6) gives an ionic yield of 1.2.

#### DISCUSSION

The preceding experiments indicate that the auxin IAA possesses no unusual lability toward ionizing frequencies of the x and  $\gamma$  range. High ionic yields characteristic of an extended chain sequence, in which the inactivation of many solute molecules is initiated by the primary ionization or excitation, were not observed. Inactivation efficiencies were of the same magnitude, irrespective of the method of auxin assay, radiation energy, auxin concentration, oxygen partial pressure,  $H^+$  concentration, or dose rate, in the ranges examined. Recrystallization of the auxin had no effect on its sensitivity. Moreover, the acid auxin extracted from the plant exhibits an initial radiosensitivity in vitro not significantly higher than synthetic IAA. We cannot, therefore, corroborate the high radiosensitivity previously ascribed to IAA (26).

Within the dose ranges examined, the decrease in auxin concentration when pure solutions are irradiated can be accurately characterized as exponential. As is indicated by equations (11) and (14), this reaction order may be attributed to competition by the products of auxin inactivation as they become an increasing fraction of the total solute. It likewise follows from the above equations that the presence of competing solutes and their inactivation products, which vary in relative reaction affinities with respect

to IAA and its products, may yield relationships between  $C_r$  and  $r$  which cannot be characterized by a single rate constant. This may explain the difference between the reaction order reported here and that indicated by Skoog's data. This interpretation is likewise suggested by our results with the native auxin obtained by chromatography in which the decrease in concentration with dose is non-exponential (fig 10). As a chromatographic eluate, the purity of the auxin used for irradiation in the above experiment may be questioned. Aside from possible introduction of contaminant by manipulative transfers, paper partition chromatography would not have separated components in the plant extract of like mobilities. The above considerations imply that the reaction order of Skoog's data may have resulted from the use of impure preparations of IAA. In addition, they suggest that the apparent exponential decrease in IAA concentration we observed on limited irradiation of auxin in the presence of added solutes might not hold at higher radiation doses.

Slightly increased ionic yields for IAA inactivation were observed with the higher molar ratios of cysteine and glutathione as cosolutes (table II). Cysteine or protein cysteinyl have been suggested as likely reaction sites for auxin function in vivo (12), the carboxyl group and ortho-hydrogen of IAA being postulated as linking with the spatially opposed amino and sulfhydryl groups. Although covalent amide and thiol ester linkages might possibly enhance IAA sensitivity, such bonds would probably not be formed spontaneously in water near neutrality. A carboxyl-amino salt linkage can also be disregarded as a causative factor, since almost identical yields were obtained on irradiating IAA as the pure acid, as the sodium salt, in the presence of ammonium or potassium sulfate, or in the presence of asparagine. It may be suggested that the lack of thiol protective effect and the slight enhancement of IAA inactivation at higher thiol concentrations could result from an accompanying IAA reaction with free thiol radicals, viz.,  $RSH \rightarrow RS\cdot$ ;  $RS\cdot + IAA \rightarrow RS - IAA + H\cdot$ . In this connection, an enhancement of a methyl linoleate oxidation by x-radiation in the presence of glutathione has been reported (22).

The observations on the protective effects of added substances are pertinent to the radiosensitivity of IAA in vivo. Though the auxin has no unusual sensitivity when irradiated as a pure solute, table II indicates that its pZ, or affinity for activated water, is high among those low molecular weight cosolutes examined. In broad terms, it appears more readily oxidized by x-radiation. On the basis of the net auxin inactivation, the generalization would apply even to glutathione and cysteine, whose lability (17, 23) and protective action (22, 24) have been demonstrated elsewhere. Yet, a number of the solutes do protect the auxin when in suitable molar ratios. In this respect, the major protein component of leaf cytoplasm (28), Fraction I protein, with its relative pZ of ca  $10^4$ , is highly protective. Similarly, in Dale's study of the protective action of various cosolutes (2), both the

apoenzyme and prosthetic group of D-amino acid oxidase had the highest radical affinities of the many compounds examined. If we consider that free auxin occurs in leaf tissues at concentrations on the order of  $10^{-8}$  gm moles per kg, surrounded by a heterogeneous mixture of potentially reactive solutes, the proteins alone being in over  $10^4$  mole excess, it appears improbable that the absorption of low doses of ionizing radiation would result in significant auxin depletion by direct molecular inactivation. Indirectly substantiating this conclusion are the results obtained by irradiating IAA in the presence of coleoptile (table II) and leaf (fig 8) extracts. The diluted aqueous extracts, containing only a small fraction of the plant's soluble components, showed marked protective action. We therefore believe that an explanation other than direct auxin photolysis probably accounts for the lowering of auxin levels (26) in plants exposed to low doses of ionizing radiation.

Emphasis has been placed recently on the ratio of solute yields (R) obtained by irradiating dilute solutions in the presence and absence of oxygen. Since identical dosimetry was used in this study to evaluate the effects of oxygen saturation and air evacuation on IAA yields, the ratios of yields were calculated. Table III lists the R values obtained at four initial IAA concentrations. A ratio between 3 and 4 is not inconsistent with mechanisms proposed (6, 17) to account for solute oxidations by ionizing radiation. Hydroxyl radicals are formed in the absence of dissolved  $O_2$  when water is irradiated by  $\gamma$ - or hard x-rays. In the presence of  $O_2$ , the solute could be oxidized not only by OH radicals but also by concomitantly formed hydroperoxyl radicals and ions. This would yield a theoretical R value of 4 (Amphlett in (6)). A lower R value may be attributed to the back reactions plus pairwise combination of like radicals in zones of high free radical concentration (6, 14). The ionic yields obtained by irradiating aerated solutions of IAA at low dose rates (fig 9) approach the above theoretical value. Figure 9 likewise suggests that, at an initial IAA concentration of  $29 \mu M$ , the radical recombination rates attain and maintain a steady state for a wide range of dose rates above ca 20r/min.

Although IAA apparently disappears in the presence of  $H_2O_2$  or organic peroxides, Siegel and Weintraub (25) have shown that no actual destruction of the auxin takes place. The "inactivation" derives

from the interference by the peroxides with both biological and colorimetric assays for IAA. Their observations raised the possibility that the peroxide produced in irradiated water would cause spurious values for IAA destruction through anomalous assay values. However, it will be shown elsewhere that a)  $H_2O_2$  is probably not involved in auxin destruction by ionizing radiation, b) the peroxide levels found under our irradiation conditions are well below the concentrations where assays are affected, and c) structural alteration of the IAA molecule does occur.

### SUMMARY

The in vitro sensitivity of indoleacetic acid (IAA) to x- and  $\gamma$ -radiation has been re-examined by a kinetic approach. No unusual lability of the auxin was observed on varying the auxin concentration or purity, oxygen concentration,  $H^+$  concentration, radiation energy, dose rate, or method of assay. Ionic yields were near unity. Acid auxin extracted from the plant exhibits an intrinsic radiosensitivity not significantly higher than synthetic IAA. The effects of several cosolutes and plant extracts on IAA destruction were examined to determine the relative radical affinity of the auxin. The above observations indicate that an explanation other than direct auxin photolysis probably accounts for the lowering of auxin levels in plants exposed to low doses of ionizing radiation.

An approximation is described for determining initial ionic yields where changes in solute concentration are nonlinear with respect to radiation dose.

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TABLE III

RATIO OF INITIAL IONIC YIELDS BETWEEN OXYGEN SATURATED AND EVACUATED SOLUTIONS OF IAA

INITIAL CONC	$(M/N)_{r \rightarrow 0}$		R
	+ $O_2$	- $O_2$	
$\mu\text{mols/ml}$			
0.0355	1.29	0.42	3.1
0.089	1.33	0.37	3.6
0.832	1.16	0.32	3.6
4.29	1.10	0.29	3.8

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## THE CORRELATION OF DIFFERENT ASPECTS OF AUXIN ACTION<sup>1</sup>

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An auxin has been defined by Thimann (10) as "an organic substance which promotes growth (i.e., irreversible increase in volume) along the longitudinal axis, when applied in low concentrations to shoots of plants freed as far as practical from their own inherent growth-promoting substance. Auxins may, and generally do, have other properties, but this one is critical."<sup>2</sup> This definition emphasizes the aspect of auxin activity which was first studied by plant physiologists and which becomes particularly obvious when a stem or petiole responds with curvature to unilateral illumination. It was this curvature that first aroused the interest of Charles Darwin (2) and led to his classical studies on the power of movement in plants. This same curvature led Boysen-Jensen (1), thirty years later to his experiments with decapitated oat coleoptiles in which he demonstrated the capacity of the curvature stimulating influence to diffuse through a gelatin barrier and finally to the experiments of Went (12) who proved that agar on which

the coleoptile tips had been placed contained a substance which caused curvature in decapitated coleoptiles.

It is therefore logical from a purely historical point of view to emphasize that aspect of auxin action which manifests as elongation growth. But, as Thimann points out in the same paper, the effects of auxin on plant cells are numerous and are by no means confined to stimulating growth in length. Stimulation of cell division in the cambium, initiation of adventitious roots, inhibition of growth of axillary buds, stimulation of parthenocarpic development in fruits are all aspects of the action of auxin. It is this multiplicity of effect which makes the action of an auxin so difficult to define and its mechanism so hard to explain. Nor is the problem made any simpler by the fact that there are many unrelated chemical compounds which function as auxins.

In the present work the technique of cultivating sterile segments of sunflower hypocotyl on nutrient agar was utilized (3). Because the segments were sterile their behavior could be studied over a period of several weeks which made possible the observation of other more slowly induced auxin effects besides the rapidly manifested effect of stem elongation. These

<sup>1</sup> Received October 9, 1954.

<sup>2</sup> More recent definition of auxin was given in *Plant Physiol.* 29: 307, 1954. This does not differ in any essential feature from the earlier definition given by Thimann.