THE PHOTOCHEMICAL INACTIVATION OF INDOLEACETIC ACID SENSITIZED BY NON-PROTEIN COMPONENTS OF PLANT TISSUES

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Summary

A study has been made of the IAA-destroying activity of the brei of etiolated pea epicotyls other than that due to the enzyme IAA oxidase. The protein-free dialysate sensitizes the destruction of IAA in the light but not in the dark. Blue-violet light is most effective, followed by blue-green, green, red, and yellow.

The kinetic behaviour of the whole dialysate has been studied in detail. The apparent K_s is 3×10^{-4}M and the reaction is first order with respect to IAA. The net pH optimum is 5·5. The temperature optimum is 25°C at high light intensities. At low light intensities there is no dependence on temperature over the range 8-36°C. It is concluded that there is a photochemical reaction followed by one or more dark reactions. The activity is reduced by heating or by exposing to visible or ultraviolet light. The inactivation of IAA probably involves an oxidation without the intermediate formation of hydrogen peroxide.

In the etiolated pea seedling the active material is much more abundant in the buds than in the stems, roots, or cotyledons. It is present in higher concentration in etiolated than in green pea seedlings.

The dialysate contains at least four active components. A major component depends for its activity on a heavy metal which has not yet been identified. A technique is described for the resolution and detection of the active compounds on paper chromatograms. Preliminary fractionation procedures have been devised.

I. INTRODUCTION

In recent years considerable attention has been given to the enzyme systems which produce and destroy the native auxin, indole-3-acetic acid (IAA) in plant tissues. The enzyme complex IAA oxidase has been studied in detail. It has been shown to be light-stimulated (Galston 1950; Galston and Baker 1951), although it has no absolute requirement for light.

In pure systems it has been demonstrated that riboflavin sensitizes the oxidation of IAA in the light (Galston 1949; Galston and Baker 1949*a*, 1949*b*; Galston 1950). This work has stimulated the appearance of several papers (Ferri 1951*a*, 1951*b*; Brauner 1952, 1953*a*, 1953*b*) demonstrating that a miscellany of fluorescent substances can catalyse the photo-oxidation of IAA.

This paper deals with a group of substances of low molecular weight which sensitize the photochemical inactivation of IAA and which occur in plant tissues. In the brei of etiolated pea epicotyls the magnitude of this reaction is comparable with that of IAA oxidase.

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II. Methods

(*a*) *Tissue Preparations*

Seeds of *Pisum sativum,* variety Alaska, were surface-sterilized in hypochlorite solution, washed, and soaked in tap water for 2 hr. They were sown in vermiculite in stainless-steel flats and grown in a total darkness room at 25-26°C with occasional blue light of intensity less than 1 f.c. or in a red room at 26-27°C in continuous illumination with orange-red light (Corning filter No. 348) of an intensity of 0·1 f.c.

For the preparation of breis, epicotyls of seedlings 6-10 days old were cut with scissors into an equal weight of ice-cold M/60 phosphate buffer, pH 6·6, and homogenized for 1 min in a cold Waring Blendor. The juice was strained through muslin and centrifuged in the cold for 15 min at 20,000g. The supernatant ("whole cytoplasm") was used as the source of IAA oxidase, and of the group of dialysable substances, described later, which photoinactivate IAA.

For storage in the lyophilized state, several large batches of whole cytoplasm were prepared by disintegrating epicotyls in water in an Eppenbach colloid mill at 0°C (5 g tissue per ml water), during which process the pH remained at approximately 6·5. The debris was separated in a basket centrifuge and the juice centrifuged for 15 min at 20,000g. The supernatant was then reduced to dryness under reduced pressure from the frozen state, using the lyophil apparatus of Campbell and Pressman (1944), and stored in a brown bottle over phosphorus pentoxide at 0°C. As required, the lyophilized whole cytoplasm was dissolved in M/15 phosphate buffer, pH 6·6.

(*b*) *Growth Experiments*

For growth experiments, pea seedlings were cultivated in total darkness at 25-26°C or in continuous red light at 26-27°C, according to the requirements of the experiment. Epicotyls were selected 6-7 days after sowing so that the third internodes were 1-1½ in. long. As in the method used by Galston and Hand (1949), the apical bud was broken off at the crook, the epicotyls inserted into the guillotine cutter, the distal 3 mm removed, and the subjacent 5-mm sections were cut, washed in distilled water, randomized, and used in the growth test. A single section was cut from each plant because, in preliminary experiments, when two sections were used, a bimodal frequency distribution curve for responsiveness to growth substances resulted. Test solutions contained 2 per cent. sucrose, M/150 phosphate buffer (pH 6·1), growth substance, and other addenda as stated. Approximately 20 sections were placed in each petri dish containing 20 ml of solution, and incubated in total darkness at 25°C, or 8 in. beneath a bank of three 40-W white fluorescent lights while fanned with temperature-conditioned laboratory air. Final lengths of the sections were measured after a growth period of 8-12 hr, over which time the growth rate is sensibly linear (Galston and Hand 1949).

(*c*) *Measurement of Reaction Velocity*

The reaction velocities were determined by measuring the rates of disappearance of IAA, using the modification by Tang and Bonner (1947) of

Salkowski's colorimetric method. The colour is not stable, rapidly rising to a maximum and then slowly fading. Not only the rate of development and fading but also the maximum intensity reached depend on the room temperature. In routine determinations the photoabsorption was measured when it was judged, from the room temperature and time-temperature calibration curves, that the optical density had reached its maximum. In every batch a measured time interval elapsed between adding the reagent and reading the colour of each sample, and several standards were included in each batch.

A standardized reaction system contained:

0·5 ml M/15 phosphate buffer, pH 6·6,
0·5 ml 10^{-3}M IAA,
a limiting concentration of dialysate or IAA oxidase,
addenda, and
water to a total volume of 2·0 ml.

All solutions were saturated with air. The IAA was added at zero time. For light experiments solutions were loaded into 6 by 1 in. test tubes selected for uniformity. Tubes were placed in a rack backed with aluminium paint, mounted in a fixed and reproducible position with respect to a 40-W G.E. "4500" fluorescent lamp. The solutions were approximately 2·5 cm from the surface of the lamp and received approximately 700 f.c. The light intensity of the fluorescent tube was very uniform to within 8 in. of each end. Temperature-conditioned laboratory air was fanned over tubes so that, during a 30-min run, the temperature did not vary by more than ± 0·1°C. Under these conditions the standard deviation for the measurement of the rate of photolysis of IAA was approximately 2 per cent.

III. Results

Earlier workers (Tang and Bonner 1948; Galston and Baker 1951) have reported that dialysis of whole brei of pea epicotylis grown in weak red light led to a substantial increase in IAA oxidase activity, owing to the removal of a naturally occurring inhibitor. It was also observed (Galston 1950; Galston and Baker 1951) that light enhanced the activity of whole brei more than that of dialysed brei, and the suggestion was made that light accomplished this effect by "removing" (causing to dissociate) the natural inhibitor.

Wagenknecht and Burris (1950), however, found that about one-third of the activities of homogenates of bean roots or etiolated pea epicotyls was lost upon dialysis. The ash was inactive in restoring the activity. The whole dialysate was not tested.

In the author's experience, dialysis consistently diminished the rate of destruction of IAA by breis from epicotyls of pea seedlings grown either in darkness or in red light. The loss was fractionally much greater in the light than in the dark, i.e. light enhanced the activity of whole brei more strongly than that of dialysed brei. Adding back the concentrated dialysate restored the original activity. In addition it was found that the dialysate *alone* had a high capacity for destroying IAA in the light.

(*a*) *Existence of IAA-photolysing Activity of the Dialysate*

Epicotyls from 8-day, dark-grown pea seedlings were blended in a minimum volume of M/60 phosphate buffer, pH 6·6, strained through muslin, and filtered. Ten ml brei were placed in each of three cellophane bags and dialysed at 0-2°C with shaking against 10 ml, 50 ml, or two changes each of 750 ml M/60 phosphate buffer, pH 6·6. As a control, 10 ml brei placed in a test tube was also mounted on the shaker. After dialysis the volumes of brei were all adjusted to 11·0 ml. The 50 ml dialysate was frozen and the water removed by vacuum distillation, the final volume being adjusted to 11·0 ml. Residual activities of the dialysed breis were determined in the light, and compared with those of undialysed brei, of "whole" brei reconstituted by adding back the dialysate to the equivalent of its original concentration, and of the dialysate alone. Figure 1 shows that dialysis leads to a loss of activity which may be quantitatively restored by adding back the dialysate. As previously reported (Goldacre, Galston, and Weintraub 1953) this is partially attributable to a dissociable co-factor for the IAA oxidase system. However, the dialysate itself has independent activity (curve 5), of magnitude comparable with that of the IAA oxidase (curve 4).

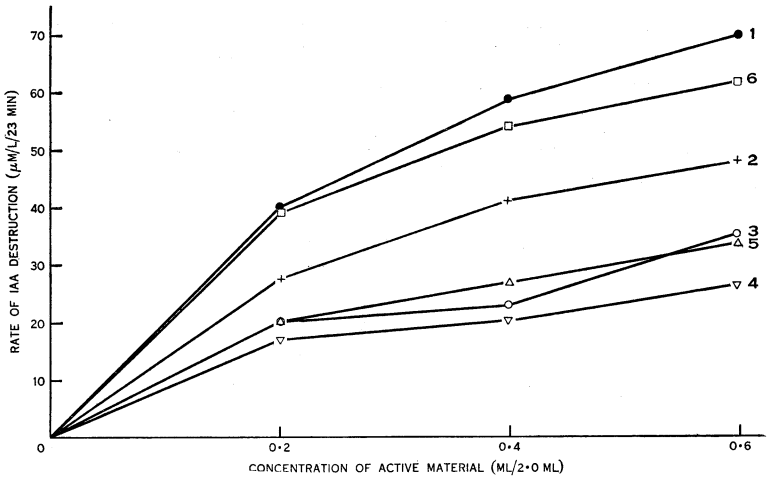

Fig. 1.—Effect of dialysis of pea epicotyl brei.
(1) Whole brei.
(2) Residue after dialysis against an equal volume of buffer.
(3) Residue after dialysis against five volumes of buffer.
(4) Residue after dialysis against 5600 volumes of buffer.
(5) Dialysate from (3).
(6) Residue plus dialysate from (3).
Initial IAA concentration 250 μM/l, temp. 26·5°C.

(*b*) *Destruction of the Biological Activity of IAA*

It is important to know whether the product of the destruction of IAA by substances in the dialysate is devoid of auxin activity. Auxin activity was determined by measuring the growth rate of pea epicotyl sections. Dialysate was

prepared by dissolving 1 g lyophilized pea epicotyl brei in 5 ml M/15 phosphate buffer, pH 6·1, and dialysing overnight at 0-2°C against 15 ml phosphate buffer. Four series of petri dishes were loaded with 2 per cent. sucrose, M/100 phosphate buffer (pH 6·1), a range of IAA concentrations, in two of the series, a 1 in 20 dilution of the above dialysate, and water to a total volume of 20 ml. Sixteen epicotyl sections were floated in each dish. Two series, one with dialysate and one without, were incubated on a shaker in the dark for 10 hr at 24°C, and a similar two series were placed on a shaker 6 in. below a bank of three 40-W white fluorescent lamps, with a fan blowing laboratory air (24°C)

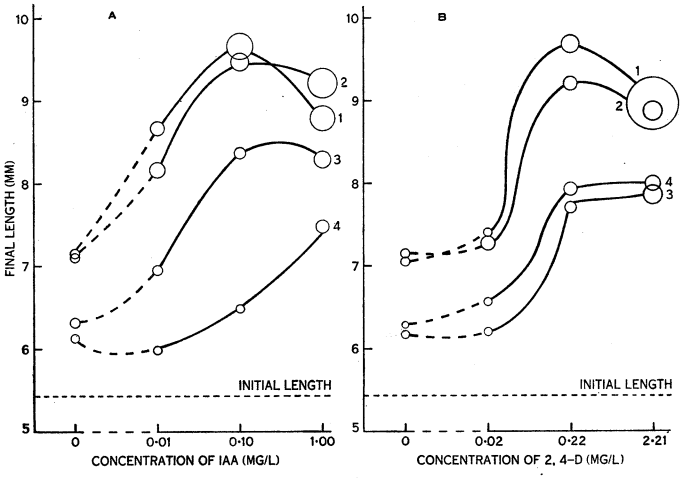

Fig. 2.—Effect of the dialysate on the growth of pea epicotyl sections in the light and in the dark under the influence of (*A*) IAA, (*B*) 2,4-D. Curve 1, dark, no dialysate; 2, dark, dialysate; 3, light, no dialysate; 4, light, dialysate. Incubated 10 hr at 24°C. Radii of circles indicate standard errors in the final lengths.

over them. Figure 2*A* shows that, as previously reported (Galston and Hand 1949), the sections made less IAA-induced growth in the light than in the dark. Moreover, in the presence of the dialysate, growth in the light is markedly inhibited, while there is no significant effect on the growth of those sections incubated in the dark. As a control against a non-specific toxic effect of the dialysate on growth in the light (e.g. the formation of an inhibitory substance in the light) the experiment was repeated using 2,4-dichlorophenoxyacetic acid as the auxin. Growth was again reduced in the light, but in this case there was no significant effect of the dialysate in the light or in the dark (Fig. 2*B*). It may be concluded that since the growth reduction produced by the dialysate is specific for IAA-induced growth, and specific for growth in the light, the dialysate is destroying the biological activity of IAA in the light.

Figure 3 shows the loss of biological activity of IAA as a function of the concentration of dialysate. The destruction rate increases with concentration.

(*c*) *Non-identity with Free Riboflavin*

Galston (1949) and Galston and Baker (1949*a*, 1949*b*) have described detailed work on the photosensitized destruction of IAA by free riboflavin. Since riboflavin occurs in appreciable concentration in these seedlings, it was necessary to ascertain whether the photolytic activity of the dialysate could be accounted for in terms of its riboflavin content. The dialysate was yellow in colour and fluoresced yellow-green as well as blue under ultraviolet light.

A riboflavin solution was prepared (4 μg/ml) so as to have approximately the same activity as a stock dialysate. Aliquots of the two solutions, both buffered at pH 6·6, were placed in matched thin-walled glass tubes and exposed 4 in. from a Keese ultraviolet lamp (model 909 T.P.) for 60 min. Initial and final activities were determined. Whereas the activity of the riboflavin was diminished to 28 per cent., that of the dialysate was diminished to 60 per cent. after exposure to an equal dose of ultraviolet light. This suggests that the riboflavin of the dialysate is not responsible for a major fraction of its activities, unless in the crude extract it is partially protected from destruction by ultraviolet light.

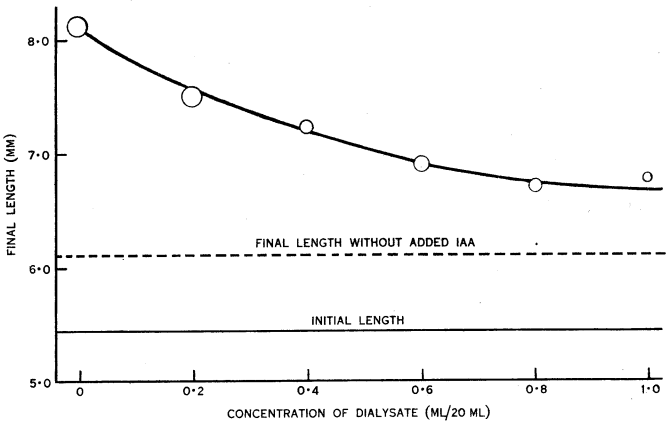

Fig. 3.—Effect of dialysate concentration on the destruction of the biological activity of IAA. Epicotyl sections incubated for 10 hr in the light at 25°C. Initial IAA concentration 0·10 mg/l. Radii of circles indicate standard errors in the final lengths.

In another type of experiment the dialysate was chromatographed alongside spots of the riboflavin solution of comparable activity. Whatman No. 1 paper was used with water as solvent. The developed chromatograms were examined under ultraviolet light. The riboflavin produced a strong yellow-green fluorescence at $R_F = 0{\cdot}33$ but the corresponding spot in the dialysate was barely discernible. Experiments to be described later, using a technique for detecting IAA-destroying activity directly on the developed chromatogram, also showed that the bulk of the activity did not correspond with the riboflavin spot. It thus became of interest to study the nature and properties of these naturally occurring substances (or substance), which are not riboflavin and which destroy IAA in the light.

(*d*) *Kinetics*

Biological catalysts are generally thought of as being proteinaceous in nature. In the above connection, however, riboflavin, a small molecule of biological origin, acts catalytically in the photodestruction of IAA (Galston 1949). The active component of the dialysate may react directly with IAA to change its identity or it may sensitize its decomposition in light. Since a catalyst might be responsible for greater activity physiologically than a substance which reacts stoichiometrically with IAA, it became an important question to determine whether the dialysate destroyed IAA in a catalytic fashion. The evidence and arguments pertinent to this question are presented in a later section after the kinetic behaviour of the system has been defined, but it may be said here that it is concluded that the dialysate reacts catalytically.

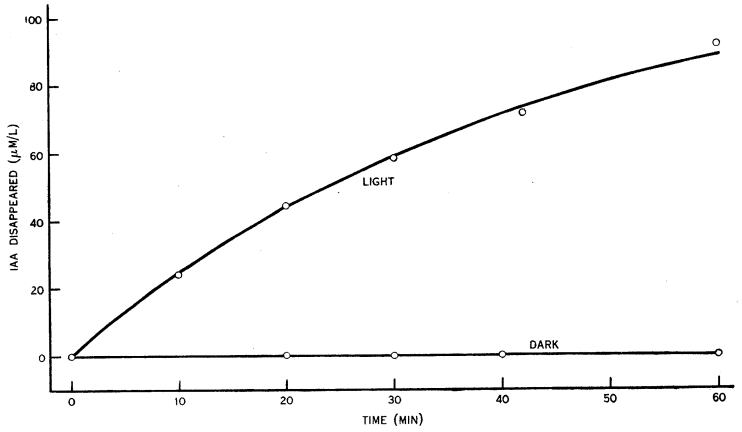

Fig. 4.—Course of IAA destruction by the dialysate, showing dependence on light. Initial IAA concentration 250 μM/l, temp. 25°C, pH 6·6.

(i) *Progress Curve.*—As a preliminary to the measurement of reaction rates, it is necessary to know the time course of the reaction under standard conditions. Two series of tubes were loaded with 0·5 ml M/15 phosphate buffer (pH 6·6), 0·5 ml dialysate, 0·5 ml water, and, at zero time, 0·5 ml 10^{-3}M IAA. One series was incubated in the light at 25°C, the other in the dark at 25°C. At time intervals, 8 ml Salkowski reagent were added to the tubes and residual IAA determined. Figure 4 shows that there is rapid destruction of IAA in the light but no destruction in the dark. With activities of this magnitude the reaction rate is practically constant for 20-30 min. In subsequent experiments, the IAA concentration change effected in this period was routinely taken as a measure of the reaction velocity, incubations all being made in the light.

(ii) *The Effect of IAA Concentration.*—The effect of IAA concentration on the reaction velocity is shown in Figure 5. The velocity is approximately proportional to concentration only below about 50 μM and the increment decreases in a hyperbolic manner, not approaching a limiting velocity even at 500 μM. Limitations of the analytical method make it impractical to exceed this value.

Let us assume for the moment that the dialysate acts as if it contains a single or a single dominant active component. According to the concept of enzyme action of Michaelis and Menten (see Wilson 1950), an enzyme and its substrate form an unstable intermediate which dissociates into free enzyme and reaction products. This premise leads to a formulation which relates reaction velocity to substrate concentration by the equation:

$$\frac{1}{v} = \frac{K_S}{V} \cdot \frac{1}{[S]} + \frac{1}{V},$$

where v = measured reaction velocity,

V = limiting velocity,

$K_s = \frac{k_2+k_3}{k_1}$, the enzyme-substrate "dissociation" constant,

$[S]$ = substrate concentration.

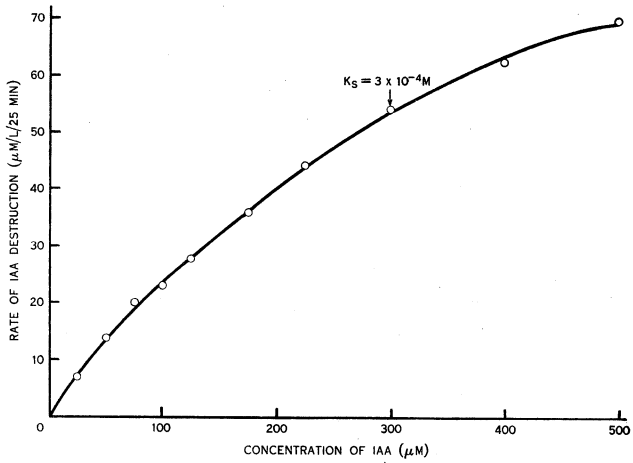

Fig. 5.—Effect of IAA concentration on the rate of its destruction by the dialysate. Temp. 25°C, pH 6·6.

By plotting $1/v$ against $1/[S]$ a straight line should result with slope equal to K_s/V and intercept $1/V$. This has been done in Figure 6 using the data of Figure 5, and the straight line fitted does not depart from the experimental data by amounts greater than can be accounted for by the expected error in the measurement of v. Thus a linear relation exists, and it may be taken that the kinetics of the dominant reaction are in accord with the concept of a catalyst-substrate intermediate complex, the dissociation constant of which from Figure 6 is approximately 3×10^{-4}M. This value has the same order of magnitude as that of IAA oxidase but the saturating concentration is much higher than that for the enzyme (Tang and Bonner 1947; Goldacre 1951*a*).

(iii) *Effect of Dialysate Concentration.*—The dialysate was prepared by dissolving 1 g lyophilized brei in 5 ml M/60 phosphate buffer, pH 6·6, and dialysing this solution against 20 ml buffer. Total reaction volume was 2·0

ml and initial IAA concentration 250 μM. The reaction velocity as a function of dialysate concentration is shown in Figure 7. Activity is proportional to

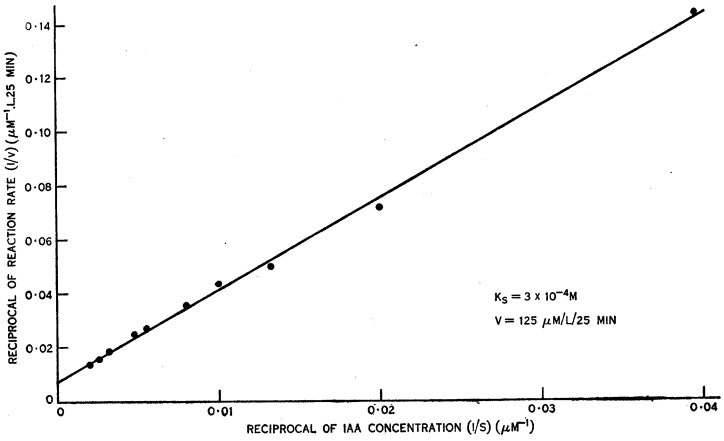

Fig. 6.—"Lineweaver-Burk" plot of the data of Figure 5.

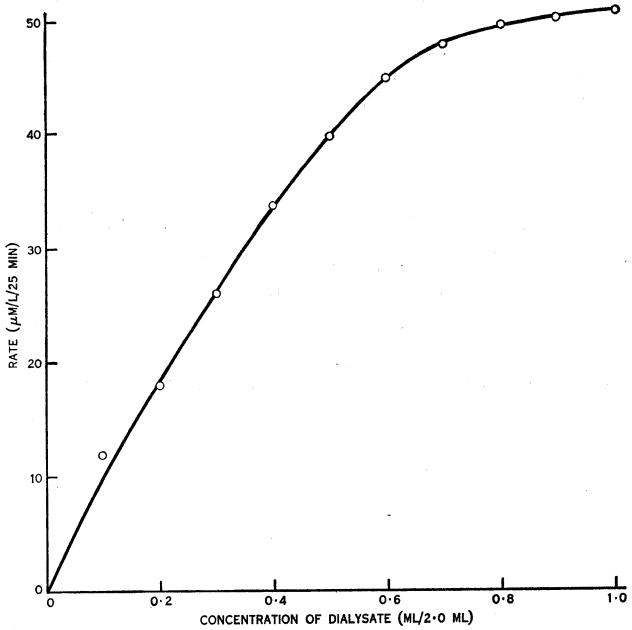

Fig. 7.—Effect of dialysate concentration on the rate of destruction of IAA. Initial IAA concentration 250 µM/l, temp. 26°C, pH 6·6.

dialysate concentration at low concentrations, but rapidly falls off. At high concentrations, presumably light intensity becomes limiting.

(iv) *Effect of pH.*—A series of McIlwain's phosphate-citrate buffers was prepared to cover the range from 3 to 7 and beyond this range the pH was adjusted with 0·2N HCl or 0·1N $NaHCO_3$. Buffer (1·0 ml), 0·5 ml dialysate or 0·5 ml water, and 0·5 ml 10^{-3}M IAA were incubated in the light at 27°C for 24 min. Aliquots of 1 ml were pipetted into 4 ml Salkowski reagent and the residual IAA determined. The pH was determined on the residual reaction mixture using the glass electrode.

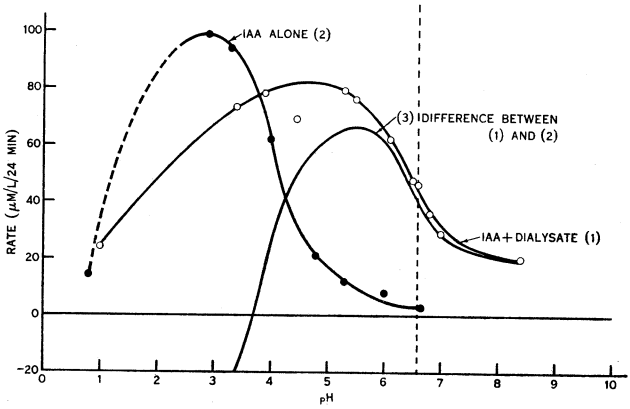

Fig. 8.—Effect of pH on the rate of destruction of IAA. McIlwain's phosphate-citrate buffers, 0·2N HCl, and 0·1N $NaHCO_3$ for extreme values. Initial IAA concentration 250 μM/l, temp. 26·5°C.

Figure 8, curve 2, shows that at acid pH IAA is destroyed by light even in the absence of dialysate. Dolk and Thimann (1932) have shown that plant growth substance isolated from *Rhizopus* culture medium, and subsequently identified as IAA, is half decomposed on standing for 3 hr at room temperature in 1N HCl in presumably low-intensity laboratory light. Algéus (1946) has also demonstrated that lowering the pH or exposing to light during storage of "pure" solutions of IAA results in losses. However, IAA itself does not absorb the visible light to which it is exposed, and its photodestruction was presumably sensitized by some pigmented impurity. All samples of IAA tested showed rapid photodestruction below pH 5, even after repeated purification (solution in NaOH and precipitation by slow addition of dilute HCl, and by recrystallization from aqueous ethanol). It was therefore necessary to carry out this control with IAA alone.

In the presence of the dialysate IAA was rapidly destroyed at considerably higher pH's (Fig. 8, curve 1). The optimum occurred between pH 4 and 5. There is little activity above pH 8.

By subtracting the smoothed curve for IAA alone from that for IAA + dialysate, curve 3 of Figure 8 is obtained. The optimum occurs at pH 5·5 and activity falls off sharply on both sides. Below pH 3·8 the activity for IAA alone is reproducibly higher than that for IAA plus dialysate. A possible explanation for this phenomenon is that, in this range, although the IAA combines more

effectively with the active material of the dialysate than with the pigmented impurity in the IAA sample, the catalyst-substrate complex decomposes more slowly. Thus below pH 3·8 the dialysate partially protects the IAA from destruction by the contaminating pigment.

Curve 2 of Figure 8 shows a reproducible optimum for IAA alone between pH 2 and 3. These results with Salkowski's reagent are not in accordance with data obtained using other methods of estimation, e.g. experiments using the growth of pea epicotyl sections as a measure of residual IAA show continuously increasing destruction with decreasing pH. Thus the optimum in curve 2 seems to be a spurious effect due to incomplete specificity of the Salkowski reagent, i.e. below pH 2-3, although the biological activity of IAA is destroyed, it is converted to a product which gives a positive Salkowski reaction.

Routine determinations were carried out at pH 6·6 because the destruction of IAA in the absence of dialysate was very low there. In addition this is the pH optimum for IAA oxidase, and many previous experiments using whole brei have been carried out at this pH.

(v) *Effect of Temperature.*—The reaction velocity of a purely photochemical reaction is independent of temperature, providing that the absorption coefficient is constant over the temperature range. Experimentally it has been shown that for most reactions known to be of this kind the temperature coefficient lies between 1·00 and 1·05 (Kistiakowski 1928). When the initial photoactivation is followed by a dark reaction which derives its activation energy thermally, the temperature coefficient may be higher, and may approach that for the dark reaction when the reaction velocity of the dark reaction limits the overall rate.

The effect of temperature on the rate of photolysis of IAA was determined, somewhat crudely, for high and low light intensities in the following way. A series of 50-ml beakers containing the reaction mixtures was placed in inverted petri dish lids and backed by white glazed tile. The whole was placed in a metal trough equipped with an overflow pipe. Water from a series of temperature-controlled tanks was led by a series of rubber tubes into the petri dishes, and permitted to overflow continuously, thus water-jacketing the reaction beakers. For high light intensity a bank of three 40-W "white 4500" fluorescent lamps was suspended 8 in. above the surface of the reaction mixtures, where the intensity was 500 f.c. For low light intensity the light of the laboratory (10 f.c.) was used. Temperatures remained constant within the reaction vessels to ± 1°C.

Figure 9 shows that at low light intensity there is very little dependence of the rate on temperature. At high light intensity, between 8° and 25°C there is an increase in average rate with temperature, which becomes more marked with the time of progression of the reaction. This suggests that the initial light reaction is followed by one or more dark reactions. Above 25°C there is a rapid decline in average rate with increasing temperature, especially at longer incubation times, suggesting that the catalyst is undergoing more rapid destruction at higher temperatures.

(vi) *Effect of Light.*—As shown in Figure 4, the dialysate sensitizes the destruction of IAA only in the light. Experiments to determine the relationship between activity and absorbed light energy did not yield precise reproducible results, probably because of the crudeness of the optical systems available. It may be said, however, that within the ranges used (up to 730 f.c. incident intensity from a "white" fluorescent lamp, or up to 1600 f.c. from a tungsten "photoflood" lamp after filtering through 6 in. of water) the activity increased with increasing incident energy.

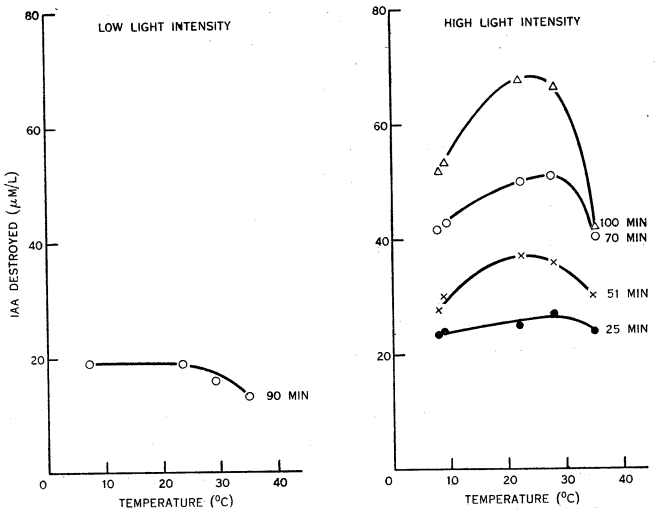

Fig. 9.—Effect of temperature on the photodecomposition of IAA by the dialysate. Initial IAA concentration 250 μM/l, pH 6·6.

A crude action spectrum was determined in the following way. A series of 5-cm dia. petri dishes was loaded with 2·0 ml M/15 phosphate buffer (pH 6·6), 1·0 ml dialysate, and (at zero time) 1·0 ml 10^{-3}M IAA. The dishes were placed inside blackened containers covered with Corning glass filters of the appropriate transmittance. The reaction vessels were set 8 in. below a bank of three "white" fluorescent lamps, and after 25 min incubation the residual IAA was determined. The relative transmitted energies from this light source through the various filters were determined by means of a blackened thermopile with a sensitive galvanometer. Table 1 shows these data. If it can be assumed that, for each wavelength, the amount of chemical change is proportional to the incident light energy in quanta, the relative destruction of IAA per quantum calculated for each wave band gives a measure of photochemical effectiveness. It may be seen from the last column of Table 1 that the relative order of effectiveness is violet > blue > green > red > yellow.

(*e*) *Stability of the Dialysate*

(i) *To Heat.*—Dialysate (0·5 ml) prepared from lyophilized whole brei was added to a series of tubes containing 0·5 ml M/15 phosphate buffer (pH 6·6)

TABLE 1

EFFECT OF LIGHT QUALITY ON THE RATE OF PHOTODECOMPOSITION OF IAA BY THE DIALYSATE

Corning Filter	Transmittance Maximum, (m μ)	Relative Incident Light		IAA Decomposed (μM/1/25 min)	Relative IAA Decomposed per Quantum
		Energies*	Quanta†		
Colourless glass	—	100	100	38	100
Blue-violet No. 511	380-440	9	7	17	640
Blue-green No. 428	400-540	39	34	35	271
Green No. 401	480-600	20	20	15	197
Yellow No. 352	560	73	75	18	63
Red No. 243	600-700	37	44	16	95

*Arbitrary units. Transmitted energies determined with a blackened thermopile and a sensitive galvanometer.

†An average value, using wavelength at centre of stated transmittance band. Relative numbers of quanta = relative energies × wavelengths.

and 0·5 ml water. Tubes were fitted with glass bulb condensers to avoid loss of water, placed in a hot water-bath maintained either at 80°C or 97°C, and at time intervals were withdrawn into an ice bath for rapid cooling. After equilibration to room temperature, 0·5 ml 10^{-3}M IAA was added and the resi-

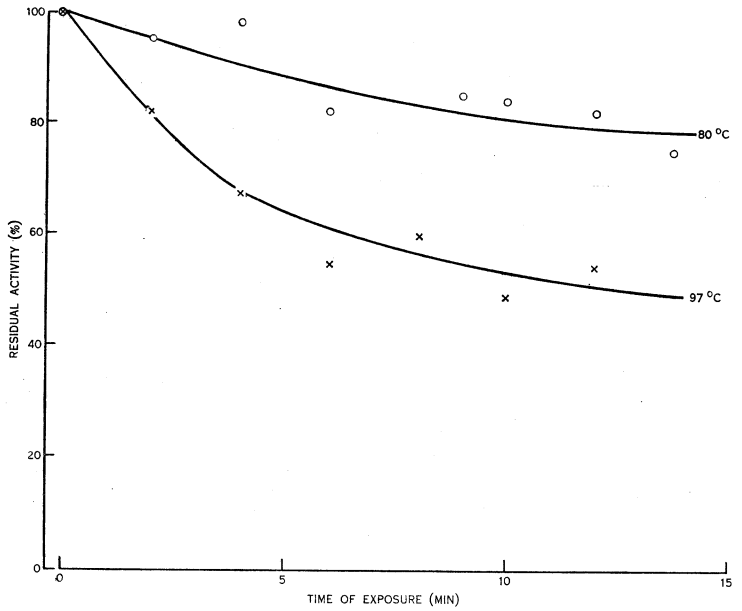

Fig. 10.—Stability of the dialysate to heat. Initial IAA concentration in assay 250 µM/l, temp. 25°C, pH 6·6.

dual activity of the dialysate was determined. Figure 10 shows that there is considerable heat inactivation of the dialysate. In 12 min approximately 50 per cent. of the activity is lost at 97°C and 20 per cent. at 80°C.

(ii) *To pH.*—Dialysate (1·0 ml) was placed in each of six beakers. The pH's were adjusted at the glass electrode over a range of values with 2N H_2SO_4 or 1N KOH, and the beakers kept in the dark at 27°C for 8½ hr. The pH's were checked, readjusted to pH 6·6, and the volumes were made up to 2·0 ml. Residual activity was then determined. Figure 11 shows that, under these conditions of incubation, the dialysate is quite stable between pH 1 and 7. Above pH 8 the activity begins to deteriorate.

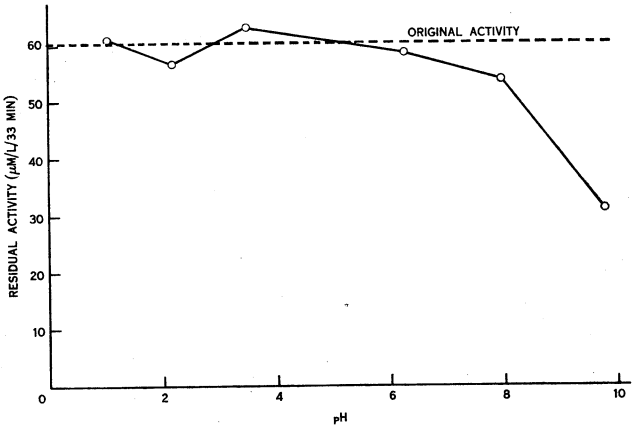

Fig. 11.—Stability of the dialysate to pH. Exposure in the dark 8½ hr at 27°C at shown pH's. Residual activity determined at pH 6·6, 23°C, and initial IAA concentration 250 μM/l.

(iii) *To Light.*—Dialysate (0·5 ml) was added to a series of tubes containing 0·5 ml M/15 phosphate buffer, pH 6·6, and 0·5 ml water. The stoppered tubes were placed on the aluminium-painted rack beneath the 40-W fluorescent lamp used in standard light experiments, and at time intervals tubes were withdrawn into a dark cupboard. Then 0·5 ml 10^{-3}M IAA was added and the residual activity of the dialysate determined. Figure 12 shows that pre-exposure to the white light produces a rapid decay in activity. The rate of decay, however, decreases rapidly with time, and the residual activity appears to approach an asymptote which, in this experiment, occurs at 50 per cent. loss of activity (or, referring to a calibration curve for activity versus dialysate concentration, approximately 70 per cent. loss of IAA-destroying units). This may be due to the dialysate containing a mixture of active components not all of which are photo-labile.

IAA affords the dialysate some protection against photolysis. Dialysate in two concentrations was incubated in the light for 5·5 hr at pH 6·6 and 25°C, with and without 500 μM/l IAA. The four solutions were then adjusted to

pH 3·0 and extracted four times with water-saturated ether to remove the residual IAA. The pH's were readjusted to 6·6 and the solutions made up to volume. Residual activities of the dialysate were then determined, each at two concentrations, and compared with the original activity. Figure 13 shows that while there is great loss of activity in all cases, especially in dilute solution, the presence of IAA during the pre-exposure period reduces the loss appreciably.

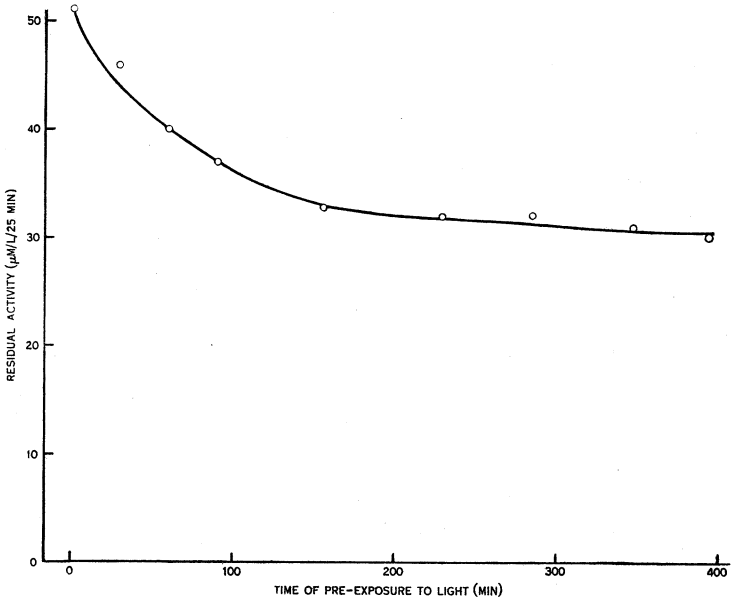

Fig. 12.—Stability of the dialysate to light (dialysate pre-exposed to 700 f.c. from a white fluorescent lamp at pH 6·6). Initial IAA concentration 250 μM/l, temp. 24°C, pH 6·6.

(*f*) *The Question of Catalyst or Reactant*

In a previous section attention was drawn to the importance of distinguishing whether the constituents of the dialysate which photoinactivate IAA act as a catalyst or a reactant. It has tentatively been accepted that the action is catalytic, and now the evidence will be considered. Useful criteria for deciding this question are the following:

(i) At the completion of a reaction, a catalyst should be recoverable with little or no loss of activity.

(ii) A reactant consumes a stoichiometric quantity of substrate so that at equilibrium the amount of substrate consumed should be directly proportional to the initial concentration of reactant. A catalyst may be expected to consume more than an equivalent number of moles of substrate.

(iii) In a simple system of reactants, the initial reaction velocity is proportional to the concentration of each reactant raised to the power of the order of the reaction with respect to each reactant, according to the collision theory

of reaction rates. In a catalysed system, on the other hand, where the substrate forms an intermediate catalyst-substrate complex in accordance with the Michaelis-Menten concept, a plot of the initial rate against substrate concentration gives a rectangular hyperbola, and a plot of the reciprocals of these values gives a straight line (Wilson 1950).

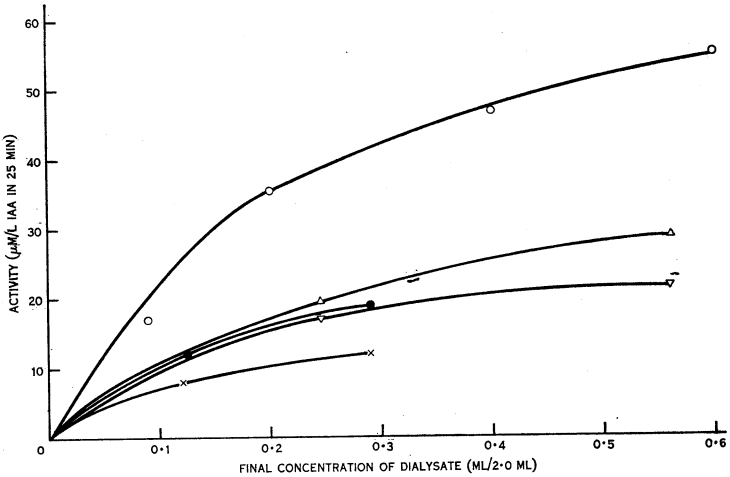

Fig. 13.—Protection of the dialysate by IAA from destruction by light. Temp. 25°C, initial IAA concentration 250 μM/l, pH 6·6. (O) Unexposed dialysate; (△) full concentration dialysate pre-exposed in presence of 500 μM/l IAA; (▽) full concentration dialysate pre-exposed in absence of IAA; (●) half concentration of dialysate, pre-exposed in presence of 500 μM/l IAA; (X) half concentration dialysate, pre-exposed in absence of IAA (see text for details).

A number of experiments has been carried out with these criteria in view.

(i) Light is essential for effecting any decomposition of IAA in the presence of dialysate. As indicated in Figure 12, the dialysate is rapidly inactivated in light so that, even in the absence of IAA, it is never possible to recover the activity quantitatively at the end of an experiment. However, referring to Figure 13, it is clear that, when irradiated in the presence of IAA, there is no greater loss in residual activity of the dialysate than when irradiated in the absence of IAA. On the contrary, IAA exerts some protective action.

(ii) It has never been possible to effect 100 per cent. destruction of IAA by prolonged incubation in the light with the dialysate preparations used, presumably because of the rapid photodestruction of the dialysate itself. In fact, in preliminary experiments a rough proportionality existed between maximum amount of IAA destroyed and the initial dialysate concentration (Fig. 14). Superficially this would seem to suggest that there is a stoichiometry between IAA used and dialysate provided. However, it may be shown that if the dialysate is pre-illuminated in the absence of IAA, then added to IAA, the rate (i.e. the slope of the progress curve) is equal to or less than that at the correspond-

ing time interval in Figure 14. This attests that the limit is approached due to the destruction of dialysate, not by reaction with IAA but by exposure to light.

(iii) If the dialysate acted as a reactant, then the reaction velocity v should be given by

$$v = k[\text{IAA}]^n.[\text{D}]^m,$$

where n = order of reaction with respect to IAA,

m = order of reaction with respect to dialysate constituent,

at all concentrations of IAA and dialysate. However, a linear plot of v against [IAA] and [D] in both cases results in a curve concave to the concentration axis (Figs. 5 and 7), which could only be obtained if the order of reaction were less than one.

On the other hand, a "Lineweaver-Burk" plot of activity *versus* IAA concentration data closely approximates to a straight line (Fig. 6), which is in agreement with the concept of the action of a catalyst.

The most reasonable interpretation of these experiments is that the dialysate contains a photoreceptor which sensitizes the destruction of IAA in a catalytic manner.

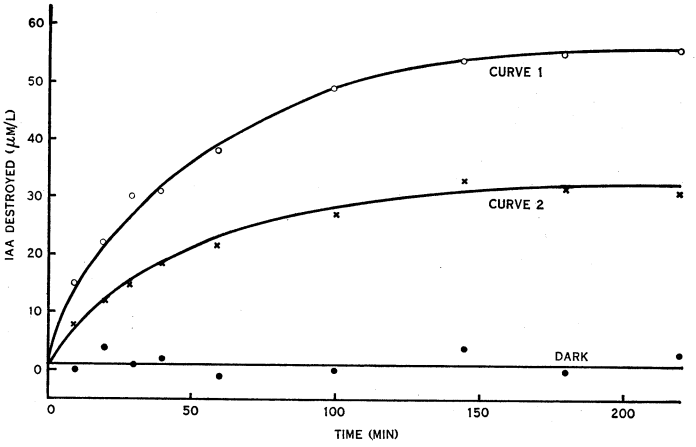

Fig. 14.—Relation between maximum IAA destroyable and dialysate concentration. In curve 1, dialysate volume = 10 per cent. of reaction mixture; in curve 2, 5 per cent. Initial IAA concentration 250 μM/l, temp. 25°C, pH 6·6.

(*g*) *The Requirement for Oxygen*

The most common photosensitization reactions are oxidations (Blum 1941). It is already known that IAA undergoes oxidation catalysed by IAA oxidase, and photo-oxidation in the presence of riboflavin, and it is of interest to determine whether this dialysate-catalysed reaction is oxidative in nature.

Thunberg tubes were loaded with 1·0 ml dialysate, 1·0 ml M/15 phosphate buffer (pH 6·6), and 1·0 ml water in the main arm and 1·0 ml 10^{-3}M IAA in the side-arm. The tubes were filled with oxygen, air, or argon. At zero time the IAA was tipped in and the tubes placed in the light in the usual way. After a

period of incubation aliquots were removed and residual IAA determined. This was repeated at two successive stages of purification to be described later. From Table 2 it is clear that the activity of crude dialysate shows no dependence on oxygen tension. After partial purification a relative though not absolute dependence appears. It seems that a requirement for oxygen exists, but that in the whole dialysate there is present an alternative hydrogen acceptor, in concentration high enough to account for the observed loss of IAA. The first purification step removed enough of this acceptor to make it become rate-limiting during the reaction, but did not remove all.

TABLE 2

EFFECT OF OXYGEN TENSION ON THE RATE OF PHOTODECOMPOSITION OF IAA BY THE DIALYSATE

Activity values are comparable along horizontal but not along vertical lines

Preparation	Incubation Time (min)	IAA Destroyed (μM/l) in		
		Argon	Air	Oxygen
Whole dialysate	26	51	54	51
Purification 1*	50	35	55	62
Purification 2†	40	49	64	70

*Whole brei was saturated with ammonium sulphate, set at 0-2°C overnight, and the precipitated proteins filtered off on diatomaceous earth. The filtrate was extracted three times into 1/20 weight of phenol. Three volumes of ether were added to the phenol solution, which was then extracted three times with 1/10 volume of M/15 phosphate buffer, pH 6·6. This aqueous fraction now contained most of the original activity and was diluted 1 in 5 before use in this experiment.

†Two ml of the above fraction were placed on a column of powdered cellulose 2·2 cm dia. by 50 cm long and chromatogrammed in water, the eluate being collected in a fraction cutter. A fraction at approximately R_F = 0·8-0·9, which was yellow in colour, fluoresced blue, was shaken into phenol and, after adding excess ether, back into 5 lots of 0·5 ml water. This was used as the active preparation.

Many photosensitized oxidations have been shown to involve the primary formation of hydrogen peroxide from oxygen and water through the action of the light-activated pigment (Blum 1941). It has already been shown that hydrogen peroxide is an intermediary oxidant in the destruction of IAA by IAA oxidase (Goldacre 1951*b*). The following experiment was carried out to determine whether hydrogen peroxide is involved in the photo-oxidation catalysed by the dialysate. Two series of dialysate concentrations were incubated in the light at pH 6·6 with 250 μM/l IAA. One series contained crystalline beef liver catalase in concentration in excess of that required to inhibit IAA oxidase completely. Figure 15 shows that catalase is not inhibitory. It may be concluded that free hydrogen peroxide is not an essential intermediate.

(*h*) *The Action of Inhibitors*

The dialysate is strongly inhibited by reagents that sequester heavy metals, e.g. KCN, H_2S, NaN_3, NH_2OH, 8-hydroxyquinoline, sym-diphenyl carbazide.

Owing to their interference in the Salkowski reaction, many of the chelating agents could be tested only at very low concentrations. As H_2S also interfered strongly, any precipitated sulphide was filtered from the dialysate through sintered glass and the H_2S was blown off with nitrogen before testing for activity.

From Table 3 and Figure 16 it is clear that a major fraction of the activity of the dialysate depends on the presence of a heavy metal. The ashed dialysate has no activity, and this observation, together with the fact that the activity is heat- and light-labile suggests that the metal may be in coordination complex with an organic moiety. The fact that activity is not second order with respect to the dialysate (Fig. 7) speaks against an interaction of an organic molecule with a substantially free ion.

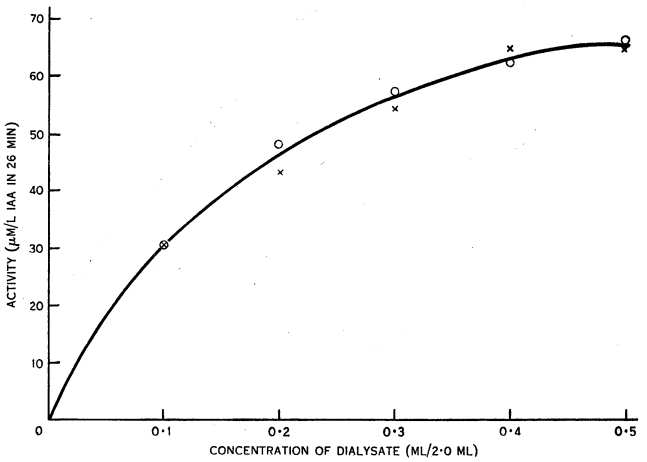

Fig. 15.—Effect of catalase on the photolysis of IAA by the dialysate. O, with catalase; X, without catalase. Initial IAA concentration 250μM/l, temp. 24°C, pH 6·6.

Albert and Gledhill (1947) have made a study of the specificities and sensitivities of a large series of chelating agents at pH 7. Of those suitable for use in the above experiment, the specificities are not such as to permit positive identification of the metal. It may be said that the metal is neither calcium nor magnesium, since 8-hydroxyquinoline inhibits but fluoride does not; nor is it copper, since benzoin oxime does not inhibit.

The addition of Cu^{++}, Co^{++}, Fe^{++}, Fe^{+++}, Mg^{++}, Zn^{++}, or Mn^{++} at 10^{-4}M concentrations to the whole dialysate produced no increase in activity. The free concentrations of native ion may already have been saturating. Cu^{++}, Co^{++}, and Mn^{++} at this concentration produced some inhibition. Attempts to remove the native metal before testing the effect of adding back these ions, were not successful.

(*i*) *Distribution of the Active Material*

(i) *In Tissues of Etiolated Pea Seedlings.*—Pea seedlings grown for 9 days in total darkness were dissected into apical buds, stems, cotyledons, and roots.

Weighed amounts of each fresh tissue were cooled and ground with sand in a measured volume of M/15 phosphate buffer (pH 6·6) in a cold mortar. Aliquots from the supernatants were placed in cellophane bags and dialysed overnight at 0°C against equal volumes of buffer. The dialysates were then assayed for activity in the usual way. Figure 17 shows that, per unit fresh weight of tissue, the apical buds have manifold greater activity than stems and roots, which have two to three times the activity of the cotyledons.

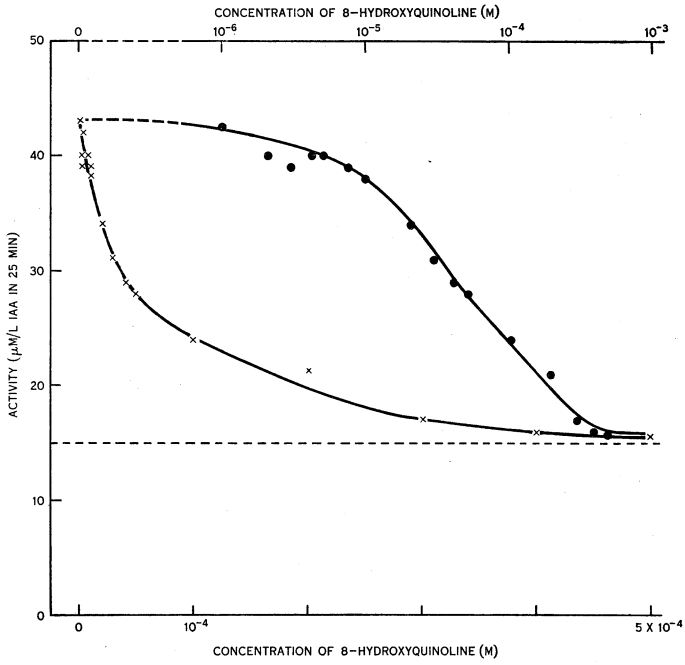

Fig. 16.—Residual activity of the dialysate as a function of concentration of 8-hydroxyquinoline. Dialysate incubated 15 min with 8-hydroxyquinoline before adding IAA. Initial IAA concentration 250 μM/l, temp. 24°C, pH 6·6. Data plotted logarithmically and arithmetically; ● = log plot, X = arithmetic plot.

(ii) *In Tissues of Green Plants.*—When a similar experiment was carried out with leaves, stems, stem tips (including leaf and stem in region where the stem had not yet elongated), and roots of pea seedlings grown for 15 days in a glass-house, substantial "apparent activity" was noted. However, subsequent experiments in which (*a*) incubations were carried out in the dark or (*b*) Salkowski reagent was added immediately after adding the dialysate to the reaction mixture, showed that only a small fraction of the "apparent activity" was in fact due to light-activated catalytic activity. The bulk of the "apparent activity" is attributed to either an interference in the Salkowski reaction or to an instantaneous dark reaction with IAA by some component of the dialysate. Similar results were experienced with green leaves of spinach, oat, and cocklebur, catalytic activity being relatively greater in younger tissue.

(*j*) *Preliminary Fractionation Behaviour*

(i) *Solubilities.*—Two-ml aliquots of dialysate, at pH 6·6, made acid with dilute HCl, or alkaline with dilute NaOH, were shaken with an equal volume of diethyl ether, *n*-butanol, light petroleum, or chloroform. The organic solvent

TABLE 3

EFFECT OF INHIBITORS ON THE ACTIVITY OF THE DIALYSATE

Substance	Concentration (M)	Activity (%)	Biologically Interesting Ions Sequestered
None	—	100	
KCN	0·0125	28	
	0·0025	47	
NaN_3	0·0025	41	
	0·0005	61	
	0·0001	87	
H_2S*	Saturated*	4	Fe^{++}, Fe^{+++}, Cu^{++}, Zn^{++}, Mn^{++}, Co^{++}
NaF	0·025	98	Ca^{++}, Mg^{++}
	0·005	96	
8-Hydroxyquinoline	0·00025	62	Mn^{++}, Zn^{++}, Fe^{++}, Fe^{+++}, Co^{++}, Cu^{++}
	0·00005	72	
Benzoin oxime	0·001	106	Cu^{++}
	0·0002	98	
α,α′-Dipyridyl	0·0025	82	Fe^{++}
	0·0005	75	
	0·00025	70	
Sym-diphenyl carbazide	0·00025	63	Zn^{++}, Cu^{++}
	0·0001	73	
Diethyl dithiocarbamate	0·00001	82	Mn^{++}, Fe^{++}, Fe^{+++}, Zn^{++}, Co^{++}, Cu^{++}
	0·000002	102	
Sulphanilamide	0·0025	91	
	0·0005	96	
Hydroxylamine	0·00125	75	Fe^{++}
	0·00025	82	
Guaiacol	0·00025	41	
	0·00005	49	
$MnCl_2$	0·0025	85	
	0·0005	80	

* H_2S from a cylinder was saturated with water vapour and passed through a volume of dialysate set in a brine-ice bath in the dark for 3 hr. The dialysate was filtered through sintered glass; the H_2S was blown out with nitrogen (scrubbed with alkaline pyrogallol and water) at 0°C in the dark until the effluent gas failed to darken lead acetate paper. Residual activity was then measured.

layer was separated off, reduced to dryness under reduced pressure, and the residue taken up in 1·5 ml M/15 phosphate buffer (pH 6·6), 0·5 ml 10^{-3}M IAA was added, and activities determined in the light. In no case was any measurable activity obtained. The yellow colour and most of the fluorescence also remained in the aqueous phase.

It was possible to extract the active material of the dialysate into phenol or into acetone after saturating the aqueous phase with ammonium sulphate.

Twenty ml of dialysate plus 40 ml saturated aqueous ammonium sulphate were extracted twice with 5 g phenol. Five volumes of freshly distilled ether were added to the phenol extracts and shaken with 15 ml, then 5 ml, water. The aqueous extract was found to contain 80 per cent. of the original activity, and most of the colour and fluorescence.

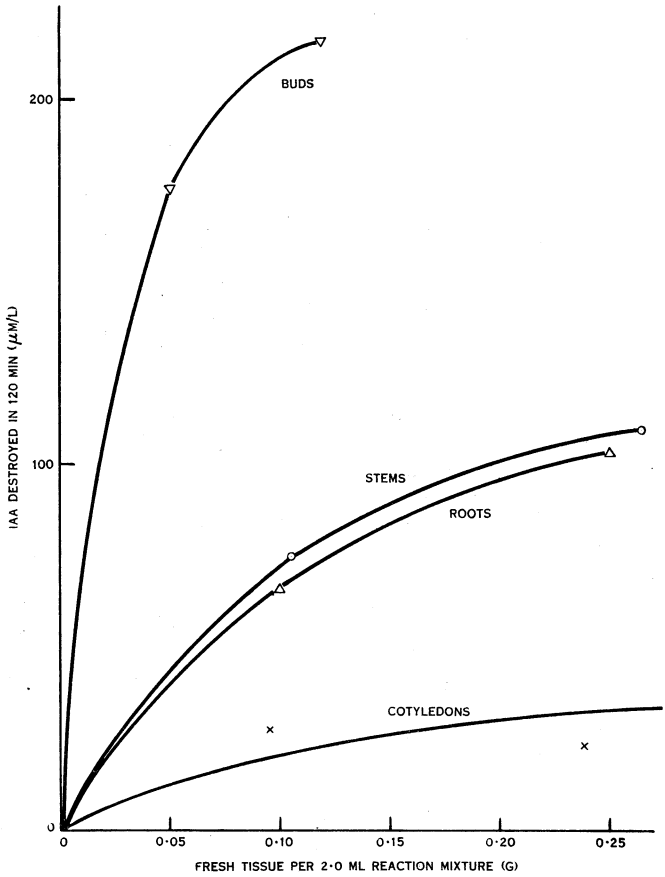

Fig. 17.—Distribution of activity of the dialysate in etiolated pea seedling tissues. Initial IAA concentration 250 μM/l, temp. 24°C, pH, 6·6.

Ten ml of dialysate were saturated with solid ammonium sulphate and extracted twice with 20 ml acetone. The acetone extract was evaporated to dryness at reduced pressure and room temperature. The residue was dissolved in 10 ml M/15 phosphate buffer (pH 6·6) and assayed for activity. About 80 per cent. of the original activity was recovered, as well as most of the colour and fluorescence.

Both extraction procedures are useful for the preliminary purification and concentration of the active material. Phenol is preferable because of the greater ease of returning the active material to the aqueous phase.

(ii) *Chromatography.*—Of the many adsorbents tested, silicic acid + "Hyflo supercel" (3 + 1) and powdered cellulose proved most useful, particularly the latter, because simultaneous runs on an analytical scale could be made on filter paper, with approximately the same relative rates of movement. The locations of active spots on filter paper were determined in the following manner. After the chromatogram was developed it was air-dried and momentarily dipped in a solution of 10^{-3}M IAA in water-saturated ether. After evaporation of the ether the papers were suspended horizontally in an atmosphere saturated with water vapour beneath a bank of fluorescent lamps. After incubating for an hour, the paper was sprayed with a modified Salkowski reagent (M/100 ferric chloride in 6 per cent. perchloric acid) and placed in a 100°C oven for 30-60 sec. The active spots appear white on a pink background.

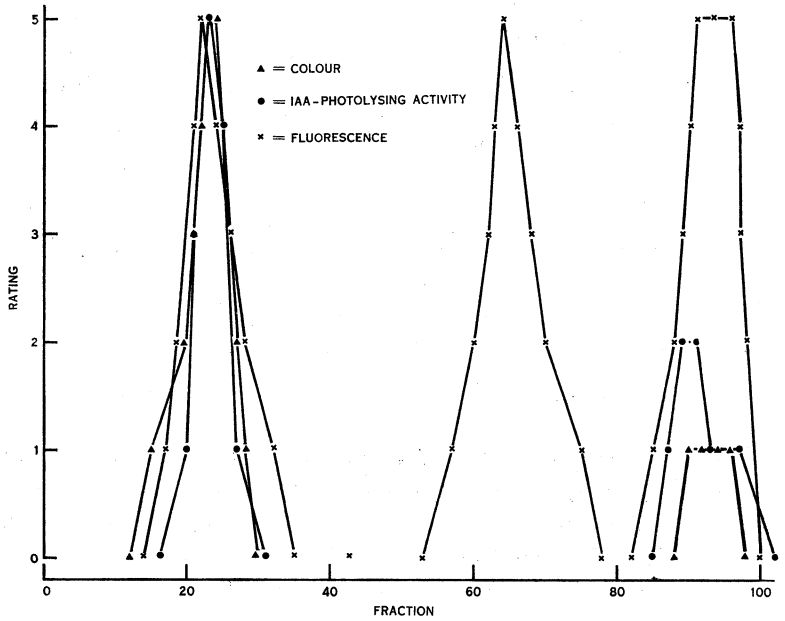

Fig. 18.—Fractionation of dialysate on a powdered cellulose column, using water as solvent. Fractions numbered, randomized, and subjectively rated 0 to 5. ▲, Colour; ●, IAA-photolysing activity; X, fluorescence.

A column 2·2 by 50 cm was filled with dry powdered cellulose, under suction, and was washed well with water. Two ml concentrated dialysate (prepared by extraction into phenol and re-extraction into water) were placed on the column, which was developed with water. The first 80 ml were discarded, then 2·8-ml fractions were collected. The fractions were numbered, randomized, and subjectively evaluated for colour and for intensity of fluorescence under ultraviolet light, each being assigned a rating from 0 to 5. The

fractions were also roughly assayed for IAA-photolysing activity by placing a drop on filter paper, drying, dipping the paper into a water-saturated ether solution of IAA, incubating in the light for 1 hr, then spraying with the modified Salkowski reagent. Figure 18 shows that the IAA-photolysing activity was confined to two peaks, both of which were coloured and fluorescent. The slower-moving peak (fraction 90), which was of lesser activity, fluoresced yellow-green and, when rechromatogrammed on paper, travelled identically with riboflavin in several solvents. Although both fractions containing the pigmented bands sensitize the decomposition of IAA in visible light (Table 4), no absorption maxima could be detected in the visible region, presumably because the concentrations were too low. Fraction 22 showed a maximum at 265 mμ and a minimum at 242 mμ, but these features cannot with certainty be attributed to the active constituent.

TABLE 4

SEPARATION OF THE ACTIVE COMPONENTS OF THE DIALYSATE BY CHROMATOGRAPHY ON PAPER

Spot	R_F in		Fluorescence under Ultraviolet	IAA-destroying Activity
	Water	Butanol-pyridine-water (10:5:6)		
1	0·32	0·45	Yellow-green	+
2	0·65	0·35	Blue	−
3	0·85	0·1 to 0·2	Blue	++
4	0·85	0·45	—	++
5	0·85	0·70	—	++
6	0·85	0·55	Blue	−

Three similar fluorescent bands were obtained by chromatographing the original concentrated dialysate on paper, using water as a solvent. Most of the activity resided in the fastest-moving spot ($R_F = 0{\cdot}85$), none in the centre spot ($R_F = 0{\cdot}65$), and a small amount in the slowest spot ($R_F = 0{\cdot}32$), which is probably riboflavin. It was possible to further resolve the first spot by chromatographing at right angles in a solvent mixture of *n*-butanol-water-pyridine in the proportions 10 : 5 : 6 by volume. Three active spots separated, only one of which was accompanied by detectable fluorescence (Table 4). A second fluorescent spot was devoid of measurable activity. Thus the dialysate is shown to contain at least four compounds active in photolysing IAA. Only two of these are accompanied by fluorescence in the procedures used.

Attempts were made to distinguish which of these spots was the metal-containing component. Developed two-dimensional chromatograms were sprayed with 5×10^{-4}M 8-hydroxyquinoline before dipping the papers into the IAA solution, but in no case was the activity of any spot destroyed. It was not possible to put enough of the dialysate on to the paper to test after eluting. It

seems that either during the process of chromatography the dissociable metal has been separated from its organic moiety or else that the reaction proceeds so rapidly on the paper that even an almost totally inhibited compound can still sensitize the destruction of IAA. Table 1 gives a crude action spectrum showing maximum activity at short wavelengths, with perhaps a second smaller peak in the red. Since this activity is a composite due to at least four substances, it would be desirable to resolve them and study their activities separately.

Brauner (1953*b*) has demonstrated that an anthocyanin (violanin) and two flavanols (viola-quercitrin and quercetagetin) are without photolytic activity toward IAA, and that an alcoholic solution of chlorophyll has slight activity.

Owing to the limitation of time, no further work has been done on the isolation of these active components.

IV. Discussion

This paper is principally descriptive in character. For simplicity in reference, many of the details of behaviour of the pea epicotyl dialysate have been discussed in the appropriate section where the result is reported.

Etiolated pea epicotyls have been shown to yield a group of at least four substances of low molecular weight which photoinactivate IAA at a rate comparable with that of IAA oxidase in the same tissue. Kinetic data indicate that the action is catalytic, and that the affinity for IAA is of the same order as that between IAA oxidase and IAA. The activity of dialysed IAA oxidase preparations is considerable in the dark and may be approximately doubled by exposure to 700 f.c. from a "white" fluorescent lamp. The dialysate has no activity in the dark, so that its light-dark differential is greater than that of IAA oxidase.

At this stage it is not possible to assign, with any degree of certainty, a physiological role to constituents of the dialysate, nor to IAA oxidase. It has been determined (Foster, McRae, and Bonner 1952) that the dissociation constant for the complex formed between IAA and the intracellular receptor site with which it reacts before evoking the growth reaction, is approximately 10^{-7}M. Since this is three orders of magnitude lower than that for IAA oxidase or the dialysate, the two latter systems would compete unfavourably against the receptors for combination with free IAA when the growth receptors are unsaturated (i.e. where a positive growth response to IAA is obtained). Thus in a tissue continuously supplied with IAA, the equivalent concentration of enzyme or of the photolysing substances of the dialysate would have to approach 1000 times that of the growth receptors in order that a change in the activity of the former would be reflected in an immediate growth response. On the other hand, in tissues where growth is limited by supraoptimal concentrations of IAA, these two systems may be expected to destroy free IAA and reduce the inhibitory effects.

It is important to consider whether the active substances of the dialysate may enter into the light-induced responses of plants known to involve changes in auxin concentration. Isolation and chemical identification of the components would permit their ready assay in the tissues and their study in purified *in vitro*

systems, leading to experiments that might determine their physiological action in plant tissues.

V. Acknowledgments

The author gratefully acknowledges the advice of Professor J. F. Bonner and Dr. A. W. Galston during the course of this work, which was carried out at the California Institute of Technology in 1951 during the tenure of a C.S.I.R.O. Studentship.

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