THE DESTRUCTION OF INDOLE-3-ACETIC ACID BY PLANT TISSUES

Thesis by

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ABSTRACT

This paper concerns those systems present in the epicotyls of eliolated pea seedlings which inactivate the plant hormone indole-3-acetic acid (I.A.A.). It consists of two sections which deal with (a) the enzyme system collectively known as I.A.A. oxidase and (b) a group of dialyzable substances which sensitize the photodestruction of I.A.A.

I.A.A. oxidase, which behaves as a flavoprotein coupled to a peroxidase is shown to have a partial cofactor requirement. Two alternative systems are postulated. The two systems are differentiated on the basis of their response to Mn^{++} and to 2,4-dichlorophenol (D.C.P.) and by their change in relative concentrations upon exposure of the seedlings to red light. D.C.P. is shown to increase the activity of I.A.A. oxidase at low concentrations. The mechanism of the effect is studied in detail. Although D.C.P. is a powerful inhibitor of catalase, which inhibits I.A.A. oxidase, it is concluded that the enhancement of I.A.A. oxidase by D.C.P. is not due to its inhibition of catalase. Probably D.C.P. is acting similarly to the native cofactor. D.C.P. increases the <u>in vivo</u> destruction of I.A.A. by some tissues, not by others. It is suggested as a useful tool for studying altered I.A.A. level in a tissue.

The dialyzate from epicotyl brei contains at least four components which sensitize the inactivation of I.A.A. in the light but not in the dark. Blue light is the most effective. The kinetics of the action of the dialyzate has been studied. The active material resides principally in the buds.

Preliminary methods of purification have been explored. Some possible physiological roles of the I.A.A. oxidase and the dialyzate have been discussed.

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INTRODUCTION

A. Indole-3-acetic Acid as a Native Plant Hormone

Indole-3-acetic acid (I.A.A.) was first isolated by Salkowski (1) in 1885 from the products of the putrefaction of eggwhite and from urine. It was later synthesized (2, 3). During the fractionation of auxins a and b from urine, Kögl, Haagen-Smit and Erxleben (4) isolated a third substance active in the Avena test and identified it as I.A.A. Subsequently I.A.A. was isolated in pure form from other sources, e. g. from yeast plasmolysate (5) and from the culture medium of Rhizopus (6). At the time this compound was not considered to be of importance as a native auxin in higher plants.

It was next shown by various workers (7, 8, 9) that several plant tissues give greatly increased yields of auxin upon alkaline hydrolysis. The auxin obtained from the alkaline hydrolysis of cornmeal was isolated and identified as I.A.A. (8, 10). The material which on hydrolysis yields this auxin was purified from mature corn kernels (10) and shown to yield 95% of the total auxin extractable from the alkali-treated kernels. The authors referred to this precursor as a form of "bound I.A.A." The question has arisen (11) as to whether the auxin liberated might be an artifact owing to the treatment of tryptophan-rich proteins with alkali. However, it was pointed out (12) that whereas by this treatment, the maximum yield from casein, a tryptophan-rich protein, was 0.001% and from tryptophan itself 0.008% (11), the yield from

whole grains of sugar corn was 0.084%. It was concluded (12, 10) that the auxin liberated from the grain was not derived from tryptophan but from a bound form of the auxin. While the fraction isolated from corn (10) may be a chemical progenitor of I.A.A., there is no evidence to show that it is a physiological precursor. On the contrary, by following the concentration of free I.A.A. and bound I.A.A. during the development of the corn endosperm, it was concluded that the bound I.A.A. was a secondary product formed from free I.A.A. (13).

It is important to know the nature of the free auxins in growing tissues, but owing to the extremely low concentrations it is not generally feasible to identify them by isolation. Indirect methods have been used, principally molecular weight by diffusion, differential destruction in acid and alkali and inactivation by light (14). The interpretation of results obtained by these methods using crude extracts must be treated with some caution because the presence of other substances in the extracts may interfere in the following ways. (a) Estimation of the molecular weight of the auxin of Avena coleoptiles by different workers has yielded results which are at variance with each other (15, 10, 17); naturally-occurring inhibitors of similar molecular weights may diffuse with the auxin in the agar plates and produce spurious values in the bio-assay (17, 18, 19). (b) Treatment of extracts with alkali may, in addition to destroying auxin a, release I.A.A. from water-soluble "precursors" (7, 20). (c) Though the destruction of I.A.A. by acid is used as a method of identification,

the reaction is dependent on the presence of oxygen (21), the partial pressure of which is not usually controlled. (d) Although auxin a-lactone and I.A.A. are stable in visible light, the presence of traces of α- or β-carotene sensitizes the photodestruction of auxin a-lactone (22), while riboflavin (23), several other fluorescent compounds (24) and a series of unknown compounds occurring in etiolated pea epicotyls (this paper) sensitize the destruction of I.A.A. by visible light. Thus careful purification of the extracts and standardization of the procedures are prerequisites to the use of these indirect methods. Even so, there may exist in the tissues unknown auxins the behaviors of which in these tests are unpredictable.

Although earlier workers using the above methods concluded that the predominant auxin of various tissues is auxin a (15), it now appears that the auxin of certain tissues is predominantly I.A.A. e.g. in the foliage and in crown gall of tomato (25), in spinach (26), in Macrocystis (27), in radish (19), in Avena coleoptile tips (17, 28, 29) in tomato stem tips (30), and in pineapple leaves (31). It was also shown (17) that the distribution of ether-extractable auxin in the Avena coleoptile paralleled the distribution of an enzyme which produces auxin (presumed I.A.A.) from tryptophan, and that the regeneration of the physiological tip of a decapitated coleoptile was accompanied by an increase in the concentration of this enzyme.

The first direct demonstration of the presence of free

I.A.A. in a fresh plant tissue was made by Haagen-Smit et al (32) in 1946. One hundred kg. of corn kernels in the milk stage (15 days after fertilization) were extracted with 95% ethanol. From the extract a crystalline product was isolated and identified as I.A.A. The concentration in the kernels was calculated to be 2.7×10^{-5} gm. per 1 gm. fresh weight. This was the only case thus far recorded of the isolation of free hormone from a fresh plant tissue.

Since then a variety of physical methods of fractionation combined with biological assay for auxin activity has shown that a major component of the auxin of several plant tissues behaves as I.A.A. (see table 1.). Although only in one case was positive identification made, each of these fractionation procedures imposes a specificity requirement on the molecule met by I.A.A. In most cases other auxins were also present. It may be safely concluded that I.A.A. is an important free auxin in higher plants.

Recent experiments leading to better understanding of the relation between structure and activity of the auxins strengthen this notion. The study (37, 38, 39, 40) of a large number of analogs of I.A.A. has revealed the structural requirements for activity in this series. Kinetic studies (41) on the growth of Avena coleoptile cylinders have confirmed that compounds in this series act at the same intracellular site and that under suitable conditions compounds with weak auxin activity (for example 2,3,5-triiodobenzoic acid) act as auxin antagonists in the presence of I.A.A. It has been shown in

Table 1

Identification of I.A.A. as an auxin of fresh plant tissues.

Author	Ref	Tissue	Fractionation Method
Holley *et al.*	21	cabbage leaves	counter current distribution
Bennett-Clark *et al.*	33	etiolated sunflower seedlings	solvent fractionation and paper chromatography
Luckwill	34	unripe asparagus fruits young broccoli leaves	paper chromatography
Linser	35	germinating cabbage	chromatography on alumina
Haagen-Smit *et al.*	32	immature maize kernels	isolation of pure compound
von Denffer *et al.*	36	maize kernels	paper electrophoresis after chromatography on alumina

several cases that under suitable conditions such compounds antagonize the effects not only of added I.A.A. but also of the native auxin (42, 43, 44, 45), suggesting that the native auxin has structural properties in common with this experimental series and probably is I.A.A. itself.

Three related indole components with growth activity or potential growth activity have been identified in neutral, ether soluble fractions of plant extracts, namely indole-3-acetaldehyde, the ethyl ester of I.A.A. and indole-3-acetonitrile. Larsen (46) extracted from etiolated pea seedlings a neutral growth substance which was converted aerobically by soil organisms or by raw milk containing an active Schardinger enzyme, into an acid growth substance, which was more active in the Avena test and which was found to have the molecular weight and acid-base stability characteristics of I.A.A. The neutral compound was therefore tentatively identified as indole-3-acetaldehyde. A similar preparation from pineapple leaves or Taraxacum roots was shown (31) to be irreversibly inactivated by dimedon, which is a specific coupling agent for aldehydes, and reversibly inactivated by sodium bisulphite. It also was oxidized to I.A.A. by soil or by Schardinger enzyme. Indole-3-acetaldehyde prepared (a) in low yield by degradation of tryptophan with ninhydrin (46) or (b) by synthesis from indole (47) showed activity in the Avena curvature (46) or straight growth (47) tests, but it was considered (47, 48) that the activity was principally if not entirely due to the enzymatic conversion of the aldehyde to I.A.A. Indole-3-acetaldehyde may be considered therefore a precursor to I.A.A. (48).

The ethyl ester of I.A.A. was extracted from immature corn kernels with ethanol, isolated and identified (49). Although less active in the Avena test than I.A.A., it is about 100 times as effective in inducing fruit-set in tomatoes.

Jones et al. (50) extracted 500 kg. of cabbage with carbon tetrachloride and after fractionation obtained a crystalline product which was identified as indole-3-acetonitrile. The compound was also syntnesized. The nitrile is more active than the acid in the straight-growth Avena test, approximately as active in the Avena curvature test and is of variable relative activity in other tests. It is polarly transported in the Avena coleoptile, a little more rapidly than I.A.A. The question arises whether the nitrile itself has activity or whether it must first be converted by the tissue to I.A.A., its greater activity being attributed to a greater rate of penetration by the uncharged molecule. The authors incline to the belief that the nitrile has activity in its own right because (a) coleoptile cylinders floated in the nitrile during the experimental period accumulated only traces of I.A.A., and (b) indole-3-acetamide, a likely intermediate in the hydrolysis of the nitrile to the acid is itself active in the straight-growth test. These observations do not constitute proof and the question must be regarded as still open.

Thus we see that the predominant auxin of various tissues is either indole acetic acid itself or a related compound readily converted into I.A.A. (indole acetaldehyde, ethyl indoleacetate or indole acetonitrile). Although other compounds

with nigh auxin activity have been isolated from biological sources, no auxin other than a substituted indole has so far been isolated from fresh plant tissue.

B. The Physiological Role of I.A.A.

The occurrence of free I.A.A. in growing plant tissues is now well established. Its role in the development and functioning of the plant can best be studied by observing the effects of exogenously applied I.A.A., and by imposing treatments that will influence the internal concentration of this auxin. The literature concerning the effects of applied I.A.A. and other auxins is very voluminous (see for example refs. 51, 52). No attempt will be made to survey it here. However, certain of the more fundamental effects will be mentioned.

The action of I.A.A. is quite nonspecific. Although there are quantitative differences, by and large higher plants react similarly to I.A.A. Applied I.A.A. promotes cell enlargement (53) and active water uptake (54), inhibits the development of lateral buds (55, 56), stimulates cambial activity (57), induces root primordia (15) but inhibits root elongation (16), induces development of parthenocarpic fruit (58, 59), delays abscission of leaves or fruit (52), and induces or inhibits the breaking of dormancy (52). It may be involved in tropisms (15, 60) and in the induction of flowering (61).

Treatment of soybeans (42) with 2,3,5-triiodo benzoic acid under conditions where it acts as an auxin antagonist produces

the symptoms of diminished free I.A.A. concentration in the tissues, namely shortening of internodes, loss of apical dominance, premature abscission of apical leaves and buds. It also greatly augments the flowering response due to photoperiodic induction and can under threshold conditions induce flowering in plants which would otherwise remain vegetative (62). Similarly, irradiation with X-rays was found (63, 64) to destroy I.A.A. in vitro and in vivo, the plants showing reduced internodal growth and bolting of the lateral buds.

Zinc deficient tomato plants were found to have a very low free auxin level (65, 66) and characteristically produce growth with very short internodes. Although zinc undoubtedly is concerned with biochemical processes besides auxin synthesis, treatment with I.A.A. had a marked effect on restoring the lengths of the internodes.

Thus it may be seen that either an increase or a decrease in the concentration of I.A.A. in the plant may produce profound changes in its morphogenesis and physiology.

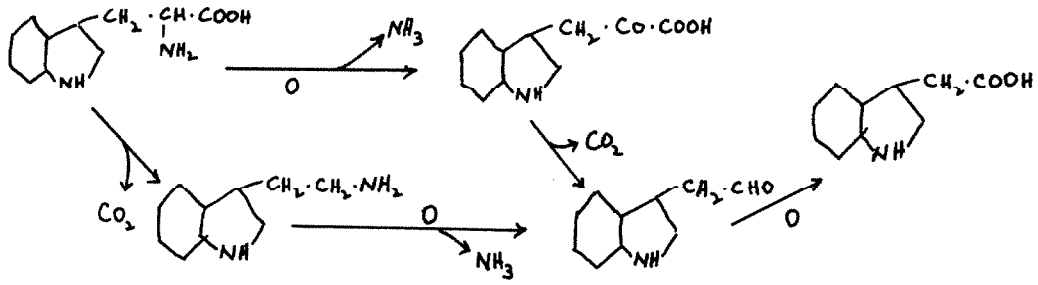
C. The Metabolism of I.A.A. in Plant Tissues

Since I.A.A. exerts such a profound influence over the growth and development of higher plants it is important to study the factors which may influence its concentration in a tissue, namely the systems which produce it and which destroy it.

From isolated Avena coleoptile tips placed onto blocks of agar for several hours, more auxin can be obtained by diffusion

than can be extracted from the tips in the first instance by solvents (67). It seems that the tip has the capacity to synthesize auxin from a biologically inactive precursor. The first suggestion that tryptophan might be a biological precursor of I.A.A. was made by Thimann (68) who studied the formation of the auxin by cultures of Rhizopus suinus and found a dependence on the tryptophan content of the medium. Tryptophan in agar applied unilaterally to an Avena coleoptile produces curvature but only after 2 to 3 hours' lag, suggesting that it is being converted into an active compound (15, 69). Woody stem cuttings treated at the base with tryptophan produce root primordia in the same way as when treated with I.A.A. (15).

The conversion of L-tryptophan to auxin (identified by indirect methods as I.A.A.) has now been directly studied in several higher plant tissues. Wildman et al. (70) showed that discs or breis of spinach leaves enzymatically convert tryptophan to a substance active in the Avena curvature test. The process is oxidative and is inhibited by bisulphite and cyanide. Identification of the intermediates is not clear. If cyanide and bisulphite are acting as keto fixatives, a carbonyl-containing intermediate such as indole-3-pyruvic acid or indole-3-acetaldehyde is suggested:



However, neither discs nor breis of spinach leaves were able to convert either tryptamine or indole-3-acetaldehyde to I.A.A. Although indole-3-pyruvic acid is converted to I.A.A. it is not clear from the data that the process is enzymatically mediated. The activity of this enzyme is comparatively very low.

Gordon and Nieva (31) confirmed the conversion of tryptophan to I.A.A. by pineapple leaf discs and also reported that the discs and an enzyme obtained from them convert indole-3-pyruvic acid, tryptamine and indole-3-acetaldehyde to I.A.A. Furthermore, indole-3-acetaldehyde was detected as an intermediate in the oxidation of indole pyruvic acid and of tryptamine.

The presence of this enzyme system has now been demonstrated in many higher plant tissues (71). It is noteworthy that in cases of zinc deficiency the production of I.A.A. by the tomato plant is markedly reduced (65, 66). The cause is indirect, zinc being essential for the synthesis of tryptophan (66). Thus, even though the free tryptophan content of the tissue may be 10^7 times greater than that of I.A.A., I.A.A. synthesis is limited by tryptophan concentration.

It has been well known for some time that auxins are rapidly inactivated in plant tissues whether used in the growth process or not (72, 73, 74). Van Overbeek (74) noted that there was reduced loss of the auxin diffused from Avena coleoptiles into agar blocks if the cut cells were first cleaned off with wet filter paper. It was suggested that the

diffusing auxin was partly destroyed by an enzyme liberated from the cut cells. He observed that the basal regions of the coleoptile destroyed I.A.A. faster than the tips and that the basal region had a more active peroxidase. He also found (75) that dwarf corn contains less auxin and has a higher auxin-destroying activity than normal corn. The peroxidase activity was also higher in dwarf corn and a causal relation was suggested. The enzymatic nature of the inactivation process was first demonstrated by Larsen (76,77). He found that the press-juice of Phaseolus seedlings contained a thermolabile component which destroyed the native auxin of Phaseolus, or of maize kernels, and synthetic I.A.A. He showed that the active material could be precipitated with 60% alcohol and that the inactivation reaction required oxygen.

Subsequently the enzyme system I.A.A. oxidase was prepared from etiolated pea epicotyls (78, 79, 80) and characterized. The properties of this system are reviewed in the section dealing with this enzyme.

EXPERIMENTAL AND RESULTS

A. General Materials and Methods

Tissue Preparations. Seeds of Pisum sativum, variety Alaska, were surface-sterilized in a 20% solution of "Purex" for ten minutes, washed and soaked in tap-water for two nours. They were sown in vermiculite in stainless-steel flats and grown in a total darkness room at 25° to 26° C. (with occasional green or blue light of intensity less than one foot-candle) or in a red room at 26° to 27° C. in continuous illumination with orange-red light (Corning filter #348) of an intensity of 0.1 foot-candle.

For the preparation of breis, epicotyls of seedlings usually six to ten days old were cut with scissors into approximately an equal weight of ice-cold M/60 phosphate buffer, pH 6.6, and homogenized for one minute in a cold Waring blendor. The juice was strained through muslin and centrifuged in the cold for fifteen minutes at 20,000 g. The supernatant was used as "whole cytoplasm," the source of the enzyme system I.A.A. oxidase, and of the group of dialyzable substances to be described which photoinactivate indole-3-acetic acid.

Several large batches of whole cytoplasm were prepared by grinding epicotyls in water in an Eppenbach colloid mill at 0° C. (five g. fresh tissue per ml. water) during which process the pH remained at approximately 6.5. The use of buffers was avoided in preparations to be lyophilized where the high salt

concentration would result in protein losses. The debris was separated in a basket centrifuge and the juice centrifuged for fifteen minutes at 20,000 g. The supernatant was then reduced to dryness from the frozen state *in vacuo*, using the lyophil apparatus of Campbell and Pressman (81) and stored in a brown bottle at 0° C. over phosphorus pentoxide. As required, the lyophilized whole cytoplasm was dissolved in M/15 phosphate buffer, pH 6.0.

Growth Experiments. For growth experiments pea seedlings were grown in total darkness at 25° to 26° C. or in continuous red light at 26° to 27° C. according to the requirements of the experiment. Epicotyls were selected six to seven days after sowing so that the third internodes were one to one and a half inches long. Following the method of Galston and Hand (82), 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% sucrose, M/150 phosphate buffer pH 6.6, growth substance and other addenda as stated. Approximately 20 sections were placed in each flask containing 10 mls. of solution and incubated in total darkness or red light at constant temperature, or eight inches beneath a bank of three white fluorescent lights while fanned with temperature-

conditioned laboratory air, according to the requirements of the experiment. Final lengths of the sections were measured after a growth period of eight to twelve hours over which time the growth rate is sensibly linear (82). A dissecting microscope with an ocular micrometer was used to measure the section lengths.

Determination of Indole-3-Acetic Acid. The reaction velocities were determined, in most cases by measuring the rates of disappearance of indoleacetic acid (I.A.A.), using Tang and Bonner's (78) modification of Salkowski's (1) colorimetric method. Routinely, four volumes of the reagent:

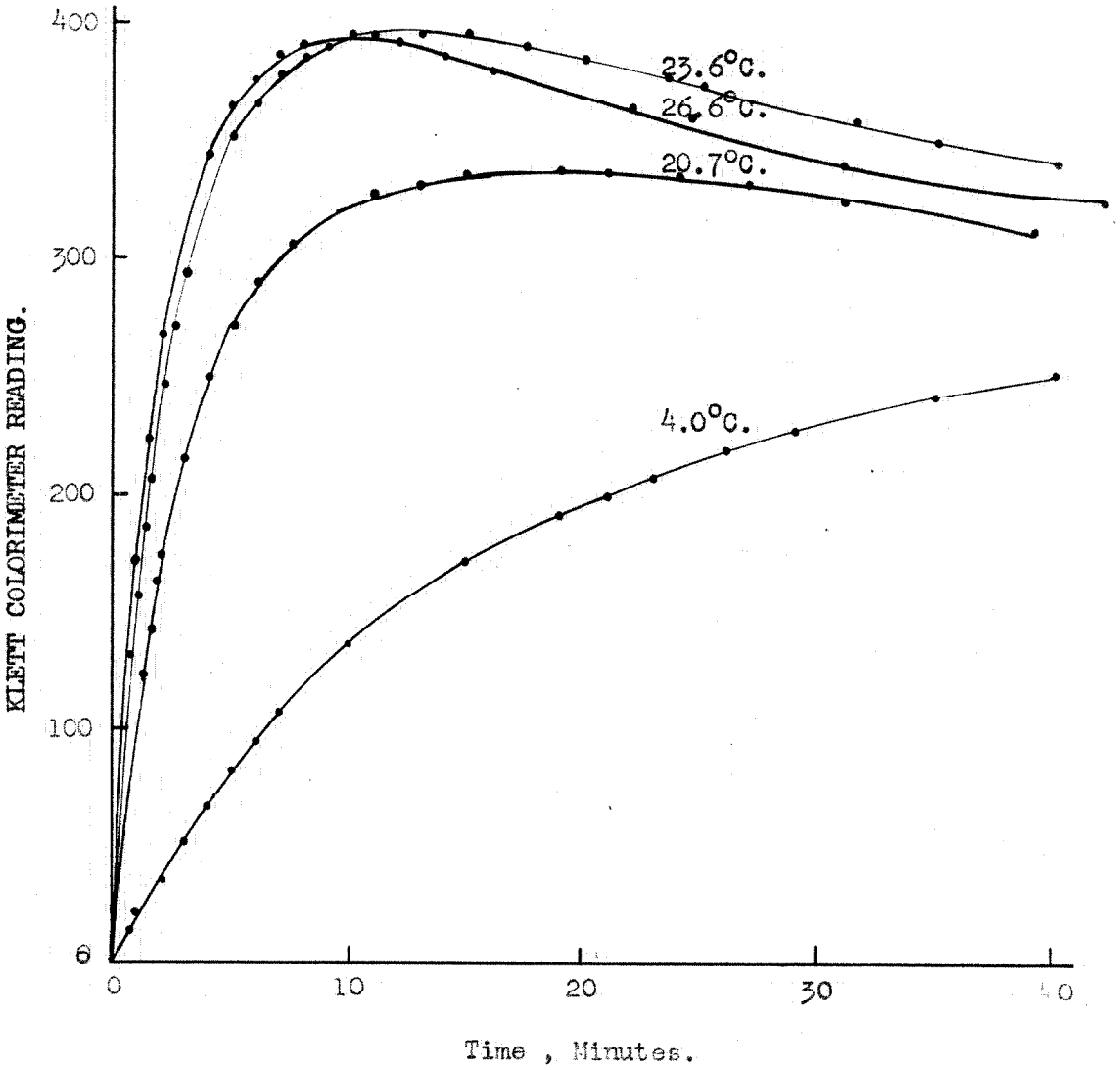
15 mls. 0.5 M $FeCl_3$

500 mls. distilled water

300 mls. H_2SO_4, S.G. 1.84

were added to the aqueous solution containing I.A.A. and the intensity of the red color measured on the Klett-Summerson photoelectric colorimeter using the 540 mμ filter and setting zero with a mixture of four volumes reagent to one volume water. The color is not stable, rapidly developing to a maximum intensity and then slowly fading. Not only the rate of development and fading but the maximum intensity reached depend upon the room temperature, as shown in figure 1. These data were obtained by temperature-equilibrating 2 mls. of 2.5 x 10^{-4}M I.A.A. and 8 mls. Salkowski reagent at each of the temperatures shown, mixing at zero time and following the rates of color development in the colorimeter, replacing the tubes in the thermostat between readings. In routine determinations,

Figure 1.



The rate of development and fading of the Salkowski color as a function of temperature. I.A.A. concentration 2.5×10^{-4} M. Klett filter 540 mμ.

the photoabsorption was measured when it was judged, from the room temperature and the relationships shown in figure 1, that the intensity of the color had reached its maximum plateau. In every batch a measured time interval elapsed between adding the reagent and reading the color of each sample. Several standards were always included in each batch. Optical density corrections were always made for the brei and any absorbing addendum. Many oxidizing or reducing agents interfere in the Salkowski reaction. Where the interference is slight, suitable corrections may be made by including the substance into a series of standards, but where the interference is serious it is not quantitatively reliable to make the correction.

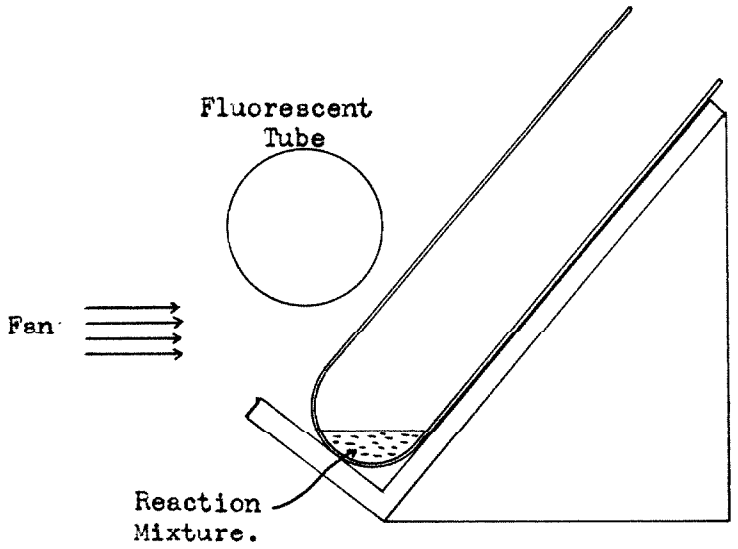
Beer's Law holds up to I.A.A. concentrations above 2.5×10^{-4} M, where the standard deviation of a determination is approximately 1.5% of the mean.

Measurement of Reaction Velocity. Except where specifically stated, the measurement of the activity of I.A.A. oxidase or of the dialyzate was carried out in the standardized reaction system in 6 x 1 inch test tubes:

0.5 ml. M/15 phosphate buffer, pH = 6.6
0.5 ml. 10^{-3} M I.A.A.
a limiting concentration of enzyme, or dialyzate
addenda
distilled water to a total volume of 2.0 ml.

The I.A.A. was added at zero time. All solutions were saturated with air and since it was found (83) that shaking the tubes in an inclined position did not enhance the rate, oxygen was

not limiting in this system. For dark experiments the mixtures were incubated in a temperature-controlled darkroom equipped with a fan, manipulations being carried out in weak (less than 1 f. c.) orange-red light (Corning #348), to which the enzyme is relatively insensitive (84). For light experiments 6 x 1 inch test tubes were selected for uniformity before loading. Up to ten tubes were placed in an aluminum-painted rack which was mounted in a fixed and reproducible position with respect to a 40 watt G.E. "4500" white fluorescent lamp. The solutions were approximately 2.5 cm from the surface of the lamp and received approximately 700 foot candles. The light intensity at the surface of the fluorescent tube was very uniform to within eight inches of each end. A fan was used to circulate temperature-conditioned laboratory air over the tubes to maintain uniform temperature. Temperatures of the solutions during a thirty minute run did not vary by more than $\pm$ 0.1°C.



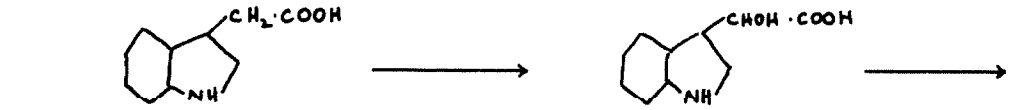
Using this system good reproducibility was obtained. For example, ten tubes were each loaded with 1.0 ml. M/15 phosphate buffer, pH 6.6, 0.5 ml. whole pea epicotyl brei dialyzate, to be described later, and which photo-destroys I.A.A. and, at zero time, 0.5 ml. 10^{-3}M I.A.A. Tubes were incubated in the light at 25° C. for 25 minutes, then 8 mls. Salkowski reagent added and residual I.A.A. determined. The values for I.A.A. disappeared were 51.5, 54.0, 54.9, 55,8, 55.3, 54.2, 54.0, 55.9, 55.9, 51.5 μM/l, giving a mean of 54.30 μM/l and standard deviation of 1.09 μM/l or 3.12%. In routine experiments the two end positions in the racks were avoided, where possible, and when the values for these tubes are omitted, the mean is 55.00 μM/l and the standard deviation 0.84 μM/l or 1.53%.

The reaction was permitted to proceed for a length of time during which the reaction velocity was found by previous trial (figure 1) to be constant (usually twenty to thirty minutes), at the end of which 8 mls. Salkowski reagent were pipetted in, well mixed and residual I.A.A. determined.

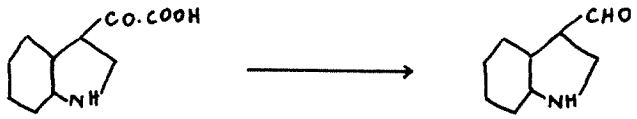
B. I.A.A. Oxidase

Reiteration of Known Properties of the Enzyme. In 1947 Tang and Bonner (78) first described an enzyme system, from the epicotyls of etiolated peas, which oxidizes and destroys the biological activity of I.A.A. They studied the kinetics of the system, found the pH optimum to be 6.0 to 6.7 and the half-maximum substrate concentration to be 25 mg/l I.A.A.

This corresponds to an apparent Michaelis constant of 1.4×10^{-4} M under these experimental conditions. One mole of O_2 is consumed and one mole of CO_2 is produced per mole of I.A.A. disappeared (determined with the Salkowski reagent) and it is stated that the indole nucleus remains uncleaved (as determined with the Hopkins-Cole reagent). It was concluded that only the sidechain is attacked, the product being non-acidic carbonyl-containing and ether soluble, presumably indole-3-aldehyde (78, 80). Indole-3-aldehyde has not been positively identified by isolation or other means, and as far as the writer is aware there has been no extensive study as to the specificity of the Hopkins-Cole reagent for the indole nucleus. However, if it may be tentatively assumed that only the sidechain is oxidized, resulting in the formation of indole-3-aldehyde, it may be considered as a working hypothesis that there are three reactions involved, probably



indole-3-acetic acid. indole-3-glycolic acid.



indole-3-glyoxylic acid. indole-3-aldehyde.

No identification of intermediates has been made.

The enzyme is inhibited by low concentrations of cyanide. Subsequently it was demonstrated (80, 85) that the enzyme was

also strongly inhibited by azide, sulfide, hydroxylamine and a variety of chelating agents. This suggested that the enzyme depended for its activity on a heavy metal. To distinguish a possible iron enzyme from a copper enzyme Tang and Bonner (78) tested for photoreversibility of carbon monoxide inhibition and reported a positive result. However Wagenknecht and Burris (80) were unable to obtain a carbon monoxide inhibition and, as they point out, the first experiment was carried out with oxidation in air as a control for comparing the rate of oxidation in 95% carbon monoxide + 5% oxygen. In this range a marked response is obtained to the partial pressure of oxygen, so that the "inhibition" was probably due to reduced oxygen tension. The "photoreversal" is explainable by the subsequent discovery (86) that the uninhibited I.A.A. oxidase is light-activated. Wagenknecht and Burris (80), on the basis of inhibitions obtained with sodium diethyldithiocarbamate, potassium ethyl xanthate and thiourea suggest that the metal associated with the enzyme is copper. However, at pH 6.5 the actions of these chelating agents are not specific enough to make a definite conclusion (87).

Tang and Bonner (79) described an increase in activity upon dialysis of the crude brei or upon precipitating of the enzyme with 40% acetone and redissolving in buffer. A heat-stable non-competitive inhibitor which combines reversibly with the enzyme was found in the whole brei. Exposure of the etiolated pea seedlings to small doses of light produces a rapid increase in the concentration of inhibitor. It is

particularly abundant in green leaves. The distribution of the enzyme in other tissues was studied. Even after acetone precipitation, little or no activity could be found in green leaves although activity was found in roots of some green plants (79, 80). Galston and Baker (86) subsequently were able to demonstrate strong activity in breis from the green leaves of peas after precipitating the enzyme several times with acetone. In the present work it is shown that discs of green leaves which give inactive breis are very active in destroying I.A.A., which suggests that in the tissue the inhibitor is spatially separated from the enzyme.

It has been demonstrated that catalase strongly inhibits the oxidation of I.A.A. by the pea enzyme (88, 89) and that guaiacol, a peroxidase substrate, gives an inhibition which is competitive by the Lineweaver-Burk treatment (101). It has been concluded that a peroxide-peroxidase system is involved in the oxidation of I.A.A. A strong activation of the enzyme by light has been observed (84, 80). The action spectrum for this process was determined on clear, concentrated whole pea epicotyl brei (84) and found to be very similar to that for the photooxidation of I.A.A. by free riboflavin (84), previously described (23) and to the visible absorption spectrum for riboflavin. The brei is light-activated after dialysis but the heated dialyzed brei no longer has any activity in the light (90), suggesting that the photoreceptor for the enhanced oxidation of I.A.A. is a non-dialyzable, heat labile substance with a flavin absorption spectrum, i.e., a flavoprotein.

The increased destruction of I.A.A. in light is thought to be due to the accelerated activity of a peroxide-producing system. This is suggested because hydrogen peroxide is known to arise from molecular oxygen by specific flavoprotein oxidases. Also, pigment-sensitized photo-oxidations are frequently attributed to the intermediate formation of hydrogen peroxide (91). Since the oxidation of I.A.A. appears to be peroxidative in nature, with H_2O_2 frequently rate-limiting (88, 92), the stimulation of an H_2O_2-producing system by light should result in a greater rate of disappearance of I.A.A. This notion is strengthened by the observations (92, 85) that the inhibition of catalase is reversed by light. Table 2 reproduced from reference (92) shows this result. Thus, as a working hypothesis, the enzyme system is envisioned (88, 90, 92) as consisting of two coupled successive oxidations, one a light-activated flavine oxidase producing H_2O_2 and the other a peroxidase utilizing the H_2O_2 produced by the flavine enzyme.

It would appear that I.A.A. itself or a primary oxidation product of I.A.A. must be the substrate for the flavine oxidation for the following reasons. (a) Exhaustively dialyzed or acetone precipitated enzymes actively destroy I.A.A. but are devoid of flavine oxidase substrates such as xanthine, amino acids, etc., which could serve for the production of H_2O_2. (b) If the peroxide arose from oxidation of an exogenous substrate, then two moles of oxygen would be consumed per mole of I.A.A. oxidized. For example, if the prosthetic group of the flavin oxidase were flavin-adenine dinucleotide (FAD),

Table 2

Reversal of the Catalase Inhibition of I.A.A.-oxidase by Light

mls 1:100 catalase per 4 ml reaction mixture	I.A.A. destroyed, γ/ml	
	dark	light
0	12.7	17.3
0.1	10.4	16.2
0.3	4.3	16.1
1.0	0	12.3
1.6	0	9.5

Reaction mixtures contained 2.0 ml acetone-precipitated I.A.A. oxidase in buffer, 0.4 ml stock I.A.A. (250 γ/ml) and water to a total volume of 4.0 ml. "Light" was provided by approximately 300 f.-c. of "daylight" fluorescent illumination.

and the end-product of the oxidation indole-3-aldenyde

$$XH_2 + FAD \dashrightarrow FAD.H_2 + X$$

$$FAD.H_2 + O_2 \dashrightarrow FAD + H_2O_2$$

$$2\ H_2O_2 + \text{I.A.A.} \dashrightarrow \text{indole-3-aldenyde} + CO_2 + 2\ H_2O$$

whereas in fact one mole of oxygen is consumed and one mole of carbon dioxide produced per mole of I.A.A. oxidized (78).

(c) In this <u>in vitro</u> system, total destruction of I.A.A. is not attained, as would be expected if H_2O_2 formed in one reaction were quantitatively required for the second. Two coupled oxidations involving one mole of oxygen are tentatively formulated (88, 90, 92).

(1) I.A.A. + O_2 —(flavin enzyme)→ indole-glycolic acid + H_2O_2 —(peroxidase)→ indole glyoxylic acid → indole-3-aldenyde + CO_2

or

(2) I.A.A. + H_2O_2 —(peroxidase)→ indole glycolic acid + H_2O —(flavin enzyme, O_2)→ indole glyoxylic acid + H_2O_2 → indole-3-aldenyde + CO_2

The activity of the enzyme system is increased by the addition of low concentrations of H_2O_2 in the dark, or by the

addition of crystalline horseradish peroxidase in the light (92). This indicates that H_2O_2 limits the reaction in the dark; in the light, H_2O_2 production is increased and peroxidase becomes limiting. The I.A.A. oxidase system can be imitated by coupling the H_2O_2 produced from the oxidation of hypoxanthine by the flavine enzyme xanthine oxidase, to crystalline horseradish root peroxidase.

Some fractionation of the system has been made (92), the separate fractions having little activity but the reconstituted system having abundant activity. The fractions are referred to as "peroxidase," which has activity toward the conventional peroxidase substrates, an "apoenzyme" precipitated in 0.22 saturated ammonium sulphate at pH 2.5 and which has no peroxidase activity, and a "coenzyme" split from the "apoenzyme" by acid treatment according to the method applied to flavoproteins by Warburg and Christian (93). The "coenzyme" showed some of the solubility properties of flavine and appeared to contain significant quantities of riboflavin by the Lactobacillus caseii assay.

There are in the literature four other examples in which coupled oxidase-peroxidase reactions carry out successive steps in the oxidation of the same substrate. (a) Dihydroxymaleic acid oxidase (94), which is identical with peroxidase, and which catalyzes the coupled reactions:

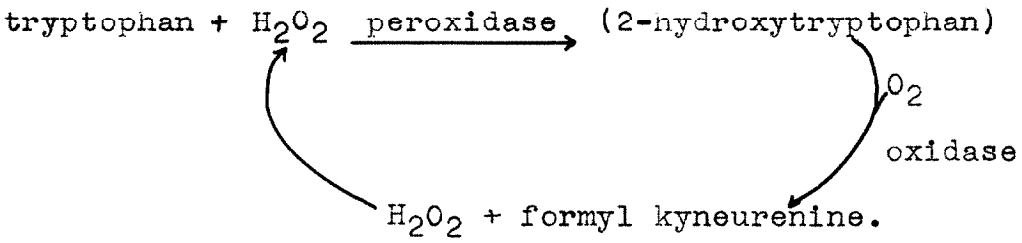
$$\text{dihydroxymaleic acid} + Fe^{+++}\ \text{peroxidase} \longrightarrow \text{diketosuccinic acid} + Fe^{++}\ \text{peroxidase}$$

$$Fe^{++}\ \text{peroxidase} + O_2 \longrightarrow Fe^{+++}\ \text{peroxidase} + H_2O_2$$

H_2O_2 + dihydroxymaleic acid + Fe^{+++} peroxidase ⟶

diketosuccinic acid + Fe^{++} peroxidase

(b) The coupled tryptophan peroxidase-oxidase system of rabbit liver (95) which is provisionally formulated:



(c) Keilin and Hartree (96) have described a similar coupling, through peroxide, of two steps in the oxidation of ethanol. In their system, catalase catalyzes the oxidation of ethanol to acetaldehyde by means of peroxide generated in the further oxidation of acetaldehyde catalyzed by xanthine oxidase.

(d) The glycolic acid oxidase of plants (97) which has been shown to contain riboflavin phosphate, catalyzes the oxidation by molecular oxygen of glycolic acid or lactic acid to glyoxylic or pyruvic acid respectively, with the formation of hydrogen peroxide. The peroxide then oxidizes the glyoxylic or pyruvic acids nonenzymatically to formic or acetic acids and carbon dioxide.

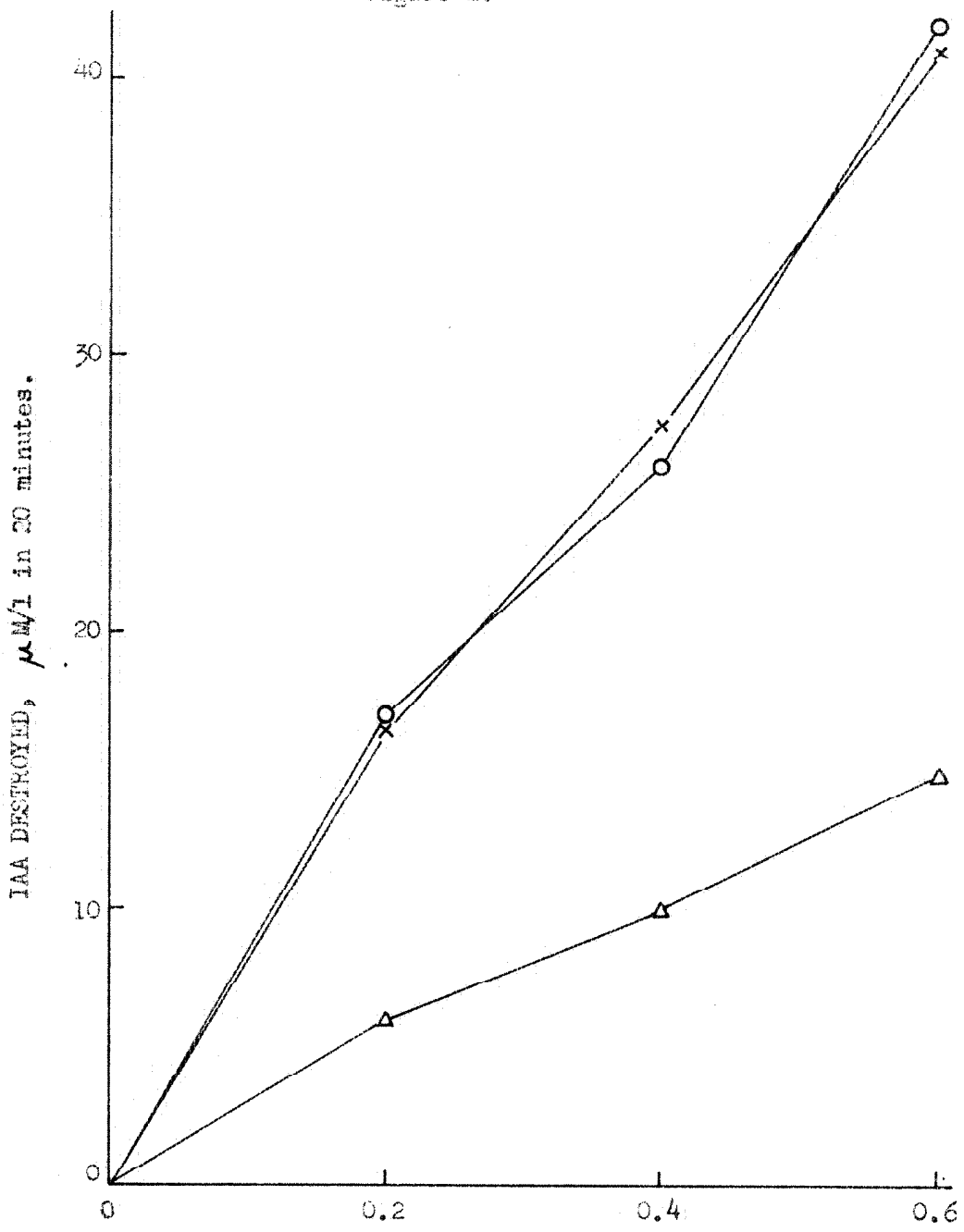
It is clear that while a good deal is now known about the I.A.A. oxidase system, the complexities of its structure and mechanism are not well understood. This section of the present paper deals with some studies which attempt to analyze further the nature of the system.

The Effect of Dialysis. Previous workers (79, 86) have

observed that dialysis of whole brei of red-grown pea epicotyls produces a marked increase in activity. Acetone precipitation of the enzyme similarly had a salutory effect. This was attributed to the removal of a naturally-occurring inhibitor. The enzyme was stated to have no cofactor requirement (78). Wagenknecht and Burris (80), however, observed that dialysis of whole brei from bean roots or etiolated pea epicotyls led to a one-third loss of activity. The activity was restored by adding 10^{-3} M manganese. The ash of whole brei was inactive in restoring the activity and the dialysate was not tested.

In the present study, it was found that the exhaustive dialysis of crude breis leads to partial though not complete loss of the enzymatic activity, the activity being restored when the dialysate is concentrated and added back. Epicotyls of 8-day dark-grown peas were blended in half their weight of cold M/60 phosphate buffer, pH 6.6, strained through muslin and centrifuged. 10 ml. crude brei were placed in a cellophane bag and dialyzed in the cold for 16 hours against 1000 ml. M/1000 phosphate buffer pH 6.6. The volume of the dialyzed brei was measured and an aliquot of the undialyzed brei adjusted to the same dilution. The dialysate was frozen and evaporated under high vacuum to the same volume. The ability to destroy I.A.A. in the dark was now tested for the whole brei, the dialyzed brei, and the dialyzed brei plus an equivalent volume of concentrated dialysate. Figure 2 shows that about 70% of the activity is lost on dialysis but is restored when the dialysate is added back.

Figure 2.



The effect of dialysis on the IAA oxidase activity of crude brei. Measured in dark at 24.2°C.

O = undialyzed brei.

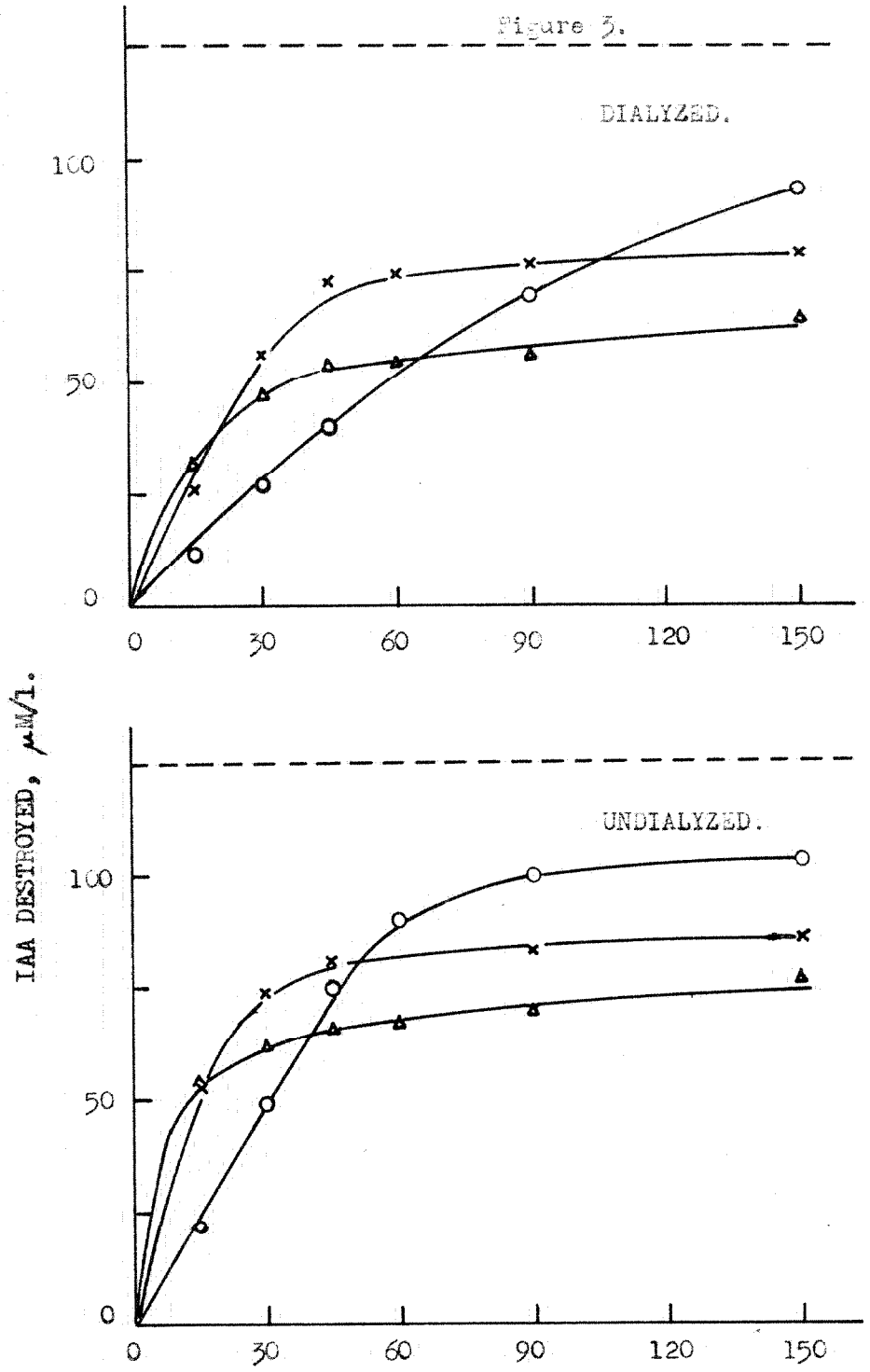
Δ = 10 ml. brei dialyzed against 1000 ml. M/1000 buffer.

x = dialyzed brei + concentrated dialysate.

The fractional loss of activity on dialysis is greater in dark-grown peas than in red-grown peas and less in old red-grown peas than in young red-grown peas. It seems that in addition to the inhibitor there is also present a dialyzable cofactor. Whether dialysis leads to an increase or a decrease in activity presumably depends on the relative concentrations of these two materials.

The Effect of Enzyme Concentration. When initial rates are measured, the relation between activity and enzyme concentration is linear at moderately low concentrations (78, 80, 83). After prolonged incubation, however, this is no longer true. Figure 3 shows progress curves for three concentrations each of dialyzed and whole breis from red-grown seedlings. In both cases the higher the enzyme concentration, the higher is the initial rate. The rate of decrease in velocity is much faster however in the case of higher enzyme concentrations so that in time the total amounts of I.A.A. destroyed are in inverse order to the initial rates. There are two likely explanations for this effect. (a) Since peroxide is produced during the oxidation of I.A.A. and since traces of peroxide are frequently observed to destroy the activity of enzymes (96), it may be that the initial high activity produces peroxide in amounts more damaging to the enzyme than in the case when the initial activity is low. (b) If, as indicated in the reaction scheme proposed earlier, peroxides formed in one reaction are coupled to the second oxidation step, any subversion of the peroxide to

Figure 3.



The effect of enzyme concentration on the time course of IAA destruction.

○ = 1 ml. enzyme in 16 ml. reaction mixture.
× = 3 ml. enzyme in 16 ml. reaction mixture.
△ = 5 ml. enzyme in 16 ml. reaction mixture.

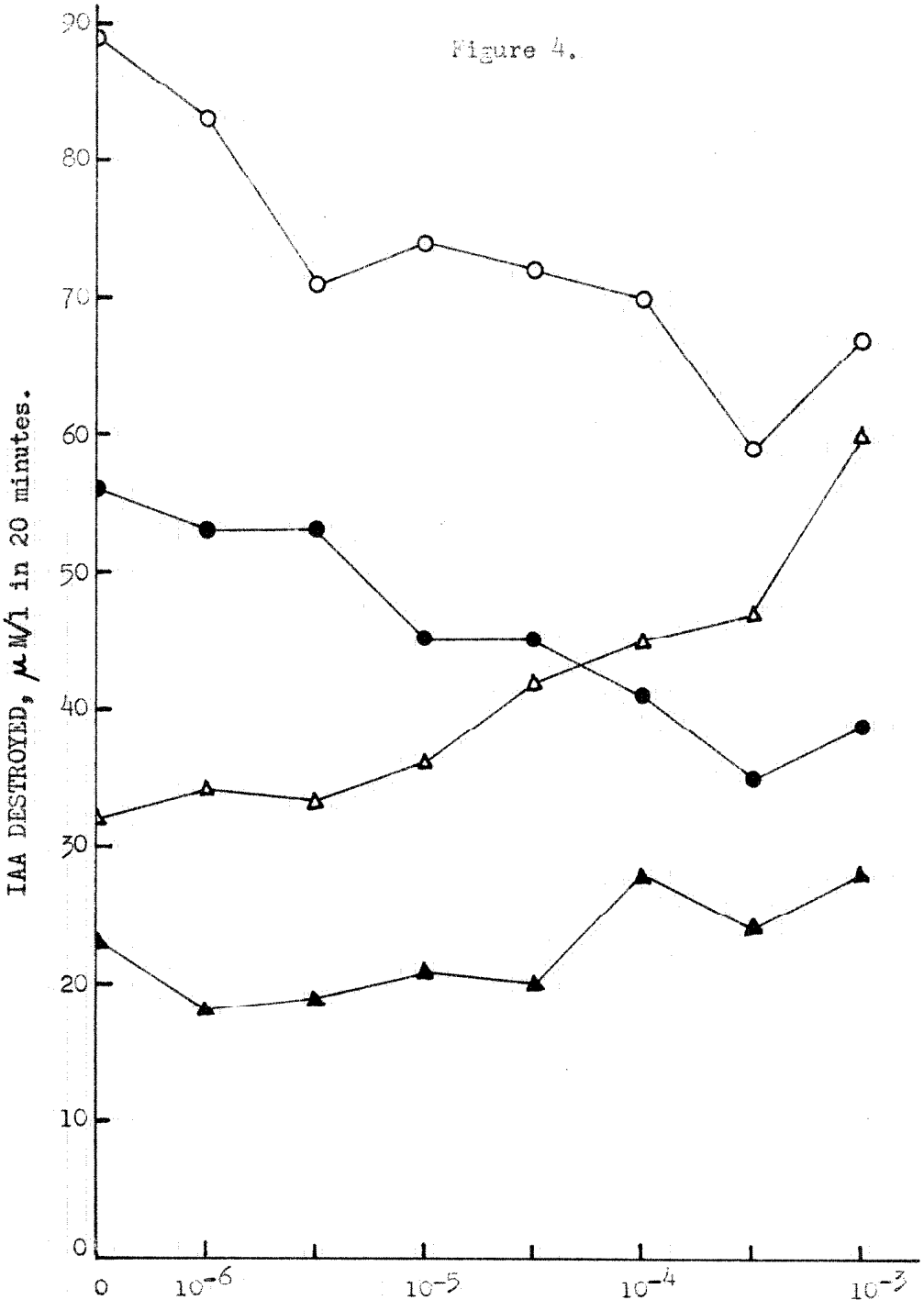
Initial IAA concentration = 125 μM/l. Run in dark at 26°C.

other reactions would reduce stoichiometrically the final amount of I.A.A. which will be destroyed. If H_2O_2 is produced at a greater rate initially, it may be that fractionally more is lost to other reactions (e.g., catalase, or interaction with the protein present) than when the reaction rate is lower. No similar data are available for other oxidase-peroxidase systems. This result points up the need to define the nature of the progress curve before reaction rates are measured.

The Effect of Manganese. Wagenknecht and Burris (80) report that the Mn^{++} ion at concentrations from 0.0005 M to 0.001 M increased the oxygen uptake of crude or salt-fractionated bean-root enzyme. With the pea epicotyl enzyme, the effect was variable however. Galston and Baker (86) have obtained strong inhibitions of an acetone-precipitated enzyme from pea epicotyls by low concentrations of manganese. The reduced activity was partially overcome by blue light. Experiences of others (83, 98) have found little consistency in the behavior of manganese.

An attempt was made to reconcile these divergent experiences. It was consistently found, as shown in figure 4, that at concentrations between 10^{-6} and 10^{-3} M, manganese inhibited the activity of undialyzed brei but promoted the activity of dialyzed brei. Above 10^{-3} M, manganese also inhibits dialyzed enzyme. No explanation can be offered at present for the enhancement. Although it was not evident in the present studies Galston and Baker (86) report that the manganese inhibition is reversible by blue light. Since light promotes the production

Figure 4.



MOLAR CONCENTRATION OF $MnCl_2$.

The effect of manganese on the activities of dialyzed and undialyzed IAA oxidase in the light and in the dark.

○ = undialyzed, light. △ = dialyzed, light.

● = undialyzed, dark. ▲ = dialyzed, dark.

Initial IAA concentration = 250μM.

of peroxide it has been suggested (86) that the inhibition by manganese may be due to the catalase-like activity of this ion in the presence of peroxidase and a trace of a peroxidase-substrate, as described by Kenten and Mann (99).

The Possibility of Two Parallel Systems. From its behavior toward various specific inhibitors and to light, and from fractionation experiments, it is now clear that the I.A.A. oxidase consists of a complex of enzymes. There are in addition certain aspects of the behavior of this complex which suggest that for at least a portion of the reaction chain, two or more enzymes act in parallel. The observations leading to this interpretation are presented below.

As described in an earlier section, dialysis removes a cofactor from the system. However, exhaustive dialysis fails to remove more than 60 to 90% of the activity. Workers who have used acetone-precipitation (79, 83, 86, 92) or ammonium sulphate precipitation (80) have prepared an active enzyme yet have presumably removed the dialyzable cofactor since neither of these procedures precipitates the cofactor out of the dialysate. Two explanations seem possible. **(a)** If the cofactor, e.g. a hydrogen carrier, exists in two forms, one may be dialyzable and the other firmly bound to the enzyme as in the case of the diphosphopyridine nucleotide of crystalline muscle phosphoglyceraldehyde denydrogenase (100). This possibility seems unlikely however because in a large number of experiments, dialyzed brei from red-grown plants were always found to have a greater residual activity than did dialyzed

brei from dark-grown plants. If only one form of the co-factor is removed by dialysis the residual activity should depend only on the relative proportions of the two forms of the cofactor at the time of dialysis. (b) There may be two enzymatic processes contributing to the destruction of I.A.A., one cofactor-dependent, the other without a cofactor requirement. They may be envisioned as performing the same reaction, or competing reactions, each acting either on I.A.A. or on a degradation product of it.

Other observations seem to lend support to the second alternative. Figure 4 shows the differential effect of manganous ions on dialyzed and undialyzed breis. As far as the concentration range of manganese has been taken, the activity of manganese-stimulated dialyzed brei never exceeds the residual activity of the combined manganese-inhibited plus manganese-stimulated whole brei, but approaches it. Apparently, while Mn^{++} stimulates the non-dissociable enzyme at low concentrations, it inhibits the cofactor-dependent enzyme.

As described in a later section, 2,4-dichlorophenol (D.C.P.) produces a marked stimulation of the activity of I.A.A. oxidase (figure 6). While the mechanism of this effect is by no means clear, certain observations are pertinent to this discussion. Although the residual activity of dialyzed brei may be a small fraction of the activity of whole brei, the final elevated activities in the presence of D.C.P. are approximately the same whether the brei is dialyzed or undialyzed (figure 15). This suggests that D.C.P. is substituting in some way for the

natural cofactor but in a more effective manner. The facts that brei from dark-grown plants undergoes greater relative loss of activity upon dialysis than does brei from red-grown plants (figure 15, etc.) and that brei from dark-grown plants is relatively more enhanceable by D.C.P. than is brei from red-grown plants (figure 9) lends some support to this notion. Thus the D.C.P. effect seems to be another differential response distinguishing between the cofactor-dependent and the cofactor-independent activities of the brei.

The most reasonable interpretation of these data seems to be that there are alternative enzymatic mechanisms contributing to the destruction of I.A.A. Both mechanisms however must be dependent upon the activity of a peroxidase since it is possible to produce total inhibition of the system by catalase (88) or by low concentrations of cyanide (78).

The Effect of 2,4-dichlorophenol. It was earlier reported (102) and confirmed (89) that 2,4-dichlorophenoxyacetic acid (2,4-D) enhances the activity of I.A.A. oxidase. Subsequently in a cooperative work with Dr. R. L. Weintraub and Dr. A. W. Galston it was found that not all samples of 2,4-D have this effect but that 2,4-dichlorophenol (D.C.P.), the most probable contaminant, gave a marked enhancement at concentrations as low as 10^{-7} to 10^{-5} M (see figure 6). Although active samples of 2,4-D had previously been recrystallized to constant melting point from hot water and from aqueous alcohol and subjected to potentiometric titration, U.V. spectrophotometry and polarographic examination, no D.C.P. was detected. When

the enzyme, I.A.A. oxidase, was used to estimate the D.C.P.-like activity in a 2,4-D sample previously reported to produce enhanced activity (89), approximately 0.15% (mole/mole) was found. Subsequent analysis with the reagent of Folin and Ciocalteau (103) gave a value of 0.3% (mole/mole) assuming all the phenol present to be 2,4-dichlorophenol. Less than 0.02% D.C.P. was found in the inactive 2,4-D samples. This implicates D.C.P. circumstantially as the compound responsible for the reported increase in activity.

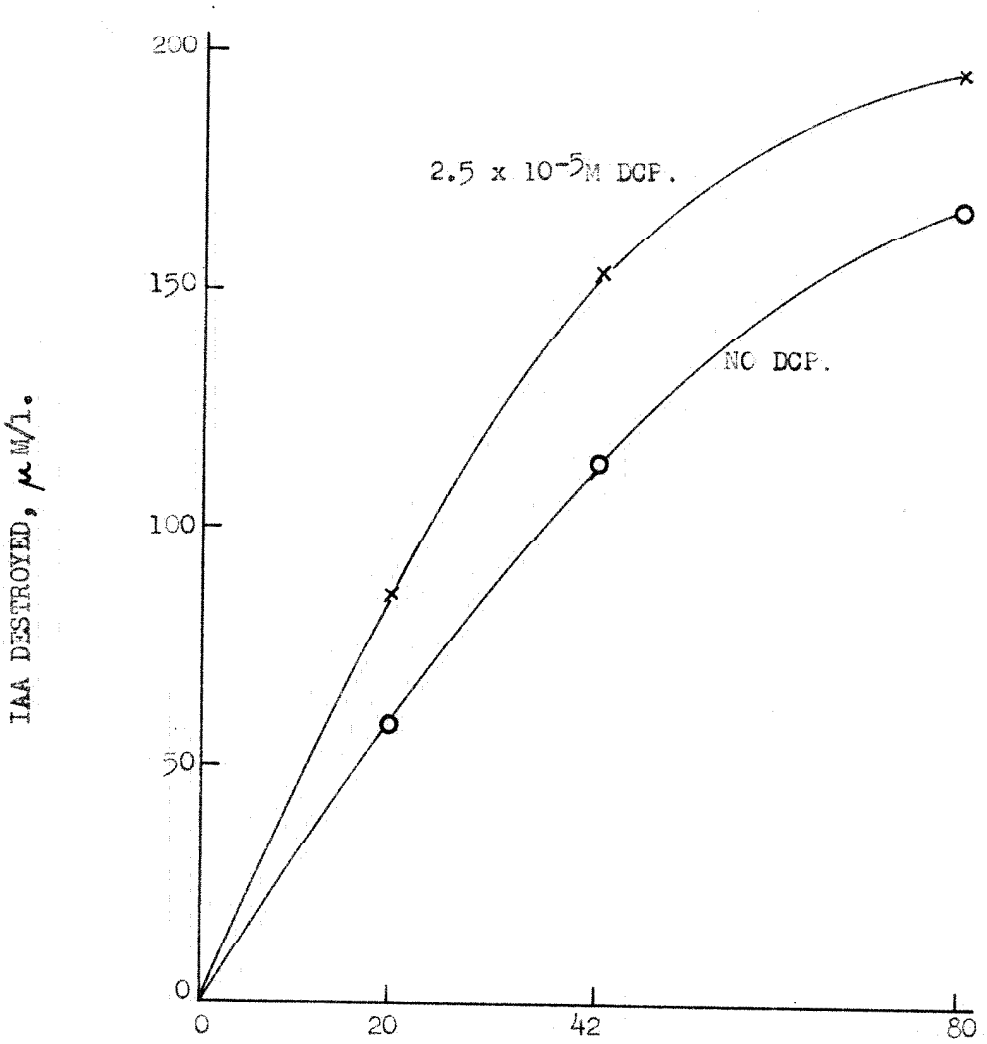
The effect has been studied in detail since this compound might be a useful tool in obtaining information on the structure and mechanism of the I.A.A. oxidase system.

(a) Progress Curve.

Figure 5 shows progress curves for I.A.A. oxidase incubated in the dark with and without D.C.P. There is no obvious qualitative difference in the time courses of reaction-rate change between the two curves. This is also borne out in figure 7 .

No loss of I.A.A. due to D.C.P. occurs in the absence of enzyme or in the presence of boiled enzyme. Thus the enhancement is enzymatically mediated. When D.C.P. was preincubated with whole pea epicotyl brei before adding I.A.A. immediately followed by Salkowski reagent, there was no loss of I.A.A. This shows that the effect is not due to the nonenzymatic action of any product of the independent action of the brei on the D.C.P., either by destruction of the I.A.A. or by interference in the Salkowski reaction.

Figure 5.



Progress curves for IAA oxidase in the dark with and without DCP. Enzyme is whole brei from 7-day red-grown pea seedling epicotyls. Initial IAA concentration was 250 μM/l.

(b) The Effect of D.C.P. Concentration

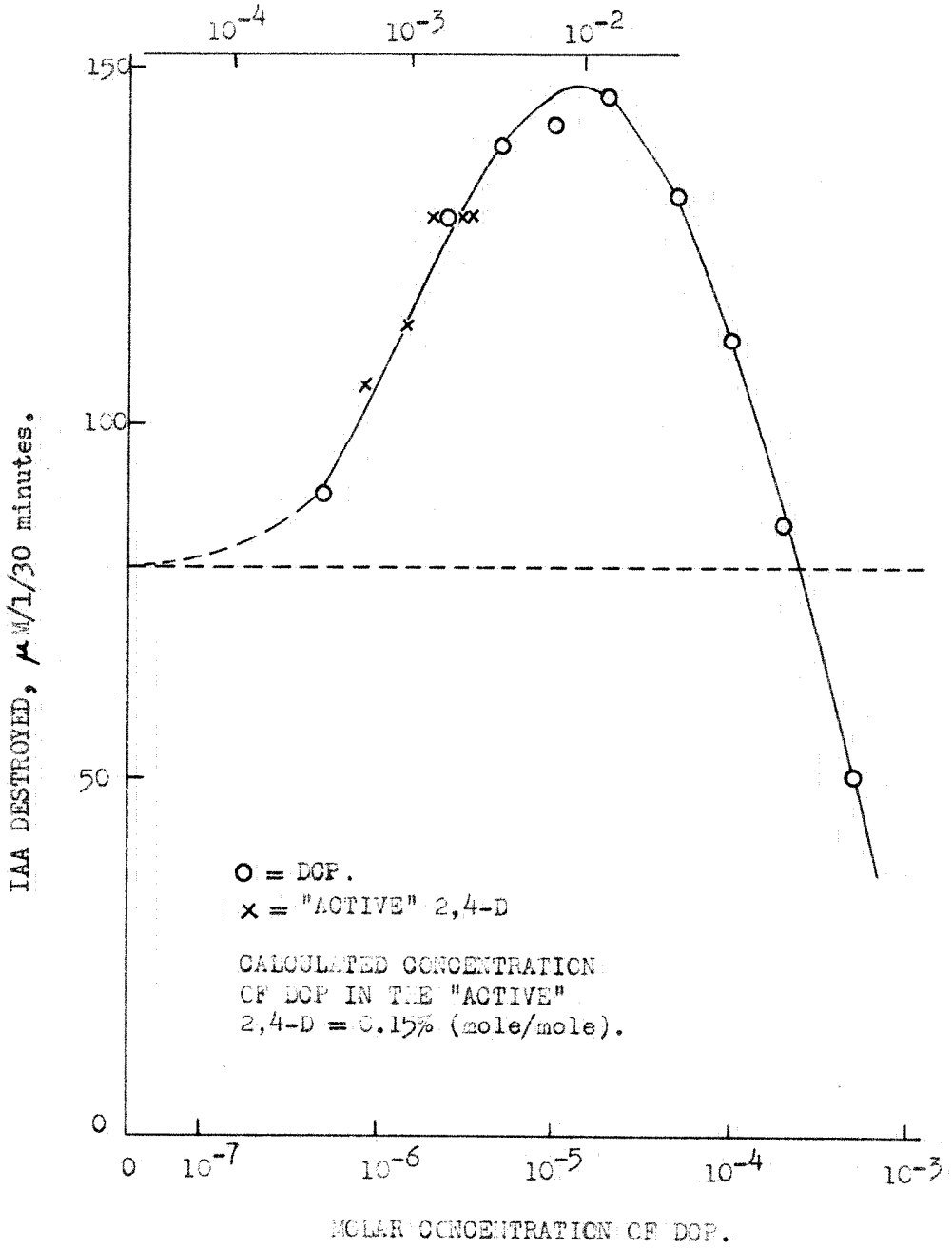
Figure 6 shows that the greatest increase in activity occurs in the region 2 to 3 x 10^{-5}M D.C.P. Although the sensitivities of various enzyme preparations differ (e.g. according to previous exposure of the plants to light) the optima always occur at this concentration. Appreciable enhancement is measurable at 10^{-7}M. At concentrations above 3 x 10^{-5}M the stimulation diminishes and above 2 x 10^{-4}M strong inhibition occurs. Maximum increases in activity from 20% to 1200% have been observed, depending on the enzyme preparation and concentration conditions.

(c) The Effect of D.C.P. on Gas Exchange

The D.C.P.-enhanced destruction of I.A.A. by I.A.A.-oxidase has been followed manometrically. Warburg flasks were loaded with 1.0 ml. dialyzed brei from 6-day dark-grown pea epicotyls (sidearm), 0.5 ml. M/15 phosphate buffer pH 6.64, 2.0 ml. 10^{-2}M I.A.A. where stated, 0.5 ml. 2 x 10^{-4}M D.C.P. where stated and water to a total volume of 4.0 ml. A duplicate series contained KOH in the center wells. Brei was tipped in at zero time. Figure 7 shows that although this concentration (2.5 x 10^{-5}M) of D.C.P. had no effect on the endogenous oxygen uptake, that due to the oxidation of I.A.A. is increased fivefold by the presence of D.C.P. Carbon dioxide evolution was measured simultaneously making correction for retention by the method of Johnson(104). A corresponding increase due to D.C.P. was observed. The R.Q. (0.87) was not significantly different from that for the basal oxidation of

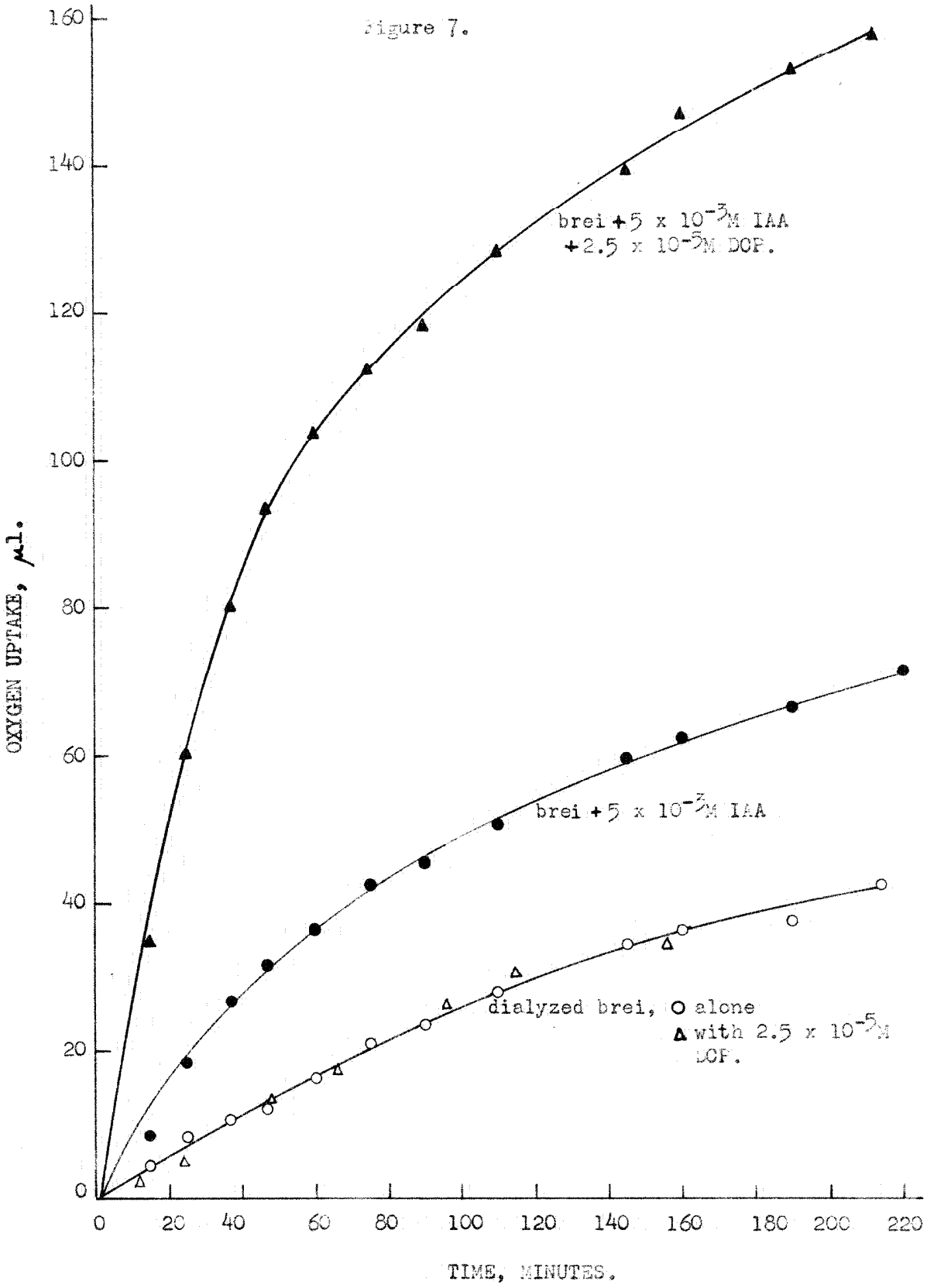
Figure 6.

MOLAR CONCENTRATION OF "ACTIVE" 2,4-D.



The effect of DCP concentration on activation of IAA oxidase and enzymatic assay for DCP in an "active" sample of 2, 4-D. Whole brei from 7-day dark-grown pea epicotyls. Initial IAA concentration = 250 μM. Incubated 30 minutes in dark at 25.8°C.

Figure 7.



The effect of 2,4-dichlorophenol on the enzymatic oxidation of IAA by dialyzed pea epicotyl brei. Temperature = 30.0°C.

I.A.A. (0.83) but owing to the large correction for CO_2 retention at pH 6.64 and the high endogenous gas exchange no precise significance is attached to these values.

(d) The Effect of Enzyme Concentration

Figure 8 shows that the greatest relative increase in activity occurs at the lowest enzyme concentrations. Brei prepared from plants grown in complete darkness is markedly more ennanceable than that prepared from plants grown in weak red light. This is not merely due to the higher basal activity of brei from red-grown plants but appears to be an intrinsic difference between the two preparations. If the plot is made of the ratio of the activities in the presence and absence of 3×10^{-5}M D.C.P. against the basal activity, the enzyme from dark-grown peas shows itself to be substantially the more enhanceable (figure 9).

(e) Possible Mechanisms of Action

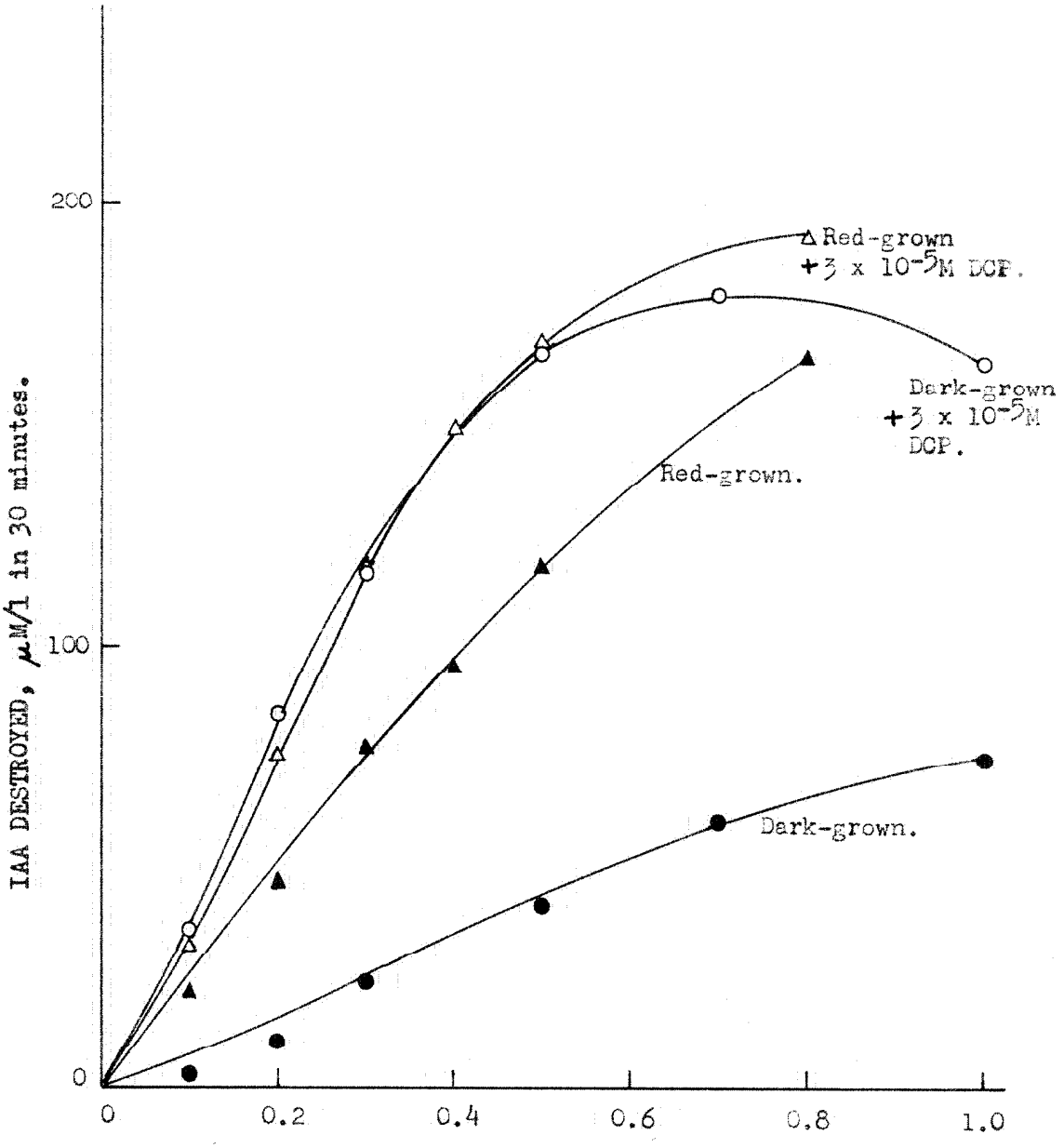
From figure 7 it may be calculated that in 212 minutes the presence of 25 μM/l D.C.P. evoked an extra destruction of 875 μM/l I.A.A. It is therefore not acting in a stoichiometric fashion (e. g. by removal of reaction product). Exogenous substances which increase the activity of a biological system catalytically are generally considered to act in one of the following ways.

(1) to act as an oxidation-reduction carrier,

(2) to reverse a naturally-occurring inhibition,

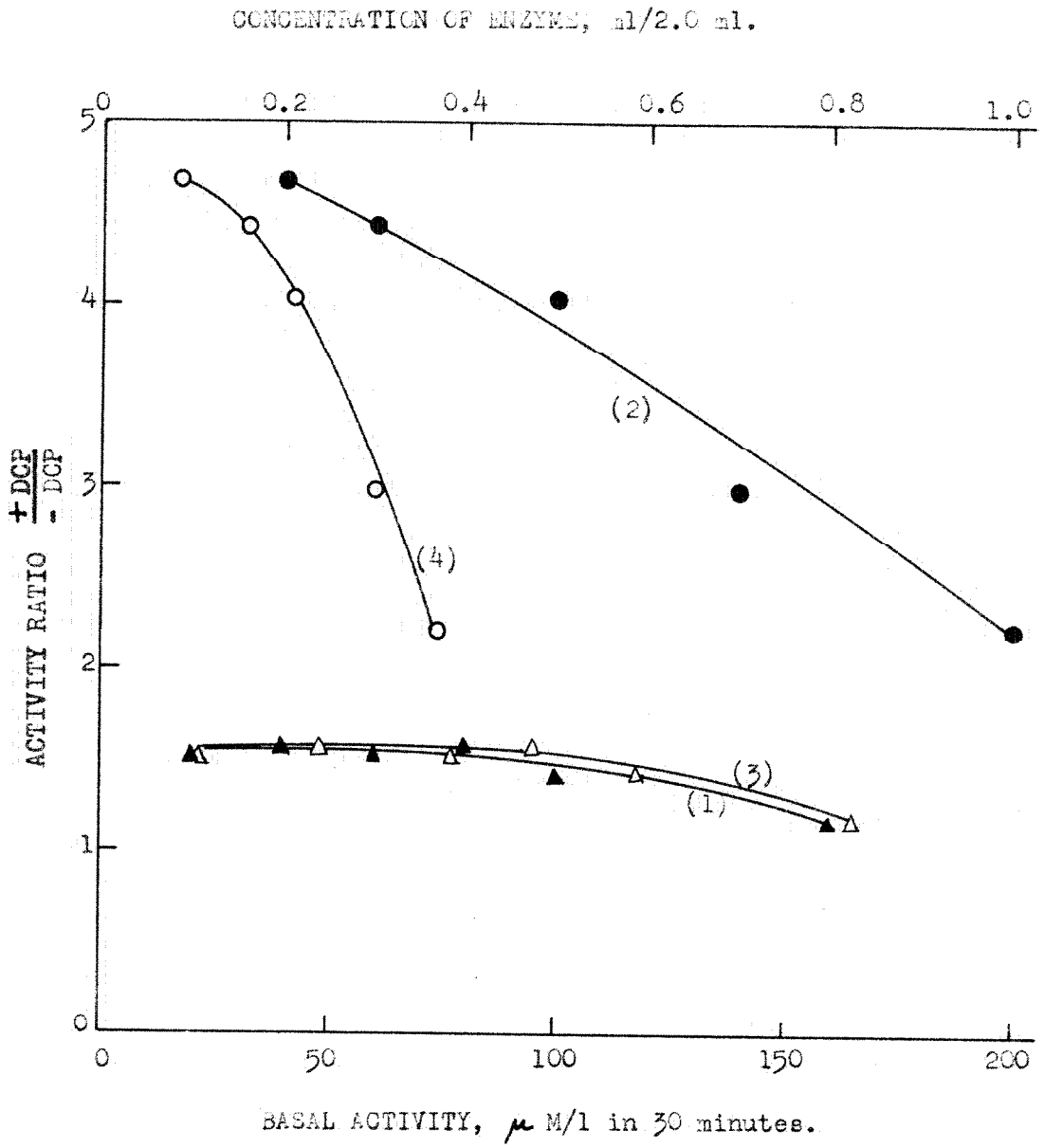
(3) to replace, with greater activity, a structurally-related naturally-occurring cofactor,

Figure 8.



The effect of brei concentration on the enhanceability of its IAA oxidase activity by 3×10^{-5}M DCP. Eight-day red-grown and dark-grown pea epicotyls blended in small volume M/60 phosphate buffer pH 6.6. Trichloracetic acid precipitable material = 5.68 mg/ml. for red-grown brei and 4.06 mg/ml. for dark-grown brei. Assayed in dark at 25.5°C. initial IAA concentration = 250 μM.

Figure 9.



DCP enhancement as a function of enzyme concentrations and basal activities for red- and dark-grown peas. Calculated from data of figure 2200.

Curve (1) refers to enzyme from red-grown epicotyls (upper abscissa).
Curve (2) refers to enzyme from dark-grown epicotyls (upper abscissa).
Curve (3) refers to enzyme from red-grown epicotyls (lower abscissa).
Curve (4) refers to enzyme from dark-grown epicotyls (lower abscissa).

(4) to interact directly with an enzyme, in some way increasing its activity.

These possibilities will be treated in order.

As will be described in a later section a large series of compounds related to D.C.P. gives accelerated I.A.A. oxidase activity. Of these some of the most effective, e. g. 2,4,6-trichlorophenol, are incapable of reversible oxidation and so could not act as hydrogen carriers.

Since the D.C.P. effect is not lost upon dialysis of the brei, if the action is through reversing a naturally-occurring inhibition, the inhibitor must be nondialyzable.

Reversal of the Catalase Inhibition by D.C.P. It has previously been reported (88) and confirmed (92) that catalase strongly inhibits I.A.A. oxidase. It is known that crude and acetone-precipitated I.A.A.-oxidase preparations contain catalase. Further, it was shown that an impure 2,4-D sample showing stimulatory activity reversed the catalase inhibition and was somewhat inhibitory both to sheep liver catalase and to pea epicotyl catalase (14). This suggested that the increased activity of the I.A.A. oxidase may be due to the inhibition of the endogenous catalase by D.C.P.

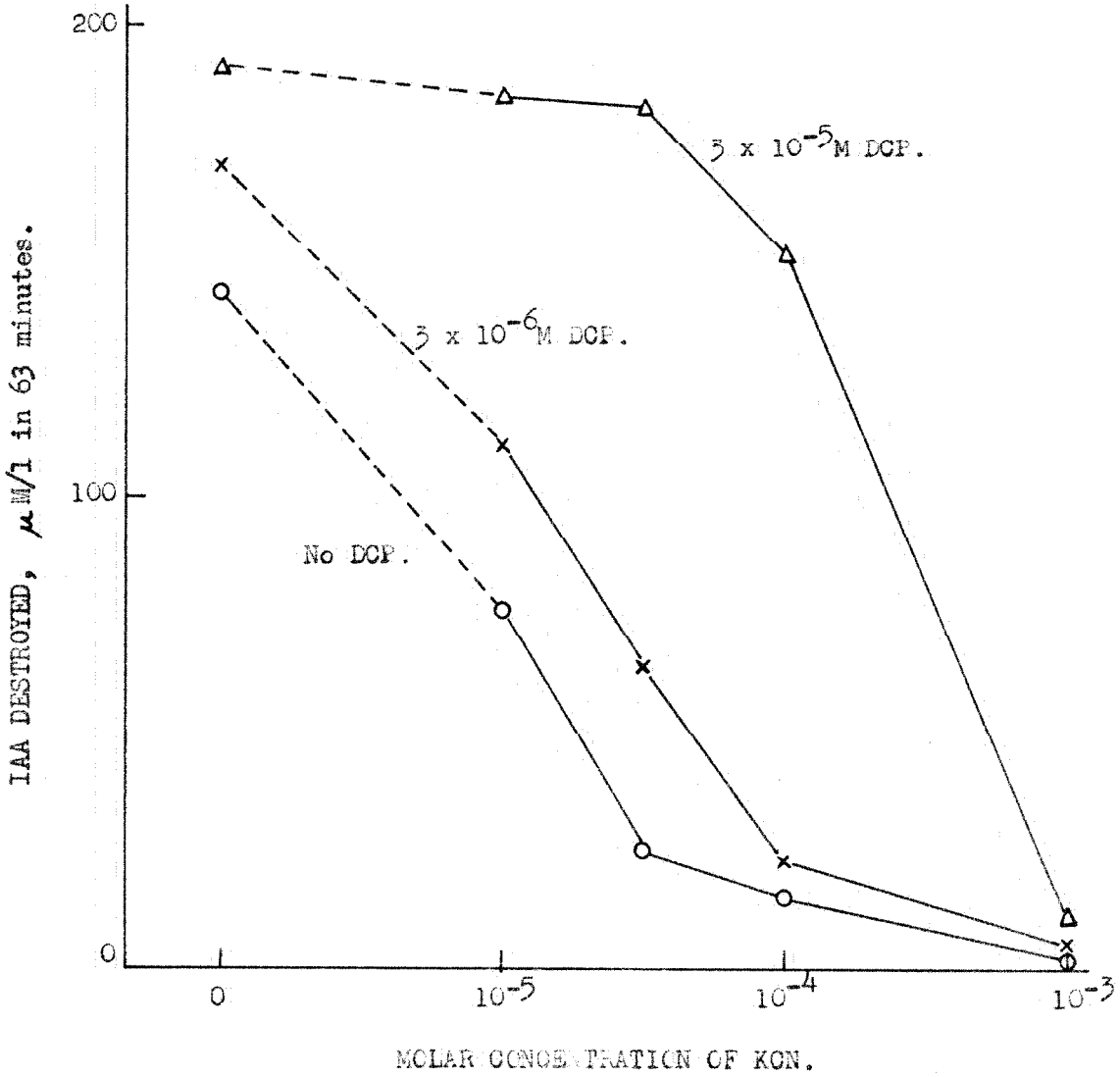
Figure 2, ref. (88) shows that the inhibitory effect of added catalase on I.A.A. oxidase is counteracted by D.C.P., the effect being greatest, percentagewise, at highest catalase concentrations where peroxide would be strongly rate-limiting. D.C.P. is able to restore activity even when this activity would otherwise be totally inhibited by catalase.

This suggests that D.C.P. is acting in some way to make peroxide more available to the I.A.A. oxidase system, e.g., by inhibiting the catalase or by stimulating the peroxide-producing reaction. A series of experiments were therefore run to determine whether D.C.P. had any effect on catalase or on analogs of the components of the I.A.A. oxidase system.

The effect of D.C.P. on I.A.A. oxidase in the presence of cyanide is shown in figure 10. At concentrations of cyanide which completely inhibit the enzyme (10^{-3}M), D.C.P. fails to produce an increase in activity. (It may be recalled that when I.A.A. oxidase is totally inhibited by catalase, D.C.P. restores substantial activity). This would indicate that although the D.C.P. enhancement is not limited by the peroxidase at low cyanide concentrations, it is limited at higher cyanide concentrations, i.e., the D.C.P. probably does not influence the peroxidase itself but some reaction which is channelled through the peroxidase step. However, this interpretation is complicated by the fact that at partially-inhibiting cyanide concentrations, D.C.P. appears to reverse the inhibition. Thus at 3×10^{-5}M KCN, while the control activity has dropped from 143 to 25, activity in the presence of 3×10^{-5}M D.C.P. has remained almost unchanged (191 to 183).

The Innibition of Catalase by D.C.P. A concentrated suspension of "crystalline" beef liver catalase supplied by Worthington Biochemical Sales Co. was diluted 1 to 300 with water and used as stock. From the value of the optical densities at 280 and 405 mμ, the concentration of this stock

Figure 10.

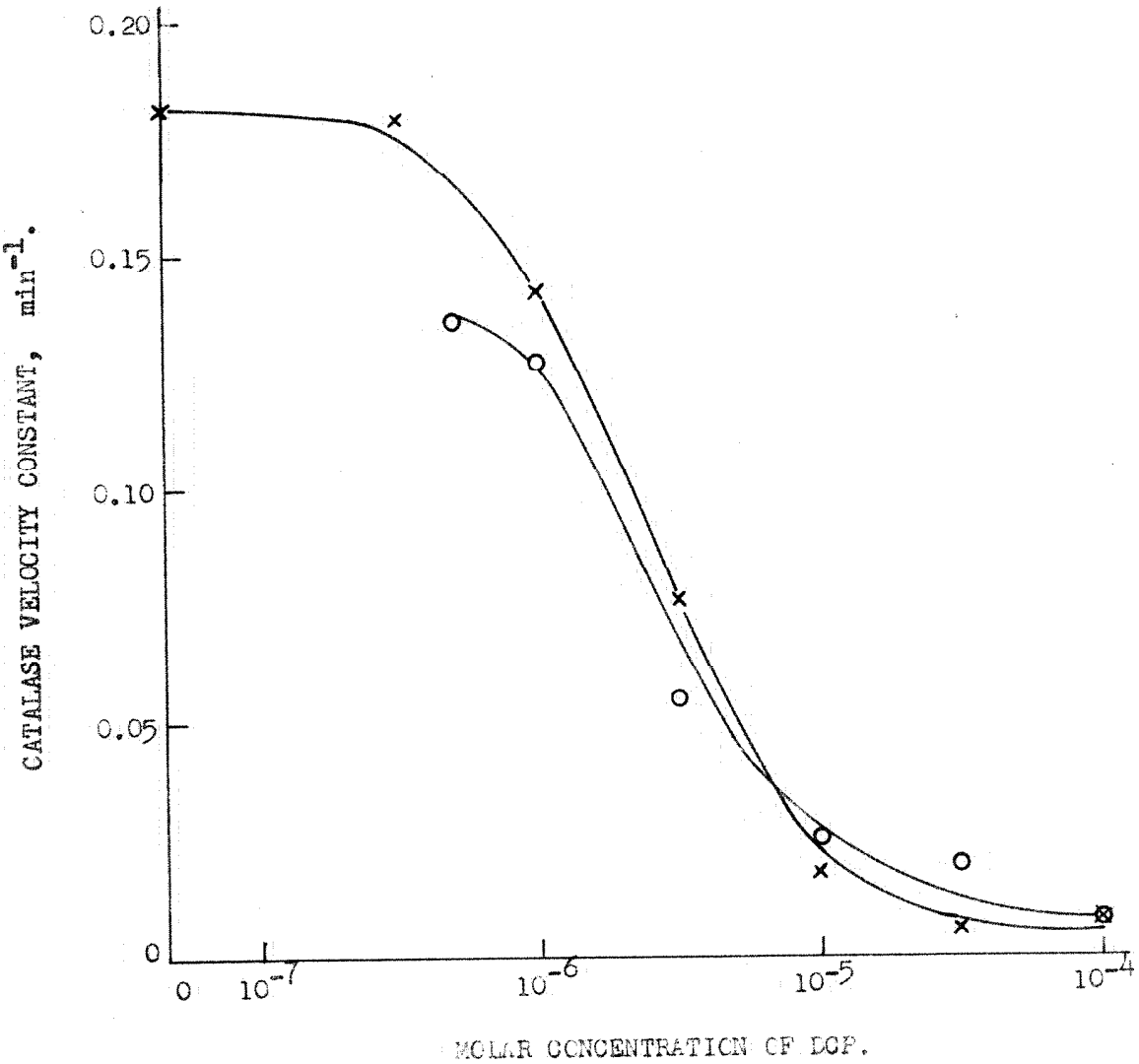


Effect of DCP in the presence of cyanide. Initial IAA concentration = 250 μM/l. Temperature = 25.5°C., in dark.

solution was found to be approximately 1.3×10^{-7}M. 0.1 ml. of catalase solution was added to each of a series of flasks with 18.9 ml. 0.1 M phosphate buffer pH 6.9 containing known concentrations of D.C.P. The solutions were incubated for 30 minutes in an ice bath after which a vial containing 1.0 ml. 0.1 M H_2O_2 was dropped into each flask and well mixed. 2 ml. aliquots were withdrawn at 30 second intervals into 5 N H_2SO_4, according to the rapid method of Bonnichsen, Chance and Theorell (105) and titrated with 0.01 N $KMnO_4$. Figure 11 shows that D.C.P. is a potent inhibitor of catalase. Half inhibition occurs at 2×10^{-6}M and complete inhibition at 10^{-4}M.

Since the final catalase concentration was approximately 5.2×10^{-8}M, it was important to ascertain whether the reduced activity was due to nonspecific denaturation of the protein or in fact was a mass action combination between the D.C.P. and the enzyme. If a reversible catalase-D.C.P. complex is formed the equilibrium should be approachable from both directions. A 1 to 300 dilution of the original catalase was made in 10^{-4}M D.C.P. at which concentration complete inhibition is produced. After 30 minutes equilibration at 0°C., 0.1 ml. aliquots were placed in a series of flasks with 18.9 ml. buffer containing concentrations of D.C.P. less than 10^{-4}M. After incubating in an ice bath for 30 minutes at the new final D.C.P. concentrations, H_2O_2 was added and the velocity constants determined as before. Figure 11 shows that after diluting out the D.C.P. the activity is restored

Figure 11.



The reversible inhibition of beef-liver catalase by DCP. Catalase concentration approximately 5.2×10^{-8}M. Temperature = 0°C. (x) designates experiment in which final DCP concentration was reached directly. (o) shows experiment in which catalase was incubated with 10^{-4}M DCP and the final concentrations obtained by diluting out.

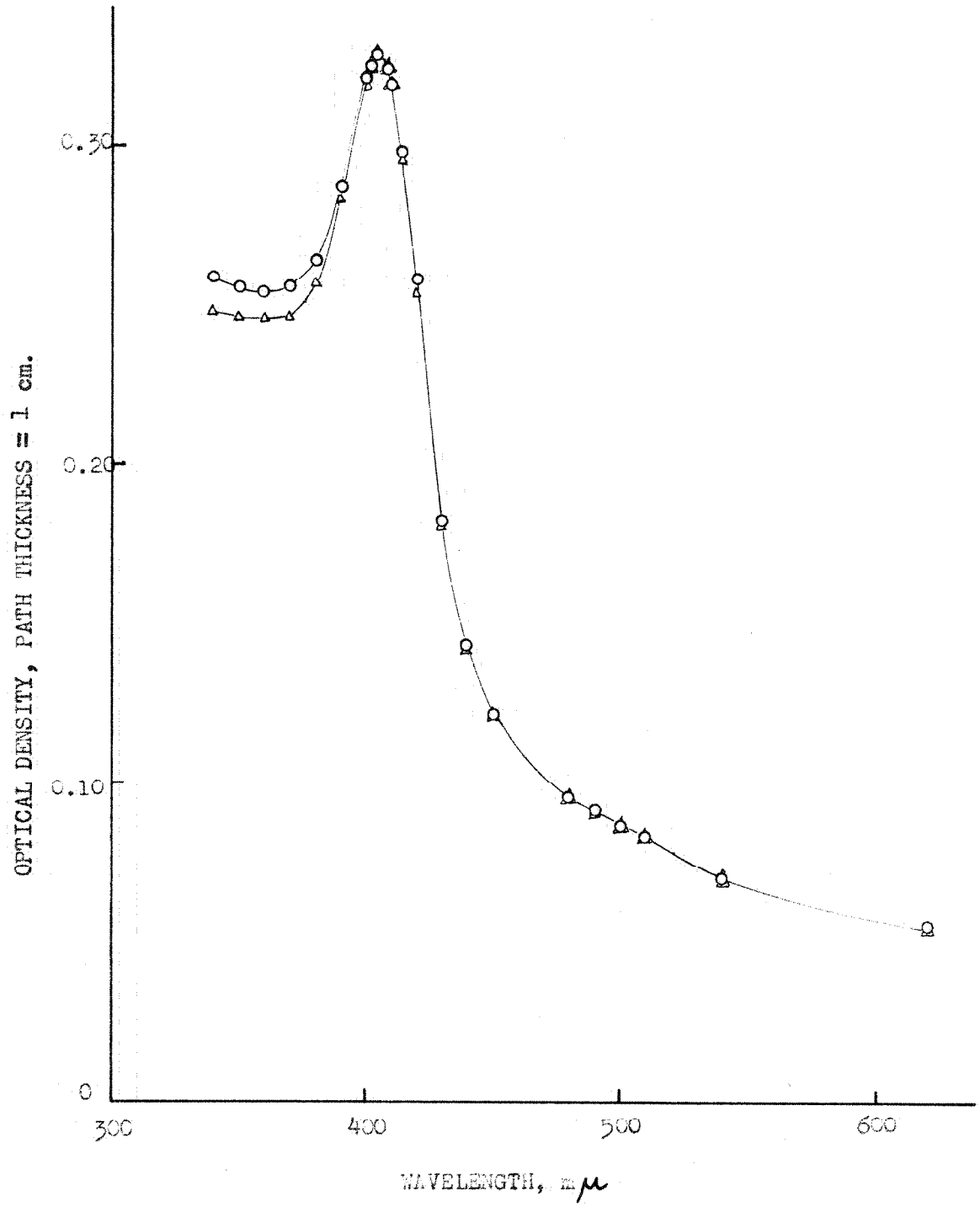
and that a given concentration of D.C.P. elicits approximately the same inhibition whether added directly or whether added in high concentration first then diluted out. It may be concluded that the inhibition of catalase by D.C.P. is reversible in character.

D.C.P. similarly was found to inhibit the catalase activity of pea epicotyl brei, the concentrations required being of the same order.

Catalase and the other heme enzymes show very characteristic absorption spectra. Additional information on the nature of the inhibition might be obtainable then by examining the absorption spectra in the presence and absence of inhibiting concentrations of D.C.P. Approximately 4×10^{-7}M catalase in M/60 phosphate buffer, pH 6.6 gave the curve shown by the circles in figure 12 using the Beckman model DU spectrophotometer. The same catalase concentration, containing 10^{-4}M D.C.P. gave the curve shown by the triangles. This concentration of D.C.P. alone gave no measurable absorption in this wavelength range. It is clear that there is no qualitative or quantitative change due to the presence of D.C.P. Probably the inhibition is due to interaction with the apoenzyme rather than with the heme prosthetic groups.

The Effect of D.C.P. on Other Heme Enzymes. D.C.P. was tested against several other heme systems namely peroxidase, cytochrome oxidase and haemoglobin for two reasons: (a) to determine whether the inhibition of catalase is specific or is also manifested against other heme enzymes, (b) since the

Figure 12.



O = 4 x 10^{-7}M beef liver crystalline catalase, pH 6.6
Δ = 4 x 10^{-7}M beef liver crystalline + 10^{-4}M DCP, pH 6.6.

best evidence indicates that the I.A.A. oxidase system contains a peroxidase component, it was important to ascertain whether the D.C.P. had any inhibitory or perhaps stimulatory effect on the peroxidase.

Brei from 6-day red-grown pea epicotyls diluted finally 1 in 2000 was used as the source of the peroxidase. In this range enzyme concentration was rate-limiting. Activity was determined by the method of Ponting and Joslyn (100) using guaiacol as a substrate and measuring the change in optical density with time at 420 mμ with a Coleman Junior spectrophotometer. Phenol, 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 3-nitrophenol, 2-carboxyphenol and 4-carboxyphenol at 10^{-4} M and 10^{-3} M and 2,4,6-trichlorophenol at 10^{-4} M and 4×10^{-4} M failed to produce any inhibition or stimulation. 2,4-dimethyl phenol at 10^{-3} M inhibited about 40% but was without effect at 10^{-4} M. However, it should be remembered that in these experiments H_2O_2 is present in excess while the concentration during the *in vitro* oxidation of I.A.A. is very low and probably rate-limiting. Existing methods do not permit the measurement of peroxidase activity under these conditions with crude preparations.

As a source of cytochrome oxidase mitochondria were prepared from 5-day red-grown mung bean (*Phaseolus aureus*) hypocotyls according to the procedure of Millerd *et al.* (107). Using 0.5 mg./ml. cytochrome C as substrate and 0.0125 M ascorbic acid to keep it in the reduced form the oxygen uptakes were determined in Warburg manometers at 30°C. D.C.P.

at 10^{-5} M or 10^{-4} M had no effect.

A hemoglobin solution was prepared by lysing with saponin the packed red cells of human blood and diluting 1 to 5 with water. By measuring the optical densities at 542 and 576 mμ the concentration was calculated to be 2.2×10^{-3} M from the known molar extinction coefficients (108). An aliquot of fully oxygenated hemoglobin was placed in the Van Slyke blood gas analysis apparatus with or without dichlorophenol. Dissolved gases were removed by evacuation and expulsion, and by re-evacuation the oxygenated hemoglobin was permitted partially to dissociate. After equilibrating against a measured oxygen tension the gas phase was expelled and the bound oxygen measured by liberating with ferricyanide. It was found that for a given partial pressure of oxygen in the gas phase, the concentration of bound oxygen was uninfluenced by D.C.P. in concentrations up to 2.8×10^{-3} M.

Thus of the four heme systems tested only catalase was inhibited by D.C.P. under these experimental conditions. Together with the observation that an inhibiting concentration of D.C.P. does not alter the visible absorption spectrum of crystalline catalase, this suggests that the D.C.P. does not interact with the heme prosthetic groups but rather with the apoenzyme of catalase. Further it permits one to understand that low concentrations of D.C.P. may in fact enhance I.A.A. oxidase and not inhibit, since the peroxidase moiety is not innibited.

The Effectiveness of D.C.P. Analogs. In order to obtain some

information on the manner in which D.C.P. might enter into the two biological systems which it affects so potently, the structural requirements for activity were examined by testing a series of analogs of D.C.P. against both I.A.A. oxidase and crystalline catalase.

For the I.A.A. oxidase experiments, tubes were loaded with 0.5 ml. M/15 phosphate buffer pH 6.6, 0.5 ml. undialyzed brei of dark-grown pea epicotyls, substance to be tested to give the final concentrations shown in table 3 and water to a volume of 1.5 ml. After 20 minutes' incubation 0.5 ml. 10^{-3} M I.A.A. was added and the tubes incubated 30 minutes in the dark at 25.5°C. This experiment was broken up into several consecutive experiments owing to the physical limitation of the number of treatments that can be made simultaneously. Activities are expressed as a percentage of that of the control in each case.

From table 3 it seems that the basic structure required for activity is a hydroxyl-substituted benzene ring (i.e., phenol), the activity of which may be increased or decreased by certain substituents. Cyclohexanol, 2-naphthol, 1-naphthol (even 2,4-dichloro-1-napthol), 4-pyridyl carbinol and p-benzoquinone have no stimulatory activity. Methylation of the phenol leads to loss of activity (2,4-dichloroanisole). Halogen substitution in the 2,4 or 6 positions increases both the maximum enhanceability and the sensitivity. While 3-chlorophenol and 2,5-dichlorophenol may produce maximum enhancement equal to that produced by 2,4-dichlorophenol, the

Table 3

The Effect of a Series of D.C.P. Analogs on I.A.A. Oxidase

Expt.‡ No.	Compound	Concentration, M	Activity*	Percent Activity	Maximum† Enhancement (percent)	Optimum† Concentration, M
1a.	control	----	51	100	----	----
	phenol	10^{-5}	85	167	143	10^{-4}
		3×10^{-5}	113	222		
		10^{-4}	124	243		
		3×10^{-4}	111	218		
		10^{-3}	75	147		
	2-chloro-phenol	10^{-5}	124	243	190	7×10^{-5}
		3×10^{-5}	140	274		
		10^{-4}	143	281		
		3×10^{-4}	111	218		
		10^{-3}	57	112		
	4-chloro-phenol	10^{-5}	115	226	190	3×10^{-5}
		3×10^{-5}	145	284		
		10^{-4}	138	270		
		3×10^{-4}	106	208		
		10^{-3}	61	119		
	2,4-dichloro-phenol	10^{-5}	143	280	185	2×10^{-5}
		3×10^{-5}	140	274		
		10^{-4}	131	256		
		3×10^{-4}	75	147		
		10^{-3}	20	38		

Footnotes: ‡Experiments 1a. to 1e. carried out consecutively on the same batch of enzyme from 7-day dark-grown pea epicotyls. Experiments 2 and 3 carried out on separate preparations from 9-day and 10-day respectively dark-grown epicotyls. A control with D.C.P. is included in each batch of enzyme for comparison.

* μM/l I.A.A. destroyed in 30 minutes' incubation in the dark at 25.5°C.

† Interpolated by plotting activity versus concentration data.

Table 3 (Continued)

The Effect of a Series of D.C.P. Analogs on I.A.A. Oxidase

Expt. No.	Compound	Concentration, M	Activity*	Percent Activity	Maximum Enhancement (percent)	Optimum Concentration, M
1b.	control	----	49	100		
	2,4-dinitrophenol	10^{-5}	49	100	0	
		$3x10^{-5}$	49	100		
		10^{-4}	48	98		
		$3x10^{-4}$	49	100		
		10^{-3}	48	98		
	2,4-dichloroanisole	10^{-6}	48	98	0	
		$3x10^{-6}$	48	98		
		10^{-5}	52	106		
	2,4-dichloro-1-naphthol	$7x10^{-6}$	41	84	0	
		$2x10^{-5}$	44	90		
		$7x10^{-5}$	48	98		
		$2x10^{-4}$	25	51		
	4-carboxyphenol	10^{-5}	51	104	> 190	$> 10^{-3}$
		$3x10^{-5}$	59	120		
		10^{-4}	87	178		
		$3x10^{-4}$	125	255		
		10^{-3}	144	294		

Table 3 (Continued)

The Effect of a Series of D.C.P. Analogs on I.A.A. Oxidase

Expt. No.	Compound	Concentration, M	Activity*	Percent Activity	Maximum Enhancement (percent)	Optimum Concentration, M
1c.	control	----	47	100		
	2,4,6-trichlorophenol	10^{-6}	55	117	202	5×10^{-5}
		3×10^{-6}	68	139		
		10^{-5}	115	245		
		3×10^{-5}	142	302		
		10^{-4}	127	270		
	2,4,6-triiodophenol	2×10^{-6}	67	143	198	3×10^{-5}
		7×10^{-6}	113	241		
		2×10^{-5}	140	298		
		7×10^{-5}	126	268		
		2×10^{-4}	72	153		
	2,3,5-triiodobenzoic acid	1.6×10^{-6}	47	100	0	
		4.8×10^{-6}	47	100		
		1.6×10^{-5}	47	100		
	4-chlorobenzoic acid	7.5×10^{-6}	51	100	0	
		2.5×10^{-5}	41	87		
		7.5×10^{-5}	47	100		
		2.5×10^{-4}	48	102		

Table 3 (Continued)

The Effect of a Series of D.C.P. Analogs on I.A.A. Oxidase

Expt. No.	Compound	Concentration, M	Activity*	Percent Activity	Maximum Enhancement (percent)	Optimum Concentration, M
1d.	control	----	47	100		
	4-bromophenol	10^{-5}	138	294	200	$3x10^{-5}$
		$3x10^{-5}$	141	300		
		10^{-4}	135	287		
		$3x10^{-4}$	109	232		
		10^{-3}	70	149		
	3-nitrophenol	10^{-5}	43	92	> 49	$>10^{-3}$
		$3x10^{-5}$	44	94		
		10^{-4}	45	96		
		$3x10^{-4}$	60	128		
		10^{-3}	70	149		
	2,4-dimethyl phenol	10^{-5}	44	94	inhib.	
		$3x10^{-5}$	8	17		
		10^{-4}	0	0		
		$3x10^{-4}$	0	0		
		10^{-3}	0	0		

Table 3 (Continued)

The Effect of a Series of D.C.P. Analogs on I.A.A. Oxidase.

Expt. No.	Compound	Concentration, M	Activity*	Percent Activity	Maximum Enhancement (percent)	Optimum Concentration, M
1e.	control	- - -	45	100		
	pyridyl-4-carbinol	10^{-5}	45	100	- - -	
		$3x10^{-5}$	45	100		
		10^{-4}	46	102		
		$3x10^{-4}$	45	100		
		10^{-3}	47	104		
	2-carboxy phenol	10^{-5}	44	98	- - -	
		$3x10^{-5}$	48	106		
		10^{-4}	46	102		
		$3x10^{-4}$	44	98		
		10^{-3}	47	104		
	4-phenyl phenol	$2x10^{-6}$	50	111	67	10^{-5}
		$7x10^{-6}$	72	160		
		$2x10^{-5}$	73	162		
		$7x10^{-5}$	42	93		
		$2x10^{-4}$	15	33		
	2-methoxy phenol	10^{-5}	11	24	inhib.	
		$3x10^{-5}$	5	11		
		10^{-4}	-1	-2		
		$3x10^{-4}$	1	2		

Table 3
(Continued)

The Effect of a Series of D.C.P. Analogs on I.A.A. Oxidase

Expt. No.	Compound	Concentration, M	Activity*	Percent Activity	Maximum Enhancement (percent)	Optimum Concentration, M
2.	control	---	19	100		
	2,4-dichlorophenol	10^{-5}	128	674	772	3×10^{-5}
		3×10^{-5}	146	769		
		10^{-4}	113	595		
		3×10^{-4}	55	289		
		10^{-3}	4	21		
	3-chlorophenol	10^{-5}	24	126	>900	$>10^{-3}$
		3×10^{-5}	42	221		
		10^{-4}	94	495		
		3×10^{-4}	146	769		
		10^{-3}	169	890		
	2,5-dichlorophenol	10^{-5}	40	210	874	4×10^{-4}
		3×10^{-5}	69	363		
		10^{-4}	134	705		
		3×10^{-4}	165	870		
		10^{-3}	125	658		
3.	control	---	80	100		
	2,4-dichlorophenol	3×10^{-5}	144	180	80	
	cyclohexanol	10^{-6}	80	100	0	
		10^{-5}	80	100		
		10^{-4}	79	99		
	p-benzoquinone	10^{-6}	40	50	inhib.	
	1-naphthol	10^{-5}	0	0	inhib.	
		10^{-6}	72	90		
	2-naphthol	10^{-5}	0	0	inhib.	
		10^{-6}	82	102		

concentrations required are much greater. 4-phenyl, 4-carboxy and 3-nitrophenols have some activity but less than that of phenol itself. 2,4-dinitro- and 2-carboxy- phenols are inactive.

An interesting point, to which reference will be made later, is that of all the phenols tested, with the exception of 2,4-dinitrophenol and 2-carboxyphenol, those which are not stimulatory are actually inhibitory. Further, as far as the study has been carried, all compounds which enhance at low concentrations also inhibit at higher concentrations. The possible significance of these observations is discussed on page 69.

Table 4 shows the effect on some members of the same phenol series on the activity of crystalline beef liver catalase. The catalase solution was incubated at 0°C. with the phenol, and the peroxide was tipped in at zero time. Velocity constants were determined by the rapid titrimetric method of Bonnichsen, Chance and Theorell (105). A striking correspondence may be seen between the ability of a phenol to inhibit catalase and its stimulating effect on I.A.A. oxidase. Exceptions are 2,4-dinitrophenol which inhibits catalase slightly without having any effect on I.A.A. oxidase, phenol which stimulates I.A.A. oxidase without being inhibitory to catalase and 2,4-dimethyl phenol which inhibits catalase and also inhibits I.A.A. oxidase. The last compound, however, was also inhibitory to the pea epicotyl peroxidase.

An interesting correlation observed by Dr. Galston is

Table 4

The Inhibition of Crystalline Beef Liver Catalase by a Series of Phenols

Reaction mixture contains 25 ml. 0.1 M phosphate buffer, pH 6.9, 0.25 ml. of the phenol solution, 0.25 ml. 1:1000 crystalline catalase solution and after incubation at 0°C, 1.0 ml. 0.1 N H_2O_2 tipped in. Rapid titrimetric method of Bonnichsen, Chance and Theorell (105).

Expt. No.	Compound	Concentration, M	Catalase k, min^{-1}	Percent Activity
1.	control	---	0.104	100
	phenol	10^{-3}	0.101	97
	2-chlorophenol	10^{-3}	0.024	23
	4-chlorophenol	10^{-3}	0.031	30
	2,4-dichlorophenol	10^{-5}	0.047	45
		10^{-4}	0.022	21
		10^{-3}	0.007	7
	2,4,6-trichlorophenol	2.5×10^{-4}	0.086	84
	2,4-dinitrophenol	10^{-5}	0.104	100
		10^{-4}	0.084	81
2.	control	---	0.148	100
	3-nitrophenol	10^{-5}	0.136	92
		10^{-4}	0.104	70
	2,4-dimethylphenol	10^{-5}	0.116	78
		10^{-4}	0.087	59
3.	control	---	0.092	100
	2-carboxyphenol	10^{-5}	0.092	100
		10^{-4}	0.085	92
	4-carboxyphenol	10^{-5}	0.084	91
		10^{-4}	0.044	48
	4-phenylphenol	10^{-5}	0.087	94
4.	control	---	0.261	100
	3-chlorophenol	10^{-5}	0.235	90
		10^{-4}	0.092	32
	2,5-dichlorophenol	10^{-5}	0.160	69
		10^{-4}	0.041	15

that the order of activity in enhancing I.A.A. oxidase roughly follows the strength of smell in the phenolic series.

Separation of I.A.A. oxidase and catalase. The following observations have already been presented. Catalase inhibits I.A.A. oxidase. D.C.P. greatly enhances the activity of I.A.A. oxidase at 10^{-6} to 10^{-4} M (figure 6). D.C.P. strongly and specifically inhibits catalase (from beef liver or from pea epicotyls) at 10^{-6} M to 10^{-4} M (figure 11). There is a good though not complete, correspondence between the enhancement of I.A.A. oxidase and the inhibition of catalase by a series of phenols (tables 3 and 4). This evidence suggests that the increase in I.A.A. oxidase activity elicited by D.C.P. may be due to its inhibition of the endogenous catalase activity. However two experiments clearly remain to be done, namely, (a) to separate the catalase from the I.A.A. oxidase of the brei and determine whether the basal activity rises and whether the D.C.P.-enhanceability is lost, and (b) to determine the endogenous catalase velocity constant and to compare it with that of added catalase required to produce a given inhibition. In this way a calculation may be made of the percentage stimulation to be expected if all the endogenous catalase were inhibited by D.C.P.

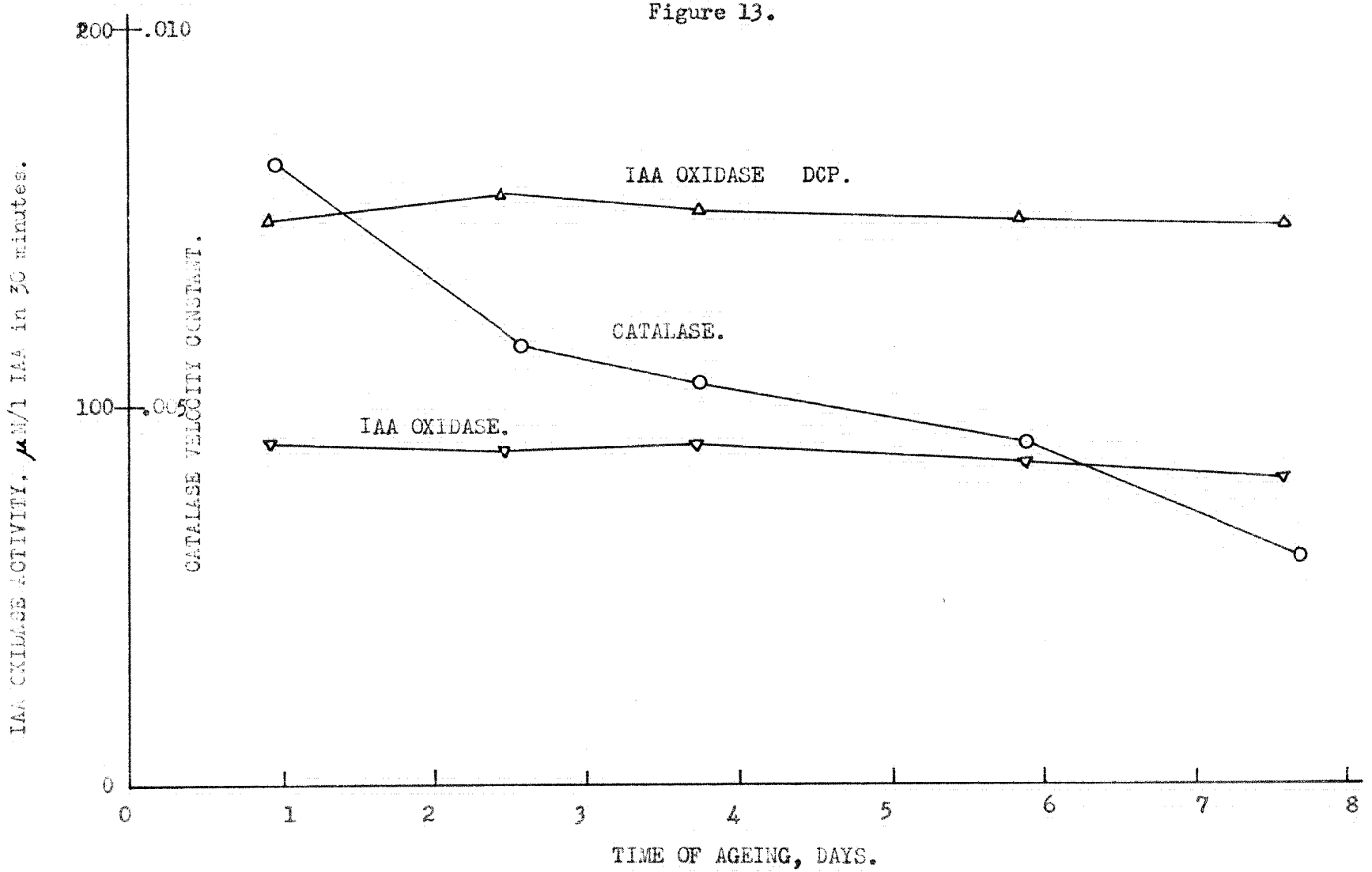
Unlike animal catalase, pea epicotyl catalase is rather labile. During storage in the crude state it loses activity much faster than does I.A.A. oxidase. Thus aging produces a differential change in the concentration of these two enzymes. Epicotyls from 7-day dark-grown peas were blended in a minimum volume of M/60 phosphate buffer, pH 6.6 and

permitted to age under toluene at 0 to 25°C. Treatment with toluene had no effect on the I.A.A. oxidase activity. At time intervals 1 ml. aliquots were withdrawn for assay of the catalase velocity constant per ml. (by the method of Von Euler and Josephson, 109). At the same time intervals 0.5 ml. aliquots were used to determine the I.A.A. oxidase activity at zero and at optimal D.C.P. concentration. Figure 13 shows that the catalase activity decreased by 50% in 6 days. However the basal I.A.A. oxidase activity and its maximum enhanceability by D.C.P. (which consistently occurred at 2 x 10^{-5}M) remained constant over this period, i.e., these two values were independent of the endogenous catalase level. This would suggest that the D.C.P. is not acting by inhibiting the endogenous catalase.

In a subsequent experiment a less equivocal separation of the I.A.A. oxidase and catalase activities was effected. It was found that the I.A.A. oxidase activity in etiolated pea epicotyls residues entirely in the soluble fraction. If precautions are taken to preserve the integrity and osmotic well-being of the cytoplasmic particles, the catalase activity is entirely particle-bound, principally in the fraction behaving ultracentrifugally as mitochondria (see table 5).

One hundred g. 9-day dark-grown pea epicotyls were cut, cooled to 0 to 5°C. and ground for a few minutes in a cold mortar with sand and an equal weight of 0.5 M sucrose. After straining through muslin and centrifuging for five minutes at 5000 g. to remove the sand and cell debris the volume of "whole

Figure 13.



The effect of ageing pea epicotyl brei on the activities of catalase and IAA oxidase and on the maximum enhanceability of IAA oxidase by DCP.

brei" was 144 ml. and pH 6.2. This was centrifuged in the cold at 20,000 g. for 30 minutes to bring down the "mitochondrial" fraction. The mitochondria were washed with 0.5 M sucrose and resuspended in 0.5 M sucrose so as to duplicate their original concentration in the whole brei. The supernatant was centrifuged in the refrigerated "Spinco" supercentrifuge at 50,000 g. for 120 minutes to bring down the "microsome" fraction. The pellet was similarly resuspended in a volume of sucrose such as to restore its concentration to that originally in the whole brei. The three fractions were then assayed for catalase activity, and for I.A.A. oxidase activity. Table 5 shows that all of the I.A.A. oxidase activity but none of the catalase activity resides in the soluble fraction. The effect of D.C.P. was then tested on this catalase-free fraction. Table 6 shows that removal of the endogenous catalase does not destroy the ability of D.C.P. to increase the I.A.A. oxidase activity

It must be concluded that the endogenous catalase activities of whole breis are not great enough to produce a significant inhibition of the I.A.A. oxidase. Figure 14 shows residual I.A.A. oxidase activity as a function of the velocity constant of added crystalline catalase in the reaction mixture. It is seen that in order to produce 50% inhibition (i.e., a potential 100% stimulation) the velocity constant must be approximately 3.0 per minute, whereas that in the pea breis, after dilution in the reaction mixture is of the order of 0.04 per minute.

Table 5

The distribution of catalase and I.A.A. oxidase activities in pea epicotyl brei.

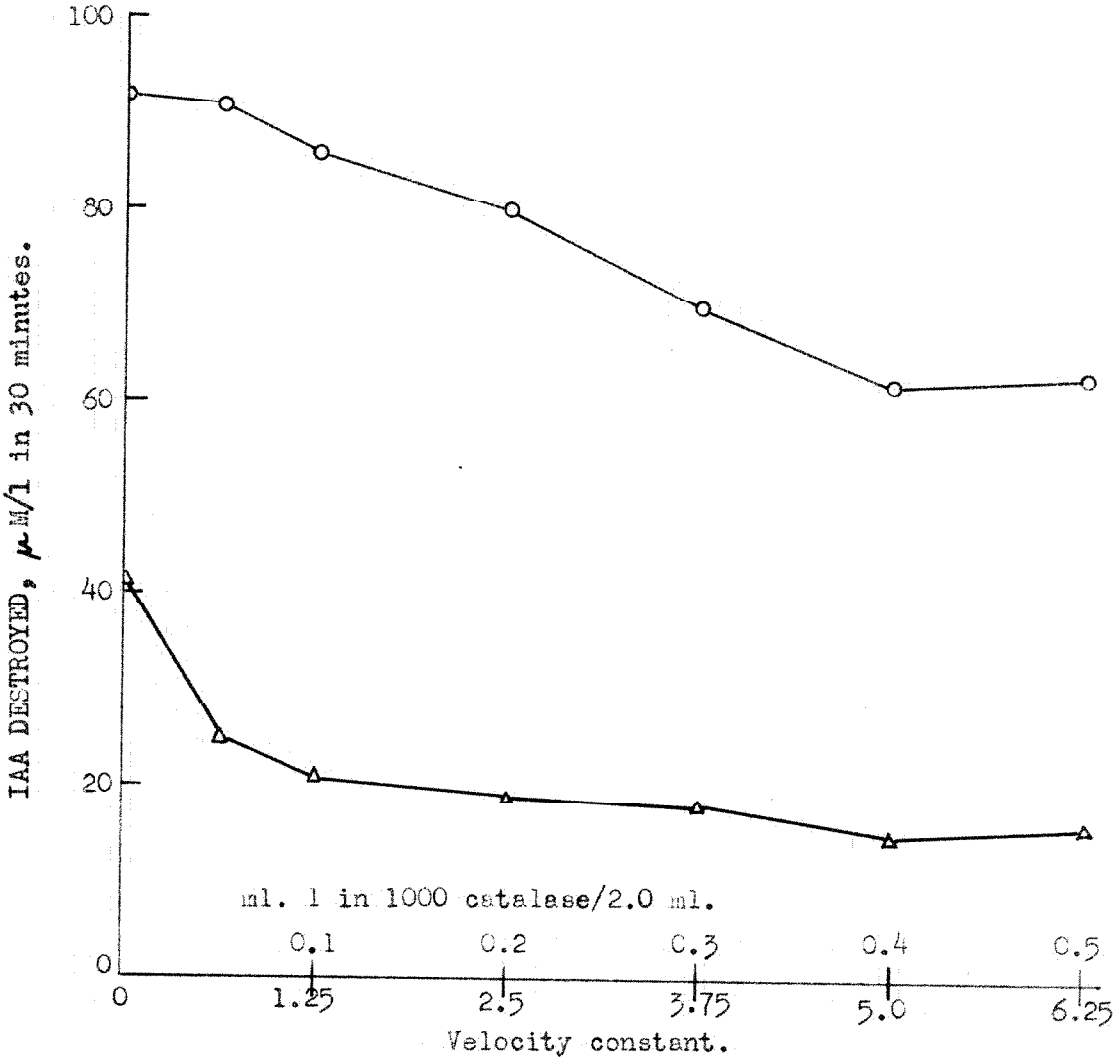
Fraction	I.A.A. oxidase activity (μM/l/30 mins./ml.)	Catalase activity ($min.^{-1}$/ml.)	Protein N (γ/ml.)
Soluble	35	0	129
Mitochondria	0	0.0092	74
Microsomes	0	0.0034	83

Table 6

The effect of D.C.P. on the I.A.A. oxidase of the catalase-free "soluble fraction" prepared by ultracentrifugation.

Concentration of D.C.P.	Activity (μM/l/30 mins.)
0	33
$3x10^{-4}$ M	72
$3x10^{-5}$ M	146
$3x10^{-6}$ M	112

Figure 14.



CATALASE CONCENTRATION IN REACTION MIXTURE.
The effect of catalase activity on the inhibition of IAA oxidase. Stock crystalline beef liver catalase, k = 25,000 per min. per ml. Initial IAA concentration = 250 μM/1 Temperature = 25°C, in dark.

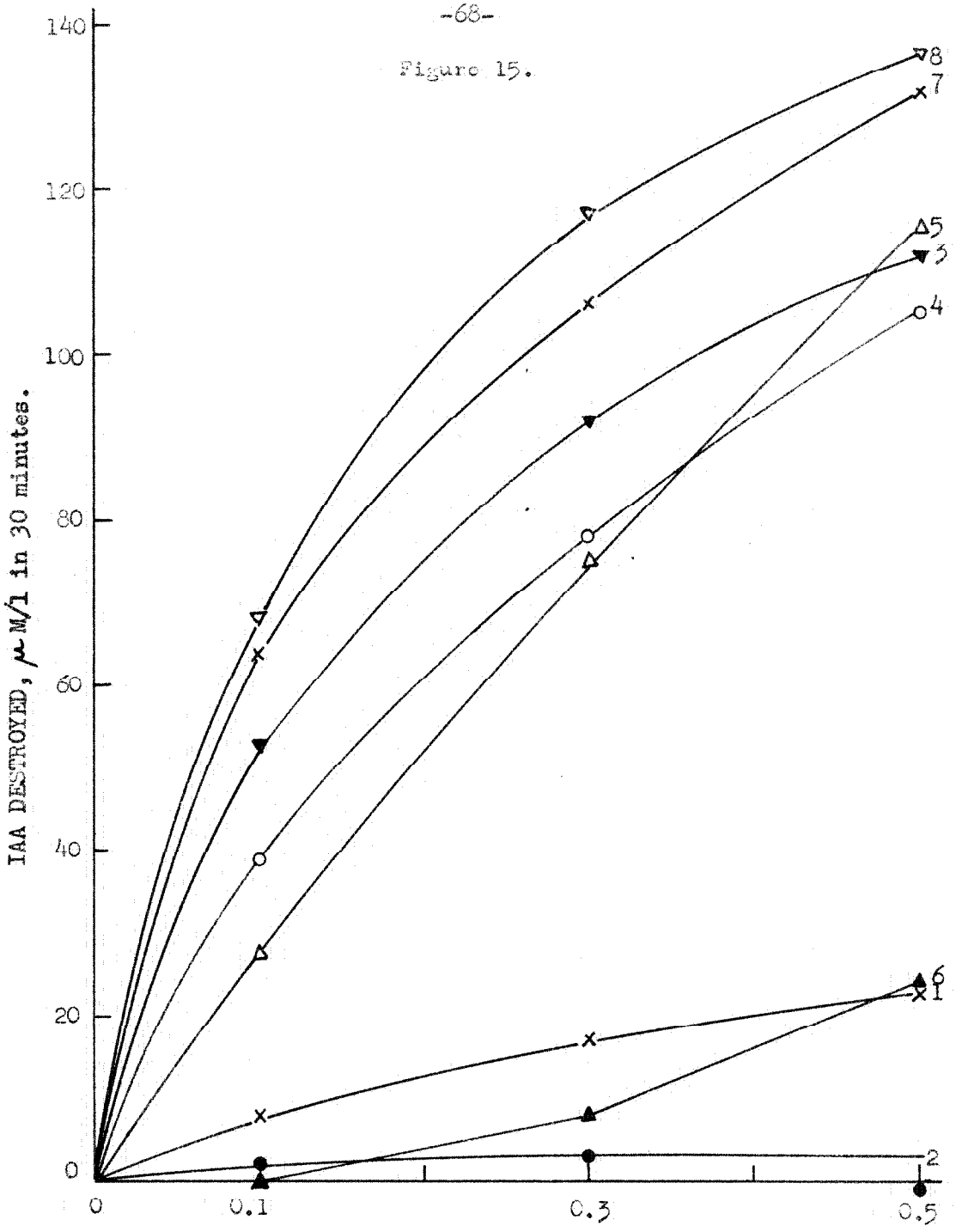
From tne experiments described in this section it may be concluded that D.C.P. does not stimulate I.A.A oxidase by inhibiting the endogenous catalase. However, as indicated in a previous section, D.C.P. seems to act so as to make H_2O_2 more available for the oxidation of I.A.A. This is perhaps brought about by action on the peroxide-producing moiety.

Does D.C.P. Act as a Cofactor? The possibility remains that D.C.P. increases I.A.A. oxidase activity by duplicating the action of the natural cofactor. While no direct decisive experiment has been made, a number of observations do substantiate this notion.

Dialysis of whole brei removes a cofactor and reduces the activity. Although the residual activity may be a small fraction of tne activity of whole brei, the final elevated activities in the presence of D.C.P. are approximately the same whether the brei is dialyzed or undialyzed (figure 15). Thus the activation is not mediated through the cofactor-independent enzyme but rather it appears that D.C.P. is in some way substituting for the natural cofactor, but in a more effective way. Consistent with this interpretation are the observations that breis from dark-grown epicotyls lose on dialysis a greater fraction of their activity than do breis from red-grown epicotyls and that dark-grown brei is relatively more enhanceable by D.C.P. than is red-grown brei (figure 9).

Table 3 shows that a large number of phenolic compounds behave similarly to D.C.P. If they are acting as cofactors of

Figure 15.



The effect of exposure of the pea seedlings to light and dialysis of the brei on the DCP-enhancement of IAA oxidase activity.
Initial IAA concentration = 250 μM/l. Run in darkness at 25°C.
Curve 1 whole dark-grown brei.
Curve 2 dialyzed dark-grown brei.
Curve 3 whole dark-grown brei + 3 x 10^{-5}M DCP.
Curve 4 dialyzed dark-grown brei + 3 x 10^{-5}M DCP.
Curve 5 whole red-grown brei.
Curve 6 dialyzed red-grown brei.
Curve 7 whole red-grown brei + 3 x 10^{-5}M DCP.
Curve 8 dialyzed red-grown brei + 3 x 10^{-5}M DCP.

greater or lesser efficacy, there probably is some structural feature which they have in common with the natural cofactor. Thus it is not surprising that of the substituted phenols listed in table 3 with the exception of 2,4-dinitrophenol and 2-carboxyphenol those compounds which do not stimulate actually inhibit the I.A.A. oxidase activity of the whole brei. On the cofactor hypothesis this would be interpreted to mean that although inhibiting phenols have the structural requirements for approaching the enzyme site, they are ineffective there and block the action of the natural cofactor. It is interesting in this connection that as far as the concentration ranges have been extended, those compounds which stimulate at low concentrations also inhibit at higher concentrations (table 3).

It would clearly be a desirable project to isolate and identify the natural cofactor.

In Vivo Effect of D.C.P. on I.A.A. Destruction. It is of some interest to determine whether the I.A.A. oxidase activities of tissues can be artificially raised by treatment with D.C.P., and if so to observe the physiological consequences of this treatment.

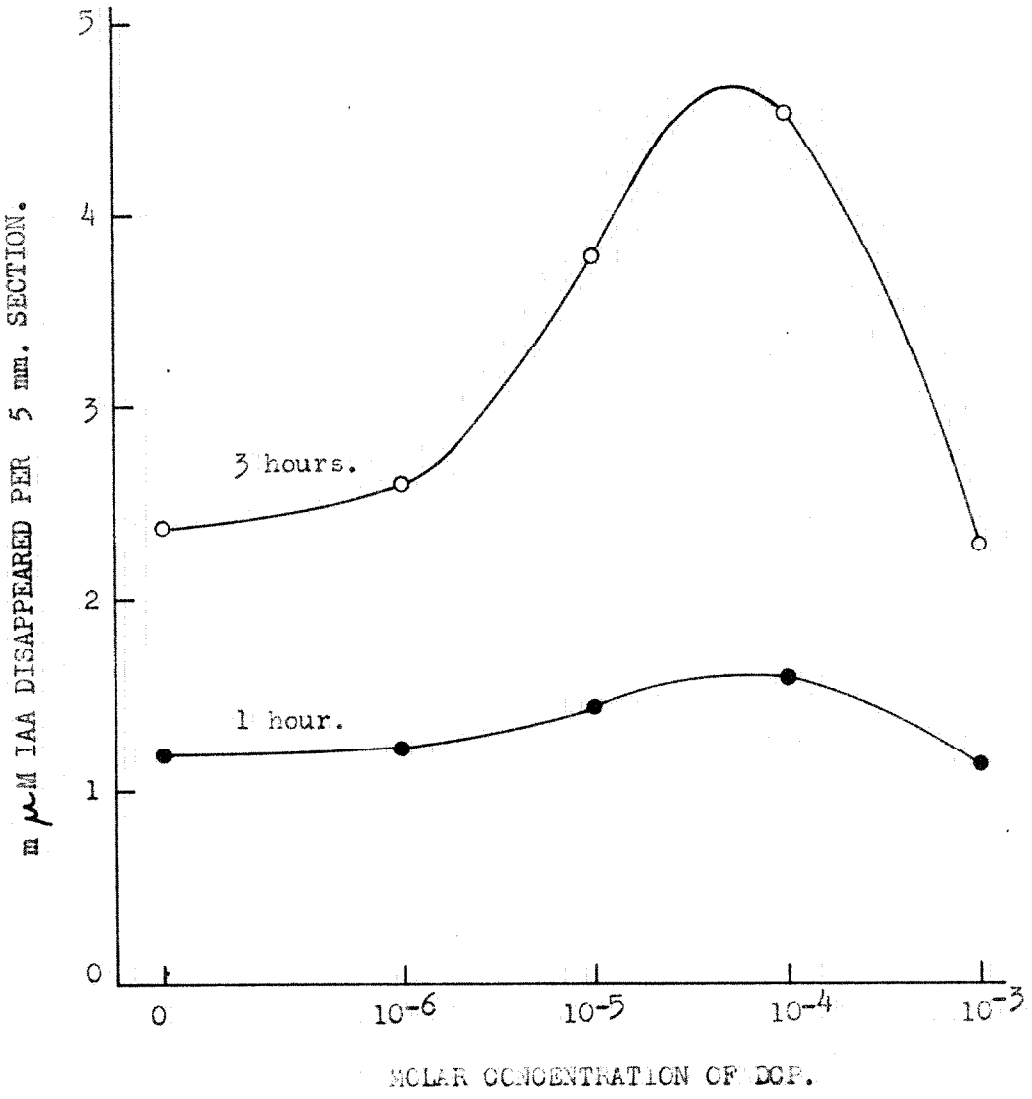
Pea seedlings were grown for seven days in weak red light at 26°C. The apical 5 mm. of the stems were discarded and three successive 5 mm. sections were cut, washed in distilled water and blotted dry. Eighty sections (approximately 1.3 g. wet weight) were placed in each of five 50 ml. conical flasks containing 2.0 ml. M/60 phosphate buffer pH 6.6, D.C.P.

to give the final concentrations shown and water to a volume of 4.0 ml. Sections were gently shaken in these solutions for 10 minutes in the dark, then, at zero time 1.0 ml. 10^{-3}M I.A.A. was added. Figure 16 shows that D.C.P., between 10^{-5}M and 10^{-4} M in the external solution, doubled the rate of disappearance from solution of I.A.A. in three hours. Thus D.C.P. is able to penetrate into the cell relatively rapidly and stimulate the activity of I.A.A. oxidase in situ. As in the case of the free enzyme the optimum is below 10^{-4} M and strong inhibition occurs at higher concentrations.

Similar experiments were conducted with tissues from other species, namely hypocotyl sections of etiolated bean seedlings, leaves of dark-grown, red-grown or green oat seedlings and leaf discs of mature green corn. Figures 17 and 18 show these results. I.A.A. was destroyed by all these tissues. In all cases higher concentrations of D.C.P.(10^{-3}M) inhibited the rate of I.A.A. destruction. Only in the pea and in the bean tissue was there an increase in the rate of destruction at lower concentrations. Similarly, breis from etiolated oat coleoptiles or leaves have very low activities whether dialyzed or not and although 10^{-3} M D.C.P. inhibits, there is no enhancement at lower concentrations of D.C.P.

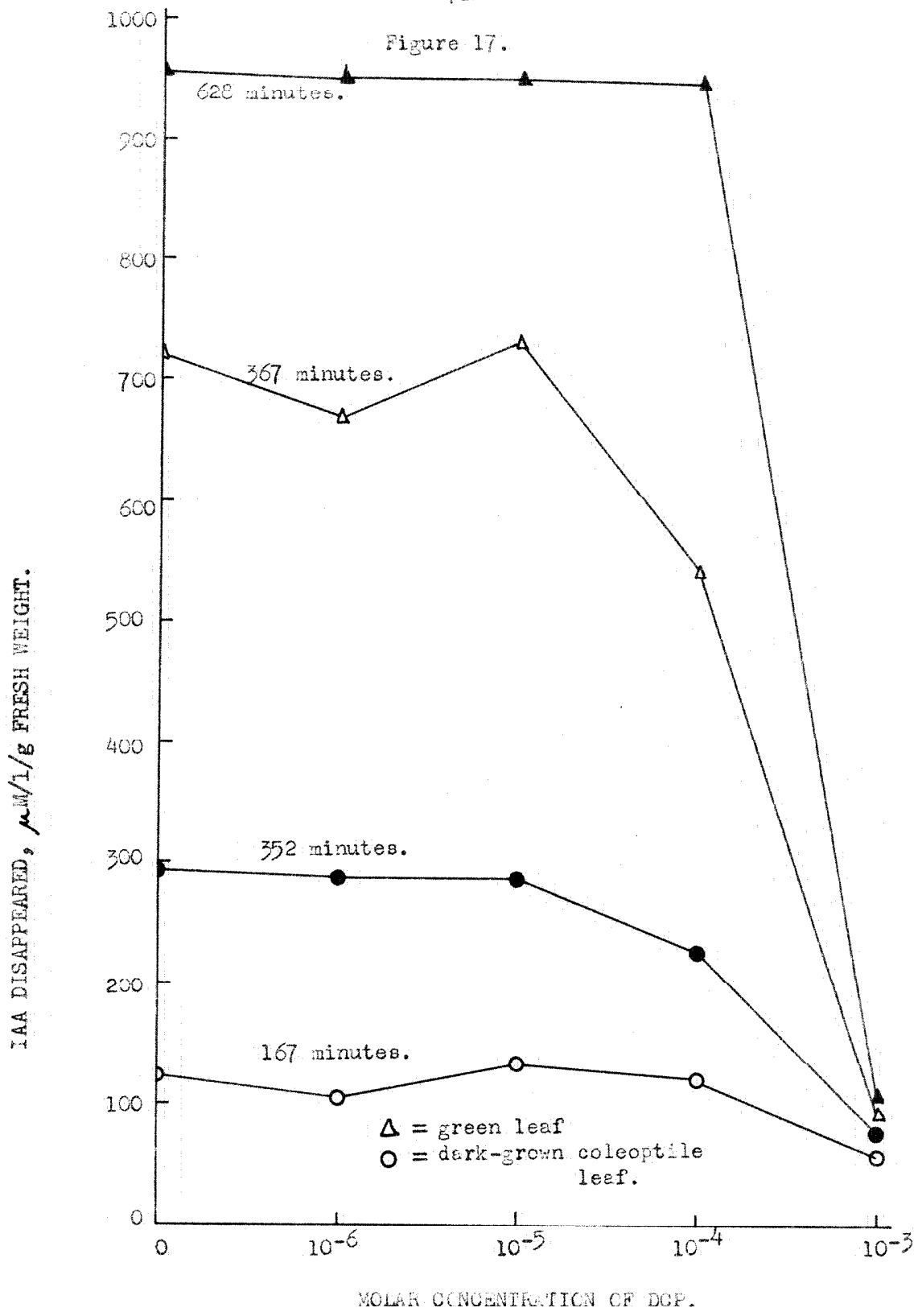
No generalizations can be made from these results but the observation is sufficiently interesting to warrant a future investigation to determine whether this increased destruction of I.A.A. induced by D.C.P. occurs only in dicotyledonous tissues. If this is the case, it may in part explain the

Figure 16.



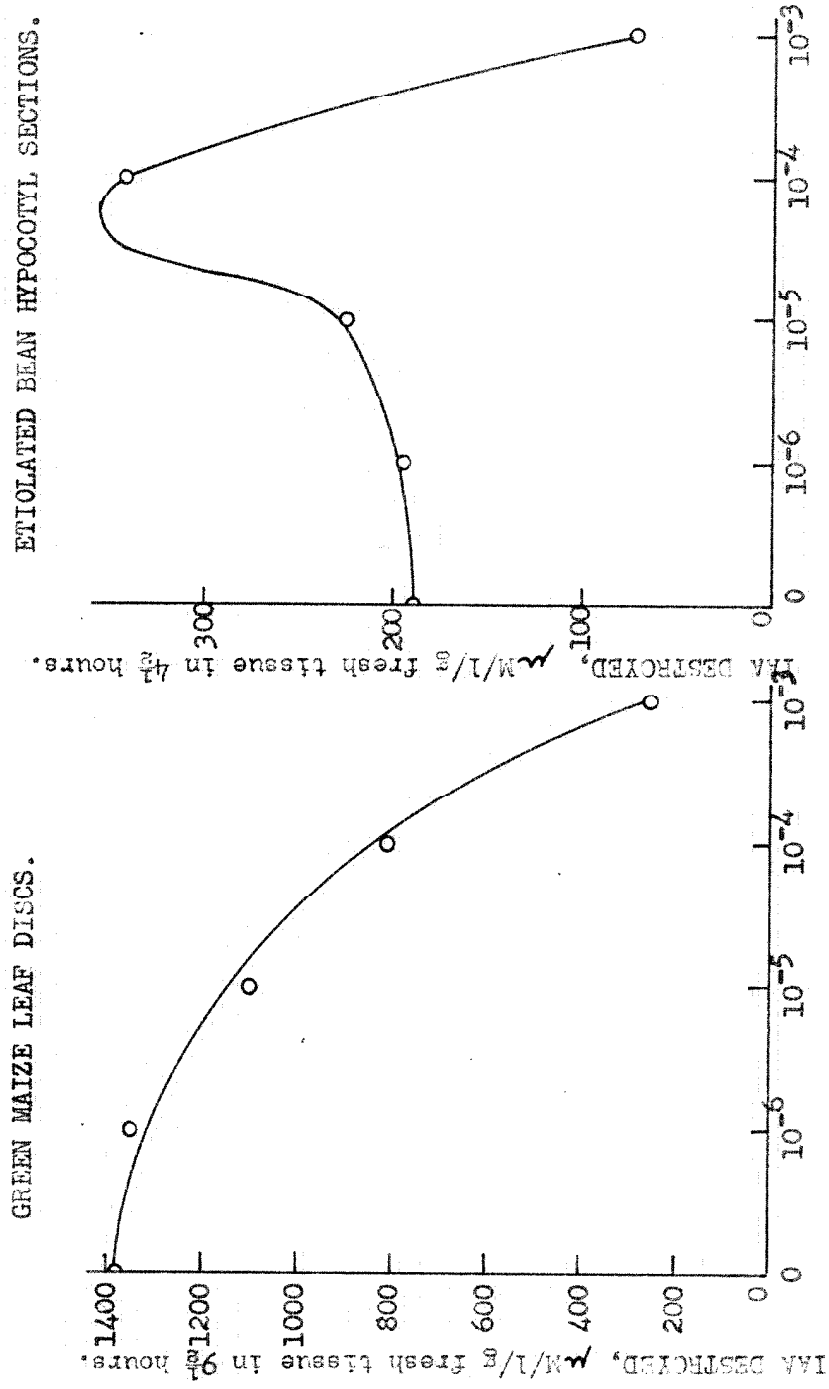
The effect of DCP on the in vivo destruction of IAA by pea stem sections. Initial IAA concentration = 200μM, pH 6.6, temperature = 25.5°C. dark. Eighty pea stem sections in 5 ml. solution.

Figure 17.



The effect of DCP on the destruction of IAA by leaf tissue of dark-grown and green Avenas. Assayed in dark, with shaking, at 25.5°C.

Figure 18.



The effect of DCP on the destruction of IAA by tissues of maize and of bean.

differential response between monocotyledons and dicotyledons to 2,4-dichlorophenoxyacetic acid, which is frequently contaminated with D.C.P. or which may be partly converted to D.C.P. in the plant (110, 111).

Summary. In this section, studies into the structure and properties of the enzyme complex referred to as "I.A.A. oxidase" have been extended and an attempt has been made to resolve some of the points of variance in the literature.

It was shown that the enzyme prepared from epicotyls of peas grown in darkness or low intensity red light has a partial, though not complete cofactor dependence. It was suggested that there are two or more alternative mechanisms for the breakdown of I.A.A. or a degradation product from it.

Altnough manganous ions have been variously reported to inhibit or to increase the activity of the enzyme, it was found here that with whole breis an inhibition was obtained and with dialyzed breis, an enhancement.

An earlier reported effect that 2,4-D enhanced the activity of I.A.A. oxidase was found to be due to a trace contamination with 2,4-dicnloropnenol (D.C.P.). The stimulatory effect of D.C.P. on the enzyme was studied in detail. Although a number of observations strongly suggested that the mechanism of the effect was through the inhibition, by D.C.P., of endogenous catalase, which was known to inhibit I.A.A. oxidase, it was shown that this was not the reason for the enhancement. The most probable explanation is that D.C.P. acts in a manner similar to the natural cofactor but that it does so

more effectively. The structural requirements for activity were studied from a series of related compounds.

The strong reversible inhibition of crystalline catalase by D.C.P. has been studied. Half-maximum inhibition occurs at 2×10^{-6} M D.C.P. Other heme enzymes, peroxidase, cytochrome oxidase and hemoglobin are not inhibited by low concentrations of D.C.P. The effectiveness of a series of substituted phenols in inhibiting catalase roughly parallels their effectiveness in increasing the activity of I.A.A. oxidase.

D.C.P. was found to enhance the *in vivo* destroying activity of pea and bean stem sections.

C. I.A.A.-photolysing Agents of the Dialysate.

Earlier workers (79, 86) have reported that dialysis of whole brei of pea epicotyls grown in weak red light led to a substantial increase in the I.A.A. oxidase activity, due to the removal of a naturally-occurring inhibitor. It was also observed (86, 90) that the activity of whole brei was enhanced to a greater extent by light than was that of dialyzed brei, and the suggestion was made that light accomplished this effect by "removing" or overcoming the effect of the natural inhibitor.

Wagenknecht and Burris (80), however, found that about one third of the activities of homogenates of bean roots or etiolated pea epicotyls was lost upon dialysis and that the original activities were restored on adding 10^{-3} M manganese.

The ash was inactive in restoring the activity and the whole dialyzate was not tested.

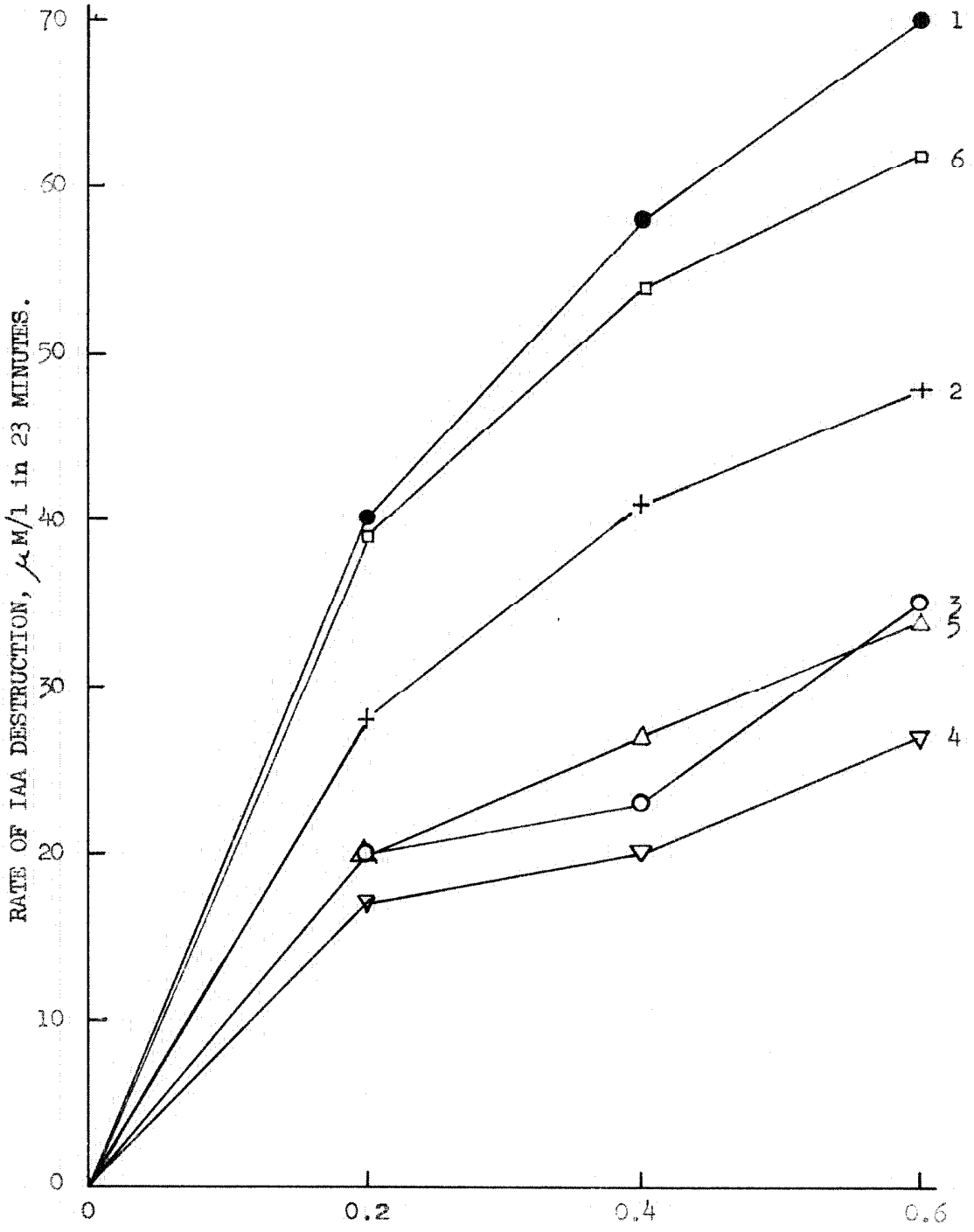
As already discussed in section B, in the present series of experiments it was consistently found that dialysis diminished the rate of destruction of I.A.A. by breis from epicotyls of dark-grown or red-grown pea seedlings. The loss was fractionally much greater when tested in the light than in the dark, that is, light enhanced the activity of whole brei more strongly than that of dialyzed brei. Adding back the concentrated dialyzate restored the original activity. However, in addition it was found that the dialyzate alone had a high capacity for destroying I.A.A. These observations are illustrated in the following experiment.

Epicotyls of eight-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 mls. brei were placed in each of three cellophane dialysis bags and dialyzed at 0 to 2° C for 16 hours against 10 ml., 50 ml, and two changes each of 750 ml. M/60 phosphate buffer, pH 6.6. As control, 10 ml. brei placed in a test tube was also mounted on the shaker. After dialysis the volumes were all adjusted to 11.0 ml. The 50 ml. dialyzate was frozen and the water removed by vacuum distillation, the final volume being adjusted to 11.0 ml. Residual activities of the dialyzed breis were determined in the light and compared with that of the un-dialyzed brei that of "whole" brei reconstituted by adding back the dialyzate to the equivalent of its original concen-

tration and that of the dialyzate alone. Figure 19 shows that: (1) dialysis leads to marked loss of I.A.A.-photolyzing activity, (2) the lost activity is quantitatively recovered in the dialyzate, (3) the dialyzate itself has independent activity, amounting to 60 to 65% of the activity of the whole brei in the light. As will be shown in a subsequent section, this fraction has no activity in the dark.

This section of this thesis deals with the properties of the components of the dialyzate active in destroying I.A.A. Destruction of the Biological Activity of I.A.A. It is of interest to determine whether the product of the destruction of I.A.A. is devoid of biological activity. Biological activity was assayed-for by measuring the growth rate of pea epicotyl sections. Dialyzate was prepared by dissolving 1g. lyophilized pea epicotyl brei in 5 ml. M/15 phosphate buffer, pH 6.1 and dialyzing overnight at 0 to 2°C. against 15 ml. phosphate buffer. Four series of petri dishes were loaded with 2% sucrose, M/100 phosphate buffer, pH 6.1, a range of I.A.A. concentrations, a 1 in 20 dilution of the above dialyzate in two of the series and water to a total volume of 20 mls. About sixteen pea epicotyl sections from dark-grown plants were floated in each dish. Two series, one with dialyzate and one without were incubated on a shaker for ten hours in the dark at 24°C. and a similar two series were placed on a shaker six inches below a bank of three 40 watt white fluorescent lamps, with a fan blowing laboratory air (24°C) over them. Figure 20 shows that, as previously reported (82),

Figure 19.



Concentration of Active Material, ml/2.0 ml.

(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 I.A.A. concentration = 250 μM/l, Temp. = 26.5°C.

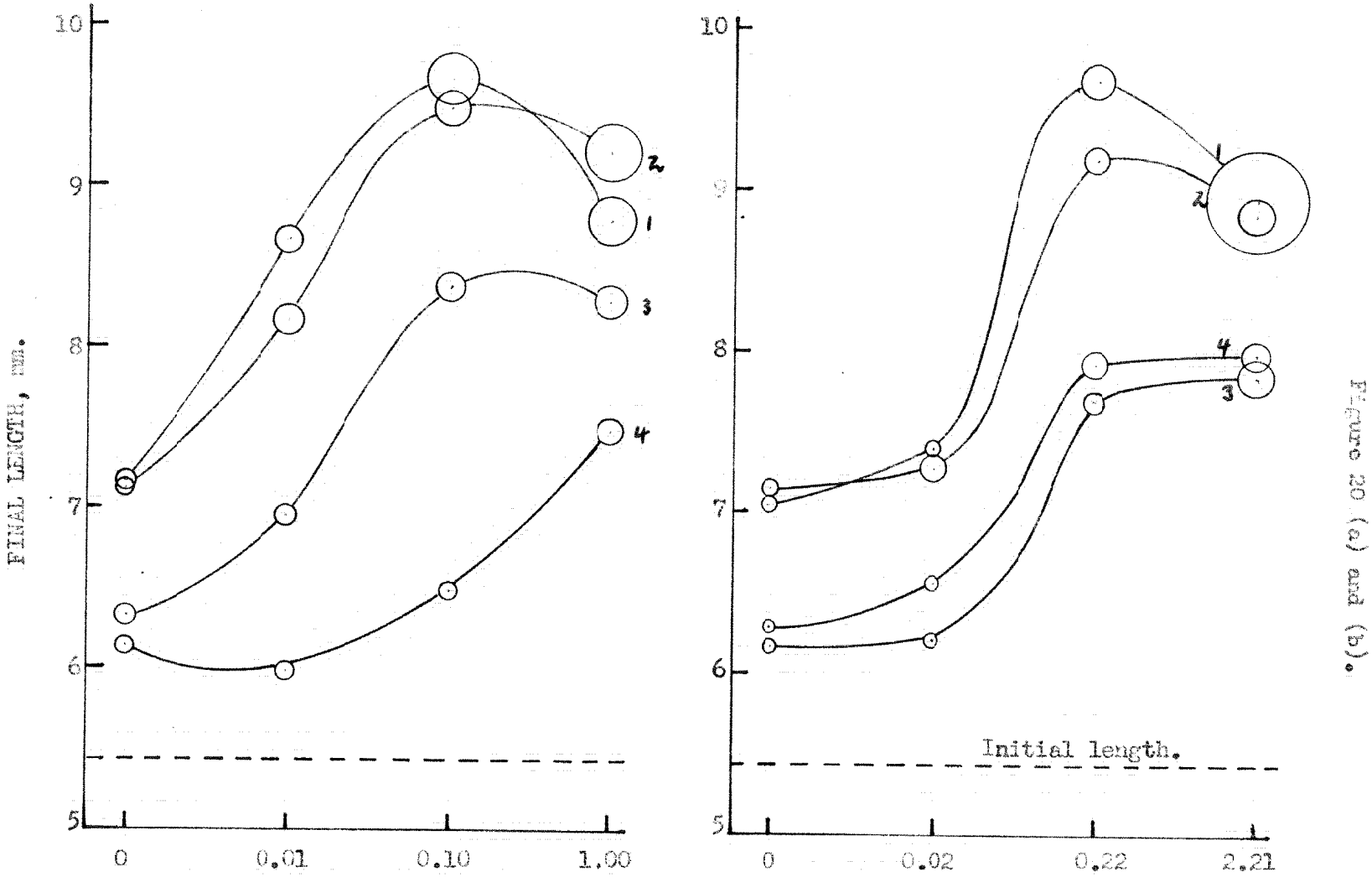


Figure 20 (a) and (b).

The effect of the dialyzate 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 dialyzate; 2 dark, dialyzate; 3 light, no dialyzate; 4 light, dialyzate.
Radii of circles indicate standard errors.

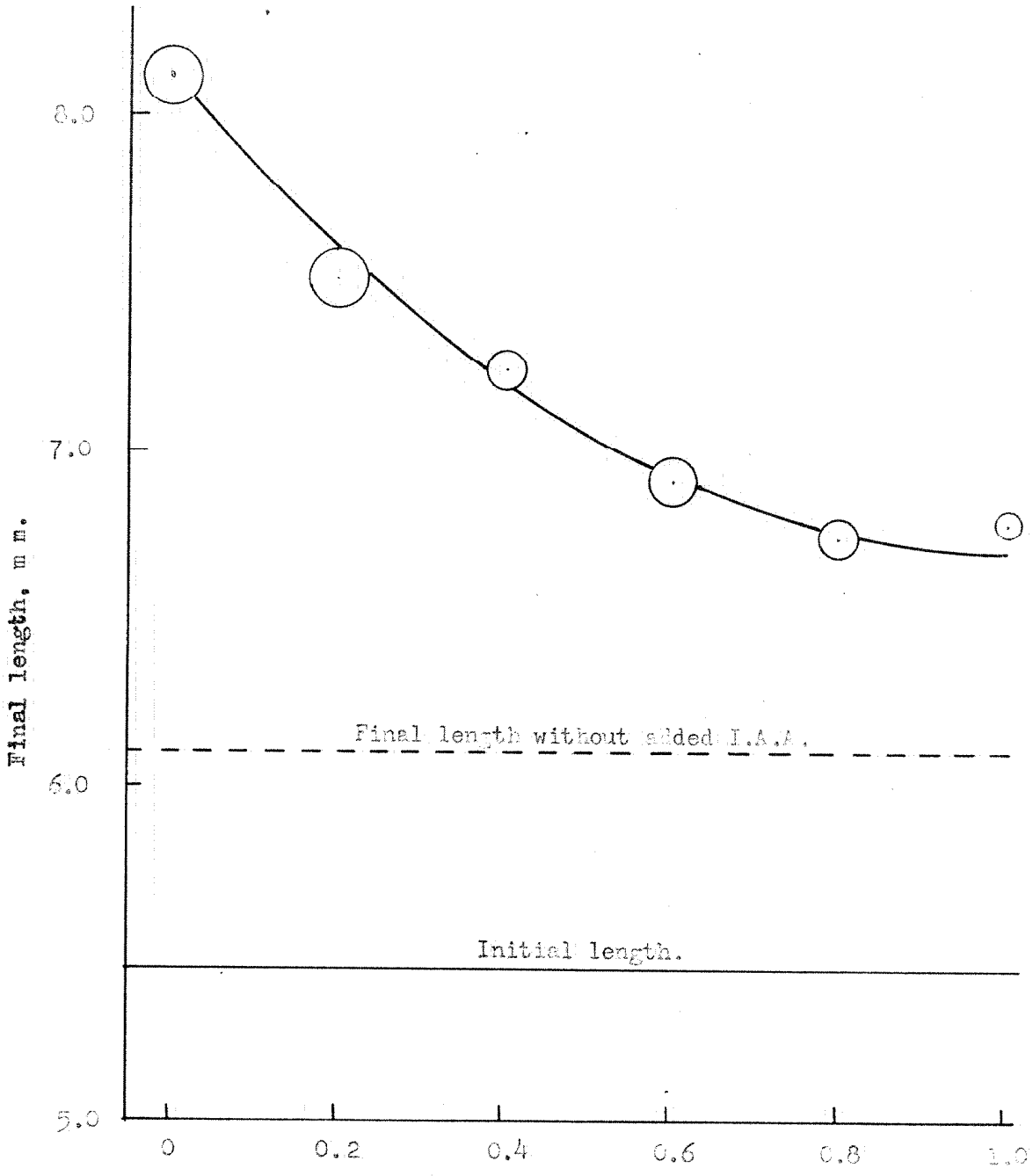
the sections made less I.A.A.-induced growth in the light than in the dark. Moreover, in the presence of the dialyzate growth in the light is markedly inhibited while there is no significant effect on the growth of those sections grown in the dark. As a control against a non-specific toxic effect of the dialyzate on growth in the light (e.g., the formation of an inhibitory substance in the light) the experiment was repeated using 2,4-D as the auxin. Growth was again reduced in the light but in this case there was no significant effect due to the presence of the dialyzate in the light or in the dark (figure 20). It may be concluded that since the growth inhibition produced by the dialyzate is specific for I.A.A.-induced growth and specific for growth in the light, the dialyzate is destroying the biological activity of the I.A.A. in the light.

Figure 21 shows the loss of biological activity of I.A.A. as a function of the concentration of dialyzate. The higher the concentration of dialyzate, the greater is the destruction of biological activity.

Non-identity with Free Riboflavin. The dialyzate was yellow in color and fluoresced yellow-green as well as blue under ultra-violet light.

In a series of papers (23, 84, 112) Galston and Baker have described a detailed work on the photosensitized destruction of I.A.A. by free riboflavin. Since riboflavin occurs in appreciable concentration in these seedlings, it was necessary to ascertain whether the photolytic activity of the dialyzate were accountable by its riboflavin content. Two kinds of

Figure 21.



The effect of dialysate concentration on destruction of the biological activity of I.A.A.
Ten hours' incubation in the light at 25°C. Initial I.A.A. concentration = 0.10 mg/l.
Radii of the circles indicate standard errors.

experiments were carried out.

One g. lyophilized pea epicotyl brei was dissolved in 10 ml. M/15 phosphate buffer pH 6.6 and dialyzed against 10 ml. buffer overnight at 0 to 2° C. Appropriate dilutions of the dialyzate were used. A stock solution of riboflavin was diluted to 4 γ/ml. so as to have approximately the same activity as the dialyzate. Aliquots of the two solutions both buffered at pH 6.6 were placed in thin-walled glass tubes selected for uniformity and exposed for 60 minutes four inches from a Keese 1.30 watt ultra-violet lamp (model 909 T.P.). The initial and final activities were determined as a function of concentration. Table 7 shows that whereas the activity of the riboflavin solution was diminished to 28%, that of the dialyzate was diminished to 60% after exposure to an equal dose of ultra-violet light. This suggests that the riboflavin of the dialyzate is not responsible for a major fraction of its activity unless in the crude state it is partially protected from destruction by ultra-violet light.

The second kind of experiment consisted of paper chromatography of the dialyzate alongside spots of the riboflavin solution of comparable activity. Whatman #1 paper was used with water as solvent. The developed chromatograms were examined under short-wavelength ultra-violet light. The riboflavin produced a strong yellow-green fluorescence at Rf = 0.33 but the corresponding spot in the dialyzate was barely discernible. Later experiments, using a technique for detecting I.A.A.-destroying activity directly on the developed

Table 7

The effect of exposure to ultra-violet light on the I.A.A.-photodestroying activities of riboflavin and dialysate.

Active Solution	Concentration,* ml./2.0 ml.	Activity, μM/1/24 mins. before U.V.	Activity, μM/1/24 mins. after U.V.	Ratio U.V. control	Mean
Riboflavin	0.1	40	11	0.27	
	0.2	66	19	0.29	0.28
	0.3	84	24	0.28	
	0.5	115	43	0.29	
Dialyzate	0.1	32	21	0.66	
	0.2	46	27	0.59	0.60
	0.3	58	31	0.53	
	0.5	73	46	0.63	

* Reaction mixture contained 0.5 ml. M/15 phosphate buffer, pH 6.6, dialyzate or riboflavin as shown, 0.5 ml. 10^{-3} M I.A.A. at zero time and distilled water to a total volume of 2.0 ml.

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 this naturally-occurring substance or substances which are not riboflavin and which destroy I.A.A. in the light.

The Question of Catalyst versus Reactant. 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 I.A.A. (23). The active component of the dialyzate may react directly with I.A.A. to change its chemical identity or it may sensitize its decomposition in light. Since a catalyst may be expected to perform a more dynamic function physiologically than a substance which reacts stoichiometrically with I.A.A., it became a pregnant question to decide whether the dialyzate destroyed I.A.A. in a catalytic fashion. The evidence and arguments pertinent to this question are presented in a later section after the kinetics of the system has been defined, but it may be said here that it is concluded that the dialyzate reacts catalytically.

Kinetics.

(a) Progress Curve.

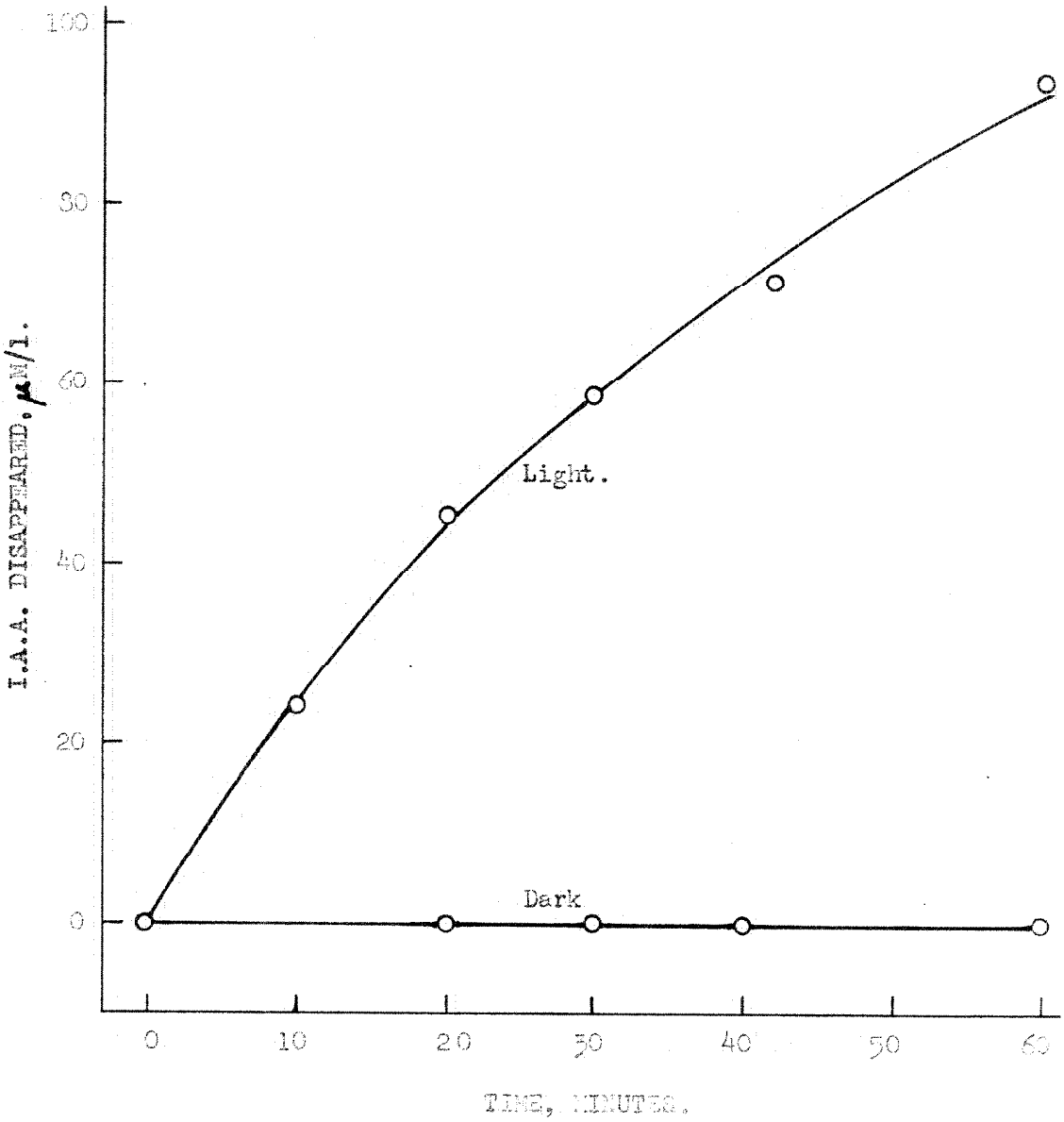
As a preliminary to the measurement of reaction rates it is necessary to know the time course of the reaction under standard conditions. Two series of tubes were loaded with 0.5 ml. M/15 phosphate buffer, pH 6.6, 0.5 ml. dialyzate, 0.5 ml. water, and at zero time, 0.5 ml 10^{-3} M I.A.A. One series

was incubated in the light at 25°C. and one in the dark at 25°C. At time intervals 8 mls. Salkowski reagent were added to the tubes and residual I.A.A. determined. Figure 22 shows that there is no loss of I.A.A. in the dark but rapid destruction in the light. With light activities of this magnitude the reaction rate is sensibly constant for the first twenty to thirty minutes. In subsequent experiments, the I.A.A. concentration change effected in this period was routinely taken as the reaction velocity, incubations all being made in the light.

(b) The Effect of I.A.A. Concentration.

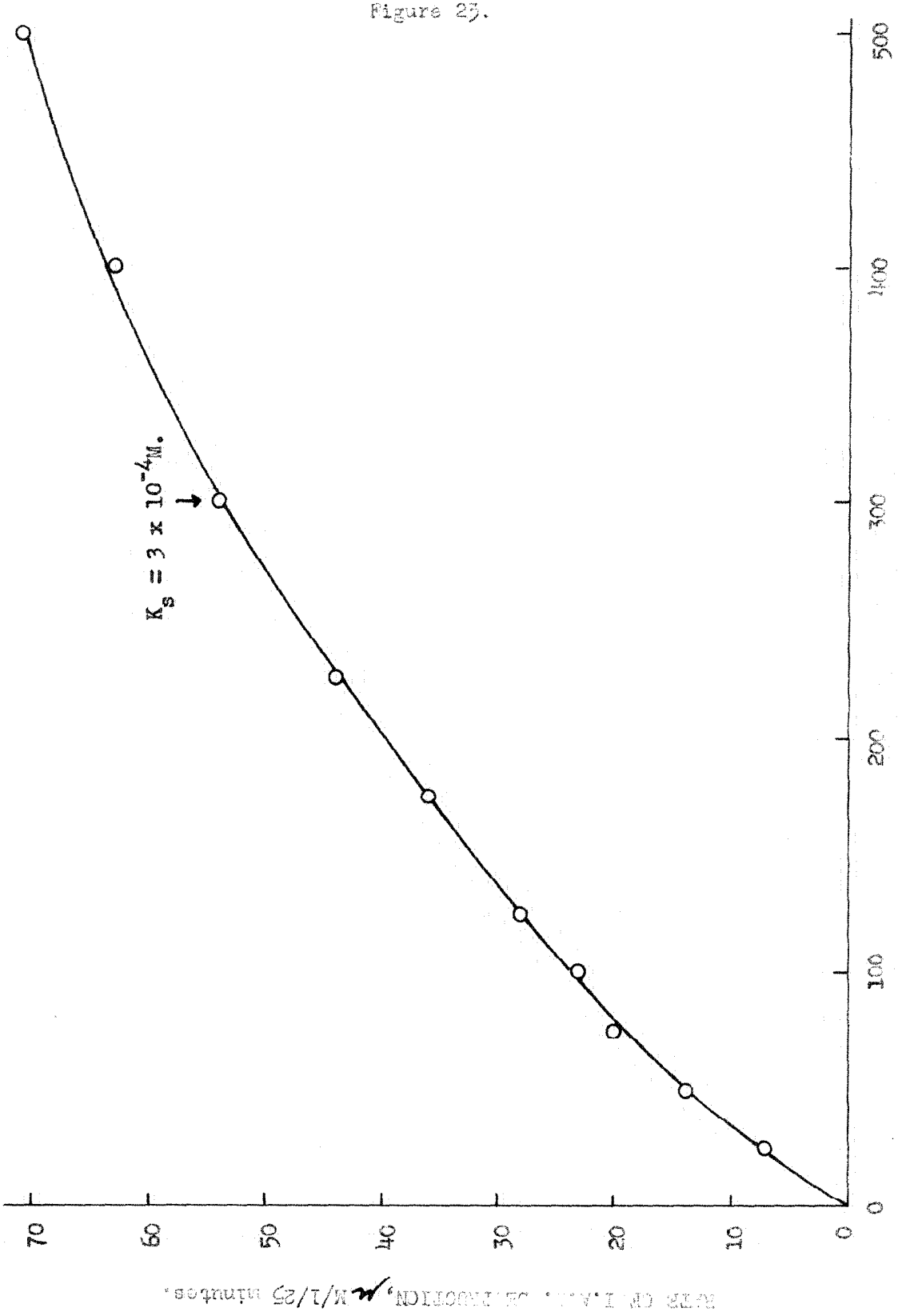
The effect of substrate concentration on the reaction velocity is shown in figure 23. The velocity is proportional to the concentration only below about 50 μM and the increment decreases in a broad hyperbola which does not, however, closely approach an asymptote even at 500 μM. Limitations of the analytical method make it impractical to exceed this value. Compared with the enzyme, I.A.A. oxidase, the affinity for the I.A.A. is approximately the same and the saturating concentration is much higher (78, 83). Let us assume for the moment that the dialyzate acts as if it contains a single or a single dominant active component. According to the Michaelis-Menten theory of enzyme action (101) 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:

Figure 22.



Progress curve. Initial I.A.A. concentration 250 μM/l, temperature = 25°C.

Figure 23.



The effect of IAA concentration on the rate of its destruction by the dialyzate.

$$\frac{1}{v} = \frac{K_s}{V} \cdot \frac{1}{(S)} + \frac{1}{V}$$

where
- v = measured reaction velocity
- V = limiting velocity
- $K_s = \frac{k_1 + k_3}{k_2}$ enzyme-substrate dissociation constant
- (S) = substrate concentration

By plotting $\frac{1}{v}$ against $\frac{1}{(S)}$ a straight line should result, with slope equal to $\frac{K_s}{V}$ and intercept $\frac{1}{V}$. This has been done in figure 24 using the data of figure 23, 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 24 is approximately 3×10^{-4} M, which is of the same order of magnitude of that of I.A.A. oxidase (calculated from 78, 79, 83, 101).

If a reacting system is represented by

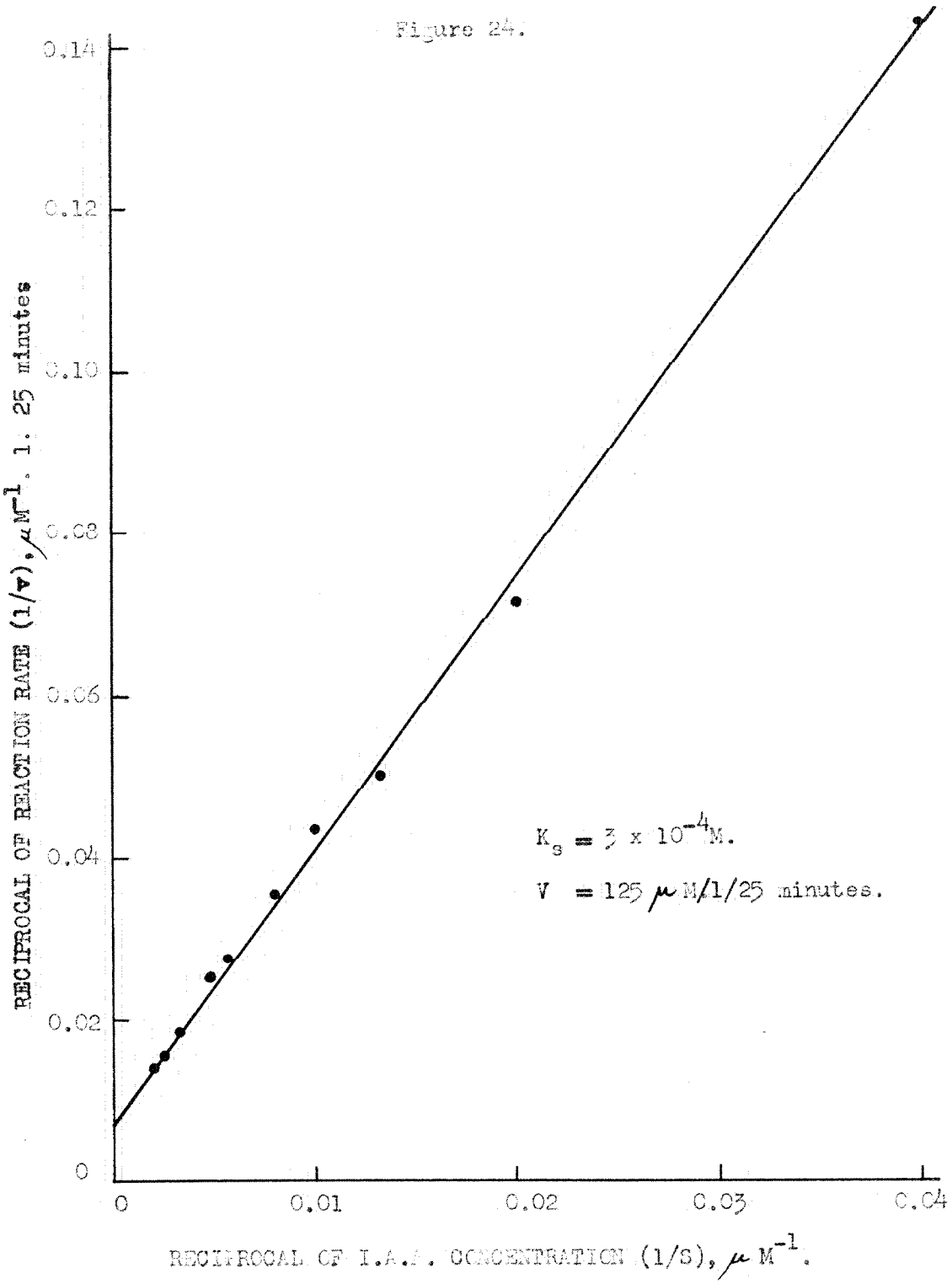
$$nA + mB + pC \rightleftharpoons \ldots\ldots\ldots$$

where n, m and p are the numbers of molecules of the species A, B, and C reacting, then the velocity v is given by

$$v = k. A^n. B^m. C^p.$$

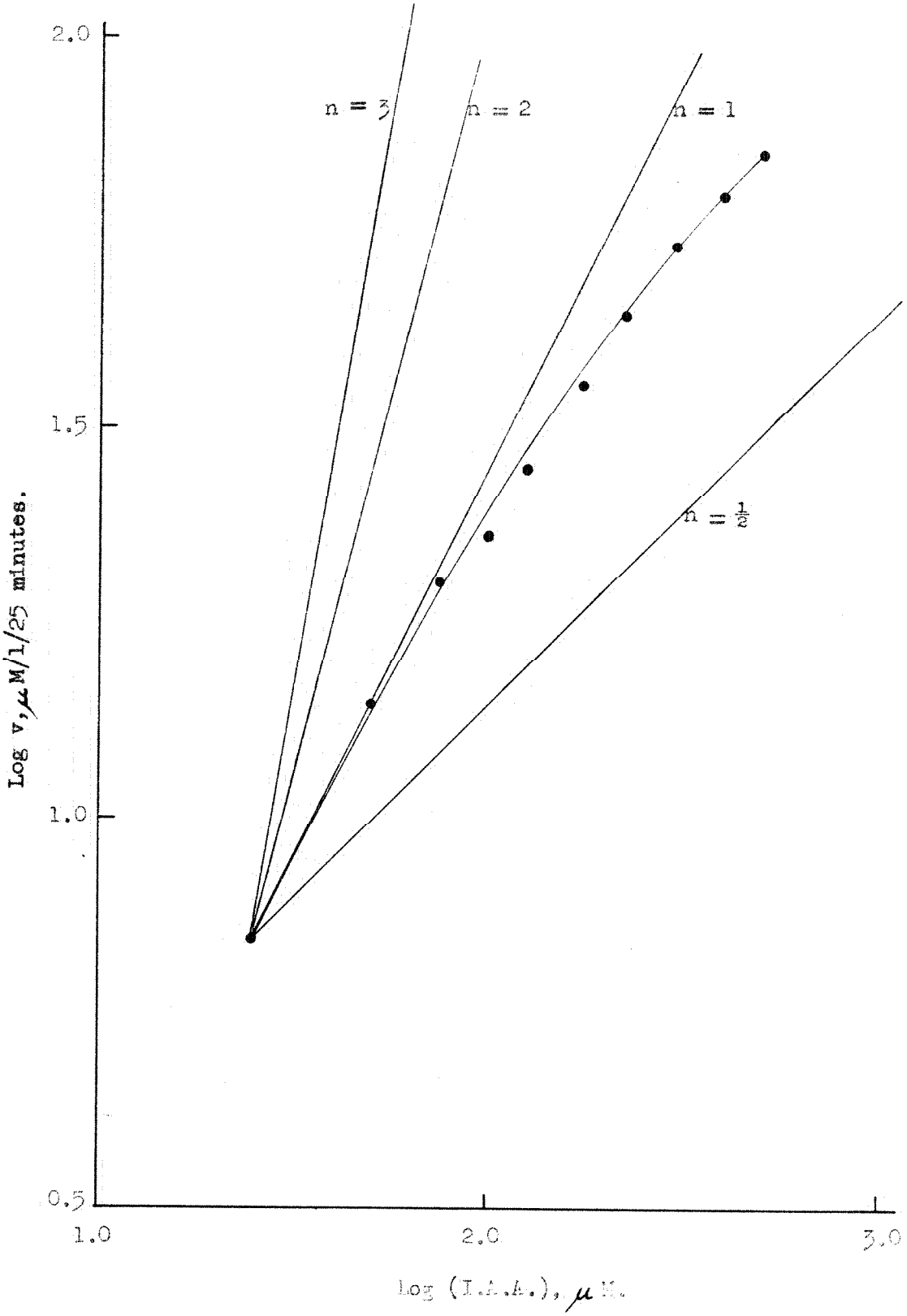
If the concentrations of B and C are kept constant, log $v = n \log A + Q$, where Q is a constant and n is the order of the reaction with respect to A and may be evaluated from the slope of the plot of log v against log A. Figure 25 shows

Figure 24.



Lineweaver-Burk plot of the data of figure 23.

Figure 25.



Order of reaction with respect to I.A.A. equals 1. Theoretical slopes for 1/2, 1, 2 and 3 order reactions drawn in.

such a plot for the data of figure 23. Since in this catalyzed reaction, I.A.A. is limiting only at low concentrations, the line is not straight and $n \longrightarrow \frac{\log v}{\log(I.A.A.)}$ as (I.A.A.) $\longrightarrow$ 0. From the tangent at log (I.A.A.) = 1, n clearly equals 1.

(c) The Effect of Dialyzate Concentrations.

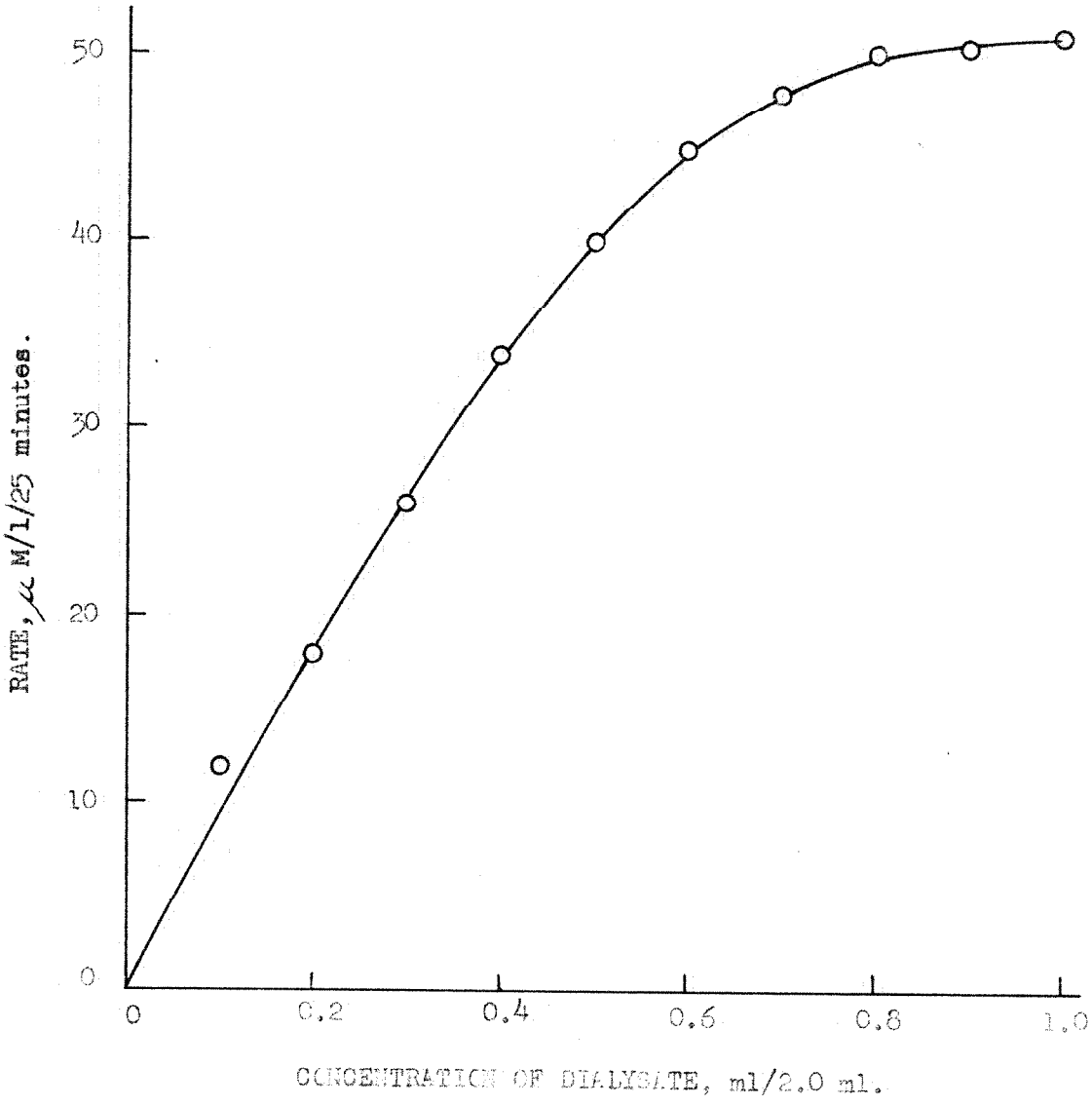
The reaction velocity as a function of dialyzate concentration, is shown in figure 26. The dialyzate was prepared by dissolving 1 g. lyophilized pea epicotyl brei in 5 ml. M/60 phosphate buffer, pH 6.6, and dialyzing against 20 ml. buffer. Total reaction volume was 2.0 ml. and initial I.A.A. concentration 250μM. Activity is proportional to dialyzate concentration only at low concentrations and rapidly falls off. At high concentrations, presumably substrate becomes rate-limiting.

(d) The Effect of pH.

I.A.A. is a weak acid with pK_a equal to 4.75. The active material of the dialyzate participates in an oxidation-reduction reaction (page 111) and has the solubility properties of a strongly polar molecule (page 124). It is to be expected that the pH would profoundly effect the configuration and hence the reactivity of both of these participants.

A series of McIlwain's phosphate-citrate buffers was prepared to cover a pH range from 3 to 7 and beyond the range the pH was adjusted with 0.2 N HCl or 0.1 N $NaHCO_3$. 1.0 ml. buffer, 0.5 ml. dialyzate or 0.5 ml. water and 0.5 ml. 10^{-3} M I.A.A. were incubated in the light at 27°C. for 24 minutes. Aliquots of 1 ml. were pipetted into 4 ml. Salkowski reagent and the residual I.A.A. determined. The pH was determined

Figure 26.



The relation between dialysate concentration and reaction rate. Initial I.A.A. concentration = 250 μ M/l. Temperature = 25.5°C.

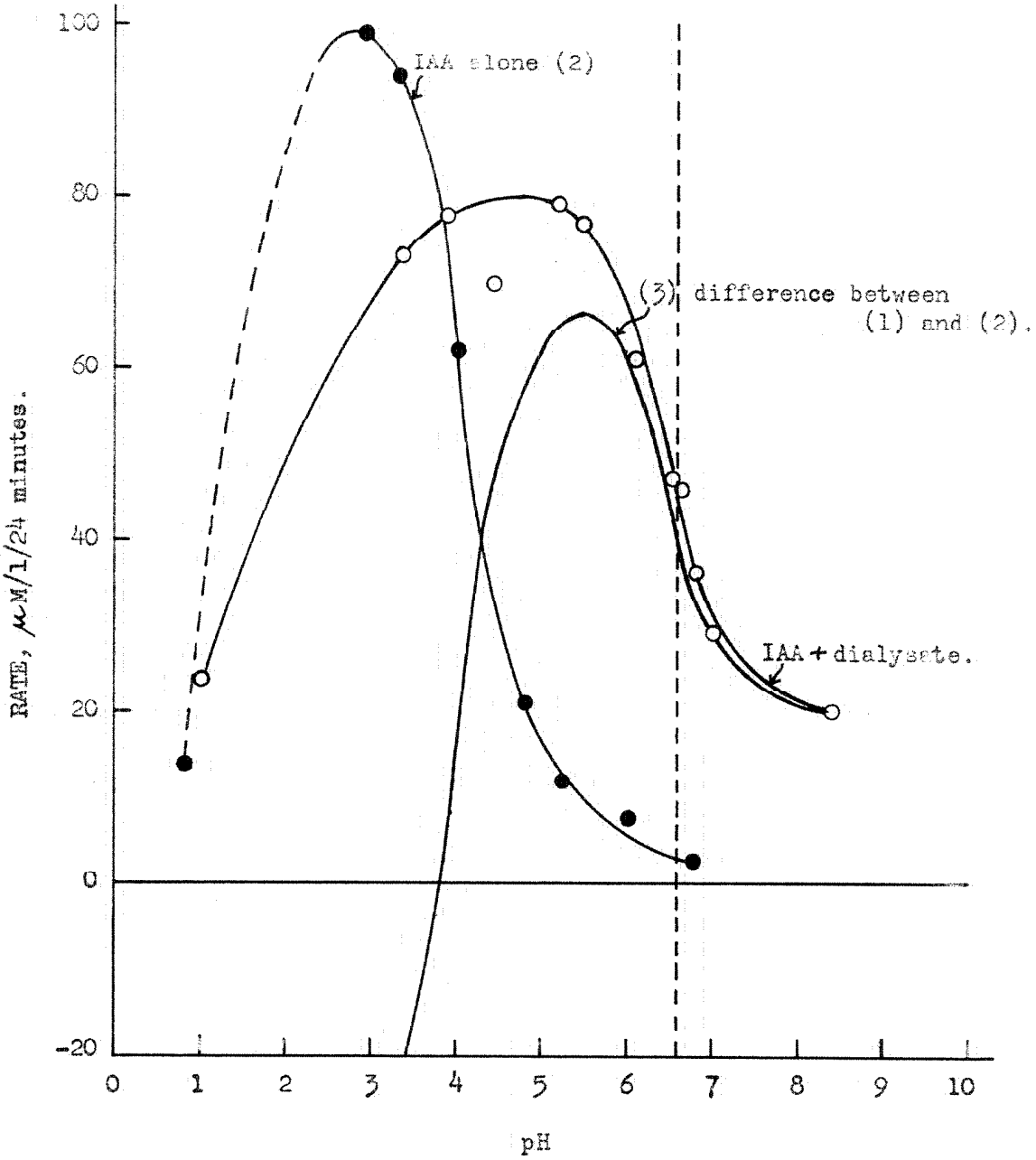
on the residual reaction mixture using the glass electrode.

Figure 27 shows that at acid pH's the I.A.A. alone is destroyed by light. Dolk and Thimann (113) have shown that a plant growth substance isolated from Rhizopus culture medium and subsequently identified as I.A.A. is 50% decomposed on standing for three hours at room temperature in N HCl in presumably low-intensity laboratory light. Algéus (114) also demonstrated that lowering the pH or exposure to light during storage of "pure" solutions of I.A.A. results in losses. However I.A.A. itself does not absorb visible light and its photodestruction is presumably sensitized by some pigmented impurity. All samples of I.A.A. tested showed rapid photodestruction below pH 5, even after repeated purification by dissolving in NaOH and precipitating by the slow addition of dilute HCl and by dissolving in ethanol and precipitating with water. It was therefore necessary to carry out this control with I.A.A. alone.

In the presence of the dialyzate I.A.A. was rapidly destroyed at considerably higher pH's. The net optimum occurred between pH 4 and 5. There is little activity above pH 8.

Subtracting the smoothed curve for I.A.A. alone from that for I.A.A.+dialyzate, curve 3, figure 27 is obtained. The optimum occurs at pH 5.5 and activity falls off sharply on both sides. Below pH 3.8 the activity for I.A.A. alone is reproducibly higher than that for I.A.A. plus dialyzate. A probable explanation for this phenomenon is that in this range

Figure 27.



The effect of pH on the activity of the dialysate.
Initial IAA concentration 250 μM. Temperature 26.5°C.
McIlwain's phosphate-citrate buffers. 0.2N HCl and 0.1N $NaHCO_3$ for extreme pH values.

although the dialyzate combines with I.A.A. more effectively than does the pigmented impurity in the I.A.A. sample, the catalyst-substrate complex decomposes more slowly. Thus below pH 3.8 the dialyzate partially protects the I.A.A. from destruction by the contaminating pigment.

Curve 2, figure 27, shows a reproducible optimum pH for I.A.A. alone at 2 to 3. However, other experiments using the growth of pea epicotyl sections as a measure of residual I.A.A. show continuously increasing destruction with decreasing pH. Thus the optimum in curve 2 seems to be a spurious effect due to the incomplete specificity of the Salkowski reagent. Below pH 2 it appears that although the biological activity of I.A.A. is destroyed, it is converted into a product which gives a positive Salkowski reaction.

Routine determinations were carried out at pH 6.6 because the destruction of I.A.A. in the absence of dialyzate was very low here. In addition this is the pH optimum for I.A.A. oxidase and many previous experiments using whole brei have been carried out at this pH.

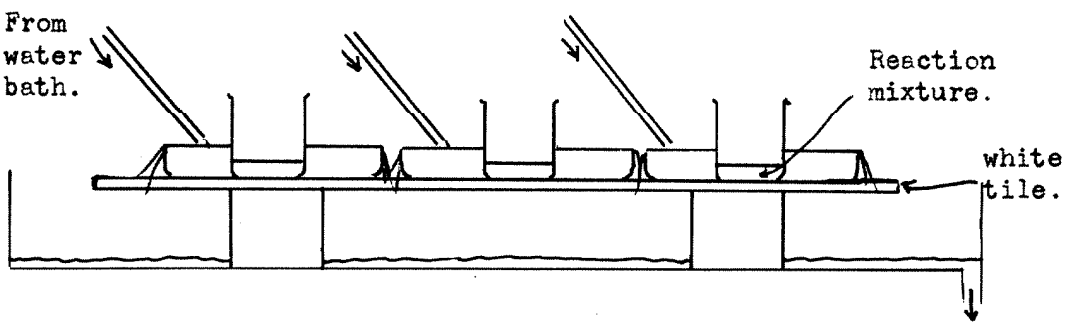
(e) The Effect of Temperature.

From a theoretical standpoint, the reaction velocity of a purely photochemical reaction is independent of temperature if it can be assumed that the absorption coefficient is constant over the temperature range. Experimentally it has been well shown that for most reactions known to be of this kind the temperature coefficient lies between 1.00 and 1.05 (115). However, where the initial photoactivation is followed by a

dark reaction which derives its activation energy thermally, the temperature coefficient for the overall reaction may be higher and may approach that for the dark reaction under conditions where the reaction velocity of the dark reaction limits that of the overall rate.

Without resorting to elaborate equipment it is difficult to control simultaneously the temperature and the intensity of irradiation of a series of solutions. The effect of temperature on the rate of photolysis of I.A.A. by the dialyzate 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 stood 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 lead by a series of rubber tubes into the petri dishes and permitted to overflow continuously, thus water jacketting the reaction-

Three fluorescent lamps.



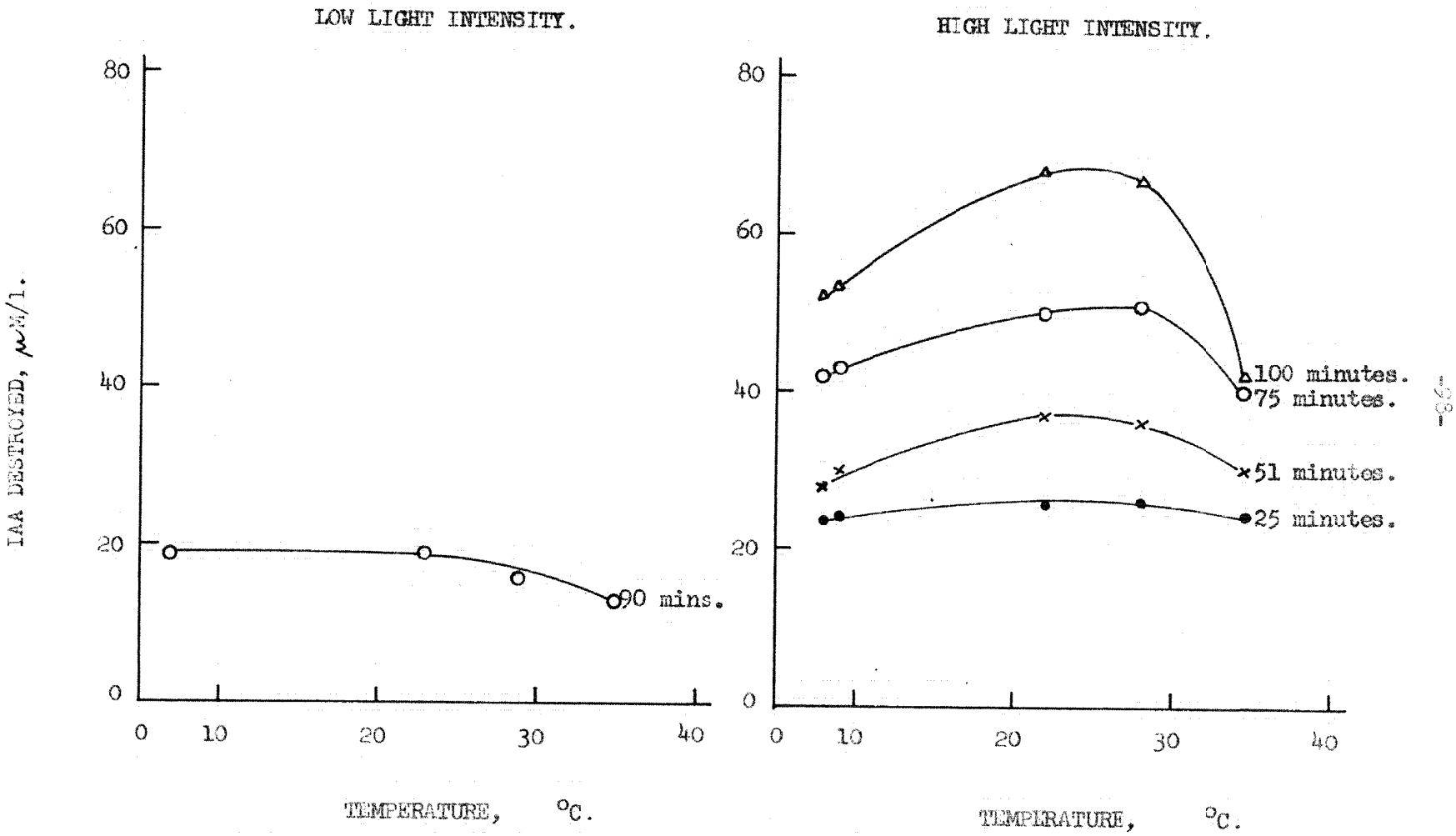
beakers. For high light intensity a bank of three 40 watt "white 4500" fluorescent lamps was suspended eight inches above the surface of the reaction mixtures, where the light intensity was 500 f.c. For low light intensity the light of the laboratory (10 f.c.) was used. At zero time 250μM/l I.A.A. were added. Aliquots were removed at time intervals for determination of residual I.A.A. Temperatures were measured within the reaction vessels at time intervals and found to be constant within $\pm$ 1°C.

Figure 28 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 rate with increasing 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. At low light intensities the light-reaction is limiting so the Q_{10} approaches 1. At high light intensities the products of the photoactivation accumulate and the secondary temperature-dependent dark reaction becomes limiting. Above 25°C. there is a rapid decline in rate, especially at longer times, suggesting that the catalyst is undergoing more rapid destruction at higher temperatures.

(f) The Effect of Light.

As shown in figures 22 and 33 the dialyzate sensitizes the destruction of I.A.A. only in the light. Experiments to determine the relationship between activity and light intensity did not yield precise reproducible results, probably because

Figure 28.



The effect of temperature on the photodecomposition of IAA by the dialyzate.

of the crudeness of the optical systems available for use. It may be said, however, that within the ranges used (up to 730 foot candles from a "white" fluorescent lamp or up to 1600 foot candles from a tungsten "photoflood" lamp filtered through 6 inches of water), the activity increased with increasing incident intensity.

A crude action spectrum was determined in the following way. A series of 5 cm. diameter petri dishes were loaded with 2.0 ml. M/15 phosphate buffer, pH 6.6, 1.0 ml. dialyzate and (at zero time) 1.0 ml. 10^{-3} M I.A.A. The dishes were placed inside blackened filter-paper box lids and covered with 6 x 6 inch corning glass filters of the appropriate transmittance. These reaction vessels were set 8 inches beneath a bank of 3 fluorescent lamps and after 25 minutes' incubation, the residual I.A.A. determined. Using a blackened thermopile with a sensitive galvanometer, the relative transmitted energies from this light source through the various filters were determined. Table 8 shows these data. If it can be assumed that for each wavelength the amount of chemical change is proportional to the incident light intensity in quanta, the relative destruction of I.A.A. per quantum calculated for each wave band gives the photochemical effectiveness. It may be seen from the last column of table 8 that the relative order of effectiveness is violet > blue > green > red > yellow.

(g) Stability of the Dialyzate.

(i) <u>To Heat</u>. 0.5 ml. dialyzate prepared from lyophilized whole brei was added to a series of tubes containing 0.5 ml. M/15

Table 8

The effect of light quality on the photodecomposition of I.A.A. by the dialyzate.

Filter	Transmittance maximum, mμ	Using fluorescent lamp as source, relative incident energies	quanta*	I.A.A. decomposed in 25 mins. μM/l	Relative I.A.A. decomposition per quantum
colorless glass	---	100	100	38	100
blue-violet #511	380-440	9	7	17	640
blue-green #428	400-540	39	34	35	271
green #401	480-600	20	20	15	197
yellow #352	560	73	75	18	63
red #243	600-700	37	44	16	95

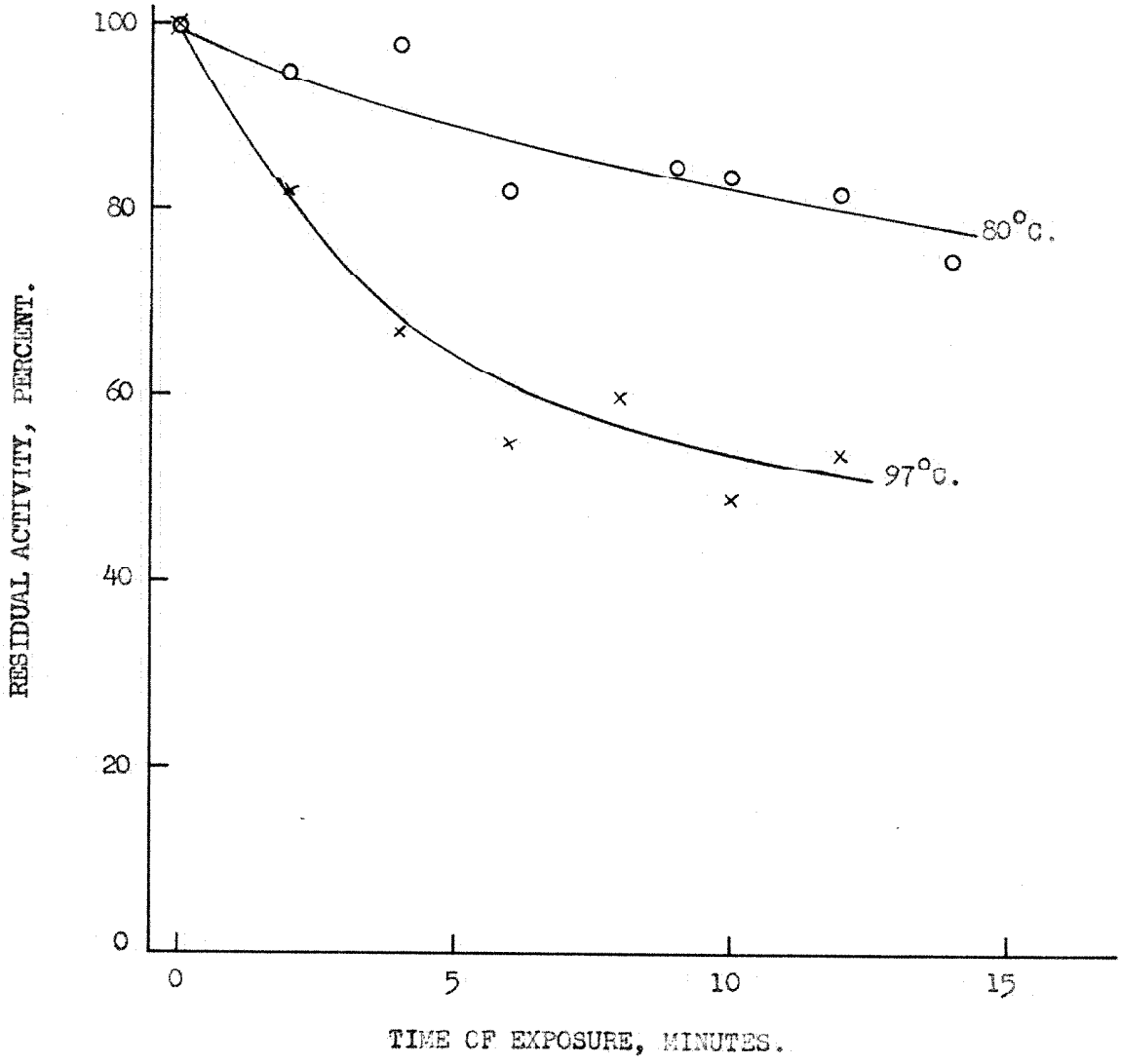
* An average value obtained by making calculations using the wavelength at center of transmitted band. Relative numbers of quanta = relative energies x wavelengths.

phosphate buffer pH 6.0 and 0.5 ml. water. The 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 withdrawn into an ice-bath for rapid cooling. After equilibration to room temperature, 0.5 ml. 10^{-3} M I.A.A. was added and the residual activity of the dialyzate was determined. Figure 29 shows that there is considerable heat inactivation of the dialyzate. In twelve minutes approximately 50 percent of the activity is lost at 97°C. and 20 percent at 80°C.

(ii) To pH. 1.0 ml. dialyzate was placed in each of six beakers. The pH's were adjusted over a range of values with 2 N H_2SO_4 or N KOH at the glass electrode and the beakers set in the dark at 27°C. for 8½ hours. The pH's were determined again, re-adjusted to 6.6 and the volumes were made up to 2.0 ml. in volumetric flasks. Residual activity was then determined. Figure 30 shows that under these incubation conditions the dialyzate is quite stable between pH 1 and 7. Above pH 8 the activity begins to drop off appreciably.

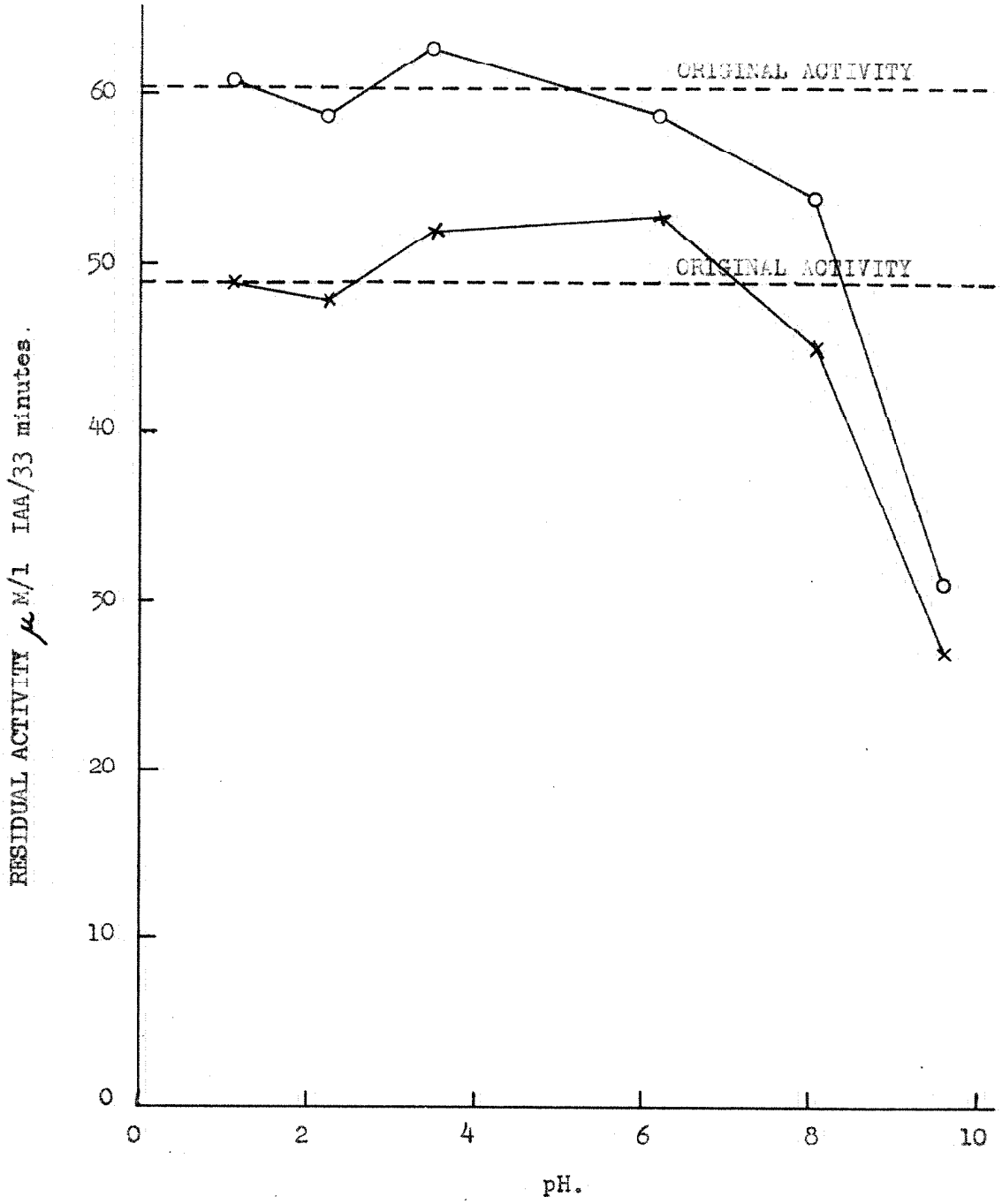
(iii) To Light. 0.5 ml. dialyzate was added to a series of tubes containing 0.5 ml. M/15 phosphate buffer, pH 6.0 and 0.5 ml. water. The stoppered tubes were placed on the aluminum-painted rack beneath the 40 watt white fluorescent lamp used standardly in light experiments and at time intervals tubes were withdrawn to a dark cupboard. Then 0.5 ml. 10^{-3} M I.A.A. was added and the residual activity of the dialyzate determined. Figure 31 shows that preexposure to the white

Figure 29.



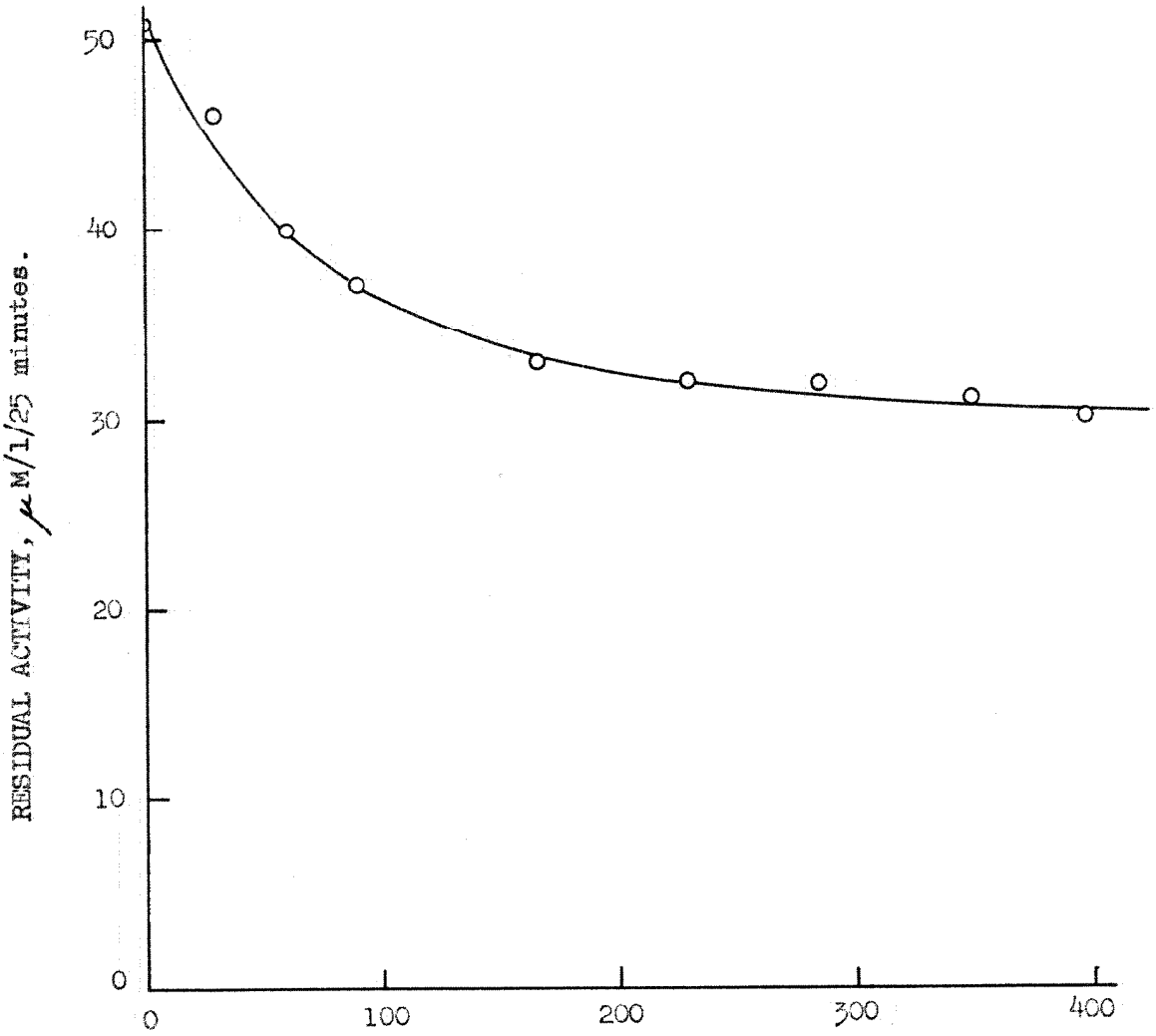
Stability of the dialyzate to heat.
Initial IAA concentration in assay = 250 μ M/l.
Temperature = 25°C.

Figure 30.



Stability of the dialyzate to pH.
8 1/2 hours exposure in the dark at 27°C. at above pH's.
Activity determined at pH 6.6, 23°C. and with initial IAA concentration of 250 μM/l.
o = 1 in 4 dilution of original dialyzate.
x = 1 in 8 dilution of original dialyzate.

Figure 31.



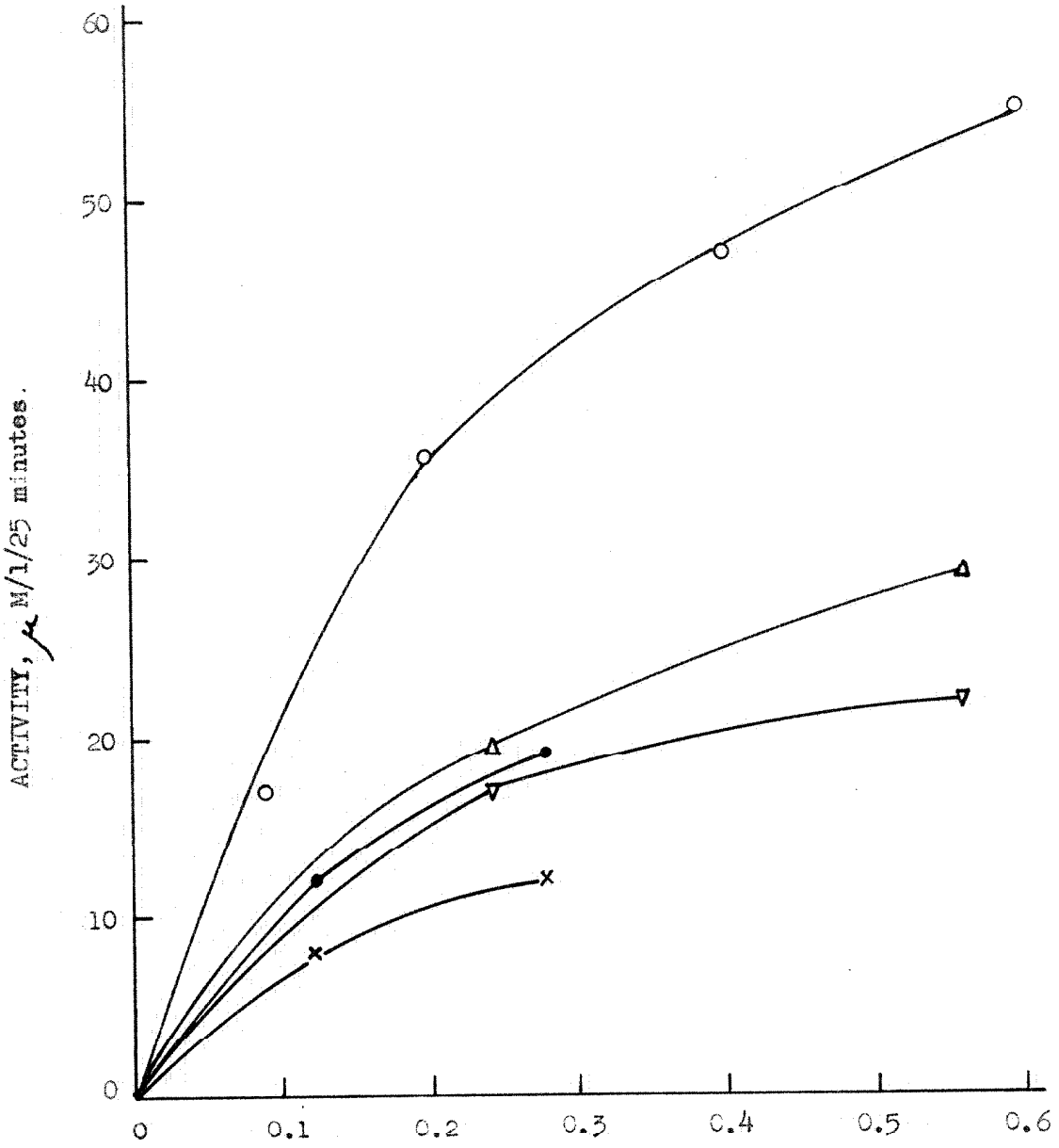
The stability of the dialyzate to light.
Initial concentration 250 μM. Temperature = 24.4°C.

light produces a rapid decay in activity. The rate of decay, however, decreases rapidly with time and appears to approach an asymptote which in this experiment occurs at approximately 50% loss of activity (or, referring to a calibration curve for activity versus dialyzate concentration, approximately 70% loss of I.A.A.-destroying units). This may be due to the dialyzate containing a mixture of active components, not all of which are destroyed by light.

I.A.A. affords the dialyzate some protection against photolysis. Dialyzate in two concentrations was incubated in the light for 5½ hours at pH 6.6 and 25°C, with and without 500 μM/l I.A.A. The four solutions were then adjusted to pH 3.0 and extracted four times with 5 ml. peroxide-free water-saturated ether to remove the residual I.A.A. The pH's were readjusted to 6.0 and the solutions made up to volume. Residual activities of the dialyzate were then determined each at two concentrations and compared with the original activity. Figure 32 shows that while there is great loss of activity in all cases especially in dilute solution, the presence of I.A.A. during the preëxposure period reduced the loss appreciably.

(iv) <u>To Ultra-violet Light</u>. As already stated on page 82 the activity of the dialyzate is rapidly destroyed by exposure to ultra-violet light (see table 7). No further quantitative data have been obtained. It is clear that unless the destruction of the active compound is photosensitized by some other pigment, the former must absorb in the ultra-violet band emitted by the Keese lamp.

Figure 32.



Protection of dialyzate by IAA from destruction by light.

- ○ unexposed dialyzate
- △ full concentration dialyzate, preexposed in presence of 500 μM/1 IAA
- ▽ full concentration dialyzate, preexposed in absence of IAA.
- ● half concentration dialyzate, preexposed in presence of 500 μM/1 IAA.
- × half concentration dialyzate, preexposed in absence of IAA.

Temperature = 25°C. Initial IAA concentration in assay = 250 μM/1.

(h) The Question of Catalyst or Reactant.

In a previous section (page 84) attention was drawn to the importance of distinguishing whether the material of the dialyzate which photoinactivates I.A.A. acts 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 without loss or with little loss of activity.

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

(iii) If a reactant, the initial reaction velocity should be proportional to the concentration of the reactant raised to the power of the order of the reaction with respect to that reactant, according to the collision theory of reaction rates. If a catalyst, which forms an intermediate catalyst-substrate complex in accordance with the Michaelis-Menten concept, then a plot of initial rate against substrate concentration should be a rectangular hyperbola and a plot of the reciprocals of these values should give a straight line (101).

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

(i) Light is essential to effecting any decomposition

of I.A.A. in the presence of the dialyzate. As indicated in figure 31 the dialyzate is rapidly inactivated in light so that, even in the absence of I.A.A. it is never possible to recover the activity quantitatively at the completion of an experiment. However, referring to figure 32, it is clear that when irradiated in the presence of I.A.A. there is no greater loss in residual activity of the dialyzate than when irradiated in the absence of I.A.A. On the contrary, I.A.A. exerts some protective action.

(ii) It has never been possible to effect 100% destruction of I.A.A. by prolonged incubation in the light with the dialyzate preparations used, presumably because of the rapid photodestruction of the dialyzate itself. In fact in preliminary experiments a rough proportionality existed between the total amount of I.A.A. destroyable and the initial dialyzate concentration. Figure 33 shows two long-time progress curves. In curve 1 the dialyzate concentration in the reaction mixture is 10% (v/v) and in curve 2, 5%, the dialyzate being prepared in the usual way from lyophilized brei. Both curves approach maximum destruction limits which are proportional to the initial dialyzate concentrations. Superficially, this would seem to suggest that there is a stoichiometry between I.A.A. used and dialyzate provided. However, it may be shown that if the dialyzate is preilluminated in the absence of I.A.A. then added to I.A.A., the rate (i.e., the slope of the progress curve) is equal to or less than that at the corresponding time interval in figure 33, which attests that the

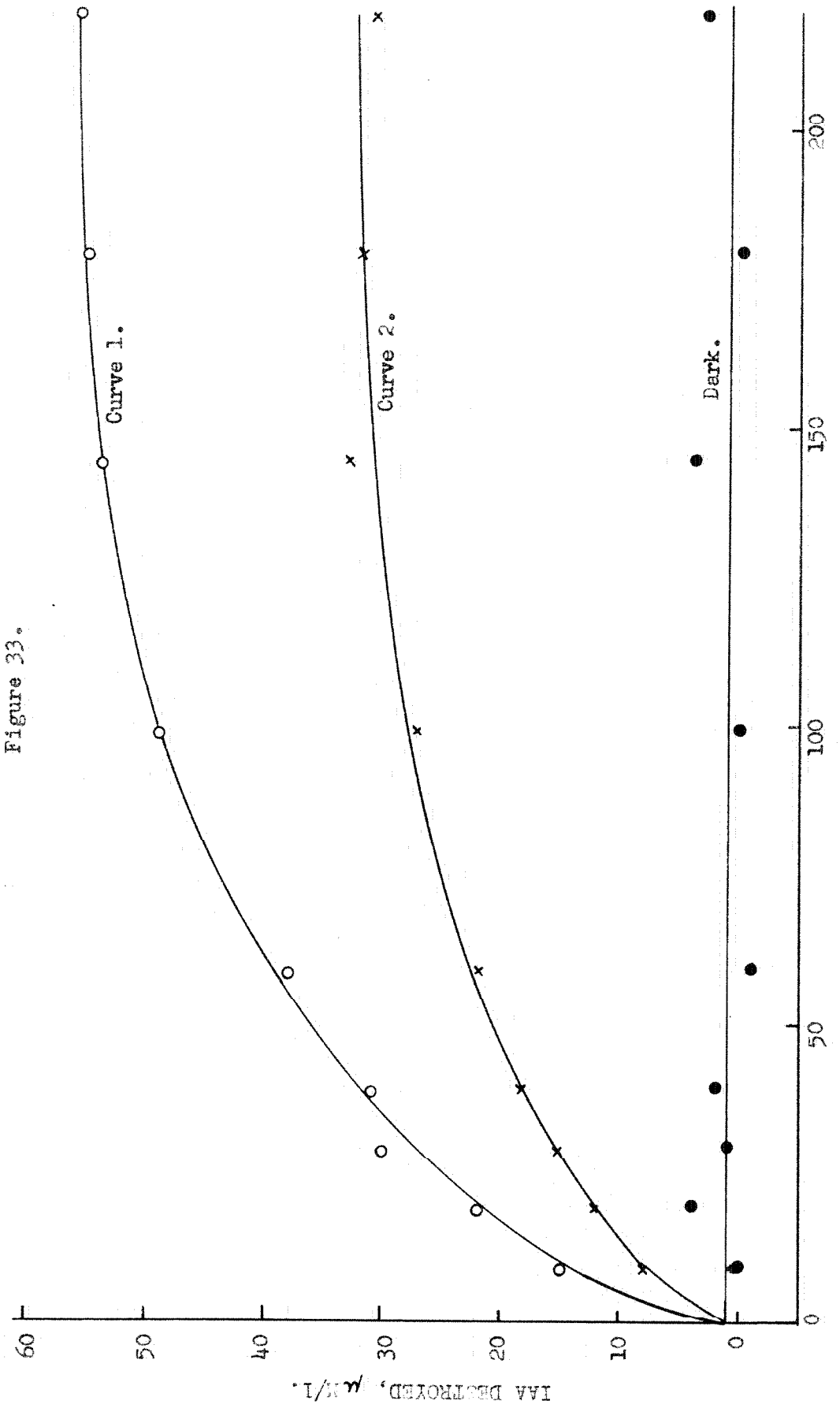

IAA DESTROYED, μM/l.
60
50
40
30
20
10
0
0
50
100
150
200
TIME, MINUTES.
Curve 1.
Curve 2.
Dark.

Figure 33.

limit is approached due to the destruction of dialyzate not by reaction with I.A.A. but by exposure to light.

(iii) If the dialyzate contained a reactant, then the reaction velocity should be directly proportional to the concentrations of I.A.A. and of the dialyzate raised to the powers of their respective orders of reaction, for all concentrations.

$$v = k. (I.A.A.)^{n}. (D)^{m}.$$

However, as shown in both cases a linear plot results in a curve concave to the concentration axis, which could only be obtained if the order of reaction were less than one. If light intensity were limiting at higher activities one would expect for a plot of activity versus dialyzate concentration a straight line which broke sharply, but for activity versus I.A.A. a straight line with no break.

On the other hand, if the dialyzate contained a catalyst which formed an intermediate catalyst-substrate complex with I.A.A. then one would expect a hyperbolic curve the plot of activity versus I.A.A. concentration, which is obtained (figure 23). Further, as shown in figure 24, a Lineweaver-Burk plot of the activity versus I.A.A. data closely approximates to a straight line, which is in agreement with the concept of the action of a catalyst.

The most reasonable interpretation of these experiments presented is that the dialyzate contains a photoreceptor which sensitizes the destruction of I.A.A. in a catalytic manner.

(j) The Requirement for Oxygen.

The most common photosensitization reactions are oxidations (91). It is already known that I.A.A. undergoes oxidation at the hands of the enzyme I.A.A. oxidase and photo-oxidation in the presence of riboflavin and it is of interest to determine whether the dialyzate-catalyzed reaction is oxidative in nature.

Thunberg tubes were loaded with 1.0 ml. dialyzate, 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 I.A.A. in the sidearm. The tubes were filled with argon, air or oxygen. At zero time the I.A.A. was tipped in and the tubes placed in the light in the usual way. After a period of incubation an aliquot was withdrawn and residual I.A.A. determined. This was repeated at two successive stages of purification to be described later. From table 9 it is clear that the activity of crude dialyzate shows no dependence on oxygen tension. However, after partial purification a relative, though not absolute dependence appears. It appears that a requirement for oxygen exists but that in the whole dialyzate there is present an alternative hydrogen acceptor in concentration high enough to account for the observed loss of I.A.A. The first purification step removed enough of this acceptor to make it become rate-limiting during the reaction, but not all. The second purification step apparently was not effective in selectively removing more of the hydrogen acceptor.

Many photosensitized oxidations have been shown to involve

Table 9

The Effect of Oxygen Tension.

Preparation	Incubation time (minutes)	I.A.A. destroyed (μM/l) in:		
		Argon	Air	Oxygen
1. Whole dialyzate	26	51	54	51
2. Purification I*	50	35	55	62
3. Purification 2†	40	49	64	70

* Whole brei was saturated with ammonium sulphate, set at 0 to 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 much of the original activity and was diluted 1 in 5 before use in this experiment.

† 2 ml. of the above fraction were placed on a column of powdered cellulose (Solka-floc) 2.2 cm. diameter x 50 cm. long and chromatographed in water, the eluate being collected in a fraction cutter. A fraction at approximately Rf = 0.8 to 0.9, which fluoresced blue, was colored yellow and which had I.A.A.-destroying activity, was shaken into phenol and after adding excess ether, back into 5 x 0.5 ml. water. This was used as the active preparation.

Activity values are comparable along horizontal but not along vertical lines.

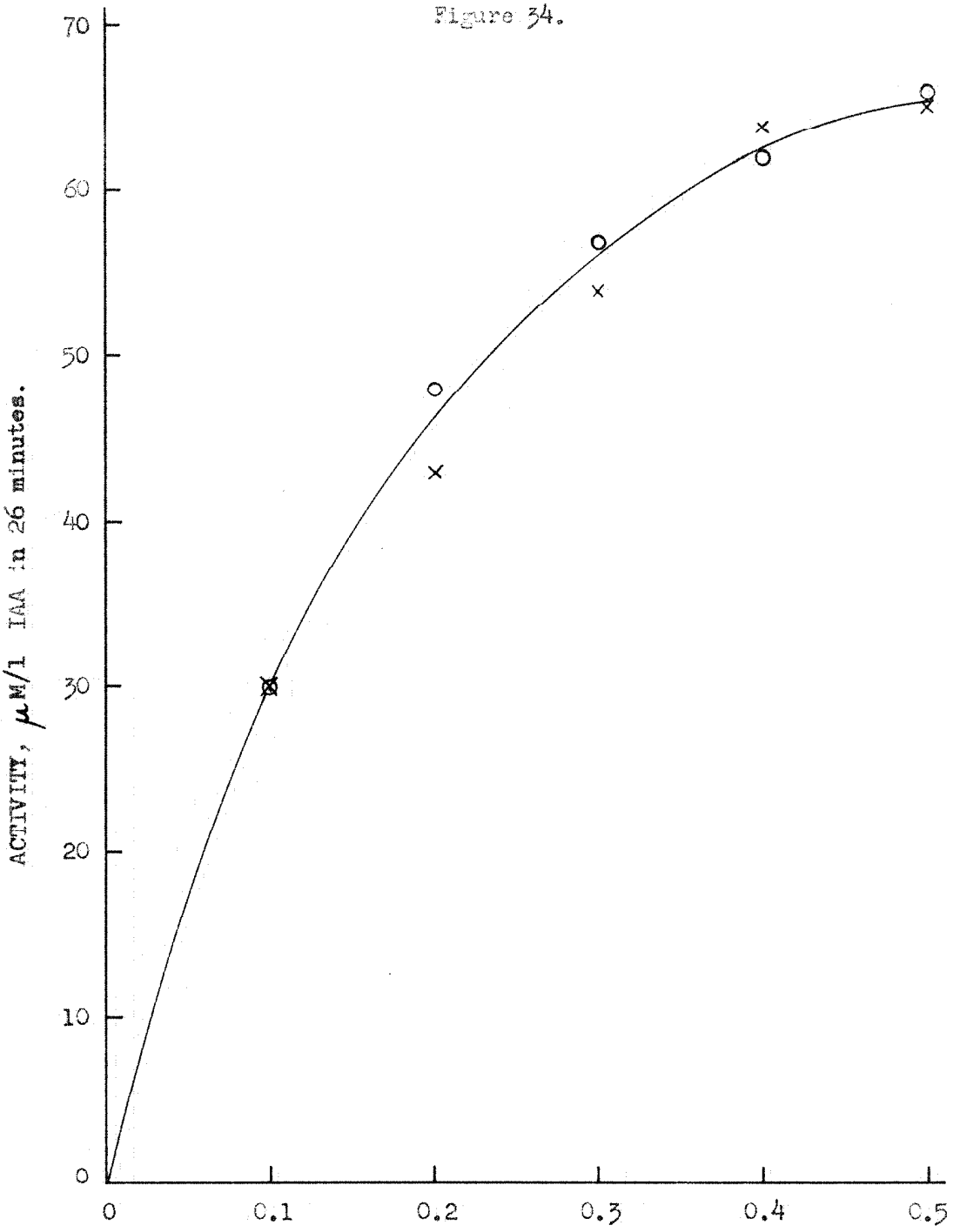
the primary formation of hydrogen peroxide from oxygen and water through the action of the light-activated pigment (91). It has already been shown (88) that hydrogen peroxide is an intermediary oxidant in the destruction of I.A.A. by I.A.A. oxidase. The following experiment was carried out to determine whether H_2O_2 is involved in the photooxidation catalyzed by the dialyzate. Two series of concentrations of dialyzate were incubated in the light at pH 6.0 with 250 μM/l I.A.A. One series contained crystalline beef liver catalase in concentration in excess of that required to completely inhibit I.A.A. oxidase. Figure 34 shows that catalase evokes no inhibition. It may be concluded that H_2O_2 is not an essential intermediate.

(k) The Action of Inhibitors.

The dialyzate is strongly inhibited by reagents that sequester heavy metals, e.g., KCN, H_2S, NaN_3, NH_2OH, 8-hydroxy quinoline, sym-diphenyl carbazide, etc. Many of the chelating agents interfered badly in the Salkowski reaction and it was necessary to use them at extremely low concentrations. Sulfide also interfered strongly, so after filtering precipitated sulfide from the dialyzate through sintered glass the H_2S was blown off with nitrogen before testing for activity.

From table 10 and figure 35 it is clear that a major fraction of the dialyzate depends upon the presence of a heavy metal for its activity. The ashed dialyzate 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

Figure 34.



The Effect of Catalase.
o = with catalase, × = without catalase.
Initial IAA concentration = 250 μM, temperature = 24°C.

Table 10

The effect of inhibitors on the activity of the dialyzate

Substance	Concentration, M	% activity	Biologically interesting ions sequestered (87)
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	

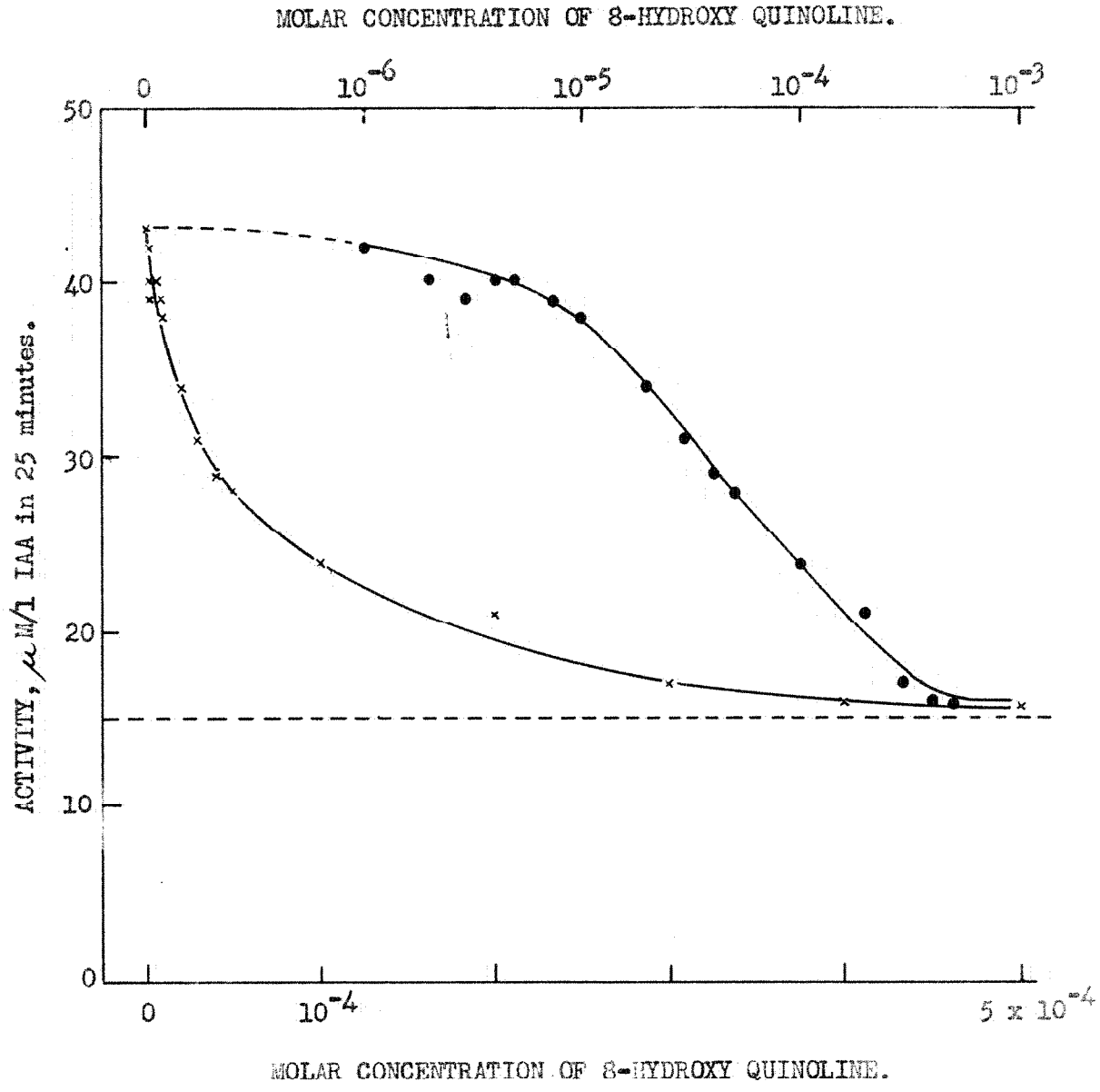
* H_2S from a cylinder was saturated with water and passed through a volume of dialyzate set in a brine-ice bath in the dark for 3 hours. The dialyzate was filtered through sintered glass and the H_2S blown out with nitrogen scrubbed with alkaline pyrogallol and water at 0°C in the dark until the effluent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

Table 10 (Continued)

The effect of inhibitors on the activity of the dialyzate

Substance	Concentration, M	% activity	Biologically interesting ions sequestered (87)
sym-diphenyl carbazide	0.00025	63	Zn^{++}, Cu^{++}
	0.0001	73	
Na 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	

Figure 35.



Residual activity as a function of concentration of 8-hydroxy quinoline. Dialyzate incubated 15 minutes with 8-hydroxy quinoline before adding IAA. Initial IAA concentration = 250 μM.
Temperature = 24°C.
• = log plot.
× = linear plot.

coordination complex with an organic moiety. The fact that activity is not second order with respect to dialyzate (figure 26) speaks against an interaction of an organic molecule with a free ion.

Albert and Gledhill (19) have made a study of the specificities and sensitivities of a large series of chelating agents under physiological conditions (pH = 7, temperature = 37°C.). Of those suitable for use in the above experiment, the specificities are not such as to permit positive identification of the metal. However, it may be said that the metal is not calcium nor magnesium since 8-hydroxy quinoline inhibits but fluoride does not, nor copper since benzoin oxime does not inhibit.

The addition of Cu^{++}, Co^{++}, Fe^{++}, Fe^{+++}, Mg^{++}, Zn^{++} or Mn^{++} at 10^{-4} concentrations to the whole brei produced no increase in activity. Presumably the free concentration of the native metal is already saturating. Cu^{++}, Co^{++} and Mn^{++} actually produced some innibition. It has not yet been successfully attempted to remove the native metal before testing the effect of adding back these ions.

Distribution.

(a) In Tissues of Etiolated Pea Seedlings.

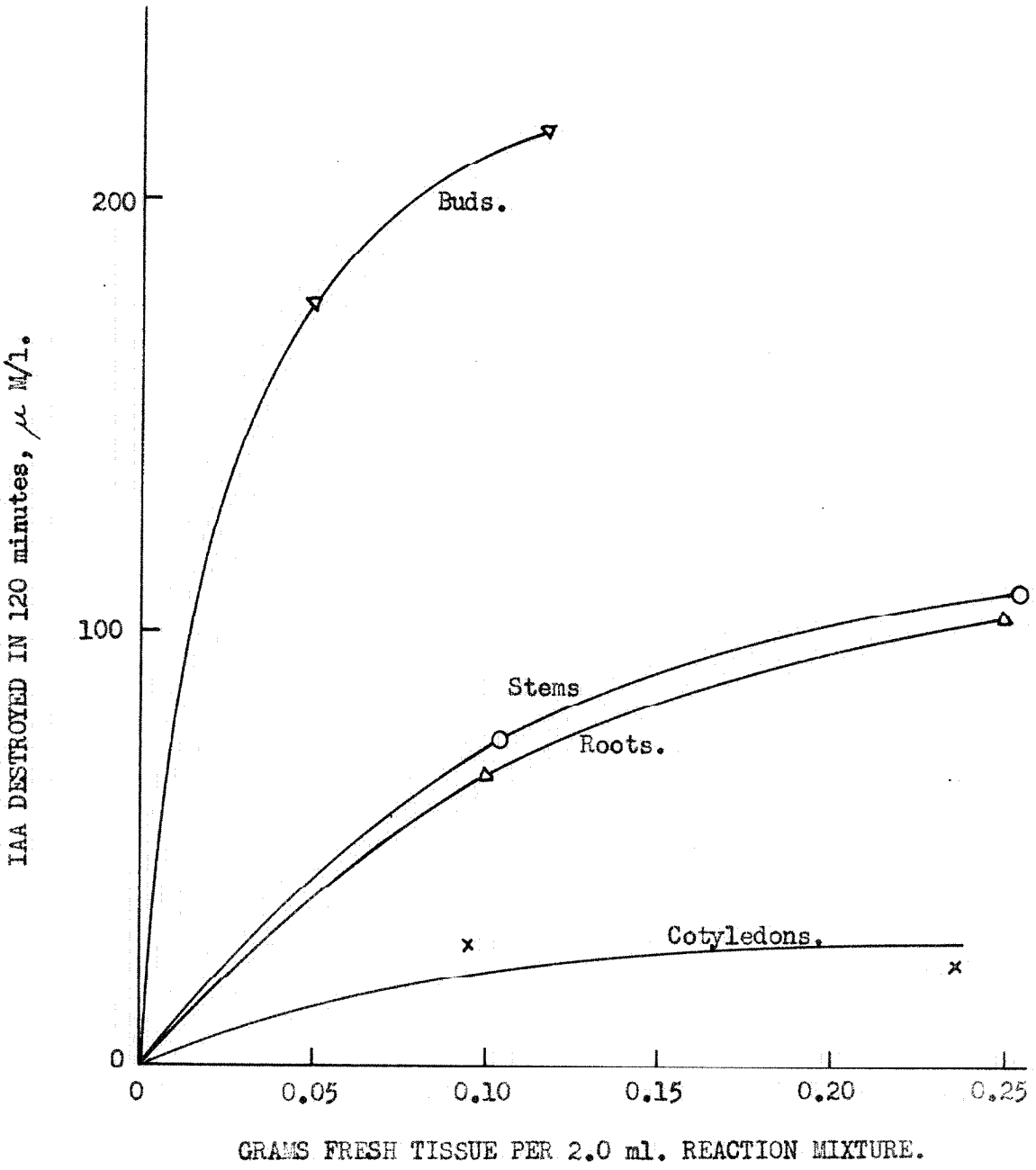
Pea seedlings grown for nine days in total darkness were dissected into apical buds, stems, cotyledons and roots. Weighed amounts of each fresh tissue were cooled and ground with sand in a cold mortar in a measured volume of cold M/15 phosphate buffer. Aliquots from the supernatants were placed

in cellophane bags and dialyzed overnight at 0°C. against equal volumes of buffer. The dialyzates were then assayed for activity in the usual way. Figure 36 shows that per fresh weight of tissue, the apical buds have manyfold greater activity than stems and roots which have 2 to 3 times the activity of the cotyledons.

(b) In Tissues of Green Pea Seedlings.

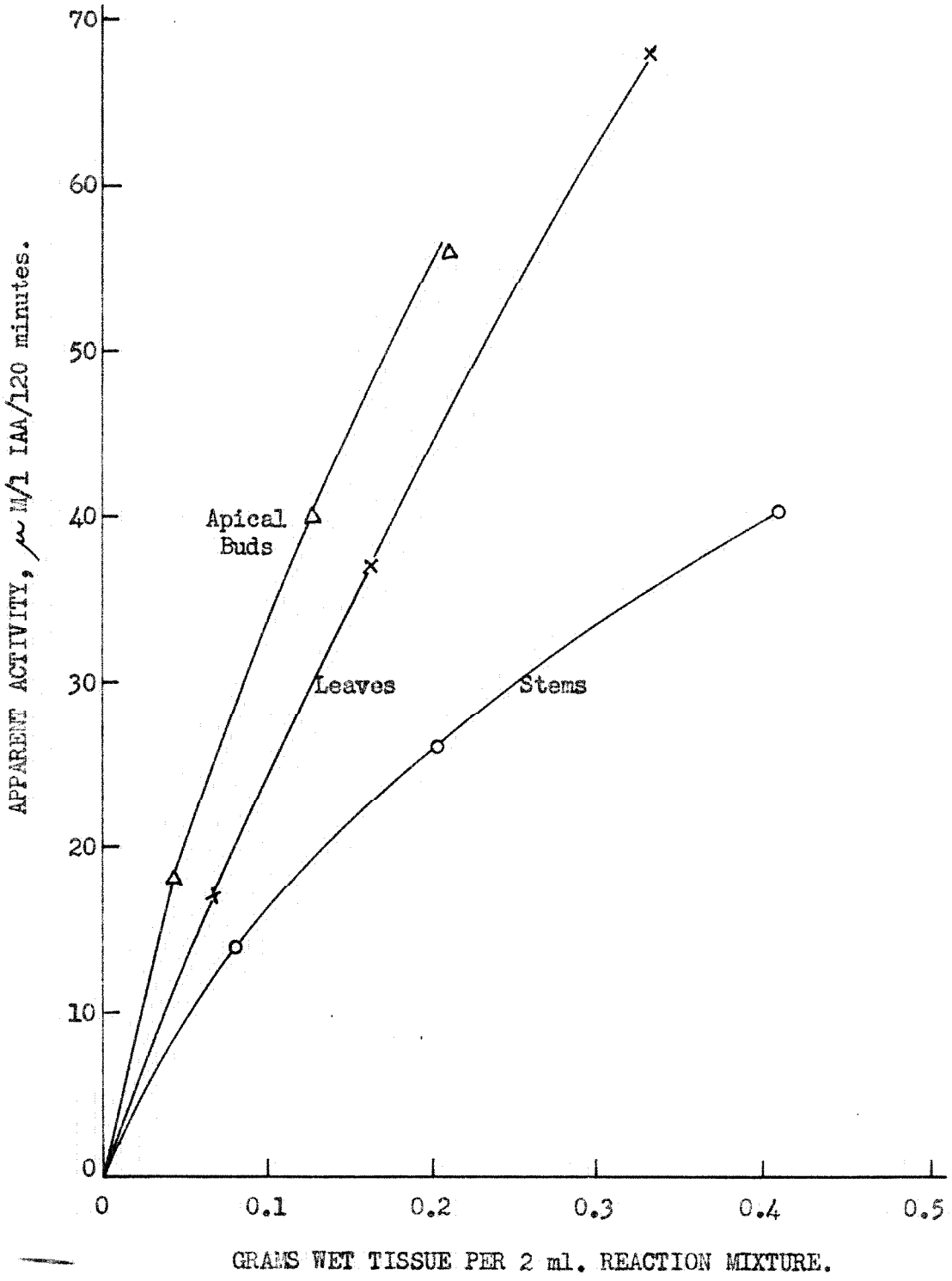
Pea seedlings were grown in vermiculite in the Dolk greenhouse for 15 days when the first three trifoliate leaves were fully developed. The epicotyls were separated into stems, leaves (including petioles and stipules) and growing tips (including leaf and stem in regions where the internodes had not yet elongated). Weighed amounts of each fresh tissue were ground with sand in a cold mortar in a measured volume of cold M/15 phosphate buffer, pH 6.6. After centrifuging, aliquots from the supernatants were placed in cellophane bags and dialyzed overnight at 0°C. against equal volumes of buffer. The dialyzates were assayed for activity in the usual way. Figure 37 shows the result of this experiment. However, further study has shown that with green tissues in general, only a small fraction of this "apparent activity" is in fact light-activated catalytic activity. For example, using the dialyzate from the green leaves prepared above, three experiments were done. Three concentrations of dialyzate were incubated in the light with I.A.A. for two hours. Another three concentrations of dialyzate were added to I.A.A. in the dark immediately prior to adding the Salkowski reagent. Figure 38 shows that

Figure 36.



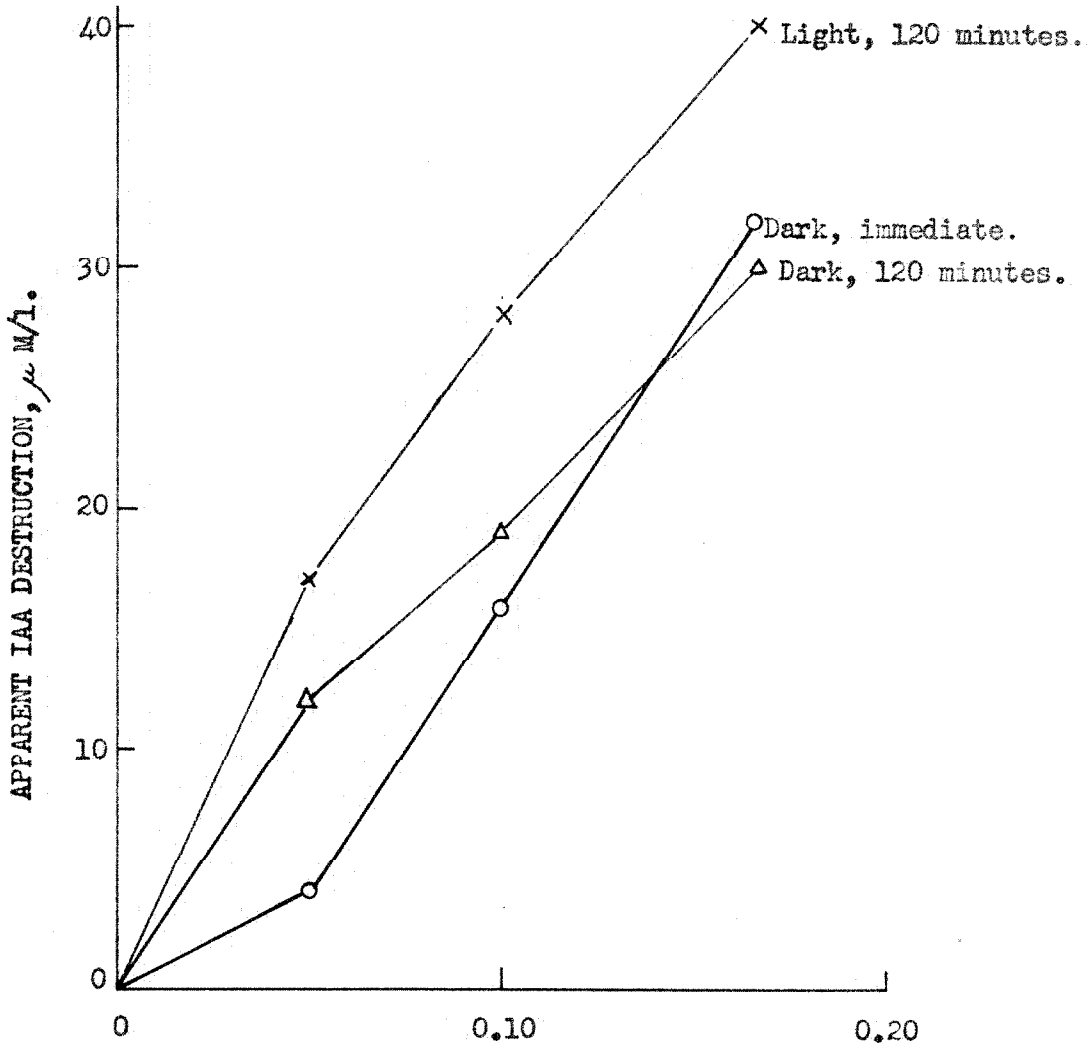
Distribution of activity of dialyzate in etiolated pea seedling tissues.

Figure 37.



Distribution of apparent activity of dialyzate in green pea seedling tissues.

Figure 38.



"Apparent" activity of green pea leaf dialyzate not due principally to a photocatalytic reaction.

most of the "activity" of the green leaf preparation is attributable to either an interference with the Salkowski reaction or to an instantaneous dark reaction with I.A.A. by some component of the dialyzate. A relatively minor activity is ascribed to catalytic photolysis. Similar results were experienced with green leaves of spinach, cocklebur and oat; catalytic activity being relatively greater in younger tissue. It is clear that the activity of etiolated tissue is much greater than that of green tissue.

Preliminary Purification Procedures. A number of studies has been made to determine the manner in which the activity of the dialyzate partitions when subjected to various fractionation procedures. These provide useful information concerning the physical and chemical properties of the active component. Owing to the limitation of time, isolation and identification have not been consummated in this work.

(a) Solubilities.

2.0 ml. dialyzate, neutral, made acid with dilute HCl or alkaline with dilute NaOH were shaken with an equal volume of diethyl ether, n-butanol, petroleum ether or chloroform. The organic solvent layer was separated off, evaporated under reduced pressure and the residues each taken up in 1.5 ml. M/15 phosphate buffer, pH 6.6. 0.5 ml. 10^{-3}M I.A.A. were added and activities determined in the light. In no case was any measurable activity obtained. The yellow color and most of the fluorescence also remained in the aqueous phase. It was found to be possible, however, to extract the active

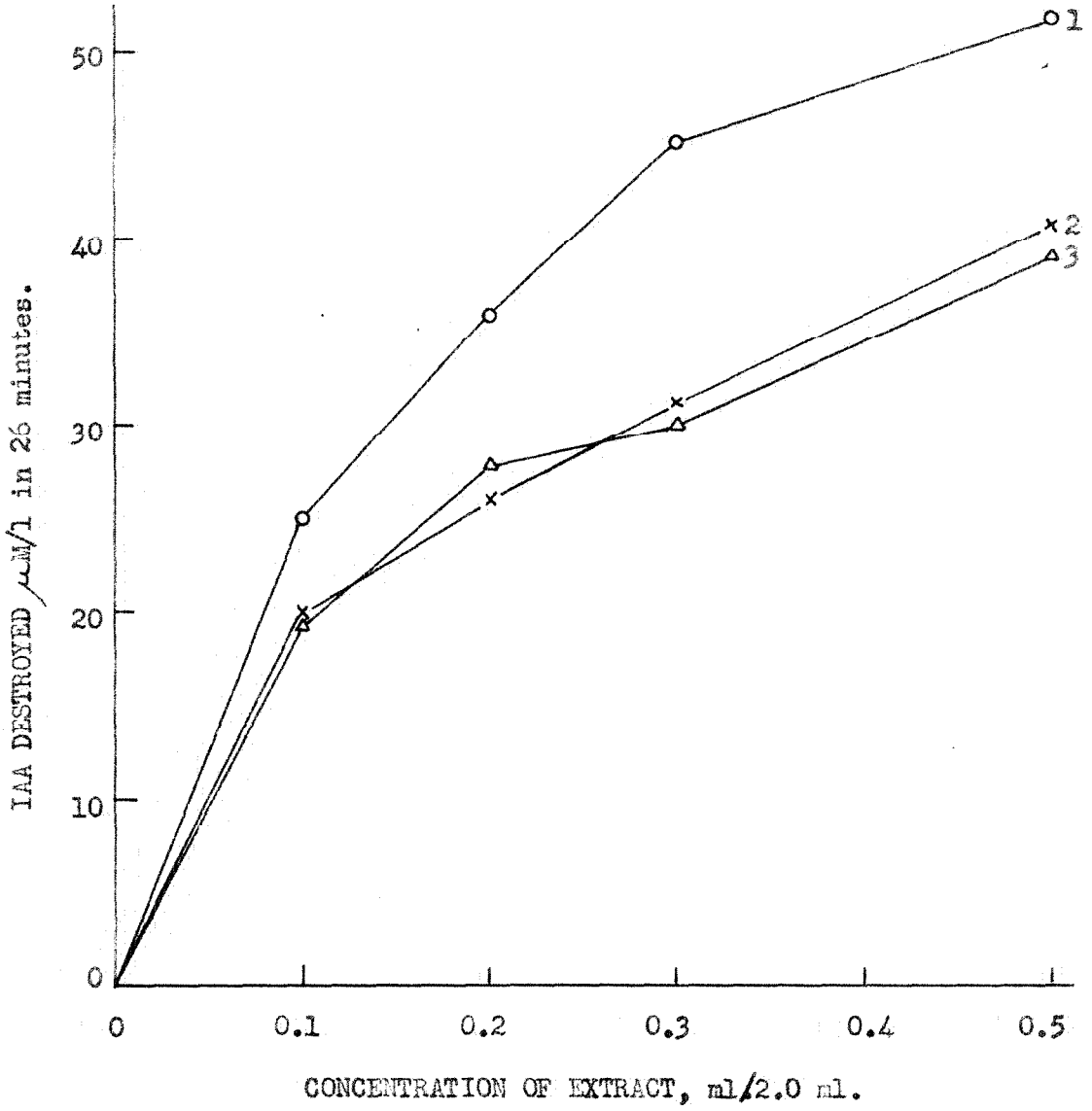
material of the dialyzate into phenol, or into acetone after saturating the aqueous phase with ammonium sulfate.

20 ml. dialyzate were placed in a separating funnel with 40 ml. aqueous saturated ammonium sulfate and extracted twice with 5 g. of phenol. Five volumes of freshly distilled ether were added to the phenol extracts and shaken with 15 ml. water then 5 ml. water. The aqueous extract was now assayed for activity at a series of concentrations. Curve 2, figure 39, shows that a large fraction of the activity was recovered.

10 ml. of dialyzate were saturated with solid ammonium sulfate and extracted twice with 20 ml. of acetone (if more than two volumes of acetone are added, water is withdrawn into the acetone phase precipitating the ammonium sulfate and leaving only one liquid phase). The acetone layer was separated off, evaporated to dryness at reduced pressure and at room temperature. The residue was dissolved in 10 ml. M/15 phosphate buffer, pH 6.6 and assayed for activity. Curve 3, figure 39, shows that a major fraction of the activity of the dialyzate was extractable into the acetone.

These two observations provide a useful procedure for the preliminary purification and concentration of the active material of the dialyzate. Phenol is preferable for this purpose because of the greater ease of returning the active material to the aqueous phase.

Figure 39.



Curve 1 = original ,dialyzate.
Curve 2 = aqueous extract of phenol extract.
Curve 3 = acetone extract.
Initial IAA concentration = 250 µM/l. Temperature = 26.5°C.

(b) Adsorbabilities.

A series of common adsorbents was tested to determine whether they might be useful in selectively taking up the active component. One ml. dialyzate in M/60 phosphate buffer, pH 6.6 was placed in each of a series of centrifuge tubes and a knife edge of the adsorbent to be tested added. After shaking a few minutes the tubes were centrifuged and 0.3 ml. supernatant taken for assay of activity in the usual way. Table 11 shows that the best adsorbents tested were charcoal, Lloyd's reagent and MgO-celite (3:1). The Amberlite cation exchange resin IR-100 and alumina adsorbed a negligible amount. There is no quantitative parallel between residual activity and residual color or fluorescence of the supernatant.

The common eluants for Lloyd's reagent (0.1 M $NaHCO_3$ at pH 7.5, 5%(v/v) pyridine at pH 8.7, 2%(v/v) 0.880 NH_4OH in water at pH 10.2 or acetone failed to yield more than 10% of the activity adsorbed to Lloyd's reagent at a single extraction.

(c) Chromatography.

Chromatographic fractionation of the dialyzate was used as a method for attempting to separate and examine some of the properties of the active components. Of the many adsorbents tested, silicic acid + Hyflo supercel (3 : 1) and powdered cellulose ("Solka-floc") proved most useful, particularly the latter, because simultaneous runs on an analytical scale were able to be made on filter paper, giving approximately the same rates of movement. The locations of the active spots on the filter paper were determined in the following manner. After

Table 11

The effectiveness of adsorbents for removing the active component of the dialyzate from solution at pH 6.6.

Adsorbent	Residual color* of Supernatant	Residual Fluorescence* of Supernatant	Ratio final : initial activity
None	+ + +	+ + +	1.00
Fuller's earth	+	+ +	0.55
Lloyd's reagent	0	+	0.11
Silicic acid	+	+ + +	0.87
MgO-celite (3:1)	+ + +	+ +	0.27
Amberlite IR-100	0	+	0.97
dry Al_2O_3	+	+ +	1.03
Powdered charcoal	0	0	0.09

* Estimated visually.

development of the chromatogram it was air-dried and momentarily dipped in a solution of 10^{-3}M I.A.A. in water-saturated ether. After evaporation of the ether the papers were suspended horizontally in an atmosphere saturated with water vapor beneath a bank of fluorescent lamps. After incubating for an hour the paper was sprayed with modified Salkowski reagent and placed in a 100°C. oven for 30 to 60 seconds. The active spots appear white on a pink background. The modified reagent consists of M/100 ferric chloride in 6% perchloric acid. Sulfuric acid of the original Salkowski reagent prevents drying of the papers leading to loss of sharpness of the spots. Higher concentrations of perchloric acid or over-heating result in charring of the paper. The same technique was used as a rough quantitative assay for the I.A.A.-destroying activity of drops taken from eluate fractions from the adsorption columns.

In order to obtain a concentrated preparation for placing on the chromatograms, deproteinized homogenate was extracted into phenol then back into water after adding excess ether, as described in a previous section.

A 12 x 1.5 cm. column of silicic acid + Hyflo (3 : 1) was dry-packed under suction and well washed with water. 2.0 ml. concentrated dialyzate were pipetted on and the chromatogram developed with water. A yellow colored band which fluoresced yellowish-blue under ultra-violet light travelled close to the front. It contained most of the I.A.A.-destroying activity and when chromatographed on paper with water as solvent (ascending) it separated into three fluorescent spots

at Rf = 0.32 (yellow fluorescent), 0.65 (blue fluorescent) and 0.85 (blue fluorescent), most of the I.A.A.-destroying activity coinciding with the fluorescence at Rf 0.85. A second band, reddish-brown in color and fluorescing blue remained at the top of the column but was readily eluted with M/1000 HCl or acetone. It had appreciable I.A.A.-destroying activity and when chromatographed on paper in water both the activity and the fluorescence moved at Rf = 0.85.

Although both fractions containing the pigmented bands sensitize the decomposition of I.A.A. in visible light (see table 8) no absorption maxima could be detected in the visible region presumably because the concentrations were too low. The leading band shows a sharp absorption maximum at 267 mμ and a minimum at 237 mμ while the band requiring elution with dilute HCl shows a maximum at 263 mμ and a minimum at 249 mμ. However, in the present crude state these spectra cannot be attributed to the active compounds.

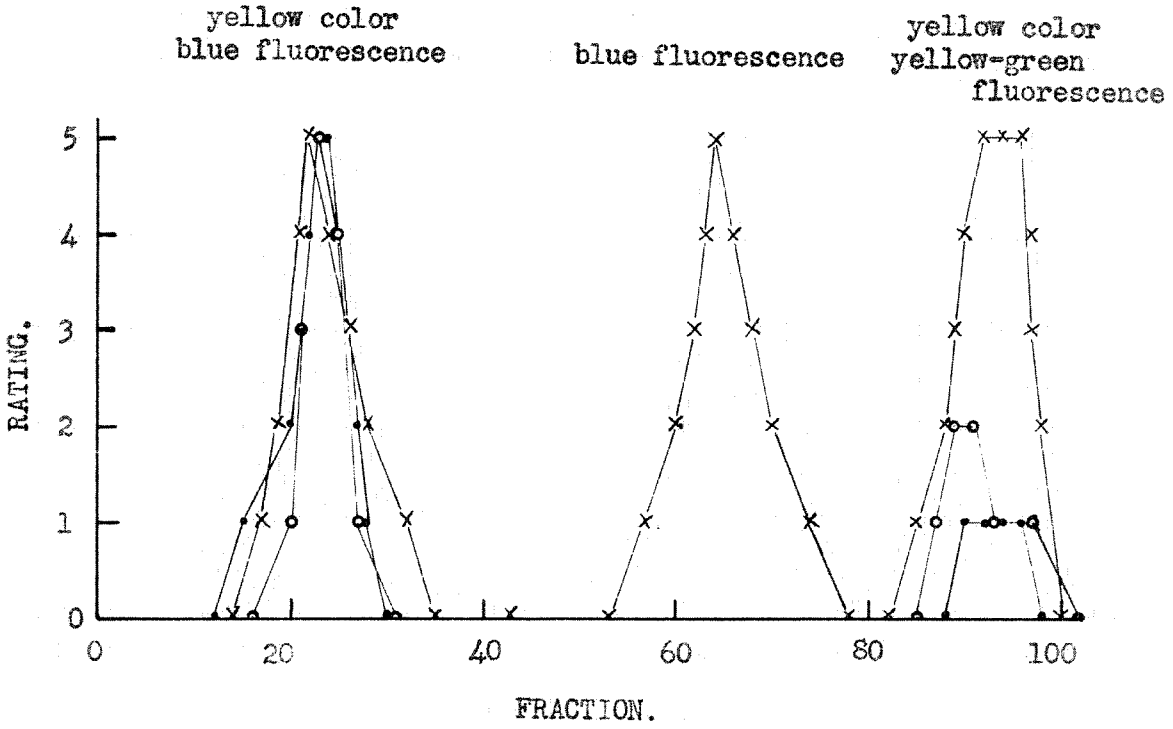
A column of powdered cellulose 2.2 x 50 cm. was filled dry under suction and well washed with water. 2 mls. concentrated dialyzate were placed on the column which was developed with water. The first 80 mls. were discarded then 2.8 mls. fractions were collected. The fractions were numbered, randomized and subjectively evaluated for color and for intensity of fluorescence under ultra-violet light assigning each a rating from 0 to 5. The fractions were also assayed for I.A.A.-photolyzing activity by placing a drop on filter paper, drying, dipping the paper into an ether solution of

I.A.A., incubating in the light for one hour then spraying with the modified Salkowski reagent. Figure 40 shows that the I.A.A. photolyzing activity was confined to two peaks, both of which were colored and fluorescent. The slower peak (fraction #90) which was of lesser activity fluoresced yellow-green and when rechromatographed on paper it travelled identically with riboflavin in several solvents. Fraction #22 was examined in the Beckman spectrophotometer but no absorption peak in the visible was obtained. The absorption curve rose sharply at 420 mμ reaching a maximum at 265 mμ and a minimum at 242 mμ.

Three similar fluorescent bands were obtained by chromatographing the original concentrated dialyzate on paper, using water as a solvent. Most of the activity resided in the fastest-moving spot (Rf 0.85), none in the center spot (Rf 0.65) and a small amount in the slowest spot (Rf 0.32), 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 fluorescence (see table 12). A second fluorescent spot was devoid of measurable activity. Thus the dialyzate is shown to contain at least four compounds active in photolyzing I.A.A. 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 chromatograms

Figure 40.



Fractionation of dialyzate on a powdered cellulose column, using water as solvent.

• = color.
o = IAA-photolysing activity.
× = fluorescence.

Table 12

Separation of the active components
of the dialyzate by chromatography on paper.

Spot	Rf in		Fluorescence under ultra-violet	I.A.A.-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	—

were sprayed with 5×10^{-4} M 8-hydroxyquinoline before dipping the papers into the I.A.A. solution but in no case was the activity of any spot destroyed. It was not possible to put enough of the dialyzate onto the paper to test the spots for activity after eluting. It seems that either the reaction is so rapid on paper that even an almost totally inhibited compound can still sensitize the destruction of I.A.A. or that during the process of chromatography the dissociable metal has been separated from its organic moiety and does not appear among the active spots.

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

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

The dialyzate contains at least four active components. A major component depends for its activity on a heavy metal.

The kinetic behavior of the whole dialyzate has been studied in detail. The apparent K_s is 3×10^{-4} and the reaction is first order with respect to I.A.A. The net pH optimum is 5.5. The temperature optimum is 25°C. at high light intensities. At low light intensities there is no temperature dependence. It is concluded that one or more dark reactions follow. The activity is unstable to heating or

to exposure to visible or ultra-violet light. The inactivation of I.A.A. 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.

Preliminary purification procedures have been devised, involving extraction into phenol or acetone and chromatography on powdered cellulose. A technique is described for detecting the presence of the active compounds on developed paper chromatograms.

DISCUSSION

In the experimental section a description was given of certain aspects of the behavior of systems which inactivate indole-3-acetic acid (I.A.A.) *in vitro*. These comprise a complex of enzymes containing two alternative members, one with a cofactor requirement and one without, and a group of dialyzable substances, resolved into four or more members which sensitize the photoinactivation of I.A.A. An important question arises, namely, what the physiological activity of these systems are. This question is not simple to answer, nor is it even clear, at this present state of knowledge, how to devise experiments to obtain this information. Certain observations provide us with some clues, which, together with a little speculative thinking may provide a basis for further experimentation.

The first question which arises is whether this enzyme system or the active components of the dialyzate, which have a relatively low affinity for the I.A.A. (apparent K_s values above 10^{-4} M) can successfully influence the growth response of a tissue by regulating the very low concentrations (10^{-7} to 10^{-10} M) of I.A.A. present in the tissues. The responses of plant tissues to auxins are among the most sensitive of biological phenomena. In experiments in which the growth of *Avena* coleoptile sections floated in a series of I.A.A. solutions was measured, it was recently calculated that the K_s for the complex between the auxin and the receptor site with

which it reacts before evoking the growth reaction is approximately 3×10^{-7} M (81). For etiolated pea stems it is slightly lower. Since this is three orders of magnitude lower than that for the enzyme, the enzyme would compete unfavorably against the receptors for the combination with the free I.A.A. for intracellular concentrations of I.A.A. at which the receptors are unsaturated. Thus in a tissue continuously supplied with I.A.A. the enzyme concentration in the cell would have to approach one thousand times that of the receptors in order that a change in the activity of the enzyme would be reflected in a changed growth response. At present there is no way of assessing this concentration ratio.

On the other hand, in tissues where the growth appears to be limited by supraoptimal concentrations of I.A.A., e.g., roots, lateral buds (56, 117), the I.A.A. oxidase may be expected to destroy free I.A.A. and reduce the inhibitory effects.

The hypothesis for the interaction of auxin with an intracellular site depends for its substantiation in the experimental data upon the assumption that the auxin concentration at the site is a linear function of the externally applied auxin concentration. An evaluation of the molar K_s further assumes that the concentration at the site is the same as that in the external medium. Thus in comparing the K_s values calculated in this way for the same auxin between two tissues a measured difference may be due to (a) a difference in the penetration

and transport of the auxin to the site or (b) to a real difference in the dissociation constants between auxin and receptor-site. For dark-grown pea stem sections floated on solutions of I.A.A., the apparent K_s is approximately 10^{-7} M. For stem sections of green peas it is approximately 10^{-5} M (116). If these differences really represent differences in the dissociation constants for the receptor site-auxin complex, then the role of I.A.A. oxidase and the effect of changing its activity, e.g., by illumination (84), in these two tissues may be quite different.

It would seem that a useful tool for studying whether I.A.A. oxidase regulates the intracellular concentration of I.A.A. would be 2,4-dichlorophenol (D.C.P.), which has been shown to increase the in vivo destruction of high concentrations of externally applied I.A.A. (figure 16). It would be expected that D.C.P. would produce symptoms of reduced auxin concentration, provided I.A.A. is a major auxin of the tissue tested. Systematic quantitative experiments have not yet been carried out on this question but a few observations may be pertinent.

Bean seedlings treated with D.C.P. either in aqueous solution applied to the hypocotyl through a wick or in lanolin applied to the leaves showed some reduction in the rate of elongation of the internodes.

Pea seedlings grown in the dark in water culture containing 10^{-5} or 10^{-4} M D.C.P. showed no significant differences from the controls in internode length, total epicotyl length,

root length or tendency for the lateral buds to develop.

In another experiment pea epicotyl sections were floated in a series of solutions containing various concentrations of I.A.A., with and without 3×10^{-5} M D.C.P. Those grown in the D.C.P. made less growth than the controls. When the experiment was repeated using as the auxin α-naphthalene acetic, which is not attacked by I.A.A. oxidase (2), a depression in growth was similarly produced by D.C.P. Thus an experiment of this kind is ambiguous because D.C.P. is inhibiting growth in another way, possibly by "uncoupling" oxidative phosphorylation.

A more specific way in which to study this question would be to look for a positive effect expected as a result of lowering the free auxin concentration, e.g., increased root elongation, bolting of lateral buds or induction of flowering in short-day plants. In a preliminary experiment it was grossly observed that roots induced to form on stem cuttings of Xanthium in Hoagland's solution produced a greater growth in length in the presence of 10^{-5} M D.C.P. No precise measurements have been made, however, and this line of work has not been pursued owing to lack of time.

Within the limits of the previous considerations concerning the ability of the enzyme to influence the growth reaction we may go on to discuss the possible functions of I.A.A. oxidase in addition to the normal maintenance of I.A.A. balance. One interesting property of the enzyme is its activation by light (84). It is therefore important to examine the

possibility that the activity either of this enzyme or of the dialyzate may enter into responses of plants to light known to involve changes in auxin concentration. Since the enzyme is activated only by blue light (84) it could act as photo-receptor only in those physiological reactions responding primarily to blue light. Similarly, in the case of the dialyzate, the shorter wavelengths are the most effective although there may be a second smaller peak in the red (table 8). Since this is a composite action spectrum due to four or more components it would be important to resolve them and study the reactions separately. At present, however, it is not known whether the active components of the dialyzate reside in the cytoplasm or in the vacuole, so it cannot yet be decided whether they are potentially able to exert a physiological effect.

It has already been proposed (84, 60) that the light-activation of I.A.A. oxidase may be of significance in the phototropic response of plants. Briefly, the main evidence for this suggestion are the following. (a) Exposure of Avena coleoptiles to phototropically active light doses results in a "redistribution" of the native auxin, a reduced concentration being found on the illuminated side (15). (b) The action spectrum for phototropic curvature (118, 119, 84) corresponds closely to the absorption spectrum of riboflavin and of flavoproteins as well as to that of carotenes (84); it is also very similar to the action spectrum for the light-activation of I.A.A. oxidase (84). (c) Flavins occur abundantly in

phototropically active organs (84). In a mutant strain of albino corn which is phototropically active, carotenes were found to be virtually absent while the flavin content was normal (120). Thus although carotenes have been shown to sensitize the photoinactivation of auxin a (22), the above observation eliminates these compounds as the receptors for the phototropic stimulus in the corn coleoptile.

Although light may in addition influence the auxin-producing and auxin transport processes, it does at present seem that the activation of I.A.A. oxidase by blue light may be of importance in initiating phototropic curvature.

If I.A.A. oxidase contributes to the regulation of the I.A.A. content of an organ it may be expected that the reduced sensitivity of tissues to I.A.A. after exposure to red light (121) could be due to a light-induced increase in the total activity of the enzyme. The enzyme would thus be of some significance in certain of the processes associated with deëtiolation (122). It has in fact been observed (85, 121) that exposure of pea seedlings to low intensity red light, which induces the reduced elongation of stems, enlargement of leaves, etc., also induces a change in the I.A.A. oxidase content such that not only the activity but also the specific activity is much increased. Despite this change, it would seem that the enzyme must play a relatively unimportant role in red-light induced deëtiolation because although the sensitivity of the growth response to I.A.A. is reduced, so

also is the response to other auxins (e.g., 2,4-D, α-napthalene acetic acid) (82), which are not attacked by the enzyme.

Biochemically, the principal studies remaining to be made on the I.A.A. oxidase system are to fractionate the enzyme complex into its components and to study independently the chemical steps in the degradation of I.A.A. This would include the isolation and identification of the cofactor.

With regard to the dialyzate, the resolution and chemical identification of the various components would permit their ready assay in the tissues and their study in clean systems, leading to experiments that may determine their physiological role in plant tissues.

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