Isolation of Indole-3-ethanol Oxidase from Cucumber Seedlings[1]

Larry E. Vickery[2] and William K. Purves
Department of Biological Sciences, University of California, Santa Barbara, California 93106

ABSTRACT

Previous work in this laboratory has shown that cucumber (*Cucumis sativus* L.) seedlings contain large amounts, relative to other indolic compounds, of extractable indole-3-ethanol (IEt); tracer studies have established that IEt is metabolized to IAA. We have now succeeded in isolating an enzyme from these seedlings which catalyzes the oxidation of IEt to indole-3-acetaldehyde (IAAld). The identification of the product as IAAld was based on solvent partitioning of the free aldehyde and its bisulfite adduct and radiochromatography following incubation of enzyme with ^{14}C-IEt. A novel, quantitative colorimetric test for IAAld was also developed utilizing the Salkowski reagent. Partial purification of the enzyme was achieved by salt gradient chromatography on Bio-Rex 70, heating the preparation to 70 C, and chromatography on Sephadex G-150. This purification procedure yielded an enzyme activity purified in excess of 3000-fold, and studies on a standardized Sephadex column suggest a molecular weight of the enzyme of approximately 105,000. The reaction was found to proceed only aerobically; and, in the absence of other electron acceptors, O_2 appears to be reduced to H_2O_2. The enzyme has nearly maximum activity from pH 8 to 11.

Several pathways have been proposed for the biosynthesis of IAA from tryptophan in higher plants and in bacteria (1, 21). Unfortunately, a majority of the evidence for the positioning of the intermediates is indirect, being based primarily on experiments in which small amounts of metabolites were analyzed chromatographically following incubation of tissues in solutions of various indolic compounds. Differing conditions of precursor concentration, incubation time, and bacterial contamination, as well as choice of plant material, have precluded any general agreement on the pathway(s) involved. In order to circumvent the problem of nonspecific conversions which might occur when precursors are exogenously supplied, we sought first to establish the natural occurrence of the intermediates, and secondly to isolate enzymes specific for the proposed reactions.

Work in this laboratory directed toward determining the presence of indolic compounds in cucumber seedlings has, over the last 4 years, resulted in the positive identification only of IEt[3] (14). This compound also appears to be found in sun-

flower (11, 16), pea (11), zucchini squash (16), and tomato seedlings (17). Two lines of evidence suggest that in cucumber, at least, IEt is formed via tryptamine: isotopic labeling studies have shown that exogenously supplied tryptamine can be converted to IEt (18); moreover, tryptamine is a potent growth promoter in the cucumber hypocotyl test while indole pyruvate and indole lactate are less active (D. L. Rayle and W. K. Purves, unpublished data). That IEt is oxidized to IAA in cucumbers is supported by the parallel in growth promotion elicited by IEt and IAA (10, 14) and by the rapid metabolism of IEt-^{14}C to IAA-^{14}C (15). It has further been shown that the conversion of tryptamine to IEt and of IEt to IAA is not dependent upon the presence of epiphytic microorganisms (18).

IAAld would seem to be a likely intermediate between IEt and IAA. Larsen first suggested IAAld as the probable immediate precursor of IAA (4), and subsequent work has supported the precursor role of this compound (12, 13, 20), although it has been isolated and chemically characterized only from pea seedlings (6, 11).

In view of the fact that IEt appears to be present in higher concentrations than other indolic compounds in cucumber, it was of interest to attempt to isolate an enzyme from these plants which would catalyze the oxidation of IEt to IAAld as a further step toward understanding the auxin economy of these seedlings. We report here the development of a simple, quantitative colorimetric test for IAAld and the application of a large scale batch extraction procedure which has enabled us to purify partially an IEt:O_2 oxidoreductase from light-grown cucumber seedlings.

MATERIALS AND METHODS

Preparation of Enzyme. Seeds of *Cucumis sativus* L. cv. National Pickling (Burpee) were sown in vermiculite and grown under light and temperature conditions as previously described (14). After 8 to 10 days the shoots were harvested and weighed. The following operations were all carried out at 4 C. The seedlings were extracted by grinding in a Waring Blendor, using 2 liters of 50 mM sodium phosphate, pH 8.0, per kg of tissue. The homogenate was squeezed through cheesecloth and centrifuged at 20,000g for 30 min. The supernatant fluid was decanted and brought to a final volume of approximately 4 liters per kilogram of tissue with 20 mM sodium phosphate, pH 7.5, hereafter referred to simply as buffer. A batch extraction procedure was carried out 2 times with the diluted supernatant fluid, as follows. Approximately 100 ml (wet volume) of Bio-Rex 70 resin (Bio-Rad Laboratories) was added to each 4-liter beaker and stirred for 2 hr. The resin was allowed to settle, and the supernatant was decanted for another extraction. The resin from each extraction was successively poured into a column of an appropriate size and washed with 20 mM buffer until the eluate was clear. Enzyme was then eluted with 0.5 M NaCl in 50 mM buffer. This concentrated enzyme solution was dialyzed overnight against 50 mM buffer, pH 7.5, centrifuged clear, and adsorbed onto a 2.1- × 15-cm Bio-Rex

[3] Abbreviations: IEt: indole-3-ethanol; IAAld: indole-3-acetaldehyde.

70 column in the same buffer. The column was eluted with a linear salt gradient, the first stage containing 500 ml of column buffer and the second containing 500 ml of buffer which was 0.5 M in NaCl. Fractions (8 ml) were collected, and aliquots were assayed with 0.4 mM IEt; those exhibiting activity equal to 50% or more of the most active fraction were combined. This solution was heated at 70 C for 15 min, rapidly cooled to 4 C, and centrifuged at 40,000g for 10 min. The supernatant fluid was dialyzed against 50 mM sodium phosphate, pH 8.0, and used for further studies. One batch was adsorbed onto a 1- × 2-cm column of Bio-Rex 70. The enzyme was eluted in 2 ml with 0.5 M NaCl in buffer and applied to a 1.5- × 46-cm column of Sephadex G-150 (Pharmacia) equilibrated with 0.1 M buffer, pH 7.5. This column was standardized for a molecular weight estimation by determining the elution volumes of 2-mg quantities of nonenzymic protein markers (Mann Research Laboratories) and Blue Dextran 2000 (Pharmacia). Fractions (2 ml) were collected at a flow rate of 15 ml/hr.

Assay Procedures. Absorbancy measurements were made with a Beckman Model DB spectrophotometer. Spectra were obtained with a Cary Model 15 recording spectrophotometer. Measurements of pH were made with a Radiometer Model 26 pH meter. Protein was determined according to the method of Lowry et al. (8).

IEt, IAA, and IAAld-bisulfite were obtained from Sigma. The latter compound was purified and recrystallized as recommended by Larsen and Klungsoyr (7). When it was necessary to liberate free IAAld, an aqueous solution of the bisulfite adduct was treated with Na_2CO_3 to bring the pH to approximately 10 and extracted 3 times with an equal volume of peroxide-free ether. The ether solution was then washed twice with small volumes of water. This solution was added to an appropriate volume of water, and the ether was removed under vacuum.

Two reagents for colorimetric tests were used. The sulfuric acid reagent (9, 19) contained 30 ml of H_2SO_4 (specific gravity, 1.84), 50 ml of H_2O, and 1.6 ml of 0.5 M $FeCl_3$; the perchloric acid reagent (2, 3) consisted of 25 ml of 70% $HClO_4$, 25 ml of H_2O, and 1.0 ml of 0.5 M $FeCl_3$. Two volumes of reagent were added to each volume of test solution; absorbance readings were made, after 30 min, against reagent blanks. Initial rates of enzyme-catalyzed reactions were extrapolated from measurements at various times.

In experiments designed to determine the pH optimum of the reaction, an enzyme solution was dialyzed overnight against 1,000 volumes of 50 mM NaCl. Aliquots of the enzyme solution (0.2 ml) were mixed with 1.2 ml of an 0.1 M solution of the appropriate buffer. The reaction was initiated by the addition of 0.6 ml of substrate. The pH was measured at 5 min and aliquots of the reaction mixture were treated with perchloric acid reagent at 10, 20, and 30 min to determine an initial rate. All reactions were carried out at room temperature (22 C).

Assay with Labeled Substrate. ^{14}C-IEt was prepared as previously described (15). 2-^{14}C-Indole (Schwarz Bioresearch, Inc., 100 μc) was converted to 2-^{14}C-IEt with a specific radioactivity of 0.2 mc per mmole at an overall yield of 65%. The purity of the product was checked by thin layer chromatography on silica gel (Eastman Chromagrams, 6061) in 3 solvent systems. The 4-ml reaction mixture for the tracer study contained 0.2 mM ^{14}C-IEt and 0.36 mg protein in 50 mM sodium phosphate, pH 8.0. After 90 min, 5 ml of 10 mM $NaHSO_3$ was added, and the pH was adjusted to between 5 and 6 with acetic acid. The reaction mixture was then extracted 3 times with 15 ml ether; these ether fractions were combined and termed the neutral fraction. The aqueous phase was adjusted to approximately pH 10 with 2 ml of 5% Na_2CO_3 and was extracted similarly for
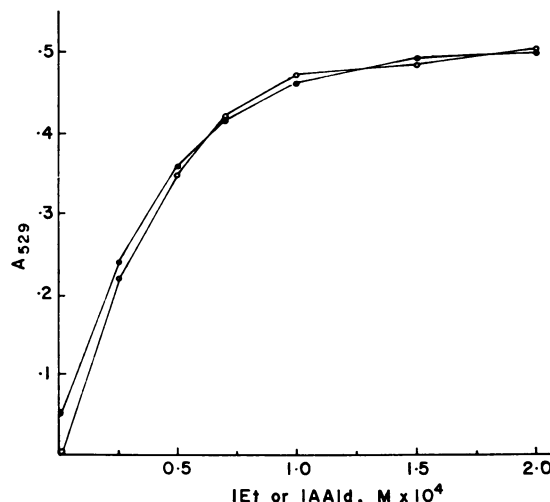

Fig. 1. Saturation curves for the colorimetric estimation of mixtures of IEt and IAAld using perchloric acid reagent. O, varying IAAld concentrations in the presence of 50 μM IEt; ●, varying IEt concentrations in the presence of 50 μM IAAld.

basics and aldehydes. The pH of the water phase was then adjusted to near 2.5, and the solution was extracted with ether for acidic compounds. Radioactivity of each fraction was determined using Spectrafluor PPO-POPOP (Amersham/Searle Corp.) and a Nuclear Chicago 720 series liquid scintillation system. Aliquots of certain fractions were also chromatographed on Eastman Silica Gel Chromagrams. These sheets were then scanned on a Nuclear Chicago Actigraph III strip-scanner.

RESULTS

Colorimetric Assay. The Salkowski test for IAA is widely used and generally regarded as being moderately specific for that compound (2, 3, 9, 19). In fact, techniques have been developed for the oxidation of IAAld to IAA for estimation of IAAld (5, 7), since the color developed by the aldehyde when treated with the Salkowski reagent under similar conditions is unsuitable for quantitative measurements. During the course of studying an enzyme believed to produce IAAld, however, positive Salkowski reactions with both the perchloric acid and sulfuric acid reagents were observed when either reagent was added to the reaction mixture. This was found to be due not to the presence of IAA, but rather, to the presence of a combination of IEt and IAAld. IAA yields a strong pink color with an absorption maximum at 529 nm; IEt alone gives a pale yellow color, while IAAld gives a very weak pinkish color with maximal absorption below 500 nm. For this reason, a quantitative assay for IAAld was developed based on its color reaction with the perchloric acid reagent in the presence of IEt. Figure 1 shows a saturation-type curve for the color developed by IEt or IAAld in the presence of increasing amounts of the other compound. It is seen that about a 4-fold excess of one component is required for maximal color development of the other. Since Beer's law does not appear to hold at high concentrations, a standard curve for up to 0.1 mM IAAld in the presence of 0.4 mM IEt is shown in Figure 2. The final color developed with IAAld is compared to that seen with IAA alone. Providing that an excess of either IEt or IAAld is present along with the component to be estimated, the technique is twice as sensitive as one based on conversion to IAA. All enzyme assays were made under conditions such that substrate was in large excess over product. Further experiments are underway to investigate both the specificity and stoichiometry of the reaction.
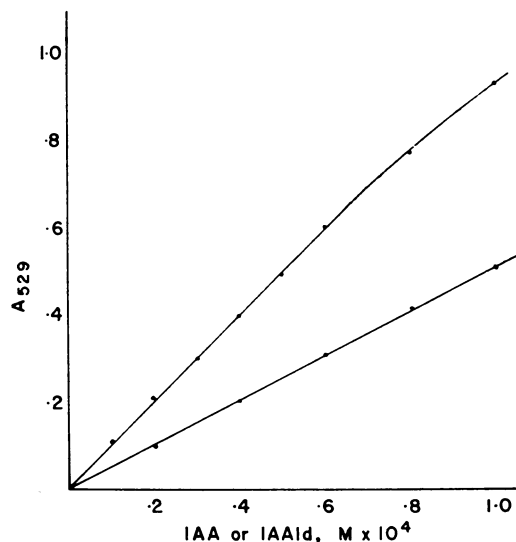
FIG. 2. Standard curve for the estimation of IAAld concentrations in the presence of 0.4 mM IEt (upper curve). Lower curve is a standard used for the estimation of IAA. Perchloric acid reagent was used.
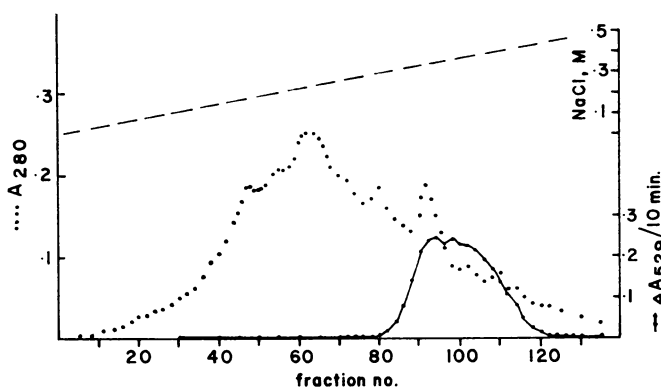
ers, and a considerable spread of activity was observed. The results of one experiment designed to assay the stability of the enzyme to heating are shown in Figure 4. The relative heat stability of the enzyme was used as a further purification step. The yields of the various steps used in the isolation are summarized in Table I. One preparation of the enzyme was also subjected to chromatography on Sephadex G-150, and this step yielded an additional purification of about 2-fold. The molecular weight estimated for IEt oxidase based on its elution volume on Sephadex G-150 is approximately 105,000 (Fig. 5).

Identification of Product. Since IAAld gives a positive Salkowski test in the presence of IEt, reactions were stopped, and the product was measured by the addition of Salkowski reagent directly to the reaction mixture after various times of incubation. Similar results were observed using either the sulfuric acid reagent (19) or the perchloric acid reagent (2), but the latter method was used for increased sensitivity. The visible absorption spectrum obtained by adding the perchloric acid reagent to the reaction mixture is compared to that of a 1:1 mixture of IEt and IAAld and to that of IAA similarly treated with Salkowski reagent in Figure 6. The curves appear quite similar except that the IEt-IAAld mixture and the reaction mixture both have a characteristic shoulder around 450 nm.

FIG. 3. Ion-exchange chromatography of IEt-oxidase of batch extractions from 500 g of tissue. For details see text.

Table I. *Purification Procedure for Cucumber IEt-Oxidase*

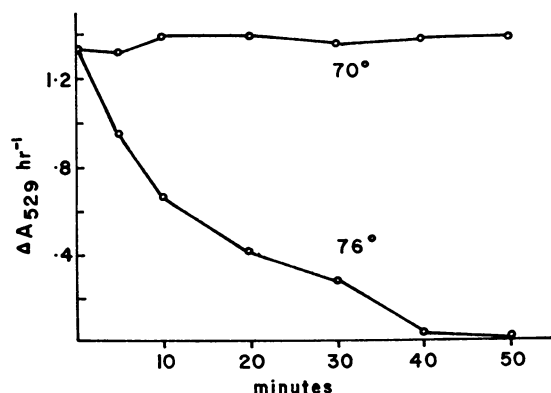| Step	Total Protein	Total Activity Units	Specific Activity	Fold Purification
	mg	*μmoles/hr*	*units/mg*	
1. Crude extract	4,850	32	0.007	1
2. Batch extract I	60	29.5	0.49	74
Batch extract II	33	6.5	0.20	30
Pool I and II	90	34	0.38	58
3. Gradient chromatography	7.5	28	3.7	570
4. Heat treatment	1.8	28	15.5	2,350

FIG. 4. IEt oxidase activity as a function of time of pre-heating at 70 and 76 C. Activity was measured at room temperature after cooling and centrifuging samples and represents an extrapolation of initial rates of conversion to 1 hr. The initial protein concentration was 40 μg/ml; IEt was 0.4 mM; sodium phosphate was 50 mM, pH 8.

Isolation of IEt Oxidase. The elution profile for salt gradient chromatography of IEt oxidase activity is shown in Figure 3. At neutral pH, the enzyme bound strongly to cation exchang-
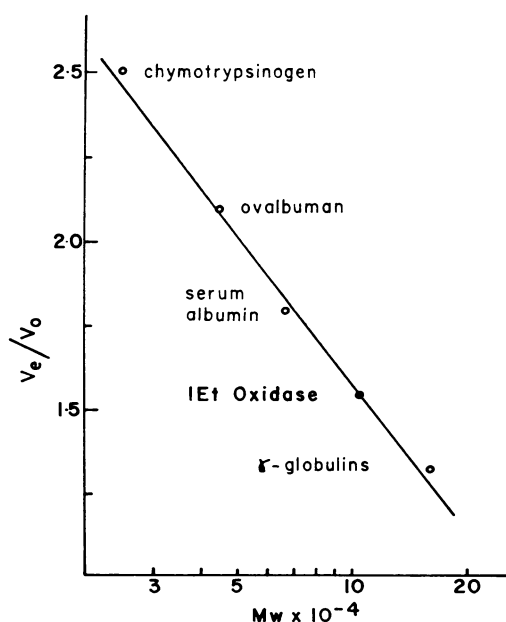

FIG. 5. Molecular weight estimation of IEt oxidase. Plot of relative elution volumes against log (molecular weight) for proteins on Sephadex G-150.

Confirmation of IAAld as the product of the reaction was obtained from tracer studies. ¹⁴C-IEt was incubated with enzyme for 90 min. NaHSO₃ was added to the reaction mixture to trap ¹⁴C-IAAld, and the remaining ¹⁴C-IEt was extracted with ether. The ¹⁴C-IAAld-bisulfite was exposed to basic pH, and the free IAAld was extracted. An additional ether extraction at acidic pH was made. The total amount of radioactivity appearing in each fraction is shown in Table II. Aliquots of each of the fractions showing significant radioactivity were chromatographed in two solvent systems. Traces of the strip scans are shown in Figure 7. All of the radioactivity extracted into the neutral fraction I after incubation with boiled enzyme chromatographs as IEt (Fig. 7, A and A'). The neutral fraction I obtained from the reaction mixture appears to contain some IAAld (Fig. 7, B and B'), but the majority of the label not associated with IEt is extracted into fraction II, which should contain basics as well as aldehydes liberated from bisulfite (Table II and Fig. 7, C and C'). Some ¹⁴C-IAAld appears to have been extracted into the neutral fraction, but complete separation of ¹⁴C-IAAld from ¹⁴C-IEt and IAA is achieved between the two solvent systems.

Characterization of the Reaction. A time course for the reaction catalyzed by IEt oxidase at 2 different enzyme concentrations is illustrated in Figure 8. The rate in the absence of oxygen was also tested in these reactions. Substrate and buffer (2.7 ml) were bubbled gently with either air or nitrogen, and 0.3 ml of an enzyme solution bubbled with nitrogen was added. A slow rate of bubbling was maintained throughout the reaction. Oxygen appears to be an absolute requirement (Fig. 8).
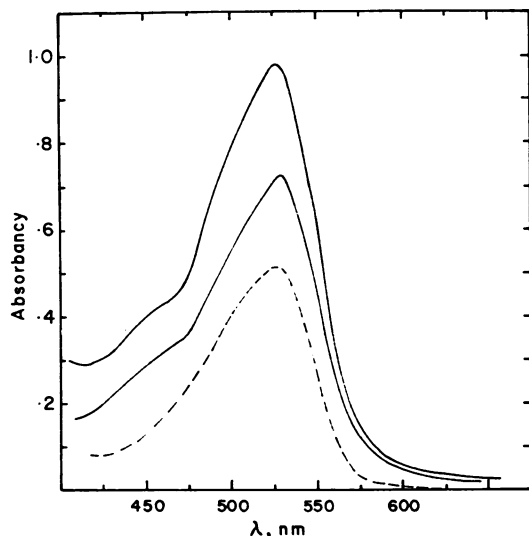
Fig. 6. Visible absorption spectra of Salkowski reaction color for IAA, IAAld + IEt, and reaction mixture. Broken line, 0.1 mM IAA; lower solid line, IAAld + IEt, each 0.1 mM; upper solid line, reaction mixture after 1 hr incubation with 0.5 mM IEt, 0.1 mg protein, and 50 mM sodium phosphate, pH 8. Reference solution in each case was H₂O + reagent.

Table II. *Total Radioactivity in Ether Extracts of ¹⁴C-IEt Reaction Mixture*

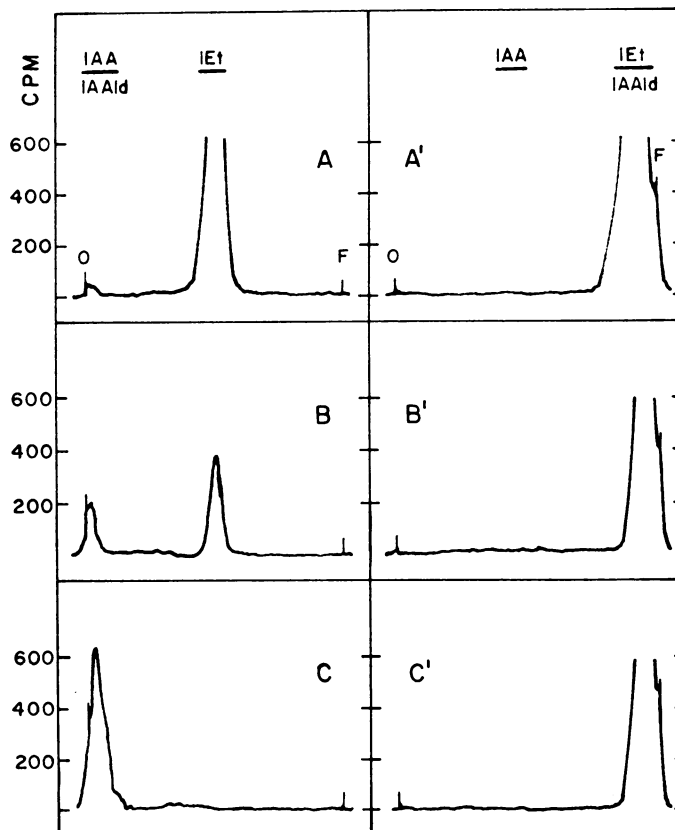| Fraction	Boiled Enzyme	Enzyme
	dpm	
I. Neutrals	3.57 × 10⁵	1.15 × 10⁵
II. Basics + aldehydes	520	2.44 × 10⁵
III. Acidics	195	425

Fig. 7. Radiochromatograms of ¹⁴C-IEt reaction mixture extracts. A, A': boiled enzyme neutral fraction; B, B': enzyme reaction neutral fraction; C, C': enzyme reaction basics and aldehydes. The solvent system for A, B, and C was ether:hexane (7:1); the solvent A', B', and C' was isopropanol:ammonia:water (10:1:1). The R_F values for IAA, IAAld, and IEt are shown at the top of the column for each solvent system. The sharp spikes at each end of the scans represent the origin (O) and the solvent front (F).
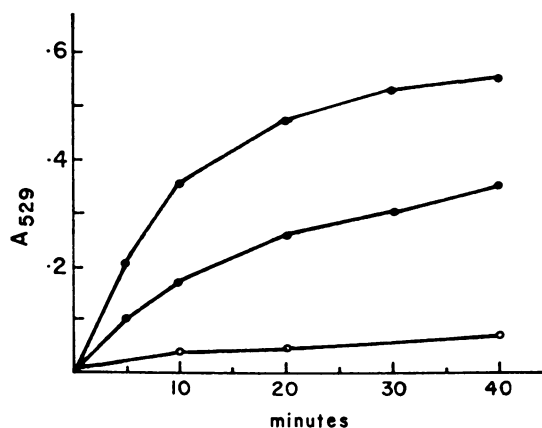
Fig. 8. Time course for IEt oxidation under aerobic and anaerobic conditions at two enzyme concentrations. ●: + O₂, upper curve 72 μg protein per ml, lower curve 36 μg protein per ml; O: − O₂, 72 μg protein per ml. IEt was 0.3 mM; sodium phosphate was 50 mM, pH 8.

A positive test for hydrogen peroxide was obtained when aliquots of the aerobic reaction mixtures were added to 1% solutions of guaiacol containing 0.1% horseradish peroxidase, as evidenced by the development of a brown color.

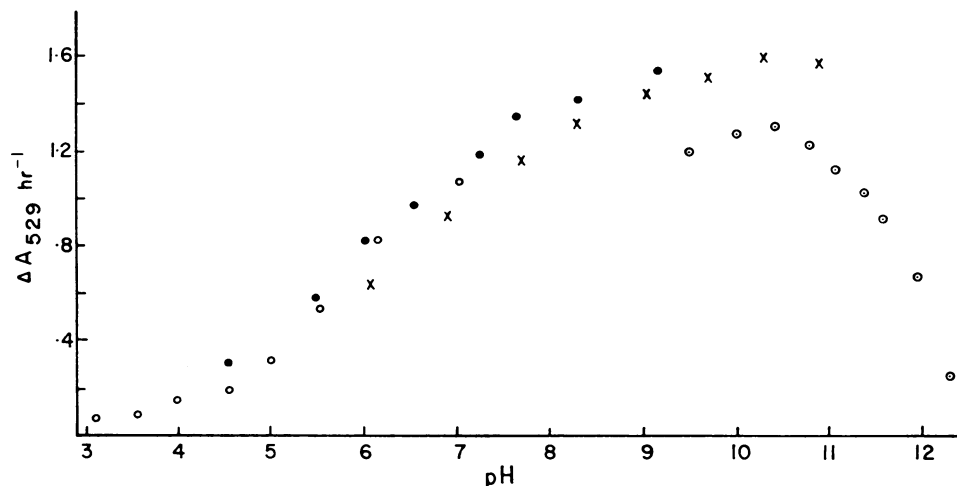The effect of pH on the rate of the reaction was measured

FIG. 9. The effect of pH on the enzyme-catalyzed oxidation of IEt. Buffer concentration 60 mM. O: sodium acetate; ●: sodium phosphate; X: sodium borate; ⊙: sodium glycinate. Protein concentration 50 μg/ml; IEt was 0.4 mM.

in four buffer systems from pH 3 to 12 (Fig. 9). A broad maximum from about pH 8 to 11 appeared. The curve is probably a composite of many effects, although other data indicate that at pH values near 8, at least, 0.3 mM IEt is sufficient to saturate the enzyme. That the rapid decrease in the rate of the reaction at high pH is due to denaturation of the enzyme is supported by the finding that pre-incubation of the enzyme at pH 11 or higher for 20 min lessens the activity observed when the reaction is carried out at pH 8.

The partially purified enzyme was observed to reduce both the 2-electron acceptor 2,6-dichloroindophenol and cytochrome c, a 1-electron acceptor. The coupled reduction of horse cytochrome c was used to test the specificity of the enzyme. No activity was detectable toward ethanol, glycolic acid, indole-3-glycolate, or indole-3-lactate.

DISCUSSION

An assay system for IAAld was developed which allows for the measurement of IAAld concentrations directly in solution. Previous techniques for the quantitative determination of IAAld have been based on a procedure involving extraction and oxidation of IAAld to IAA, which is then measured with the Salkowski reagent (5, 7). In addition to the time saving offered, the new method is twice as sensitive as methods based on conversion of IAAld to IAA. The specificity of the color test is currently being investigated. We are also testing the usefulness of this mixture technique for the location and identification of IEt and IAAld on chromatograms. When a chromatogram containing a spot of IAAld is sprayed with a solution of IEt and then with Salkowski reagent, a bright pink spot develops against a yellow background. The application of this method to the 5-hydroxy indoles is also under investigation.

Using this technique, we have succeeded in demonstrating an enzyme in cucumber seedling extracts which catalyzes the oxidation of IEt to IAAld. Several differences exist between this enzyme preparation and the enzyme(s) extracted from mung bean seedlings by Wightman and Cohen (20). The enzyme obtained from mung bean would catalyze the oxidation of ethanol, while cucumber IEt oxidase appears to be specific for IEt. This may be due to the presence of more than one oxidoreductase in the mung bean preparation, however, since the cucumber enzyme following gradient chromatography has a specific activity over 1,000 times greater than that of the mung bean enzyme. Moreover, the "alcohol dehydrogenase"

described by Wightman and Cohen (20) was associated with an "aldehyde dehydrogenase" which catalyzed the oxidation of IAAld to IAA. No IAA could be detected in reaction mixtures containing IAAld and cucumber IEt oxidase. The cofactor requirement for the mung bean enzyme which catalyzed the conversion of IEt to IAAld (and of ethanol to acetaldehyde) was not reported, but the reverse reaction from IAAld to IEt appeared to depend upon added NADH. An enzyme from oat seedlings also appears to require NADH for the conversion of IAAld to IEt (12). IEt oxidase for cucumber, however, is not reversible and shows no specific cofactor requirement other than the presence of an electron acceptor. Aerobically, oxygen is reduced to H_2O_2 during the course of the reaction; the enzyme may be a flavoprotein similar to glycolic oxidase (22). Attempts are underway to purify sufficient quantities of the enzyme to obtain absorption spectra of the oxidized and reduced (under anaerobic conditions) enzyme.

LITERATURE CITED

1. CARR, D. J., ed. 1972. Plant Growth Substances 1970. Springer Verlag, Berlin. In press.
2. GORDON, S. A. AND R. P. WEBER. 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26: 192-195.
3. HAMMENCE, J. H. 1943. The detection and estimation of auxin in organic manures. Analyst 68: 356-358.
4. LARSEN, P. 1944. 3-Indole acetaldehyde as a growth hormone in higher plants. Dansk Bot. Ark. 11(9): 1-132.
5. LARSEN, P. 1966. Quantitative determination of indole-3-acetaldehyde. Physiol. Plant. 19: 780-784.
6. LARSEN, P. AND T. AASHEIM. 1961. The occurrence of indole-3-acetaldehyde in certain plant extracts. In: Plant Growth Regulation. Iowa State University Press, Ames. pp. 43-55.
7. LARSEN, P. AND S. KLUNGSOYR. 1964. Quantitative determination of indole-3-acetaldehyde. Physiol. Plant. 17: 151-156.
8. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.
9. MITCHELL, J. W. AND B. C. BRUNSTETTER. 1939. Colorimetric methods for the quantitative estimation of indole-3-acetic acid. Bot. Gaz. 100: 802-816.
10. PURVES, W. K., D. L. RAYLE, AND K. D. JOHNSON. 1967. Actions and interactions of growth factors on cucumber hypocotyl segments. Ann. New York Acad. Sci. 144: 169-179.
11. RAJAGOPAL, R. 1967. Occurrence of indoleacetaldehyde and tryptophol in the extracts of etiolated shoots of Pisum and Helianthus seedlings. Physiol. Plant. 20: 655-660.
12. RAJAGOPAL, R. 1968. Metabolism of indole-3-acetaldehyde. II. On dismutation. Physiol. Plant. 21: 1076-1096.
13. RAJAGOPAL, R. 1971. Metabolism of indole-3-acetaldehyde. III. Some characteristics of the aldehyde oxidase of Avena coleoptiles. Physiol. Plant. 24: 272-281.
14. RAYLE, D. L. AND W. K. PURVES. 1967. Isolation and identification of indole-

3-ethanol (tryptophol) from cucumber seedlings. Plant Physiol. 42: 520–524.

15. RAYLE, D. L. AND W. K. PURVES. 1967. Conversion of indole-3-ethanol to indole-3-acetic acid in cucumber seedling shoots. Plant Physiol. 42: 1091–1093.

16. RAYLE, D. L. AND W. K. PURVES. 1968. Studies on indole-3-ethanol in higher plants. In: F. Wightman and G. Setterfield, eds., The Physiology and Biochemistry of Plant Growth Substances. Runge Press, Ottawa.

17. SCHNEIDER, E. A., R. A. GIBSON, AND F. WIGHTMAN. 1971. Biosynthesis and metabolism of 3-indoleacetic acid. I. The native indoles of barley and tomato shoots. J. Exp. Bot. In press.

18. SHERWIN, J. E. AND W. K. PURVES. 1969. Tryptophan as an auxin precursor in cucumber seedlings. Plant Physiol. 44: 1303–1309.

19. TANG, Y. W. AND J. BONNER. 1947. The enzymatic inactivation of indole-acetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. Biophys. 13: 11–25.

20. WIGHTMAN, F. AND D. COHEN. 1968. Intermediary steps in the enzymatic conversion of tryptophan to IAA in cell free systems from mung bean seedlings. 1968. In: F. Wightman and G. Setterfield, eds., The Physiology and Biochemistry of Plant Growth Substances. Runge Press, Ottawa.

21. WIGHTMAN, F. AND G. SETTERFIELD, eds. 1968. The Physiology and Biochemistry of Plant Growth Substances. Runge Press, Ottawa.

22. ZELITCH, I. AND S. OCHOA. 1953. Oxidation and reduction of glycolic and glyoxylic acids in plants. I. Glycolic acid oxidase. J. Biol. Chem. 201: 707–718.