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Indole-3-ethanol Oxidase

KINETICS, INHIBITION, AND REGULATION BY AUXINS[1]

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ABSTRACT

We report the further characterization of indole-3-ethanol oxidase from cucumber seedlings. The effects of various inhibitors suggest that the enzyme may be a flavoprotein with a metal ion and sulfhydryl groups required for full activity. Indole-3-acetaldehyde, a product of the reaction, inhibits the enzyme. This inhibition is overcome by O_2 but not by indole-3-ethanol, indicating that the kinetic mechanism of the enzyme is a ping-pong Bi-Bi. The enzyme undergoes cooperative interactions with indoleethanol, yielding Hill coefficients as high as 2.96. Gibberellins are without effect on the enzyme, but it is inhibited by several acidic indoles possessing growth-promoting activtiy and by two synthetic auxins, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid. Increasing concentrations of indoleacetic acid (IAA) brought about a slight reduction in the indoleethanol concentration producing half-maximal velocity. Increasing levels of indoleethanol decreased the concentration of IAA required for half-maximal inhibition. At low concentrations of indoleethanol, low levels of IAA activated rather than inhibited. The effect of IAA was not overcome at higher levels of indoleethanol. These results may be interpreted as showing that IAA is a noncompetitive inhibitor which binds to that conformation of the enzyme which also binds indoleethanol. The significance of these interactions for the regulation of IAA biosynthesis is discussed.

One aim of research on IAA biosynthesis and metabolism has been to determine how auxin levels are controlled in the plant. To date, more emphasis has been placed on regulation of the hormone's metabolism than on control of its rate of synthesis. There have been reports of increased IAA oxidase activity in plants after auxin treatment (6, 11); and investigators have also shown that various plant products, particularly phenolic compounds, can affect the enzyme (5, 9). In addition to oxidative degradation of the hormone, IAA also forms conjugates with aspartate (1), glucose (21), and myoinositol (10). The regulation of these reactions is another potential mechanism for controlling the concentration of auxin.

Recently, a number of enzymes involved in IAA biosynthesis have been isolated from a variety of plant sources (7, 15-17). This makes it possible to look for regulation occurring through effects on one or more of the reactions in the synthetic pathway. Vickery and Purves have isolated and partially purified an IEt[3] oxidase from cucumber seedlings (17, 18). The enzyme catalyzes the oxidation of IEt by molecular oxygen to form IAAld and H_2O_2. We have studied the kinetics of the reaction, and some of our results suggest that IEt oxidase may have a regulatory function in IAA biosynthesis.

MATERIALS AND METHODS

Plant Material. Seeds of *Cucumis sativus* L. cv. National Pickling (Burpee) were surface-sterilized in 2.5% Clorox, soaked in distilled water for 1 hr, and sown in vermiculite saturated with a tap water solution of NH_4NO_3 (400 mg/liter) and $CuSO_4$ (50 mg/liter). The seedlings were grown under a 14L10D light cycle at 26 C. After 6 to 7 days of growth, they were harvested and extracted.

Preparation of Enzyme. IEt oxidase was isolated following the methods of Vickery and Purves (17). The harvested tissue was ground in 50 mM NaP, pH 8 (1.5 kg/liter), in a Waring Blendor. The grinding was performed in two steps, the first 750 g of tissue being homogenized in the whole liter of buffer. This homogenate was then used to extract the second 750 g of seedlings. After grinding, the homogenate was squeezed through eight layers of cheesecloth, and the filtrate was centrifuged at 16,000*g* for 30 min. The supernatant was filtered through glass wool to remove lipids and diluted with an equal volume of 20 mM NaP, pH 7.5. This solution was extracted twice with BioRex 70 (BioRad) using 25 ml of resin (1:1, v/v, suspension in buffer) per liter of extract; the suspension was stirred for 2 hr. The resin was allowed to settle, and the supernatant was siphoned off for the second extraction. The two batches of resin were washed with 20 mM NaP, pH 7, until the wash buffer was clear. They were poured into a glass column and washed with additional buffer. The enzyme was eluted from the resin with 0.6 M NaCl in 20 mM NaP, pH 7, and dialyzed overnight against 15 to 20 volumes of the same buffer. The enzyme was bound to the top of a BioRex 70 column (3 × 30 cm) equilibrated with 20 mM NaP, pH 7. Inactive material was eluted from the column with 100 ml of 0.1 M NaCl in buffer, and this was followed by a linear salt gradient elution in which 1.0 liter of 0.1 M NaCl in 20 mM NaP, pH 7, was the starting buffer and 1.0 liter of 0.6 M NaCl in 20 mM NaP, pH 7, was the final buffer. Fractions (10 ml) were collected, and the active tubes were pooled and concen-

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[3] Abbreviations: DCIP: 2,6-dichloroindophenol; GA_3 and GA_7: gibberellins A_3 and A_7; IAAld: indole-3-acetaldehyde; IEt: indole-3-ethanol; NaP: sodium phosphate buffer.

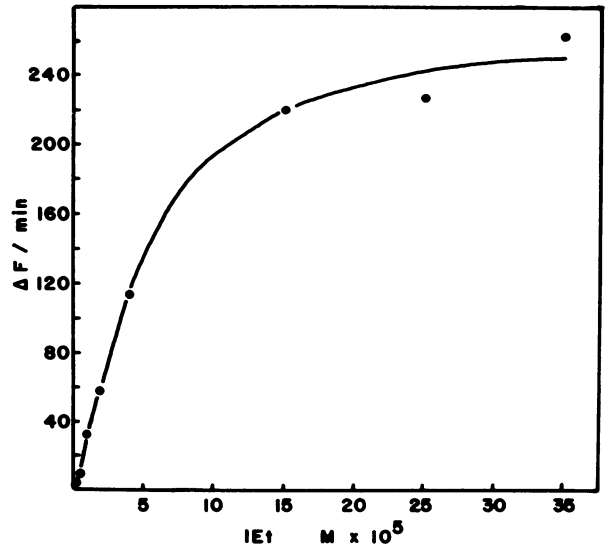

FIG. 1. Effect of IEt concentration on IEt oxidase activity. The reaction was run in 50 mM NaP, pH 9, and followed with the peroxidase-coupled fluorometric assay.

trated by ultrafiltration using a Diaflo PM-30 membrane (Amicon). All procedures were carried out at 2 to 4 C.

Assay Procedures.

1. Fractions from chromatographic steps were routinely assayed by the colorimetric method developed by Vickery and Purves (17). The reaction mixture included a 0.2-ml enzyme sample and 0.3 ml of 0.17 mM IEt in 50 mM NaP, pH 7.5. The reaction was stopped by the addition of 1.0 ml of the perchloric acid Salkowski reagent (8). In studies of inhibitors, IEt sufficient to give a final concentration of 0.5 mM was added just prior to stopping the reaction with Salkowski reagent. After 30 min in the dark, optical densities of solutions were measured at 529 nm with a Beckman DB spectrophotometer, using a boiled enzyme reagent blank as a reference.

2. The peroxidase-coupled fluorometric assay of McGowan and Muir (13) was applied in some studies. The assay mixture consisted of 0.1 ml of enzyme, 0.1 ml of 0.1 mM scopoletin, 0.1 ml of horseradish peroxidase (1 mg/ml) (Mann), 0.2 ml of H_2O, 0.5 ml of IEt, and 1.0 ml of 0.1 M NaP. The reaction was monitored by following the disappearance of scopoletin fluorescence using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation and emission wavelengths were 370 and 465 nm, respectively.

3. A spectrophotometric assay was used for kinetic studies of the enzyme (18). The reaction was coupled to the bleaching of the dye DCIP. The reaction mixture contained 0.2 ml of the enzyme, 0.1 ml of 0.5 mM DCIP, 0.2 ml of the fixed ligand, and 0.5 ml of the variable ligand in NaP, pH 7, at a final concentration of 50 mM. The reaction was followed with a Cary 15 recording spectrophotometer at 600 nm, and the initial rate was taken to be the steepest portion of the progress curve because of a lag at the beginning of the assay. This assay is based on a secondary, H_2O_2-dependent reaction of the dye and not a direct reduction by the enzyme.

RESULTS

Substrate Kinetics. The effect of IEt concentration on the reaction rate was studied using the peroxidase-coupled fluorometric assay. When the data from the lower concentration region of Figure 1 are replotted on an expanded scale, they show a sigmoidal relationship between initial velocity and substrate concentration, indicating cooperativity with respect to IEt (see also Figs. 3 and 4). This is seen more clearly in the Eadie-Hofstee plot of the same data in Figure 2. The convex curvature seen in this plot is characteristic of a positively cooperative system. The Hill coefficient determined from these data was 1.3; Hill coefficients as high as 2.9 have been calculated for some preparations of the enzyme. This property varies somewhat from one isolation to the next, and the cooperativity disappears altogether after 2 weeks' storage at 4 C. The enzyme exhibits cooperative interactions with IEt at least over the pH range from 6 to 9.

Product Inhibition. W. W. Cleland has developed graphical methods for determining the kinetic mechanism of an enzyme catalyzing a bisubstrate reaction (3, 4). Although the substrate

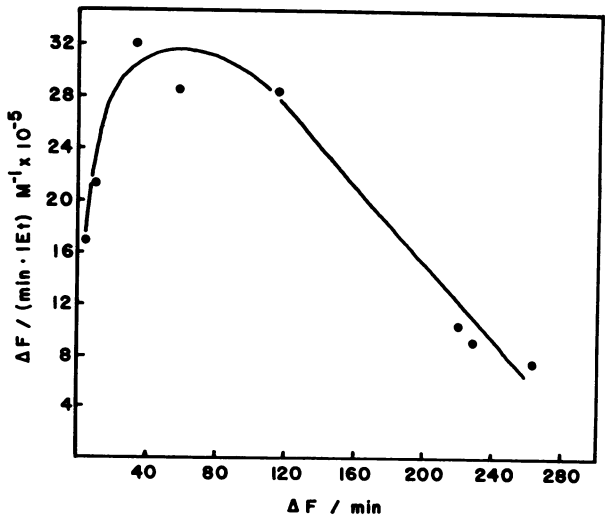

FIG. 2. Effect of IEt concentration on IEt oxidase activity. The data of Figure 1 were replotted according to Eadie and Hofstee.

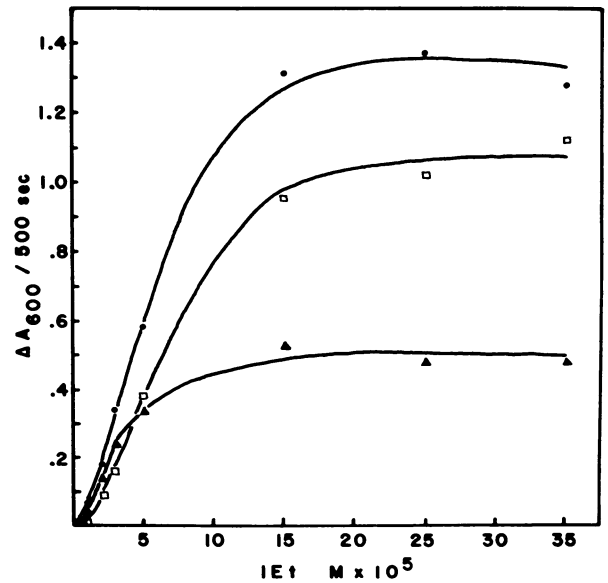

FIG. 3. Effects of IAAld and IAA on IEt oxidase. ●: No addition; □: 0.2 mM IAAld; ▲: 0.2 mM IAA. The buffer was 50 mM potassium phosphate, pH 7; and the reaction was monitored with the DCIP assay.

kinetics for cooperative enzymes are complicated by nonlinear double reciprocal plots, it may be possible to identify tentatively the mechanism type of IEt oxidase through a study of product inhibition. The definitions of competitive and noncompetitive inhibition will be taken to be that the inhibition can and cannot, respectively, be overcome by high concentrations of substrate. The effect of 0.2 mM IAAld on the enzyme is illustrated in Figure 3. IAAld inhibits the enzyme reaction, and the inhibition is not overcome by increasing the concentration of IEt. However, this inhibition is greatly decreased when oxygen is bubbled through the reaction mixture.

Inhibitor Effects. A number of compounds were tested for their effects on enzyme activity in the presence of 0.1 mM IEt. These data are presented in Table I. Mixed results were obtained when a variety of metal complexers and chelators were included in the reaction mixture. Neither EDTA nor KCN affected the reaction rate, but the enzyme was inhibited by 8-hydroxyquinoline, azide, and fluoride. Both Hg^{2+} and iodoacetate inhibited the enzyme with the heavy metal ion effect being much more pronounced.

Yagi *et al.* (20) have demonstrated that the flavoenzyme D-amino acid oxidase is inhibited by phenol and substituted phenols. IEt oxidase is strongly inhibited by 2,4-dinitrophenol and 2,4-dichlorophenol. It is also inhibited by riboflavin and FMN, a property characteristic of many flavoenzymes (19).

The possibility that the enzyme activity might be regulated by hormones led to a test of the effects of gibberellins, auxins, and related compounds (Table II). GA_3 and GA_7, both potent promoters of cucumber hypocotyl growth (2), were without effect on the rate of IEt oxidation. On the other hand, all of the synthetic auxins and acidic indoles tested were found to inhibit the enzyme, although their order of effectiveness did not correlate with their efficiencies as growth promoters. 2,4-D was by far the most potent inhibitor of the enzyme, producing complete inhibition at a concentration of 1.0 mM. IAA at the same concentration inhibited the reaction by 75%. To test the specificity of the auxin inhibition of IEt oxidase, other indoles and organic acids were tested. Tryptophan and tryptamine did not inhibit the reaction rate, and benzoic acid and glycolic acid were also without effect.

Table I. *Effects of Enzyme Inhibitors on IEt Oxidase*

Reactions were run in 0.1 mM IEt and 50 mM NaP, using the Salkowski assay. The values were corrected for the effects of the inhibitors on the assay.

	Concentration	Inhibition
	mM	%
EDTA	10.0	0
KCN	1.0	0
Na_3N	1.0	29.1
	10.0	71.2
NaF	1.0	1.1
	10.0	67.0
8-Hydroxyquinoline	17% saturated	49.6
	33% saturated	67.0
$HgCl_2$	0.01	1.1
	0.10	85.9
Iodoacetate	1.0	10.1
	10.0	23.4
2,4-Dinitrophenol	0.10	16.9
	1.0	73.0
2,4-Dichlorophenol	0.10	39.6
	1.0	82.5
Riboflavin	0.02	11.4
	33% saturated	27.5
FMN	1.0	7.6

Table II. *Effects of Hormones and Related Compounds on IEt Oxidase*

Except where indicated, the reactions were followed with the DCIP assay. Buffer was 50 mM NaP, pH 7.

	Concentration	Inhibition
	mM	%
GA_3[1]	0.25	0
GA_7[1]	0.25	0
IAA	0.20	55.2
Indole-3-propionic acid	0.20	26.0
Indole-3-butyric acid	0.20	17.0
Indole-3-lactic acid	0.20	8.8
Indole-3-glycolic acid	0.20	62.0
Glycolic acid	0.25	0
Tryptophan[1]	0.25	0
Tryptamine[1]	0.25	0
2,4-D	0.20	74.2
2,4,5-T	0.20	46.7
Benzoic acid	1.0	0

[1] Peroxidase-coupled fluorometric assay.

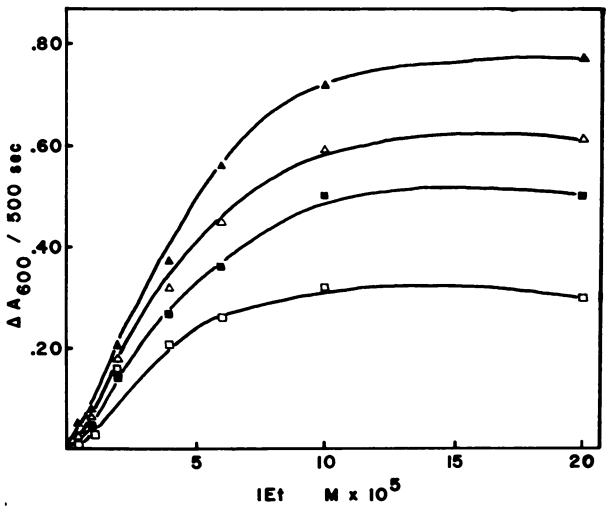

FIG. 4. Effect of IAA on IEt oxidase. ▲: No IAA; △: 0.02 mM IAA; ■: 0.05 mM IAA; □: 0.2 mM IAA. The buffer was 50 mM potassium phosphate, pH 7; and the reaction was monitored with the DCIP assay.

Characterization of the Inhibition by IAA. Figure 4 is a typical example of the kinetic plots obtained with the DCIP assay when the concentration of IEt is varied in the presence of fixed concentrations of IAA. The inhibition is not overcome by increasing the IEt concentration in the reaction mixture. Thus, the inhibition is not the simple competitive case, nor does it fit the usual pattern for an allosteric inhibitor (14). Although IAA inhibits the enzyme reaction, it shifts the substrate concentration producing half-maximal velocity ($S_{0.5}$) to slightly lower values (38 to 31 μM), suggesting that IEt binding to the enzyme is facilitated by the binding of IAA. Hill plots of the data in Figure 4 are shown in Figure 5. From the Hill coefficients (n) calculated from these plots, it can be seen that IAA decreases the cooperativity with respect to IEt, although the relationship is not simple.

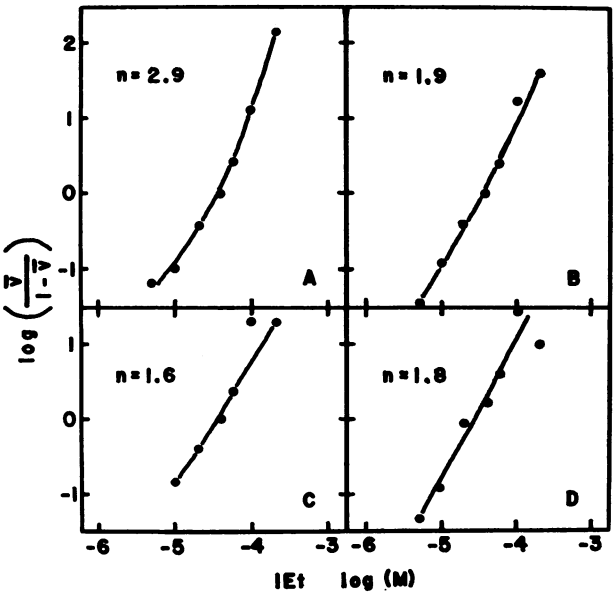

FIG. 5. Effect of IAA on IEt oxidase. The data of Figure 4 are presented in Hill plots. *n:* The Hill coefficient. A: No IAA; B: 0.02 mM IAA; C: 0.05 mM IAA; D: 0.2 mM IAA.

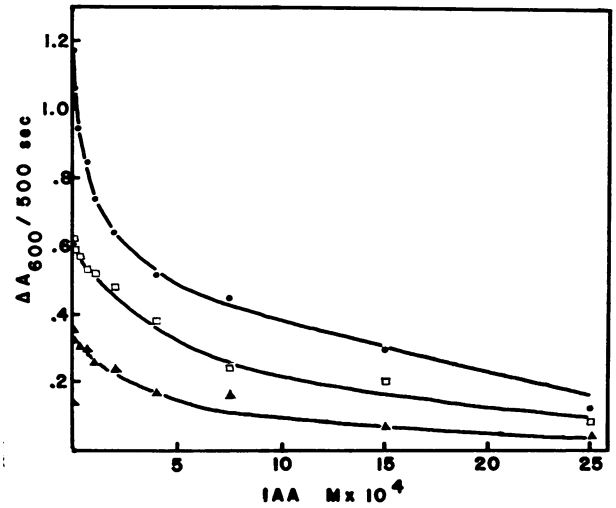

FIG. 6. Effect of IAA concentration on IEt oxidase activity. ●: 0.1 mM IEt; □: 0.04 mM IEt; ▲: 0.02 mM IEt. The DCIP assay was used to follow the reactions, and the buffer was 50 mM NaP, pH 7.

Variation of the IAA concentration in the presence of fixed concentrations of IEt generates the type of results illustrated in Figures 6 and 7. At low concentrations of both IEt and IAA, there is a marked activation of the enzyme which decreases and becomes inhibition as the concentration of IAA increases. As the concentration of IEt is increased, the low IAA activation disappears. And, as the IEt concentration is raised, the concentration of IAA producing half-maximal inhibition ($I_{0.5}$) is shifted from 1.5 mM to 0.3 mM for the concentrations of IEt used in this study. These results suggest that IAA binding is made easier by the binding of IEt.

DISCUSSION

We have reported further characterization of the IEt oxidase from cucumber seedlings. Inhibitor studies suggest that the enzyme may contain a metal ion, although the varied responses to the different chelating and complexing agents preclude any firm conclusions. The inhibitions by iodoacetate and Hg^{2+} suggest that there may be sulfhydryl groups involved in the structure of the enzyme. However, the high concentrations of iodoacetate required for inhibition seem to argue against a role for a sulfhydryl group in the catalytic mechanism.

Yagi *et al.* have suggested that phenol and its ring-substituted derivatives may inhibit flavoenzymes by binding to the ribotyl moiety of the cofactor, thus competing with the apoenzyme for the flavin (20). The inhibition of IEt oxidase by 2,4-dinitrophenol and 2,4-dichlorophenol as well as by riboflavin and FMN (19) suggests that the enzyme may have a flavin cofactor.

The study of product inhibition lends some strength to this possibility. The case in which the product of the first substrate is a competitive inhibitor for the second is characteristic of the "ping-pong" mechanism which is diagrammed for the case of IEt oxidase in Figure 8. IEt would react with the enzyme to form IAAld and a reduced enzyme; the enzyme would be reoxidized by molecular oxygen with the formation of H_2O_2. This mechanism is the type used by many flavin oxidases (12). However, the development of methods for obtaining relatively large amounts of the enzyme, purified to homogeneity, will be

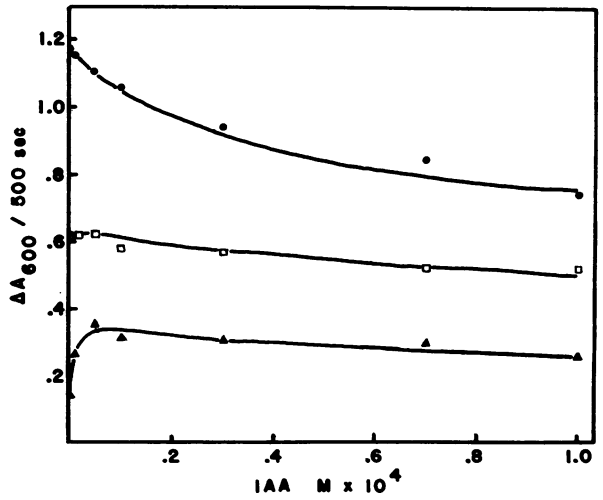

FIG. 7. Effect of IAA concentration on IEt oxidase activity. The data of Figure 6 were replotted on an expanded concentration scale. Symbols as in Figure 6.

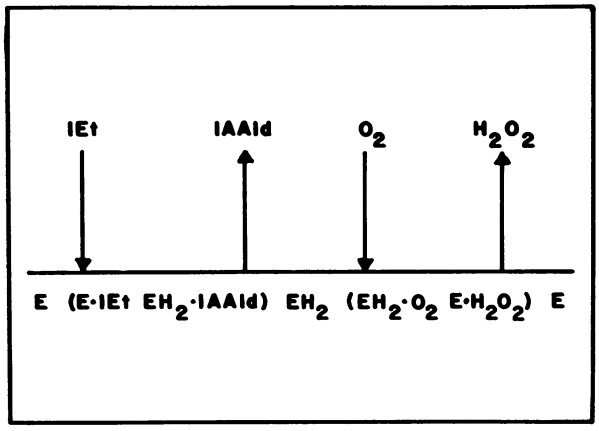

FIG. 8. Proposed kinetic mechanism for IEt oxidase.

necessary before chemical or spectral techniques can be applied to the identification of the cofactor.

IEt oxidase undergoes a relatively specific inhibition by auxins. Other enzymes in the pathway which have been checked for regulation by IAA were insensitive to the compound. These include the tryptophan decarboxylase and tryptophan transaminase from barley and tomato (F. Wightman, personal communication) and the aldehyde oxidase isolated from oats (P. Larsen, personal communication). That so many auxins in addition to IAA inhibit the enzyme is not surprising, since the auxins share common structural features. Rather, it lends strength to the idea that the inhibition is auxin-specific. It seems likely that the binding sites for IAA on the enzyme and at its point of action in growth responses are similar, although not identical, since there is not a one-to-one correlation in efficiencies of the auxins as growth promoters and as inhibitors of IEt oxidase. The fact that IAA inhibition is noncompetitive could suggest that IAA is simply mimicking the effect of IAAld. However, as can be seen in Figure 3, the two compounds at the same concentration produce quantitatively different effects.

The finding that IEt undergoes cooperative interactions with the enzyme lends some support to the idea that IEt oxidase may play a regulatory role in IAA biosynthesis. Such interactions are a common property of most of the regulatory enzymes studied thus far (14). However, the inhibition by IAA does not fit the usual pattern for an allosteric inhibitor. While the classical allosteric inhibitor shifts the $S_{0.5}$ to higher values, IAA brings about a slight decrease in this parameter. The effect of an allosteric inhibitor may be overcome by increasing the substrate concentration—not through simple competition for a binding site, but by pulling a conformational equilibrium away from an inactive, inhibitor-stabilized form of the enzyme to an active conformation (14). The inhibition due to IAA is not overcome by higher concentrations of IEt; but, instead, the binding of the inhibitor is made easier by an increase in the substrate concentration as seen from the shift of the $I_{0.5}$ to lower values. The most reasonable interpretation of these results is that IAA is a noncompetitive inhibitor which binds more strongly to the enzyme form binding IEt than it does to the inactive conformation. The increase in the Hill coefficient observed at high IAA concentrations may reflect binding to the latter form. This model also explains the activation of the enzyme at low concentrations of both inhibitor and substrate. It is an example of the same effect observed for competitive inhibitors of allosteric enzymes (14). The binding of the inhibitor pulls the conformational equilibrium over to the form which binds substrate more effectively, thus generating more substrate binding sites. Yet, at the same time, the inhibitor concentration is too low for there to be inhibition at all of the new sites; this results in a net activation.

Although the kinetic results lend themselves to interpretation in terms of a fairly simple structural model, evaluating the significance of such a system for the regulation of IAA synthesis is another matter entirely. It is not clear why a modifier of a regulatory enzyme should produce two conflicting effects—a lowering of the catalytic efficiency and a decrease in the $S_{0.5}$. The shift in the $S_{0.5}$ is small, however, and it may be that it is insignificant for the enzyme as it acts *in vivo*. The decrease in the $I_{0.5}$ for IAA binding with increased IEt levels is a larger effect. If this shift is large enough in the actual functioning of the enzyme *in vivo*, it could suggest the following relationships. At times when the IAA concentration is high in the cytoplasm, it would also be reasonable to expect the level of IEt to be high. The increased level of IAA would bring about an inhibition of the enzyme; and, at the same time, the binding of IAA would be facilitated by the high IEt concentration. The two ligands would thus act in concert—IAA inhibiting the reaction and IEt making the enzyme more susceptible to inhibition.

There are a number of questions still to be answered before a regulatory role can be confidently assigned to IEt oxidase. The position of IEt on the pathway is still not clearly established. If it is on the main pathway (as, for example, by compartmentation of certain steps), it is still not known whether the IEt oxidase reaction is the rate-limiting step in the sequence of reactions. A further problem arises in that it is not known whether the concentration of IAA *in vivo* ever becomes large enough to alter the reaction rate of the enzyme significantly. Although these obstacles do exist, it still will be surprising if the fairly specific interaction of IAA with IEt oxidase turns out in the end to be completely unrelated to the control of auxin biosynthesis in the cucumber.

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