Isolation and Characterization of Indole-3-acetaldehyde Reductases from *Cucumis sativus*

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In a continuing study of the biosynthetic pathway and regulatory mechanisms governing indole-3-acetic acid (auxin) formation, we report the isolation and initial characterization of three distinct indole-3-acetaldehyde reductases from cucumber seedlings. These enzymes catalyze the reduction of indole-3-acetaldehyde to indole-3-ethanol with the concomitant oxidation of NAD(P)H to NAD(P)⁺. Two of the reductases are specific for NADPH as second substrate, while the third is specific for NADH. The enzymes show a strong specificity for indoleacetaldehyde, with apparent Kₐ values of 73, 130, and 400 μM being calculated for the two NADPH-specific reductases and the NADH-specific reductase, respectively. Under no conditions of substrate concentration, incubation time, or assay method could the reverse reaction be observed. Chromatography on a calibrated Sephadex gel column led to estimated molecular weights of 52,000 and 17,000 for the NADPH-specific reductases, while a value of 33,000 was obtained for the NADH-specific reductase. Both NADPH-specific reductases showed a pH optimum of 5.2 with a secondary optimum at 7.0, and both enzymes were activated by increasing ionic strength. The NADH-specific reductase showed a pH optimum of 7.0 with a secondary optimum at 6.1 and was slightly inhibited by increasing ionic strength.

This study represents the first extensive purification and characterization of the enzymatic sources presumed to be responsible for the formation of indole-3-ethanol. The formation of indoleethanol and its subsequent metabolism are thought to have a regulatory role in auxin synthesis.

The biosynthesis of the plant growth hormone indole-3-acetic acid (auxin) is of current interest and has recently been reviewed by Schneider and Wightman (1). Although some controversy still exists as to the exact pathway through which indoleacetic acid is synthesized from tryptophan, the major unanswered questions concern the regulation of biosynthesis of this important hormone. Of particular interest to us is the role of indole-3-ethanol in auxin synthesis because of the possible regulatory mechanisms centered around its metabolism. A role of indoleethanol in indoleacetic acid synthesis is suggested by our observations that indoleethanol rivals indoleacetic acid as a growth promoter in the cucumber hypocotyl test (2) and that [¹⁴C]indoleethanol is rapidly converted to [¹⁴C]indoleacetic acid *in vivo* (3). The natural occurrence of indoleethanol has been demonstrated in cucumber seedlings (4) and several other plants (2, 5, 6). It has also been shown that indoleethanol can be formed *in vivo* from added tryptamine or tryptophan (7, 8) and that of some 33 plant species representing 17 lower and higher plant families tested, all could metabolize added indoleacetaldehyde to indoleethanol *in vivo* (9).

Several recent findings suggest that metabolism of indoleethanol has a regulatory role in auxin synthesis. Gibson *et al.* (7) have shown that upon the addition of large amounts of [¹⁴C]tryptamine to tomato shoots, the fluctuation in the indoleethanol pool size is far larger than that of the indoleacetic acid pool. This suggests that indoleethanol serves as a reservoir for excess auxin precursor. The isolation and characterization of indoleethanol oxidase (10, 11) has led to the proposal that indoleacetic acid regulates its own synthesis through modulation of this enzyme. This highly specific enzyme oxidizes indoleethanol to indole-3-acetaldehyde, the immediate precursor of indoleacetic acid. Indoleethanol oxidase is markedly inhibited by indoleacetic acid, leading to speculation that a feedback loop regulatory mechanism is operative at this enzyme (11).

In view of the regulatory role indoleethanol may play in auxin synthesis, it became of interest to establish the enzymatic route by which indoleethanol is synthesized. The present report deals with the isolation and initial characterization of three distinct, rather specific indole-3-acetaldehyde reductases from cucumber seedlings.

**EXPERIMENTAL METHODS**

Preparation of Indole-3-acetaldehyde—Indole-3-acetaldehyde was purchased as the bisulfite adduct from Sigma Chemical Co. and stored desiccated at -20°. Free indoleacetaldehyde was prepared from this...
product as follows. Indoleacetaldehyde bisulfite was dissolved in a volume of water one-fifth that of the final volume desired and the pH raised to approximately 10 with saturated Na₂CO₃. The solution was allowed to stand for 5 min at room temperature and the free indoleacetaldehyde then removed by three extractions with equal volumes of anhydrous diethyl ether. The ether solutions were combined with an appropriate volume of water (or buffer) and the ether removed by flash evaporation. The final concentration of free indoleacetaldehyde was determined by measuring the absorbance at 280 nm and applying Beer's law with ε₉₀ = 5400 liter·mole⁻¹·cm⁻¹ (see "Results").

Preparation of Other Reagents—Reduced NAD(P)H was obtained from Sigma Chemical Co. and stored desiccated at 20°C. NAD(P)H was determined by measuring the absorbance at 280 nm and applying Beer's law with ε₉₀ ≈ 5400 liter·mole⁻¹·cm⁻¹ (see "Results").

All spectrophotometric measurements were made with a Beckman ACTA V ultraviolet visible spectrophotometer using the double beam mode.

Preparation of Enzyme—Seeds of Curcuma sativa L. cv. National Pickling (Burpee) were sown in vermiculite saturated with tap water. The seedlings were grown for 7 days under a 14-hour light 10-hour dark cycle at 25°C, harvested, and extracted. All the following operations were carried out at 0–4°C. Harvested shoot tissue was homogenized in 50 mM sodium phosphate buffer, pH 7.5, containing 1 mM cysteine, by grinding for 90 s in a 1-gallon Waring Blender. The homogenate was filtered through eight layers of cheesecloth and the filtrate centrifuged at 11,000 × g for 30 min. The supernatant was filtered through glass wool to remove surface lipids. This filtrate was batch extracted for 2 hours with BioRex 70 cation exchange resin (50 ml of settled resin/liter of solution) in order to remove positively charged protein which competes with the reductases for binding sites on cellulose phosphate in a later step. The resin was then allowed to settle and the supernatant removed by siphon. The pH of the supernatant was slowly lowered to 4.5 with 1 N HCl and the solution allowed to equilibrate for 30 min. The solution was centrifuged at 11,000 × g for 20 min and the pellet discarded. The supernatant was then batch extracted with diethyl ether two times for 30 min. The ether solutions were evaporated to dryness and the residue concentrated to a small volume of 0.1 M sodium phosphate buffer, pH 7.5, and 12-mL fractions were collected. The protein bound to the cellulose phosphate eluted as a single peak and was pooled. Enough ammonium sulfate was added to the eluate to achieve 50% saturation, and the resulting solution was allowed to equilibrate for 30 min. Precipitated protein was removed at 11,000 × g for 20 min and resuspended in a small volume of 0.1 M sodium phosphate buffer, pH 7.5. The supernatant was then mixed with enough (NH₄)₂SO₄ to achieve 80% saturation and allowed to equilibrate for 30 min. The solution was centrifuged at 10,000 × g for 20 min, the pellet resuspended in a small volume of the same buffer, and then combined with the resuspended 0 to 50% fraction. The combined fractions were centrifuged at 37,000 × g for 30 min and the small pellet discarded.

Aliquots (23 ml) of this preparation were applied to a Sephadex G 150 gel column (41 × 110 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.5, and eluted with this buffer at a rate of 20 ml/hour with 6-ml fractions being collected. Active fractions were determined by the Salkowski reaction method and pooled, yielding three enzyme preparations labeled "T₉₀," "D," and "T." Each enzyme preparation was concentrated by ultrafiltration (Amicon Ultrafiltr) to approximately one-eighth its original volume to yield a preparation of sufficient activity to allow kinetic studies to be performed by observing a continuous decrease in absorbance at 340 nm.

Thin Layer Chromatography of Products—Preparation of product for determination of thin layer chromatography. Rₙ values were achieved as follows. A reaction mixture consisting of 2 ml of 0.05 mM indoleacetaldehyde, 0.2 ml of 0.8 mM NAD(P)H in 0.1 M sodium phosphate buffer, pH 7.5, and 0.2 ml of enzyme preparation in 50 mM sodium phosphate buffer, pH 7.5, was incubated for 3 hours at 20°C and then extracted three times with 5-ml aliquots of anhydrous diethyl ether. The ether extracts were evaporated to dryness and the residue redissolved into 1 ml of acetone. This material was applied to a silicon gel glass thin layer chromatographic plates (Eastman) and visualized using white light and a 1-mm slit width.

Preparation of product for reaction with p-dimethylaminobenzaldehyde was achieved as follows. A 50-ml reaction mixture containing 30 µmol of indoleacetaldehyde, 25 µmol of NAD(P)H, and approximately 3 ml of the appropriate enzyme preparation was incubated for 4 hours at 20°C. The reaction mixtures were extracted three times with 75-ml aliquots of anhydrous diethyl ether and the ether extract concentrated and subjected to thin layer chromatography as described above. Known indoleacetaldehyde and indoleethanol were chromatographed at the same time. Following development in ether/hexane solvent, small portions of the plates corresponding to known indoleethanol and indoleacetaldehyde were removed, sprayed with Ehrlich's reagent, and heated at 80°C for 10 min. The indoleethanol and indoleacetaldehyde color reactions on these samples allowed the positions of these compounds to be determined for the other runs, and the silica gel at these positions was removed into 10 ml of 95% ethanol. The ethanol was evaporated to a minimal volume and brought to 1 ml with 95% ethanol. The 1 ml solutions were allowed to react with 20 µg of p-dimethylaminobenzaldehyde and 0.2 ml of concentrated HCl at 100°C for 5 min. The color reactions were completed by adding 0.1 ml of 5% NaNO₂ to each of the hot solutions and allowing them to cool for 30 min. Visible spectra were obtained.

RESULTS

Determination of Molar Extinction Coefficient for Indoleacetaldehyde in Water—The notorious instability of free indoleacetaldehyde in aqueous solution necessitates that it be manufactured and stored as the bisulfite adduct (15). The method by which the free indoleacetaldehyde is obtained from the bisulfite adduct leaves uncertain the final concentration of the free indoleacetaldehyde in aqueous solution. Thus, it was advantageous to obtain the molar extinction coefficient at 280 nm for free indoleacetaldehyde in water. Brown et al. (16) have synthesized free indoleacetaldehyde and determined its molar extinction coefficient at 280 nm in 95% ethanol to be 6000 liters·mole⁻¹·cm⁻¹. In a modification of the procedure given under "Experimental Methods," indoleacetaldehyde was freed into 95% ethanol and its concentration determined using the above value of ε. A series of dilutions was made so that the indoleacetaldehyde was placed in solvent mixtures with an increasing mole fraction of water. The ε at 280 nm was determined for each dilution taking care that the spectrophotometer was blanked with the appropriate mixture of water and 95% ethanol. Fig. 1 shows the results of this experiment. Extrapolation to 100% water gives the value ε₁₀₀ = 5400 liters·mole⁻¹·cm⁻¹. This extinction coefficient was used in the subsequent experiments to determine the concentration of indoleacetaldehyde with the assumption that its value was insignificantly changed by the presence or absence of buffer salts in low concentration.

Purification and Estimation of Molecular Weights—The partial purification of the indoleacetaldehyde reductases is
described in Table I. At pH 7, all three reductases fail to bind to cation exchangers, and bind only weakly to anion exchangers such as DEAE-cellulose. This suggests that at this pH, the reductases have a net negative charge accessible to ion exchangers. Upon lowering the pH to 4.5, much unwanted protein precipitates while the reductases remain in solution. If this low pH is maintained, the reductases bind to cation exchangers such as cellulose phosphate. This behavior suggests

that the reductases have accessible acidic groups whose pKₐ values are above or near 4.5. Bound reductases are eluted with buffer of neutral pH (0.1 M sodium phosphate, pH 7.5).

Three distinct indoleacetaldehyde reductase activities are resolved by gel filtration as shown in Fig. 2. The first and third peaks are specific for NADPH as the second substrate, and these activities are referred to as Tᵣ and T₂, respectively. The second peak requires NADH as the second substrate and is referred to as the D activity. We achieve 700- to 1400-fold purifications for the various indoleacetaldehyde reductases as isolated from the gel column. Linear salt gradient DEAE-cellulose chromatography or hydroxylapatite chromatography of these separated enzyme preparations did not lead to significant further purification and were not routinely employed.

Elution of the isolated indoleacetaldehyde reductases from a calibrated Sephadex G-150 gel column led to an estimation of their molecular weights. We assume that the indoleacetaldehyde reductases bear the same relationship of molecular weight to diffusion coefficient as do the calibrating proteins, we are led to the approximate molecular weights 52,000, 33,000, and 17,000 for Tᵣ, D, and T₂, respectively. Further experiments on the physicochemical properties of these enzymes are underway.

Product Identification—Thin layer chromatograms of diethyl ether extracts of the reaction mixtures were developed and sprayed with Ehrlich's reagent as outlined under "Experimental Methods." This reagent causes colored deposits to appear at positions on the plate occupied by indolic compounds. The particular color formed is a function of the indole derivative undergoing the reaction. In ether/hexane and chloroform solvent systems, a compound corresponding to known indoleethanol is absent. Reaction mixtures containing active reductase and indoleacetaldehyde yield a strong pink

Fig. 1. Determination of the molar extinction coefficient at 280 nm for indole-3-acetaldehyde in water. In a modification of the method given under "Experimental Methods," indoleacetaldehyde was freed from its bisulfite adduct into 95% ethanol and its concentration determined spectrophotometrically using the value of e₂₈₀ obtained by Brown et al. (16) for indoleacetaldehyde in this solvent. Dilutions with water were made to known concentrations and the e₂₈₀ determined in each new solvent. Extrapolation to a solvent composition of 100% water yielded the value e₂₈₀ = 5400 liters mol⁻¹ cm⁻¹ for free indoleacetaldehyde in water.

<table>
<thead>
<tr>
<th>Purification of indole acetaldehyde reductases</th>
</tr>
</thead>
</table>
| Protein was determined by the method of Lowry et al. (19). Enzyme was assayed by the Salkowski reagent as described under "Experimental Methods." Enzyme activity is here defined as the amount of enzyme activity yielding a change in A₅₂₉ = 0.1/min when the initial concentration of indole acetaldehyde and NADPH or NADH is 0.4 mm and 0.2 mM, respectively. Absorbance at 529 nm is a measure of the indoleethanol/indole acetaldehyde ratio as shown by Vickery and Purves (10). A reaction time course was carried out with samples from each purification step in order to calculate the reaction rate during the time in which the rate was linear.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total enzyme units determined with indicated nucleotide</th>
<th>Specific activity (units/mg)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude homogenate</td>
<td>76,500</td>
<td>4,650</td>
<td>NADPH</td>
<td>0.06</td>
</tr>
<tr>
<td>2. BioRez 70 supernatant</td>
<td>59,900</td>
<td>7,730</td>
<td>NADPH</td>
<td>0.13</td>
</tr>
<tr>
<td>3. Acid precipitation supernatant</td>
<td>14,700</td>
<td>16,300</td>
<td>NADPH</td>
<td>1.10</td>
</tr>
<tr>
<td>4. Pooled cellulose phosphate-bound protein</td>
<td>3,800</td>
<td>12,500</td>
<td>NADPH</td>
<td>0.302</td>
</tr>
<tr>
<td>5. (NH₄)₂SO₄ precipitation</td>
<td>2,500</td>
<td>11,900</td>
<td>NADPH</td>
<td>4.76</td>
</tr>
<tr>
<td>6. Sephadex gel filtration</td>
<td>120</td>
<td>5,210</td>
<td>NADPH</td>
<td>45.3</td>
</tr>
<tr>
<td>Total enzyme units determined with indicated nucleotide</td>
<td>147</td>
<td>2,840</td>
<td>NADPH</td>
<td>19.3</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>53</td>
<td>4,480</td>
<td>NADH</td>
<td>84.7</td>
</tr>
</tbody>
</table>

TABLE I: Purification of indole acetaldehyde reductases
Fig. 2. Sephadex G-150 column chromatography of indoleacetaldehyde reductases. Aliquots of concentrated protein from the cellulose phosphate extraction were applied and eluted as described under "Experimental Methods." The assay reaction mixture consisted of 0.1 ml of 0.8 mM indoleacetaldehyde, 0.1 ml of 0.8 mM NAD(P)H, and 0.1 ml of column eluate. After 30 min, reactions were stopped with 0.5 ml color when mixed with perchlorate-Salkowski reagent (12). It was shown that the \( A_{280} \) was a function of the ratio of indole ethanol to indoleacetaldehyde, and this property has been used in the assay of indoleethanol oxidase (10, 11) and the indoleacetaldehyde reductases. Fig. 3B shows the visible spectra of three different indoleacetaldehyde to indoleethanol ratios developed with perchlorate-Salkowski reagent. The strong absorption peak centered at 529 nm is accompanied by a small shoulder at shorter wavelengths. This shoulder varies somewhat in shape, and the ratio of peak height to shoulder height decreases as the indoleacetaldehyde to indoleethanol ratio increases. This is probably due to the increased absorbance at shorter wavelengths contributed by reaction of excess indoleacetaldehyde with Salkowski reagent.

Fig. 3A shows the visible spectra of three solutions in which indoleacetaldehyde, NAD(P)H, and the appropriate indoleacetaldehyde reductase had been incubated and the reaction stopped with perchlorate-Salkowski reagent. The correspondences between these spectra and those of mixtures of indoleacetaldehyde and authentic indoleethanol are evident.

Reaction of various indolic derivatives with \( p \)-dimethylaminobenzaldehyde has been shown to yield colored products with distinctly different visible spectra (2, 13). Chromatogram plate areas corresponding to \( R_f \) values of suspected indoleethanol from the reaction mixtures were scraped into ethanol and reacted with \( p \)-dimethylaminobenzaldehyde as described under "Experimental Methods." The results of this experiment are shown in Fig. 4. Material derived from the reaction mixture and removed from the indoleethanol \( R_f \) yields upon reaction with \( p \)-dimethylaminobenzaldehyde a compound with a visible spectrum identical with that of the reaction product of known indoleethanol with \( p \)-dimethylaminobenzaldehyde. Reaction of chromatographed known indoleacetaldehyde with \( p \)-dimethylaminobenzaldehyde yields of Salkowski reagent and \( A_{280} \) determined against a blank consisting of the above substrates which had been incubated with the appropriate boiled enzyme preparation. ----, \( A_{280} \); ---, reaction with NADPH and indoleacetaldehyde; -----, reaction with NADH and indoleacetaldehyde.

Fig. 3. Visible spectra of Salkowski reaction mixtures. A, spectra a, b, and c are of \( T_m \), \( D \), and \( T_i \) reaction mixtures reacted with Salkowski reagent. Enzyme reaction mixtures consisting of 0.1 ml to 0.6 mM indoleacetaldehyde, 0.1 ml of 0.6 mM NAD(P)H, and 0.1 ml of the appropriate enzyme preparation were incubated for 30 min and stopped with 0.6 ml of Salkowski reagent. B, spectra of several mixtures composed of known ratios of indoleethanol to indoleacetaldehyde reacted with Salkowski reagent. Reaction mixtures consisted of 0.1 ml of indoleethanol, 0.1 ml of indoleacetaldehyde, and 0.1 ml of \( H_2O \) reacted with 0.6 ml of Salkowski reagent. Spectra a', b', and c' are of mixtures of indoleethanol to indoleacetaldehyde ratios of 1.26, 0.253, and 0.126, respectively. Spectrum d' is of indoleacetaldehyde at the same concentration as found in each of the above mixtures.
Indoleacetaldehyde Reductases

Substrate specificity of indoleacetaldehyde reductases

Reaction rates with alternate substrates are presented as the percentage of the indole acetaldehyde rate. Each datum is the average of triplicate or quadruplicate reactions. Reaction mixtures consisted of 0.5 ml of substrate, 0.2 ml of 0.84 m M NAD(P)H in 0.1 M sodium phosphate buffer, pH 7.5, and 0.2 ml of enzyme preparation. The reaction rate was determined by observing decrease in $A_{340}$ with time.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>Activity as % of indoleacetaldehyde velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole-3-acetaldehyde</td>
<td>0.33</td>
<td>100</td>
</tr>
<tr>
<td>Indole-3-aldehyde</td>
<td>0.425</td>
<td>0</td>
</tr>
<tr>
<td>Phenyl acetaldehyde</td>
<td>0.11</td>
<td>43</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>trans-Cinnamaldehyde</td>
<td>1.0</td>
<td>2</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>5.0</td>
<td>17</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>5.0</td>
<td>8</td>
</tr>
</tbody>
</table>

With the aliphatic aldehydes, both $T_H$ and $T_L$ show some activity toward these substrates. Especially in the case of butyraldehyde, this activity may result from a partial occupation of the aromatic function binding site by the terminal methyl and methylene carbons, still allowing the correct positioning of the aldehyde group.

The presence of endogenous indoleacetaldehyde in cucumber seedlings has recently been conclusively shown. The product of phenyl acetaldehyde reduction by these enzymes is phenyl ethanol, and attempts in our laboratory to show the natural presence of this compound in cucumber seedlings by steam distillation and vapor phase chromatography have been unsuccessful. Thus it seems likely that the predominant substrate for these enzymes in vivo is indoleacetaldehyde.

There is an absolute requirement for reduced NAD(P) in order to effect aldehyde reduction as assayed both by the Salkowski reaction and by thin layer chromatography of reaction mixtures. The elution profile from Sephadex G-150 indicates that there is little or no cross-reactivity between NADPH and NADH with any of the three reductases (Fig. 2). The slight contamination of the separated enzyme preparations with enzyme from neighboring peaks made direct nucleotide specificity studies difficult as did the lack of sensitivity of the reductases to product inhibition. While absolute specificity has not been shown, we conclude that there is at least a marked preference for the respective nucleotides.

Enzyme kinetic studies were performed in which the initial decrease in absorbance at 340 nm as a function of time was recorded continuously and taken as the reaction velocity. At the substrate concentrations tested, typical hyperbolic kinetic data were observed for each of the indoleacetaldehyde reductases. With the reduced nucleotide concentration held constant at 0.2 mM, apparent $K_m$ values for indoleacetaldehyde at pH 7.0 of 400 μM, 73 μM, and 130 μM were obtained for D, $T_H$, and $T_L$, respectively. The apparent $K_m$ values for the appropriate reduced nucleotides for D, $T_H$, and $T_L$ were 180 μM, 23 μM, and 12 μM when the indoleacetaldehyde concentration was held constant at 0.4 mM. The $K_m$ values for phenyl acetaldehyde

were also determined and found to be comparable to those for indoleacetaldehyde. It is interesting to note that although the D reductase shows the greatest substrate specificity (Table II), it has the highest values of $K_m$ for indoleacetaldehyde and reduced nucleotide.

Several experiments were performed which were designed to detect the reverse reaction in which reducing equivalents from indoleethanol are transferred to NAD(P)$^+$ yielding indoleacetaldehyde and NAD(P)H. Under no conditions of substrate concentration or incubation time could this reverse reaction be observed, either by Salkowski color reaction, increase in $A_{340}$, or thin layer chromatography of product. Further, none of the reductases showed any sensitivity to inhibition by product (indoleethanol, NAD(P)$^+$) with decreasing $A_{340}$ as the assay. It is a common feature of pyridine nucleotide-dependent dehydrogenases that the $K_m$ values for the alcohol and oxidized nucleotides are 10 to 1000 times higher than for the corresponding substrates. However, the limited solubility of indoleethanol in water makes it impossible to achieve concentrations such that its oxidation might be observed. Thus, the inability to observe the reverse reaction, coupled with the lack of product inhibition by indoleethanol and NAD(P)$^+$, leads us to conclude that these enzymes function as reductases in vitro. It would be surprising if they functioned in another manner in vivo.

Effects of pH and Ionic Strength—The response to pH of the indoleacetaldehyde reductases was explored in the pH range 3.6 to 9.3, using barbital-acetate buffer. Fig. 5 shows the initial velocity as a function of pH for the three indoleacetaldehyde reductases. Both $T_h$ and $T_t$ show quite similar responses to pH, with an optimum pH of 5.2. The pH optimum for the D enzyme is 7.0. All three enzymes are remarkable in that their pH curves show secondary optima.

The $K_m$ values for indoleacetaldehyde at pH 5.2 and 7.0 may be compared for the $T_h$ and $T_t$ enzymes. In the case of the $T_h$ enzyme, the $K_m$ values for indoleacetaldehyde in barbital-acetate buffer are 67 μM and 200 μM for pH 7.0 and 5.2, respectively. For the $T_t$ enzyme, the values were 133 μM and 250 μM. In both cases, the $K_m$ values for indoleacetaldehyde increased as the pH was shifted to the acid optimum. Since the initial velocities at various pH values (Fig. 5) were measured at indoleacetaldehyde concentrations near the pH 7.0 $K_m$ values for these enzymes, an increase in the $K_m$ for this substrate at lower pH values might be expected to lead to a decreased initial velocity at those pH values. The opposite behavior was observed, and we tentatively conclude that the effect of low pH on $T_h$ and $T_t$ is to increase their absolute turnover rates.

Fig. 6 shows the initial velocity as a function of the sodium chloride concentration at pH 7.5 and constant substrate concentration. We have assumed that the effects of NaCl are due to its ionic strength and are not specific to the sodium or chloride ions. It can be seen that the responses of the three reductases are distinctly different. The D enzyme is slightly inhibited at increased ionic strength, while both $T_h$ and $T_t$ are activated at NaCl concentrations up to 0.1 M. $T_t$ reaches an optimum at this concentration. Although the higher NaCl concentrations are most likely unphysiological, the overall responses to ionic strength further mark these enzymes as distinctly different. Further studies designed to elucidate the mechanism of these ionic strength effects are under way.

Recent results in our laboratory1 have indicated that one or

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DISCUSSION

This paper reports the first extensive purification and characterization of indoleacetaldehyde reductases from any organism. Several reports have appeared in which indoleacetaldehyde reducing activity has been partially characterized. Rajagopal (17) has demonstrated an indoleacetaldehyde reducing activity in "whole cytoplasm" of Avena. He found this activity to be dependent upon added reduced nucleotide, with greater indoleacetaldehyde reduction being observed with NADPH than with NADH. The whole cytoplasm preparation showed a pH optimum of approximately 5.6 with respect to this activity while an acetone powder extract of Avena exhibited an indoleacetaldehyde reducing optimum at about pH 7.6. This was taken as evidence for two separate indoleacetaldehyde reducing activities, although it is possible that the acetone extraction simply led to a shift in the pH optimum of the same enzyme. The production of indoleethanol from indoleacetaldehyde by this whole cytoplasm preparation was inhibited by high concentrations of indoleacetaldehyde (at constant reduced nucleotide concentration). This was interpreted as substrate inhibition of the reductase activity. It is difficult to know whether this observation was indeed due to an effect on the indoleacetaldehyde reductase(s) since the reductases were not purified away from enzymes possibly capable of converting the indoleacetaldehyde or indoleethanol to undetected compounds at these higher indoleacetaldehyde concentrations. The partially purified indoleacetaldehyde reductases from cucumber seedlings are not inhibited by high concentrations of indoleacetaldehyde.

Wightman and Cohen (18) have reported an NADH-dependent indoleethanol dehydrogenase from preparations of mung bean. Using the supernatant from a high speed centrifugation of mung bean homogenate, they observed indoleethanol formation from indoleacetaldehyde bisulfite and NADH by thin layer chromatography. This indoleacetic acid formation was blocked by added bisulfite which was presumed to react with indoleacetaldehyde formed from indoleethanol. However, an alternate explanation is that the oxidation of indoleethanol to indoleacetaldehyde was catalyzed by indoleethanol oxidase (10, 11) and was not due to a reversal of the indoleacetaldehyde reducing activity. If this is the case, the added NADH was either instrumental in the oxidation of indoleacetaldehyde to indoleacetic acid by an aldehyde dehydrogenase (18) or was unnecessary since the results of a control reaction without this compound were not reported.

It has been clearly established that indoleethanol is an intermediate in the synthesis of the hormone indoleacetic acid (2-6, 9). The demonstration that indole ethanol oxidase is inhibited by indoleacetic acid (11) and that indoleethanol is found in rather large amounts in vivo (4) suggest that indoleethanol plays a regulatory role in indoleacetic acid synthesis. We have now identified and partially characterized three distinct, highly specific indoleacetaldehyde reductases from cucumber seedlings. Further studies are under way concerning the intracellular localization and regulatory properties of these enzymes.

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