Kinetic Studies on Growth of Excised Root Tips of Lycopersicum esculentum 1, 2

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When isonicotinic acid hydrazide (INH) was first used for therapy of tuberculosis many patients developed peripheral neuritis resembling that of pyridoxine deficiency. Such patients exhibited increased urinary excretion of pyridoxine; administration of pyridoxine prevented the neuritis (1). In vitro studies have confirmed that INH inhibits pyridoxine-dependent bacterial decarboxylases and transaminases (2, 21, 22). This inhibition could also be released more effectively with pyridoxal than with pyridoxine (2, 21). The structures of pyridoxal and its analog are illustrated in figure 1.

Fig. 1. Structural formulas of pyridoxal (left) and isonicotinic acid hydrazide (right).

An effect of INH on several higher plants was also discovered (20); Ludwig observed in 1958 that INH inhibits the growth of tomato and pea seedlings (10). He ascribed this effect to an inhibition of auxin production in plants, since the formation of the auxin, indole acetic acid (IAA) requires decarboxylation and transamination.

The coenzymatic role of pyridoxal phosphate is known in many types of enzymatic reactions (12). In all of these reactions amino compounds, chiefly, α-amino acids are activated. It was therefore reasonable to suspect that the inhibitory effect of INH is mainly concerned with the amino acid and protein metabolism of the plant, rather than with auxin production, as Ludwig suggested. To test this hypothesis excised roots of tomato were selected in which exogenous IAA or β-indolylacetonitrile (IAN) is either without any effect or inhibitory to their growth (17), and it could be shown that the antiauxin, α-(1-naphthylmethyl-sulphide) propionic acid (NMSP), stimulated growth and enhanced survival of main axis meristems when repeatedly subcultured (16).

In the present paper an attempt is made to give a kinetic treatment of the growth of excised tomato root tips. The dynamics of root growth, whose time-dependent cellular changes reveal a highly organized growth pattern have been studied by Erickson and Goddard (5). Erickson and Sax (6, 7), and Goodwin and Stepka (8). Root inhibition studies in cellular terms have been made on intact roots by Burström (3) and on excised ones by Torrey (18, 19). However, so far as the author is aware, while the Line weaver-Burk Analysis of the kinetics of inhibition has been applied by McRay and Bonner (11) for demonstrating auxin antagonism in excised Avena coleoptile sections, this analysis has not been applied to root elongation phenomena. The difficulty in undertaking such a study lies partly in the fact that the use of the formal methods of enzyme kinetics on a highly organized (growing) system might be seriously questioned. Also, in many cases there is doubt of the usefulness of elongation as a measure of any specific type of activity. However, a purely quantitative treatment may be useful in expressing results in a more precise way: parameters can be introduced and compared with each other: more precise hypotheses can be formed, with the consideration that questions of the cellular and biochemical changes which underlie growth are not adequately answered.

► Kinetic Treatment & Terminology: In many cases an enzyme will function only in the presence of a coenzyme which is attracted to the enzyme protein; the combination of the enzyme with the coenzyme can be subjected to exactly the same treatment as enzyme substrate complex formations and, hence, the dissociation constant ($K_s$) of the enzyme-coenzyme complex can be found.

On the basis of these considerations it is possible to treat the growth response of excised and depleted tomato roots to added pyridoxal, by the formal methods of enzyme kinetics as follows:

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I  \[ E + S \xrightarrow{\text{ES}} \text{ES} \]
II  \[ \text{ES (+ any second reactant)} \xrightarrow{\text{E + prod}} \text{ES} \]

where

- E = apoenzyme within the root tip
- S = coenzyme (the transformation of pyridoxal to pyridoxal phosphate is assumed within the root)
- ES = activated holoenzyme

if

- \( s \) = concentration of free pyridoxal
- \( e \) = total apoenzyme concentration
- \( p \) = concentration of activated holoenzyme

III  \[ K_s = \frac{(e - p)s}{p} = K_m = \text{pyridoxal concentration giving half the maximal velocity} \]

This equation, however, is based on equilibrium conditions between ES, E, and S, where any effect of reaction II on the equilibrium is ignored. In cases where unusually high affinities for the substrate (CoE) are observed and the ES complex will not dissociate readily, Briggs-Haldane steady state kinetics have been shown to apply. This holds in general for the pyridoxal phosphate activated enzymes, as shown in experiments with purified enzyme preparations by Meister et al. (13). Introducing velocity constants into the separate reactions

IV  \[ E + S \xrightarrow{k_1} \text{ES} \xrightarrow{k_2} E + \text{product} \]

\( K_s \) in the Michaelis equation is replaced by \( K_m \) as follows:

\[ V = \frac{V}{1 + \frac{K_m}{s}} \]

where

V  \[ K_m = \frac{k_2 + k_3}{k_1} = K_s + \frac{k_3}{k_1} \]

and

\[ V = \text{maximum velocity of the reaction (growth) obtained when the enzyme is saturated with substrate (CoE).} \]

Reaction velocity (v) is expressed in terms of relative growth rate. During growth the length of an apical segment of the root, X, will change with time. If the instantaneous rate of growth \( dX/dt \) is divided by the length of the growing root tip one obtains:

\[ \frac{1}{X} \cdot \frac{dX}{dt} = \frac{d\ln X}{dt} \]

the relative elongation rate of an apical segment X. It has the dimensions: unit length, per unit length, per unit time. Cancelling the dimension of length from the numerator and denominator and multiplying by 100 one may speak of it as a percentage rate. According to Erickson (6) it is a useful measure wholly apart from any particular theories of the nature of growth.

The graphical analysis of the competitive inhibition follows the Lineweaver-Burk method, discussed by Dixon and Webb (4).

Taking the reciprocal of equation V

\[ \frac{1}{V} = \frac{1}{V} + \frac{K_m}{V} \cdot \frac{1}{s} \]

and plotting \( 1/V \) against \( 1/s \), if the resulting straight line is extrapolated to the left of the zero ordinate, it will cut the abscissa giving an intercept of \( \frac{1}{K_m} \). This is evident by putting \( s = 0 \) in equation VII which then becomes

\[ \frac{1}{s} = \frac{1}{K_m} \cdot \frac{1}{K_p} \]

In the presence of the inhibitor the parameter \( K_p = \text{substrate concentration giving half maximum velocity} \) is obtained graphically. \( K_i \), the dissociation constant of the enzyme inhibitor complex, is calculated from the following expression:

\[ K_i = \frac{i}{K_p \left(1 - \frac{K_p}{K_m'} \right)} \]

where \( K_m' \) is the Michaelis constant in the presence of excess of inhibitor.

**Experimental Methods**

Culture Techniques: Excised roots of *Lycopersicon esculentum* Mill. variety Bonny Best were used. The seeds were surface sterilized for 10 minutes in 1% Ca(OCl), solution, rinsed in sterile double distilled water, and germinated in the dark in petri dishes on 1% washed agar at 25°C for 64 to 66 hours. Root tips 2 mm long were then excised aseptically and transferred to a depletion medium lacking any vitamins, in 10 cm petri dishes, 8 to 10 roots per dish. They were kept in the dark at 25°C for 52 hours. First-transfer primary root tips of 2 mm were excised from these isolated roots, selected for uniformity, and transferred to 20 ml experimental media of various compositions. For each treatment usually 10 to 16 roots were used with replication at least three times in different experiments.

For depletion the incomplete modified Street medium contained per liter of twice distilled water from pyrex glass: 20 g sucrose; 200 mg CaNO₃; 360 mg MgSO₄; 80 mg KNO₃; 65 mg KCl; 200 mg Na₂SO₄; 16.5 mg NaH₂PO₄; 0.75 mg KI; 45 mg MnCl₂; 1.5 mg ZnSO₄; 1.5 mg H₃BO₃; 0.0017 mg H₂MoO₄; 0.013 mg CuSO₄; 6.1 mg ferric citrate as anhydrous com-
pounds, and 10 g washed agar. The same medium was supplemented in the experimental media with 0.1 mg thiamine HCl, 0.5 mg nicotinic acid, and various concentrations of INH and pyridoxal HCl. The pH of the medium was adjusted to 4.8 before autoclaving with a glass electrode by dropwise addition of 0.05 N HCl or 0.05 N KOH. Pyridoxal and INH solutions were sterilized separately by cold glass filtration and were added to the medium after autoclaving with the same pH adjustment.

Methods of Recording Growth: When the 2 mm long root tips were transferred to the experimental media their growth was recorded up to 48 hours. From these measurements initial growth rates were estimated. The growth rates were obtained with an automatic camera taking pictures one per hour, and increments were analyzed at 3-hour intervals. The negatives were projected with a 35 mm strip film projector on a cardboard screen at 13X the original length of the roots. Measurements made in this way were more accurate than under a dissecting microscope, since any curvature of the roots which started to appear after 24 hours could be followed with an error of ± 150 μ. In experiments with different simultaneous treatments initial length was measured under a dissecting microscope at 16X with an error of ± 100 μ. Final lengths were obtained by operating the automatic camera manually.

Results & Discussion

From figure 2 it is apparent that the depleted roots increase in length exponentially up to 48 hours when transferred to the experimental media, in the presence of externally supplied pyridoxal. In other words, the logarithm of root length is linearly related to time, or the relative rate of elongation remains constant for 2 days. The initial relative rate of elongation of the roots under these conditions was 0.96 % per day as calculated by the least squares method.

If pyridoxal is not added to the experimental medium a complex growth curve results (fig 3). One cannot, as in the case of figure 2, cite a single value of the relative elongation rate. A simple comparison can, however, be made in terms of the relative length increment in 2 days. In this case

\[
\frac{\ln X_2 - \ln X_0}{2} = 0.74 \text{ day}^{-1}
\]

as compared with relative rate 0.96 day\(^{-1}\) in the presence of pyridoxal.

The explanation of this complex growth cycle presents some difficulty. From the graph it appears that two growth phases are separated by a lag phase. This phenomenon, which is called diauxie, i.e., double growth, was observed by Monod (14) when bacteria are grown in media containing limiting amounts of two different carbohydrates. In his experiments the first growth phase corresponds to the exclusive utilization of one of the sugars followed by a period of adaptation before the other substrate is metabo-

Fig. 2 (Top). Growth of excised roots in the presence of 4.9 \times 10^{-7} \text{M} pyridoxal. Each point is the mean of ten measurements, from one experiment.

Fig. 3 (Bottom). Growth curve of excised roots, in the absence of pyridoxal. Each point is the mean of ten measurements from one experiment.
lized and growth recommences. It may be assumed that the first very brief growth phase corresponds to the remaining limiting amounts of residual vitamins in the root after depletion and is followed by an adaptation to the utilization of vitamin B₁ and nicotinic acid from the experimental medium and a possible synthesis of essential growth substances.

If the pyridoxal-induced growth rate is plotted as a function of pyridoxal concentration, the hyperbolic form of a typical substrate concentration curve is obtained in figure 4. The initial slope gives \( V \) and

\[
V = \frac{V}{K_m} = 0.096 \text{mM (10^{-6} M) DAY}^{-1}
\]

and from the final part of the curve where \( V = V' \) the maximum value of relative growth rate can be estimated. It may be noted from equation \( V \) that

\[
\frac{V}{2} = \frac{V}{K_m}
\]

when \( s = K_m \), \( v = \frac{V}{2} \) or vice versa, so that the pyridoxal concentration at which half the maximal relative growth rate is attained is numerically equal to the value of \( K_m \). Obtained by a graphical method \( K_m = 4.9 \times 10^{-8} \text{M} \). This low value would indicate an exceptionally high affinity for pyridoxal. However, the severe limitations of such data should be emphasized. According to Snell (15) the association reactions, (a) and (b) below, are not freely reversible in any instance,

(a) Pyridoxal phosphate + apoenzyme \( \longrightarrow \) holoenzyme

(b) Analog coenzyme + apoenzyme \( \longrightarrow \) analog holoenzyme

and the numerical values of the molar concentrations required for half maximum activation or inhibition, especially those for inhibitory analogs are subject to considerable variation depending upon the experimental conditions. In the present study for instance, the length of the depletion period is critical.

![Fig. 4](image-url) Relative growth rates of excised roots in presence of pyridoxal. The basal rates without pyridoxal in the medium are subtracted from the pyridoxal induced growth rates.

![Fig. 5 (Top)](image-url) Growth curve of excised roots in the presence of \( 1.5 \times 10^{-4} \text{M INH} \). Each point is the mean of ten measurements from one experiment.

![Fig. 6 (Bottom)](image-url) Growth curve of excised roots in the presence of \( 1.5 \times 10^{-4} \text{M INH} \) and \( 4.9 \times 10^{-7} \text{M pyridoxal} \). Each point is the mean of ten measurements from one experiment.
Despite such difficulties even crude approximations permit valid comparisons of affinities of pyridoxal and its analog in influencing growth.

During the depletion period the roots were not completely deprived of pyridoxal since 1.5 × 10^{-4} M INH in the experimental medium decreased the average relative increase in length from 0.74 to 0.46 per day (fig 5). However, when the same medium was supplied with 4.9 × 10^{-7} M pyridoxal, exponential growth was obtained with an 0.86 average relative rate of elongation per day, calculated by the least squares method (fig 6).

The effects of varying concentrations of pyridoxal and of INH alone and in combination on relative rates of root elongation are summarized in table I. It is apparent that INH inhibits pyridoxal induced root elongation and that inhibition increases with increasing concentrations of INH. Further, the inhibition decreases with increasing concentrations of pyridoxal.

In general, reversible inhibitors may act either on the apparent K_m or V or on both. Those which act by increasing the effective K_m are termed competitive inhibitors, since the structural analog and the true substrate (or coenzyme) tend to drive one another off the enzyme, so that the inhibitor competes with the substrate for the enzyme.

Those which have no effect on K_m but act simply on V are termed non-competitive. The effect of a competitive inhibitor is abolished in high substrate (coenzyme) concentrations, which means that V is not affected. That of a non-competitive inhibitor remains.

To determine the mode of action of INH on root elongation—was plotted against—according to the Lineweaver-Burk method, (fig 7). The lower line is that obtained in the presence of pyridoxal alone while the upper two lines are those obtained in the presence of pyridoxal but with increasing concentrations of INH. The three lines were fitted with the least squares method and the significance of slopes A and B was demonstrated with t-test.

The double reciprocal plot shows that in the presence of 5 × 10^{-5} M INH the slope K_m increased but the intercept or V remained unchanged. The effective K_m increased to a K_p of 1.77 × 10^{-7}, or the affinity of the apoenzyme for pyridoxal decreased about 30-fold. This would indicate a competitive effect. The value of K_i, 1.9 × 10^{-5}, does not suggest a great affinity of INH for its reactive site within the root.

In the presence of 1.5 × 10^{-4} M INH both the slope and intercept increased, while K_p remained the same. This would indicate a non-competitive effect. One would conclude from this plot that INH may act in more than one way, either giving a mixed type of inhibition by affecting both K_m and V or influencing two different enzymatic reactions. The difference between intercepts A and C, however, is not significant on a 20 to 30 % probability level as obtained with t-test; we may eliminate these hypotheses on statistical grounds.

Assuming that one pyridoxal phosphate-dependent rate-limiting reaction is affected by INH one may regard the observed inhibition as a competitive one. From the available data it is not possible to decide whether it is fully or partially competitive; in other words only EI or both EI and EIS complexes are formed with the inhibitor. According to Dixon (4)

![Fig. 7. Data from table I plotted according to the Lineweaver-Burk method. Curve A refers to pyridoxal alone, curves B and C refer to 5 × 10^{-5} M and 1.5 × 10^{-4} M INH in the presence of varying concentrations of pyridoxal.](image)

### Table I

<table>
<thead>
<tr>
<th>Concentration of INH (molar)</th>
<th>Conc of pyridoxal HCl (molar)</th>
<th>Conc of pyridoxal HCl in mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00</td>
<td>4.91 × 10^{-8}</td>
</tr>
<tr>
<td></td>
<td>0.00</td>
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<tr>
<td>0.00</td>
<td>0.696</td>
<td>0.814</td>
</tr>
<tr>
<td>5 × 10^{-5}</td>
<td>0.523</td>
<td>0.662</td>
</tr>
<tr>
<td>1.5 × 10^{-4}</td>
<td>0.414</td>
<td>0.517</td>
</tr>
</tbody>
</table>

* Growth rates in the absence of exogenous pyridoxal are not subtracted from the induced ones. Each rate is an average of 30 measurements.
the EIS complex when formed breaks down at the same rate as the ES complex, so a partially competitive system is completely indistinguishable from a purely competitive type in a Lineweaver-Burk plot. While in this study, in agreement with Lichtstein's observations (9), the primary site of action of INH is to compete with pyridoxal phosphate for the alopezyme, the possibility that the hydradize group of the analog might inactivate the coenzyme by the formation of a hydradzone cannot be neglected.

The role of pyridoxal in overcoming the inhibition of INH in the described system, cannot be ascribed to a general growth-promoting ability of the former compound. The pyridoxal-promoted root elongation amounts to 20% (table 1). However pyridoxal reduced the inhibition of INH from 40% to 10%. This relatively large reduction in inhibition suggests a competitive relation between the two compounds.

Returning, finally, to our initial problem one may conclude since INH had neither a zero nor a stimulatory effect on the growth of excised tomato roots that it does not affect auxin metabolism, but might well be concerned with amino acid and protein metabolism in plants.

Summary

The growth of first-transfer primary root tips of *Lycopersicum esculentum* Mill. was studied, as inhibited by isonicotinic acid hydradize (INH) and promoted by pyridoxal HCl. Continuous growth curves were obtained with an automatic camera. The elongation of the root was constant up to 48 hours in the presence of pyridoxal. Inhibition by INH was almost completely abolished with increasing concentrations of pyridoxal. The Lineweaver-Burk graphical analysis of the data points to a competitive inhibition.

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Literature Cited