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Reversal of Hydroxyproline-Induced Inhibition of Elongation of Avena Coleoptiles[1, 2]

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Summary. Hydroxy-L-proline-induced inhibition of elongation of *Avena* coleoptile segments was measured in water and in indole-3-acetic acid. This inhibition was completely reversed by L-proline.

Time-sequence experiments revealed that some time had to elapse, or some elongation had to occur, prior to the onset of hydroxyproline inhibition. The presence of sucrose supported the rate of auxin-induced elongation throughout a 24-hour period, and enhanced the effectiveness of hydroxyproline as an inhibitor of elongation.

The application of adenosine triphosphate effectively counteracted the hydroxyproline-induced inhibition of elongation; completely in water, and partially in indole-3-acetic acid. Optimal ATP concentrations were between 0.25 and 0.75 mM. Similarly, guanine-HCL, L-glutamic acid, and L-ornithine-HCL were capable of reversing the hydroxyproline inhibition. Other compounds that were tested but which proved to be less effective are tabulated.

It is suggested that hydroxyproline may exert its inhibitory effect on the protein and/or RNA synthesis necessary for elongation of the oat coleoptile by interference with the metabolism of adenosine triphosphate or other high energy compounds.

Steward et al. (17) and Pollard and Steward (14) observed that hydroxyproline supplied exogenously to cultures of carrot tissue explants was a powerful inhibitor of growth. They attributed its inhibitory action on growth and on protein synthesis to its competition with proline, since the presence of L-proline reversed the inhibition.

Cleland (2, 4) noted that auxin-induced growth of *Avena* coleoptile sections was inhibited by hydroxyproline, and that this inhibition could be completely reversed with equimolar amounts of proline. His experimental evidence indicated that hydroxyproline acted as an antagonist of some aspect of proline metabolism (4).

Recently Norris (13) reported that ATP was capable of reversing ethionine-induced inhibition of elongation of *Avena* coleoptiles. In light of these results it seemed appropriate to examine the effect of ATP on the hydroxyproline-induced inhibition of elongation of *Avena* coleoptile sections. The results of these experiments are reported here, along with the effects of various purine and pyrimidine bases, nucleosides, and amino acids.

Materials and Methods

Avena sativa L. seeds, Victory Strain (U. S. Department of Agriculture, C. I. 2020) were used in these experiments. The seedlings were grown on filter paper strips immersed in distilled water which previously had been aerated. Additional details of the growing method are described elsewhere (20). Only seedlings that were 72 hours old and that had 30 ± 2 mm coleoptiles were used.

Elongation measurements were made on coleoptiles which were isolated from the seeds and primary leaves. The second 5-mm sections were used, measuring from the apex toward the base. These sections were floated in Petri dishes containing the media (20 sections/20 ml solution). The control medium was previously aerated distilled water or 10 μM IAA. Other substances were added to these as indicated. During cutting and transferring, the sections were exposed to low intensity red neon light (wavelengths longer than 6074A). The sections were allowed to elongate in the dark for 24 hours. At the end of this period, their lengths were measured with a micrometer in the ocular of a stereoscopic microscope. All procedures were carried out at a temperature of 22 ± 1°.

The compounds used in these experiments were obtained from Nutritional Biochemicals Corporation.

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[2] A preliminary report of this work was presented to the Southern Section, ASPP, at Jackson, Mississippi in February 1966.

Results

Figure 1 shows the effects of various concentrations of hydroxy-L-proline, L-proline, and mixtures of 10 mM hydroxy-L-proline and L-proline on elongation of oat coleoptile sections in water. The amount of elongation of the coleoptile segment in water is taken as the control value, which in a 24-hour period is between 1.5 and 2.0 mm, averaging about 1.7 mm. It is apparent that hydroxyproline, at a concentration of 10 mM, inhibits elongation about 50 %, while proline alone is somewhat stimulatory, from 10 to 25 % at the higher concentrations. The third curve represents the results obtained when various amounts of proline were mixed with 10 mM hydroxyproline. It is evident that the presence of proline completely reverses the inhibitory effect of hydroxyproline, and furthermore in relatively low concentrations, e.g. the presence of 0.75 mM proline completely negates the inhibitory effect of 10 mM hydroxyproline.

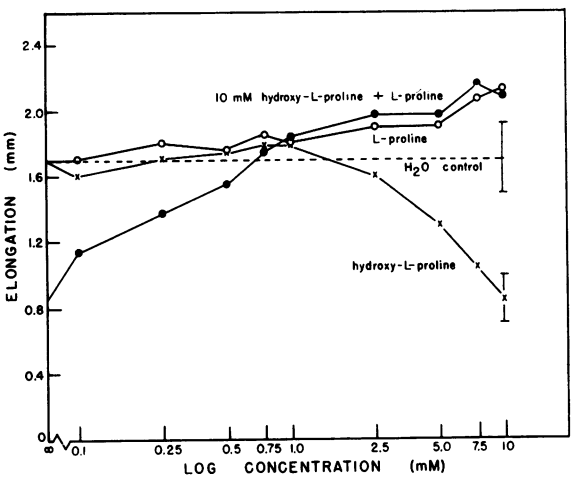

FIG. 1. Influence of hydroxy-L-proline, L-proline, and mixtures of 10 mM hydroxy-L-proline and L-proline on elongation of the second 5-mm segment of the *Avena* coleoptile in water. Standard deviations are shown for the water control and for 10 mM hydroxy-L-proline. For the hydroxy-L-proline and L-proline curves each plotted point is the average of 40 to 80 measurements, while the points on the 10 mM hydroxy-L-proline plus L-proline curve result from an average of 20 to 60 measurements.

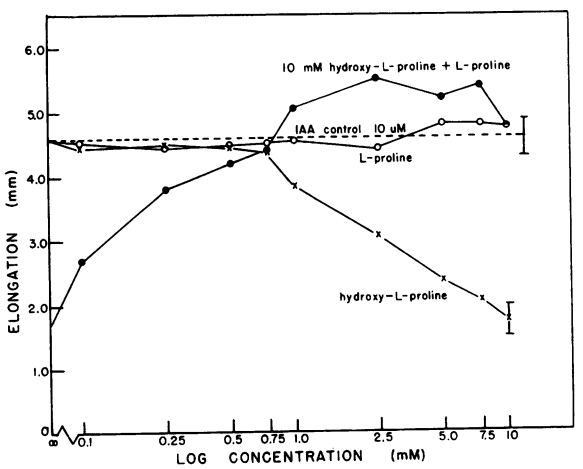

FIG. 2. Influence of hydroxy-L-proline, L-proline, and mixtures of 10 mM hydroxy-L-proline and L-proline on elongation of the second 5-mm segment of the *Avena* coleoptile in IAA. Standard deviations are shown for the IAA control and for 10 mM hydroxy-L-proline. Each plotted point is the average of 20 to 80 measurements.

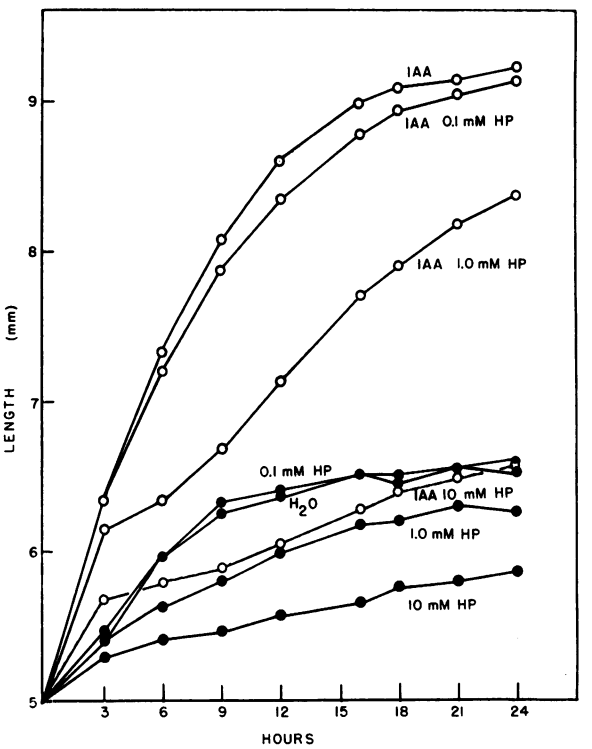

FIG. 3. Time course of *Avena* coleoptile section elongation in water (●—●), in IAA (○—○), and in various concentrations of hydroxyproline in water, and in IAA. Each plotted point is the average of 10 measurements. The figure represents the data of 1 typical experiment selected from 2 replications.

The data presented in figure 2 differ only in that the control solution was 10 μM IAA. In the presence of this concentration of exogenous IAA the average elongation for a 24-hour period was 4.6 mm as compared to 1.7 mm in water. In general the results indicate the same relationship as those shown in figure 1. The 10 mM hydroxyproline is slightly more inhibitory, inhibiting elongation by about 60 %; proline alone causes less stimulation of growth; and again the addition of 0.75 mM proline completely reverses the inhibitory effect of 10 mM hydroxyproline.

These results are in accord with the work of Cleland (4) with the exceptions that he obtained maximal inhibition with more dilute solutions of hydroxyproline (0.5 mM), and that in the absence of IAA he observed little effect of hydroxyproline, even in concentrations as high as 0.01 M. It should be noted however that Cleland's experimental medium contained 2 % sucrose and K maleate buffer at a pH of 4.6. Frequent checks of our experimental solutions revealed the pH to be in the same range. Cleland (2) has divided the response of auxin-treated coleoptile sections to hydroxyproline into 2 phases: (1) initially after addition of hydroxyproline no inhibition of elongation occurs for a time, then (2) growth becomes inhibited and the sections elongate at a low, but constant rate. He has found both the length of the lag and the amount of the subsequent inhibition to be functions of the hydroxyproline concentration (4).

Figure 3 records the time course of the *Avena* coleoptile sections elongation in water, in IAA, and in various concentrations of hydroxyproline in water, and in IAA. No substantial inhibition resulted from 0.1 mM hydroxyproline in either water or IAA. In the presence of the higher concentrations of hydroxyproline (1.0 and 10 mM) it is evident that the hydroxyproline does not exert its maximum inhibitory effect on elongation until 3 hours have elapsed. In general these observations are in agreement with those of Cleland's (4) with the exceptions previously noted.

Since Cleland (4) found that sucrose significantly enhanced the effectiveness of hydroxyproline as an inhibitor, the time course experiments shown in figure 4 were conducted. The presence of sucrose does increase the auxin-induced elongation and appears to augment the inhibitory effect of 10 mM hydroxyproline. Again it may be noted that the maximum inhibitory effect of hydroxyproline on elongation is not exerted until after elongation has proceeded for 3 hours.

The influence of various concentrations of ATP on elongation of the coleoptile both in water and in 0.01 M hydroxyproline is shown in figure 5. Standard deviations are shown on all points, and the water control as well as the 0.01 M hydroxyproline control is included. It is apparent (closed circles) that in water the addition of various concentrations of ATP resulted in stimulation of elongation of the oat coleoptile amounting to a maximum of about 30 % at an ATP concentration of 0.25 mM. Why higher concentrations of ATP inhibit elongation is not clear, however both of these observations are in accord with previously reported results (13). When the coleoptile segments were allowed to elongate in 0.01 M hydroxyproline, an inhibition of approximately 45 % resulted. When ATP was added to the hydroxyproline solution (open circles) the inhibition of elongation caused by the 0.01 M hydroxyproline was completely reversed by ATP concentrations of 0.5 and 0.75 mM.

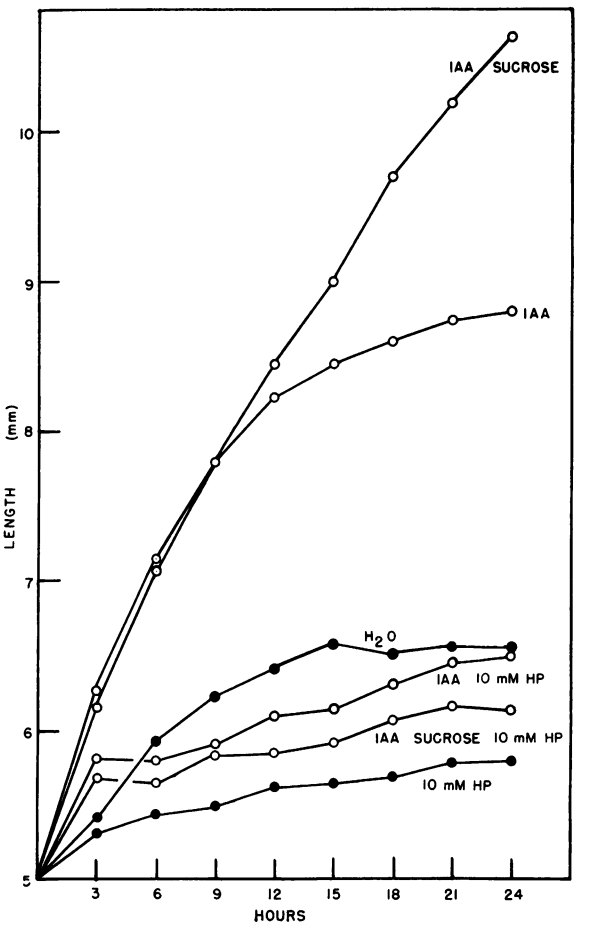

FIG. 4. Time course of *Avena* coleoptile section elongation in water (●—●), in IAA (○—○), in IAA and sucrose (○—○), and in each of these media containing 10 mM hydroxyproline. Each plotted point is the average of 10 measurements. The figure represents the data of 1 typical experiment selected from 2 replications.

The data presented in figure 6 differ only in that the control solution was 10 μM IAA instead of water. In general the results are the same as those shown in figure 5 with the exceptions that (1) ATP alone (closed circles) is not as stimulatory as it was in water; here stimulation is shown only at a concentration of 0.1 mM, while all higher concentrations of ATP inhibit elongation, (2) hydroxyproline is more inhibitory in the presence of exogenous auxin: 58 % as compared to 45 % in water, and (3) upon mixing ATP with the 0.01 M hydroxyproline in IAA, reversal of the hydroxyproline-induced inhibition of elongation was not as complete as it was in water; at an ATP concentration of 0.5 mM a 21 % reversal of the hydroxyproline-induced inhibition resulted.

Figure 7A includes data identical with that presented in figures 5 and 6 except all solutions contained 2 % sucrose. The addition of sucrose enhances elongation, especially when exogenous

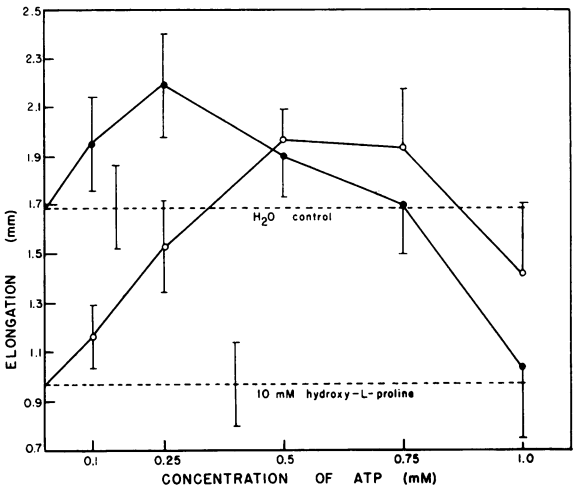

FIG. 5. Influence of ATP on the elongation of the second 5-mm segment of the *Avena* coleoptile. ○—○ in a strongly inhibitory solution (0.01 M) of hydroxy-L-proline; ●—● in water. Standard deviations are shown. Each plotted point is the average of 40 to 60 measurements.

auxin is present [this was previously shown in fig 4, and has been reported by Schneider (16) and by Cleland (4)]. Where the average 24-hour elongation of the segments in water had been about 1.7 mm, in 2 % sucrose it is 1.9 mm, and in IAA average values were elevated from 4.3 mm to 6.3 mm during the 24-hour elongation period. In the absence of exogenous auxin ATP proves to be more stimulatory to elongation when sucrose is present (75 % as compared to 30 % in fig 5). ATP completely reverses the hydroxyproline inhibition in both cases. It is also clear, contrasting the data of figure 7A with those of figures 5 and 6, that the hydroxyproline-induced inhibition of elongation is enhanced in the presence of 2 % sucrose (in water inhibition is 43 % and in 2 % sucrose it is 55 %; in IAA inhibition is 58 % while in IAA and 2 % sucrose it is 78 %). This is in accord with Cleland's work (4). In the presence of IAA and 2 % sucrose ATP reverses the inhibitory effect of hydroxyproline to about the same degree that it did in the absence of sucrose (25 %).

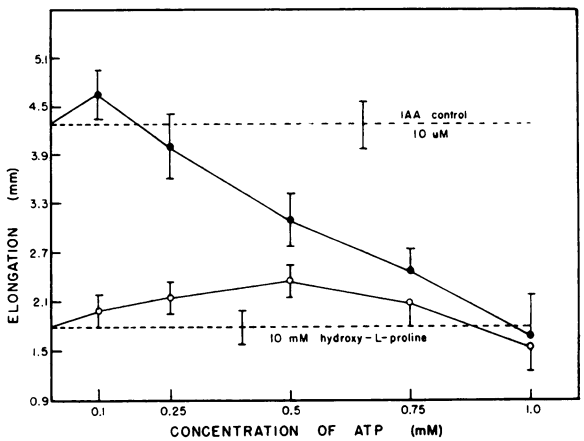

FIG. 6. Influence of ATP on the elongation of the second 5-mm segment of the *Avena* coleoptile in IAA. ○—○ in a strongly inhibitory solution (0.01 M) of hydroxy-L-proline in IAA; ●—● in IAA only. Standard deviations are shown. Each plotted point is the average of 50 to 90 measurements.

In figure 7 B, C, and D there is recorded in a comparable way the effects of guanine-HCl, L-glutamic acid, and L-ornithine-HCl. These compounds are quite stimulatory to elongation of the oat coleoptile sections in water and somewhat less stimulatory in IAA. They are also capable of reversing the hydroxyproline-induced inhibition of elongation (completely in water, and partially in IAA), exhibiting much the same pattern as that shown by ATP.

There is listed in table I various compounds that were screened with regard to their ability to stimulate elongation and to reverse the hydroxyproline-induced inhibition of elongation, but which proved to be less effective. Adenine and adenine

Table I. *Effect of Various Compounds on Elongation and in Reversing the Hydroxyproline-induced Inhibition of Elongation*

The second 5-mm segment of the *Avena* coleoptile both in water and in IAA was used. In each case 40 to 100 measurements were made.

Compound	Stimulation of elongation		Reversal of HP inhibition of elongation		Effective conc range
	H_2O	IAA	H_2O	IAA	
	%	%	%		mM
Adenine sulfate	+50	...	75	...	0.5 to 0.05
Adenine	+50	...	10	...	1.0 to 0.1
Adenosine	+10	...	...	...	1.0 to 0.1
Arginine	+10	...	20	...	1.0 to 0.1
Cytosine	...	...	10	...	1.0 to 0.1
Guanosine	...	+10	...	...	1.0 to 0.1

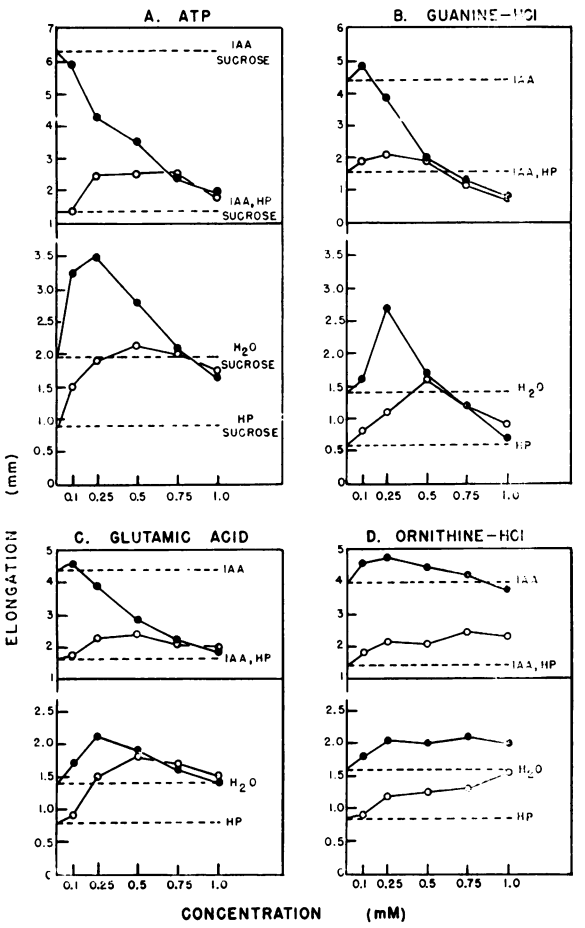

FIG. 7. Influence of various substances on the elongation of the second 5-mm segment of the *Avena* coleoptile in water, and in IAA (10 μM); and in a strongly inhibitory solution (0.01 M) of hydroxyl-L-proline in water, and in IAA. Each plotted point is the average of 40 or more measurements. A) ATP and 2 % sucrose; B) Guanine-HCl; C) L-glutamic acid; D) L-ornithine-HCl.

sulfate exhibit stimulation of elongation and some reversal of the hydroxyproline-induced inhibition of elongation in water. Adenosine and arginine show lesser effects. While of doubtful significance the estimated 10 % effects of cytosine and guanosine may represent an indication. In agreement with the preceding data the influence of various substances is more apparent in the absence of exogenous auxin (i.e. in water) than when their effects are examined on IAA-induced elongation.

Discussion

Time course experiments reported here confirm Cleland's previous observations (2, 4) that some time has to elapse, or a certain amount of elongation has to occur, prior to the onset of hydroxyproline inhibition. He obtained evidence in pre-treatment experiments which supported the latter possibility. It is clear from the data of figures 3 and 4 that hydroxyproline does not exert its maximal inhibitory effect during the initial 3-hour period of elongation. The presence of sucrose sustained the rate of auxin-induced elongation throughout the entire 24-hour period, a fact that long ago had been noted by Schneider (16), and as previously reported by Cleland (4) sucrose enhanced the effectiveness of hydroxyproline as an inhibitor of elongation.

The most striking results of these investigations were those obtained with ATP. The application of ATP is capable of stimulating the growth of *Avena* coleoptile sections, and of completely reversing the hydroxyproline-induced inhibition of elongation in water while partially reversing the inhibition in IAA. Guanine-HCl, glutamic acid, and ornithine-HCl act similarly to ATP. Undoubtedly the activity of glutamic acid and ornithine results from their convertibility to proline. Why guanine-HCl reverses hydroxyproline-induced inhibition of *Avena* coleoptile section elongation while adenine is less effective is difficult to explain. According to the work of Villa-Trevino et al. (19) opposite results might have been anticipated.

With regard to the possible mechanisms involved it may be well to examine certain characteristics of amino acid analogues. Richmond (15) has pointed out that in practice the majority of amino acid analogues inhibit growth by interfering with either protein synthesis or the function of the proteins synthesized in the presence of the analogues. In the first case they prevent or reduce the rate of formation of normal protein; in the second they replace natural amino acids in proteins (5, 9) thus rendering the proteins less effective regardless of whether they assume a structural or a catalytic role in the cell. Cleland (4) has called attention to the fact that hydroxyproline probably doesn't act in this second manner since free hydroxyproline doesn't get into proteins (14, 18). In a recent communication however Cleland (3) examined the effect of hydroxyproline on metabolism. He reported that hydroxyproline caused a slight decrease in the incorporation of proline into proteins, and that the conversion of proline to protein bound hydroxyproline was markedly inhibited by free hydroxyproline, thus hydroxyproline may inhibit elongation by preventing the normal formation of the hydroxyproline rich wall proteins. Cleland (3) also observed that free hydroxyproline was rapidly metabolized in *Avena* coleoptile tissue to free proline, probably in the cytoplasm. He found that some hydroxyproline could be directly incorporated into protein and suggested that the formation of abnormal hydroxyproline containing proteins may be a second factor leading to the inhibition of growth.

Considerable evidence has accumulated indicating that auxin-induced cell enlargement is dependent on continual protein synthesis (10, 11). Key (7) found RNA and protein synthesis to be essential for cell elongation in soybean hypocotyl tissue. He suggested that the role of auxin in regulating cell elongation may be associated with the control of RNA and/or protein synthesis, and thinks that the rate of formation of some specific RNA may be enhanced in some way by auxin thus leading to an increased supply of a limiting enzyme or enzyme system. In later work Key and Ingle (8) found that cell elongation of excised plant tissues, both endogenous and exogenous auxin-induced, appeared to depend upon the synthesis of messenger RNA. Hamilton et al. (6) have reported data which indicate a marked stimulation by IAA of the rate of synthesis of the major classes of RNA in sub-apical sections of the *Avena* coleoptile. Recently Nooden (12) has presented data supporting the idea that IAA may act by inducing new messenger RNA for enzymes that will plasticize the cell walls.

The question remains as to the manner in which ATP may reverse hydroxyproline-induced inhibition of elongation. Norris (13) in studying the interaction of ethionine and methionine found that ATP was capable of reversing ethionine-induced inhibition of elongation of *Avena* coleoptiles. Based on the work of Villa-Trevino et al. (19) he suggested that ethionine might exert an adenine- or ATP- trapping effect and thus manifest its inhibitory effects by interference with ATP metabolism. Considering that amino acids are activated by the formation of amino acid adenylates, perhaps hydroxyproline represents an ATP trap in the same sense as has been demonstrated for ethionine (1). If true this would reduce the energy available to the system thus inhibiting protein and/or RNA synthesis, and could account for the observed hydroxyproline inhibition as well as its reversal by the addition of ATP.

Acknowledgments

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