STUDIES ON 3-INDOLEACETIC ACID METABOLISM. VI. 3-INDOLEACETIC ACID UPTAKE AND METABOLISM BY PEA ROOTS AND EPICOTYLS

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For several years we have studied the metabolism of applied indoleacetic acid (IAA) by plant tissues to determine whether growth stimulation or inhibition by IAA could be in any way related to its metabolism. So far we have been chiefly concerned with IAA metabolism by pea epicotyls, and in previous publications (2, 3, 4) have suggested that the toxic action of IAA might be related to the accumulation of free IAA in the tissues which appeared in turn to be related to the rate of indoleacetylaspartic acid (IAAspA) formation. Both IAAspA formation and growth became maximal at the same concentration of applied IAA (about 0.6 \( \times 10^{-4} \) M). With lower concentrations almost all the IAA found in the tissues was present as IAAspA; only with the higher, growth inhibitory concentrations, did free IAA accumulate in the tissues to any extent.

To study more fully this apparent relationship between growth inhibition and IAA accumulation in plant tissues, we have turned our attention to pea roots. For many years it has been known that roots are over a thousand times more sensitive than epicotyls to growth inhibition by IAA. We, therefore, have attempted to determine whether this sensitivity could be related to a greater accumulation of IAA due either to an increased uptake of IAA, as compared with epicotyls, or to a less active metabolism via degradation or conjugation. Uptake in these studies was equated with loss from solution; degradation was considered to be the amount of IAA lost from solution which could not be accounted for as Salkowski reactive indole compounds in the tissues. Other workers (18) have shown that in tissue breis IAA is oxidized by the IAA-oxidase system, and it is probable that in tissues, IAA undergoes oxidative decarboxylation by the same enzyme system (9, 10).

The Salkowski reactive compounds found in the IAA treated roots are almost entirely IAA and IAAspA (2, 5, 9) although, as in epicotyls (12), small amounts of other derivatives are detectable. As shown below, we could find no evidence of the stable IAA-protein complex described by Siegel and Galston (16).

The present paper is concerned with the uptake, conjugation, and degradation of IAA in root tips. It also is concerned with a comparison between these findings and those of epicotyls, part of which has been published previously (5). A subsequent paper will deal with the relationship of these metabolic processes to the growth inhibitory action of applied IAA.

METHODS

Details of the methods used for raising plants and for incubating the tissues have been described in previous papers (4, 5). In some experiments, the tissues, after incubation, were transferred to a moist chamber which consisted of a large Petri dish containing a layer of moist cellocotton and a small Petri dish as receptacle for the tissues. The methods of determining IAA and IAAspA in the incubating medium and tissues have also been described in these papers.

The procedures used with roots differed in certain aspects from those used with epicotyls. These changes were necessary because roots are metabolically more active than epicotyls and more sensitive to their environment. These differences are as follows:

A. The sample weight of root tips was only 1/200 that of epicotyls but correspondingly smaller solution volumes were used except where specified so that in both cases the solutions lost about 50% of their IAA content during 24 hours. In most experiments twelve 5 mm root tips (about 65 mg) were added to 20 ml of solution while with epicotyls 10 g of 2-inch sections were added to 500 ml of solution. The results on roots and epicotyls were then compared on a per gram fresh weight basis.

B. It was shown in a previous paper (5) that the accumulation of IAAspA and the uptake of IAA by root tips were both greatly diminished on excision and that Ca++ and sucrose restored these processes completely. On the other hand, Ca++ and sucrose were without effect on the IAA uptake and metabolism by epicotyls. Ca++ and sucrose were therefore added only to the solutions in which root tips, but not epicotyls, were incubated.

C. The pH of the medium used with roots was higher (pH 5.2) than that with epicotyls (pH 4.6). Figure 1 shows that with roots the rate of IAA loss from solution and IAAspA accumulation are both optimal at pH 5.2. No such pH dependence could be noted with epicotyls over the same pH range.

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With epicotyls a pH of about 4.6 was selected as this pH minimizes bacterial contamination of the kind which markedly accelerates IAA destruction in solution.

D. Although antibiotics were added to the incubation medium of both roots and epicotyls (streptomycin and penicillin did not affect the metabolism of IAA), it was necessary to autoclave the medium used for roots. The presence of sucrose and the higher pH made this medium much more susceptible to bacterial contamination which was readily detected by the appearance of turbidity in the medium. The accumulation of IAA or IAAspA in the tissues was not significantly affected in contaminated media but the results on the loss of IAA from solution became most erratic. Consistently sterile conditions prevailed throughout the experimental period if roots from sterile seedlings were used; this precaution gave essentially the same results as the non-sterile tissues manipulated by the simplified procedure.

The two tissues differed in age; epicotyls were taken from 7-day old seedlings while root tips were taken from 2-day old seedlings. These tissues were selected because they were of the same stage of development as tissues used in growth studies, and thus, IAA metabolism could be directly compared to its effect on growth. The results of growth studies will be presented in a subsequent paper.

**RESULTS**

I. IAA Metabolism by Roots. A. Metabolic changes after incubation for 24 hours. Figure 2 shows that the loss of IAA from solution increases constantly over the entire concentration range studied, while the IAAspA accumulation increased similarly only up to $2 \times 10^{-4}$ M IAA; at this concentration the rate of conjugation was maximal and declined at higher concentrations. At optimum conditions of IAAspA accumulation the concentration of IAAspA reached 1.6% of the dry weight. Below $4 \times 10^{-4}$ M of applied IAA, free IAA could not be detected in the tissues, but was present at higher concentration.

B. Metabolic changes at intervals over 24 hours. Quite a different pattern of IAA metabolism is obtained during the early hours of incubation. For the short-term experiments only one-quarter the amount of solution was used; twelve tips were incubated in 5 ml solution in 50 ml Erlenmeyer flasks. This change of the procedure still provided about the same solution depth and enough IAA so that after 14 hours

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**Fig. 1.** The effect of pH on the IAA loss from solution and the accumulation of indoleacetaspartic acid by root tips incubated in $10^{-4}$ M IAA solution for 24 hours.

**Fig. 2.** The IAA lost from solution and the accumulation of indoleacetaspartic and IAA by root tips incubated in IAA solutions of various concentrations for 24 hours. The vertical lines represent twice the standard error of the mean of three experiments.
of incubation only 50% of the initial IAA was left in solution, but the accuracy of determining the IAA lost from solution was increased four-fold.

Figure 3 shows that root tips during the first half hour of incubation in $10^{-4}$ M IAA take up IAA very rapidly. The IAA lost from solution can be recovered almost quantitatively from the tissues as accumulated free IAA. At 1 hour the accumulated free IAA represents about a ten-fold greater concentration within the tissues than in the surrounding medium. The tissue IAA remains at this high level for 2 to 3 hours, after which it diminishes; by the 12th hour it has completely disappeared from the tissues. A similar proportionate high initial IAA accumulation within the tissues was observed at all concentrations of applied IAA (table I). In the presence of $10^{-3}$ M KCN the IAA content of the tissues rose only to the concentration of the external solution.

After the initial half hour of incubation, the rate of IAA uptake is greatly diminished for about five hours. During this time the amount of IAA lost from solution can no longer be accounted for completely as Salkowski reactive compounds in the tissues. This indicates that the degradative activity has commenced. However, degradation does not reach maximum activity until about the sixth hour of incubation. There was no evidence of IAAspA formation until after the second hour. This was confirmed by chromatography (fig 4). Because of the relatively long lag period required for degradative and conjuga-

tive activity to commence, it is possible to separate the initial uptake of IAA from the subsequent continuous uptake. After the initial uptake is complete the rate of subsequent uptake of IAA is dependent upon the rate of its metabolic removal from the tissues.

The initially accumulated IAA which subsequently disappears from the tissues was metabolized by conjugation and degradation as soon as these processes became active. This can be seen in an experiment in which the root tips, after 2 hours incubation in IAA solution, were transferred to a moist chamber (fig 5). Within the next 4 hours about 70% of the IAA in the tips disappeared and could be accounted for completely as IAAspA; the residual IAA in the tips was not conjugated but degraded to Salkowski unreactive compounds during the next 24 hours. It should be noted that while no degradative loss of the accumulated IAA was observed during the first 4 hours in the moist chamber, some degradative activity was apparent before the sixth hour in the previous experiment where the tips were incubated in solution (fig 3). This suggests that the relatively small amount of degradation observed during the early hours in solution may be limited to the cut surface of the tissues and that the process requires a longer period to become active within the tissues. This raises the question as to whether loss from solution can be equated with uptake or whether some extracellular oxidation may occur. At the moment there is some doubt if all the IAA degraded is first taken up by the tissues. However, there can be little secretion of enzymes into the solution as IAA loss from solution ceases when the tissues are removed; this was also found to be the case with epicotyls (4).

There was no evidence of the IAA-protein formation as described by Siegel and Galston (16). This complex was reported to form rapidly within the first 30 minutes of incubation in pea root tips treated with IAA, and to accumulate to about the same concentration as free IAA. Siegel and Galston obtained their complex by precipitating the proteins of the breis with trichloroacetic acid. The precipitated IAA-protein complex was then dissolved in $0.1 N$ NaOH; this alkaline extract gave a positive Salkowski reaction. Under the conditions of our studies any IAA

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<th>Concentration of IAA Proportioned</th>
<th>Concentration of IAA in Tissues</th>
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<th>Epicotyls</th>
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<td>0.40</td>
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1 After 2 hours incubation in various concentrations of applied IAA.
Fig. 4. Chromatogram of root tip extracts after 2, 4, 6, 8, 10, 12, 14, and 24 hours incubation in $10^{-4}$ M IAA solution. The standard, at the right, contains indoleacetic acid (IAA), IAA, and indoleacetylaspartic acid (IAAspA). The chromatogram was sprayed with Ehrlich reagent.

Fig. 5. Chromatogram of extracts of root tips incubated in $10^{-4}$ M IAA solution for 2 hours; from the left first extracted immediately, next in a moist chamber for 4, and last for 24 hours before extraction. The standard, at the right, contains indoleacetic acid (IAA), IAA, and indoleacetylaspartic acid (IAAspA). The chromatogram was sprayed with Ehrlich reagent.

present in the tissues was completely ethanol extractable; an alkaline extract of the proteinaceous residue, after ethanol extraction, gave a negative Salkowski reaction. This was true whether looked for in excised root tips incubated for 3 to 24 hours; whether Ca++ and sucrose were added to the medium, or whether intact roots or root tips were used. In our opinion, the IAA-protein complex is an artifact of the trichloroacetic acid precipitation procedure. The experimental evidence for the metabolic nature of this process is not convincing as uptake of IAA from solution by roots is also a metabolic process which would be inhibited by the same metabolic inhibitors.

II. IAA METABOLISM BY PEA EPICOTYLS. Our previous publication on the IAA metabolism of pea epicotyls (4) was primarily concerned with metabolic changes after 24 hours incubation. At low concentrations of applied IAA (below $10^{-4}$ M IAA) little free IAA was found in the tissues after 24 hours. It was not suspected at that time that free IAA might appear and again disappear during the initial hours of incubation. The time course was studied but only at 2, 4, 8, and 16 hours and with a single concentration of $1.72 \times 10^{-4}$ M IAA. This was supraoptimal for IAApA formation, so that IAA persisted in the tissues. Our recent observations with root tips have compelled us to reinvestigate the time course for epicotyls. We, therefore, have extended the experiments over a wider concentration range and closer time intervals to allow comparison with the results on roots (table 1 and fig 6).

For the short term experiments, only 2 g of epicotyls were incubated in 40 ml solution in 125 ml Erlangeneyer flasks. This change of procedure increased the accuracy of determining the IAA loss from solution without significantly affecting the experimental conditions of incubation.

Figure 6 shows that the loss of IAA from solution was about linear with time for the highest concentrations of applied IAA. For the lowest concentrations ($0.25 \times 10^{-6}$ M), however, there was a slow loss from solution over a period of several hours. This loss then almost ceased until the 6th hour when the loss again increased. During the first 2 hours with the low concentration, almost all the IAA lost from solution could be accounted for as free IAA in the tissues. With all concentrations the free IAA
Fig. 6. The loss of IAA from solution and accumulation of IAA and indoleacetylaspartic acid in epicotyls during 14 hours of incubation in 0.25, 0.5; 1.0 and $2.0 \times 10^{-4}$ M IAA solution. The degradation line represents the difference between the IAA lost from solution and the IAA and indoleacetylaspartic acid accumulated in the tissues.
content of the tissues rose over a period of 4 to 6 hours, until it reached a concentration equal to or less than that in the external solution (table I). This equilibrium was also established in the presence of $10^{-3}$ M KCN. On prolonged incubation the IAA content of the tissue decreased and disappeared by about the twelfth hour except with $2 \times 10^{-4}$ M IAA, a concentration supraoptimal for IAA spA formation. This fall is associated with the conjugative activity. The lag period of conjugation, unlike that of degradation, is independent of the concentration of IAA applied; in every case conjugative activity commenced about the second hour and became maximal at about the sixth hour.

Degradative activity, on the other hand, showed a lag period which decreased with increasing concentration of applied IAA. While some degradative loss is apparent by the 2nd hour with $2 \times 10^{-4}$ M IAA, none could be observed even at the 6th hour with 0.25 $\times 10^{-4}$ M IAA.

The results indicate clearly that after the IAA content of the tissues has attained equilibrium, the rate of the subsequent uptake, as in roots, is dependent upon the rate of the metabolic removal via conjugation and degradation. As the initial uptake of IAA, on the other hand, was found to be insensitive to cyanide, and as IAA does not accumulate in the tissues beyond the concentration in solution, it would seem that the uptake process per se is a non-metabolic process. In an earlier paper (4) it was concluded that the uptake of IAA by pea epicotyls was entirely metabolic for no IAA or IAA spA could be detected in the tissues incubated under pure nitrogen. In these experiments, the tissues and IAA solutions were placed in flasks from which the air was evacuated and replaced by pure nitrogen. At present there is no adequate explanation why, under these conditions, IAA did not enter the tissues by a physical process. In a recent modified experiment in which tissues were maintained under anaerobic conditions by placing them in flasks completely filled with boiled and cooled solution and stoppered under expulsion of any air space, the IAA content of the tissues rose to the same level as under aerobic conditions.

**DISCUSSION**

In these experiments we have attempted to set up metabolic balance studies for uptake and metabolism of IAA by pea roots and epicotyls during 24 hours incubation. The results indicate that the differences between IAA metabolism by roots and epicotyls are chiefly of quantitative nature. Both degradation and conjugation, once the mechanisms have been adapted, are more rapid with roots than epicotyls.

The uptake mechanism of IAA, however, appears to be distinctly different between roots and epicotyls. Considering the function of these two tissues, this is not surprising. In roots there is a rapid metabolic uptake leading to accumulation of IAA in the tissues in excess over the external concentration, while in epicotyls there apparently is a physical process by which IAA concentration of the tissues does not exceed that in solution (table I). The kinetics of the rapid metabolic uptake by root tips appear to be analogous to the uptake of galactosides by induced Escherichia coli bacteria as studied by Cohn and Monod (7). These workers ascribed galactoside uptake to the catalytic action of a galactoside permease system. The observed high galactoside accumulation inside the tissues results from the greater rate of the entry reaction over the exit reaction; thus the accumulated galactosides may be considered as steady state intermediates. This concept has led Miller (14) to derive the expression:

$$K_e \left( \frac{S_1}{S_0} \right) = \frac{K_e}{K_m}$$

where $(S_1)$ and $(S_0)$ are the internal and external concentrations at thermodynamic equilibrium, and $K_e$ and $K_m$ are constants characteristic of the permease system. The uptake of IAA by roots as given in table I can be seen to follow this expression if $K_e/K_m$ is taken as approximately ten.

This accumulation could only be observed during the early periods of incubation before the metabolic processes for IAA removal have been activated. Once conjugation and degradation have become adapted the IAA disappears from the tissues unless supraoptimal concentrations for IAA spA formation are applied. When an equilibrium between the internal and external IAA concentration has been attained (either actively as in roots or passively as in epicotyls) the rate of uptake slows down until the metabolic processes of IAA destruction and conjugation become adapted. The rate of continued IAA uptake is then limited by the rate of its metabolic removal from within the tissues.

The uptake of IAA by other plant tissues has been previously discussed by Albaum et al (1) and Sutter (17), who studied Nitella cells and cucumber hypocotyls, respectively, and considered IAA uptake a purely physical process. Reinhold (15) concluded from her studies with carrot root discs that two processes are involved, an initial physical uptake followed by a continued slow metabolic uptake which she attributed either to an active transport of IAA into the cells or else to its metabolic removal. Our results, as just shown, provide evidence for the latter alternative.

Both degradation and conjugation appear to be auxin inactivating processes which may occur in different areas of the tissue. This is suggested by results on the metabolism of accumulated IAA (fig 5). There is also some histological evidence in the literature that the areas of degradation and conjugation are located in separate regions. First, Jensen (13) has shown that the peroxidase activity in root sections of broad beans is limited to the root cap, epidermis, and vascular elements. Since the IAA-oxidase system is composed of a hydrogen peroxide producing system and a peroxidase (10), and since degradation of IAA by intact tissues probably takes place via the IAA-
oxidase system, it is likely that the degradative activity is limited to these areas of peroxidase activity. Second, Ebert (8) has shown that roots incubated in \(10^{-3}\) M IAA for 10 hours accumulate a Salkowski reactive compound in the central areas; little or none could be detected in the epidermis, root hair or root cap. Ebert considered this Salkowski reactive compound to be IAA bound to tissues as it persisted in the tissues even if rinsed in running water for several days. From our studies it seems certain, however, that Ebert's Salkowski reactive compound was chiefly IAAspA. We have previously reported (2) that IAAspA is resistant to leaching and metabolic degradation.

In view of these histological studies it is suggested that while degradation may be limited to the root cap, epidermis, and vascular elements, conjugation occurs primarily in the cortical areas. It could also be argued that the conjugating and degrading sites are located in different areas of the same cell, but in our opinion, histological evidence favours the former concept. However, the possibility must not be excluded that besides IAA-oxidase, other, as yet unknown, systems of degradation might exist.

If these two reactions take place in different areas of the tissue, then the accumulation of IAA may occur in one or the other areas separately. Since degradation did not attain a maximal rate over the concentration range studied, it is unlikely that IAA could be found in the degradative areas after this process has become adapted. On the other hand, as conjugative activity reaches a maximal rate with high concentrations of applied IAA (\(10^{-4}\) M for epicotyls and \(2 \times 10^{-4}\) M for root tips) it stands to reason that with higher concentrations, IAA will accumulate in the conjugating areas. It has in fact been shown that IAA which has accumulated in epicotyls incubated in \(1.72 \times 10^{-4}\) M IAA for 16 hours is subsequently completely conjugated when the tissues are placed in a moist chamber (4); there was no evidence of degradation.

Degradation in the present studies has only been determined indirectly as the IAA lost from solution which could not be accounted for as Salkowski reactive compounds in the tissues. Fang et al (9), by means of carboxyl labeled IAA, have shown that degradation involves oxidative decarboxylation and over a period of 19 hours accounts for almost all the IAA metabolized which could not be recovered from the tissues as IAA or IAAspA. Quantitatively they confirmed our results that in epicotyls over 80% of the IAA lost from solution is degraded while about 15% is retained in the tissues as free IAA or IAAspA after 24 hours incubation (4). With roots they found degradation accounted for about 93% and conjugation for only 7% of the IAA lost from solution. These latter results also agree with our experiments (5) in which \(Ca^{++}\) was absent from the medium. In the present study where \(Ca^{++}\) had been added, degradation accounted for only 74% and conjugation for 26% of the loss after 24 hours. From the present experiments, however, it is clear that the proportions between degradation and conjugation are not absolute and depend on the time of incubation and the concentration of IAA applied: during the first 6 hours in epicotyls treated with \(0.25 \times 10^{-4}\) M IAA all the IAA metabolized is conjugated; there is no evidence of degradation until after the 6th hour.

There has been considerable discussion as to the significance of degradative activity in the tissues. This reaction has been largely studied in tissue breis, where it is active, unlike the formation of IAAspA which has not yet been demonstrated in vitro. Galston (10) postulated that IAA-oxidase not only metabolizes applied IAA, but also controls the endogenous auxin level of untreated tissues thus explaining auxin phenomena, auxin rhythm, and lateral bud inhibition. Briggs et al (6), on the other hand, consider that IAA-oxidase activity is purely a cut surface phenomenon, as the amount of applied IAA recovered after transport through tissue segments was unaffected by the length of tissue through which the IAA had passed. Neither Reinhold (15) nor we (4), however, were able to find any proportionality between the area of cut surface and oxidase activity. We believe that rather than being limited to the cut surface, this process, once adapted, occurs in the areas which have been shown to possess peroxidase activity, but that because of the long lag periods found with low concentrations of applied IAA this process may play no part normally in the tissues studied.

It should be noted, that equating loss from solution with degradation, Galston and Dalberg (11) have reported a 10 to 20 minute lag period for this process to become adapted in epicotyls incubated in \(2 \times 10^{-4}\) M IAA. As seen in the present studies, loss from solution cannot be equated with destruction. For even after 2 hours incubation in this concentration of IAA, 80% of the IAA lost from solution could be found as IAA in the tissues.

These studies on IAA uptake and metabolism were carried out concurrently with studies on the inhibitory action of IAA on growth. A subsequent paper will deal with the relationships observed. It might be said at this point, however, that while the IAA content of roots has been shown (fig 2) to be considerably greater than that of epicotyls (fig 6) during the first half hour incubation in IAA, it is not believed that this difference can account for the greater inhibitory action of IAA on root growth.

**SUMMARY**

I. Root tips and epicotyl segments of pea seedlings were incubated in IAA solutions for various lengths of time. Quantitative measurements were made to determine the IAA lost from solution and the free IAA and indoleacetalaspartic acid in the tissues. Besides IAA, only indoleacetalaspartic acid occurred in the tissues to any extent as Salkowski reactive compounds; there was no evidence of any IAA-protein complex. The formation of indoleacetalaspartic acid was referred to as the conjugation
process. The loss of IAA by degradation was followed indirectly as the difference between the IAA lost from solution and the Salkowski reactive compounds of the tissues.

II. During the early period of incubation the IAA loss from solution could be almost quantitatively recovered in the tissues as free IAA, as both conjugative and degradative activity require some time to become apparent. For conjugation a period of about two hours is required for any evidence of activity, while maximal activity is not attained for 4 to 6 hours. These time relationships are independent of the applied IAA concentration. For degradation, on the other hand, the period of adaptation is inversely proportional to the concentration of applied IAA. The disappearance of IAA from the tissues appears to be related almost entirely to the conjugative activity. It is suggested that the degradative activity is limited to a small area of tissue, probably the epidermis and root cap. Both the degradative and conjugative activity are more active processes in roots than in epicotyls.

III. The uptake of IAA by roots appears to be an active, cyanide sensitive process whereby roots accumulate IAA to ten times the external concentration. The uptake of IAA by epicotyls, on the other hand, appears to be a physical process in which the IAA content of the tissues does not exceed that in solution. In both cases, the IAA content of the tissues was proportionate to the concentration of IAA applied. The rate of continuous uptake in both roots and epicotyls was limited by the rate of metabolic removal of IAA within the tissues.

IV. Because of the rapid active accumulation of IAA by root tips, the IAA content of the tips after half an hour of incubation was much greater than in epicotyls. This difference gradually decreased. After 10 hours little or no IAA was detectable in either tissue unless a concentration of IAA supraoptimal for indoleacetylaspartic acid formation was applied.

**Literature Cited**