Influence of Auxins on in vitro Incorporation of Glycine-C\(^{14}\) in Pea Shoot Proteins\(^1\) \(^2\)

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Radioactive amino acids have been utilized in the study of protein synthesis in both detached plant tissues and plant mitochondria. When a plant tissue is incubated in the presence of a radioactive amino acid, the radioactivity is rapidly accumulated in the cells (1, 7, 8, 11, 12, 13, 14). The accumulation process is dependent on respiration and varies somewhat according to the amino acid and experimental conditions. Stephenson et al. (10) demonstrated that labeled amino acids were incorporated into protein of tobacco leaves. Christiansen and Thimann (2) have found that auxin-induced growth in pea stem sections is accompanied by an increase in protein synthesis. They concluded that synthesis of new protein may be of the utmost importance in auxin-induced growth. However, auxin-induced increases in expansion are not always paralleled by increases in protein (3, 7). Cleland (7) cited a decrease in protein nitrogen in maize mesocotyl section during expansion and this decrease is not affected by auxin. Boroughs and Bonner (1) reported that there was no increase in incorporation of radioactive glycine into protein of *Avena* coleoptile sections in the presence of 17 \(\mu\)moles IAA per liter. This communication reports the observation made during a study of the incorporation of glycine-1-C\(^{14}\) or -2-C\(^{14}\) in the protein of pea shoots with or without the influence of 2,4-dichlorophenoxyacetic acid (2,4-D) or indole-3-acetic acid (IAA).

**Methods and Materials**

Alaska peas were germinated at room temperature between 2 sheets of moist paper in the dark for 4 to 5 days as described previously (4). The apical end (20 mm in length) of the shoot was removed and then used in subsequent experiments. The sectioned tissues, 30 in each treatment, were incubated in 10 ml 0.02 \(M\) potassium phosphate buffer (pH 5.2) containing various concentrations of 2,4-D or IAA, 0.1 % of glucose, and 10 \(\mu\)moles of specific labeled glycine-C\(^{14}\) (2.0 \(\mu\)c per flask). Since most antibiotics affect the synthesis of protein in plants (7), addition of this in the medium for precaution against the growth of bacteria was omitted. The flasks were continuously shaken at a speed of 40 to 50 reciprocations per minute in a water bath maintained at 25\(^\circ\). At the end of the incubation period, tissue sections were removed from the medium and washed thoroughly under running water, blotted dry, and weighed. The tissues were immediately frozen and stored in a dry ice cabinet until further work could be carried out. The frozen tissues were first pulverized in a mortar and then finely ground with water. The total homogenate was made up to 10 ml and filtered through a glass fiber filter paper disc. After thoroughly washing this residue with water, followed with a small amount of ethyl alcohol, the residue was dried under an infrared lamp before submitted to the measurement of radioactivity. The activity after correction for background and self-absorption factor, was designated as the amount of incorporation into water insoluble residue. The activity of water soluble fraction was determined by plating 0.5 ml aliquots of the filtrate in 2.5 cm stainless steel cupped planchets. The average values of duplicate measurements were recorded. Total protein was precipitated by the addition of an equal volume of 10 % trichloroacetic acid to 7 ml aliquot of the filtrate, and the solution was then heated in boiling water for 10 minutes. The trichloroacetic acid coagulable protein was filtered through a weighed glass-fiber filter disc, washed with 5 % trichloroacetic acid, dilute alkali and thioglycollic acid in that order before drying, weighing, and radioactivity counting. Total incorporation of radioactivity into this protein and its specific activity were then calculated. Glycine absorption by the pea shoots was determined from the difference between the initial and final activities of the incubating medium.

The protein was hydrolyzed with 2 \(N\) HCl in a sealed glass tube in an autoclave at 16 lb. per square inch pressure for 10 hours. Both protein hydrolysate and the deproteinized water extract of pea shoots were chromatographed separately on Whatman No. 1 paper strips with either buffered phenol or \(n\)-butanol-acetic acid-water (4:1:1) as the developing solvent. The paper chromatograms were scanned with gas-flow detector for radioactive spots, and were identified subsequently for labeled amino acids.

**Results and Discussion**

*In Vitro Incorporation of Glycine-1-C\(^{14}\) into TCA

\(^1\) Received August 12, 1964.
\(^2\) Technical Paper No. 1873, Oregon Agricultural Experiment Station.
Shoot Sections. Data in table I shows that the C\(^{14}\) from labeled glycine incorporated readily into the protein of pea shoot sections. This incorporation was linearly increased with time of incubation during the first 16 hours, with the exception of a slight initial lag. After that, the incorporation of C\(^{14}\) activity in the protein started to decline indicating that the rate of degradation of C\(^{14}\)-protein was greater than the rate of incorporation. Despite the uptakes of glycine by 2,4-D and IAA treated tissues were 25\% and 35\% less respectively, approximately an equal amount of C\(^{14}\) from labeled glycine was incorporated into the pea shoot protein from both the control and the treated sections. At 1 \(\times 10^{-4}\) M of 2,4-D or IAA, no significant differences in C\(^{14}\) incorporation were evident during the first 10 hours. The average radioactivity in the protein found between sixteenth and fortieth hours seemed to indicate a slight increase of glycine incorporation from auxin-treated tissues. Only a small fraction (less than 10\%) of the absorbed glycine was incorporated into the protein while the major portion of the activity was not recovered and was presumably lost as respiratory CO\(_2\). Parthier (8) in his study of amino acid incorporation into tobacco leaf protein observed that from 60 to 80\% of glycine-1-C\(^{14}\) was lost as CO\(_2\). In short-term experiments, approximately 17\% of glycine-1-C\(^{14}\) absorbed by excised roots was incorporated in the respired CO\(_2\) (14). The average rate of C\(^{14}\) incorporation calculated from the specific activity of protein at sixteenth hour was 0.14 \(\mu\)moles per g protein per hour for pea shoot tissues. This value is considerably less than the incorporation of C\(^{14}\)-glutamate in pea seedlings as reported by Webster (11, 12). This difference in observation could be the result of differences in experimental conditions, such as the concentration of amino acid, the pH of the medium, and the amino acid used.

The actual trichloroacetic acid precipitable protein content in the excised pea shoots decreased continuously with the time of incubation. As illustrated in figure 1, in which a log of protein content per g fresh tissue is plotted against the incubation time, we have observed 2 rates of decrease for protein with or without auxin treatment in pea shoots. During the first 10-hour period, the faster rate of decrease was mainly due to the combined results of a rapid increase in weight of the floating tissues and a steady decrease of the protein content. The second rate of decrease appeared between the period of 16 to 40 hours at which time there was no further gain in fresh weight. At this period, the influence of 2,4-D or IAA at

![Graph](image)

**Fig. 1.** The rate of decrease of protein in excised pea shoots as affected by auxin treatment. (○) Control, (×) 1 \(\times 10^{-4}\) M 2,4-D and (●) 1 \(\times 10^{-4}\) M IAA are plotted against the time of incubation.

### Table I. Incorporation of Glycine-1-C\(^{14}\) into the Proteins of Control and Auxin-Treated Pea Shoots (1 \(\times 10^{-4}\) M 2,4-D or IAA.)

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Fr wt, g</th>
<th>TCA protein</th>
<th>Glycine-1-C(^{14}) incorporation</th>
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<tr>
<td></td>
<td>Control</td>
<td>2,4-D</td>
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1 $\times 10^{-4}$ M concentration on the rate of protein degradation was noted. The rate of decrease was approximately twice over the control tissues. However, the rate of decrease of radioactivity in the proteins from auxin treated tissues was not paralleled to the rate of actual loss of protein. Galston et al. (5,6) reported that increasing 2,4-D concentration sharply reduced the quantity of heat coagulable proteins of pea stem sections which were incubated overnight. IAA was approximately as active as 2,4-D. From these results it appears clear that different observations made by many workers on the effect of auxin treatment to protein synthesis as indicated by the incorporation of labeled amino acid or to protein content as measured from the trichloroacetic acid coagulable-protein, could well be due to the differences in experimental method, as well as the time of treatment. Nevertheless, this observation is in accord with the others reported in the literature.

**Concentration of Auxins on Glycine-$C^{14}$ Utilization.** Data in table II which are the average values of 8 runs illustrates the effects of 2,4-D and IAA on the absorption of glycine, fresh weight increase, and the incorporation of $C^{14}$ from glycine in pea shoot protein. It is clear that higher concentrations of 2,4-D or IAA inhibited glycine absorption. Other than the $5 \times 10^{-4}$ M concentration, both 2,4-D and IAA induced an increase in fresh weight of pea shoot sections over the controls. The $C^{14}$ incorporation into plant protein was decreased at higher concentrations of auxin ($5 \times 10^{-4}$ M and $1 \times 10^{-4}$) and the percent of $C^{14}$ incorporation were related to the reduction of glycine uptake by the treated tissues. Despite no increase in glycine uptake by pea shoots treated with auxins at $10^{-5}$ M or $10^{-6}$ M concentration, the incorporation of $C^{14}$ from labeled glycine into protein was significantly enhanced. The increases in fresh weight as induced by auxins were not exactly paralleled with the increases of $C^{14}$ incorporation into plant protein. In all cases, total trichloroacetic acid precipitable protein content in fresh shoot sections was greatly reduced during an overnight incubation and was not noticeably altered by various concentrations of auxins. At growth inhibitory concentration of 2,4-D or IAA ($1 \times 10^{-4}$ M) the rate of protein degradation in the treated pea shoot sections was twice greater than in the control, yet the $C^{14}$ incorporation at 16- to 48-hour period showed slightly more in the proteins from treated tissues. This would suggest that the effect induced by 2,4-D on protein catabolism and synthesis may be different. However, the supply of glycine in the medium at this period due to unequal absorption between the control and treated tissues, might contribute to this observation. $C^{14}$-glycine incorporation into cell walls and tissue fragments (water-insoluble residue) was also affected by auxin treatment; it was depressed at $5 \times 10^{-4}$ and enhanced at $1 \times 10^{-4}$ M concentration. At lower concentrations ($1 \times 10^{-5}$ and $10^{-6}$ M) the influence to this incorporation was a slight inhibition for 2,4-D and no significant effect for IAA. Ray and Baker (9) also observed an increase of glucose-$C^{14}$ incorporation into cell walls by IAA.

**Kinetic Study on the Breakdown of $C^{14}$ Protein in Pea Shoot Tissues as Affected by Auxins.** Approximately 300 pea shoots were incubated in phosphate buffer containing glycine-1-$C^{14}$ for 18 hours to label the protein. At this time, all shoots were removed from the radioactive medium, washed thoroughly under running water, divided equally into 3 groups, and reincubated in either one of the following mediums: A) phosphate buffer alone, B) phosphate buffer + $5 \times 10^{-4}$ M 2,4-D and C) phosphate buffer + $5 \times 10^{-4}$ IAA. All 3 mediums also contained 0.1% glucose. Eighteen shoots were removed from each group at different intervals and analyzed for trichloroacetic acid and heat coagulable protein and its specific activity. The experiment was repeated several times using either $5 \times 10^{-4}$ or $1 \times 10^{-4}$ M concentration of auxins. From 5 separate runs of control pea shoot tissue, a steady decrease of the specific activity of the labeled protein was observed. The average rate of decrease, $k$, in this 10-hour experimental period, was $-0.075$ with a range of $-0.067$ to $-0.090$ for the control tissues (fig 2). In the presence of IAA or 2,4-D the average

| Table II. Effect of Auxin Concentration on the Absorption of Glycine, and Its Incorporation into Various Fractions of Excised Pea Shoots |
|-------------------|------------------|------------------|------------------|
|                   | Control          | 2,4-D            | IAA              |
|                   | 5 $\times 10^{-4}$ | 1 $\times 10^{-4}$ | 1 $\times 10^{-5}$ | 1 $\times 10^{-6}$ | 5 $\times 10^{-4}$ | 1 $\times 10^{-4}$ | 1 $\times 10^{-5}$ | 1 $\times 10^{-6}$ |
| Fr wt after incubation, g | 0.90             | 0.85             | 1.09             | 0.96             | 0.94             | 0.89             | 1.07             | 0.99             | 0.96             |
| Glycine absorption, cpn | 235000           | 123400           | 178400           | 224300           | 224000           | 149800           | 151500           | 199000           | 225000           |
| Protein, mg/g fr wt | 10.8             | 12.1             | 10.4             | 11.1             | 11.8             | 10.3             | 9.2              | 10.1             | 10.5             |
| Specific activity | cm/mg protein   | 912              | 471              | 670              | 1013             | 859              | 602              | 785              | 1043             | 1097 |
| Total activity in protein, cm/g fr tissue | 9850            | 5700             | 6760             | 112400           | 10140            | 6200             | 7200             | 10530            | 11520            |
| Total $H_2O$ soluble activity, cm | 24500            | 12620            | 27100            | 253000           | 22700            | 14400            | 27200            | 24400            | 23900            |
| Total $H_2O$ insoluble activity, cm | 8670            | 4510             | 9600             | 7430             | 7570             | 3650             | 10720            | 8230             | 8200             |
rates of decrease for these 2 auxins were $-0.161$ and $-0.204$ for $1 \times 10^{-4} \text{m}$ and $5 \times 10^{-4} \text{m}$ respectively. At the same concentration, the magnitude of changes was quite similar between IAA and 2,4-D. Assuming that the breakdown of protein molecule does not differentiate between isotopic and nonisotopic forms, then a steady decrease of the specific activity in protein primarily could be due to a dilution from continuous synthesis of nonisotopic molecules. In this case, the result from this kinetic study would suggest that both IAA and 2,4-D may induce an increase in protein synthesis. However, increase in the rate of breakdown with no change in the rate of synthesis could also result in a faster reduction of specific activity. As we found in previous experiment that both 2,4-D and IAA at $1 \times 10^{-4} \text{m}$ concentration induced a 2- to 3-fold loss of protein in the treated tissues over the control, therefore, a greater reduction in specific activity of protein from auxin-treated tissues would indicate to be a result of accelerated rate of degradation. However, when the experiments were carried on for longer than 5 hours, a changing trend was observed. The specific activity of protein from treated tissues was actually slightly increased with a concomitance reduction of the radioactivity in the water insoluble fraction. This observation provides further evidence of the regulatory action of auxin on the movement of metabolites in plant cells.

The nature of radioactivity in the water insoluble fraction was not determined.

Evidence obtained from paper chromatographic separation of protein hydrolysate from control and treated tissues revealed the presence of both radioactive glycine and serine, with more labeled serine in the protein from control tissues. This is in agreement with the report by Parthier that the $C^{14}$ from labeled glycine is largely incorporated into serine in tobacco leaves (8). At growth inhibitory level both 2,4-D and IAA did not alter the relative contents of labeled glycine and serine in pea shoot protein. However, at stimulatory level, a greater amount of $C^{14}$ in the protein hydrolysate was glycine. This observation suggested that auxin may control the interconversion of glycine and serine. We have never found the presence of a noticeable amount of radioactive serine in the deproteinized water extract of the pea shoot tissues. This would indicate that glycine-serine interconversion occurred only after the incorporation of glycine into plant protein and the serine is not released to the metabolic pool during the experimental period.

Summary

The influence of auxins on the incorporation of $C^{14}$-labeled glycine into the protein of excised pea shoot tissue was investigated. After an initial lag, the incorporation of glycine-$C^{14}$ proceeded at a linear rate for about 16 hours. Indoleacetic acid and 2,4-dichlorophenoxyacetic acid at concentrations which promoted the weight increase of the fresh tissue enhanced $C^{14}$-glycine incorporation into protein, whereas inhibitory levels decreased glycine absorption and consequently its incorporation into protein. The increase in fresh weight as induced by auxins were not exactly paralleled with the increase of $C^{14}$ incorporation into the plant protein. Results from time course studies on the decreases of protein content or the specific activity of the protein indicate that auxins, at inhibitory levels, induced a 2-fold greater decrease. Paper chromatographic separation of protein hydrolysates revealed the presence of radioactive serine and glycine. Auxin treatment at stimulatory levels may control the interconversion of glycine and serine.

Literature Cited

Damage to Spinach Chloroplasts Induced by Dark Preincubation with Ferricyanide 1, 2, 3

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While ferricyanide has been used as an electron acceptor for light-induced electron flow in chloroplasts since the early work of Hill and Scarsbrick (14), an oxidant this strong might be expected to have additional effects. Indeed, Lumry and Spikes (21) in a careful kinetic analysis of the Hill reaction, found that ferricyanide when used at concentrations above 1 mM seemed to inhibit the light step(s) of the Hill reaction, and to stimulate the limiting dark reaction in a manner which we would now suspect of being related to uncoupling. A rather large number of studies have been carried out in which ferricyanide was used to effect a dark oxidation of one or another component of the electron transport chain, either in chloroplasts (1, 4, 13, 16, 17, 18, 20, 22, 24) or in bacterial chromatophores (7, 8, 11, 20).

We noticed that preincubation of chloroplasts with ferricyanide in the dark, at the concentrations ordinarily employed to measure the Hill reaction, appeared to have a deleterious effect on the subsequent rate of photoreduction. The present work is an attempt to explore this and related phenomena more systematically.

A preliminary account of this work has appeared (5).

Materials and Methods

Chloroplasts were prepared from commercial spinach by grinding briefly in a Waring blender in STKM 5. The resulting homogenate was filtered through cheesecloth and centrifuged at 1200 × g in a refrigerated centrifuge. The chloroplast pellet was resuspended, and either used without washing or washed once in fresh STKM.

In experiments where chloroplasts were preincubated with ferricyanide solutions and washed prior to assay, 3 ml aliquots of untreated chloroplast sus-

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1 Received August 12, 1964.
2 Contribution number 432 from the McCormick-Pratt Institute.
3 Supported in part by grants from the National Institutes of Health (GM 03923) and from the Kettering Foundation and by a predoc toral fellowship to J. M. Brewer from the National Science Foundation. Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy by J. M. Brewer to the faculty of Philosophy of the Johns Hopkins University.
4 Present address: Department of Chemistry, Biochemistry Division, University of Illinois, Urbana, Illinois.
5 Abbreviations include: STKM, 0.4 M sucrose, 0.02 M tris-HCl, pH 8.0, 0.01 M KCl, 0.001 M MgCl2; TCP1P, trichlorophenolindophenol; CMU, p-chlorophenyl dimethyl urea; PMS, phenazine methosulfate; PII, radioactive isotope of phosphorous as orthophosphate, FeCN-K3Fe(CN)6.