Conversion of Tryptophan to Indoleacetamide and Further Conversion to Indoleacetic Acid by Plant Preparations [1, 2]

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Introduction

Since Kögl, Haagen-Smit and Erxleben (10) first reported on the growth stimulatory properties of IAA, many studies have been devoted to tracing its route of biosynthesis. In 1940, Skoog and Thimann (20) recognized that tryptophan in structural protein could be converted to IAA in the presence of proteolytic enzymes. Since then a number of investigations (6, 13, 24) has given support to the conclusion that tryptophan is the major primary precursor of auxin. A host of other indole compounds have been implicated as intermediates in the conversion of tryptophan to IAA. Considerable evidence that indoleacetaldehyde may be an intermediate has accumulated (1, 5, 11). Indoleacetonitrile (IAN) (7), indolepyruvic acid, tryptophol (11) and ethylindoleacetate (18) are among the indole derivatives that have been reported as precursors of IAA.

The hydrolysis of IAN to IAA in plant tissues (22) has led to a search for indoleacetamide (IAm) as an intermediate. Wightman (23) found IAm to have some auxin activity and to be hydrolysed in vivo to IAA by tomato and pea. He has concluded that IAm does not occur as a free intermediate in the conversion of tryptophan via IAN to auxin. Others (2, 3, 4, 21) have given passing notice to IAm as a possible biological intermediate. On several occasions, IAm was found to be an artifact due apparently to the propensity of esters of IAA to form amides in the presence of ammonia. Thus, Zenk (25) reported that 57 % of 1-(indole-3-acetyl) β-*d*-glucose was converted to IAm during paper chromatography.

Recently it has been found that peroxidase catalyzes the oxidative decarboxylation of methionine with the formation of 3-methylthiopropionamide as the product (16, 17). Several other amino acids also undergo a similar reaction.

We have recently reported the peroxidase-catalyzed decarboxylation of tryptophan to IAm (19). This has subsequently been confirmed (9). The work reported in this paper gives further details of this reaction and some observations on the further metabolism of IAm.

Materials and Methods

Peroxidase Preparations. Horse-radish (*Armoracia lapathifolia*, Gilib) peroxidase (HRP) with an RZ of 3.0 was purchased from C. F. Boehringer and Son. A stock solution was made up containing 5 μg per ml. This solution was stable to freezing and thawing over a period of many weeks. Cabbage peroxidase preparations were made by homogenizing the plant material with one-half its weight of cold distilled water, filtering the homogenate through cheesecloth and centrifuging at low speeds in a clinical centrifuge to remove the debris and heavy particles. Dialysis of the supernatant solution against distilled water for 24 hours removed potent inhibitors of the oxidative decarboxylation activity of cabbage peroxidase.

Assay for Peroxidase Activity. Peroxidase activity was assayed by a modification of the guaiacol method described by Maehly (14). An amount of peroxidase test solution or standard HRP containing about 0.01 to 0.02 μg of peroxidase and 0.1 ml of a 0.01 M guaiacol solution were combined in a colorimeter tube containing 5 ml of 0.02 M potassium phosphate buffer pH 7.0. Then 0.04 ml of 0.04 M H_2O_2 was added, and the optical density of the solution at 470 mμ determined at 1-minute intervals in a colorimeter. In order to minimize temperature effects, the solution was removed from the instrument between readings. Under the conditions of the test procedure the oxidation rate is proportional to the enzyme concentration. Using a known quantity of HRP as a standard, the peroxidase equivalent of other preparations was determined by comparing the respective activities in the guaiacol test.

In Vivo Experiments. Cabbage (*Brassica oleracea* var. *capitata*, L.) seedlings were grown on moist gauze in the dark. The plants were used when 5 to 15 days old. Immediately before use the plants were selected and the stems cut under water just above the roots. The stems were then transferred to short test tubes which contained the labeled substrate in 0.3 to 0.5 ml of water. A stream of air was passed across the seedlings to aid uptake by increasing the rate of transpiration. Under these conditions the radioactive material was almost completely absorbed in less than 3 hours. At this point 2 ml of water was added to the test tube so that there would be sufficient water for the remainer of the experiment. In some cases the solution with radioactive substrate was taken up almost to dryness, then the same volume of distilled water as the original solution was added to the vessel, and this was replenished as the water was taken up in the course of the experiment. No difference in the products of metabolism was observed between these 2 methods. No degradation products were found in a

[1] Received September 28, 1964.

[2] This work was supported by National Science Foundation grant GB1142.

control vessel of radioactive solution with no seedlings.

When tryptophan was infiltrated, the incubation time was from 7 to 20 hours under constant illumination. When IAm-C^{14} was used, the incubation time was 12 hours. In all cases water was replenished as it was utilized.

At the end of the infiltration period, the seedlings were rinsed thoroughly with distilled water and then homogenized in a Potter-Elvehjem homogenizer with 5 ml of cold water, 100 mg of unlabeled tryptophan, and 10 ml of acetone. The homogenizer was kept cold by frequent immersion in a dry ice-acetone bath. The homogenate was filtered through celite on sintered glass, and the filtrate reduced in volume under vacuum to near dryness. The residue was extracted with 10 ml of acetone which was clarified by centrifugation. The clear extract was concentrated to 0.2 ml for use in paper chromatography and radioactive strip counting procedures.

Synthesis of IAm-2-C^{14}. IAA-2-C^{14} was purchased from the Nuclear Chicago Corporation. Eighteen μmoles, specific activity 2.82 μc/μmole, were dissolved in 2 ml of methanol. A trace of concentrated HCl was added, and the solution allowed to stand at room temperature for 6 hours, then stored at 5° overnight. The volatile components were removed by evaporation in vacuo. One ml of concentrated NH_4OH was added to the residue and this left in a refrigerator at 5° for 2 weeks. This period was much longer than turned out to be necessary. Excess ammonia was removed by drying under vacuum, and the impure product dissolved in 1 ml of distilled water. The day before use 0.2 ml containing 10 μc of C^{14} was purified by 1-dimensional paper chromatography with the following solvent systems: A) CCl_4 : water (50 : 1) in an atmosphere produced by a mixture of CCl_4 : acetic acid : water (1 : 1 : 1) ; B) isopropylalcohol : concentrated NH_4OH : H_2O (8 : 1 : 1). The zone corresponding to the amide was eluted with acetone. The acetone was removed by drying and the residue of pure labeled amide dissolved in the desired volume of water. An alternate method of elution was to place the paper strip containing the amide directly into the desired volume of water and heating to elute the amide. Unlabeled IAm was added to the solution to obtain the desired specific activity. The criterion for purity of the synthesized labeled IAm was co-chromatography with carrier amide in several solvent systems. In each case only 1 radioactive spot was found coinciding with the spot giving a positive Ehrlich's reagent test.

Materials. DL-tryptophan-2-C^{14}, and DL-tryptophan-3-C^{14} were purchased from New England Nuclear Corporation. Indoleacetamide and indoleacetonitrile were obtained from Calbiochem. Pyridoxal-5'-P was purchased from Sigma Chemical Company.

Results

Horse-Radish Peroxidase and Tryptophan-C^{14}. Previous work with pure horse-radish peroxidase had shown that the ratio of IAm production in the presence of enzyme to that in the absence of enzyme was 2.6 (19). Lowering the amount of pyridoxal-P in the reaction mixture has been found to increase the enzymatic to nonenzymatic ratio. Reaction mixtures were made up consisting of 100 μmoles potassium phosphate buffer at pH 7.0, 0.5 μmole $MnCl_2$, 2 μmoles DL-tryptophan-2-C^{14} containing 2 μc C^{14}, and either 0.1 μmole or 0.3 μmole of pyridoxal-P in the presence or absence of 2 μg of HRP. The final volume was 2 ml. After 90 minutes of incubation 100 mg of unlabeled tryptophan were added and the mixture concentrated to near dryness on a rotary evaporator. The residue was extracted with several portions of acetone and the extracts filtered. After filtering the extracts were concentrated and equivalent amounts of each reaction mixture chromatographed on paper using 2 solvent systems for comparison. One was an acidic system of isopropylalcohol : acetic acid : water (5 : 1 : 1) and the other, a basic one of *n*-butanol saturated with a 1 : 4 mixture of NH_4OH and water.

After chromatography the products were detected by use of a paper strip counter. Marker spots of known compounds were run alongside for purposes of identification.

The results demonstrated that at low concentrations of pyridoxal-P, no detectable nonenzymatic conversion takes place. The larger peak attributed to the amide on chromatograms developed with the acidic solvent was considered the result of some overlapping with IAA which was present in low concentration. IAm was the only indole product present in sufficient concentration to be detected with a *p*-dimethyl-aminobenzaldehyde spray.

Cabbage Preparations and Tryptophan-C^{14}. Cabbage seedlings 5 to 15 days of age were infiltrated with DL-tryptophan -3-C^{14} and then extracts prepared for paper chromatography as described in Methods. After development by the CCl_4 : acetic acid : water solvent system described by Wightman (23), the radioactive compounds were found to be IAA, IAN, and 1 or more unidentified products believed to result from the further metabolism of IAA. No IAm could be detected.

Cabbage seedling homogenates were found to contain naturally occuring inhibitors of peroxidase activity which were removed by dialysis. After such treatment the conversion of tryptophan to IAm could be shown with these preparations. A small amount of IAA was always formed in these reactions. This was the same as had been reported when purified HRP was the enzyme used (19). Amounts of dialyzed cabbage homogenates were used that contained equivalent amounts of peroxidase activity to those reactions which had been carried out with the purified HRP.

Experiments with IAm-C^{14}. Cabbage seedlings infiltrated with IAm-2-C^{14} hydrolyzed a significant portion of the amide in 12 hours. Figure 1 is a reproduction of the tracing of the strip chart obtained from a chromatograph developed by a benzene : acetic

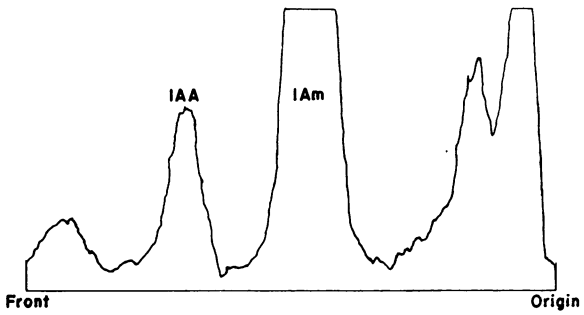

FIG. 1. Strip chart tracing of radioactive compounds formed from indoleacetamide-2-C^{14} (IAm) by cabbage seedlings.

acid: water (2:1:1) solvent. There was no non-enzymic conversion of amide to products. The peaks near the origin and those at the solvent front are believed to be due to products formed by further degradation of the IAA.

Homogenates of cabbage seedlings were capable of hydrolyzing IAm. Dark-grown cabbage seedlings from 12 to 15 days old were homogenized with ice water. The homogenate was centrifuged at low speed and the supernatant solution used as the source of enzyme. An aliquot was heated in boiling water for 15 minutes, and another aliquot was dialyzed against distilled water at 5° for 24 hours. An aliquot of the supernatant solution, the boiled homogenate, and the dialyzed homogenate were incubated with 0.9 μmole IAm-2-C^{14} containing approximately 2.5 μc of C^{14} for 4 hours. At the end of the incubation time the reaction mixtures were dried and acetone extracts were chromatographed as usual. The results are shown in figure 2. The solvent was the chloroform: acetic acid: water mixture. After dialysis the homogenate no longer can deamidate IAm. Boiled homogenate is almost devoid of activity. The radioactive peaks near the solvent front are not identified, however similar peaks are formed when IAA-2-C^{14}, pyridoxal-P, Mn^{++}, and HRP are incubated together. The possibility that IAm is degraded to other products than IAA has not been excluded; however, in the presence of HRP, pyridoxal-P, and Mn^{++} there is no reaction with IAm.

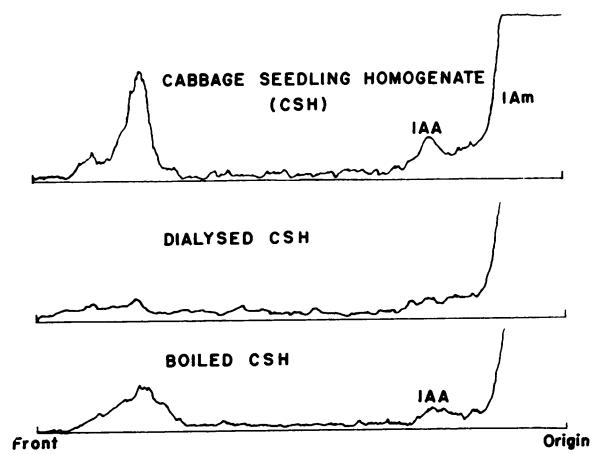

FIG. 2. Strip chart tracings of metabolism of indoleacetamide-2-C^{14} by cabbage seedling homogenates (CSH).

Discussion

The present report has shown that plant preparations can produce IAA from tryptophan through the intermediate IAm. The yields in the reactions described were quite low. However, since IAA is produced in rather low concentrations in vivo this may indicate that the amount of amidase present for the production of IAA from IAm is very small. Since after dialysis or heating no deamidation takes place, the enzymic nature of this process is indicated. Since peroxidase was required when the pyridoxal-P concentration was low, it would suggest that at physiological pyridoxal-P concentrations there is no non-enzymic conversion of tryptophan. The fact that IAm was not observed in the in vivo feeding experiments can be due to 2 factors. First, since we know that natural inhibitors of the peroxidase reaction are present and must be removed to demonstrate the decarboxylation to amide, it is consistent with the small amount of IAA required that only a certain few cells may have sufficiently low inhibitor concentration to produce the amide by decarboxylation. Secondly, if the deamidation rate is rapid compared to the decarboxylation, the amide concentration would be very low.

The major product of the in vitro HRP catalysed decarboxylation of tryptophan is IAm. However, a small amount of IAA is always found in the reaction mixture (19). This has also been observed in the present work with dialyzed cabbage preparations. Incubation of IAm with the standard HRP reaction mixture does not yield any IAA. Previous work on the decarboxylation of methionine to 3-methyl-thiopropionamide by peroxidase (16, 17) had also shown that some 3-methyl-thiopropionic acid was formed during this reaction.

A reaction mechanism to explain the amide formation has been proposed (17). One of the intermediates that has been suggested is a Schiff base consisting of pyridoxal-P and the amide. In figure

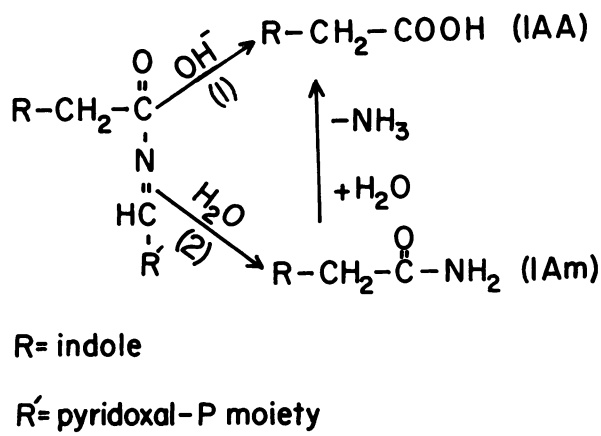

FIG. 3. Proposed reaction mechanism to explain formation of small amounts of IAA during the decarboxylation of tryptophan by peroxidase.

3 an extension of the previous mechanism is now advanced to explain the concomitant appearance of some free acid as well as amide. Since the carbonyl carbon has some positive character, a nucleophilic attack by OH would yield free acid and the pyridoximine derivative which would spontaneously hydrolyze to pyridoxal-P. This is not to infer that under in vivo conditions the IAA would arise in the same fashion.

From the viewpoint of comparative biochemistry some recent work by Magie, Wilson and Kosuge (15) lends support to the in vivo importance of the pathway described in this paper. These workers studied the metabolism of tryptophan by *Pseudomonas savastonoi*, a plant pathogen responsible for tumorous growth on certain fruit trees. Whole cell and cell-free preparations were shown to convert tryptophan first to IAm and then to IAA. The cell-free preparations possessed a high peroxidase activity.

Summary

Horse-radish peroxidase and cabbage preparations with peroxidase activity catalyse the oxidative decarboxylation of tryptophan to indoleacetamide. Pyridoxal-5'-phosphate and Mn^{++} are required cofactors in this conversion. The further conversion of indoleacetamide to indoleacetic acid can be demonstrated in cabbage seedlings and cabbage seedling homogenates. If the homogenates are dialyzed, there is a complete loss of amidase activity.

No indoleacetamide could be detected after feeding DL-tryptophan-3-C^{14} to cabbage seedlings. Cabbage seedling homogenates contained potent inhibitors of the decarboxylation reaction which were removed by dialysis. An explanation is advanced as to how these observations would explain the lack of indoleacetamide formation in vivo.

Small amounts of free indoleacetic acid are formed from tryptophan during the decarboxylation even when purified horse-radish peroxidase is the enzyme used. There is no reaction between free indoleacetamide and horse-radish peroxidase in the presence of pyridoxal-5'-phosphate and Mn^{++}.

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