ACTA CHEMICA SCANDINAVICA 7 (1953) 289–296

Nitrogen Metabolism of the Alder (*Alnus*) The Absence of Arginase and Presence of Glutamic Acid Decarboxylase

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The predominating free amino acid of the roots and root nodules of the alder species *Alnus glutinosa* and *Alnus incana* is L(+)-citrulline [1]. In summer, the nodules contain citrulline to about 0.2—0.3 % of the dry weight, but in winter even as much as 2 % of the dry weight. In addition to citrulline, the roots of *A. glutinosa* contain in winter considerable amounts of arginine and ornithine which are not found in the nodules.

The role of citrulline in the nitrogen metabolism of alder is not yet known in detail, but judging from the previous results [1] it can be expected to occur as a very central compound of the nitrogen store.

The question now arises as to which are the enzymatic reactions through which the citrulline nitrogen becomes utilized by the plants. A possible reaction mechanism might be the Krebs-Henseleit urea cycle [2] through which the synthesis of arginine and the elimination of nitrogen take place in the liver of animals. The simultaneous occurrence in abundance of all three amino acids of this cycle in free form in the roots of *A. glutinosa* implies that the synthesis of arginine in alder could proceed along this line as it has been proved to proceed in *Neurospora* [3].

In the above mentioned work [1] we were unable to find urea except in uninoculated roots of alder and even then only in minimal amount, about 0.005 % of the dry weight. Hence, it does not seem likely that mobilization of citrulline nitrogen would take place through urea.

Since, however, the absence of urea might also be due to its further rapid decomposition, we thought it important to estimate the arginase in the nodules of alder. Unless the alder possesses arginase activity, citrulline nitrogen cannot, of course, be mobilized through the urea cycle.

The root nodules of *A. incana*, but not those of *A. glutinosa*, also contain abundantly γ-aminobutyric acid, in autumn up to 0.2 % of the dry weight [1]. The only enzymatic reaction so far known which leads to the formation of this amino acid is decarboxylation of L-glutamic acid.

By the manometric technique, whereby one of the two decomposition products of the reaction, CO_2, is determined, this reaction has been demonstrated and the particular enzyme activity has also been determined in many plant tissues [4,5]. The other decomposition product, γ-aminobutyric acid, has also been isolated and identified [6] when split by the use of a plant born enzyme.

Steward *et al.*[7,8] have found only a minimal activity of L-glutamic acid decarboxylase in potato tubers which contain abundantly γ-aminobutyric acid. In this case they held it possible that γ-aminobutyric acid is a precursor of glutamic acid and not its decarboxylation product. In further support for this hypothesis they mention the fact that γ-aminobutyric acid is not found in the protein hydrolysates and — after having disappeared from the potato during the protein synthesis — it is no longer set free as γ-aminobutyric acid, on the hydrolysis of the potato protein.

It was therefore interesting to examine whether L-glutamic acid decarboxylase activity is detectable in the nodules of grey alder. As, in another connection [9], we had found considerable amounts of free γ-aminobutyric acid in all parts of pea at every period of growth we made the same experiment with leaves of pea, and also, for the sake of comparison, with carrot, from which Schales [5] prepared his soluble L-glutamic acid decarboxylase.

ARGINASE EXPERIMENT

Arginase has been studied in detail with preparations of animal origin, which show an optimal activity at about pH 9.8[10]. The optimal pH for yeast-[11] and jack-bean [12] arginase also lies on the alkaline side. In animal preparations, of which information is available, the enzyme commences to lose its stability already at pH 8, being very unstable at the optimal pH but quite stable between pH 6 and 8.

We have therefore carried out the arginase experiments at pH 7.8 where the activity of arginases of animal origin is known to be about 60 % of the maximal. We have used $MnSO_4$ as an activator, adding it as required to make the final reaction mixture about 0.01 *M* in respect to manganese.

Preparation of plant tissue suspension and arginine solution. The nodules, leaves, and roots of grey alder were taken from nature in August. A pipettable suspension was prepared from the nodules and leaves by crushing about 20 g fresh tissue with 100 ml cold *M*/15 phosphate buffer, pH 7.8. The activator was added on crushing. The roots were cut in thin slices. L(+)-arginine (B.D.H.) was dissolved in *M*/15 phosphate buffer pH 7.8 to make a solution of concentration 20 mg/ml.

Performance of the experiment. 2 ml of the plant tissue suspension and 2 ml of arginine solution were mixed and kept under toluene at about + 20° C, occasionally stirring. The

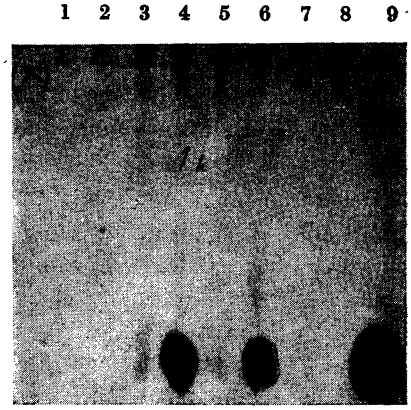

Fig. 1. Absence of arginase activity from the alder. One-dimensional paper chromatogram developed with n-*butanol from samples taken after reaction time of 1 hour. Development of colour with* p-*dimethyl aminobenzaldehyde reagent which gives a yellow spot with urea and citrulline.*

1. *Crushed leaves of A. incana*
2. *The same + arginine*
3. *Crushed leaves of A. incana + crushed cow's liver*
4. *The same + arginine*
5. *Crushed cow's liver*
6. *The same + arginine*
7. *Arginine*
8. *Ornithine*
9. *Urea*

(Citrulline control missing, but the location of citrulline marked with x. Crushed liver contained traces of citrulline.)

reaction was followed by determining paper chromatographically the possible formation of both reaction products, ornithine and urea.

Preparation of paper chromatograms. A 10 μl spot of mother liquor was pipetted on Whatman no. 1 paper straight from the reaction mixture. Four chromatograms were prepared from each sample, two by developing with water-saturated *n*-butanol for 2 days and two by developing with water-containing phenol (500 g phenol + 183 ml distilled water) for 36 hours in the atmosphere made by placing 100 ml of 0.5 % ammonia at the bottom of the chamber. In each run traces of HCN were used in the atmosphere.

One of the butanol and phenol chromatograms was used for identification of ornithine, the other for that of urea.

Ornithine was examined by developing the colour with ninhydrin whereby even 1 μg of ornithine could be detected with certainty corresponding to a 10 μl spot when a concentration of 0.1 mg/ml is used. In this way even the decomposition of *ca.* 1 % of arginine to ornithine could have been demonstrated.

Urea was shown by using *p*-dimethyl aminobenzaldehyde (Dent [13]) for the development of colour in a slightly modified [1] form. A spot of about 0.3—0.5 μg urea is detectable by this reagent.

The samples were taken 1, 4, and 24 hours after the start of the reaction.

RESULTS

Arginase activity could not be noted in any tissues, crushed leaves, nodules, or roots, of *Alnus incana*. Formation of neither ornithine nor urea could be shown paper chromatographically.

For control an experiment was made with cow's liver using the same technique. The chromatograms developed with ninhydrin were then rather blurred due to many impurities in the crushed liver, but in the chromatograms sprayed with *p*-dimethyl aminobenzaldehyde a very intensive formation of urea due to the crushed liver could clearly be shown.

In order to make sure that the crushed alder tissues do not contain any arginase inhibitor, an experiment was also made with a mixture of alder (*A. incana*) leaves and liver crushed together. No inhibitory effect on liver-arginase could be demonstrated with the crushed leaves of alder (see Fig. 1).

Since a formation of even a minute amount of ornithine could have been detected with the very sensitive and specific paper chromatographic method, the arginase activity of the alder tissues examined must be considered as being very low or nil.

Many bacteria contain arginine desimidase [14–16] which splits off an imino group from arginine forming citrulline. The alder tissues did not affect formation of citrulline under the conditions used in our experiments.

L(+)-GLUTAMIC ACID DECARBOXYLASE

Okunuki [4] and Hasse [6] have found that L(+)-glutamic acid decarboxylase has been entirely localized in insoluble cell particles of the cellular tissues examined by them, but Schales [5] again has found that it is easily extractable *e. g.* from carrot with phosphate buffer. In this work, therefore, we used in all experiments unfractionated crushed plant tissue, cell-free phosphate buffer extract and washed suspension of cell residue, obtained by centrifugation.

In this qualitative study we thought it most suitable to demonstrate the reaction by paper chromatographic determination of γ-aminobutyric acid; the manometric technique is less specific because glutamic acid can catalyze the formation of CO_2 in the crushed material even through reactions other than mere decarboxylation (*e. g.* by accelerating cell respiration).

γ-Aminobutyric acid is a completely specific reaction product which can hardly be formed from glutamic acid in any other way than through decarboxylation. To our knowledge the only method for its microdetermination is paper chromatography.

The decarboxylation experiment was made according to the instructions of Schales [5].

Experimental material. All the plant material was grown in the greenhouse and harvested as follows:

1.series: Leaves and nodules of grey alder. Harvested 28 July.
2.series: Harvested 17 Oct.

Nodules of grey alder. The test plant about 2 years old, grown in N-free sand, leaves just falling.

Root of carrot. Variety "Nantes" fully developed, but parts still green in colour.

Leaves of pea. Test plants grown in pots in N-free sand inoculated with an effective strain of root nodule bacteria. Age approximately 10 weeks; been in flower for approximately 1 week.

Preparation of plant tissue suspension. 4 g of plant tissue was rubbed to a homogeneous pipettable suspension in 20 ml of ice-cold *M*/15 phosphate buffer pH 5.60. pH of the suspension was approximately 5.70.

Preparation of L*(+)-glutamic acid solution.* 250.0 mg of L(+)-glutamic acid (Light & Co.) dried *in vacuo* was dissolved in approximately 15 ml of ~ 0.175 *N* NaOH in a beaker and the pH of the solution obtained was adjusted to 5.8 by dilute HCl. The solution was quantitatively washed into a 25 ml measuring flask and made up to volume.

Performance of the experiment. 5 ml centrifuge tubes, calibrated to 2.0 ml, were placed in ice and the following reagents pipetted to them:

A. 2.0 ml suspension + 1.0 ml water.
B. 2.0 ml suspension + 1.0 ml glutamic acid solution.
C. Precipitate, which was obtained by centrifuging the 2.0 ml suspension 3 times with cold phosphate buffer, pH 5.60, and finally making up the volume to 2.0 ml with phosphate buffer pH 5.60 + 1.0 ml water.
D. The precipitate as in experiment C made up to 2.0 ml + 1.0 ml glutamic acid solution.
E. 2.0 ml clear mother liquor obtained by centrifuging the suspension + 1.0 ml water.
F. 2.0 ml mother liquor + 1.0 ml glutamic acid solution.

The volume of the mother liquor used in experiments E and F corresponds to a slightly greater volume of suspension than the one used in experiments A—D.

Solutions A, C, and E are controls to solutions B, D, and F respectively, the latter ones containing 3.33 mg glutamic acid/ml at the start of the experiment.

After all the solutions had been pipetted, they were shaken and transferred from the ice-bath to a thermostat at 37° C where they were occasionally stirred. The tubes were open during the experiment. The samples, 10 μl each, were pipetted on Whatman no. 1 paper, in series 1 after 1 hour, in series 2 after 30 min, 1 hour and 2 hours, counting from the moment the samples were placed in the thermostat. The samples were pipetted by two persons in 4 minutes. The spot was dried on the paper by a current of warm air in about 30 sec. Whatman no. 1 paper was used in 10.2 mm wide strips.

The chromatograms were prepared by running with water-saturated phenol for 36 hours in a neutral atmosphere containing some HCN. Drying was effected by keeping the papers in a current of air of about 60° C for 10 min. in order to evaporate the major part of phenol and then overnight at room temperature.

The colour reaction was performed by drawing the strips through an acetone solution containing 0.25 % ninhydrin and by allowing the colour to develop overnight at room temperature.

A rough semiquantitative determination of the γ-aminobutyric acid split off was made by visually comparing the spots with a reference series with known amounts of γ-aminobutyric acid.

RESULTS

Series 1, which was performed at the end of July, gave very similar results both in regard to leaves and nodules of *A. incana*. The unfractionated suspension of both tissues and the washed insoluble cell residues decarboxylated distinctly L(+)-glutamic acid. On the other hand, the mother liquor which was centrifuged clear showed no noticeable activity of glutamic acid decarboxylase. The quantitativeness of the results is not good enough for the estima-

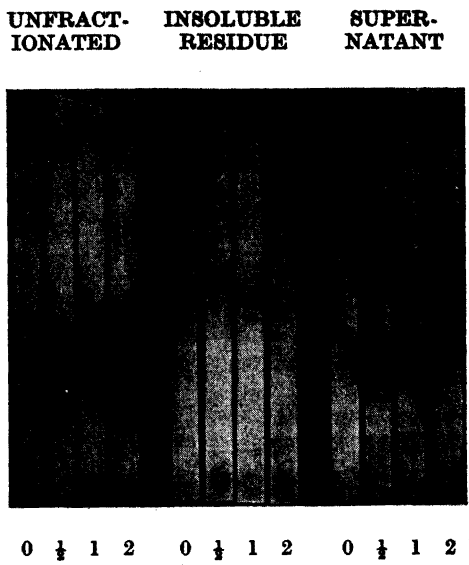

Fig. 2. L(+)*-glutamic acid decarboxylase in the root of carrot. One-dimensional phenol chromatograms for demonstrating γ-aminobutyric acid. Reaction time 0—2 hours at 37° C at pH 5.70.*

tion of the actual enzyme activities, but it can be judged from them that the activities of the unfractionated suspension and the corresponding washed suspension of cell particles are of the same order and relatively weak; about 0.3—0.5 mg γ-aminobutyric acid was split off per ml per hour.

Another experiment, which was carried out in October just when the leaves were falling, gave with the nodules of alder a similar result to the first one but the activities were weaker still; only about 0.1 mg γ-aminobutyric acid was split off per ml per hour, the smallest amount still detectable with certainty.

The activity of the suspension of carrot root was somewhat higher — about 0.3 mg γ-aminobutyric acid/ml/hour was split off — and the activity of the suspension of pea leaves much higher still; noticeably over 1.0 mg γ-aminobutyric acid/ml/hour was split off. A more definite comparison was impossible because the reference series did not contain spots of such high concentrations.

Furthermore, the glutamic acid decarboxylase of carrot and pea — contrary to that of the alder — was s o l u b l e, the washed cell residue containing only traces of activity (Figs. 2 and 3). Thus, this enzyme varies in different plant species from completely insoluble to completely soluble, as also noted in the previous studies.

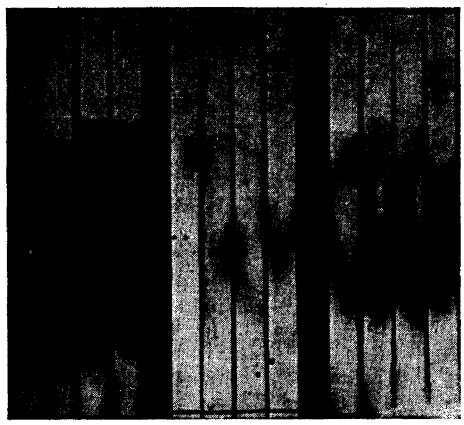

Fig. 3. L(+)*-glutamic acid decarboxylase in pea leaves. Reaction conditions the same as in Fig. 2.*

DISCUSSION

The absence of arginase activity from the alder tissues suggests that the citrulline nitrogen found abundantly in them cannot become utilized *via* urea and so the citrulline must have some other reaction possibilities.

The glutamic acid decarboxylase activity was lower than expected. Although the determinations are semiquantitative only, they seem to indicate that this activity is further lowered from the summer value when the tree prepares itself for the winter rest. An experiment of this kind cannot, of course, conclusively prove whether γ-amino butyric acid is formed *via* decarboxylation or otherwise. Even a low glutamic acid decarboxylase activity suffices to explain the abundant occurrence of γ-aminobutyric acid in the nodules of alder in the autumn [1], providing that the reactions consuming it are retarded very much in the autumn. No direct correlation seems to exist between the concentration of γ-aminobutyric acid and the glutamic acid decarboxylase activity in the plant tissues. The nodules of grey alder contain in October about 0.2 % γ-aminobutyric acid of the dry weight [1], the leaves of pea only about 0.05 % of the dry weight [9] yet the decarboxylase activity in the pea leaves is over 10 times that in the alder. This need not, however, mean that the formation of γ-aminobutyric acid in both cases were not

effected by decarboxylase, because, as indicated above, the enrichment depends both on the velocity of formation and consumption.

SUMMARY

The leaf, root, and nodule tissues of *Alnus incana* were examined for the occurrence of arginase. No arginase activity was found.

The occurrence of L(+)-glutamic acid decarboxylase was examined in the leaf and nodule tissue of *A. incana*. Some activity was noted in summer but very little in autumn at the time the leaves are falling, the activity seeming to concentrate principally in the insoluble cell particles. In comparative semiquantitative experiments carrot and pea possessed a greater activity, the enzyme being easily extractable with phosphate buffer.

The significance of the results is discussed. Utilization of citrulline in the absence of arginase does not seem likely *via* the urea cycle. Formation of γ-aminobutyric acid as a product of glutamic acid decarboxylation is probable although the activity of the corresponding decarboxylase in the leaf and nodule tissue of alder is weak.

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Received December 8, 1952.