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On the Reactions and Biosynthesis of Azetidine-2-carboxylic Acid

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The hydrolysis of azetidine-2-carboxylic acid is studied.

β-Naphthoquinone sulphonate is presented as a sensitive and specific reagent for compounds containing the =CH—NH—CH= group.

Homoserine, which does not occur free in *Convallaria majalis*, is found in the acid hydrolysates of the "peptide fraction".

Uniformly labelled aspartic acid is infiltrated into the leaves of *Convallaria majalis* and the incorporation of radioactivity is followed. No activity is found in azetidine-2-carboxylic acid. Aspartic acid is readily decarboxylated to both α- and β-alanine. From the other compounds glutamic acid is primarily labelled. The activity accumulates in asparagine, glutamine and an unknown glutamic acid derivative. In the "peptide" fraction hydrolysates aspartic acid, glutamic acid and serine are labelled, whereas in the protein hydrolysates only aspartic acid is found to be labelled.

The metabolic role of azetidine-2-carboxylic acid is discussed.

Only a few years ago Miettinen *et al.*[1] for the first time isolated and identified homoserine as a free amino acid in pea *(Pisum sativum)*. According to earlier experiments [2] based only on chromatographical evidence it was known to occur in *Neurospora*. Later homoserine has been found to be widely distributed in the plant kingdom [3].

Though homoserine is rather a "newcomer" among the free amino acids in plants, some of its derivatives have been known for a long time. Kitagawa and Tomita [4] as far back as 1929 isolated canavanine, α-amino-γ-guanidinoxybutyric acid, from *Canavalia ensiformis*. Recently canavanine was also found in *Colutea arborescens* [5]. Pantonine, another homoserine derivative, was isolated from *Escherichia coli* [6]. Because there was already strong evidence for the importance of homoserine and its derivatives in the metabolism of plants, the recent discovery of azetidine-2-carboxylic acid in some *Liliaceae* plants

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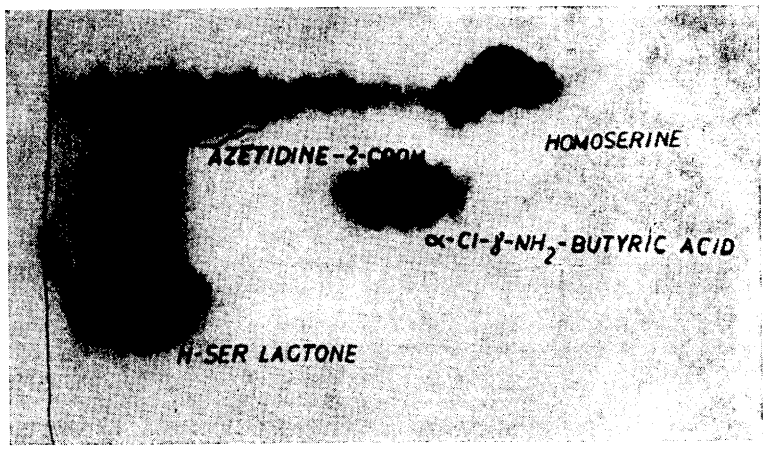

Fig. 1. The products of hydrolysis of azetidine-2-carboxylic acid by means of 6 N hydrochloric acid (sealed tube, 20 h, 100°C).

was very interesting [7–9]. This imino acid may be related to α,γ-diaminobutyric acid [10,11] or to its deamination product, aspartic-α-semialdehyde. This known intermediate in the reduction of aspartic acid to homoserine [12,13] is found to occur in yeast [14]. Hence the possibility that azetidine-2-carboxylic acid may be formed from aspartic acid in the leaves of *Convallaria majalis* was examined.

HYDROLYSIS OF AZETIDINE-2-CARBOXYLIC ACID

When azetidine-2-carboxylic acid is subjected to hydrolysis with hydrochloric acid three new spots on the chromatograms are observed in addition to that corresponding to the small amount of unchanged compound (Fig. 1) [15,7]. Two of these spots were identified as homoserine and γ-amino-α-chlorobutyric acid [9], the relative amount of the latter being [15] only about 5 %. The third compound, homoserine lactone [15], formed secondarily from homoserine, is evidently identical with spot S_3 of Fowden, which he in turn describes as α-amino-γ-chlorobutyric acid but which could not, however, be synthesized. This compound was isolated in crystalline form as the hydrochloride. It gave the typical lactone reaction with ferric chloride and hydroxylamine, could easily be hydrolysed to homoserine by barium hydroxide and was finally found identical with the synthetic product of Armstrong [16]. The optical rotation determined as a function of time (Fig. 2) was in good agreement with Armstrong's results. In contrast to the observation of Fowden that azetidine-2-carboxylic acid is stable against alkalies it was found that 4 N sodium hydroxide caused some decomposition already on standing at room temperature. Only homoserine was formed during alkaline hydrolysis. Ammonia and acids, however, did not cause decomposition at this temperature.

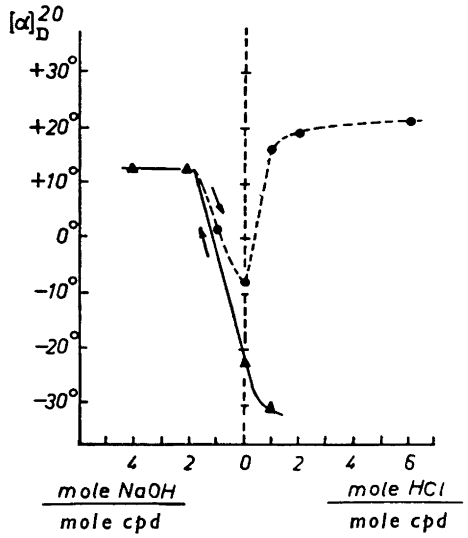

Fig. 2. The optical rotation of homoserine (---) and its lactone (———) as a function of pH.

β-NAPHTHOQUINONESULPHONATE REAGENT

Müting [17] and Giri and Nagabushanan [18] detected that α-naphthoquinonesulphonate gives, with most of the amino acids and their derivatives, colors varying from green to bluish when the paper chromatogram is dipped into the reagent. Only proline was found to give a red color reaction. Because azetidine-2-carboxylic acid also gave an intensive red color reaction, several other secondary amines were studied in respect to this reagent. Hydroxyproline, piperidine-4-carboxylic acid, sarcosine and N-phenylglycine developed a strong red color, whereas the red color caused by piperidine-2-carboxylic acid and its 5-hydroxyderivative was somewhat weaker. With peptides and N-acetylderivatives of amino acids the normal bluegreen color was obtained. The reaction is about as sensitive to secondary amines as ninhydrin and therefore seems to be a good specific reagent for the compounds containing the = CH—NH—CH= group. As the only exception so far known 1-amino-*cyclo*propane-1-carboxylic acid developed a weak red color [19].

ON THE BIOSYNTHESIS OF AZETIDINE-2-CARBOXYLIC ACID

The amount of azetidine-2-carboxylic acid in young *Convallaria majalis* plants was about 20 % or even more of the ethanol soluble nitrogen compounds. When the plants started to turn yellow in the fall, the relative amounts of most of the free amino acids increased (Fig. 3). The only noticeable exception was azetidine-2-carboxylic acid which had almost disappeared from the completely yellow plants. This is in good agreement with the results of Kemple and Macpherson [20] who noticed that the amount of all protein amino acids increased in the ethanol soluble fraction during the drying of grass. All the nitrogen

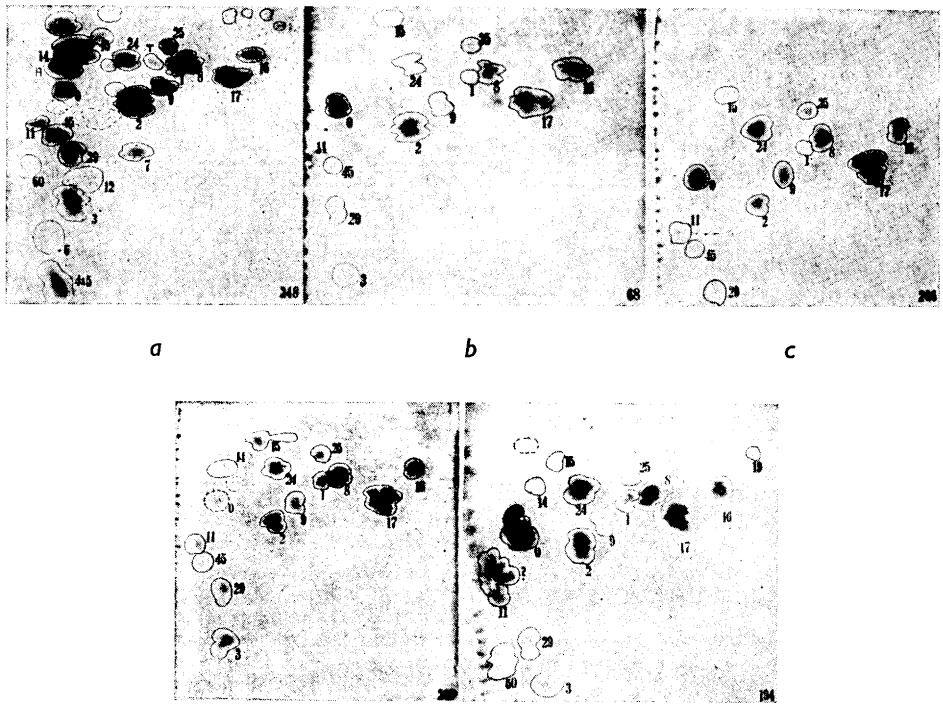

Fig. 3. Free amino acids of *Convallaria majalis*. *a*, Seeds; *b*, young leaves; *c*, partly yellow leaves; *d*, completely yellow leaves; *e*, soft part of ripe berries. 1 = gly, 2 = ala, 3 = val, 4 & 5 = leu + ileu, 6 = β-ala, 8 = ser, 9 = threo, 11 = prol, 12 = tryptophane, 14 = arg, 15 = lys, 16 = asp, 17 = glu, 24 = glu-NH_2, 25 = asp-NH_2, 29 = γ-AB, 45 = ethanolamine, 60 = piperidine-2-carboxylic acid, 0 = azetidine-2-carboxylic acid, ? = unknown compound.

liberated from proteins could be found in the free amino acids, amides, peptides and volatile bases.

During the disappearance of azetidine-2-carboxylic acid no homoserine or other "new" amino compounds were formed. However, great amounts of the imino acid were detected in the soft parts of ripe berries, while almost none was found in the seeds. It may be of interest to mention here that 1-aminocyclopropane-1-carboxylic acid also exists only in ripe fruits [19,21]. Two unknown amino compounds, which could not be detected in the other parts of the plants, were also found in the berries of *Polygonatum officinale*. One of them was isolated by fractionation on a Dowex 50 cation exchange resin column and identified as tyramine. This amine was found [22] in *Viscum album* already 1916. Later it was isolated from *Sarothamnus* [23,24] and chromatographically identified in some other plants [24,25]. The other unknown compound

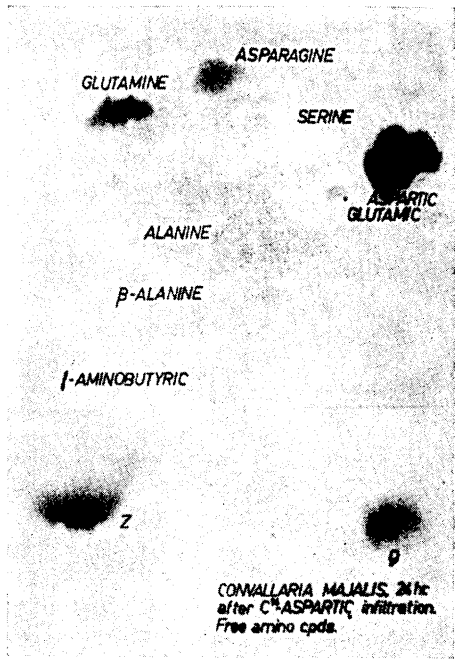

Fig. 4. Radioautogram of the amino compound fraction of *Convallaria majalis*. 24 h after uniformly labelled aspartic acid infiltration.

marked with a question mark in Fig. 3 e has not yet been identified. Both the place on the chromatogram and the graybluish color reaction with ninhydrin are characteristic to many amines.

Virtanen *et al.*[26] noticed that homoserine, which did not occur either in free state or bound in pea seeds, was rapidly formed during germination. Already after 72 h this compound was the most abundantly appearing free amino acid. After five days the relative amount of homoserine had still increased while glutamic acid and serine had decreased. The young stem and root now contained far greater amounts of homoserine than did the seed. Examination of *Convallaria* in this respect was very difficult because the seeds do not easily germinate. With a few seeds it could, however, be shown that azetidine-2-carboxylic acid behaved like homoserine during the germination of seeds.

The observation of Fowden and Steward[27] that azetidine-2-carboxylic acid was found in all parts of *Convallaria*, but in some other species only in leaves or in seeds, respectively, may be due to the changes in concentration of this compound in different parts of plants during various stages. It is interesting that in the leaves of *Fritillaria meleagris* γ-methyleneglutamic acid but no azetidine-2-carboxylic acids was found whereas in the seeds of *Fr. imperialis* the situation was quite the opposite.

The phosphorylation of the β-carboxyl group of aspartic acid is now known to be the primary reaction in threonine biosynthesis in yeast. β-Aspartyl-

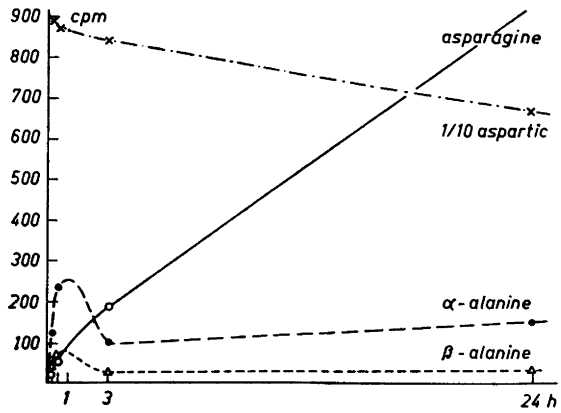

Fig. 5. Relative activities of aspartic acid and its direct biosynthetic products in *Convallaria majalis* after uniformly labelled aspartic acid infiltration.

phosphate can quantitatively be reduced to homoserine by means of a specific enzyme isolated from baker's yeast [13]. A few years later [28] it was noticed that incubation of rat liver homogenate with DL-2-^{14}C-homoserine induced labelling into α-ketobutyrate, α-hydroxybutyrate, α-aminobutyrate and propionate, but no labelling was found in threonine, methionine, alanine and α-keto-γ-hydroxybutyrate. Therefore, in this case the primary reaction would be identical with the dehydration of threonine and serine. However, α-keto-γ-hydroxybutyric acid is now known to occur in higher plants [29].

The cyclization of aspartic β-semialdehyde is the most likely reaction leading to azetidine-2-carboxylic acid. A similar type of reaction is known in the conversion of δ-aminolaevulinic acid to the porphyrin ring [30]. 4-Carb-

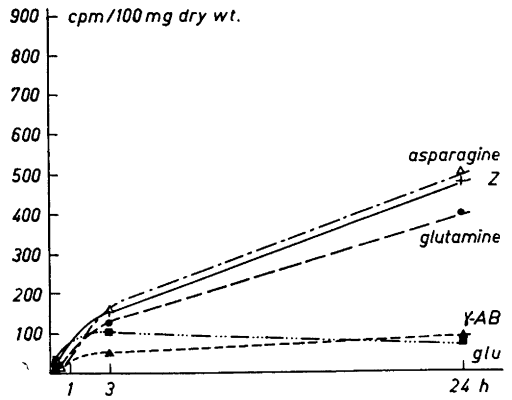

Fig. 6. Total radioactivity changes in *Convallaria majalis* after uniformly labelled aspartic acid infiltration.

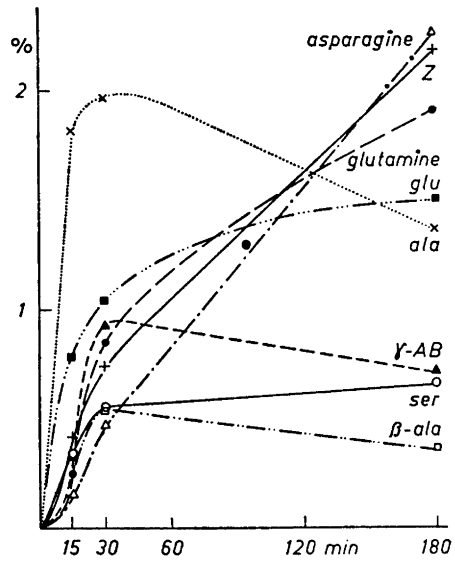

Fig. 7. The percentage of radioactivity in the individual amino compounds calculated from the total ethanol soluble activity in *Convallaria majalis* after uniformly labelled aspartic acid infiltration.

oxyl-azetidinone is also known to be formed synthetically from asparagine in phosphate buffer[31] (pH 6.7; 100°C). Uniformly labelled aspartic acid was therefore infiltrated into the leaves of young *Convallaria majalis* plants grown in the greenhouse. Similar methods were used as described earlier[32]. After half an hour there was a slight minimum in the percentage of activity of the amino compound fraction as compared to the total ethanol soluble activity. After 24 h the activity of this fraction was still 90 % of the total soluble one. No radioactivity could be found in azetidine-2-carboxylic acid (Fig. 4). Both carboxyl groups of aspartic acid were readily decarboxylated α- and β- alanine respectively, being formed (Fig. 5). The β-carboxyl was more rapidly removed. The activity of both decarboxylation products had a peak after about one hour showing that they were rapidly metabolised further. From the other compounds glutamic acid was primarily formed. After short lag periods activities in both asparagine and glutamine increased countinuously. The same was due to an unknown compound, Z, which yielded glutamic acid as the only active component on acid hydrolysis (Figs. 6 and 7).

In the acid hydrolysates of the proteins only aspartic acid was found labelled, but when the "peptide" fraction was subjected to acid hydrolysis aspartic acid, glutamic acid and serine were found to be labelled (Fig. 8). Practically all the amino acids, except azetidine-2-carboxylic acid, were found to occur in the hydrolysates. The percentage of the activity of aspartic acid had a peak after half an hour, glutamic acid being the main labelled product after 24 h. It was very interesting to find that though azetidine-2-carboxylic acid was absent in this fraction, inactive homoserine was always found in the hydrolysates. This cannot arise from azetidine-2-carboxylic acid during hydrolysis, because no α-chloro-γ-aminobutyric acid could be detected on

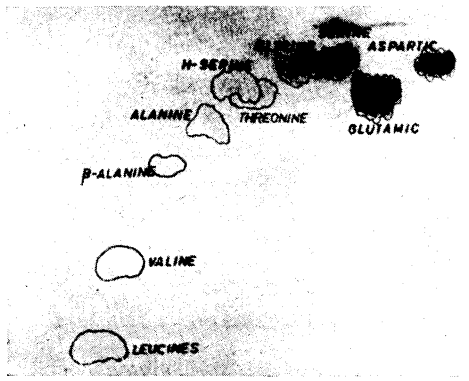

Fig. 8. A ninhydrin sprayed chromatogram of amino acids arising from the "peptide" fraction of *Convallaria majalis* in acid hydrolysis. The darkened spots were labelled after uniformly labelled aspartic acid infiltration.

the chromatograms. The experiments performed in order to show the synthesis of azetidine-2-carboxylic acid from inactive homoserine by the method of Miettinen and Virtanen [33] failed, but this could be due to the great amount of azetidine-2-carboxylic acid originally present, making comparisons very difficult. Recently, bound homoserine was found in the ethanol insoluble fraction of cowberries and cranberries [34]. Even in this case no free homoserine was found, but the berries of cowberries contained a cyclic four-carbon amino acid, 1-amino-*cyclo*propane-1-carboxylic acid [19]. It may also be mentioned here that strains of *Neurospora* requiring homoserine for growth also grow with canavanine [35].

That no radioactivity was found in azetidine-2-carboxylic acid after ^{14}C-aspartic acid infiltration may partly be due to the small amount of labelled aspartate available as compared to the relatively great amount of azetidine-2-carboxylic acid originally present in the plant thus causing great dilution of the possible active synthetic product with inactive material. However, it is most evident that the rate of synthesis of azetidine-2-carboxylic acid is much lower in the leaves than in young sprouts and that this compound may arise from the compound present in the "peptide" fraction which yields homoserine on acid hydrolysis and which in turn may be formed from aspartic acid *via* aspartic-β-semialdehyde.

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