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THE ACCUMULATION OF γ-AMINOBUTYRIC ACID IN BEAN CALLUS TISSUE

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Abstract—Bean callus tissue contains enzyme systems which convert both L-glutamic acid and L-ornithine to γ-aminobutyric acid (γ-AB). The accumulation of γ-AB in callus tissue grown with limited access to air is probably due to the non-occurrence of transamination in these growth conditions.

INTRODUCTION

IN PLANTS, γ-AB is generally regarded as arising from glutamic acid by decarboxylation.[1-3] The decarboxylase which effects this reaction requires pyridoxal phosphate as co-factor, and has been purified from plants,[4] bacteria,[5,6] yeasts,[7] and animals.[8] Alternative routes for the formation of γ-AB from other precursors have, however, been reported. A strain of *Pseudomonas fluorescens* was found to contain enzyme systems which convert pyrrolidine and putrescine to γ-AB,[9] and the oxidation of proline by D-amino acid oxidase preparations, low in catalase activity, was also found to lead to the formation of γ-AB,[10] The further metabolism of γ-AB involves transamination to yield succinic semialdehyde, which is then converted to succinic acid to undergo final oxidation through the Krebs cycle.[2,11,12]

It has been reported earlier that plant cell tissue cultures, grown under conditions of limited access to air, showed a high concentration of γ-AB in the callus tissue,[13] and the studies described here deal with the mechanism of the accumulation of this amino acid in bean callus. The results indicate that γ-AB is formed by decarboxylation of glutamic acid and also by a hitherto undescribed route involving oxidative deamination of ornithine, followed by oxidative decarboxylation of the keto acid formed. Pyridoxal phosphate was found to act as a co-factor in the latter reaction. The reason why anaerobic conditions lead to the accumulation of γ-AB appears to be the lack of transamination reactions under such conditions.

RESULTS AND DISCUSSION

Experiments were first carried out to determine which amino acids could act as precursors of γ-AB. Acetone powders prepared from bean callus grown with either free or limited access

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to air, were incubated separately with L-glutamic acid, L-ornithine, L-citrulline, L-arginine or L-proline, together with pyridoxal phosphate in 0·2 M phosphate buffers of pH values ranging from 4·4 to 11·8. The incubation was carried out in Warburg manometers (gas phase air) and after incubation the mixtures were analysed for γ-AB by paper chromatography. It was found that γ-AB was only formed from glutamic acid and ornithine, and that the intensity of the γ-AB spot was greater using the acetone powder prepared from the callus grown under conditions of limited access to air. Subsequent experiments were, therefore, carried out using callus grown under the conditions described in the Experimental section.

The reaction with glutamic acid was accompanied by the evolution of carbon dioxide, and the optimum pH for the decarboxylase was found to be 5·8. In the case of ornithine, the reaction occurred with both the uptake of oxygen and the evolution of carbon dioxide. Using oxygen as gas phase, and following the reaction with and without alkali in the centre well of the manometer vessels, it was found that oxygen uptake occurred over a wide range of pH values and was optimal at pH 9·2, whilst the evolution of carbon dioxide was optimal at pH values between 5·2 and 5·8. Paper chromatographic analyses of the incubation mixtures at the different pH values showed that the intensity of the γ-AB spot was highest at pH 5·8. However on spraying a duplicate chromatogram with *o*-aminobenzaldehyde reagent, a yellow spot of R_f value corresponding to α-oxo-δ-aminovaleric acid (prepared by the action of L-amino acid oxidase of *Crotalus adamanteus* venom on L-ornithine), was obtained. The intensity of this spot showed a progressive increase from pH 5·8 and was maximal at pH 9·2. These results indicated that the first step in the conversion of ornithine to γ-AB is α-deamination to the α-keto acid, followed by oxidative decarboxylation of this product. Cyclization of the α-keto acid would result in the formation of Δ^1-pyrroline-2-carboxylic acid, which reacts with *o*-aminbenzaldehyde to form a yellow dihydroquinazolinium compound[14] (Fig. 1).

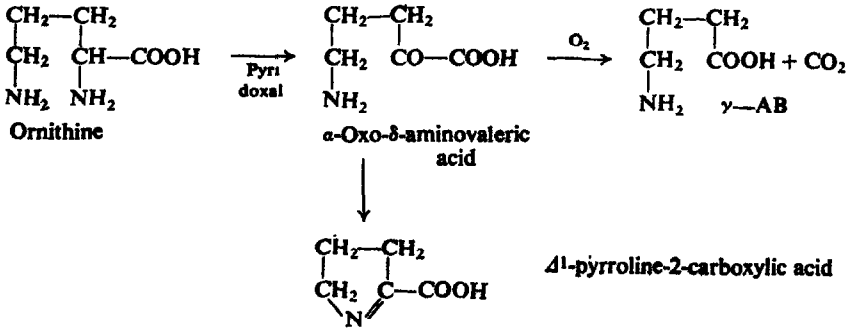

FIG. 1.

Additional evidence for the identification of α-oxo-δ-amino valeric acid was the identical R_f values on silica gel plates of the 2,4-dinitrophenylhydrazone derivatives of the keto-acids formed by the action of the bean callus acetone powder and *Crotalus adamanteus* venom respectively on L-ornithine at pH 9·2. Using the bean callus acetone powder no free ammonia could be detected by the Conway micro-diffusion method in the reaction solution, indicating that this product was bound in some way during the reaction.

In addition to γ-AB and α-keto-δ-aminovaleric acid, another spot was sometimes observed

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to appear on incubation of the acetone powder–ornithine–pyridoxal phosphate mixtures. This spot fluoresced blue when viewed under u.v. light, had an R_f value slightly lower than that of γ-AB in butanol-acetic acid–water, gave a yellow colour with ninhydrin–$CoCl_2$, an orange colour with a saturated solution of vanillin in ethanol : conc. HCl (1:3), and no colour with either isatin, 2,4-dinitrophenylhydrazine followed by 10% Na_2CO_3, *p*-dimethylaminobenzaldehyde or diazotized sulphanilic acid. Since its production was erratic it could not be obtained in sufficient quantity for further examination. It appears likely that it is not an intermediate in γ-AB formation, but perhaps a transformation product of Δ^1-pyrolline-2-carboxylic acid.

Course of the reaction. The acetone powder preparation of bean callus was incubated with L-ornithine at pH 5·8 and aliquots of the reaction mixture analysed by paper chromatography at 30-min intervals for a period of 5½ hr. Duplicate chromatograms were run, one of which was sprayed with ninhydrin–$CoCl_2$ and the other with *o*-aminobenzaldehyde. It was found that α-oxo-δ-aminovaleric acid was first formed and then γ-AB. The concentration of the latter increased with time whilst that of the keto acid decreased as the ornithine was used up. In a separate experiment it was found that 38% of the ornithine, estimated by the method of Gale,[5] disappeared in the first hour.

Pyridoxal phosphate as co-factor. The rate of oxygen uptake of the acetone powder–ornithine mixtures was decreased five-fold at pH 5·8 and twenty-fold at pH 9·2 if no addition of pyridoxal phosphate was made, indicating as expected that the latter compound was a co-factor for the deaminase. Analysis of the reaction mixture by paper chromatography showed the presence of a spot with a purple fluorescence when viewed under u.v. light and having the same R_f value as pyridoxamine, intensity of the pyridoxamine spot was greater at pH 9·2 than at 5·8. This observation explains the absence of detectable free ammonia mentioned above.

Accumulation of γ-AB. A sample of autoclaved coconut milk used for the preparation of the culture medium, was analysed for its amino acid content and showed the presence of small amounts of methionine and proline, and appreciable amounts of aspartic acid, glutamic acid, glycine, serine, threonine, alanine, tyrosine, phenylalanine, valine, leucine, isoleucine, ornithine, lysine, histidine and γ-AB. The relatively large amounts of γ-AB in coconut milk may *per se* contribute to the high concentration of this acid in the callus tissue, but the negligible amounts of γ-AB in the callus tissue grown with free access to air indicates either that the amino acid is not taken up by the callus under these conditions of growth, or that it is taken up and transformed to other products. To decide between these alternatives, a comparison was made of the amounts of γ-AB in bean callus grown for 3 weeks under four different conditions. In the first two the callus was grown on coconut milk medium with either free access of air for the whole period or for the first week only. In the other two the callus was grown on coconut milk medium with free access of air for 1 week and limited access of air for the next week, after which the callus was transferred to a medium without coconut milk and grown either with free access or with limited access of air for the last week.

The results obtained (Table 1) show that γ-AB only accumulates along with other amino acids in the callus grown under conditions of limited access to air. On transferring the callus to a medium, devoid of coconut milk but containing sucrose, the accumulated γ-AB is transformed to other products under aerobic conditions, but is apparently unchanged when the amount of available air is limited. These results suggest that the accumulation of γ-AB in conditions of limited access of air is due to the reduction in transamination which is the first step in the utilization of γ-AB. The probable reasons for this reduction are the lack of keto

TABLE 1. EFFECT OF AIR ON ACCUMULATION OF γ-AB IN 3-WEEK BEAN CALLUS

Growth conditions*		Total	Amount of
week 2	week 3	amino acids†	γ-AB
CM+	CM+	0·9	None
CM−	CM−	3·7	Large
CM−	S+	0·9	Trace
CM−	S−	3·6	Large

* All were grown for the first week on coconut milk (CM), in air (+), S is a coconut milk-free medium containing sucrose (2%) and co-factors only,[15] (−) shows limited access of air.

† Values for total amino acids estimated with ninhydrin,[16] expressed as μmoles/g callus in terms of β-phenylalanine.

acids and the low pH both unfavourable for transamination, caused by the accumulation of carbon dioxide under these growth conditions.[13] It is obvious that under such conditions many other reactions might be inhibited, and points once more to the importance of aeration in obtaining standard tissue cultures.

EXPERIMENTAL

Preparation of acetone powders. Bean callus was grown on the coconut milk medium described previously,[15] for a period of 1 week with free access to air, after which the caps of the bottles were made airtight and growth allowed to proceed in conditions of limited air for a further 2 weeks. At the end of this period of growth, the callus tissue from several bottles was frozen at −20° C for 20–22 hr. The frozen callus was well ground in a cooled mortar with ballotini beads in 0·2 M KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, until no intact cells were visible on microscopic examination. The suspension was squirted through a pipette into 5 times its volume of cold acetone and allowed to stand for 15 min with occasional stirring. The acetone was then removed by filtration and the residue washed successively with 100 ml aliquots of cold acetone, acetone:ether (1:1) mixture and ether.

Reactions with amino acids. The acetone powder (100 mg) was suspended in 0·2 M phosphate buffer of the required pH (2·5 ml) containing the relevant amino acid (10 μM) and pyridoxal phosphate (1 μM). The reaction mixture was incubated at 30° for 1 hr, the acetone powder removed by centrifuging and an aliquot of the supernatant taken for chromatography.

Chromatography. Paper chromatographic analyses were carried out using butanol:acetic acid:water (6:1:2 v/v) as developing solvent. In two-dimensional analyses aqueous phenol was used as the second solvent. The spray reagents generally used were 0·4% ninhydrin in 95% ethanol containing 0·2% cobalt chloride, and 0·1% *o*-aminobenzaldehyde in absolute ethanol.

Thin-layer chromatographic analyses of 2,4-dinitrophenylhydrazones was carried out on silica gel plates using toluene:ethyl acetate (60:40 v/v) as solvent.

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Preparation of o-aminobenzaldehyde. *o*-Aminobenzaldehyde was prepared by reduction of *o*-nitrobenzaldehyde as described by Mann and Wilkinson.[17]

Note added in proof. Prof. L. Fowden, F.R.S. has informed us that the first stage in the formation of γ-AB from ornithine can proceed non-enzymically. Further investigation of this system seem warranted.

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