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# CHANGES IN THE FREE AMINO ACIDS OF SPIRODELA POLYRHIZA (L.) SCHLEIDEN DURING GROWTH INHIBITION BY L-VALINE, L-ISOLEUCINE, OR L-LEUCINE. A GAS CHROMATOGRAPHIC STUDY

A. C. BORSTLAP

Botanisch Laboratorium, Utrecht

SUMMARY

By means of gas-liquid chromatography the contents of free amino acids of the duckweed *Spirodela polyrhiza* have been determined after growth inhibition by L-valine, L-isoleucine and L-leucine. The most remarkable changes were when growth was inhibited with (1) valine: accumulation of α-aminobutyric acid, decrease in the isoleucine content and increase in that of leucine, (2) isoleucine: decrease in the leucine content and increase in that of valine, (3) leucine: decrease in both the valine content and isoleucine content. It is concluded that valine inhibits isoleucine synthesis by blocking acetohydroxy acid synthetase, isoleucine inhibits leucine synthesis, and leucine inhibits a common enzymatic step of the valine-isoleucine pathway.

## 1. INTRODUCTION

The biosynthetic pathways of the branched-chain amino acids are closely linked up with each other. The synthesis of valine and leucine proceeds through the common intermediate α-ketoisovaleric acid which is formed from pyruvic acid and active acetaldehyde by three enzymes. These enzymes also catalyze analogous reactions of the isoleucine pathway. The biosynthetic routes for valine, isoleucine and leucine have therefore three enzymes in common (Rodwell 1969).

From previous experiments it has been tentatively concluded that growth inhibition of *S. polyrhiza* by a branched-chain amino acid is brought about by inhibition of one of the common enzymes (Borstlap 1970). If this is true it is to be expected that during growth inhibition by one of the branched-chain amino acids the amounts of the other two amino acids will be growth limiting. This supposition has now been tested by measuring the contents of the free amino acids in cultures of *S. polyrhiza* after growth had been inhibited with valine, isoleucine or leucine.

Amino acids were determined as their N-TFA n-butyl esters by gas-liquid chromatography according to Gehrke *et al.* (1968). This method is a fast, sensitive and reliable alternative to the classical ion-exchange column chromatography, but it has so far been employed in a few laboratories only. In the present paper some details on the application of this method are described.

*Abbreviations used*: TFAA, trifluoroacetic anhydride; TFA, trifluoroacetyl-; EGA, ethylene glycol adipate; OV-17, phenyl methyl silicone; DMCS, dime-

thyl dichlorosilane. Isoleucine, asparagine, glutamine, α-aminobutyric acid, α-amino-iso-butyric acid, γ-aminobutyric acid, and homoserine are abbreviated as Ile, Asn, Gln, Abu, Aib, γAbu, and Hse, respectively; the other amino acids by their first three letters.

## 2. MATERIAL AND METHODS

### 2.1. Plants

Axenic cultures of *Spirodela polyrhiza* were grown as described previously (BORSTLAP 1970). The basic medium employed has been described by LACOR (1968). It contained 1% sucrose and 36 mg/l $Na_2HPO_4.2H_2O$. When a culture consisted of about 40 fronds, 1 ml of a sterile $10^{-2}$ M amino acid solution was added to the medium (100 ml). At various times after the addition of the amino acid, cultures were harvested, rinsed with tap water, lyophilized at -10°C, and stored in a freezer until used for analysis.

### 2.2. Chemicals and materials

Amino acids were purchased from the British Drug Houses Ltd., Poole, Dorset, U.K., from Fluka A.G., Buchs, Switzerland or from E. Merck A.G., Darmstadt, G.F.R. Cellulose (microcrystalline), silicagel G and TFAA were obtained from E. Merck A.G. The support materials A.W. Chromosorb W, 80–100 mesh, and the same material but treated with DMCS were products of Johns-Manville Corp., New York 16, N.Y., U.S.A. Stabilized grade EGA was obtained from Analabs Inc., North Haven, Conn., U.S.A. Chloroform, methylene chloride and n-butanol, "Analar reagents" from the British Drug Houses Ltd., were refluxed with $CaCl_2$ and distilled before use. HCl-gas, purchased from J.T. Baker Chemicals N.V., Deventer, The Netherlands, was passed through concentrated $H_2SO_4$ before bubbling into the butanol. Tubes with teflon-lined screw cap were obtained from Corning Glass Works Co., No. 9826.

### 2.3. Extraction of free amino acids

A lyophilized culture was homogenized with a pestle and mortar in 80% (v/v) ethanol. The homogenate was transferred to a round bottom flask and evaporated to dryness on a rotating evaporator. Demineralized water and chloroform were added to the residue in equal amounts. For quantitative analysis by gas-liquid chromatography 2 μmoles of α-amino-iso-butyric acid were added as an internal standard. The water-chloroform mixture was centrifuged so as to clearly separate the two phases; the waterphase was siphoned off and placed on a 20 × 0.6 cm cation exchange column (Dowex 50W–x8, 20–50 mesh, $H^+$ form). After passage of the waterphase the column was rinsed with 25 ml of demineralized water, after which the amino acids were eluted with 50 ml of 2N-ammonia. The eluate was collected and concentrated to a few milliliters on a steam-bath.

### 2.4. Thin-layer chromatography

A mixture of 10 g cellulose and 4 g silicagel G was blended with 60 ml of demineralized water. The slurry was applied to glass plates (20 × 20 × 0.3 cm) to a thickness of 0.25 mm. The plates were dried at room temperature. Amino acids were chromatographed in two dimensions by the ascending technique. The solvent system for the 1st dimension was n-butanol – acetone – ammonia – water (10:10:5:2, by volume) and for the 2nd dimension isopropanol – formic acid – water (20:1:5, by volume) (PILLAY & MEHDI 1970). Between the two runs the plates were dried in an oven at 40 °C. To make the amino acid spots visible the plates were sprayed with a 0.5% (w/v) ninhydrin solution in acetone and heated for 5 minutes at 100 °C.

### 2.5. Quantitative gas-liquid chromatography of amino acids

#### 2.5.1. Preparation N-TFA n-butyl esters of amino acids

An amino acid fraction, or a standard mixture of amino acids was pipetted into a tube with teflon-lined screw cap and dried by passing a stream of dry filtered air over it at 100 °C in a test tube heater. Next, 1 ml 3N-HCl in n-butanol was added to the sample. The esterification took place in the closed tube for 25 minutes at 100 °C (ROACH & GEHRKE 1969b). Excess of butanol was *just* removed at 100 °C using a stream of dry filtered air. 0.1 ml of methylene chloride was added and evaporated to ensure complete azeotropic removal of water. Finally, 0.1 ml of methylene chloride and 0.1 ml of TFAA were added to acylate the butyl esters for at least 30 minutes at room temperature. The samples were analysed within 4 hours after addition of TFAA.

#### 2.5.2. Columns

The support material, dried at 140 °C for 16 hours (ROACH & GEHRKE 1969a), was put in a round bottom flask and covered with chloroform. An appropriate amount of EGA was dissolved in chloroform and then added to the flask with the support material. The chloroform was slowly removed with a rotating evaporator at 60 °C and reduced pressure. The coated support was packed in a spiral-shaped glass column (1.8 m × 4 mm I.D.). The column was conditioned at 220 °C and 40 ml/min $N_2$ for about 30 hours.

#### 2.5.3. Apparatus and chromatographic conditions

A Hewlett-Packard gaschromatograph (model 700–00) with flame ionization detectors and equipped with a strip chart recorder (model 7128A) and a temperature programming unit (model 240) was used. The conditions were as follows:

| | |
|---|---|
| column temperature: | initial 60 °C, final 220 °C |
| program rate: | 3 °C/min |
| injection port temperature: | 250 °C |
| detector temperature: | 250 °C |
| carrier flow, nitrogen: | 40 ml/min (as a rule) |
| air flow to detector: | 450 ml/min |
| hydrogen flow to detector: | 30 ml/min |
| sensitivity: | $5 \times 10^2$ (as a rule) |

Peak areas were measured with a digital integrator (Kent, Chromalog 2) at ½ sample/sec and slope sensitivity 8.

### 2.5.4. Calculations

Relative molar responses (RMR's) of the amino acids have been determined with respect to α-amino-iso-butyric acid, which was used as an internal standard. When equimolar amounts of an amino acid and internal standard are chromatographed, the RMR of the amino acid (a.a.) with respect to internal standard (i.s.) simply follows from the ratio of their peak areas:

$$RMR_{a.a./i.s.} = \frac{\text{peak area}_{a.a.}}{\text{peak area}_{i.s.}}$$

To amino acid samples of unknown composition an exact amount (N moles) of internal standard was added. After analysis, the total amount of a given amino acid in the sample could be calculated as follows:

$$\text{moles}_{a.a.} = \frac{\text{peak area}_{a.a.}}{\text{peak area}_{i.s.}} \times \frac{N}{RMR_{a.a./i.s.}}$$

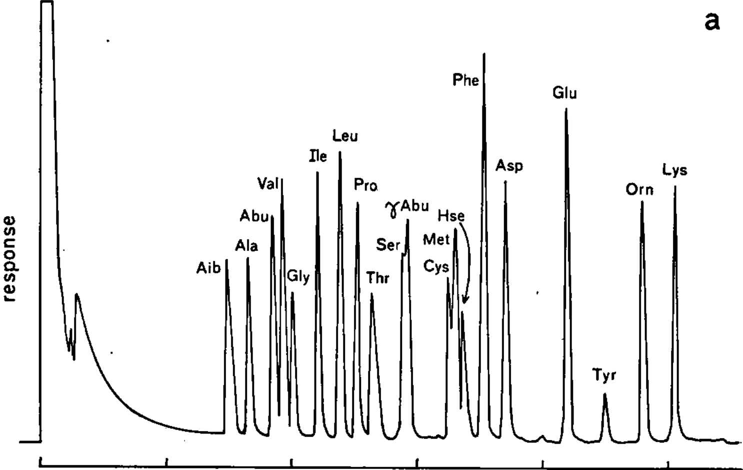
## 2.6. Mass spectrometry of the N-TFA α-aminobutyric acid n-butylester

To 3 cultures of *S. polyrhiza* (about 40 fronds and 100 ml medium per culture) L- valine was added to a final concentration of $10^{-4}$ M. After 5 days the cultures were harvested and lyophilized. The free amino acid fraction was prepared from the cultures and the amino acids were derivatized to their N-TFA n-butylesters. The derivatives were fractionated on a 0.325% EGA column (see section 3.1.). The column was installed in a Packard gaschromatograph (model 835), equipped with argon ionization detectors. The fraction supposed to contain the derivative of α-aminobutyric acid was collected at the detector outlet in a small U-shaped glass tube. During collection the glass tube was immersed in a dry ice-acetone mixture. After collection the derivative was immediately dissolved in anhydrous methylene chloride. Its mass spectrum was determined with an MS-9 AEI mass spectrometer at 70 eV and an ion source temperature of about 85°C. This mass spectrum was compared with that of L-α-aminobutyric acid (from Fluka A.G.) at 56 eV.

# 3. RESULTS

## 3.1. Separation of amino acids

The gas-liquid chromatographic method for the analysis of amino acids, as developed by GEHRKE *et al.* (1968), is a dual column system, in which EGA and OV-17 are used as the stationary phases. Sixteen of the protein amino acids can be chromatographed on an EGA-column, while for the analysis of arginine, histidine, tryptophan and cystine an OV-17 column is needed. In the present study only the EGA-column was used.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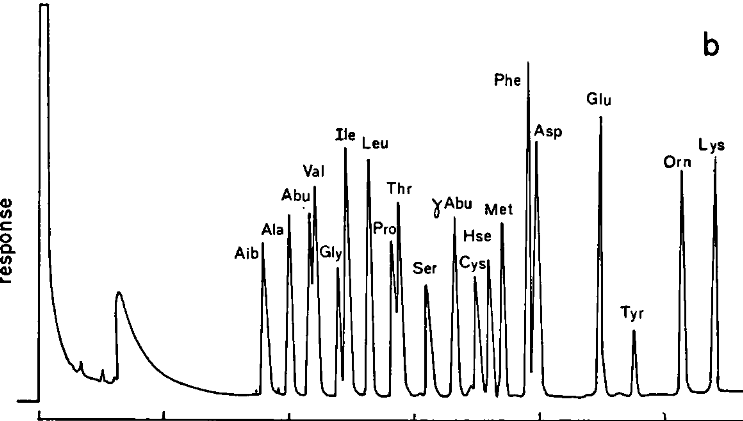
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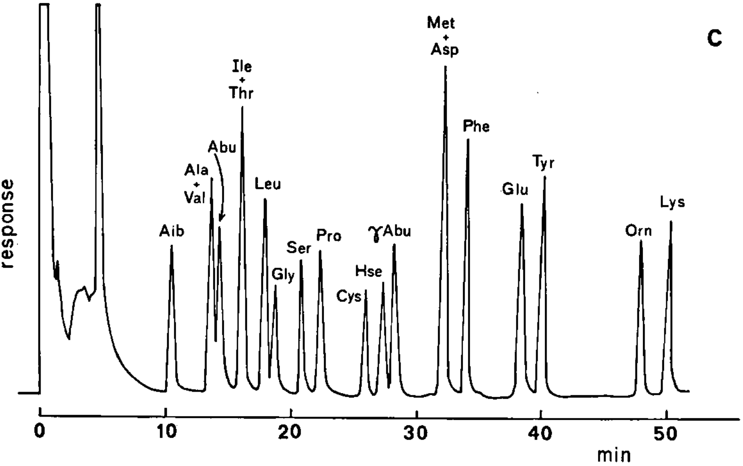
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According to ROACH & GEHRKE (1969a) a column containing 0.65% (w/w) EGA coated on dried, A.W. Chromosorb W, 80–100 mesh, gives excellent separation. This holds, at least, for the protein amino acids. When this column is used for the analysis of free amino acids from higher plants, separation problems can be encountered, owing to the presence of non-protein amino acids. A possible solution is then to change the stationary phase content of the column packing, because the retention times for the various amino acids are differently affected by changes of the stationary phase content (STEFANOVIC & WALKER 1967).

In the present investigation, the following columns were used:
column 1: 0.325% (w/w)EGA on A.W. Chromosorb W, 80–100 mesh.
column 2: 0.65 % (w/w)EGA on A.W. Chromosorb W, 80–100 mesh.
column 3: 0.65 % (w/w)EGA on A.W. Chromosorb W, 80–100 mesh, DMCS-treated.

Typical chromatograms of a standard amino acid mixture obtained with these columns are depicted in *fig. 1*. Raising the stationary phase content from 0.325% to 0.65% resulted in shifts in the peak pattern. As a consequence columns 1 and 2 have different separation characteristics. Column 1 gives better separations of (1) α-aminobutyric acid and valine, (2) glycine and isoleucine, (3) proline and threonine and (4) phenylalanine and aspartic acid, whereas column 2 is better for the separation of (1) valine and glycine, (2) serine and γ-aminobutyric acid and (3) cysteine, methionine and homoserine.

STEFANOVIC & WALKER (1967) have shown that the shifts in the peak pattern mentioned above continue when the liquid load is increased to 2%. From *fig. 1b* and *c* it appears that these shifts also occur, and to a high degree, when DMCS-treated Chromosorb W was used as the support material.

It should be emphasized that the separation characteristics of the EGA-columns, as illustrated in *fig. 1*, only hold for the given conditions of flow rate of the carrier gas, the rate of temperature program, and the amounts of amino acid derivatives injected. Moreover, the peak pattern was slightly influenced by the age of the column.

### 3.2. Relative molar responses of amino acids

Relative molar responses (RMR's) of the amino acids were determined with respect to α-amino-iso-butyric acid. These values were converted to the RMR's with respect to glutamic acid, in order to compare them with the values given by ROACH & GEHRKE (1969b). Moreover, the theoretical RMR's were calculated, assuming that the response in flame ionization detection is directly proportional to the number of ionizable carbon atoms (BAUMANN 1967). The RMR's of 22 amino acids are listed in *table 1*. No significant differences were found whether

Fig. 1. Gas chromatograms of an equimolar standard amino acid mixture. a, b and c represent chromatograms obtained with column 1,2 and 3 respectively. The injected mixture contained about 50 nmoles of each amino acid. Flow rate of carrier gas for a and c 40 ml/min, for b 30 ml/min.

Table 1. Relative molar responses of amino acids with respect to glutamic acid.

| | Column 1[a]) | Column 2[a]) | Column 3[a]) | Roach & Gehrke[b]) (1969b) | Theoretical Value |
|---|---|---|---|---|---|
| Aib | 0.63 | 0.62 | 0.64 | – | 0.64 |
| Ala | 0.54 | 0.53 | 0.58 | 0.52 | 0.55 |
| Abu | 0.65 | 0.63 | 0.64 | – | 0.64 |
| Val | 0.75 | 0.73 | 0.75 | 0.73 | 0.73 |
| Gly | 0.45 | 0.43 | 0.49 | 0.43 | 0.45 |
| Ile | 0.82 | 0.82 | 0.84 | 0.83 | 0.82 |
| Leu | 0.85 | 0.84 | 0.87 | 0.83 | 0.82 |
| Thr | 0.65 | 0.63 | 0.71 | 0.64 | 0.64 |
| Pro | 0.72 | 0.72 | 0.75 | 0.72 | 0.73 |
| Ser | 0.50 | 0.51 | 0.61 | 0.54 | 0.55 |
| γAbu | 0.65 | 0.65 | 0.67 | – | 0.64 |
| Cys | 0.51 | 0.53 | 0.57 | 0.45 | 0.55 |
| Met | 0.70 | 0.70 | 0.73 | 0.56 | 0.73 |
| Hse[c]) | 0.53 | 0.57 | 0.59 | – | 0.64 |
| Phe | 1.13 | 1.16 | 1.18 | 1.14 | 1.09 |
| Asp | 0.91 | 0.92 | 0.91 | 0.91 | 0.91 |
| Asn | 0.88 | 0.88 | 0.94 | – | 0.91 |
| Glu | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Gln | 1.01 | 0.95 | 0.97 | – | 1.00 |
| Tyr | –[d]) | 0.58 | 1.10 | 0.95 | 1.09 |
| Orn | 0.72 | 0.72 | 0.76 | – | 0.73 |
| Lys | 0.84 | 0.82 | 0.86 | 0.86 | 0.82 |

[a]) Each value represents an average of at least 5 independent determinations.
[b]) Columnfilling used by Roach & Gehrke: 0.65% EGA coated on A.W. Chromosorb W, 80–100 mesh.
[c]) The low response of homoserine, as compared with the theoretical RMR, was probably due to impurities.
[d]) Tyrosine was very poorly eluted from column 1; the response varied from 0.10 to 0.35, depending on the age of the column.

they were determined with column 1 or column 2, except for tyrosine. In general, the values agree with the theoretical values, and with the values given by ROACH & GEHRKE (1969b). There are some inconsistencies. In contrast to the results of Roach & Gehrke, the RMR's found for cysteine and methionine were closer to the theoretical values, and for tyrosine a good response was only found in column 3.

The RMR's obtained with column 3 were in general higher than those found with columns 1 and 2. This was especially so for glycine, alanine, serine, threonine and cysteine.

### 3.3. Free amino acids from S. polyrhiza

Qualitative analysis of free amino acids from *S. polyrhiza* by means of gas-liquid chromatography was checked by thin-layer chromatography. Thus the amino acids listed in *table 2* have been identified by both methods. *Fig. 2* shows typical gas chromatograms of the free amino acids from a normal culture and from a culture after growth inhibition with valine.

Table 2. Changes in the contents of free amino acids in S. polyrhiza after growth inhibition with L-valine, L-isoleucine or L-leucine. Column 1 and 2 were used for gas chromatographic analysis of the amino acids. Amino acid contents are expressed as nmoles per mg dry weight.

| | control | L-valine | | L-isoleucine | | L-leucine | |
|---|---|---|---|---|---|---|---|
| | number of days after addition of the amino acid | | | | | | |
| | 0 | 1 | 3 | 1 | 3 | 1 | 3 |
| Ala | 9.4 | 13.6 | 14.3 | 13.0 | 11.2 | 14.3 | 17.3 |
| Abu | 0.0 | 6.1 | 14.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| Val | 3.0 | 64.9 | 72.4 | 9.4 | 12.0 | 0.7 | 0.5 |
| Gly | 2.3 | 2.6 | 3.7 | 3.0 | 1.8 | 2.8 | 2.6 |
| Ile | 0.7 | 0.1 | 0.3 | 70.9 | 63.4 | 0.4 | 0.5 |
| Leu | 0.7 | 16.7 | 19.0 | 0.3 | 0.5 | 66.6 | 82.7 |
| Pro | 0.6 | 1.4 | 1.8 | 0.5 | 1.1 | 0.5 | 2.3 |
| Thr | 2.3 | 3.9 | 3.0 | 4.3 | 3.6 | 4.1 | 5.6 |
| Ser | 7.3 | 15.9 | 19.4 | 8.8 | 9.0 | 12.0 | 14.3 |
| γAbu | 7.3 | 8.8 | 10.4 | 7.4 | 11.2 | 5.7 | 14.5 |
| Hse | 0.2 | 1.4 | 1.5 | 0.5 | 0.5 | 0.4 | 0.9 |
| Asp + Asn | 43.9 | 69.5 | 125.9 | 42.9 | 52.3 | 83.2 | 142.7 |
| Glu + Gln | 44.0 | 93.5 | 96.6 | 64.4 | 49.9 | 97.3 | 111.8 |
| Orn | 1.6 | 1.5 | 3.4 | 0.8 | 1.3 | 1.3 | 2.7 |
| Lys | 2.2 | 2.0 | 3.2 | 1.7 | 1.9 | 1.6 | 2.9 |

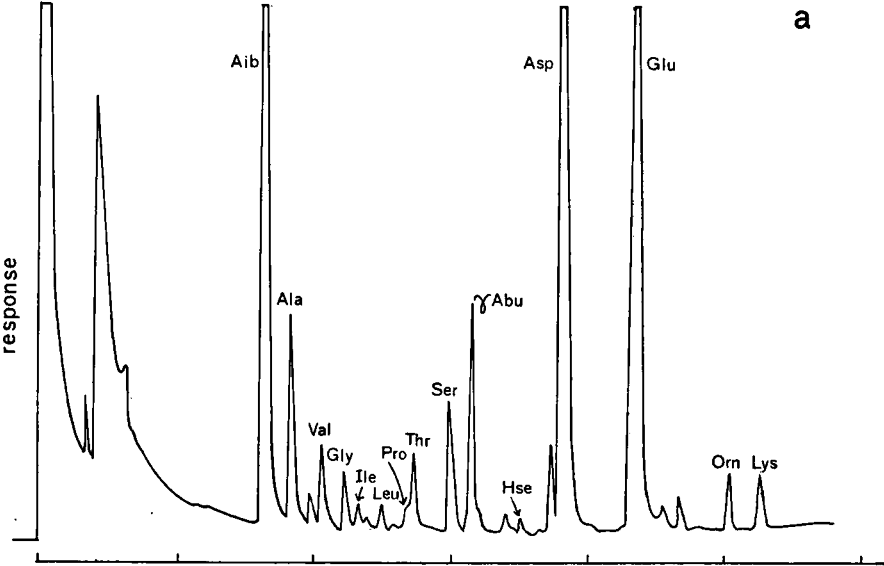
The identity of the peaks in the gas chromatograms was determined on the basis of co-chromatography with reference compounds. Due to slight variations in the retention times of the amino acid derivatives (see above), this was the only reliable method.

The presence of asparagine and glutamine was detected by thin-layer chromatography. These compounds are converted to their corresponding amino acids during the esterification in butanol-HCl. Retention times and RMR's of the amides were found to be identical with those of the corresponding amino acids.

### 3.4. Changes in amino acid contents after growth inhibition with L-valine, L-isoleucine or L-leucine

The growth of *S. polyrhiza* can be inhibited by each of the branched-chain amino acids. *Fig. 3* shows the course of growth after addition of the amino acids. With the concentrations used, valine and leucine yielded an almost complete arrestment of growth, while isoleucine only partly reduced the growth rate. The amounts of free amino acids were measured at various times after addition of the growth inhibiting amino acid. The results are given in *table 2*.

The contents of the amino acids alanine, threonine, proline, serine, γ-aminobutyric acid, aspartic acid plus asparagine and glutamic acid plus glutamine invariably increase during the growth inhibition. The increases were higher according as the growth inhibitions were stronger. When growth was inhibited with valine there was a strong accumulation of α-aminobutyric acid and leucine, whereas the isoleucine content diminished. During growth inhibition by isoleu-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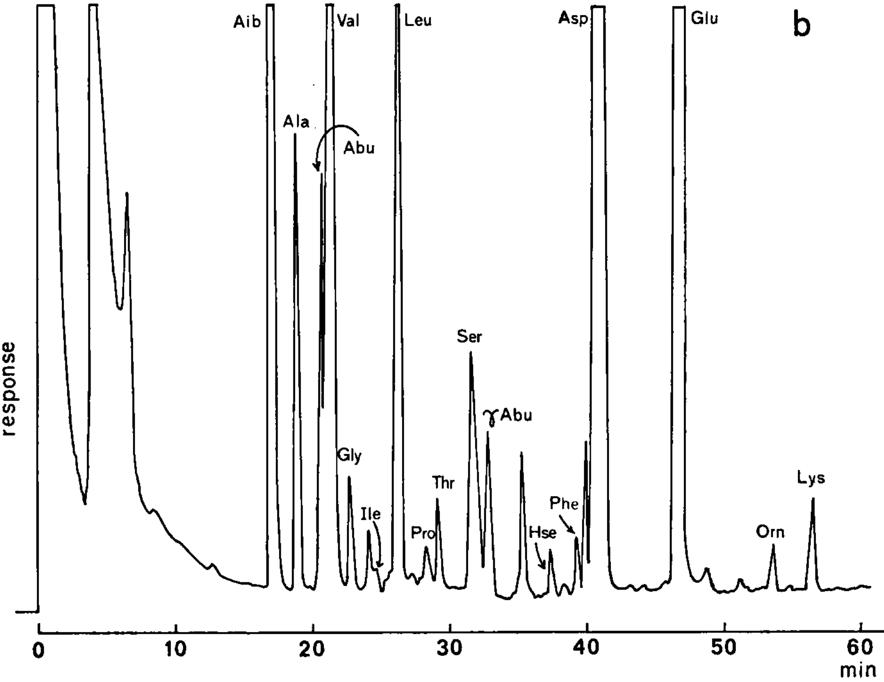
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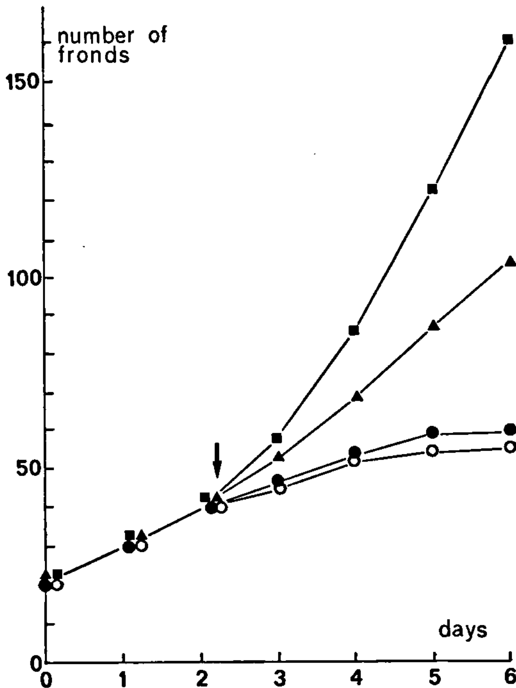


Fig. 3. Effect of addition of a branched-chain amino acid on the course of growth of *S. polyrhiza* cultures. Four groups of three cultures were grown. The points represent average values for each group. To one group (—■—) no amino acid was added. To the other three groups L-isoleucine (—▲—), L-valine (—●—) and L-leucine (—○—) were added to the medium to a final concentration of $10^{-4}$M at the time indicated by the arrow.

cine there was a marked increase in the valine content, while the leucine content slightly decreased. Growth inhibition by leucine was attended by a decrease in the valine content and isoleucine content.

### 3.5. Identification of α-aminobutyric acid

As will be discussed, the observed accumulation of α-aminobutyric acid offers a reasonable explanation for the way in which valine inhibits the growth of *S. polyrhiza*. Up to now the occurrence of this amino acid in some higher plants has been indicated on the basis of paper chromatographic evidence only (see e.g.: DENT, STEPKA & STEWARD 1947; VIRTANEN & MIETTINEN 1953). In the present study special attention has therefore been given to the identification of this compound.

The presence of α-aminobutyric acid in *S. polyrhiza* after growth inhibition with L-valine has been revealed by thin-layer chromatography and ion-exchange chromatography, and by gasliquid chromatography with column 1 and 3. The amino acid always co-chromatographed with the reference compound. During the gas chromatographic separation of the N-TFA *n*-butyl esters of the amino

Fig. 2. Gas chromatograms of free amino acids from *S. polyrhiza* cultures. α-Amino-iso-butyric acid was included as an internal standard. a. Culture of 40 fronds grown under standard conditions; column 2. b. Culture grown to 40 fronds, then L-valine added to the medium to a final concentration of $10^{-4}$M and harvested after 1 day; column 1.

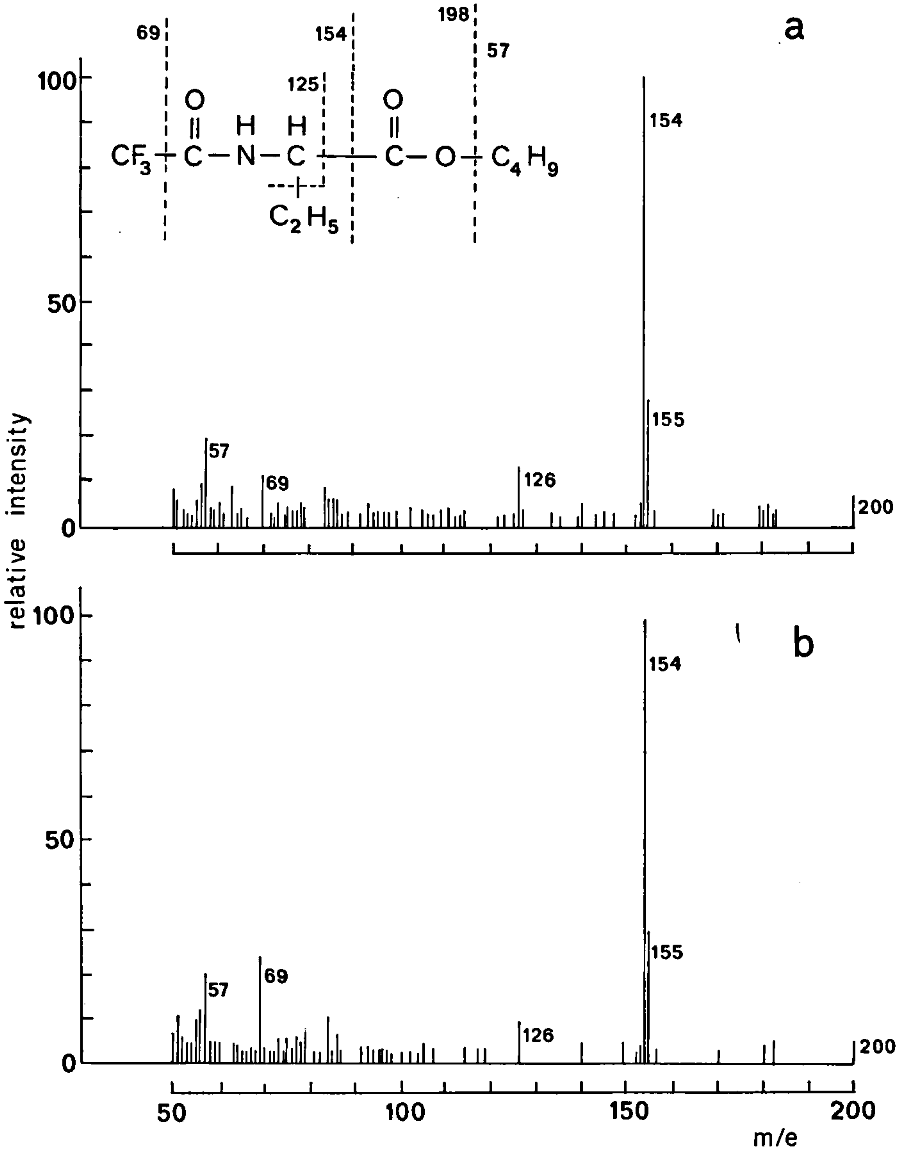


Fig. 4. Mass spectra of N-TFA α-aminobutyric acid n-butyl ester. a. Spectrum of the reference compound. b. Spectrum of the compound prepared from *S. polyrhiza* cultures.

acids the compound supposed to be the derivative of α-aminobutyric acid was isolated. As can be seen in *fig. 4*, the mass spectrum of this compound corresponds with the mass spectrum of the N-TFA *n*-butyl ester of α-aminobutyric acid.

Mass spectrometric data of N-TFA *n*-butyl esters of amino acids have been reported by GELPI *et al.* (1969). According to these authors the most characteristic ion of the derivatives of aliphatic amino acids results from the loss of the esterified carboxylic group with retention of the positive charge on the "amine fragment". This ion is accompanied by another relatively intense ion, one mass unit higher, which is most likely derived from the protonated molecule in an analogous way. In *fig. 4* these ions are represented by peaks at m/e = 154 and m/e = 155 respectively. Other peaks were found at m/e = 57; m/e = 69; m/e = 126 (m/e = 125 + $H^+$) and m/e = 200 (m/e = 198 + $2H^+$).

## 4. DISCUSSION

In the isoleucine pathway, acetohydroxy acid synthetase condenses α-ketobutyrate with active acetaldehyde to form α-aceto- α-hydroxybutyrate. This reaction was apparently blocked during growth inhibition of *S. polyrhiza* with valine, for appreciable amounts of α-aminobutyrate (the amination product of α-ketobutyrate) had accumulated in the plants. The same phenomenon has been found with *Escherichia coli* K-12: α-aminobutyrate appeared in the culture fluid when the bacterium was incubated in the presence of valine. This has been taken as further evidence for the view that in *E. coli* K-12 valine inhibits growth by preventing the formation of α-aceto- α-hydroxybutyrate (LEAVITT & UMBARGER 1962). The accumulation of α-aminobutyric acid in *S. polyrhiza* observed is therefore of crucial importance. It justifies the conclusion that valine inhibits growth by blocking the isoleucine synthesis, in spite of the fact that the isoleucine pool was not completely exhausted.

Although acetohydroxy acid synthetase is blocked during growth inhibition with valine, large amounts of leucine were found in the plants. There is good reason to suspect that this leucine was formed from the exogenously supplied valine, as $^{14}C$-valine, fed to the duckweed, was found to be converted to $^{14}C$-leucine (Borstlap, manuscript in preparation).

The decrease in the contents of both valine and isoleucine during inhibition with leucine indicates that excess of leucine blocks a common enzymatic step of the valine-isoleucine pathway. MIFLIN (1969) has demonstrated that acetohydroxy acid synthetase from barley seedlings is inhibited by leucine. If this enzyme is blocked in *S. polyrhiza* during growth inhibition by leucine, threonine deaminase must be blocked as well since α-aminobutyrate did not accumulate.

When the growth of *S. polyrhiza* was inhibited with isoleucine, the leucine content slightly decreased, but there was a conspicuous increase in the valine content. Obviously, excess of isoleucine does not inhibit a reaction of the valine-isoleucine pathway, but merely a reaction of the leucine synthesis. Growth inhibition would then be caused by shortage of leucine.

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