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GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE AMINO ACIDS IN FERMENTATION BROTH DURING GROWTH OF *CLOSTRIDIUM ONCOLYTICUM* M 55

G. GAMERITH*,*
Institut für Med. Biologie und Humangenetik, Universität Graz, Harrachgasse 21/8, A 8010 Graz (Austria)
and
G. F. X. ZUDER, A. GIULIANI and H. BRANTNER
Hygiene-Institut, Universität Graz, Universitätsplatz 4, A 8010 Graz (Austria)
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SUMMARY

The amino acid concentration profiles in the fermentation broth during the germination, growth and sporulation of *Clostridium oncolyticum* were investigated. Amino acids were isolated after deproteinization by cation exchange and derivatized to their N(O,S)-trifluoroacetyl *n*-propyl esters. The analytical method allowed the separation of more than 30 derivatives within 10 min on a glass column filled with 0.65% ethylene glycol adipate on Chromosorb W AW 80–100 mesh.

C. oncolyticum was cultivated anaerobically on a complex medium, in which sixteen amino acids were detected. Significant changes in the concentration of several amino acids were observed during bacterial growth.

Metabolization of Phe, Leu, Tyr, Pro, Met and Trp begins either in the exponential phase or with the onset of sporulation. Glx and Lys concentrations increase during the fermentation, and α-aminobutyric acid, γ-aminobutyric acid and a little pipecolic acid are excreted in particular during the sporulation phase. Ornithine concentration increases before the exponential phase begins, but then, during the exponential growth, the concentration decreases. The concentrations of the other amino acids remain constant. With other cultivation conditions different concentration profiles were observed.

Our results indicate that Phe and Leu may represent amino acids that are metabolized by the Stickland reaction, an important energy-yielding metabolic path of anaerobic proteolytes. Furthermore, a synthetic medium for *C. oncolyticum* may contain Phe and Leu as the sole sources of energy and nitrogen.

* Present address: Institut für Biotechnologie, Mikrobiologie und Abfalltechnologie, Technische Universität Graz, Technikerstrasse 4, A 8010 Graz, Austria.

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INTRODUCTION

Gas–liquid chromatography (GLC) is a convenient method for the analysis of amino acids, particularly when large numbers of samples are involved. The method is highly sensitive, fast and inexpensive, but requires the preparation of volatile derivatives prior to analysis.

Many investigations have dealt with the preparation of amino acid derivatives suitable for GLC determination[1,2]. Although a single-step method to prepare cyclic derivatives[3] has recently been described, methods involving esterification of the carboxylic groups and subsequent acylation of the other functional groups have gained most attention to date[4–9]. To maintain the advantages of GLC over other routine methods for amino acid analysis, a simple, inexpensive and rapid derivatization technique must be employed. A method that fulfills these requirements better than those previously described, has been developed by Gamerith[10].

This paper describes the application of this method to biological samples. The purpose of these investigations was the developement of a synthetic medium required for further investigations on the metabolism and the oncolytic properties of *C. oncolyticum* M 55[11].

Although the overall pathways of amino acid biosynthesis and degradation in bacteria are reasonably well known, little is known about the amino acid metabolism of *Clostridia* species[12].

Amino acids have been shown to facilitate germination either alone, as with various bacillus species[13], or together with other germinants. Germination of *Clostridium* spores may be triggered by alanine[14], methionine[15] or cysteine[16]. The role of the amino acid during germination is not clear, although it does not seem to be metabolized.

Almost nothing is known about changes in the amino acid metabolism during the growth cycle of *Clostridia*, although alterations in enzyme activities prior to sporulation have been demonstrated[17].

In a first set of experiments, the results of which are presented here, we examined the concentrations of the free amino acids in the fermentation broth during the batch fermentation of this species. The free amino acids were isolated from the fermentation broth by cation exchange and derivatized to their TFA *n*-propyl esters. Separation was achieved on 0.65% ethylene glycol adipate (EGA) on Chromosorb W AW.

Significant changes in the amino acid concentrations were detected in the medium. The concentration profiles correspond well with the cell number, and changes can be correlated with the different growth phases.

The results will be used in the development of a synthetic medium, and provide important information on the metabolism and, therefore, on the oncolytic effects of this species.

MATERIALS AND METHODS

Acetyl chloride, *n*-propanol, ethyl acetate, ammonia and acetic acid were obtained from Merck (Darmstadt, F.R.G.) and were of analytical grade. Methylene chloride, gold label quality, was from Aldrich (Beerse, Belgium) and trifluoroacetic

anhydride (TFAA), reagent grade, from Pierce (Rotterdam, The Netherlands). Pipecolic acid, 3-methylhistidine and cystathionine, all A-grade, and α-amino caprylic acid, B-grade, were obtained from Calbiochem (San Diego, CA, U.S.A.). Phosphoethanolamine was purchased from Fluka (Buchs, Switzerland) and the other, chromatographically pure, amino acids from Serva (Heidelberg, F.R.G.). The cation-exchange resin Dowex 50 W X 8, 100–200 mesh, was obtained from Biorad (Vienna, Austria). Chromatographic materials, including the stock packing of ethylene glycol adipate (EGA), were purchased from Supelco (Crans, Switzerland).

Gas chromatography was performed using a Hewlett-Packard gas chromatograph HP 5880A equipped with flame ionization detectors and an automatic sampler HP 7672A.

For derivatization, 100 × 16 mm I.D. glass tubes with PTFE-lined screw caps were used. Tubes were heated in an aluminium block heater (Pierce). An adaptor allowed the simultaneous evaporation of ten tubes on the rotavapor.

Cultivation of C. oncolyticum M 55

The cells were cultivated anaerobically in a complex medium containing 4% caseine peptone, 0.5% meat extract and 0.5% sodium chloride at 37°C. The methods for cultivation and spore preparations are published elsewhere[18].

Sample preparation

At various times during the growth cycle, samples were taken and, after the optical density had been measured, the cells removed by centrifugation. Then an appropriate volume of internal standard, usually 100 μl of a 2.5 m*M* α-aminocaprylic acid solution, was added to 200 μl of the sample. Proteins in the solution were then precipitated with 50 mg/ml of sulphosalicylic acid and the amino acids were isolated by a cation-exchange method similar to that described by Adams[5]. About 0.2 g of the exchange resin in the hydrogen form was placed in a Pasteur pipette above a plug of glass wool. The sample was allowed to pass slowly through the column, which resulted in the adsorption of the amino acids on the resin. Then the resin was washed with 2 *M* acetic acid and deionized water. The amino acids were then eluted with 1 ml of 4 *M* ammonia and 2 ml of deionized water, which were allowed to drip into the reaction tubes. The resin was regenerated with 1 *M* hydrochloric acid after each run. The solution containing the amino acids was evaporated first with dry air and then on the rotavapor until completely dry.

Derivatization

The amino acids were esterified with 400 μl of 3.5 *M* acetyl chloride in *n*-propanol by heating the tubes to 110°C for 25 min. After cooling to room temperature, the excess reagent was evaporated at 40°C on the rotavapor. Acylation was achieved by adding 600 μl of 70% TFAA in methylene chloride and heating to 150°C for 5 min. The tubes were again cooled to room temperature and evaporated at 30°C on the rotavapor. To the dry residue, 1 ml of ethyl acetate was added, and 3 μl of the solution were used for injection.

A detailed description of the derivatization procedure has been published previously[19].

Chromatography

The samples were analysed on a 2 m × 2 mm I.D. glass column filled with 0.65% EGA coated on Chromosorb W AW, 80–100 mesh. The injector temperature was 250°C and the detector temperature was set to 300°C. The carrier gas was nitrogen with a flow-rate of 20 ml/min. The flame ionization detector was supplied with 450 ml/min of air, and 30 ml/min of hydrogen. The chart speed was 1 cm/min and the attenuation usually 32. The oven temperature was initially 115°C for 1 min, was then increased by 5°C/min to 120°C and then by 20°C/min to 220°C, and finally held at this temperature for 10 min. Quantitation was performed using internal standardization with α-aminocaprylic acid. A calibration mixture was included in each batch of ten, to compensate for response fluctuations of a few amino acids caused by the derivatization procedure.

RESULTS

Chromatography

The chromatogram of a standard solution is shown in Fig. 1. There, separation of 28 compounds is achieved within 10 min. Additionally, the chromatographic data of the following compounds are known: phosphoethanolamine (PEA), pipecolic acid (Pip), hydroxyproline (Hyp), citrulline (Cit), dipicolinic acid (DPA), 3-methylhisti-

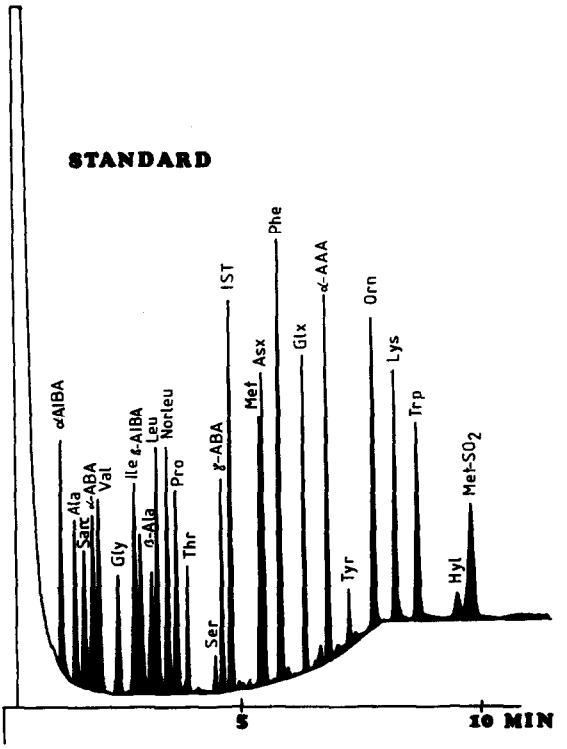

Fig. 1. Chromatogram of an amino acid standard solution. The chromatographic setpoints are given in the text. The peaks were obtained by injecting a solution containing 0.25 nmol/μl of each amino acid, except Hyl (0.14 nmol/μl).

TABLE I

REPRODUCIBILITY WITH BIOLOGICAL SAMPLES

Each value is the mean of 30 determinations, obtained from the experiment described in Fig. 4.

Amino acid	*Mean C.V. (%)*	*Amino acid*	*Mean C. V. (%)*
Ala	9.4	γ-ABA	4.2
α-ABA	8.4	Met	3.9
Val	8.1	Asx	13.9
Gly	7.7	Phe	6.8
Ile	8.7	Glx	13.7
Leu	4.5	Tyr	13.7
Pip	13.5	Orn	18.1
Pro	12.2	Lys	4.3
Thr	18.3	Trp	11.6
Ser	20.6		

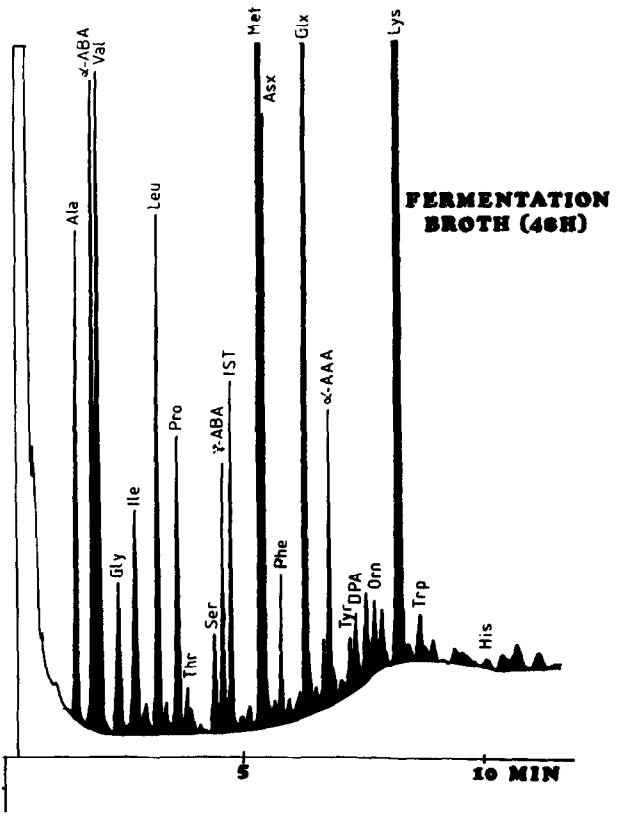

Fig. 2. Chromatogram of a sample obtained after 48 h of cultivation. The same chromatographic parameters as in Fig. 1 were employed. The α-aminoadipic acid was added as second internal standard. The peaks, however, were only quantified with α-aminocaprylic acid.

dine, 1-methylhistidine diaminopimelic acid (DAPA), histidine, cystathionine (CTT) and homocystine (HCys). CTT and HCys are eluted only with a final oven temperature of 240°C. Not all these amino acids are well separated from those included in the calibration mixture. Pipecolic acid elutes at the position of norleucine (the reason why another internal standard has to be used), hydroxyproline coelutes with aspartic acid and the separation of DAPA, 1-methylhistidine and methionine sulphone (Met-SO_2) is not complete. However, accurate quantification of 35 compounds in a single 13-min run (if the higher final oven temperature is used) is potentially possible.

Separation capability changes with the age of the column. With an older column, separation of Ile from β-aminoisobutyric acid (β-AIBA) and of Pro from Thr becomes poorer, but Met is completely separated from Asx and Asx is partially separated from Hyp, which allows the additional determination of Hyp. The retention time of DPA increases with an older column; thus, coelution with tyrosine may occur.

Standard deviations with standard solutions exceed 2% only with a few amino acids, in particular the hydroxylated amino acids[20]. With biological samples, the reproducibility decreases as a result of the sample clean-up procedure. The mean coefficients of variation may increase by up to 20% and are listed in Table I. During these investigations, it became clear that the cation-exchange is crucial with respect to reproducibility.

Fig. 2 represents the chromatogram obtained from a fermentation broth.

Concentration limits for quantitation range from 1 to 5 mg/l for most amino acids. The linearity of the response relative to the internal standard was demonstrated with DAPA. The absolute response as well as the relative response, which is shown in Fig. 3, is linear over more than a 100-fold concentration range.

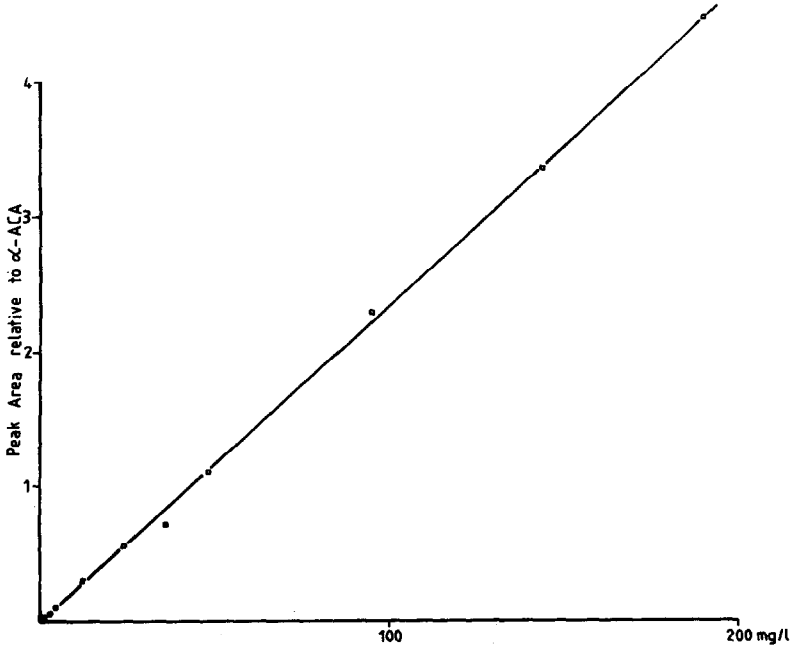

Fig. 3. Response of diaminopimelic acid relative to α-aminocaprylic acid.

Free amino acids in the fermentation broth

The initial concentrations of the amino acids of the medium usually employed for cultivation are given in Table II. Because the behaviour of Ser and Tyr in the standard differs from that in the biological samples, no absolute concentrations of these two amino acids are given. Conclusions for these amino acids are based on relative values only.

During bacterial growth, the concentrations of several amino acids change significantly. The amino acids can be assigned to a few groups by their concentration profiles. The concentration of one group remains almost constant over the whole fermentation time. To this group belong Ala, Val, Gly, Ile, Asx, His and probably Arg.

The concentrations of another group decrease during the fermentation. To this group of the metabolized amino acids belong Phe, Leu, Pro, Ser, Tyr, Met and Trp. The last group includes the amino acids Glx and Lys, the concentrations of which increase and α- and γ-aminobutyric acid (γ-ABA), which are initially not present in the medium, but are excreted in considerable quantities, particularly during the stationary phase and the sporulation. Also, small amounts of pipecolic acid are present in the broth after 48 h of cultivation. The other amino acids that can be determined by this method, such as CTT and HCys, were not present in detectable amounts. Although we did not run a complete series on a silicone phase, there is evidence from some runs on 2% OV 17–1% OV 210, that Arg concentrations do not change significantly with the cultivation method employed.

The concentration profiles of several amino acids are shown in Fig. 4a and b. The values are the means of six determinations of samples obtained from two parallel cultures, each sample having been analysed in triplicate.

In addition, the course of the optical density is plotted to show the correlation

TABLE II

FREE AMINO ACIDS IN THE CULTURE MEDIUM

The values are the mean initial concentrations of six experiments. The standard deviations are probably due to hydrolytic effects during autoclaving of the peptone-containing medium.

Amino acid	*Concentration (mg/l)*	*Standard deviation*
Ala	370.7	64.9
Val	531.0	116.8
Gly	303.0	55.8
Ile	421.6	75.8
Leu	1763.6	328.0
Pro	357.0	70.1
Thr	445.7	100.9
Met	361.1	60.6
Asx	360.5	64.5
Phe	947.6	162.6
Glx	537.2	106.5
Orn	128.3	49.0
Lys	1348.5	321.8
Trp	280.7	81.0
His	114.6	38.4

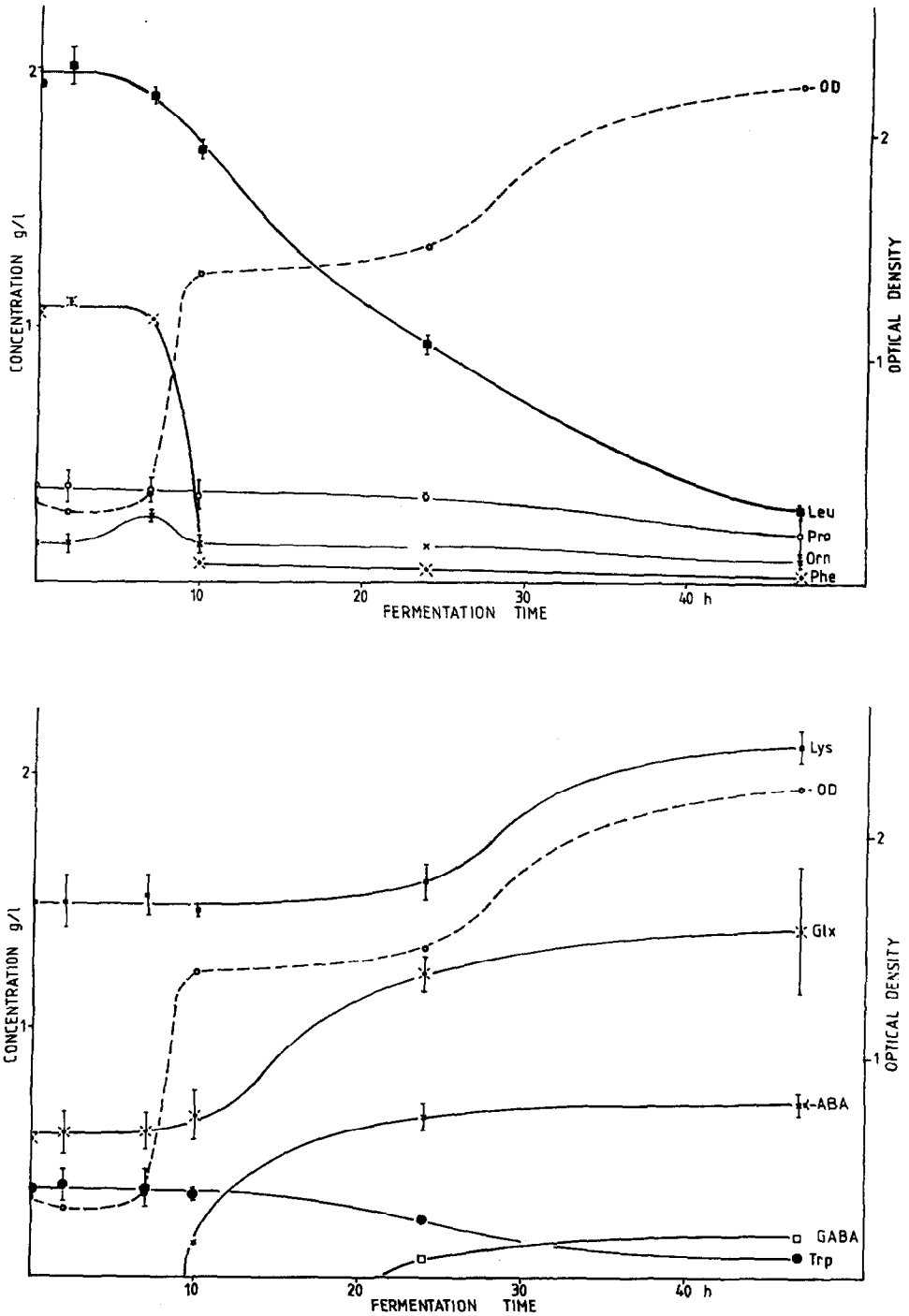

Fig. 4. Concentration profiles of amino acids in the fermentation broth. The growth was observed by measuring the optical density at 580 nm. The bars represent the standard deviation.

TABLE III

AMINO ACID PROFILES WITH CARBON DIOXIDE

Values are given in mg/l.

Amino acid	*Fermentation time (h) and optical density*						
	0 *0.290*	*2* *0.190*	*5* *0.153*	*8* *0.177*	*10* *0.324*	*12* *0.806*	*26* *1.405*
Ala	418.5	428.3	306.3	457.5	393.9	414.2	431.6
α-ABA	0	0	0	0	0	19.9	677.1
Val	584.2	632.5	476	637.7	547.5	600.4	1134.8
Gly	523.6	313.9	307.4	519.4	304.6	347.7	468.8
Leu	1838.0	1846.7	1579.9	1972.7	1697.3	1685.3	1794.4
Pro	460.3	443.5	325.5	517.7	377.5	400.4	478.6
Phe	1104	1117.7	1070.1	1102.7	1102.4	747.2	67.6
Glx	583.8	614.5	470.6	671.5	608.7	617.8	1327.7
Orn	121.6	179.2	157.5	109.5	256.0	330.0	149.9
Lys	1513.4	1456.0	1432.1	1498.0	1601.7	1356.7	2074.0

between the concentration profiles and the bacterial growth. These findings were found to be reproducible not only under exactly the same experimental conditions, but also using another cultivation technique, which included continuous measurement of pH, rH and microcalorimetric control[18].

Only when nitrogen was replaced by carbon dioxide as the gas maintaining an anaerobic environment did changes in the concentration profiles occur. The values are given in Table III.

Preliminary investigations using other nutrient broths established large differences in the final amino acid concentrations after 48 h of cultivation. The only common feature is the decrease in the Phe concentration. This needs to be investigated further.

DISCUSSION

Chromatography

Separation speed is higher than with any other method with packed columns described previously. Other GLC separations usually require at least 15 min[5,10], or even longer[6–9]. Although several liquid chromatographic methods have been described[21–25], none of them allows the separation of as many compounds in less than 15 min. Simultaneous derivatization of ten samples is easily performed, resulting in high time efficiency. Sensitivity is somewhat less than can be achieved by high-performance liquid chromatographic methods with fluorescence detection, which allow detection is subpicomole amounts[26]. In many cases, however, a sensitivity allowing quantitation in the ppm range is sufficient for biological investigations. Further, the sensitivity can be enhanced by nitrogen-selective detection[10,27].

The arginine derivative does not elute from this column, but can be determined on silicone phases[10]. So far, the TFA *n*-propyl ester of cysteine or cystine cannot be determined. Derivatization of cysteic acid, however, results in the appearance of a small peak very early in the chromatogram, which is probably due to degradation to

dehydroalanine, as has been demonstrated with the HFB isobutyl ester of this amino acid[28].

Perhaps the removal of the acylating reagent prior to injection prohibits cysteine or cystine determination, since this accounts for partial loss of an acyl group from arginine and histidine, and probably also from serine, tyrosine and citrulline. In spite of these effects, evaporation of the anhydride is considered to be advantageous in most cases, because the baseline becomes much better.

Elution of the histidine derivative with its low response on this column was verified by loading experiments.

The increase of the baseline caused by column bleeding may be electronically corrected.

Asparagine and glutamine are converted into the corresponding dicarboxylic acids during the esterification, and the amides cannot be determined by any method that includes acid-catalysed esterification.

The decrease in the reproducibility in the analysis of biological samples was caused mainly by the cation-exchange step. Serine, threonine and tyrosine were particularly affected, as were glutamic and aspartic acid, though to a lesser extent. The sample clean-up procedure by cation exchange should be more carefully controlled or even replaced by another method, *e.g.* that described by Husek *et al.*[29]. Furthermore, the cation exchange results in an almost complete loss of dipicolinic acid[30], an important compound in the sporulation of bacteria.

Free amino acids during fermentation

Proteolytic micro-organisms are capable of obtaining their energy from amino acids by coupling the oxidation of one amino acid with the reduction of another amino acid, a process known as the Stickland reaction. The amino acids that are preferentially reduced are Gly, Pro, Hyp and Orn, as well as a few others to a lesser extent. Most other amino acids may be oxidized, and there is evidence that a specific combination of amino acids being reduced and oxidized exists in a given species. Recently, the participation of Phe as electron acceptor and Leu or another amino acid as electron donor has been demonstrated in the metabolism of various *Clostridia* species[31].

The findings described in this study therefore constitute evidence that, when grown on a medium containing only amino acids and peptides as carbon and nitrogen sources, Phe and Leu are the participants in the Stickland reaction necessary for energy production in *C. oncolyticum*. The less rapid decrease of the Leu concentration compared with that of Phe may be explained by assuming the reaction mechanism proposed by Bader[31], in which two amino acid molecules (Phe) must be reduced for the oxidation of one molecule of electron donor (Leu). Of course, transport mechanisms of amino acids through the cell membrane should also be considered when explaining the changes of nutrient amino acid concentrations. In preliminary experiments with other media, the same rapid decrease in Phe concentration was always observed, but Leu may be replaced by a few other amino acids. Also, when carbon dioxide was used instead of nitrogen to maintain the anaerobic environment, Leu was not metabolized during vegetative growth. The amino acid used as the electron donor may therefore depend on the environmental conditions.

Synthetic media have been prepared for several *Clostridium* species[32,33], but

not for *C. oncolyticum*. Until now, amino acids have not been used as the single carbon and nitrogen source, but only for triggering the germination of the spores. From our study, we expect that *C. oncolyticum* will grow on a synthetic medium containing Phe and Leu as the sole carbon and nitrogen source. Leu may probably be replaced by some other amino acids.

From previous investigations it is known that single amino acids in a minimal medium do not enable the growth of *C. oncolyticum*, whereas supplementation of the medium with several dipeptides, in particular glycyl-phenylalanine, resulted in a low, but significant increase in the optical density[34]. This fits well with the findings presented in this paper, and there is no doubt that a synthetic medium for this species will have to contain Phe and a second amino acid as energy and nitrogen source. The development of a synthetic medium is an important step in the investigations of the metabolic and oncolytic properties of this species.

Comparison of the amino acid concentration profiles and the optical density reveals a strong correlation between the amino acid concentrations and the growth cycle. No doubt, the rapid decrease of Phe during the exponential phase points to this amino acid as a growth-limiting factor inducing the stationary phase and, perhaps, also sporulation[35].

Changes in the intracellular amino acid concentrations have been demonstrated during sporulation of *Bacillus licheniformis*[36], and differences in the activities of enzymes involved in the amino acid metabolism between vegetative and sporulating cells of *Clostridium pasteurianum* have also been reported[17]. From our observations of the fermentation broth, there is strong evidence for significant changes in the amino acid metabolism during the growth cycle of this proteolytic bacterium. In general, some amino acids are metabolized, particularly during the exponential phase, but Trp, Pro, Ser and Thr do not seem to be metabolized before sporulation begins. Some others, which represent metabolic end-products under the conditions used, are excreted into the medium. The aminobutyric acids are not excreted before the stationary phase. Orn accumulates before the exponential phase, but an uptake must occur during exponential growth, since the concentration in the medium decreases. The excretion of some amino acids may be explained by interconversion of amino acids, as demonstrated with *Clostridium sporogenes*, which can convert Leu into Val[37]. It will, however, be necessary to investigate the intracellular concentrations before drawing any reliable conclusions about metabolic changes during the growth cycle. Then, the role of the amino acids during germination may also be evaluated.

Using various media, or carbon dioxide instead of nitrogen for anaerobic environment, we found different amino acid concentration profiles. Only the rapid Phe decrease was always observed. This points to a high metabolic versatility of this micro-organism, and various regulation processes induced by different initial amino acid concentrations may be involved. With *C. sporogenes*, the inhibitory effect of Pro, Tyr or pyruvate on the glycine reductase has been demonstrated[38]. Furthermore, our results confirm the statement made by Turton *et al.*[39], that standardization of the medium and environmental conditions is a necessity when identifying anaerobic bacteria by their fermentation products.

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