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CHANGES IN THE AMINO ACID COMPOSITION OF TOBACCO CELLS IN SUSPENSION CULTURE*

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Abstract—Changes in the content of free and protein amino acids of tobacco cells in suspension culture during the growth were determined. The principal free amino acids throughout the culture period were glutamine, asparagine, and γ-aminobutyric acid. Glutamic acid, glycine, and lysine decreased in the lag period, followed by the increase in the log period, and then decreased towards the stationary phase. By contrast alanine, serine, valine, leucine, and proline increased in the lag period, followed by the decrease in the log period. The contents of glutamic acid and hydroxyproline in the protein fraction changed markedly during the growth. The glutamic acid content increased in the early period of the log phase and then gradually decreased whereas the hydroxyproline content decreased in the log period and then abruptly increased.

INTRODUCTION

SEVERAL investigators have examined the amino acid composition of cultured plant tissues such as *Kalanchoe*,[1] Jack bean,[1] *Haplopappus*,[1] papaya,[1] Jerusalem artichoke,[2] and ginkgo[3] from various points of view. In studies on nitrogen metabolism of potato and carrot tissues, Steward *et al.* reported that rapidly proliferating tissues had a much lower content of free amino acids and amides than non-growing tissues.[4] Krikorian and Steward[1] showed that cultured peanut cells grown in the light had slightly more free amino acids that those grown in the dark, although no difference was observed in the amount of alcohol-insoluble nitrogen. They also showed that the glutamine content was greater in the light than in the dark, whereas the asparagine content was greater in the dark than in the light.

In green leaves of growing plants, considerable variations of the free amino acid content with leaf age have been reported and the content is usually higher in young leaves which are growing rapidly than older, non-growing ones.[5] However, in plant cell cultures the variation patterns of the amino acid content during the culture period have not been elucidated. In order to see the overall relationship of protein biosynthesis, cell division and cell enlargement to the biosynthetic activities of individual amino acids in plant cells, a preliminary investigation on the changes in the amino acid composition of tobacco cells in suspension culture during the growth were carried out and this paper deals with the results.

RESULTS

The growth rate of tobacco cells in suspension culture is shown in Fig. 1. The period of rapid growth is preceded by a 2-day lag period. The stationary phase is observed after

* Part I in a projected series "Nitrogen metabolism in plant tissue culture".

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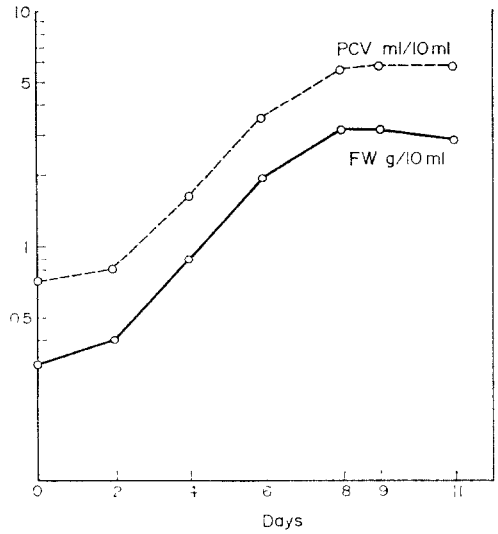

FIG. 1. GROWTH CURVES OF TOBACCO CELLS GROWN IN SHAKE CULTURE.
PCV: packed cell volume, FW.: fresh weight.

8 days. During the growth period, the fresh weight of the cells increased up to about 10 times the original value.

The free amino acid pool of the cells at 2-day intervals of growth is shown in Table 1. The total free amino acid content appeared to decrease a little during the lag period, then increased rapidly in the log period until day 6. The principal free amino acids throughout the culture period were glutamine, asparagine and γ-aminobutyric acid, although the contents were fairly variable with the culture age. The content of amides, especially glutamine, was very high during growth. The content of glutamine decreased during the lag period, followed by the increase during the log period up to 23·8 μmoles /g.fr. wt. (equivalent to 7% of dry wt.) at day 6, thereafter decreased rapidly. On the other hand, the asparagine content increased during the lag period, followed by little changes in the log period, and then increased abruptly during the stationary phase. The content of γ-aminobutyric acid increased only in the lag period and a gradual decrease was observed in the latter period.

The contents of glutamic acid, glycine and lysine decreased in the lag period followed by an increase in the log period. By contrast, alanine, serine, valine, leucine and proline increased in the lag period followed by the decrease in the log period. The variations of the other amino acids did not follow any recognisable pattern, except that the contents of threonine, phenylalanine, aspartic acid, histidine, arginine and tyrosine were profoundly elevated during the stationary phase.

The free amino acid content in the medium of the liquid culture of tobacco cells is shown in Table 2. The total free amino acid content in the medium was highest at the latter period of the log phase. Amides were also richest in the medium. Aspartic acid and γ-aminobutyric acid increased in the last period of the culture growth.

The amino acid composition of the alcohol-insoluble fraction (bulk protein) of tobacco cells is shown in Table 3. The total protein amino acid content was highest at the early period of the log phase. The relative contents of glutamic acid and hydroxyproline changed

TABLE 1. AMOUNT OF FREE AMINO ACIDS AND AMIDES DURING GROWTH IN SHAKE CULTURE

Amino acid or amide	Amount of free amino acids and amides after (μmoles/100 g fr.wt.)						
	0 days	2 days	4 days	6 days	8 days	9 days	11 days
Aspartic	13·5	13·7	19·5	20·9	18·4	21·0	36·3
Threonine	29·9	27·2	23·2	21·7	19·8	37·1	61·5
Serine	33·0	50·2	47·4	37·4	26·5	28·6	40·0
Asparagine	116·0	205·0	149·0	232·0	202·0	293·0	730·0
Glutamine	1280·0	985·0	2030·0	2380·0	1400·0	1280·0	1070·0
Proline	4·3	23·8	17·3	1·9	3·1	4·4	4·9
Glutamic	50·0	43·7	62·5	65·5	71·9	70·7	67·3
Glycine	46·3	36·2	49·1	70·6	67·7	55·1	32·4
Alanine	45·1	91·8	69·3	59·8	46·4	53·4	31·4
Valine	11·1	31·8	27·7	23·1	11·8	19·5	21·1
Methionine	3·1	2·5	2·5	4·4	2·3	1·3	1·9
Isoleucine	9·3	6·4	8·6	5·5	5·6	15·5	12·2
Leucine	13·2	25·8	30·3	20·2	9·6	12·5	8·0
Tyrosine	4·3	20·0	3·6	4·7	5·3	8·9	19·2
Phenylalanine	7·3	0·8	4·9	6·8	2·9	12·3	51·5
β-Alanine	9·4	9·5	14·4	12·4	13·3	13·3	9·6
γ-Aminobutyric	100·0	181·0	177·0	134·0	122·0	140·0	76·4
Lysine	45·3	22·2	28·7	41·9	39·8	41·3	46·4
Histidine	12·7	16·8	19·3	16·8	12·0	17·2	29·6
Arginine	6·6	3·3	5·3	9·1	5·1	9·0	17·2
Total	1840	1797	2790	3169	2086	2134	2367

TABLE 2. FREE AMINO ACIDS AND AMIDES IN THE MEDIUM OF LIQUID CULTURE

Amino acid or amide	Amount of free amino acids and amides after (μmoles/1000 ml of medium)						
	0 days	2 days	4 days	6 days	8 days	9 days	11 days
Aspartic	0·7	0·7	2·4	5·1	5·0	4·0	7·5
Threonine	0·3	1·0	1·2	1·1	1·4	2·3	0·5
Serine, Glutamine, Asparagine	12·7	81·9	118·0	150·0	138·0	80·5	46·8
Proline	−	−	+	+	−	−	−
Glutamic	−	2·7	4·1	3·2	2·1	0·5	0·5
Glycine	3·1	1·4	2·6	4·0	10·5	5·1	0·6
Alanine	0·9	1·2	3·1	5·0	15·9	4·9	+
Valine	+	0·7	1·8	2·0	1·8	+	−
Methionine	0·2	0·3	0·5	1·3	1·1	0·5	−
Isoleucine	0·2	1·1	0·7	1·0	1·5	2·2	+
Leucine	0·5	2·1	2·7	3·2	2·2	1·2	+
Tyrosine	−	−	0·3	1·0	1·1	+	+
Phenylalanine	−	−	0·3	1·8	0·5	+	+
β-Alanine	−	−	0·4	0·8	+	+	−
γ-Aminobutyric	0·7	1·2	2·9	9·4	14·2	13·8	18·8
Lysine	+	+	+	2·1	2·3	+	+
Histidine	−	+	+	0·4	+	+	+
Arginine	−	−	+	+	+	−	−
Total	19·3	94·3	141·0	191·4	197·6	115·0	74·7

markedly during the culture growth. The glutamic acid content increased in the early period of the log phase followed by the gradual decrease in the latter period. On the contrary, the hydroxyproline content decreased in the early period of the log phase followed by the abrupt increase in the latter period up to 8% molar ratio of the total protein amino acid content in the 11-day culture. The relative contents of the other amino acids did not show any remarkable change.

TABLE 3. PROTEIN AMINO ACIDS OF TOBACCO CELLS GROWN IN SHAKE CULTURE

Amino acid	Amount of protein amino acids of tobacco cells after (μmoles/100 g fr. wt. tissue)						
	0 days	2 days	4 days	6 days	8 days	9 days	11 days
Aspartic	8·76	9·49	9·40	9·71	8 85	9·03	8·74
Threonine	5·69	5·47	5 47	5·37	5·30	5·33	5·23
Serine	7·84	7·13	7·10	7·26	8·27	7·44	7·70
Glutamic	6 17	9·87	10·27	9·64	8·80	8.38	7·82
Proline	6·17	5·98	5·78	5·67	5·57	6·08	6·09
Glycine	7·95	8·18	8·29	8·18	8·59	7·86	8·13
Alanine	7·65	8·11	8·43	8·21	8·80	7·54	7·27
Cysteine	0·73	0·74	0·34	0·81	0·79	2·06	0 98
Valine	6·65	6·45	6·93	6·48	6·63	6·18	6 40
Methionine	2·07	1·92	1·84	1·85	1·64	1·63	1·35
Isoleucine	5·06	4·83	4·98	4 56	4 72	4·35	4·18
Leucine	8·80	8 58	8·78	8·27	8·16	7·86	7 14
Tyrosine	3·73	3·17	3·17	3·45	3·81	3·98	4·12
Phenylalanine	3·55	3·44	3·65	3·48	3 44	3·32	2·95
Lysine	8·13	7·74	7·24	7·59	6·68	7·82	7 70
Histidine	2·62	2·56	2·26	2·21	2 44	2·29	2·27
Arginine	3·88	4·05	4·00	3·58	3·12	3·51	2·95
Hydroxyproline	4·47	2·19	1·98	3·58	4·29	5 24	8·87
Total	4975	8874	8868	7393	6672	7686	6971

The values are given as a percentage of the content (mole) of each individual amino acid of the total amino acids detected by the analysis.

DISCUSSION

The very high content of amides in the free amino acid pool was conspicuous in the present experiment. Steward *et al.* showed that the nitrogen rich substances such as asparagine, glutamine, and arginine, which were rich in the non-growing cells, were maintained at very much lower levels in the actively growing cells.[4] The high nitrogen basal medium used in our experiment and a better contact of the medium to each suspended cells seem to have resulted in a very high content of amides in tobacco cells.

Asparagine has been reported to increase when plant cells are in a catabolic state and is rich in the dark grown cells comparing with the light grown ones.[1] In the present experiment, the abrupt increase of asparagine content in the stationary phase may be concerned with the degradation of protein.

γ-Aminobutyric acid was found to be present in the next high amount in both the tissue and the medium. The decarboxylation of glutamic acid and the transamination of succinic semialdehyde with an amino acid are two main metabolic ways which lead to the formation

of γ-aminobutyric acid.[6,7] Wickremasinghe *et al.* have shown that there is an accumulation of γ-aminobutyric acid in tissue cultures of rose, bean, sycamore and *Haplopappus* when the air supply is limited.[8] In addition, Naylor and Tolbert have demonstrated a large accumulation of γ-aminobutyric acid in barley leaves in a nitrogen atmosphere in darkness.[9] γ-Aminobutyric acid in green tobacco leaves was reported to be rapidly oxidized to organic acids of tricarboxylic acid cycle both in the light and in the dark.[10] These indicate that γ-aminobutyric acid is an important intermediate through which glutamate is oxidized to organic acid and a suppression of the oxidation may result in the large accumulation of γ-aminobutyric acid.

However, tobacco cells in the present experiment were grown in a 3 l.-flask shake culture and the air would be freely accessible through the usual cotton filter. Since glutamic acid decarboxylase activity was found to be very high in tobacco cells,[11] the activities of the further γ-aminobutyric acid metabolism, whose oxidation to succinic acid and final oxidation through the Krebs cycle, must concern with the pool size of γ-aminobutyric acid.

The higher content of hydroxyproline in the protein fraction of rapidly growing plant cells than in comparable resting or non-growing cells was first shown by Steward *et al.*[4] The cell cultures of *Agave*, *Acer*, *Haplopappus* and tobacco were also shown to contain hydroxyproline in the protein fraction.[1] Moreover, a high hydroxyproline content is characteristic of plant cell wall proteins.[12–14] Recently, Chrispeels has shown that there is a large increase in the protein-bound hydroxyproline content of the wall, when carrot disks are incubated for a long time.[15] Tobacco cells were also found to have a large amount of hydroxyproline in the protein fraction and this amount, as expected, changed markedly during the growth.

EXPERIMENTAL

The Culture System

A plant tissue culture derived from the stem of *Nicotiana tabacum* L. var. 'Bright Yellow' was used. The cells have been reproducibly subcultured in our laboratory for 2 yr. Suspension culture of tobacco cells was grown in the liquid medium of Linsmaier & Skoog inorganic salts[16] supplemented with 2,4-dichlorophenoxyacetic acid (0·2 mg/l.) and thiamine–HCl (0·4 mg/l.). Kinetin and myoinositol were omitted. Sucrose was added at 30 g/l. and the pH was adjusted to 5·8. The cells were grown at 28° in the dark in a 700-ml shake culture. Under these conditions, the cells were white and homogeneous. Fifty-ml samples of this culture were withdrawn aseptically at 2-day intervals.

Growth Assay

The average packed cell volume and fr. wt. of two 10-ml samples were used as the growth assay. Packed cell volume (PCV): Ten ml of the suspension was centrifuged at 2000 *g* for 5 min and the volume of the sedimented cells was expressed as PCV (ml)/10 ml culture.

Fresh weight. The cells were harvested by filtration on nylon cloth and weighed and the weight was expressed as fr. wt. (g)/10 ml culture.

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Amino Acid Analysis

The filtered and weighed cells were ground in a mortar with 70% alcohol and centrifuged. The sediment was further extracted thrice with 70% alcohol and centrifuged again. The extracts were combined and evaporated *in vacuo* to dryness. The residue was dissolved in 0·2 N sodium citrate buffer, pH 2·2. The solution was used for amino acid analysis with a Beckman Model 120C Amino Acid Analyzer. The alcohol-insoluble sediment was hydrolyzed with distilled 6 N HCl at 110° for 24 hr in a sealed test-tube. The hydrolyzate was used for amino acid analysis. Sufficient ethanol was added to the medium sample to give a concentration of 70% alcohol. Glutamine and asparagine in the free amino acids were separated using 0·3 N lithium citrate buffer, pH 2·80. Hydroxyproline in the hydrolyzate of the protein fraction was determined by the method of a physiological fluid analysis.

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