Accumulation of Glutamine by Suspension Cultures of *Symphytum officinale*

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A callus was induced from the veins of a leaf of *Symphytum officinale*, comfrey, on a medium containing the inorganic elements reported by Murashige and Skoog with addition of 3% sucrose, 0.5 mg/liter 2,4-D and 0.3–3.0 mg/liter kinetin.

Suspension cultures of this cell line obtained from the callus were shown to accumulate a large amount of L-glutamine intracellularly. The effect of growth hormones and nutrients on accumulation of the amino acid has been examined in suspension cultures. The most suitable concentrations of 2,4-D and kinetin for glutamine accumulation were 0.3 mg/liter each. The presence of potassium nitrate as a nitrogen source was beneficial for growth and ammonium nitrate stimulated the accumulation of glutamine. High levels of these nitrogen sources in the medium were required for obtaining a high level of glutamine. The concentration of glutamine accumulated reached to approximately 20% of dry cell weight when *S. officinale* was incubated in the medium containing 0.495% of ammonium nitrate and 0.570% of potassium nitrate which corresponded to three times higher levels than those in a Murashige and Skoog’s medium.

Most of the amino acid was found intracellularly but a small amount was excreted into the medium in the later stages of the incubation. Addition of a cationic surfactant, cetyltrimethylammonium bromide, to the cultures caused to increase the amount of the amino acid in the culture filtrate.

The contents of free amino acids in leaves of *S. officinale* were compared with those in the callus. The level of glutamine in the callus was 260 times higher than that in the intact plant.

Glutamine is one of the storage amides in plants as well as asparagine. Steward et al.1,2) reported that the levels of free amino acids and amides in proliferating cells were much lower than those in non-growing cells.

However, during the course of investigations of metabolic products in the cultured plant cells, a large amount of free glutamine was found to accumulate in cultured *Symphytum officinale* callus cells. Its level was much higher than that in an intact plant.

In this report, we describe the cultural conditions for the growth of the cells and for accumulation of glutamine in the suspension culture.

**MATERIALS AND METHODS**

**Cell line.** The cell line used for this study was induced and maintained as follows. In 1970, *Symphytum officinale*, comfrey, was collected at Hiratsuka Agricultural Experimental Station in Japan. A callus was derived from the veins of a leaf of this plant on an agar medium containing the inorganic elements reported by Murashige and Skoog3) with addition of 3% sucrose, 0.5 mg/liter 2,4-D and 0.3–3.0 mg/liter kinetin. After the callus had been maintained on the same medium as above for a year, it was transferred in a liquid medium containing the same components to obtain a fine suspension culture. The cell line in suspension was maintained for three months by weekly transfer of one tenth cultured cells to a fresh medium having the same composition.

**Medium.** The medium used mainly in this experiment was Murashige and Skoog’s,3) containing 30 g of sucrose, 0.5 mg of 2,4-D, and 0.3–3.0 mg of kinetin per liter.
Cultivation. A well-conditioned suspension culture, 7 to 10 days old, was used as the inoculum culture. Five ml of the culture was transferred to 50 ml of the medium in a 250 ml Erlenmeyer flask and it was incubated for 7 to 12 days at 28°C on a 180 rpm-rotary shaker.

Determinations. Cultured cells were harvested by filtration through Miracloth (Chicopee Mills Manufact. Co., Milltown N.J., U.S.A.), After drying at 105°C overnight, the weight of the dried cells was determined. Cell growth was expressed as mg of dry cell weight per ml of the medium.

Glutamine in a culture medium, extracellular glutamine, was assayed by ninhydrin method after separation by paper chromatography employing the following solvent system; n-butanol—acetic acid—water (12: 3: 5, v/v). The spot stained by ninhydrin was excised within 1 hr, and extracted with methanol containing 0.4% of a saturated cupric nitrate solution for 20 min. Glutamine was determined by the optical density of the extract at 506 nm. Total glutamine was assayed as follows. After a cultured whole broth was homogenized with a Waring blender and centrifuged, the glutamine concentration in the supernatant fluid was determined as described above. Intracellular glutamine was indicated by the difference between total and extracellular glutamine.

RESULTS

Callus formation

The callus was induced on the Murashige and Skoog's medium containing 3% sucrose. Table I shows the results of callus formation of S. officinale under different conditions of kinetin and auxins. Combinations of kinetin and IAA as an auxin in the medium gave differentiated tissues instead of callus cells. Callus cells were induced frequently on the medium containing 0.3~3.0 mg of kinetin and 0.5 mg of 2,4-D per liter and the growth of the cells was abundant.

Accumulation of L-glutamine

The callus of S. officinale was able to grow well not only on the agar medium but also in the liquid medium. It was observed that cells grown on both media accumulated a large amount of a ninhydrin positive compound intracellularly. The compound was isolated and identified as L-glutamine by the following methods. The cultured whole broth was homogenized and centrifuged. The supernatant fluid was passed through a column of a strongly acidic cation exchange resin, Diaion SK-1, and after the column was washed with water, L-glutamine was eluted with 0.2 N ammonium hydroxide. Ethanol was added to the concentrate of the eluate to give white crystals of L-glutamine. The mp, Rf values in paper chromatography, [a]D and IR-spectrum of the compound were identical to those of authentic L-glutamine. It was also identified as glutamine with an automatic amino acid analyzer.

Table I shows the relationship between levels of growth hormones, kinetin and 2,4-D, and the amounts of cell mass and of L-glutamine accumulated. The optimum level of 2,4-D was 0.1~0.3 mg/liter for the production of both cell mass and the amide. For glutamine accumulation, 0.3 mg of kinetin per liter was suitable, but 3.0 mg was favorable for cell growth as shown in this figure.

<table>
<thead>
<tr>
<th>Kinetin (mg/liter)</th>
<th>None</th>
<th>0.2</th>
<th>2</th>
<th>10</th>
<th>20</th>
<th>0.5</th>
<th>1</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>(±)</td>
<td>(±)</td>
<td>(±)</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>0.03</td>
<td>0</td>
<td>(±)</td>
<td>(±)</td>
<td>(±)</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>0.3</td>
<td>0</td>
<td>(++)</td>
<td>(++)</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>3.0</td>
<td>0</td>
<td>±</td>
<td>±</td>
<td>(++)</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

Growth: −, no; ±, poor; +, good; ++, excellent; ( ), differentiated.

Table I. Callus Induction from the Veins of a Leaf of S. officinale
Accumulation of Glutamine by Plant-cell Cultures

FIG. 1. Effects of 2,4-D and Kinetin Levels in the Medium on the Growth and the Glutamine Accumulation of the Suspension Culture of S. officinale.

Cultivation was performed for 7 days as described in the text. Basal medium: Murashige and Skoog’s medium containing sucrose 3%.

Effects of inorganic nitrogen sources

Among several inorganic nitrogen compounds tested as nitrogen sources in the medium, both ammonium nitrate and potassium nitrate, which have been used in the Murashige and Skoog’s medium, gave interesting results for the suspension culture of S. officinale. Namely, ammonium nitrate was favorable for accumulation of the amide but was not for cell growth. On the other hand, potassium nitrate was effective for cell growth as shown in Fig. 2. The highest amount of cell growth, 19.0 mg/ml as dry cell weight, was obtained by the incubation in the medium containing potassium nitrate alone as a nitrogen source for a week (Fig. 2).

Other nitrogen sources, that is, ammonium sulfate, ammonium chloride, diammonium hydrogen phosphate and urea were shown to have no positive effects on cell growth and glutamine accumulation (Table II).

Figure 3 shows the effects of the concentrations of ammonium nitrate and potassium nitrate in the medium on the growth and the glutamine accumulation. The maximum amount of L-glutamine (2.9 mg/ml as total glutamine, about 20% of dry cell weight) was obtained by the incubation in the medium containing potassium nitrate alone as a nitrogen source for a week (Fig. 2).

TABLE II. EFFECT OF INORGANIC NITROGEN SOURCES IN THE MEDIUM ON GROWTH AND GLUTAMINE ACCUMULATION OF S. OFFICINALE SUSPENSION CULTURE

<table>
<thead>
<tr>
<th>N-Source (%)</th>
<th>Final pH</th>
<th>Cell growth (mg/ml)</th>
<th>Total glutamine (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>0.2</td>
<td>5.4</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6.0</td>
<td>19.0</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>0.2</td>
<td>5.8</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>5.8</td>
<td>10.6</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.2</td>
<td>4.6</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.6</td>
<td>5.7</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.2</td>
<td>4.4</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.5</td>
<td>5.1</td>
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<tr>
<td>(NH₄)₂HPO₄</td>
<td>0.2</td>
<td>5.0</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6.8</td>
<td>2.6</td>
</tr>
<tr>
<td>NH₂CONH₂</td>
<td>0.2</td>
<td>5.8</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.9</td>
<td>8.8</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>4.4</td>
<td>9.5</td>
</tr>
</tbody>
</table>
Effects of NH₄NO₃ and KNO₃ Levels in the Medium on the Growth and the Glutamine Accumulation of S. officinale Suspension Culture.

Cultivation was performed for 7 days as described in the text. Basal medium: Murashige and Skoog's medium without KNO₃ and NH₄NO₃ and with complement of sucrose 3%, kinetin 0.3mg/liter and 2,4-D 0.5mg/liter.

-■-, total glutamine; ○---○, cell growth.

Accumulation occurred when the concentrations of ammonium nitrate and potassium nitrate were 0.495 and 0.570%, respectively. These concentrations of the two nitrogen sources were three times higher than those in the basal Murashige and Skoog's medium.

Time course of the glutamine accumulation

Figure 4 shows the typical time course of glutamine accumulation by suspension culture of S. officinale. After the lag period in an early stage of incubation, cell mass increased markedly as drawn with the block line and reached to the stationary phase at 12 day incubation. The intracellular level of L-glutamine accumulated increased gradually to the maximum of 12~14 day culture accompanied with cell growth and then this level decreased slowly. On the other hand, the amide was slowly excreted in the medium after 12 days of incubation.

Effect of surfactant

It has been shown that the addition of surfactants in the media of microbial4) and plant cell cultures5) results in the leakage of their

<table>
<thead>
<tr>
<th>Time of addition (days)</th>
<th>Cell growth (mg/ml)</th>
<th>Glutamine accumulated (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Extracellular</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8.3</td>
<td>1.01</td>
</tr>
<tr>
<td>10</td>
<td>10.8</td>
<td>1.36</td>
</tr>
<tr>
<td>12</td>
<td>10.9</td>
<td>2.16</td>
</tr>
<tr>
<td>Not added (8-day culture)</td>
<td>10.5</td>
<td>0.00</td>
</tr>
<tr>
<td>■ (10-day culture)</td>
<td>12.3</td>
<td>0.01</td>
</tr>
<tr>
<td>■ (12-day culture)</td>
<td>14.4</td>
<td>0.05</td>
</tr>
<tr>
<td>■ (14-day culture)</td>
<td>15.3</td>
<td>0.16</td>
</tr>
</tbody>
</table>
metabolites in the media from the inside of the cells.

A cationic surfactant, cetyltrimethylammonium bromide, was added to the culture of *S. officinale* cells at a later stage of the incubation. With the surfactant, the extracellular glutamine level was greatly increased as listed in Table III. For example, by the addition of 1 mg/ml of the surfactant after 12 days, most of the intracellular glutamine was excreted in the medium, but the total amount of the amide was not increased. Since the cell growth was decreased by the addition of the surfactant (1 mg/ml) as shown in Table III, it seemed reasonable to assume that the cells were almost dead.

**Comparison of free amino acid and amide contents between the intact plant and the callus**

The free amino acid and amide contents in the callus cells of *S. officinale* were determined with an automatic amino acid analyzer, Japan Electric Optics Lab., JLC-5AH., (Table IV).

Contents of glutamic acid, asparagine, aspartic acid and alanine in the leaves were higher than those of other amino acids. However, these levels were much lower than those in callus cells. The content of glutamine in the callus tissue was particularly high, 924 μmoles/g dry cells, i.e. 260 times higher than that in the intact plant.

### DISCUSSION

In general, as Steward\(^1,2\) reported, it has been believed that larger amounts of nitrogen-rich free amino acids such as asparagine and glutamine are accumulated in the non-growing tissue cells of higher plants but much smaller amounts in the proliferating state like cultured cells. Weinstein *et al.*\(^6\) also recognized that the levels of total free amino acids in the callus tissues were lower than those in the intact plant tissues.

Koiwai *et al.*\(^7\) analyzed amino acid composition of tobacco cells and showed the content of glutamine was the highest among free amino acids and amides in the suspension cultured cells.

In this paper we described the accumulation of very high level of L-glutamine in the cultured cells of *S. officinale* in suspension. The amount of the amide accumulated reaches up to 20% of dry cell weight, corresponding to about 260 times higher than that in the intact leaves.

It was also found that the addition of high levels of potassium nitrate and ammonium nitrate to the medium as nitrogen sources caused the accumulation of a large amount of L-glutamine in the cells.

The difference between the results described above and those reported by Steward *et al.*\(^1,2\) and Weinstein *et al.*\(^6\) seems to be cleared by paying attention to the amounts of the nitrogen sources in the media used. White's medium
used by Steward et al.$^{1,2)}$ and Weinstein et al.$^{6)}$
contains 0.8 mM of potassium nitrate and 1.3
mM of calcium nitrate as nitrogen sources:
the nitrogen concentration of the medium cor-
responds to only 0.25 mg/ml as glutamine. On
the other hand, the amounts of ammonium
nitrate and potassium nitrate in the medium
which we used for glutamine accumulation by
*S. officinale* suspension culture are three times
higher than those of Murashige and Skoog’s
medium, and the nitrogen content corresponds
to over fifty times higher than that of White’s
medium.

Acknowledgements. We wish to thank Miss Y. Seki
and Mrs. S. Ito for their helpful assistance. Thanks
are also due to Mr. N. Yamaguchi for analysis of
amino acids by automatic analyzer.

REFERENCES

1) F. C. Steward, J. F. Thompson, and J. K. Pollard,

2) “Plant Physiology,” Vol. VB, ed. by A. D.
Krikorian and F. C. Steward, Academic Press

3) T. Murashige and F. Skoog, *Physiol. Plant.,* 15,
473 (1962).

Technol.,* 40, 614 (1962).

5) M. Misawa, S. M. Martin and I. A. Veliky, oral
presentation, X-International Congress for Micro-

6) L. H. Weinstein, W. Tulecke, L. G. Nickell and
H. J. Laurencot, Jr., *Bull. Boyce Thompson Inst.,*
1959, 239.

7) A. Koiwai, M. Noguchi and E. Tamaki, *Phyto-
chem.,* 10, 561 (1971).