

Some Aspects of the Regulation of Arginine Biosynthesis in Soybean Cell Cultures¹

Received for publication March 19, 1973

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ABSTRACT

The levels of the activities of argininosuccinate synthetase and argininosuccinate lyase were measured in soybean (*Glycine max* L. var. Mandarin) cell suspension cultures grown in the presence or absence of exogenous arginine. In some experiments, actinomycin D or cycloheximide were also added to the cultures, at critical stages of their growth. The results obtained led to the conclusion that activity of argininosuccinate synthetase is subject to significant inhibition by levels of arginine similar to those found to occur within the cells. Argininosuccinate lyase activity appeared to be enhanced, when arginine levels were increased above those occurring physiologically. Both enzymes appeared to be subject to inactivation, possibly via proteolysis.

The regulation of arginine biosynthesis in microorganisms and in animals has received a considerable amount of attention in the past (8, 13-15). These studies have shown that in both groups the activity of enzymes involved in the pathway is regulated by the levels of key metabolites and also by the rate of synthesis and degradation of the enzymes. By comparison with these studies, very little is known of the mechanisms used by plants to regulate the biosynthesis of arginine. This communication presents an attempt to demonstrate some of the mechanisms which appear to exist in soybean cells for controlling the activities of two enzymes involved in arginine biosynthesis, argininosuccinate synthetase, and argininosuccinate lyase.

MATERIALS AND METHODS

The soybean culture used in these studies (*Glycine max* L. var. Mandarin), was obtained from Dr. O. L. Gamborg of the Prairie Regional Laboratory, Saskatoon. Cultures were grown routinely in B5 medium as described by Gamborg (1, 2). The average size of inoculum for the 100-ml cultures used in the experiments described in the text was 2.4 g fresh weight (equivalent to 150 mg dry weight). Additions of arginine, cycloheximide, or actinomycin D were made by dissolving these compounds in B5 medium, adjusting the pH to 5.5, and then filter sterilizing and adding to the cultures.

Soybean cell extracts used as a source of enzymes were ob-

tained by homogenizing the cells in a Braun MSK homogenizer (Canadian Laboratory Supplies Ltd.). Plant cells were collected on a disk of Miracloth over a Buchner funnel and washed with approximately 100 ml of 0.1 M, pH 7.9, Tricine buffer. They were then homogenized using a 1:2:4 ratio (w/v/w) of plant cells-Tricine buffer-glass beads (1.0-1.05 mm diameter). Homogenization was carried out for 90 sec, during which time the suspension was cooled to approximately 4 C with the aid of liquid CO₂. At the end of this time, breakage of the cells appeared complete, when a sample was viewed microscopically. The homogenate was then filtered through a Miracloth disk into a cooled Buchner flask. Finally, it was spun for 10 min at 20,000g in the SS34 head of the Sorval RC2 refrigerated centrifuge, and the supernatant was used for further experiments.

Dry weight estimations were made on 2-ml aliquots of the cultures. These were filtered on glass fiber discs, and the discs were washed thoroughly with distilled water. The discs plus cells were then lyophilized for 24 hr and finally weighed to a constant weight.

The assay of argininosuccinate synthetase and argininosuccinate lyase was carried out essentially as previously described (10, 12). Thus each assay contained 1.0 μmole of carbamyl-¹⁴C-labeled citrulline equivalent to 0.028 μmole of citrulline. Argininosuccinate synthetase activity was determined by adding the amounts of ¹⁴C-arginine and ¹⁴C-argininosuccinate produced in each assay. Argininosuccinate lyase activity was determined solely from the amount of ¹⁴C-arginine produced in each assay. An enzyme unit is defined as that amount of enzyme which catalyzes the production of 1 nmole of arginine or argininosuccinate plus arginine (in the case of the synthetase enzyme) per min.

In order to obtain samples for the estimation of arginine in the soluble fraction of the cells, the cells were collected, homogenized, and the homogenates were filtered and centrifuged as described for the preparation of enzyme extracts. These supernatants were processed according to the method of Gehrke *et al.* (3), and the final solution of soluble amino acids was analyzed with an automatic amino acid analyzer. The results obtained were corrected for the loss of arginine occurring during the picrate deproteinization process, using the data of Pellizzari *et al.* (6).

The protein content of enzyme solutions was estimated by the method of Lowry *et al.* (5), using bovine serum albumin as a standard.

Actinomycin D (Dactinomycin) was a generous gift from Merck, Sharp & Dohme Co. Cycloheximide was obtained from Sigma Chemical Co., Sephadex G-50 from Pharmacia of Canada, Montreal. All other chemicals were of the highest purity available from commercial sources.

¹ This work was supported by a grant-in-aid of research from the National Research Council of Canada.

RESULTS

Patterns of Growth and Enzyme Production. When the density of the inoculum added to 100 ml of B5 medium was varied in order to obtain a variety of growth rates, it was found that the activity of the synthetase and lyase enzymes present in extracts of these cultures also varied. Thus, while the synthetase enzyme was present for the duration of the growth period, the lyase enzyme was present for a limited period of time only (Figs. 1 and 2). The particular inoculum used here (2.5 g), the heaviest used in any of the experiments carried out, was used in all subsequent experiments, since it yielded reproducible results in terms of growth and enzyme patterns.

Effect of Increasing the Intracellular Arginine Levels. Studies were also carried out in which cells were grown in the presence of 2.1 mM arginine. The greatly decreased levels of synthetase activity present in the extracts prepared from these cells suggested that repression of the synthesis of this enzyme had taken place (Fig. 1). When the extracts were passed through G50 Sephadex however, a significant increase in the activities of both synthetase and lyase enzymes was observed (Figs. 1 and 2). An experiment in which cells were grown in medium containing U-¹⁴C-arginine, together with unlabeled arginine in a total concentration of 2.1 mM, showed that it was rapidly taken into the cells during the first 24 hr of growth. This was via an active uptake, which was inhibited by the addition of cyanide to the medium. Thus the depression of the levels of activities of the enzymes obtained from cells grown in the presence of exogenous arginine was probably due to the presence of increased levels of arginine in these cells. On the basis of these results, it seemed likely that arginine was inhibiting the activity of the synthetase enzyme, since the amounts of argininosuccinate and arginine produced in the enzyme assays were both greatly decreased. Inhibition of the lyase enzyme by arginine should not have decreased the amount of argininosuccinate produced in the assay.

Effect of Physiologically Occurring Concentrations of Arginine on the Enzyme Assay. The amount of arginine present in extracts of the soluble amino acids obtained from soybean cultures from 0 to 96 hr of growth was found to vary between 0.05 and 0.18 μ mole/g fresh weight. Experiments designed to test the effect of arginine concentrations of this order on the synthetase enzyme assay utilized extracts from the 72- to 96-hr stage of growth where lyase activity was undetectable. These experiments showed (Fig. 3) that the inhibition produced varied from 14 to 51% for 0.05 to 0.18 mM concentrations of arginine. The effect of the far higher intracellular concentrations of arginine produced during growth in exogenous arginine must be to increase the level of inhibition of the synthetase enzyme even further (Fig. 1).

Effect of Increasing the Intracellular Arginine Levels and Adding Actinomycin D or Cycloheximide. In Figure 4 we can see the effect of growth in 2.1 mM arginine, coupled with the addition of 70 μ g/ml of actinomycin D to the cultures at the 24-hr stage of growth. This leads to a maintenance of the levels of the lyase enzyme for up to 96 hr. Without the inhibitor, the enzyme has normally disappeared by this time, even in the presence of added arginine (Fig. 2). The effect of adding 50 μ g/ml of cycloheximide to cells growing in medium containing 2.1 mM arginine is shown in Figure 5. This treatment also leads to a preservation of lyase activity.

DISCUSSION

Any attempt to explain the regulation of argininosuccinate synthetase and argininosuccinate lyase levels under *in vivo*

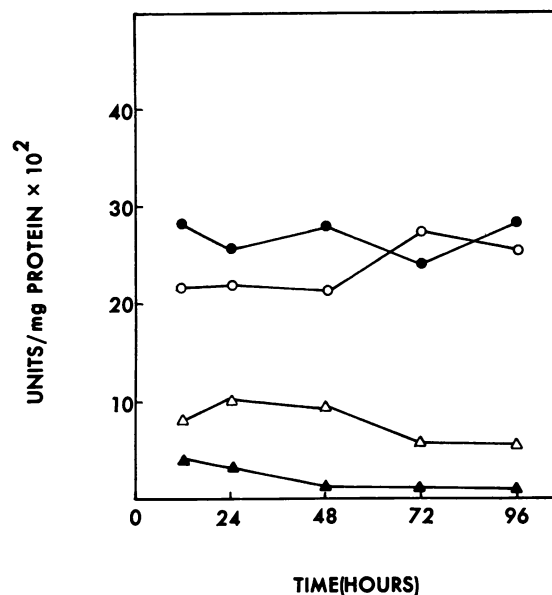


FIG. 1. Argininosuccinate synthetase activity present in extracts produced from soy bean cells which were grown in either B5 medium alone, or in medium containing 2.1 mM arginine. Activity in extracts from cells grown in B5 medium only (Δ); activity after passing a portion of each of the previous extracts through Sephadex (\circ); activity in extracts from cells grown in B5 medium plus 2.1 mM arginine (\blacktriangle); activity after passing a portion of each of the previous extracts through Sephadex (\bullet).

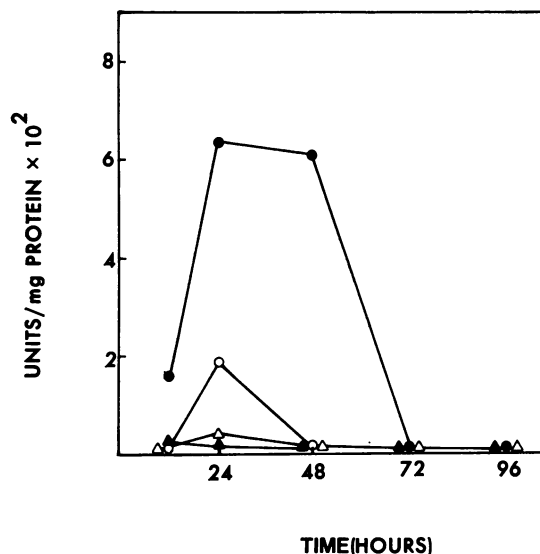


FIG. 2. Argininosuccinate lyase activity present in extracts produced from soybean cells which were grown in either B5 medium alone, or in medium containing 2.1 mM arginine. Activity in extracts from cells grown in B5 medium only (Δ); activity after passing a portion of each of the previous extracts through Sephadex (\circ); activity in extracts from cells grown in B5 medium plus 2.1 mM arginine (\blacktriangle); activity after passing a portion of each of the previous extracts through Sephadex (\bullet).

conditions should be made with reference to the arginine levels apparently existing *in vivo*. These vary between 0.05 and 0.18 μ mole/g fresh weight. The amount of citrulline occurring under *in vivo* conditions in the cells is certain to be much less than 0.05 μ mole/g fresh weight, assuming that citrulline

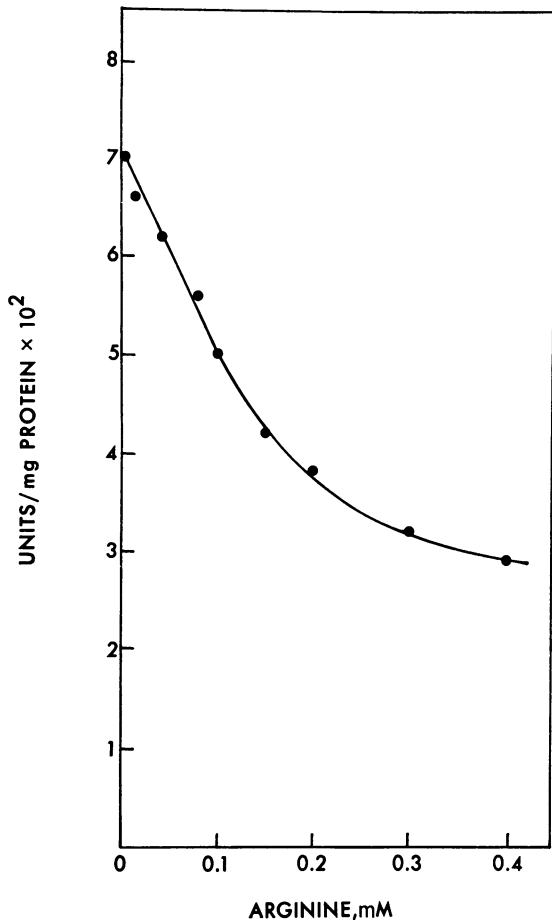


FIG. 3. Effect of arginine on the activity of argininosuccinate synthetase. Arginine was added to the enzyme assays, to give a final concentration indicated by the value on the abscissa.

exists only as a transitory intermediate in arginine biosynthesis. Thus the 0.028 μ mole of citrulline present as ¹⁴C-labeled citrulline in the enzyme assays is perhaps the nearest approach that can be made to a "physiological" level of citrulline under the conditions used. Enzyme assays containing this amount of citrulline are readily inhibited by physiological concentrations of arginine (Fig. 3), a finding similar to that made for *Neurospora crassa* by Wampler and Fairly (16), who reported that arginine was a competitive inhibitor of the argininosuccinate synthetase from *Neurospora*.

The increase in synthetase activity seen upon Sephadex treatment of enzyme preparations must be responsible for the increase in lyase activity obtained by removing arginine (Fig. 2). Since more argininosuccinate is being produced, the lyase will display more activity in the coupled assay. However, not only is there a much larger increase in activity of the lyase enzyme from cells grown in the presence of exogenous arginine, when they are Sephadex treated in order to remove residual arginine, but the activity apparently is present for a much longer period of time during the growth cycle (Fig. 2). The lyase enzyme has been shown to be capable of binding arginine and using this to produce argininosuccinate (11). The apparent protection of the enzyme in the presence of higher intracellular levels of arginine can be most readily explained by invoking substrate protection. Similar results have been obtained by Schimke *et al.* (9), with tryptophan oxygenase, and tryptophan. Schimke *et al.* found that the protection of tryptophan oxygenase by tryptophan caused an increase in the levels of enzyme activity occurring in rat liver. This in-

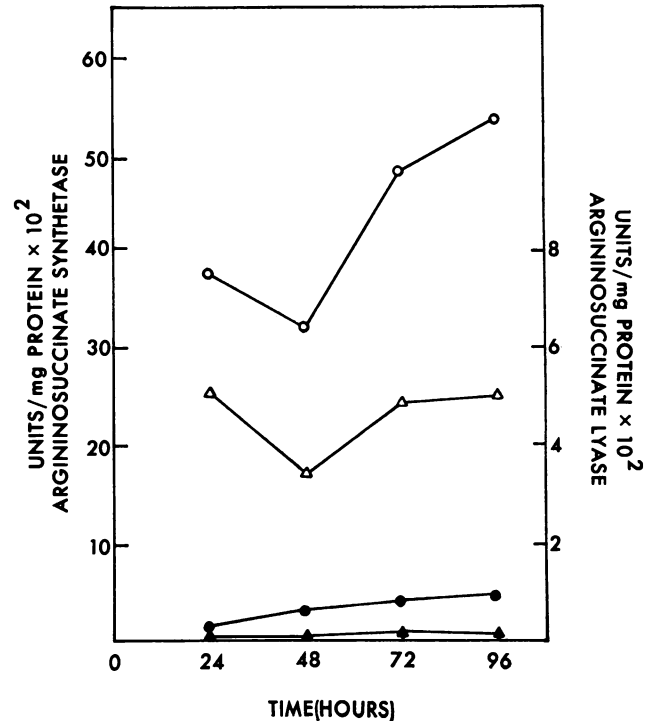


FIG. 4. Argininosuccinate lyase and argininosuccinate synthetase activities present in extracts produced from soybean cells which were grown in B5 medium containing 2.1 mM arginine and 70 μ g/ml actinomycin D. Lyase activity before (\blacktriangle) and after (\triangle) Sephadex treatment. Synthetase activity before (\bullet) and after (\circ) Sephadex treatment.

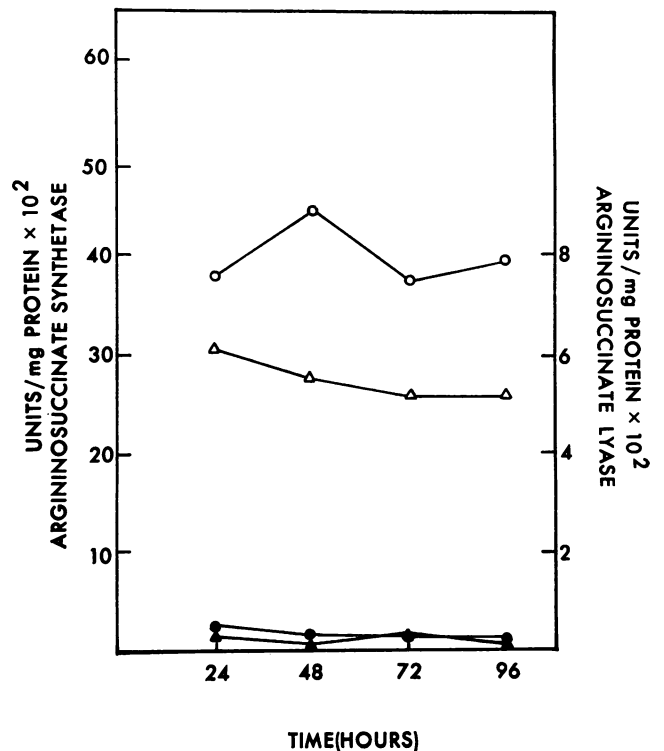


FIG. 5. Argininosuccinate lyase and argininosuccinate synthetase activities present in extracts produced from soybean cells which were grown in B5 medium containing 2.1 mM arginine and 50 μ g/ml cycloheximide. Lyase activity before (\blacktriangle) and after (\triangle) Sephadex treatment. Synthetase activity before (\bullet) and after (\circ) Sephadex treatment.

crease was shown to be independent of enzyme protein synthesis and was due solely to a decrease in the rate of degradation of the enzyme. The idea that this might be the role played by excess arginine in the soybean cells, is strengthened by the results of the experiments conducted in the presence of arginine plus actinomycin D or cycloheximide (Figs. 4 and 5). Actinomycin D presumably acts by blocking the formation of any mRNA produced after 24 hr, which acts as the template for an inactivator protein. In Figure 4, we see that the activity of the lyase enzyme is preserved as far as 96 hr of growth. The synthetase activity appears to rise rapidly after 48 hr (Fig. 4), indicating that this enzyme may be subject to some degree of inactivation under normal conditions, but that this is compensated for by a continual synthesis of new active enzyme.

The growth of cells in medium containing added arginine plus cycloheximide yields results which appear to be complementary to those of the actinomycin D experiment (Fig. 5). Thus if the necessary mRNA is not produced until after 24 hr of growth, then the addition of cycloheximide at 24 hr would be expected to inhibit the production of any protein produced on this template. Similar results to these discussed here have been obtained when using actinomycin D or cycloheximide to study the turnover of enzymes of amino acid metabolism in animal tissues. Kenney (4) and Reel and Kenney (7) showed that these compounds inhibited the turnover of tyrosine transaminase in hepatoma cell cultures. These authors concluded that the degradative phase of the turnover of the transaminase was correlated with the synthesis of a protease by the cells.

The results presented in this communication demonstrate the necessity for protein synthesis to take place, in order that inactivation of argininosuccinate lyase may occur. They do not, however, enable a distinction to be made between the alternative of a degradative protein (a protease) and an inhibitor protein, which binds to the active site of the lyase enzyme, being the cause of this inactivation.

In contrast to the synthetase enzyme which appears to be subject to direct feedback inhibition by arginine, previous studies of the lyase enzyme isolated from pea cotyledons have

failed to reveal any form of ligand modification of its activity (11).

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