Plant Physiol. (1971) 48, 261–264

Influence of Cycloheximide on the Synthesis and Utilization of Amino Acids in Suspension Cultures[1]

Received for publication February 22, 1971

JOHN S. FLETCHER[2] AND HARRY BEEVERS
Division of Natural Sciences, University of California, Santa Cruz, California 95060

ABSTRACT

Cells from 4-days old suspension cultures of Paul's Scarlet rose were incubated with acetate-U-^{14}C for 10 minutes. After washing, cells were incubated for 2 hours in growth medium in the presence and absence of cycloheximide. The ^{14}C content of individual amino acids in the soluble form and in protein were determined at the end of the 10 minute pulse and at intervals thereafter in control cells and those treated with cycloheximide. During the period following the pulse there was a 3-fold increase in the ^{14}C content of protein in control cells; no such increase occurred in the presence of cycloheximide.

In the control cells there was a net increase in the ^{14}C content of eight individual amino acids during this period. For six of these, the corresponding increases in the presence of cycloheximide were curtailed by the following amounts: arginine 98%, lysine 94%, isoleucine 93%, threonine 82%, valine 49%, and proline 36%. This is interpreted to mean that the synthesis of each of these amino acids was slowed down when their incorporation into protein was prevented.

Experiments with microorganisms have shown that the rate of synthesis of individual amino acids is closely regulated by the amount present in the cell (19, 20). Commonly, an exogenous supply of an amino acid will prevent endogenous synthesis by inhibition of specific enzymes in the synthetic pathway (end product inhibition) or by repression of synthesis of the enzymes required (20).

Although it has been shown that key enzymes isolated from plants are inhibited by amino acids (2, 18) evidence for the operation of such control by end product inhibition in living cells of higher plants is based primarily on isotope competition experiments (5, 6, 15–17). These experiments have required several hours for completion, and therefore, have not distinguished between control arising from end product inhibition, a rapid response in microorganisms, and that due to enzyme repression, a slow response (4, 20).

Davies has pointed out that a second complication with some isotope competition studies has been the failure to consider the influence of high concentrations of exogenously supplied nonradioactive amino acids on the size and makeup of the protein precursor pools (4). The necessity for such consideration is emphasized by the work of Holleman and Key (14) in which they estimated that exogenously supplied leucine (5 mM solution) caused a 64-fold expansion of the protein precursor pool of leucine in soybean hypocotyls during a 5-hr incubation period. It is apparent that if this occurred during an isotope competition experiment, the nonradioactive amino acids entering the tissue from an exogenous source would seriously dilute amino acids synthesized endogenously from a radioactive precursor. Such dilution would reduce the specific radioactivity of the amino acids in the protein precursor pools and lead to a decreased incorporation of ^{14}C into the protein of the treated tissue. It follows that in those isotope competition studies, where no consideration was given to changes in the soluble amino acids (5, 6, 15), the reduced incorporation of ^{14}C into the protein may not be due directly to the regulation of amino acid synthesis.

The purpose of the present investigation was to determine if the synthesis of amino acids is subject to rapid control when protein synthesis is stopped. To achieve this, suspension cultures of Paul's Scarlet rose were exposed to a pulse of acetate-U-^{14}C and its incorporation into soluble- and protein-bound amino acids was followed in control cells and in samples treated with cycloheximide, an inhibitor of protein synthesis (7, 8, 11).

MATERIALS AND METHODS

Suspension cultures of Paul's Scarlet rose were grown as previously described (9). The investigation was performed on cells harvested after 4 days of growth, at a time when the cells were undergoing rapid cell division (9). The sterility of the cells and the medium used in the labeling experiments was established by plating each separately on Difco Bacto Nutrient Agar and incubating at 25 C for 96 hr.

Ten grams of cells were aerated for 10 min in 20 ml of growth medium containing 20 μc of acetate-U-^{14}C (53 μc/μmole). The material was held in a 30-ml coarse fritted glass filter funnel connected to an air line. Following the pulse, the medium was drawn off through the bottom of the funnel, and the cells were washed several times with sterile growth medium. Approximately 1 g of tissue was removed and homogenized in 15 ml of 80% (v/v) ethanol to serve as the zero time sample. The remaining cells were divided into two equal portions and placed in separate funnels. One portion, the control cells, was aerated in sterile medium for the remainder of the experiment. The second portion, the treated cells, was aerated in medium containing cycloheximide (50 μg/ml). This high concentration of cycloheximide was used to ensure an immediate and drastic inhibition of protein synthesis. The cells were incubated in medium removed from the growing cultures at harvesting. Five milliliters of medium were used for each gram of cells.

Samples (5 ml) were pipetted from both funnels at selected

[1] This research was supported by the United States Atomic Energy Commission Contract AT 11-1-330.

[2] Present address: Department of Botany and Microbiology, The University of Oklahoma, Norman, Okla. 73069.

time intervals and handled as previously described for the zero time sample. Pipetting of plant suspensions did not yield samples of uniform weight. Therefore, the protein amino acid content was determined for each sample, and these values were used to express the observed data in terms of equal sample weights (1 g).

The alcohol extracts recovered from the samples were fractionated and the distribution of ^{14}C in the individual soluble amino acids and those in the protein were determined as previously described (9). The amount of ^{14}C incorporated into the amino acids of the cycloheximide-treated cells was compared with that of the control cells.

RESULTS

In cells exposed to a 10 min pulse of acetate-U-^{14}C (Fig. 1), 15 amino acids became labeled in both soluble and bound fractions (Table I and III). Immediately following the pulse, a total of 258,000 cpm of ^{14}C were present in the soluble amino acids and slightly over 40,000 cpm in the protein. Except for leucine, each amino acid was more heavily labeled in the soluble phase than in the bound phase. Protein bound leucine contained approximately 10 times more ^{14}C than the soluble leucine. At zero time, leucine accounted for 70% of the total ^{14}C present in protein, but only 1% of the total ^{14}C in the soluble amino acids. In contrast to this, glutamate was heavily labeled in the soluble phase (75% of total ^{14}C), but moderately labeled in the protein (17% of total ^{14}C).

In cells exposed to cycloheximide following the pulse, only a small amount of ^{14}C (4600 cpm) was incorporated into the protein during the first 20 min and none thereafter (Fig. 1). In contrast to this, in the control cells, 19,800 cpm of ^{14}C entered the protein during the first 15 min following the pulse, and an additional 51,800 cpm accumulated during the subsequent 105 min. Increased amounts of ^{14}C in 12 amino acids accounted for the rise of ^{14}C in the protein of the control cells (Table III). The

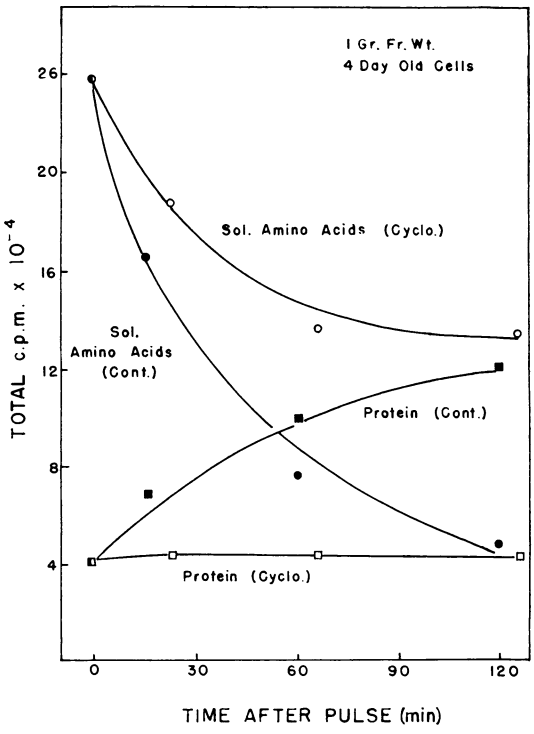

FIG. 1. Influence of cycloheximide on the ^{14}C content of the protein and soluble amino acids in 4-day cells following a 10 min pulse of acetate-1,2-^{14}C.

Table I. *Distribution of ^{14}C in the Soluble Amino Acids of Control Cells following a 10-Min Pulse of Acetate-1,2-^{14}C*

Samples were 1 g fresh weight.

Amino Acid	Time after Pulse			
	0 min	15 min	60 min	120 min
	cpm			
Glutamate	127130	77415	25245	10485
Glutamine	11470	8330	3340	1064
Proline	4080	4360	2600	1820
Arginine	230	235	140	85
Aspartate	19310	16965	7545	1850
Methionine sulfoxide	1590	1125	640	635
Threonine	1065	1195	915	555
Isoleucine	185	230	315	330
Lysine	75	60	15	0
Homoserine	535	510	295	140
Asparagine	1460	3265	5415	5650
Alanine	3020	4095	2140	1800
Serine	625	600	595	365
Valine	470	605	510	405
Leucine	1800	875	210	415
Glycine	250	360	100	10
Phenylalanine	55	0	4	0
Tyrosine	25	50	110	150
Histidine	0	0	0	0

Table II. *Distribution of ^{14}C in the Soluble Amino Acids of Cycloheximide-treated Cells following a 10-Min Pulse of Acetate-1,2-^{14}C*

Samples were 1 g fresh weight.

Amino Acid	Time after Pulse			
	0 min	20 min	65 min	125 min
Glutamate	127135	78475	47800	39925
Glutamine	11470	9490	7650	10945
Proline	4080	5790	5540	6040
Arginine	230	345	800	205
Aspartate	19310	21230	11795	11710
Methionine sulfoxide	1590	525	1135	1015
Threonine	1065	1555	1420	1495
Isoleucine	185	365	355	570
Lysine	75	90	140	115
Homoserine	535	345	220	205
Asparagine	1460	4305	6970	12250
Alanine	3015	3345	2130	2080
Serine	625	1230	1355	1930
Valine	470	595	620	1065
Leucine	1800	2635	2445	3940
Glycine	250	180	145	140
Phenylalanine	55	95	0	70
Tyrosine	25	65	0	90
Histidine	0	0	0	0

individual increases ranged from a 4-fold increase in glutamate-^{14}C to a 1400-fold increase in valine-^{14}C. During the same time period, the protein in the treated cells experienced only minor changes in its ^{14}C composition (Table III).

Despite the almost total inhibition of protein synthesis in the treated cells, the level of ^{14}C in the soluble amino acids dropped rapidly immediately following the pulse and in 2 hr it was approximately one-half of that at zero time (Fig. 1). In the control cells there was an even more rapid disappearance of

Table III. *Distribution of ^{14}C in the Protein of Control Cells and Cycloheximide-treated Cells following a 10-Min Pulse of Acetate-1,2-^{14}C*

Samples were 1 g fresh weight.

Amino Acid	Time after Pulse			
	Control			Cycloheximide
	0 min	15 min	120 min	125 min
	cpm			
Glutamate and glutamine	4650	10410	18720	5995
Proline	650	2465	6750	1150
Arginine	700	2015	5070	800
Aspartate and asparagine	940	3170	8260	1355
Methionine sulfoxide	65	380	1080	180
Threonine	75	860	4090	260
Isoleucine	485	670	3180	310
Lysine	318	1415	4115	490
Alanine	205	1225	2870	365
Serine	0	190	740	105
Valine	0	335	1430	105
Leucine	19090	22235	22835	19540
Glycine	0	0	10	0
Phenylalanine	0	0	1	0
Tyrosine	0	0	0	
Histidine	0	0	2	0

Table IV. *Calculation for Percentage Inhibition of Threonine Synthesis*

	Control	Cycloheximide
	cpm	
^{14}C in soluble threonine at zero time	1065	1065
^{14}C in protein-bound threonine at zero time	75	75
Total ^{14}C in threonine at zero time	1145	1145
^{14}C in soluble threonine at 120 min	555	1495
^{14}C in protein-bound threonine at 120 min	4090	260
Total ^{14}C in threonine at 120 min	4640	1755
Increase of ^{14}C in threonine between 0 and 120 min	3500	615
Percentage inhibition of threonine synthesis = 82%		

^{14}C from soluble amino acids; after 2 hr only one-seventh of that present at zero time remained. However, of the 209,000 cpm of ^{14}C which were lost from the soluble amino acids in control cells, only 81,600 cpm appeared in protein, a discrepancy of some 127,000 cpm. It is significant that a similar amount of ^{14}C (122,000 cpm) was lost from the soluble amino acids of treated cells, in which protein synthesis was halted, during the same period. Clearly some component(s) of the soluble amino acid fraction labeled during the pulse was utilized in both treated and control cells in reactions not leading to protein synthesis. Inspection of Tables I to III shows that the only component whose behavior fits this pattern is glutamate. The losses of ^{14}C from this single constituent go far towards accounting for the total loss of ^{14}C from the total amino acid fraction which is not recovered in protein.

In the control cells, the ^{14}C content of the other soluble amino acids also decreased steadily after the pulse. However, in contrast to the pattern observed for glutamate, the amount of ^{14}C which disappeared from the soluble fraction of most of the other amino acids was either matched or exceeded by ^{14}C recovered in the corresponding amino acid in the protein fraction. Exceptions to this were glycine and asparagine. None of the soluble glycine-^{14}C was incorporated into protein, and there was a continuous accumulation of ^{14}C in soluble asparagine.

In the treated cells the soluble amino acids behaved quite differently. Many of the amino acids either retained their initially high amounts of ^{14}C or gained additional ^{14}C after the pulse (Table II). The increase in ^{14}C was most pronounced in soluble asparagine and serine. After 120 min, twice as much asparagine-^{14}C and five times as much serine-^{14}C were present in the treated cells as compared to the control cells. Since serine and glycine are presumably closely linked metabolically

Table V. *Effect of Cycloheximide Treatment on Incorporation of ^{14}C into Amino Acids*

Tests were made in the 2-hr period following a 10-min exposure to acetate-U-^{14}C.

Amino Acid	Net Increase in ^{14}C			
	Control	Cycloheximide	Inhibition	Enhancement
	cpm		%	
Arginine	4245	75	98	
Lysine	3720	210	94	
Isoleucine	2845	210	93	
Threonine	3500	615	82	
Valine	1365	695	49	
Proline	3840	2465	36	
Leucine	4065	4300		5
Asparagine	4190	10790		260
Serine	485	1410		290

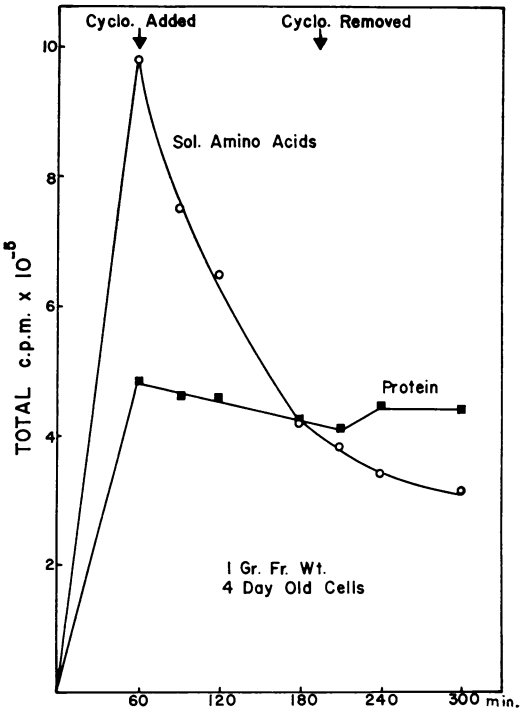

FIG. 2. Changes in the ^{14}C content of the protein and soluble amino acids of cells subjected to the following sequential treatments: 0 to 60 min: acetate-2-^{14}C; 60 to 210 min: acetate-2-^{14}C plus cycloheximide; 210 to 300 min: *no* acetate-2-^{14}C or cycloheximide (growth medium only).

(3, 10), it was surprising to find only a small amount of ^{14}C in glycine during the later stages of the experiment when ^{14}C accumulated in serine. Some of the other amino acids such as glutamate, glutamine, aspartate, and alanine showed steady losses of ^{14}C.

To determine if the inhibition of protein synthesis had any influence on amino acid synthesis, an analysis was made of the total ^{14}C incorporation into individual amino acids between 0 and 120 min in the control and treated cells. Table IV shows a sample of the calculation, with threonine as the example. It is clear that the incorporation of ^{14}C into threonine in the period 0 to 120 min was drastically reduced in the cells exposed to cycloheximide. No such reduction could be demonstrated for ^{14}C incorporation into glutamate, aspartate, methionine, or alanine, since no net incorporation of ^{14}C into these amino acids occurred in control cells. For five other amino acids, however, total ^{14}C incorporation in the period 0 to 120 min was strongly affected by the concurrent cycloheximide exposure. As shown in Table V, the inhibition was particularly severe for arginine, isoleucine, lysine, and threonine, but the synthesis of labeled valine and proline was also markedly affected. In contrast, the synthesis of serine-^{14}C was increased by a factor of 2.9, and the accumulation of soluble asparagine was increased by a factor of 2.6. Incorporation into leucine was virtually unaffected.

As shown in Figure 1, the total ^{14}C in protein after a short pulse of acetate-^{14}C remained essentially constant after the addition of cycloheximide. In a further experiment cells were exposed to acetate-2-^{14}C for 60 min (Fig. 2). Under these conditions a much heavier labeling of the protein was achieved, and, when cycloheximide was added, a steady decline in ^{14}C content of the protein was evident at a rate of roughly 5% per hour. This was accompanied, as expected, by a rapid fall in ^{14}C in soluble amino acids. At 180 min the cells were thoroughly washed to remove cycloheximide and resuspended in growth medium. During the subsequent 2 hr the loss of ^{14}C from protein was arrested and it appeared that net synthesis was resumed for a limited period. The estimate of protein turnover by this method is somewhat higher than that determined in other systems by different methods (1, 12, 13).

DISCUSSION

Cycloheximide treatment had the anticipated effect of inhibiting protein synthesis; the incorporation of each labeled amino acid was rapidly brought to a virtual halt (Table III). If there were no secondary repercussions, it might then have been anticipated that incorporation of ^{14}C into free amino acids would continue at the same rate as in control cells but accumulate there rather than in protein. This was not the case. For three of the amino acids, arginine, lysine and isoleucine, the net incorporation of ^{14}C after the pulse was reduced by over 90% by cycloheximide treatment and that into threonine, valine, and proline was strongly curtailed (Table V). Most of the ^{14}C that was incorporated into these amino acids in the presence of cycloheximide was recovered in the free amino acids. We believe that the increase in ^{14}C in these free amino acids observed in the first 20 min of exposure to cycloheximide reflects an increase in the size of their protein precursor pools due to inhibition of their incorporation into protein. Thus, we ascribe the drastic and rapid curtailment of total ^{14}C incorporation into these amino acids to the regulation, by end product inhibition, of their respective syntheses.

Direct proof of these contentions would, of course, require information about the concentrations of amino acids in the protein precursor pools and the demonstration that, when protein synthesis was stopped, increases occurred which were sufficient to inhibit the appropriate enzymes. Such increases would not necessarily be reflected in significant over-all increases of concentrations of total free amino acids, since the protein precursor pools are believed to represent only small fractions of the total cellular contents and are segregated in special compartments (1, 14, 17). Measurements of concentrations of amino acids in the protein precursor pools would also be required to sustain the possibility that, rather than simple end product inhibition, more subtle controls over amino acids synthesis were being exerted.

The possibility that cycloheximide was acting as a general metabolic inhibitor is discounted for the following reasons. Firstly, in the concentrations used in the labeling experiments, cycloheximide had no effect on the basal respiratory rate, as shown by measurements of O_2 consumption of cells suspended in dilute phosphate buffer (unpublished). Secondly, the effect of cycloheximide was selective; incorporation of ^{14}C into some components, *e.g.*, serine and asparagine, was actually stimulated, and that into leucine was essentially unaffected (Table V). Finally, utilization of labeled amino acids, *e.g.*, glutamate, by reactions not leading to incorporation into protein was not affected by the cycloheximide treatment.

LITERATURE CITED

1. Bidwell, G. S., R. A. Barr, and F. C. Steward. 1964. Protein synthesis and turnover in cultured plant tissue: Sources of carbon for synthesis and the fate of the protein breakdown products. Nature 203: 367–373.
2. Bryan, J. K. 1969. Studies on the catalytic and regulatory properties of homoserine dehydrogenase of *Zea mays* roots. Biochim. Biophys. Acta 171: 205–216.
3. Cohen, G. 1967. The Regulation of Cell Metabolism. Holt, Rinehart, and Winston Inc., New York.
4. Davies, D. D. 1968. The metabolism of amino acids in plants. *In:* E. J. Hewitt and C. V. Cutting, eds., Recent Aspects of Nitrogen Metabolism in Plants. Academic Press, New York. pp. 125–135.
5. Dougall, D. K. 1965. The biosynthesis of protein amino acids in plant tissue culture. I. Isotope competition experiments using glucose-U-^{14}C and the protein amino acids. Plant Physiol. 40: 891–897.
6. Dougall, D. K. 1966. Biosynthesis of protein amino acids in plant tissue culture. II. Further isotope competition experiments using protein amino acids. Plant Physiol. 41: 1411–1415.
7. Ennis, H. L. and M. Lubin. 1964. Cycloheximide: Aspects of inhibition of protein synthesis in mammalian cells. Science 146: 1474–1476.
8. Felicetti, L., B. Colombo, and C. Baglioni. 1966. Inhibition of protein synthesis in reticulocytes by antibiotics. II. The site of action of cycloheximide, streptovitacin A, and pactamycin. Biochem. Biophys. Acta 119: 120–129.
9. Fletcher, J. S. and H. Beevers. 1970. Acetate metabolism in cell suspension cultures. Plant Physiol. 45: 765–772.
10. Fowden, L. 1965. Origins of the amino acids. *In:* J. Bonner and J. E. Varner, eds., Plant Biochemistry. Academic Press, New York. pp. 361–390.
11. Grollman, A. P. 1966. Structural basis for inhibition of protein synthesis by emetine and cycloheximide based on analogy between Ipecac alkaloids and glutarimide antibiotics. Proc. Nat. Acad. Sci. U.S.A. 56: 1867–1874.
12. Hellebust, J. A. and R. G. S. Bidwell. 1963. Protein turnover in wheat and snapdragon leaves—preliminary investigations. Can. J. Bot. 41: 179–182.
13. Hellebust, J. A. and R. G. S. Bidwell. 1964. Protein turnover in attached wheat and tobacco leaves. Can. J. Bot. 42: 1–12.
14. Holleman, J. M. and J. L. Key. 1966. Inactive and protein precursor pools of amino acids in the soybean hypocotyl. Plant Physiol. 42: 29–36.
15. Joy, K. W. and B. F. Folkes. 1965. The uptake of amino acids and their incorporation into the proteins of excised barley embryos. J. Exp. Bot. 16: 646–666.
16. Oaks, A. 1965. The soluble leucine pool in maize root tips. Plant Physiol. 40: 142–148.
17. Oaks, A. 1965. The effect of leucine on the biosynthesis of leucine in maize root tips. Plant Physiol. 40: 149–155.
18. Oaks, A. 1965. The synthesis of leucine in maize embryos. Biochim. Biophys. Acta 111: 79–89.
19. Roberts, R. B., D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. S. Britten. 1955. Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. No. 607.
20. Umbarger, H. E. 1963. The integration of metabolic pathways. Annu. Rev. Plant Physiol. 14: 19–42.