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EFFECTS OF PHENOLIC INHIBITORS ON GROWTH, METABOLISM, MINERAL DEPLETION, AND ION UPTAKE IN PAUL'S SCARLET ROSE CELL SUSPENSION CULTURES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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BY

MAUREEN L. CROAK

Norman, Oklahoma

1972

EFFECTS OF PHENOLIC INHIBITORS ON GROWTH, METABOLISM, MINERAL DEPLETION, AND ION UPTAKE IN PAUL'S SCARLET ROSE CELL SUSPENSION CULTURES

APPROVED BY

Elroy L. Rice

John A. Fletcher

Norman Boke

James Estes

Simon H. Wender

DISSERTATION COMMITTEE

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EFFECTS OF PHENOLIC INHIBITORS ON GROWTH, METABOLISM, MINERAL DEPLETION, AND ION UPTAKE IN PAUL'S SCARLET ROSE CELL SUSPENSION CULTURES

CHAPTER I

INTRODUCTION

Competitive biochemical interactions between plants have been reviewed recently by several investigators (Muller, 1966; Rice, 1967; Went and Sheps, 1969; and Kefeli and Kadypov, 1971) and in many instances interference through biochemical suppression has proved to be of ecological significance in determining pattern, composition, and succession in plant communities. The chemical compounds responsible for biochemical suppression are primarily secondary plant substances including phenolics, alkaloids, flavonoids, and terpenoids.

Many investigators have noted changes in seed germination, growth, and seedling development as a result of biochemical suppression, but few investigators have pursued the mechanisms by which cellular alterations are effected in the susceptible plant. Muller, Lorber, and Haley (1968) found

that the terpene, cineole, retarded cell elongation, cell division, and differentiation, but enhanced cell enlargement in roots of various herbs. This compound also reduced lateral root development and respiration. Einhellig et al. (1970), reported that the coumarin, scopoletin, had no effect on respiration in seedlings of tobacco, sunflower, and pigweed. However, they did find a reduced shoot:root ratio and reduced CO_2 exchange rates in inhibited seedlings, suggesting that a reduced net photosynthetic rate was contributing to the reduction in growth.

There have been attempts to establish a mode of action of phenols and coumarins as growth regulators. Hare (1964) reported that monohydric phenols accelerated decarboxylation of IAA, while dihydric phenols inhibited decarboxylation of IAA. Chlorogenic acid, a dihydric phenol, resulted, therefore, in a high IAA concentration (Griffin and Sondheimer, 1960; Zenk and Muller, 1963), whereas p-coumaric and ferulic acids increased IAA decarboxylation resulting in reduced growth (Zenk and Muller 1963). Stimulation or inhibition may be dependent upon concentration. Imbert and Wilson (1970) found that low concentrations of externally applied scopoletin stimulated IAA oxidase preparations from sweet potato, while high concentrations inhibited it.

Several investigators have noted increased levels of phenols and coumarins in plants suffering from mineral deficiencies, but the relationship between increased levels of

these compounds and suppression of growth remains unclear (Wender, 1970; Armstrong et al., 1971; Lehman and Rice, 1972). Tilberg (1970), working with Scenedesmus, noted a small decrease in phosphate uptake in cells treated with salicylic acid, while trans-cinnamic acid had no effect. Trans-cinnamic acid did inhibit oxygen evolution, however, and both compounds resulted in increased ATP levels in the cells. Olmsted and Rice (1970) found that treatment of Amaranthus retroflexus seedlings with either chlorogenic acid or gallotannic acid resulted in a decrease in the uptake of K^+ and Ca^{2+}.

Although plant tissue cultures have been used extensively to study the biosynthesis and degradation of phenols, coumarins, flavonoids, and related compounds (Chan and Staba, 1965; Gamborg 1966a, 1966b; Gamborg and Keeley, 1966; Ellis and Towers, 1970; Schafer and Wender, 1970; Fritig, Hirth, and Ourisson, 1970) few studies have been designed to study the effect of these compounds on growth, basic metabolism, and mineral nutrition. Lee and Skoog (1965a, 1965b) found that trans-cinnamic acid inhibited tobacco callus growth and had weak bud-forming activity. In contrast, ferulic acid and other dihydroxyphenolpropanoic acid derivatives failed to induce buds and, in certain concentrations, stimulated tobacco callus growth. They concluded however, that those phenols tested played only a secondary role in promoting or inhibiting growth through their action on IAA activation and inactivation.

Plant tissue cultures do provide an ideal system with which to study the cellular effects of inhibitors. Cells may be grown on a completely defined minimal medium under controlled conditions. In the present study, an attempt was made to assess the cellular effects of two commonly named plant inhibitors, ferulic and cinnamic acids, on Paul's Scarlet Rose cell-suspension cultures. The influence of these chemicals on growth, carbon metabolism, mineral depletion, and ion uptake was of particular concern.

CHAPTER II

MATERIALS AND METHODS

Cell cultures and growth--Suspension cultures of Paul's Scarlet rose cells, originally isolated from stem tissue by Walter Tulecke, were used in this investigation (Nickell and Tulecke, 1959). Cultures were propagated every 14 days by transferring 0.25 - 0.50 g fresh weight of tissue to 250 ml Erlenmeyer flasks containing 80 ml of a minimally defined medium (Nesius, Uchytil, and Fletcher, 1972) at pH 5.5. Cells were incubated at approximately 25 C in the dark on a New Brunswick rotary shaker at 180 rpm.

Appropriate amounts of the test compounds were added to the basal medium to make concentrated stock solutions and the pH adjusted to 5.5. Test solutions were filter-sterilized through 0.22 μ Millipore filter paper. Aliquots of the stock solution were added aseptically to appropriate volumes of the basal medium immediately before inoculation to obtain the desired final concentrations. The test compounds were obtained from the following sources: chlorogenic acid and scopoletin, Sigma Chemical Co.; cinnamic acid, Fisher Chemical Co.;

ferulic acid, General Biochemicals; and p-coumaric acid, Calbiochem.

Increments in growth were determined daily throughout the growth cycle. Cells were filtered through Miracloth on a Millipore filter apparatus and fresh weights were recorded immediately. Dry weight of the tissues was determined after a minimum desiccation period of 24 hr at 100 C.

Metabolism of glucose-UL-^{14}C--Glucose-UL-^{14}C (specific activity 29 mc/mmole) was obtained from ICN-Tracerlab. One gram samples of 5-day-old cells were incubated for 210 min in 25 ml Erlenmeyer flasks containing 5 ml of fresh medium with 4 µc of glucose-UL-^{14}C added. New medium containing one quarter (0.01462 M) the normal amount of sucrose (0.05848 M) was used in this experiment. Cells were incubated at approximately 25 C on a desk rotary shaker throughout the incubation period. Cells treated with either ferulic acid (10^{-4} M) or cinnamic acid (10^{-5} M) were preincubated in medium supplemented with the inhibitor for 15 min prior to introduction of the labelled glucose. To determine the rate of uptake of the label, 10 µl aliquots of medium were removed at zero time and every half hour for the first 120 min and subsequently every 60 min until the experiment was terminated. At the end of the incubation period, cells were filtered through Miracloth, washed thoroughly with deionized water, and homogenized in hot 80% aqueous ethanol (v/v).

The techniques of Fletcher and Beevers (1970) were used to separate the ethanol homogenate into the following fractions: chloroform soluble compounds (lipids), positively charged compounds (amino acids), negatively charged compounds (organic acids), neutral compounds (sugars), and alcohol-insoluble residue (protein). The protein present in the insoluble material was hydrolyzed and assayed by the method of Fletcher and Beevers (1970). Individual amino acids and protein amino acids were separated and identified by the chromatographic techniques of Morris and Thompson (1965). The organic acid fraction was separated into malate, citrate, and succinate by gradient elution from a Dowex 1-X10 resin column (Fletcher and Beevers, 1970) on a Gilson fraction collector. Amounts of individual organic acids were estimated by titration of the various fractions with 0.05 N NaOH to a phenolphthalein endpoint.

Alpha-amino nitrogen content of the alcohol-insoluble residue was determined with the ninhydrin-hydrindantin assay of Moore and Stein (1954). These values were used to express observed data in equal sample weights of one gram.

^{14}C determinations of the various fractions were made on a Beckman LS-100 liquid scintillation counter. The scintillation fluid consisted of 6 g of 2,5-diphenyloxazole and 100 g of napthalene per liter of 1,4-dioxane.

Mineral depletion--To determine the effect of ferulic acid on the capacity of cells to remove selected macronutrients

from the media, cells were incubated in basal medium containing 10^{-4} M ferulic acid for 14 days. On days 3,5,7,9,12, and 14 of the growth cycle, a 1 ml aliquot of medium was removed from each of six test and six control cultures with a sterile pipette. The sample was then placed in a sterile vial and stored at 5 C. In order to observe patterns of uptake in a single cell culture, aliquots were removed from the same flask throughout the 2 wk period. At the end of the 14-day growth cycle, fresh and dry weights were determined for each culture.

The amount of phosphorus in the medium was determined spectrophotometrically by a modified Fiske-SubbaRow method (1925). Concentrations of magnesium, calcium, and potassium in the medium were determined on a Perkin-Elmer Model 303 atomic absorption spectrophotometer.

To determine the effect of ferulic acid on the depletion of micronutrients from the media, cultures were prepared as described previously. However, on days 3,5,7,9,12, and 14, individual cultures were harvested and the medium collected in a 100 ml vacuum graduated cylinder. A sufficient number of flasks was harvested to collect a minimum of 300 ml of medium for each treatment. Fresh and dry weights were determined for each culture. The volume of the composite medium was reduced to approximately 40 ml in vacuo at 40 C. Protein was precipitated from the reduced fraction with 20% trichloroacetic acid and the extract centrifuged at 0 C for 20 min at 10,000 g. The pellet was discarded and the volume of the supernatant

further reduced to 20 ml and digested by boiling with 10 ml of 10% perchloric and 60% nitric acid (v/v). The resulting digest was brought to a known volume of 10 ml with deionized water. Concentrations of iron, manganese, and molybdenum were determined from this sample on the atomic absorption spectrophotometer.

Curvilinear regressions of the second degree were used to plot data from these experiments.

Uptake of ^{86}Rb--^{86}Rb (specific activity 124 mc/g) was obtained from ICN Chemical and Radioisotope Division. Techniques used to measure ^{86}Rb uptake were modified from Epstein, Schmid, and Rains (1963) and Hodges, Darding, and Weidner (1971). Cells of the appropriate age were filtered through Miracloth on a Millipore filter apparatus. Fifty milligrams of tissue were incubated at pH 5.5 in 10 ml of either 0.5 mM $CaCl_2$ (control) or 10^{-4} M ferulic acid in 0.5 mM $CaCl_2$ (test) for approximately 1 hr on a desk rotary shaker.

Experiments were initiated by filtering the tissue through a 0.22 μ Millipore filter and washing several times with 0.5 mM $CaCl_2$. Cells were then placed in 10 ml salt solutions (pH 5.5) ranging in concentration from 0.01 mM to 100 mM $^{86}RbCl$ and incubated on a desk rotary shaker at 25 C for the desired time interval. At concentrations below 0.2 mM RbCl the absorption period was terminated by collecting the cells on 0.22 μ Millipore pads and rinsing them rapidly (60 sec) with an ice-cold wash solution (5 mM RbCl in 0.5 mM $CaCl_2$).

At higher salt concentrations (0.5 mM - 100 mM), the cells were retained in the Millipore filter apparatus for an additional 30 min in approximately 20 ml cold (5 C) wash solution and stirred frequently throughout the desorption period. At the end of the 30-min desorption period, cells were again filtered, washed thoroughly with cold wash solution, and placed into tared planchets. Dry weights of the tissue were determined after 12 hr at 105 C. Dried cells were dispersed with a dilute soap solution, dried under an infrared lamp, and counted for radioactivity on a Nuclear Chicago gas flow counter. Each sample was counted twice for a minimum 10-min period. Aliquots of the stock solutions were counted at the same time.

Linear and curvilinear regressions of the second degree were used to plot the data.

CHAPTER III

RESULTS

Cell cultures and growth--New cultures were initiated by transferring 0.25 - 0.50 g of 14-day tissue to flasks each containing 80 ml new medium. Following inoculation, fresh weight of the culture increased slowly until day 4, after which there was a rapid increase in fresh weight until day 7 (Fig. 1). This rapid increase in fresh weight represents the logarithmic phase of cell division characteristic of these cultures. From days 7 to 10, the cultures underwent a slower rate of increase in fresh weight. This phase is primarily one of cell expansion. After day 10, the cultures demonstrated essentially no increase in fresh weight.

The cultures increased in dry weight from approximately 14 mg at day 0 to over 750 mg by day 14. This represented more than a 54-fold increase in dry weight of the culture through the growth cycle.

Incubation of the cell cultures in varying concentrations of test compounds throughout the 14-day growth cycle revealed that these cultures exhibited distinct tolerances to the different growth inhibitors tested (Table 1). All test

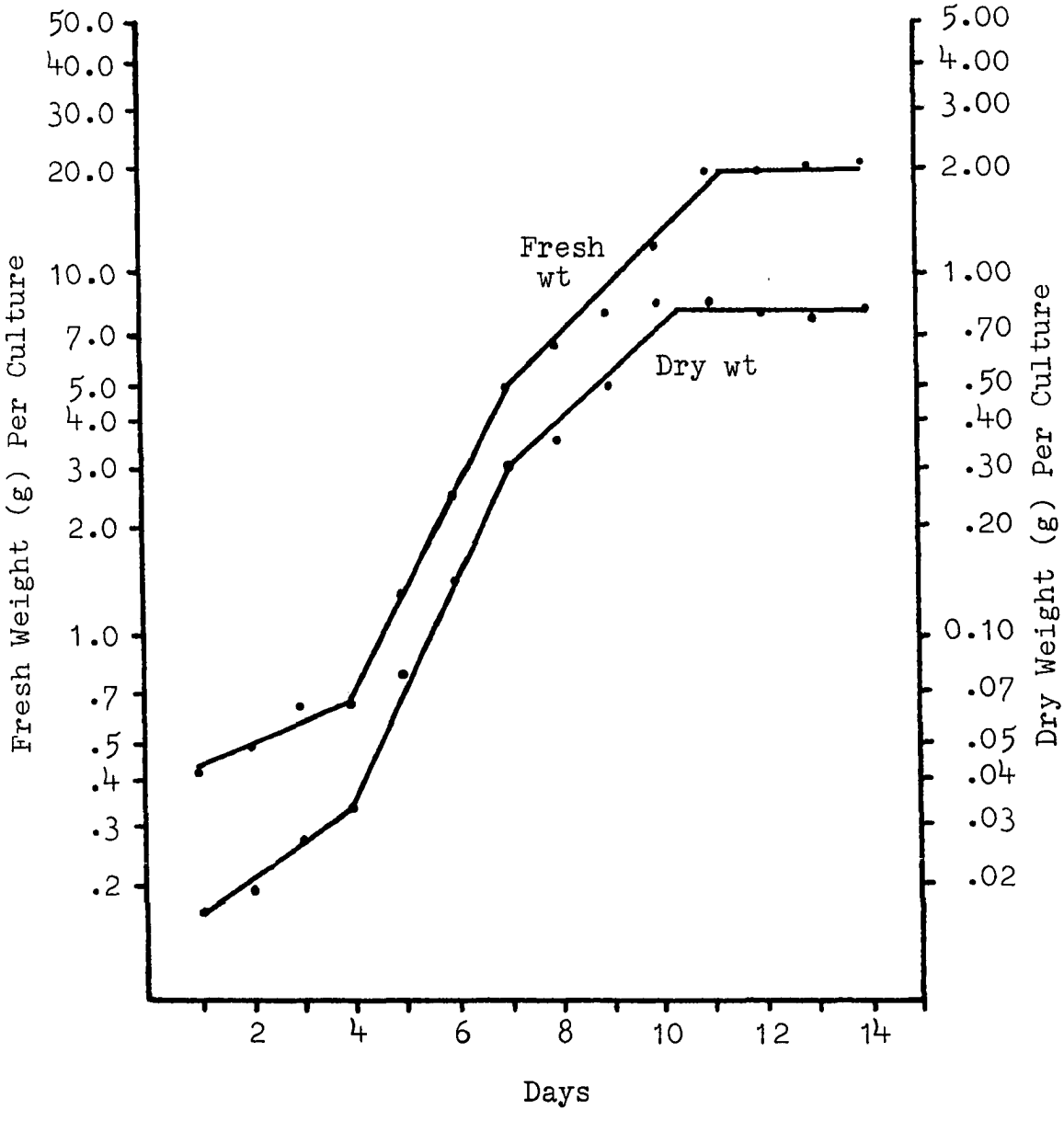


Figure 1. Changes in fresh weight and dry weight during growth of cell cultures.

Table 1. Effect of varying concentrations of test compounds on growth of Paul's Scarlet rose cell suspension cultures.

Compound	Wt.(g)	Age (days) 7 fresh	7 dry	14 fresh	14 dry
Control		4.9871	.3025	19.5075	.7569
Chlorogenic acid					
	10^{-3} M	.7469[a]	.0424[a]	2.6689[a]	.1604[a]
5 x	10^{-4} M	6.0606	.3586	16.9108	.7536
	10^{-4} M	2.8950	.1532	9.2275	.4675
	10^{-6} M	3.1474	.1392	23.8433	.7423
Cinnamic acid					
5 x	10^{-4} M	.1225[a]	.0039[a]	.0735[a]	.0062[a]
	10^{-4} M	.1725[a]	.0055[a]	.1166[a]	.0049[a]
5 x	10^{-5} M	1.4078[a]	.0549[a]	-	-
	10^{-5} M	1.4587[a]	.0703[a]	15.4247	.5628
P-coumaric acid					
	10^{-4} M	1.7641[a]	.0870[a]	10.3726[a]	.4654[a]
	10^{-5} M	3.8761	.2097	18.7055	.7319
Ferulic acid					
	10^{-3} M	.0862[a]	.0101[a]	-	-
	10^{-4} M	1.2026[a]	.0707[a]	6.8336[a]	.4185[a]
	10^{-5} M	4.1996	.2231	19.9087	.6787
	10^{-6} M	3.9337	.2070	21.1565	.7418
Scopoletin					
	10^{-3} M	.1642[a]	.0205[a]	-	-
	10^{-5} M	2.1483	.1183	22.2784	.7708
	10^{-6} M	3.7441	.1977	22.0089	.7084

[a]Significantly different from the control tissue during that growth period.

compounds except chlorogenic acid were completely inhibitory to the growth of the cultures at a 10^{-3} M concentration, resulting in death of the cells prior to the end of the growth cycle. At concentrations below 10^{-4} M, ferulic acid and p-coumaric acids had no measureable effect on the growth of the cells. Chlorogenic acid and scopoletin were ineffective as growth inhibitors when present in concentrations below 10^{-3} M. Cinnamic acid was the most effective inhibitor in retarding growth of the cultures, the lowest effective concentration being 10^{-5} M.

Incubation of cells in 10^{-4} M ferulic acid over the 14-day growth cycle resulted in changes in the growth kinetics of the cell cultures (Fig. 2,3). The logarithmic growth of the control cells occurring between days 3 and 7 has been previously described by Fletcher et al. (1970) as a phase of cell division, whereas the subsequent growth from day 7 to day 10 was shown to be a phase of cell expansion. In the present study, treatment of the cells with ferulic acid resulted in an extension of the division phase to day 9 and the expansion phase was still in progress at the conclusion of the experiment on day 14.

Metabolism of glucose-UL-^{14}C--Treatment of the cells with 10^{-5} M cinnamic acid during the 210-min incubation period in glucose-UL-^{14}C resulted in a significant decrease in incorporation of ^{14}C into the alcohol-insoluble residue (primarily

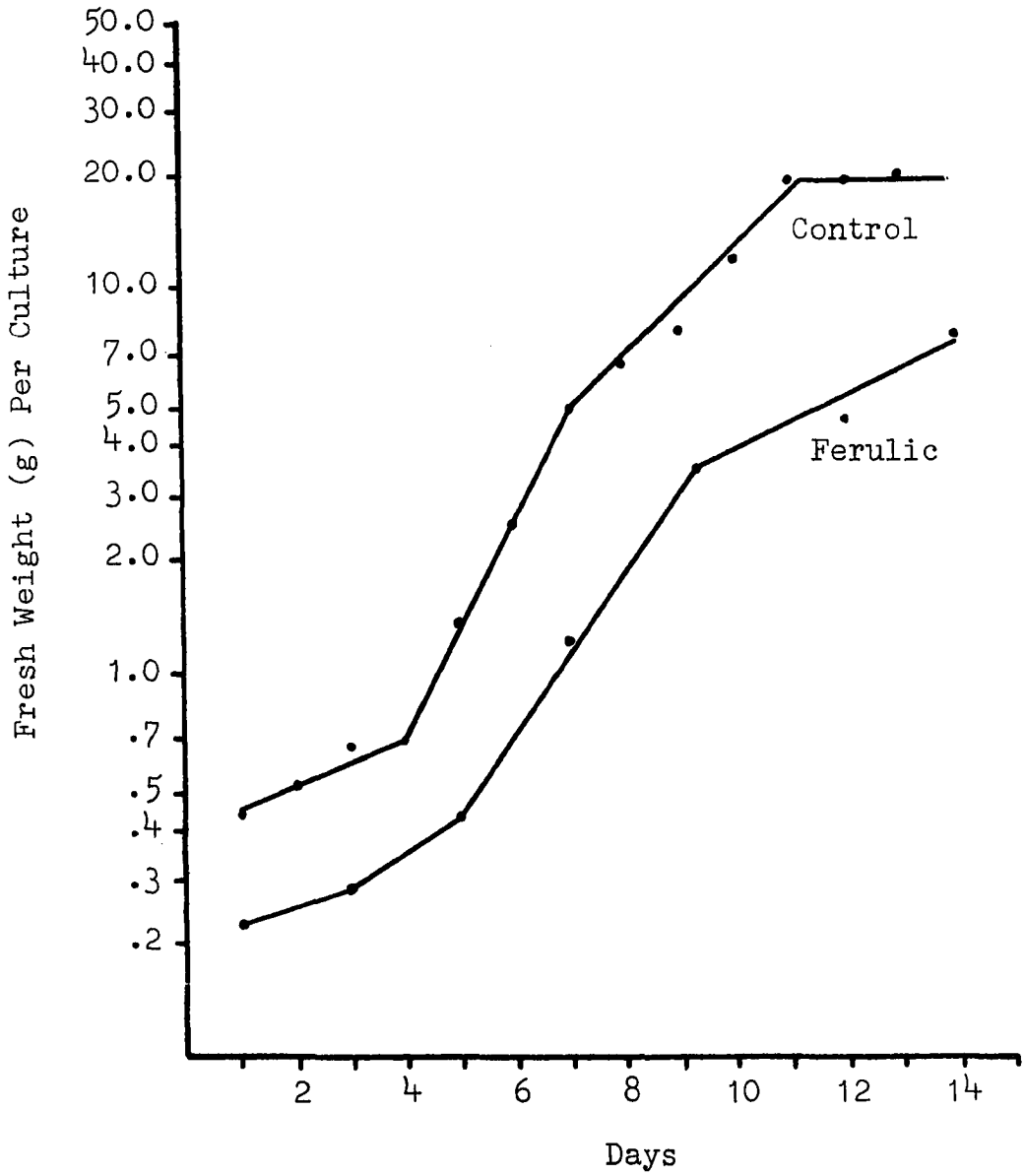


Figure 2. Effect of 10^{-4} M ferulic acid on changes in fresh weight during growth of cell cultures.

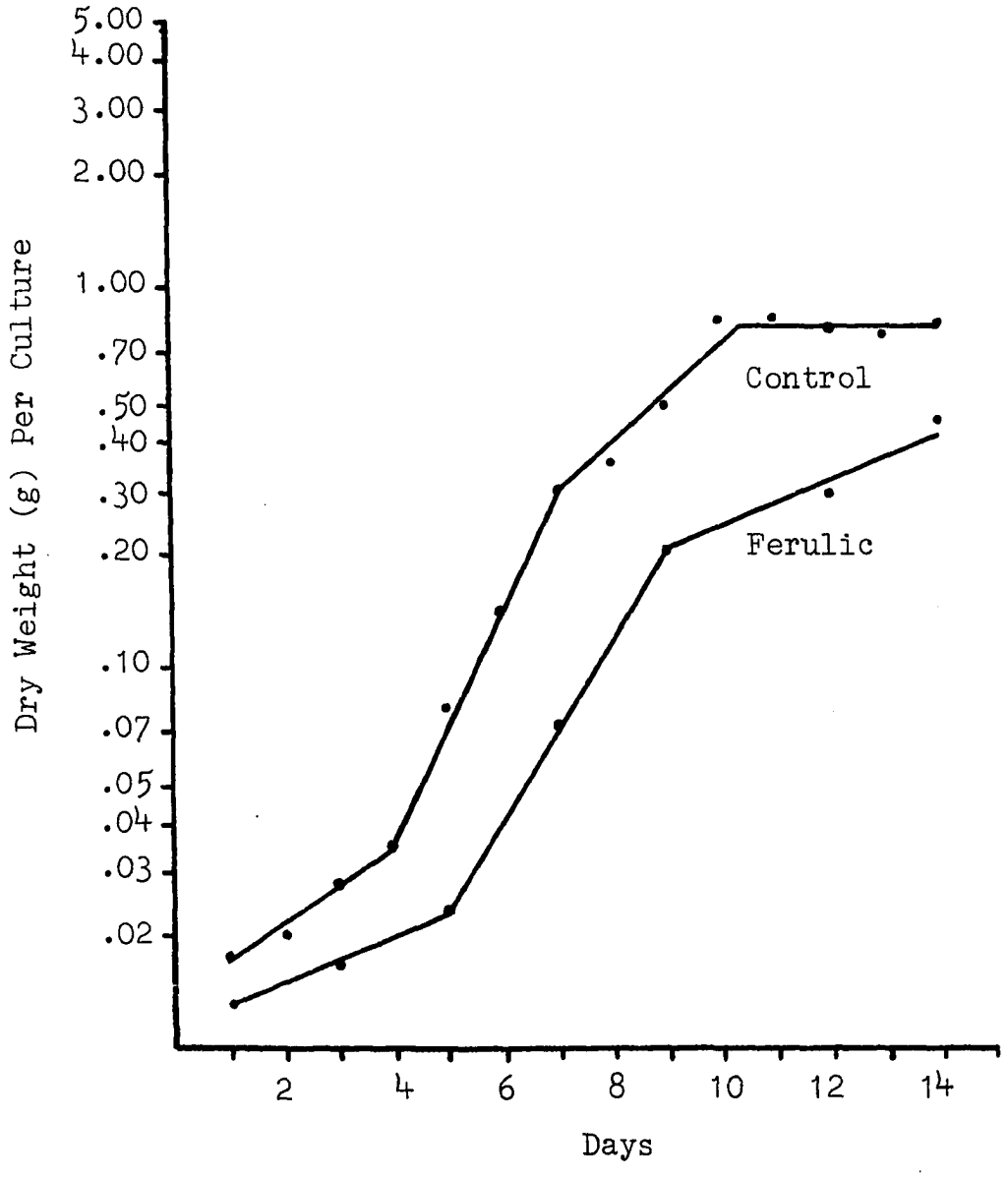


Figure 3. Effect of 10^{-4} M ferulic acid on changes in dry weight during growth of cell cultures.

protein) (Fig. 4). Concomitantly, ^{14}C incorporation into soluble amino acids was significantly increased. Evidence for decreased incorporation into protein was further substantiated by decreased ^{14}C incorporation into protein amino acids.

To determine whether this inhibition of protein synthesis might be a result of the reduced synthesis of a particular amino acid, an analysis of the percent distribution of ^{14}C in soluble amino acids was made in the control and treated cells (Table 2). No significant increase or reduction of incorporation of ^{14}C into a particular amino acid was observed.

A reduction of total incorporation of ^{14}C from glucose into the organic acid fraction occurred in the cinnamic acid-treated cells. To determine the distribution of the label within the constituents of this fraction, the percent distribution of ^{14}C in succinate, malate, and citrate was determined (Table 3). A significantly greater amount of the ^{14}C appeared in succinate as compared with control cells. This was coupled with a simultaneous reduction of ^{14}C into malate and citrate. Incorporation of ^{14}C into soluble lipids was also reduced in cells treated with cinnamic acid (Fig. 4).

Cells treated with 10^{-4} M ferulic acid exhibited a different labeling pattern from control and cinnamic acid-treated cells. A significantly greater portion of the label appeared in the soluble lipid fraction (Fig. 4). This increase

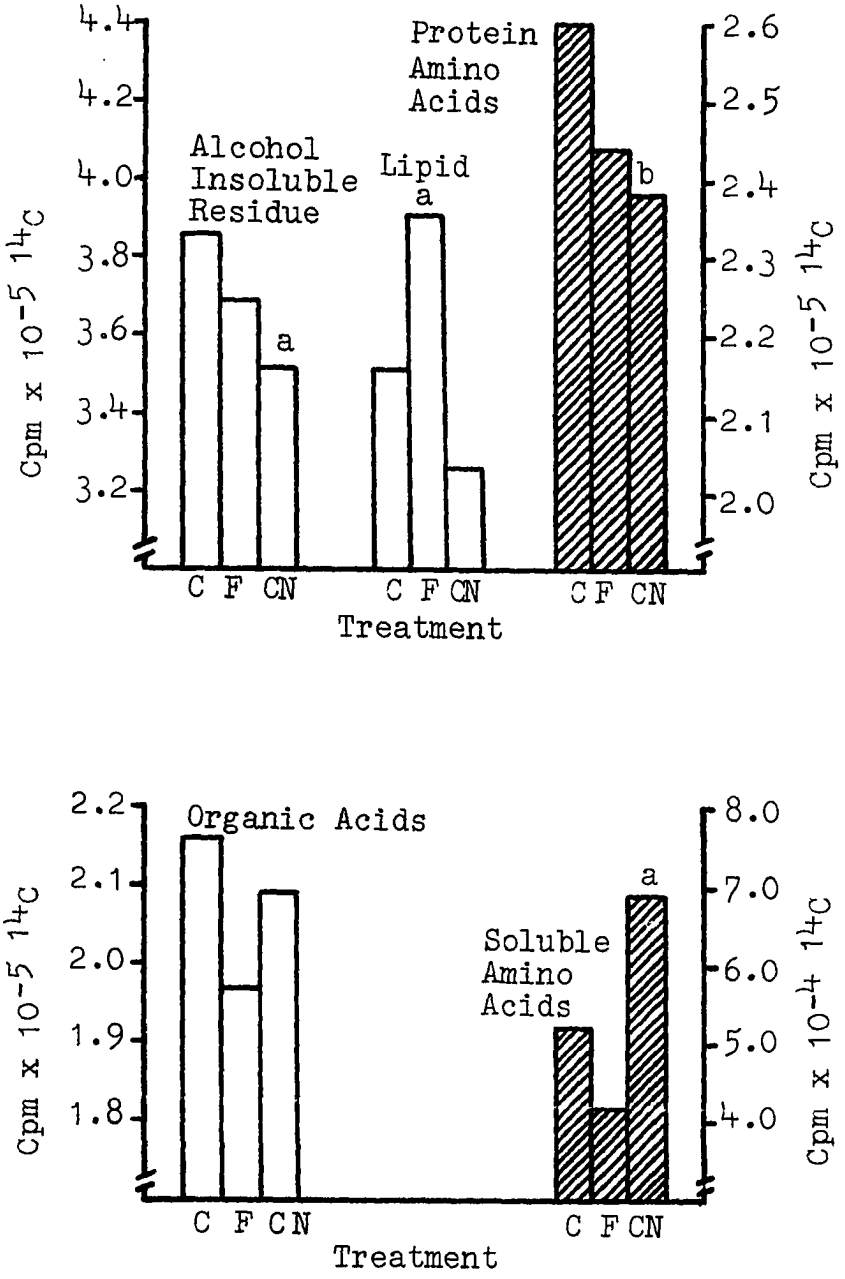


Figure 4. Distribution of ^{14}C in various cell fractions following 210 min incubation of cells in glucose-UL-^{14}C medium containing 10^{-4} M ferulic acid or 10^{-5} M cinnamic acid. Data = mean of three replicates. C, control; F, ferulic acid; CN, cinnamic acid. a = significantly different from the control at 5% level; b = significantly different from the control at 10% level.

Table 2. Percent distribution of ^{14}C in soluble amino acids following 210 min incubation of cells in glucose-UL-^{14}C medium containing ferulic or cinnamic acid. Each figure is the mean of three replicates.

Amino Acid	Control	10^{-4} M Ferulic	10^{-5} M Cinnamic
Aspartate	4.59	3.49	3.39
Serine	12.93	11.44	12.25
Glycine	3.59	3.25	3.76
Glutamate	27.24	22.88	24.03
Asparagine	2.04	2.29	2.34
Threonine	3.22	3.47	3.02
Homoserine	1.64	1.79	1.56
Glutamine	2.68	2.61	1.90
Alanine	21.65	21.87	24.59
Tyrosine	3.97	5.02	5.12
γ-Aminobutyric	5.43	7.45	5.92
Proline	3.40	3.57	4.01
Methionine	3.18	5.98	2.38
Valine	1.95	2.20	1.78
Phenylalanine	1.55	1.97	1.66
Leucine/Isoleucine	1.42	2.15	1.71

Table 3. Percent distribution of ^{14}C in selected organic acids following 210 min incubation of cells in glucose-UL-^{14}C medium containing ferulic or cinnamic acid. Each figure is the mean of three replicates.

Organic Acid	Control	10^{-4} M Ferulic	10^{-5} M Cinnamic
Succinate	7.97	8.71	10.16[a]
Malate	81.51	76.80	77.97
Citrate	5.29	5.66	4.91

[a]Significantly different from the control, .05 level.

was accompanied by decreased incorporation of ^{14}C into the remaining fractions: protein, organic acids, and amino acids.

Although ^{14}C incorporation into total protein and soluble amino acids was reduced, no significant alteration in the percentage distribution of ^{14}C in the constituent amino acids was noted in either of these fractions (Table 2,4). In contrast, changes in the distribution of ^{14}C in organic acids were apparent when compared with control cells. Incorporation of ^{14}C into succinate was slightly enhanced and incorporation into malate slightly reduced (Table 3).

Mineral depletion--During the 14-day growth cycle, the concentration of magnesium and potassium was decreased to less than 20% of the initial level in the control medium; phosphorus decreased to 6% and calcium to 57% of the original value (Fig. 5-8). Rates of removal of these macronutrients calculated on a μmole/flask/day basis indicated that, except for magnesium, the maximum rate of decrease of macronutrients per flask occurred between days 7 - 9 of the growth cycle (Table 5). Maximum rates of removal of calcium per gram fresh weight of tissue occurred, however, between days 3 - 7 (Table 6). In contrast, the highest rate of removal of potassium, magnesium, and phosphorus occurred early in the growth cycle, shortly after initiation of the new cultures. Similar patterns of depletion occurred when removal of macronutrients from a single flask was considered.

Table 4. Percent distribution of ^{14}C in the protein amino acids following 210 min incubation of cells in glucose-UL-^{14}C medium containing ferulic or cinnamic acid. Each figure is the mean of three replicates.

Amino Acid	Control	10^{-4} M Ferulic	10^{-5} M Cinnamic
Aspartate	9.84	9.63	9.40
Serine	7.61	7.68	7.47
Glycine	10.15	9.32	9.57
Glutamate	10.69	12.19	11.62
Asparagine	1.96	2.88	2.48
Threonine	4.14	3.86	4.05
Alanine	10.93	10.90	10.44
Tyrosine	5.40	5.59	6.75
Valine	8.84	8.09	8.57
Phenylalanine	9.89	9.72	9.81
Leucine/Isoleucine	14.64	13.94	14.27
Proline	4.16	4.18	4.04
Methionine	1.75	1.75	1.52

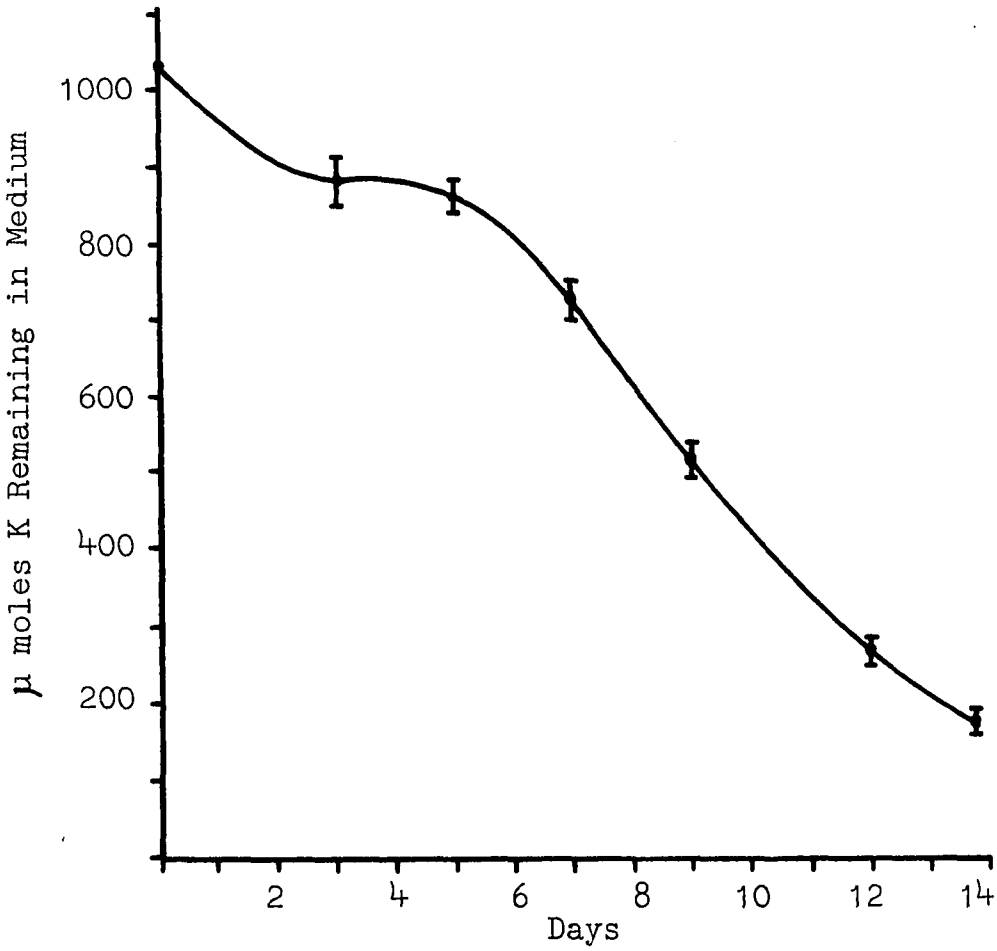


Figure 5. Removal of potassium from medium during growth of cell cultures. Each point represents the mean of six flasks.

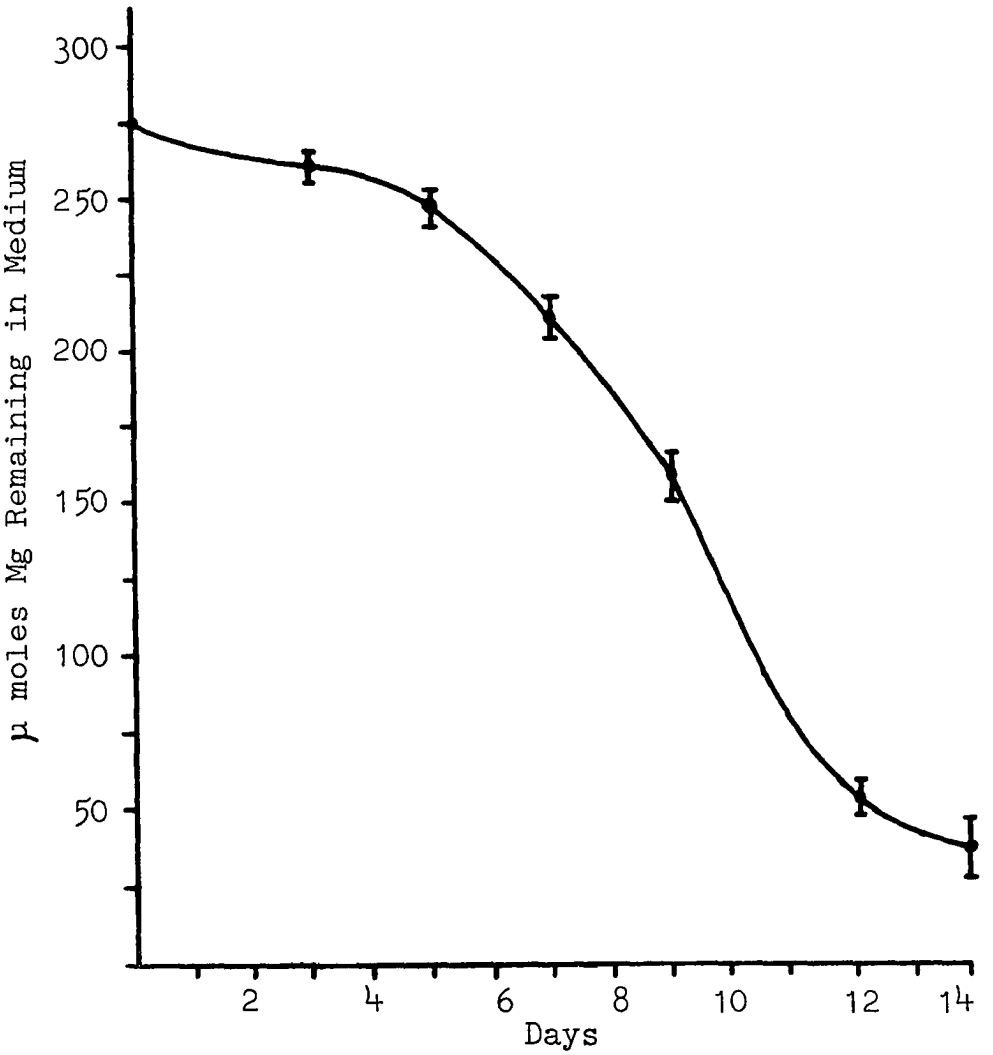


Figure 6. Removal of magnesium from medium during growth of cell cultures. Each point represents the mean of six flasks.

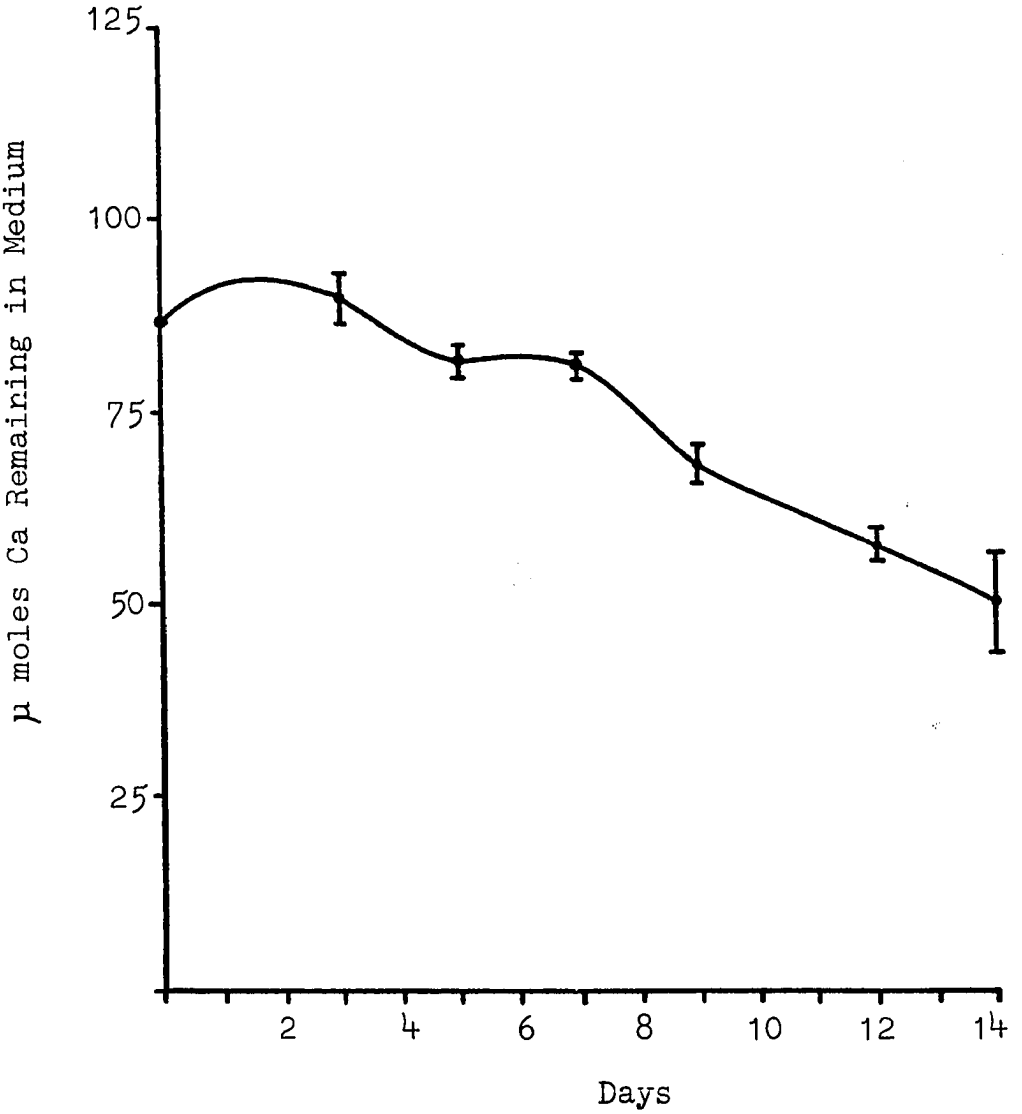


Figure 7. Removal of calcium from medium during growth of cell cultures. Each point represents the mean of six flasks.

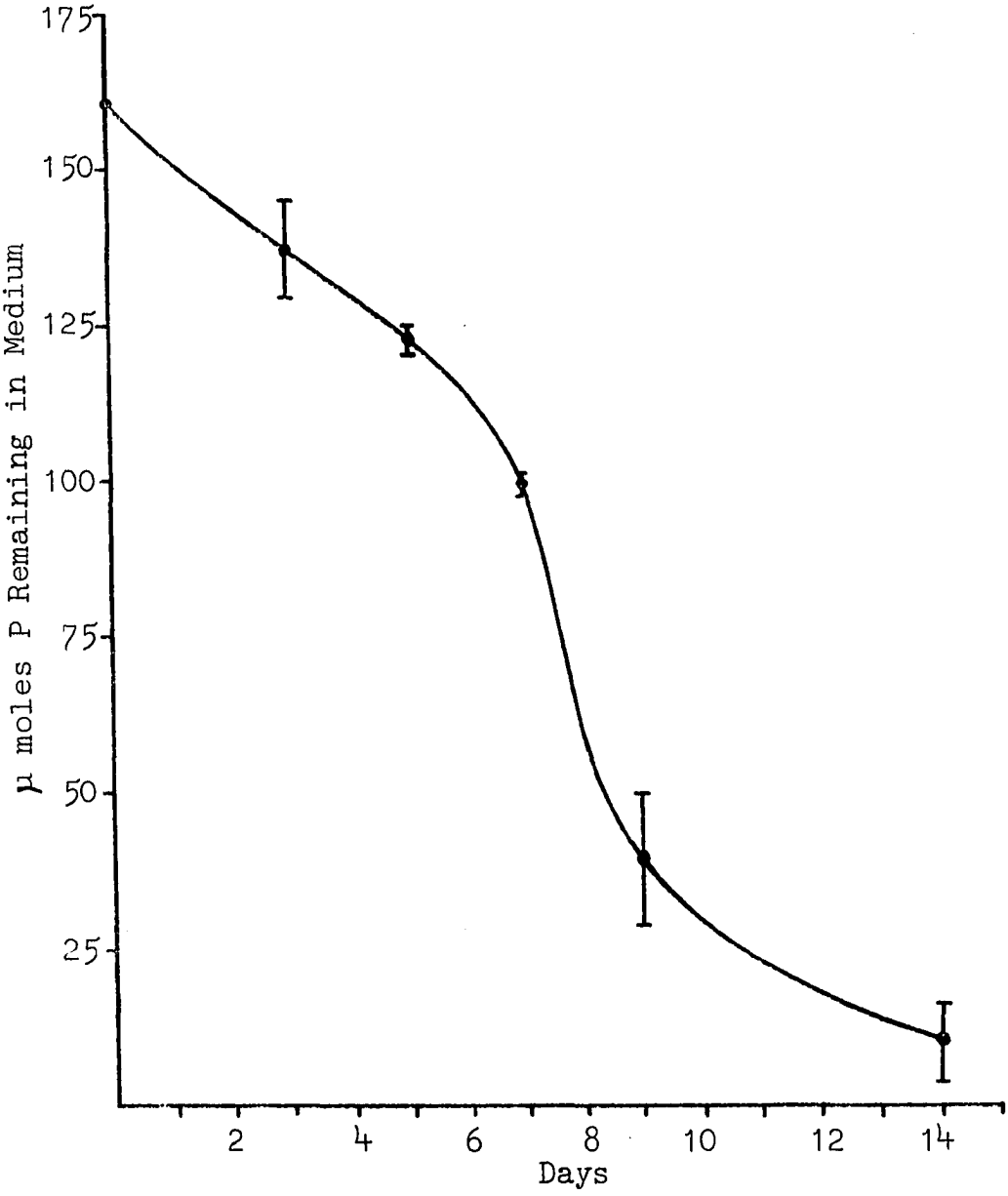


Figure 8. Removal of phosphorus from medium during growth of cell cultures. Each point represents the mean of two flasks.

Table 5. Rate of decrease of macronutrients in media containing 10^{-4} M ferulic acid in μ moles/flask/day.

Days	Ca[a]		K[a]		P[b]		Mg[a]	
	C[c]	F[d]	C	F	C	F	C	F
0 - 3	-1.34	-.74	53.40	38.66	7.28	8.12	4.92	3.52
3 - 5	4.37	-3.26	4.37	-45.36	6.76	-5.16	5.03	-6.06
5 - 7	.34	9.13	69.79	71.02	11.96	.52	18.36	14.25
7 - 9	6.15	.54	107.31	22.12	30.61	2.63	23.92	6.46
9 - 12	3.57	.55	83.32	66.86	7.37	4.19	37.18	19.20
12 - 14	2.66	6.86	47.62	30.95	2.79	2.23	4.88	44.37

[a]Figures are the mean of six flasks
[b]Figures are the mean of two flasks
[c]C, control cells
[d]F, cells treated with ferulic acid

Table 6. Rate of decrease of macronutrients in media containing 10^{-4} M ferulic acid in μ moles/g/day.

Days	Mg[a]		Ca		K		P[b]	
	C[c]	F[d]	C	F	C	F	C	F
0 - 3	9.74	12.88	-2.65	-2.70	105.72	141.61	14.42	29.74
3 - 5	6.25	-14.38	5.43	-7.72	6.63	-107.58	6.11	-12.24
5 - 7	9.13	11.85	.17	7.59	34.71	59.05	4.10	.43
7 - 9	4.80	1.86	1.24	.16	21.56	6.35	4.31	.76
9 - 12	3.10	4.22	.30	.12	6.96	.70	.45	.92
12 - 14	.30	6.49	.16	1.03	2.89	4.53	.17	.33

[a]Magnesium, calcium, and potassium figures are the mean of six flasks.
[b]Phosphorus figures are the mean of two flasks.
[c]C, control
[d]F, 10^{-4} M ferulic acid

Treatment of cells with 10^{-4} M ferulic acid over the 14-day growth period decreased the depletion of macronutrients per flask (Fig. 9-12). For example, the concentration of potassium in the medium decreased to 61% of the initial level and magnesium to 33%, while the concentration of calcium and phosphorus in the medium decreased to 68% and 82% of the original value. Maximum rates of removal of calcium and potassium per flask occurred between days 5 - 7, whereas maximum rates of removal of magnesium per flask occurred late in the growth cycle, between days 12 - 14 (Table 5). In contrast, the highest rate of depletion of phosphorus occurred early in the growth cycle, between days 0 - 3. When rates of removal were calculated on the basis of fresh weight, cells treated with ferulic acid consistently depleted macronutrients, except phosphorus, at a higher rate on days 5 - 7 than control cells (Table 6).

The overall pattern of removal of macronutrients through the growth cycle was also altered by treatment of the cells with ferulic acid. Test cultures showed a consistent loss of macronutrients between days 3 - 5 with a subsequent increase in the rate of depletion (Fig. 9-12). The total depletion of macronutrients on a fresh weight basis was greater in cells treated with ferulic acid than in control cells with the exception of phosphorus (Table 7).

Patterns of depletion of iron and manganese from control and test cultures containing 10^{-4} M ferulic acid were

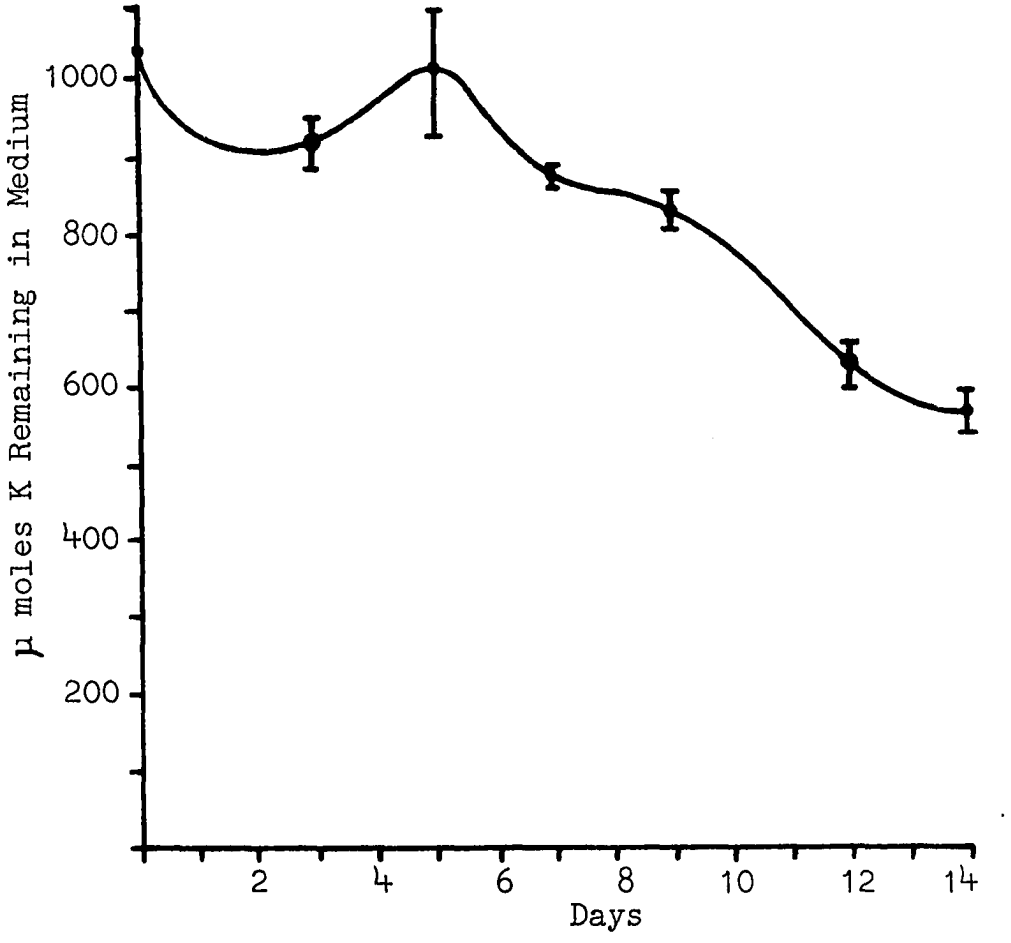


Figure 9. Effect of 10^{-4} M ferulic acid on removal of potassium from medium during growth of cell cultures. Each point represents the mean of six flasks.

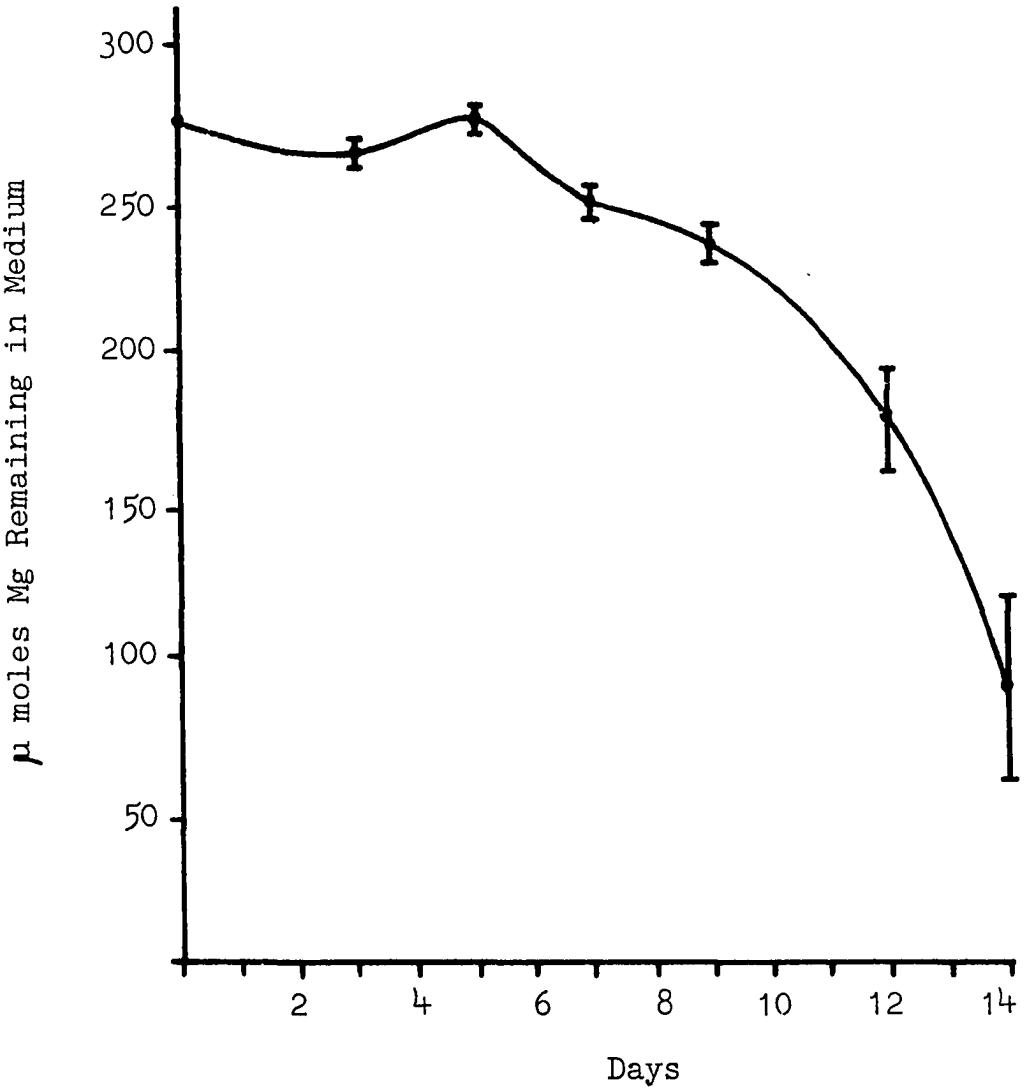


Figure 10. Effect of 10^{-4} M ferulic acid on removal of magnesium from medium during growth of cell cultures. Each point represents the mean of six flasks.

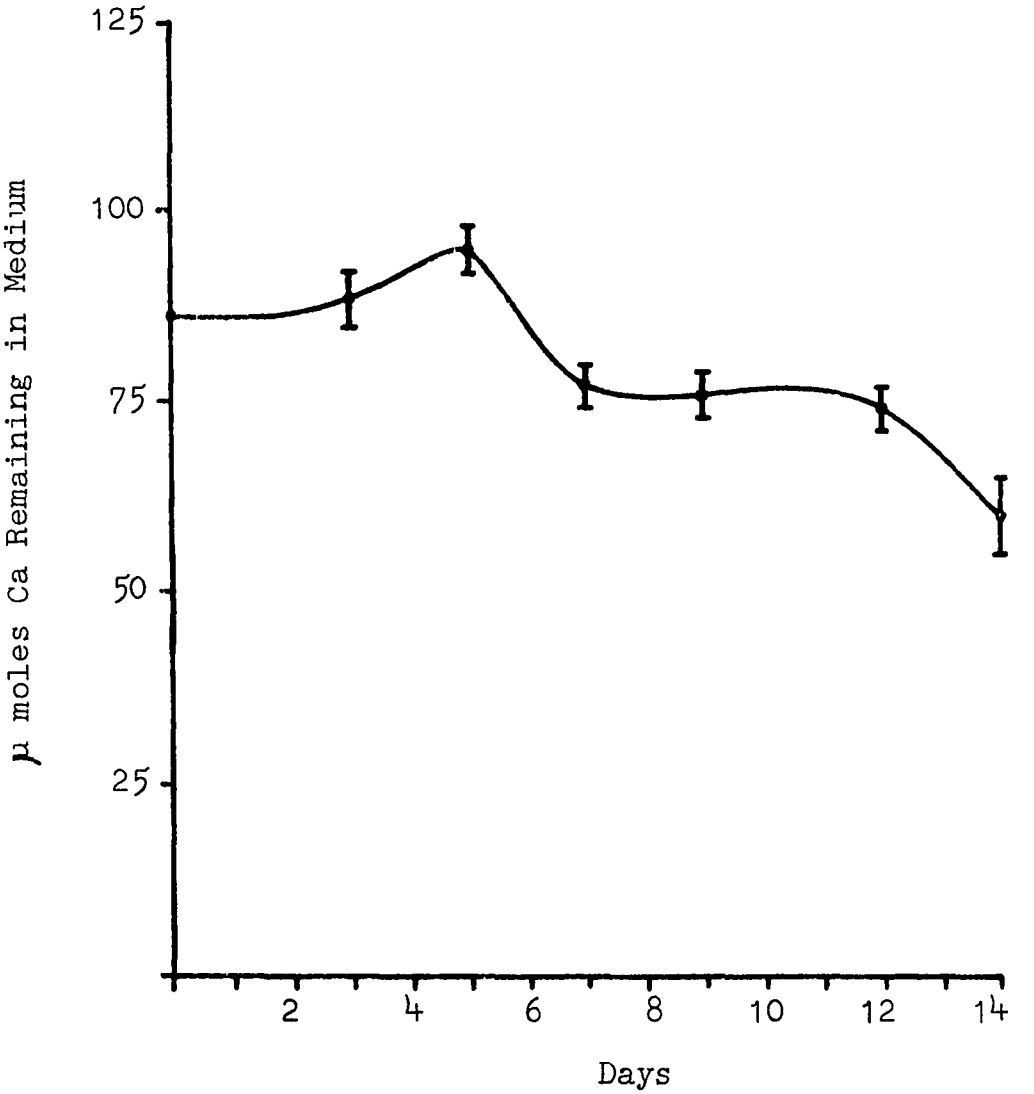


Figure 11. Effect of 10^{-4} M ferulic acid on removal of calcium from medium during growth of cell cultures. Each point represents the mean of six flasks.

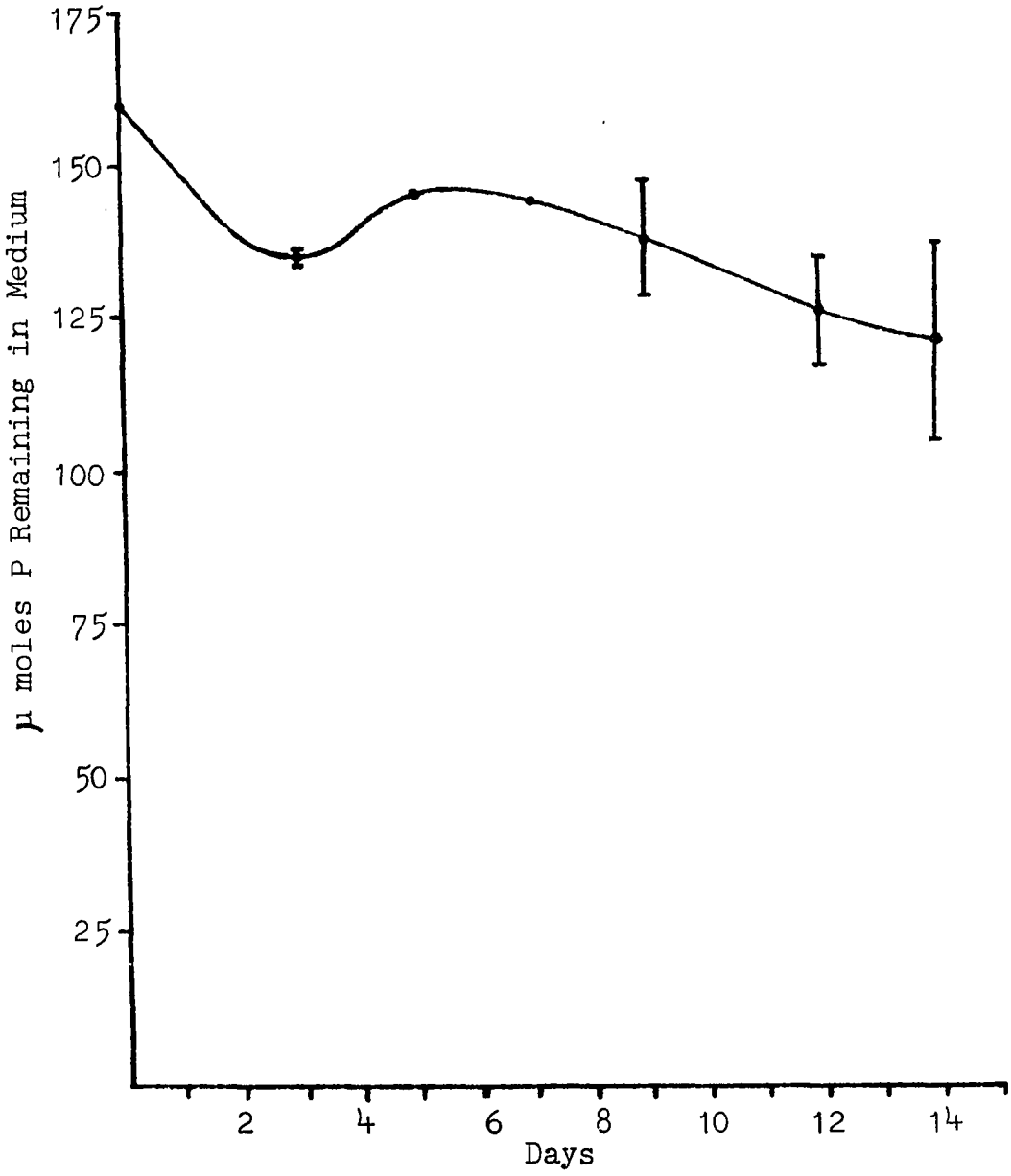


Figure 12. Effect of 10^{-4} M ferulic acid on removal of phosphorus from medium during growth of cell cultures. Each point represents the mean of two flasks.

Table 7. Total depletion of macronutrients in medium containing 10^{-4} M ferulic acid in μ moles/g/14 days.

Ion	Control	10^{-4} M Ferulic
K	52.36	69.36
Mg	14.20	27.24
Ca	2.19	3.80
P	8.93	5.47

similar throughout the 14-day growth cycle (Fig. 13,14). The concentration of manganese and iron in the control medium decreased to 28% and 45% of the original concentration as compared with 38% and 26%, respectively, in test cultures. The maximum rate of removal of iron and manganese per flask in both control and test cultures occurred between days 12 - 14 (Table 8). This rate was greater in cells treated with ferulic acid. In both test and control cultures, the greatest rate of removal of iron per gram fresh weight occurred between days 0 - 3, whereas the greatest rate of removal of manganese per gram fresh weight occurred between days 3 - 5 and again, these rates were higher for cells treated with ferulic acid. The concentration of molybdenum in the test medium decreased to 59% of the initial concentration as compared to 31% in the control medium (Fig. 15). In both control and test cultures, the highest rate of decrease of molybdenum per flask occurred between days 12 - 14 and was greater in control cells. However, rates of decrease calculated on a fresh weight basis indicated that the greatest rate of decrease of molybdenum occurred between days 0 - 3 in cells treated with ferulic acid.

^{86}Rb uptake--Both 5-day and 11-day cells showed dual patterns of absorption of ^{86}Rb (Fig. 16). At low external salt concentrations, cells reached a maximum rate of absorption at 0.02 mM RbCl, while at high external salt concentrations cells had not reached a maximum rate of absorption of

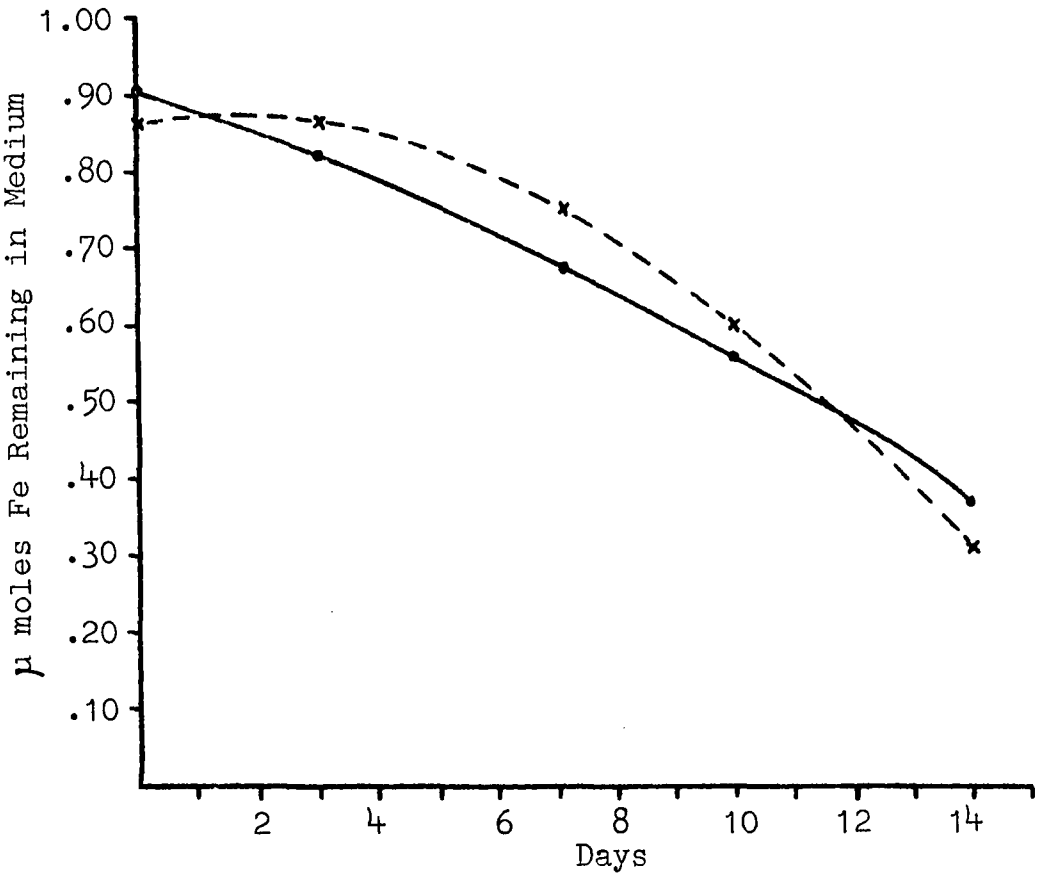


Figure 13. Effect of 10^{-4} M ferulic acid on removal of iron from medium during growth of cell cultures. ·———·, control; x- - - -x, cells treated with ferulic acid.

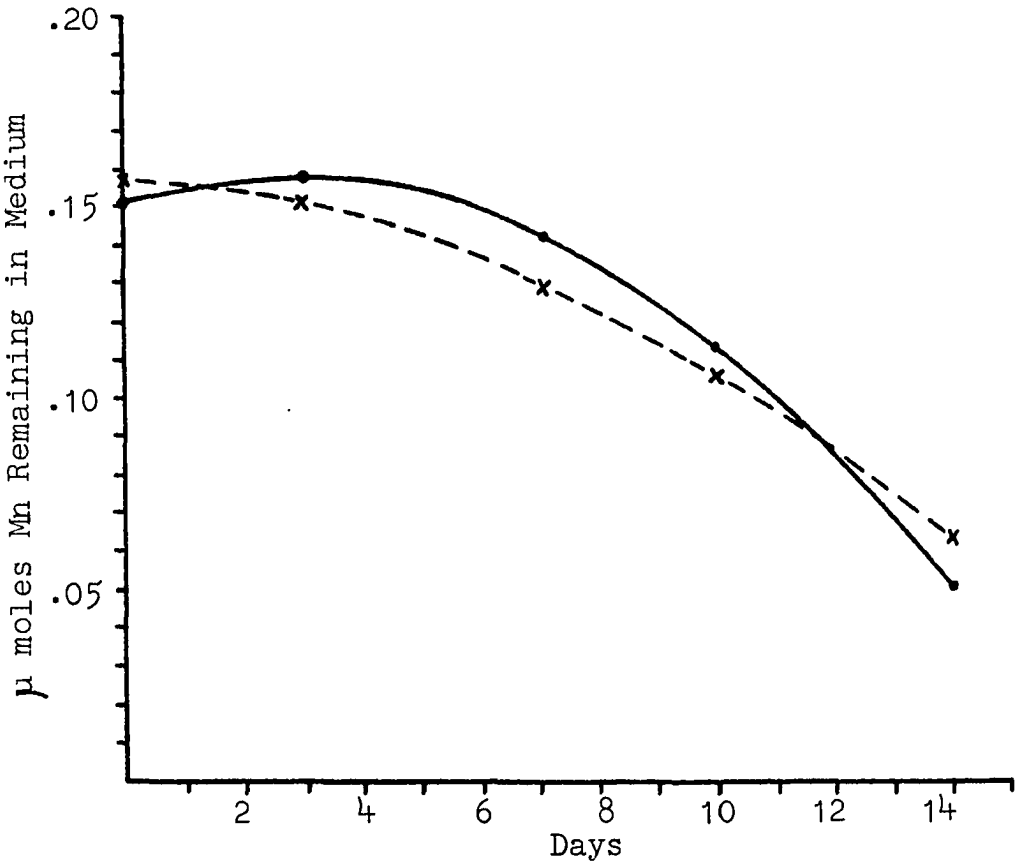


Figure 14. Effect of 10^{-4} M ferulic acid on removal of manganese from medium during growth of cell cultures. ·———·, control; x- - - -x, cells treated with ferulic acid.

Table 8. Rate of decrease of micronutrients in medium containing 10^{-4} M ferulic acid in μ moles/g/day and μ moles/flask/day.

	μ moles/g/day						μ moles/flask/day					
	Fe		Mo		Mn		Fe		Mo		Mn	
Days	C[a]	F[b]	C	F	C	F	C	F	C	F	C	F
0-3	.057	.006	.006	.015	-.005	.008	.029	-.002	.003	.004	-.002	.002
3-5	.029	.051	.005	.012	.002	.011	.030	.021	.005	.005	.002	.005
5-7	.013	.027	.003	.004	.002	.005	.040	.033	.009	.005	.006	.006
7-9	.007	.014	.001	.001	.001	.002	.040	.050	.010	.005	.009	.007
9-12	.002	.015	.008	.001	.001	.002	.040	.067	.013	.005	.013	.010
12-14	.003	.011	.001	.001	.001	.002	.055	.076	.014	.006	.017	.011

[a] C, control cells

[b] F, cells treated with 10^{-4} M ferulic acid

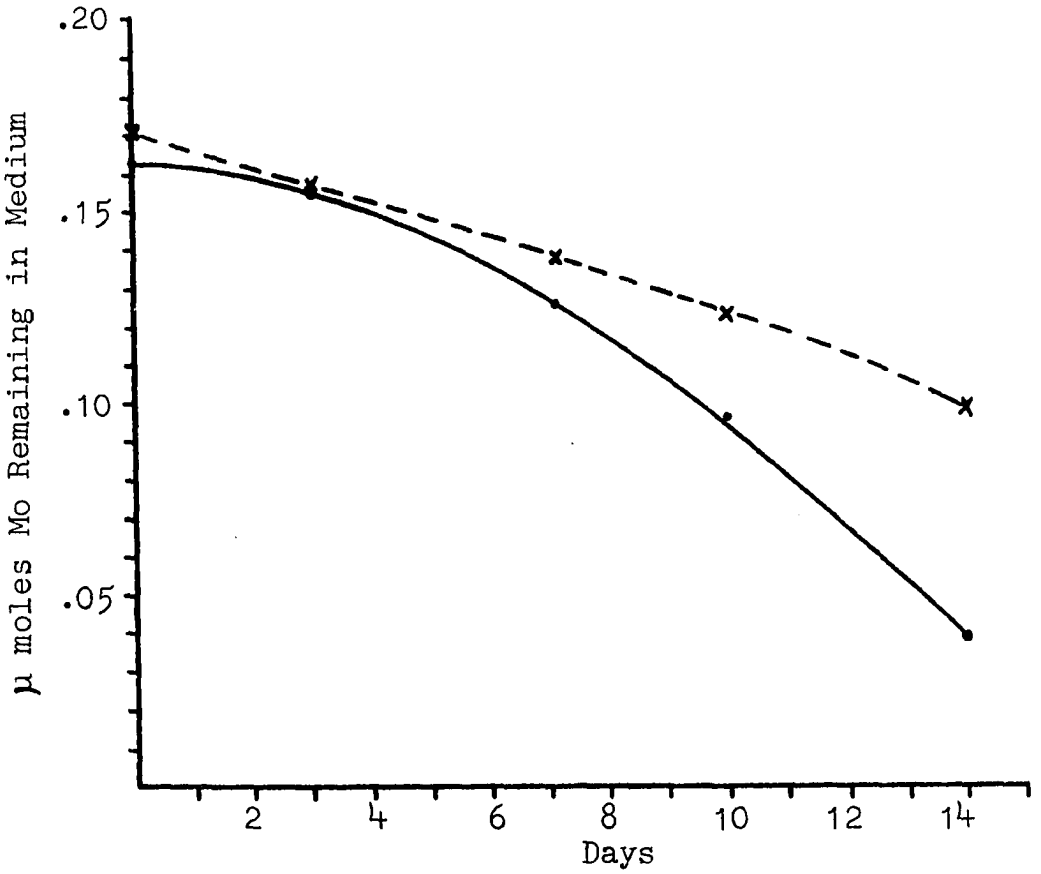


Figure 15. Effect of 10^{-4} M ferulic acid on removal of molybdenum from medium during growth of cell cultures. •———•, control; x - - - -x, cells treated with ferulic acid.

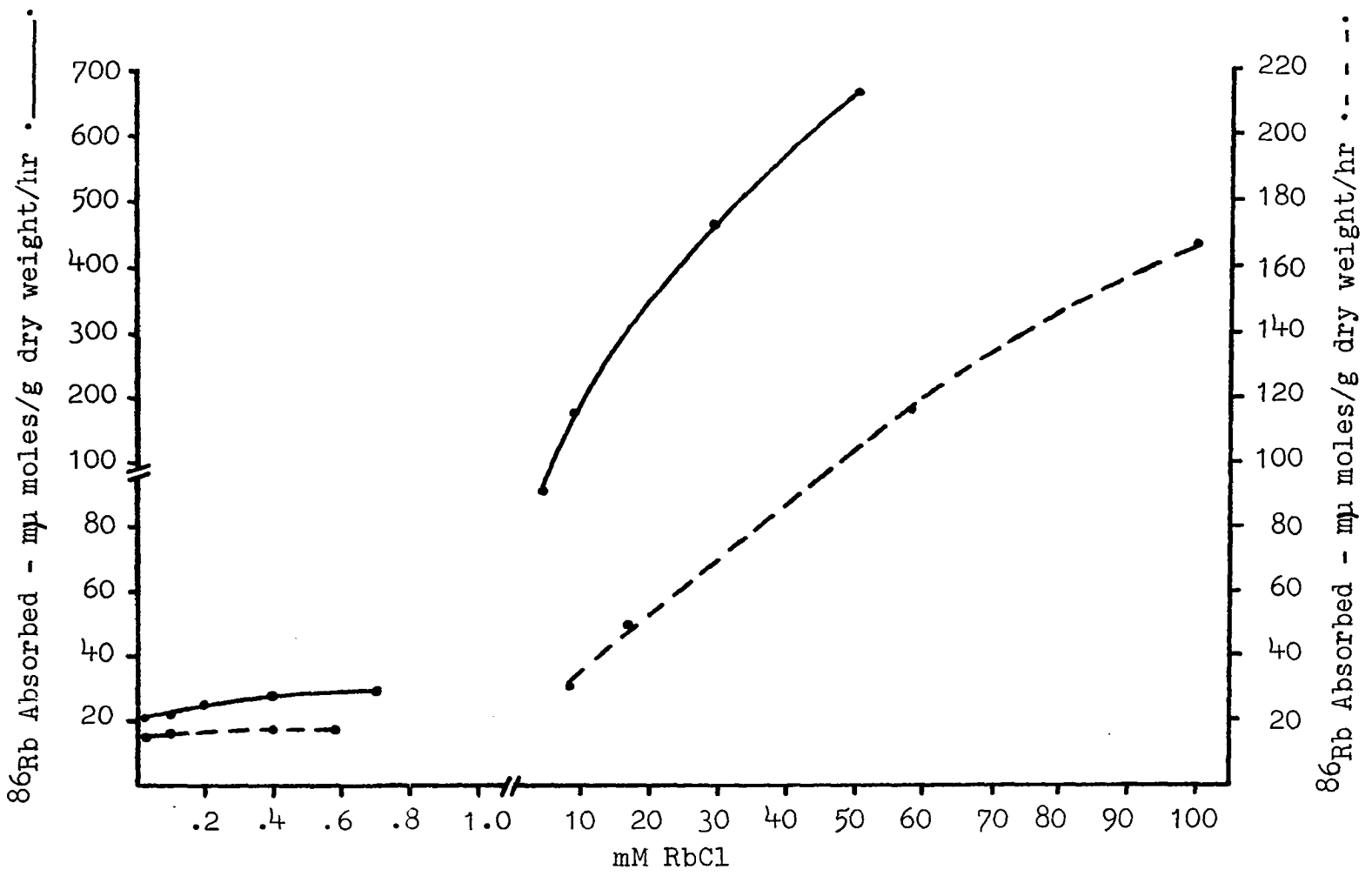


Figure 16. Rate of ^{86}Rb absorption in 5 day and 11 day cells as a function of the concentration of RbCl, over the range 0.02 mM to 100 mM. ·——·, 5 day cells; x- - - -x, 11 day cells.

^{86}Rb at external concentrations of 50 - 100 mM RbCl. Cells of all ages, and at both high and low concentrations of RbCl, attained a linear rate of absorption after approximately 10 min incubation in the solutions (Fig. 17-28). The rate of absorption of ^{86}Rb varied with age of the tissue and the concentration of the bathing medium. For example, the rate of absorption of ^{86}Rb from 5.0 mM RbCl solutions increased markedly (1.7 fold) when 4-day and 5-day-old cells were compared, while the rate of uptake from 0.2 mM RbCl increased only slightly (Fig. 17-24). In contrast, the rate of absorption of ^{86}Rb from both 0.2 mM and 5.0 mM RbCl solutions decreased significantly in older 10-day cells as compared to younger cells (Fig. 25-28). Rates of absorption from 5.0 mM solutions of RbCl were consistently higher than rates of absorption from 0.2 mM solutions at all ages.

Incubation of 4-day and 5-day cells in 10^{-4} M ferulic acid during the 10-min absorption period resulted in inhibition of uptake of ^{86}Rb from both 0.2 mM and 5.0 mM solutions (Fig. 17-24). However, inhibition of uptake was consistently greater at high external salt concentrations (5.0 mM RbCl). In contrast, incubation of 10-day cells in the same concentration of inhibitor had very little effect on the rate of ^{86}Rb absorption at either high or low external salt concentrations. In fact, ferulic acid appeared to stimulate absorption of ^{86}Rb from 5.0 mM RbCl solutions.

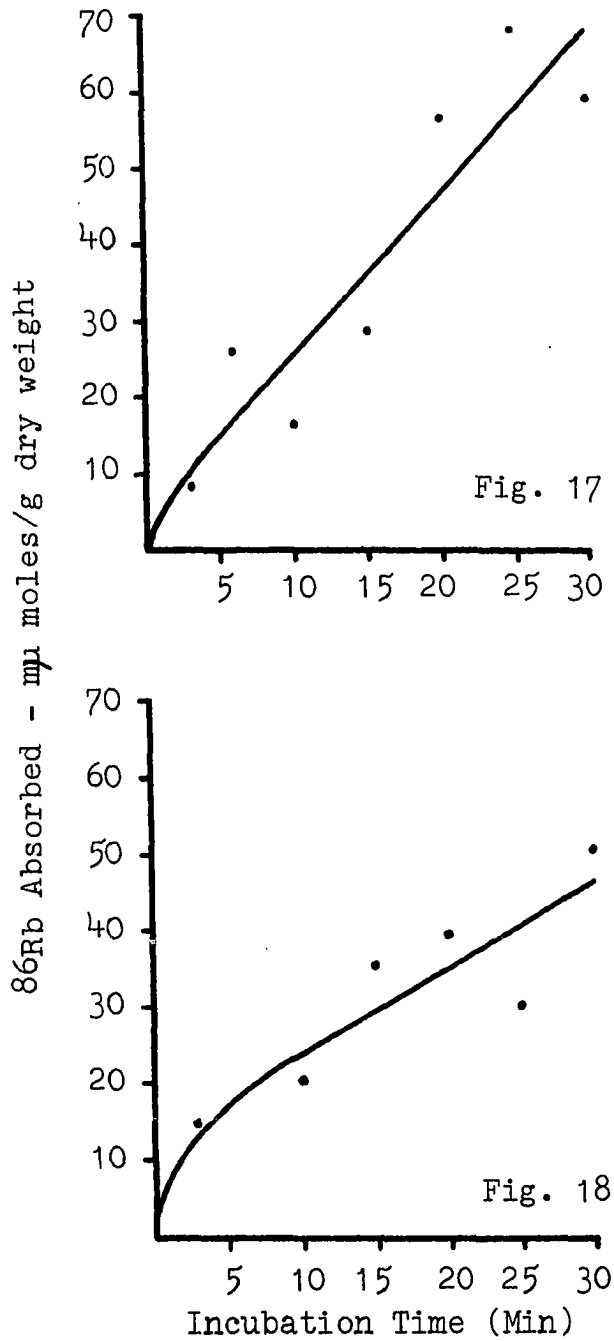


Figure 17. Rate of uptake of ^{86}Rb from 0.2 mM RbCl by normal 4 day cells. Control rate = 11.16 mμ moles/g dry wt/hr.

Figure 18. Effect of 10^{-4} M ferulic acid on rate of uptake of ^{86}Rb from 0.2 mM RbCl by 4 day cells. Test rate = 8.75 mμ moles/g dry wt/hr.

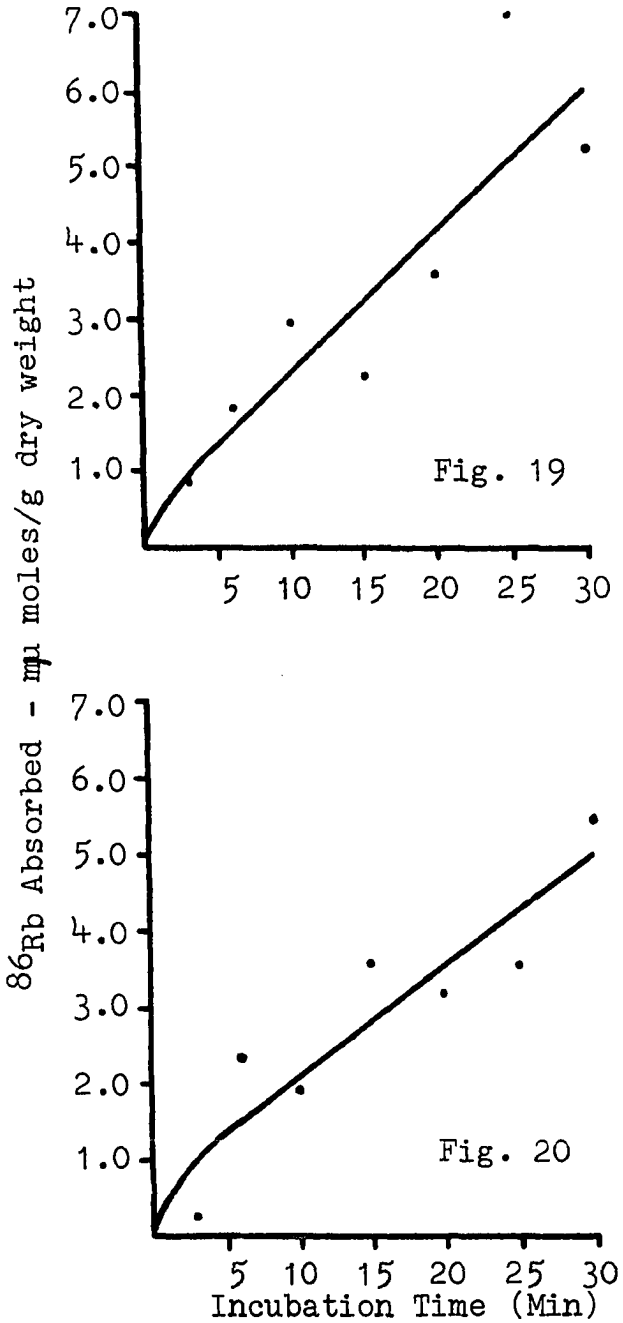


Figure 19. Rate of uptake of ^{86}Rb from 5.0 mM rbCl by normal 4 day cells. Control rate = 129.25 mμ moles/g dry wt/hr.

Figure 20. Effect of 10^{-4} M ferulic acid on rate of uptake of ^{86}Rb from 5.0 mM RbCl by 4 day cells. Test rate = 70.02 mμ moles/g dry wt/hr.

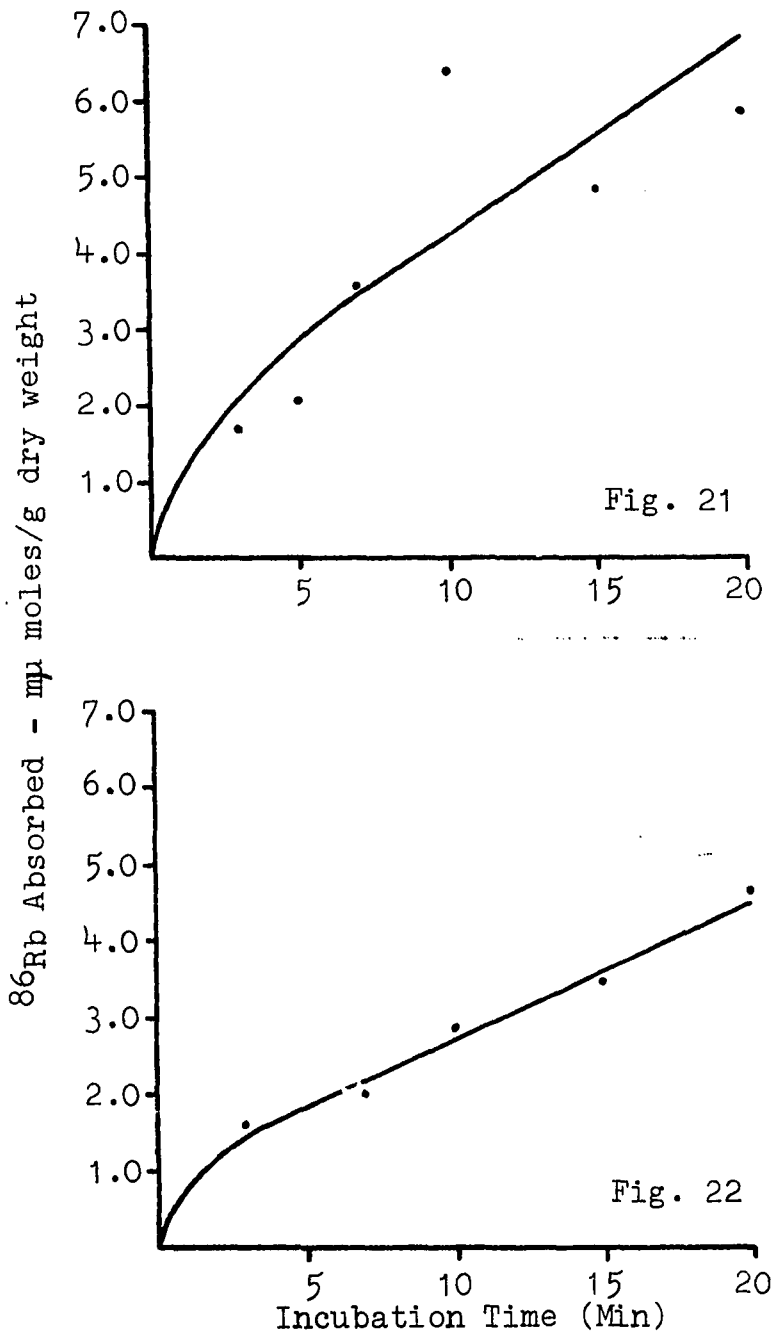


Figure 21. Rate of uptake of ^{86}Rb from 0.2 mM RbCl by normal 5 day cells. Control rate = 14.17 mμ moles/g dry wt/hr.

Figure 22. Effect of 10^{-4} M ferulic acid on rate of uptake of ^{86}Rb from 0.2 mM RbCl by 5 day cells. Test rate = 10.81 mμ moles/g dry wt/hr.

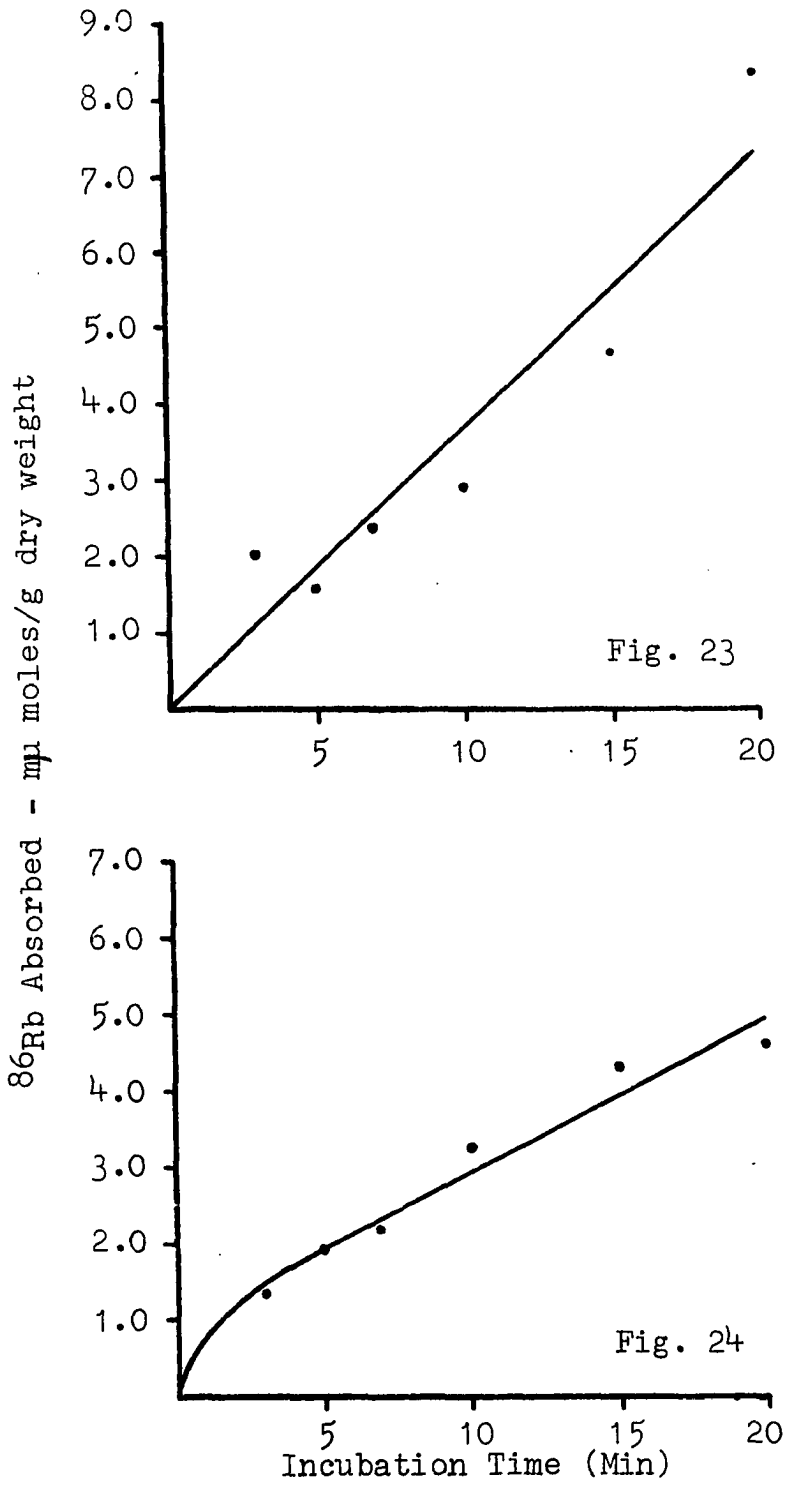


Figure 23. Rate of uptake of ^{86}Rb from 5.0 mM RbCl by normal 5 day cells. Control rate = 224.32 mμ moles/g dry wt/hr.

Figure 24. Effect of 10^{-4} M ferulic acid on rate of uptake of ^{86}Rb from 5.0 mM RbCl by 5 day cells. Test rate = 122.82 mμ moles/g dry wt/hr.

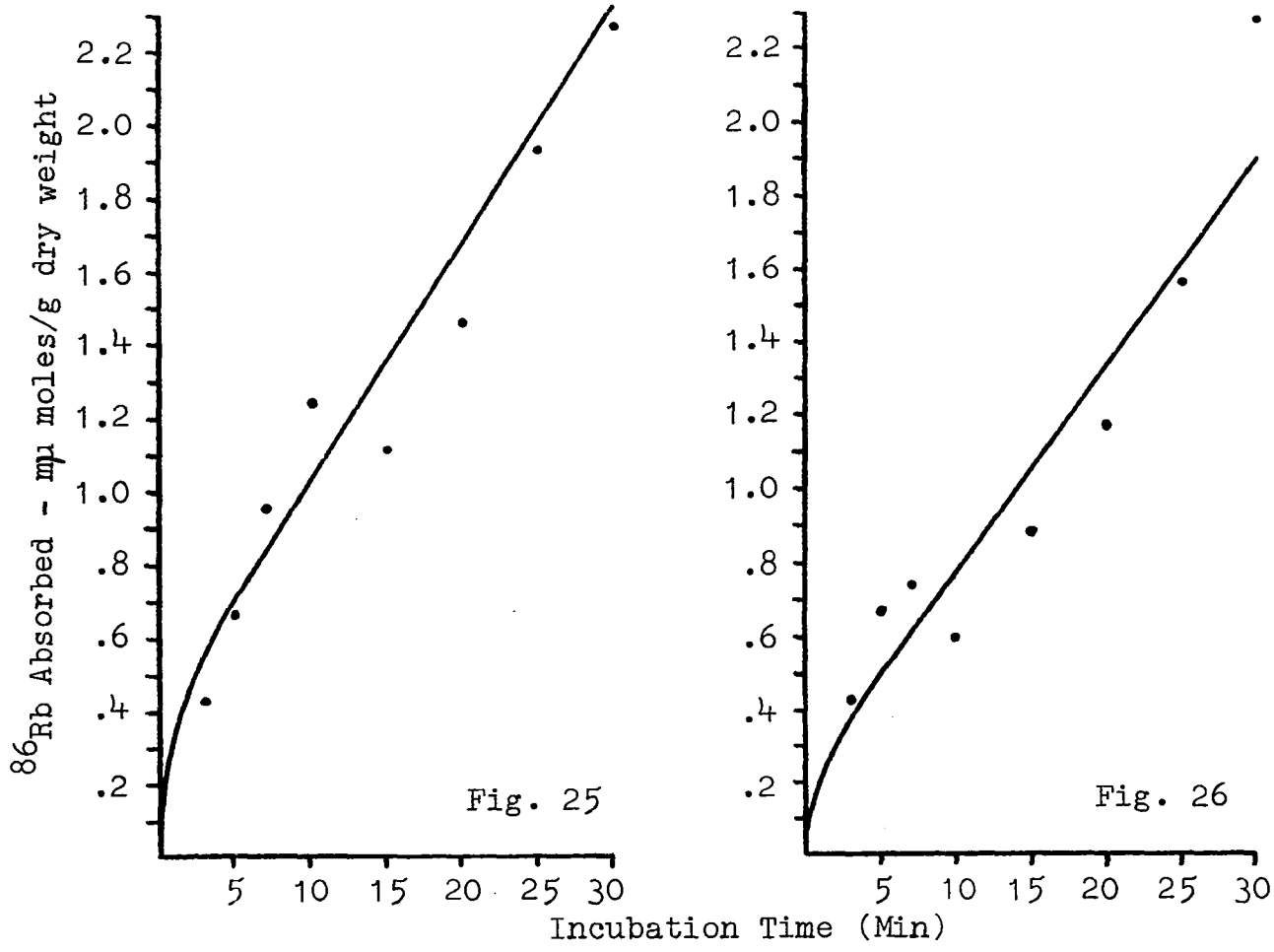


Figure 25. Rate of uptake of ^{86}Rb from 0.2 mM RbCl by normal 10 day cells. Control rate = 3.51 mμ moles/g dry wt/hr.

Figure 26. Effect of 10^{-4} M ferulic acid on rate of uptake of ^{86}Rb from 0.2 mM RbCl by 10 day cells. Test rate = 3.39 mμ moles/g dry wt/hr.

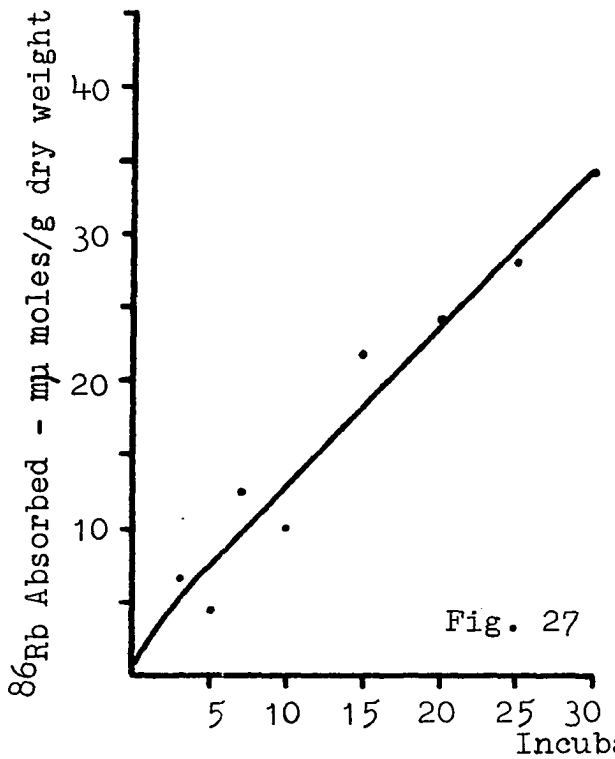


Fig. 27

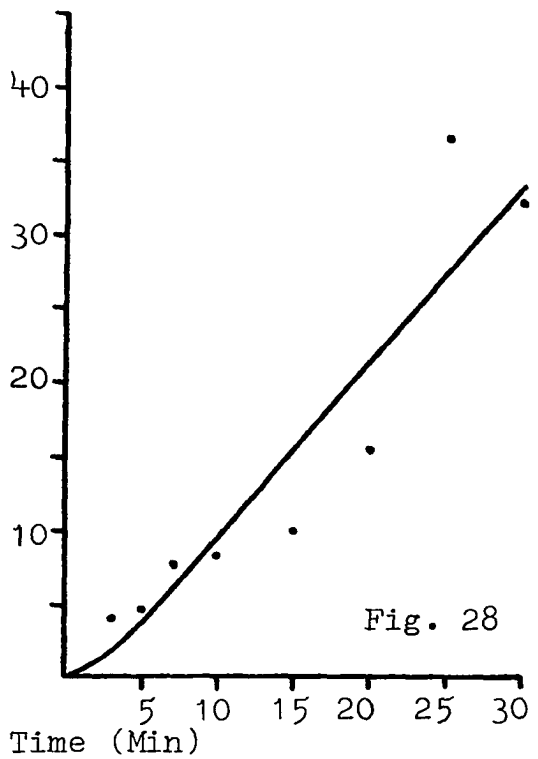


Fig. 28

Figure 27. Rate of uptake of ^{86}RbCl from 5.0 mM RbCl by normal 10 day cells. Control rate = 64.85 mμ moles/g dry wt/hr.

Figure 28. Effect of 10^{-4} M ferulic acid on rate of uptake of ^{86}RbCl from 5.0 mM RbCl by 10 day cells. Test rate = 70.86 mμ moles/g dry wt/hr.

Correlations between observed values of absorption and those obtained by means of linear and curvilinear regression analysis were consistently high (Fig. 16-28).

CHAPTER IV

DISCUSSION

Chlorogenic acid, scopoletin, p-coumaric acid, cinnamic acid, and ferulic acid play an important role in the biochemical suppression of one plant by another (Bonner and Galston, 1944; Bonner, 1946, 1950; Rice, 1965a, 1965b; Wilson and Rice, 1968; Olmsted and Rice, 1971; Lodhi and Rice, 1971). In these experiments, cinnamic, p-coumaric, and ferulic acids were very inhibitory to the growth of rose-cell suspension cultures when present in low concentrations, while chlorogenic acid and scopoletin were effective as inhibitors only at high concentrations. The failure of the latter compounds, when provided in low concentrations, to inhibit growth of tissue culture cells may indicate that their inhibitory effects operate in whole plants on processes not common to tissue culture systems. Einhellig and Kuan (1971), in fact, reported that scopoletin prevents stomatal opening in sunflower seedlings.

In most instances, the mechanisms by which these compounds bring about a reduction in growth are unknown. Ferulic and cinnamic acids may play a secondary role in inhibiting

growth through their action on IAA activation and inactivation (Lee and Skoog, 1965). However, present studies indicate that they may also have a more direct influence on basic cellular processes.

Exposure of 5 day cells to ferulic and cinnamic acids significantly altered the distribution of ^{14}C in various cell constituents, particularly protein, lipids, and amino acids. The reduction in incorporation of ^{14}C into protein, if maintained throughout the 14 day growth cycle, would certainly lead to a reduction in the growth of the cultures. Although the mode of action by which these compounds reduce protein synthesis was not determined from these studies, the data suggest that ferulic and cinnamic acids do affect protein synthesis in different ways. In cells treated with cinnamic acid, ^{14}C incorporation into all soluble amino acids was actually enhanced; thus, the reduction in protein synthesis was not due to the lack of supply of a particular amino acid but apparently due to inhibition of the mechanism of protein synthesis.

In contrast, there was a reduction of incorporation of ^{14}C into soluble amino acids, protein, and organic acids with a concomitant increase of incorporation into lipids in cells treated with ferulic acid. The reduced radioactivity observed in these fractions isolated from ferulic acid treated cells can be attributed to an apparent diversion of acetate-^{14}C into lipid synthesis rather than the TCA cycle and

subsequent pathways leading to amino acids and protein. These alterations in the flow of carbon into cellular constituents when cells are treated with cinnamic and ferulic acids may shed some light on these compounds as inhibitors of growth, seed germination, and seedling development. Such alterations would be particularly significant during seed germination and seedling development when storage reserves are being mobilized and resynthesized into other compounds necessary for growth.

Mineral depletion and ion uptake studies provide evidence that these inhibitors may have other routes of inhibition. Treatment of cells with ferulic acid altered the pattern of removal of macronutrients through the growth cycle. During the first 3 days of the cycle, cells treated with ferulic acid consistently depleted macronutrients at a greater rate than control cells. In contrast, on days 3 - 5, cells treated with ferulic acid showed a consistent loss of all the macronutrients. Subsequently, on days 5 - 14, there was a positive accumulation of ions. An entirely different pattern of uptake emerges when rates of removal are based on depletion per flask. In this case, the observed reduction in the rate of absorption is due to reduced growth of test tissues rather than actual inhibition of ion uptake. Similar results were reported for the effects of chlorogenic and gallotannic acid on uptake of K^+ and Ca^{2+} by pigweed seedlings (Olmsted and Rice, 1970).

Two systems of ion uptake, as described by Welch and Epstein (1969) and Laties (1969), operate in the

ion-accumulation process in rose-cell suspension cultures. Young cells (4 and 5 day) incubated in a ferulic acid solution showed inhibition of ^{86}Rb uptake in both systems, inhibition being greater in system 2 operating at high concentrations. These data may partially explain observations discussed in the previous paragraph on losses of ions during the earlier phase of the growth cycle. The lack of evidence for inhibition of either system in 10-day cells, or perhaps even slight stimulation, also correlates well with data on mineral depletion in which older cells treated with ferulic acid accumulated nutrients at a faster rate than control cells of the same age. The high rate of depletion of macronutrients in 0 - 3 day cells may be due to the fact that these cells are physiologically old when they are transferred from 14-day cultures and thus behave more like older 10- to 11-day cells than young, actively metabolizing 5-day cells.

The significance of greater inhibition of system 2 in young, actively growing cells is difficult to assess in terms of importance to whole plants under field conditions as concentrations involved in system 2 are not within the range of concentrations normally present in the soil and available for plant roots (Laties, 1969).

The mechanism of ferulic acid inhibition and stimulation of ion uptake in young and mature tissues is unknown. However, Hodges et al. (1971) reported a similar differential sensitivity to Rb uptake with age in oat root sections treated

with the antibiotics gramicidin and nigericin. The inhibition or stimulation may be related to alterations in carbon metabolism discussed previously. Young cells with high demands for energy may be limited in their capacity for ion uptake by available ATP.

The significant increase of incorporation of ^{14}C into the soluble lipid fraction in cells treated with ferulic acid may also offer some clue to the inhibitory mode of action of ferulic acid on ion uptake in young cells. That is, some alteration in lipid composition of membranes could contribute to reduced uptake in cells of this age. Further studies are necessary, however, to investigate these possibilities.

CHAPTER V

SUMMARY

Cinnamic, p-coumaric, and ferulic acids were very inhibitory to the growth of rose-cell suspension cultures when present in low concentrations, while chlorogenic acid and scopoletin were effective as inhibitors only at high concentrations.

Treatment of 5-day cells with 10^{-4} M ferulic acid and 10^{-5} M cinnamic acid resulted in altered patterns of incorporation of ^{14}C from glucose-UL-^{14}C into the following cell constituents: amino acids, organic acids, protein, and lipids. Although both inhibitors decreased incorporation of ^{14}C into protein as compared with control cells, the mechanisms of inhibition of protein synthesis are apparently different for the two compounds. A significant portion of the label from glucose-UL-^{14}C appeared in the soluble lipid fraction in cells treated with ferulic acid, apparently at the expense of incorporation into other cellular fractions.

The effect of incubation of cells with 10^{-4} M ferulic acid on the rate of depletion of Mg^{2+}, Ca^{2+}, K^{+}, P, Fe^{3+}, Mn^{2+}, and Mo^{3+} from the medium during the 14-day growth cycle

varied with age of the cells and the ion under consideration. In general, rates of uptake were higher than control rates in older cells and in very young cells and less than control rates in cells 3 - 5 days old.

The degree of inhibition of uptake of ^{86}Rb also varied with age in cells treated with 10^{-4} M ferulic acid. Young (4 - 5 day) cells showed approximately 50% inhibition at higher concentrations of RbCl (system 2) and approximately 25% inhibition at lower concentrations of RbCl (system 1). In contrast, the rate of ^{86}Rb uptake in 10-day cells was not significantly altered by incubation in ferulic acid at either system 1 or system 2.

It appears, therefore, that the inhibition of growth of Paul's Scarlet rose cells by ferulic and cinnamic acids was due in part to a reduction in protein synthesis and uptake of minerals in physiologically active concentrations.

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