Effects of the Host-Selective Toxins of *Alternaria alternata* f. sp. *lycopersici* on Suspension-Cultured Tomato Cells

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ABSTRACT

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Tomato cell cultures were used to investigate the metabolic effects of the host-selective toxins produced by *Alternaria alternata* f. sp. *lycopersici*, a pathogen of tomato. Toxin concentrations of approximately 1 μM were sufficient to strongly inhibit cell expansion, accumulation of dry matter, and cell division in suspension cultures. Growth-inhibitory concentrations of toxin did not inhibit respiration, uptake of [^{3}H] leucine or its incorporation into protein, or uptake of [3-^{14}C] pyruvate or its incorporation into lipids. The toxins also did not induce potassium ion leakage. The toxins did inhibit uptake of [^{3}H] uridine and [^{3}H] thymidine but apparently did not inhibit net synthesis of RNA or DNA; the inhibition of uridine and thymidine uptake probably was not the cause of growth inhibition. The toxins apparently did not induce pyrimidine shortage, because toxin sensitivity was unaffected by supplementing cultures with pyrimidines or aspartate. Our results in cultured cells do not support the theory that these toxins inhibit aspartate carbamoyltransferase, an enzyme involved in pyrimidine biosynthesis.

Additional keywords: *Lycopersicon esculentum, L. peruvianum*.

Tomato cultivars (*Lycopersicon esculentum* Mill.) of the genotype *asc*/*asc* are susceptible to the stem canker disease caused by *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici* (4,16). This fungus produces toxins, AAL toxins, which are essential for pathogenicity (14). In fact, the purified toxins alone can produce leaf necrosis and most other symptoms of the disease. The AAL toxins occur in two structurally similar forms, TA and TB (31); each consists of tricarballylic acid (1,2,3-propanetricarboxylic acid) joined by ester linkage to a 19-carbon amino alcohol (3). The AAL toxins are host-selective toxins (30) since they are highly toxic only to those tomato cultivars that are susceptible to stem canker disease (14). Disease-resistant (*asc*$^+$/asc$^+$) cultivars are "toxin resistant"; that is, they are up to 1,000-fold less sensitive to the induction of disease symptoms by the toxins than are disease-susceptible (*asc*/*asc*) cultivars (4,14,32).

Because the AAL toxins are essential for pathogenicity, understanding their action is crucial to an understanding of the disease. McFarland (24) and Gilchrist (13) have recently suggested that the AAL toxins inhibit aspartate carbamoyltransferase (ACTase; EC 2.1.3.2), an enzyme involved in pyrimidine biosynthesis. The evidence for this theory is that the toxins inhibit tomato seedling ACTase in vitro and that certain pyrimidine precursors can protect tomato leaves from toxin damage. It is not known, however, whether the toxins have metabolic effects that are consistent with ACTase inhibition.

In the present work, we have investigated the AAL toxins' effects on a variety of metabolic processes, including macromolecular syntheses. We studied processes that would presumably be affected by ACTase inhibition and also processes that might be affected if the toxins had some other site of action. We have used cultured tomato cells because, for metabolic studies, cell cultures have some advantages over plants. For example, cultured cells can be uniformly exposed to the toxins and to radioactive tracers and can be easily sampled. A potential drawback is that findings in cultured cells may not apply to intact plants. Unlike most cells in the intact plant, cells in culture are rapidly growing, undifferentiated, and nonphotosynthetic. Nonetheless, cell cultures have been used successfully to study several toxins involved in plant diseases. These toxins are T toxin, an unusual respiratory uncoupler (12,30); cercosporin, a photosensitizer (7,8,30); and phaseolotoxin, which inhibits ornithine carbamoyltransferase (EC 2.1.3.3) (13 and references therein; 19). Each of the toxins has essentially the same effects on cultured cells that it has on plants.

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MATERIALS AND METHODS

Plants. The *L. esculentum* cultivars Earlypak-7 and Ace were from D. G. Gilchrist. From N. G. Hogenboom we received seeds of the family 741505. This family, which includes plants 741505-31, -32, -45, and -46, is a panmictic F_3 population of *F. peruvianum* (L.) Mill./*L. esculentum* hybrids; its ancestry is 50% *L. peruvianum*, 25% *L. esculentum* cultivar Pearson, and 25% *L. esculentum* cultivar Moneymaker (18; N. G. Hogenboom, *personal communication*). The genotype of Pearson is (*asc*/*asc*) (4,16), but those of the Moneymaker and *L. peruvianum* ancestors are unknown. A self-fertilization of plant 741505-46 produced plants 741505-46 ⊗ C and -46 ⊗ E. A cross of 741505-31 with 741505-32 produced plant 741505-(31×32)A, and a self-fertilization of this plant produced plants 741505-(31×32)A ⊗-16a, -114, -117, -127, and -146. Of the 741505 plants and progeny used in this work, only two still exist: 741505-46 ⊗ E (as a cell culture only) and 741505-45.

Plant cell cultures. The culture medium was 2D/1P (37), unless noted otherwise. Other media were 2I/2D/1P (2D/1P plus 2 mg of indoleacetic acid per liter) and 2D/1P CCM (2D/1P with 10% [v/v] processed coconut milk [17]). Media were adjusted to pH 6.0 with KOH before autoclaving; indoleacetic acid was filter-sterilized.

Unless noted otherwise, cultures were incubated in the dark at 27 C and flask cultures were on a gyrotory shaker at 125 rpm. Callus was initiated from leaves (37). Suspension cultures, 50 ml of liquid medium in a 125-ml Erlenmeyer flask closed with a plastic foam plug (Identi-Plug, Van Waters Scientific), were started from established callus. Growth of suspensions was usually expressed as the settled-cell volume, i.e., the volume of gravity-settled cells as a percentage of total culture volume. Stock cultures were diluted 2- to 11-fold when the settled-cell volume, visually estimated after settling for 10 min, reached about 50%.

Toxins. AAL toxin preparations were from D. G. Gilchrist and colleagues and have been described in detail elsewhere (11). Toxins were obtained from culture filtrates of *A. a. lycopersici* isolates AS27 and AS27-3 (5), a single-spore isolate from AS27.

A cell-free culture filtrate was used for some experiments with leaves. For all other experiments, toxins were purified from culture filtrates by one of three methods. In method 1, toxins were extracted from the culture filtrate with *n*-butanol, and the butanol was exchanged back into water on a rotary evaporator. These toxin preparations were not analyzed for purity. Method 2 consisted of barium acetate precipitation, butanol extraction, and gel permeation chromatography (3,31). The purified toxins were in 50 mM acetate buffer (32 mM sodium acetate, 18 mM acetic acid). TLC analysis (31) of equivalent toxin preparations revealed only two ninhydrin-positive spots, corresponding to TA and TB (24). Method 3 (5), using reversed-phase chromatography and gel permeation chromatography, yielded >99% pure TA (5) in water. All toxins were stored at −20 C. Toxins purified by method 1 were used in the experiment of Figure 2A (see below) and in most of the pyrimidine and aspartate supplementation experiments; for one aspartate experiment, toxin was purified by method 3. Toxins purified by method 2 were used in all other experiments unless otherwise noted.

We express toxin activity as the titer; a titer of 1 is defined as the lowest concentration that induces necrosis in a leaf bioassay using leaflets of the toxin-sensitive cultivar Earlypak-7. This concentration is on the order of 2×10^{-8} M (10 ng/ml [5,32]). The cell-free culture filtrate had a titer of 2,500; titers of the purified toxin preparations ranged from 28,000 to 2,000,000. (The precision of measurement of each titer is approximately within a factor of two.)

Leaf bioassay and cell growth assay of toxin activity. In a leaf bioassay similar to that previously described (14,32), excised leaflets were allowed to take up toxin solution through the petiole. The highest toxin dilution that induced necrosis was determined.

The cell growth assay was normally done in 16- × 300-mm flat-bottomed culture tubes (Bellco Glass, Vineland, NJ) with metal caps. Culture volume was 10 ml: 7.0–7.5-ml culture medium; 0.5–1.0 ml of toxin, or another test compound, or both; and 2 ml of inoculum. Test compounds included tricarballylic acid (Aldrich Chemical Co., Milwaukee, WI), aspartic acid, and various pyrimidines. Solutions of toxin and other test compounds were adjusted to pH 6.0 with KOH, diluted as needed with water, and filter-sterilized. The inoculum was from a stock culture at 40–60% settled-cell volume (containing expanding but nondividing cells). Tubes were incubated nearly horizontal, on a tube roller at 60 rpm. Settled-cell volume was measured daily using a 20-min settling time. To determine the 50%-inhibitory toxin concentration, we used a series of toxin concentrations twofold to 10-fold apart; the growth rate at each concentration was taken as the slope of the exponential growth curve (a plot of log [settled-cell volume] vs. time).

Cell death was detected by staining with phenosafranine (38).

In the cell growth experiment with 23 mM aspartate, extracellular aspartate was measured daily in samples of culture filtrate. Aspartate was determined after reversed-phase HPLC, using the basic procedure of Jones and Gilligan (20); mobile phase solvents were methanol and 50 mM sodium acetate, pH 6.2.

Growth of cultures for metabolic experiments. For the experiments described in the four sections below, cultures with an initial settled-cell volume of about 10% were prepared from stock cultures of 30–60% settled-cell volume. Cultures were in Erlenmeyer flasks with 125–150 mm sidearms, with 40 ml of culture in a 125-ml flask, 80–100 ml in a 250-ml flask, or 160–230 ml in a 500-ml flask. Settled-cell volume in the sidearm was measured after 30 min. Unless otherwise noted, toxin was added to one of two replicate cultures after about 4 days, when the settled-cell volume was 18–27%.

For dry weight determinations cells were collected by centrifugation, washed with water, and dried at 60 C. For cell counts, a sample of culture was treated with chromic acid to disperse cell aggregates (33), and the cells in 30 randomly selected microscope fields were counted (17). Cell number was calculated from the mean of the 30 counts; percent error (standard error/mean) averaged 12%.

Uptake and incorporation of thymidine, uridine, and leucine. Experiments lasted for 4–25 hr after toxin addition. For 4-hr experiments, [methyl-^{3}H]thymidine, [6-^{3}H]uridine, or L-[4,5-^{3}H]leucine (New England Nuclear) was added to cultures immediately after toxin was added; samples were collected (see below) about every 20 min thereafter. For longer-term experiments, at the times indicated, a culture aliquot of 5–6 ml was transferred to a 25-ml flask to which isotopically labeled compound was added; aliquots were then incubated for 1 hr. All incubations with isotopes were done in a reciprocal-shaking waterbath (100–110 strokes per minute, stroke length 1 inch) at 27 C in ordinary room light; suspensions were not kept axenic.

Whole cells and material insoluble in trichloroacetic acid (TCA) were collected as described by Cox et al (6) except that TCA precipitates also were washed with ethanol and ether. Cells and TCA precipitates were digested for at least 16 hr at 40–45 C in 0.2 ml of water and 1 ml of Protosol (New England Nuclear) before scintillation counting. Fractionation of TCA precipitates (6) indicated that ≥95% of the radioactivity derived from uridine and leucine was in RNA (base labile) and in protein (stable in hot acid), respectively.

Respiration. Cultures were used in respiration experiments after growth for 12–36 hr, when the settled-cell volume was still only about 10%. (Older and denser cultures, with settled-cell volumes around 18–27%, used oxygen too rapidly for accurate measurement.) Respiration rates of culture aliquots (3 ml) were measured with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH) at 27 C. The oxygen concentration in air-saturated culture medium was taken to be 240 μM (2). Aqueous KCN was adjusted to pH 7.0 and filter-sterilized; salicylhydroxamic acid (SHAM) was dissolved in acetone. Appropriate volumes of water and acetone were added to control cultures.

Uptake of pyruvate and incorporation into lipids. Cells were incubated with [3-^{14}C] pyruvate (sodium salt, New England Nuclear), and uptake was measured as described above for thymidine, uridine, and leucine labelings. To determine incorporation of ^{14}C into total lipids, cells were collected by centrifugation; lipids were extracted from the cell pellet (34) and then spotted on filters for scintillation counting. In the long-term experiment we used four replicate cultures, adding toxin to two of them. (Toxin was desalted before use by passing over a C_{18}-bonded silica cartridge and eluting toxin in water [32].) Four aliquots were taken from each culture, at 15 min, 1 hr, 3 hr 15 min, and 4 hr after toxin addition. Each aliquot was incubated with [^{14}C] pyruvate for 4 hr.

Potassium ion leakage. Cells were suspended in 10^{-2} M $CaCl_2$ as described by Murphy and Wilson (26) and then incubated under nonsterile conditions on a reciprocal shaker at room temperature in ordinary room light. Extracellular K^+ was measured with a potassium-sensitive electrode (26).

RESULTS

AAL toxin resistance of leaves and cell cultures from different genotypes of tomato. To study the effects of the AAL toxins on suspension-cultured cells, we needed a tomato genotype that was toxin sensitive and that grew well in culture. We screened 54 genotypes for these traits, first testing the toxin resistance of leaves and then, in some genotypes, testing the growth characteristics and toxin resistance of suspension cultures. In 10 genotypes, we determined the toxin resistance of both the leaf and the culture, and these data are compared in Table 1. Eight genotypes are hybrids (50% *L. peruvianum*/50% *L. esculentum* in genetic background) from our laboratory; Ace (*asc*$^+$/*asc*$^+$) and Earlypak-7 (*asc*/*asc*) are *L. esculentum* genotypes that were used extensively in previous research with the AAL toxins (4,24). In seven of the 10 genotypes, the resistance level of the leaf and that of the culture were similar. The remaining three genotypes include two, Ace and

741505-46 ⊗C, in which the leaf was much more resistant than the culture, and one, 741505-(31×32)A⊗-114, in which the culture was much more resistant than the leaf. We do not know if the differences in toxin resistance among the hybrid genotypes were determined by the alleles *asc*$^+$ and *asc*, because genetic barriers prevented crossing the hybrid plants with *L. esculentum* plants of known *asc* genotype.

The hybrid 741505-46⊗ E ("46 ⊗ E") was chosen to study the toxins' metabolic effects on cultured cells and was used in all the work described below. Suspension cultures of 46 ⊗ E grew vigorously, had well-dispersed cells, and were toxin sensitive (Table 1). The cultivars Earlypak-7 and Ace proved unsuitable for our work. They grew poorly in culture, as pure *L. esculentum* genotypes often do (36).

TABLE 1. Toxin resistance of suspension cultures and leaves from different genotypes of tomato

Genotype	Toxin resistance[a]	
	Culture[b]	Leaf
741505-45	>250 (7)[c]	≥625 (2)
741505-(31×32)A ⊗-114	60-160 (1)	1 (1)
741505-46 ⊗C	40 (1)	>625 (1)
741505-46 ⊗E	1-8 (9)	10 (1)
741505-(31×32)A ⊗-127	6 (1)	1-7 (1)
741505-(31×32)A ⊗-16a	4 (1)	≤7 (1)
Earlypak-7	3 (1)[d]	1 (11)
Ace	2 (1)[d]	150-2000 (6)
741505-(31×32)A ⊗-117	<2 (1)	1-7 (2)
741505-(31×32)A ⊗-146	<2 (1)	1-2 (2)

[a]For cultures, resistance is expressed as the toxin titer that inhibits the growth rate by 50% in the cell growth assay. For leaves, resistance is expressed as the minimum toxin titer needed to induce necrosis in the leaf bioassay. Values from 1 to 10 may not represent truly different levels of resistance because of the possible twofold imprecision in the toxin titers (see Materials and Methods) and because the various genotypes were tested in different experiments. Toxins used for 741505-45, −46 ⊗C, and −46 ⊗ E were cell-free culture filtrate for leaves, and toxin purified by method 1 or method 2 for suspension cultures. In each of the other genotypes, the leaf and the culture were tested using toxin purified by the same method, which was either method 1 or method 2.

[b]Culture media: 2D/1P, 2I/2D/1P, and 2D/1P CCM for 741505-45 and −46 ⊗E; 2D/1P CCM for 741505-46 ⊗C, Earlypak-7, and Ace; 2D/1P for all other genotypes.

[c]Number of experiments in parentheses.

[d]The high toxin sensitivities of Ace and Earlypak-7 cultures were confirmed with four different toxin preparations in a second experiment. This experiment is not included in the table because it was not designed to give precise 50%-inhibitory concentrations.

With long maintenance in culture, 46 ⊗ E became increasingly toxin resistant, even though stock cultures were not exposed to toxin. For 46 ⊗ E cells kept in culture for less than 2 yr, the 50%-inhibitory toxin concentration was a titer of 4 ± 2 (average ± standard deviation in the nine experiments reported in Table 1; Figure 1A is an example). To completely prevent growth, titers of 15–70 were usually required. In 2- to 5-yr-old cultures, however, the 50%-inhibitory concentration was a titer of 32 ± 18 (12 experiments), and titers of 50–200 were needed for complete inhibition. As the cells became more resistant we used higher toxin concentrations in our experiments in order to inhibit growth sufficiently.

Growth-inhibitory toxin concentrations also killed 46 ⊗ E cells, but slowly. In a cell growth assay with just enough toxin to inhibit growth completely (a titer of 130, in the 3-yr-old culture used) cell death began sometime between 24 and 50 hr after inoculation. The number of dead cells increased gradually until by 10 days after inoculation, >99% of the cells had died. (In the control culture at 10 days, about 85–90% of the cells remained alive.)

Light did not affect toxin sensitivity. The degree of toxin-induced growth inhibition was the same whether the cell growth assay was done in continuous fluorescent light (about 360 footcandles) or in the dark.

Cell expansion, cell division, and accumulation of dry matter. Our strategy in investigating the site of action of the toxins was first to determine how soon growth became inhibited after toxin was added to exponentially growing cells. We then compared this to the times when various metabolic processes were affected. The likeliest primary site of action would be a metabolic process that was inhibited well before growth was inhibited.

In a typical 46 ⊗ E culture growing without toxin, settled-cell volume and dry weight increased roughly in parallel (Fig. 1B). Cell number (not shown) increased exponentially, and faster than either settled-cell volume or dry weight did, during approximately the first 4 days of culture (from about 6×10^5 to about 5×10^6 cells per milliliter of culture). After that, the cells stopped dividing but continued to expand and to accumulate dry matter.

In most of our growth and metabolic experiments, toxin was

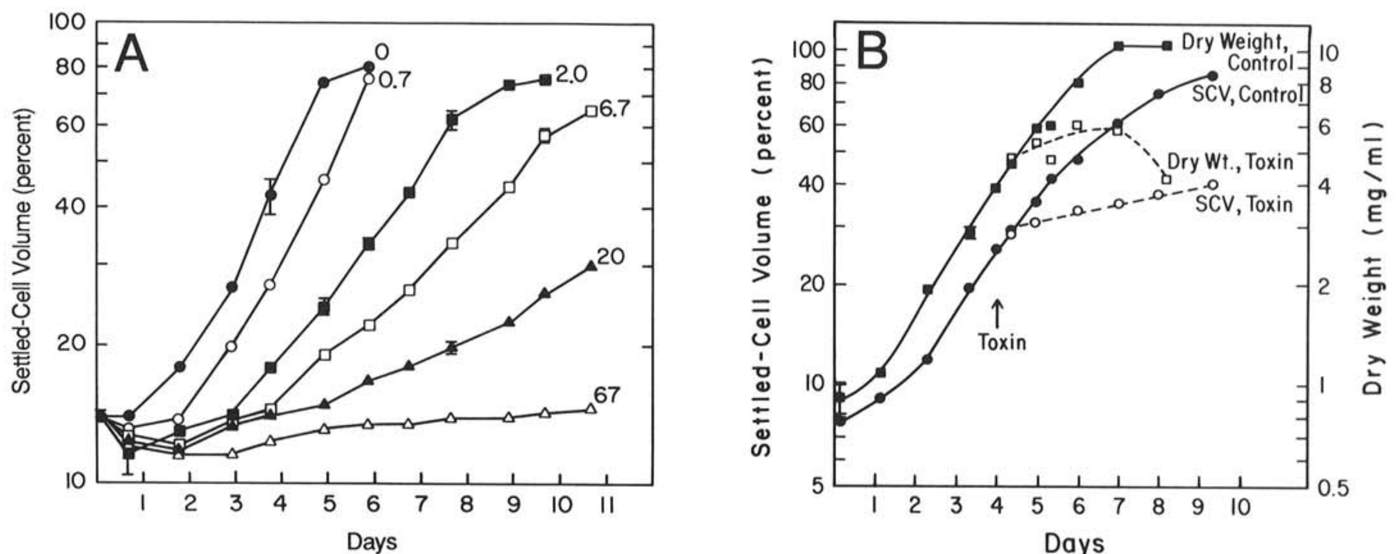

Fig. 1. Growth inhibition of 46 ⊗E cells by AAL toxins. **A,** Effects of various toxin concentrations on settled-cell volume increase in the cell growth assay. Cells were inoculated, at a density of approximately 10^6 cells per milliliter, into medium that contained toxin at titers of 0 to 67 as indicated. Each growth condition was tested in duplicate. Vertical bars show range of replicate measurements; where data symbols are shown without bars, variation was within the dimensions of the symbol. **B,** Effects of toxin on settled-cell volume (SCV) and dry weight increase. Cells were inoculated at a density of approximately 6×10^5 cells per milliliter into medium without toxin. Toxin (titer 150) was added at the midlog stage of growth (arrow). Vertical bars (see above) indicate variability between the control culture and the toxin-treated culture before toxin addition.

added to a culture at about the midpoint of the exponential increase in settled-cell volume, i.e., at "midlog" (Fig. 1B). Toxin concentrations were one to two times those needed to completely prevent growth in cultures in which toxin was present throughout incubation (as in Fig. 1A); titers ranged from 15–200, depending on the age and toxin sensitivity of the culture used. Added at midlog, such toxin concentrations inhibited growth strongly but not completely.

Within 20 hr after toxin was added to midlog cultures a new exponential rate of settled-cell volume increase was established, and this rate was maintained for at least 4 days (Fig. 1B). The new rate was 4–39% of the control rate (average 22%, $n = 15$). Our data were not precise enough to show exactly when growth inhibition began or how quickly it reached its maximum. To estimate the time of onset of inhibition, we extrapolated the exponential growth curves for the periods before and after toxin addition and determined their intersection point. The calculated intersection times in the 15 cultures ranged from 4 to 15 hr after toxin addition. (In the experiments of Fig. 1B and 2A, B, and C, the calculated intersection times were 12, 6, 4, and 14 hr after toxin addition, respectively.) The average intersection time was 9 hr after addition (standard deviation, 3 hr). Therefore, growth inhibition began sometime between 0 and 9 hr after toxin was added, and the primary metabolic target must have been affected within this time.

Within our limits of detection, the toxins did not affect any growth process sooner or more severely than another. Dry weight increase, like cell expansion, was inhibited by about 80% after toxin was added to midlog cultures (Fig. 1B and two experiments not shown). Cell division was close enough to completion in midlog cultures that it was not affected by toxin addition. But when toxin was added to 2-day-old cultures, cell division, cell expansion, and dry matter accumulation all were strongly inhibited within 24 hr, and stopped within about 32 hr.

DNA, RNA, and protein synthesis. By incubating midlog cultures for 4 hr with [^{3}H] thymidine, [^{3}H] uridine, or [^{3}H] leucine, we determined the time course of uptake of these precursors and of their incorporation into DNA, RNA, and protein, respectively (data not shown). Both uptake and incorporation of thymidine (5×10^{-5} M) were linear, as were uptake and incorporation of leucine (3×10^{-3} M). The rate of uptake of uridine (5×10^{-5} M) also was essentially constant over the 4 hr. In contrast, the uridine incorporation rate increased continuously—about eightfold overall—during the first 3 hr; after that there was little change. We attribute this rate increase to increases in the specific activities of the UTP and CTP pools as the cells took up more [^{3}H] uridine (35).

During these 4-hr experiments, the presence of growth-inhibitory concentrations of toxin had no effect on the uptake of thymidine or uridine, or on the incorporation of thymidine, uridine, or leucine into macromolecules. The only effect of toxin was to decrease the rate of leucine uptake by about 20% during the fourth hour of incubation.

To determine the longer-term effects of the toxins on DNA, RNA, and protein synthesis, toxin was added to midlog cultures, and aliquots of culture were removed at intervals and incubated for 1 hr with tritium-labeled precursor. The toxins reduced thymidine uptake within 12 hr (possibly as early as 6 hr) after toxin addition (Fig. 2A); we do not know whether the uptake inhibition preceded or followed growth inhibition. Thymidine incorporation into DNA, however, remained unaffected for the first 12 hr—that is, until after growth inhibition had begun. By 25 hr after toxin addition, both uptake and incorporation of thymidine were reduced, but uptake was affected much more than incorporation was.

The toxins also considerably reduced uptake and incorporation of uridine, roughly simultaneously, about 6 hr after toxin addition (Fig. 2B); this may be either before or after the onset of growth inhibition. Inhibition of both processes increased during the next 19 hr, but uptake was always inhibited more than incorporation was.

In contrast to the results with thymidine and uridine, leucine uptake was only slightly decreased by the toxins (Fig. 2C). Leucine incorporation was essentially unaffected for at least 24 hr, which is well past the onset of growth inhibition.

Respiration. Before testing the toxins' effects on respiration, we determined the effects of two known respiratory inhibitors: KCN, an inhibitor of the cytochrome pathway; and SHAM, an inhibitor of the alternative respiratory pathway (23). Respiration was affected by both these compounds; thus the cells appear to possess

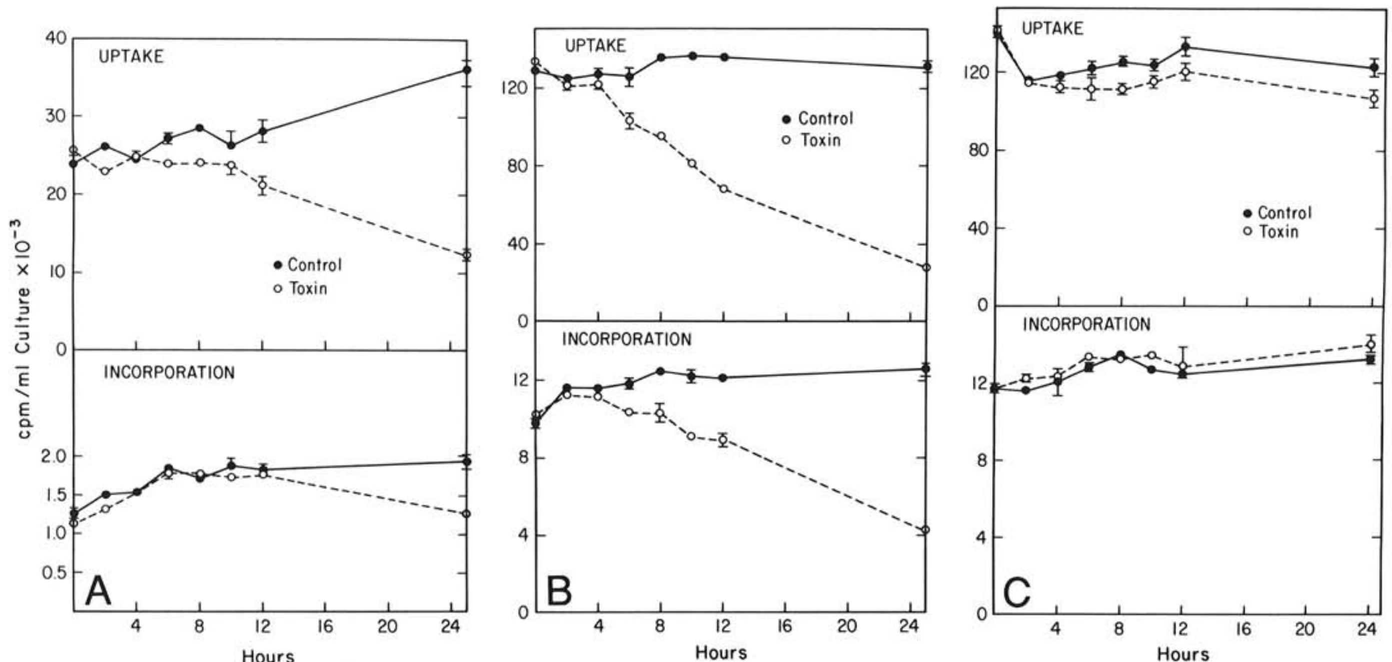

Fig. 2. Effects of toxin on uptake of [^{3}H] thymidine, [^{3}H] uridine, and [^{3}H] leucine, and on their incorporation into DNA, RNA, and protein, respectively. Toxin was added to midlog cultures at zero time. At the indicated times, aliquots were removed from the cultures and incubated with tritium-labeled compound for 1 hr; uptake and incorporation into TCA-precipitable material were then measured. Vertical bars (see Fig. 1A) indicate variability between duplicate measurements on a single aliquot of culture for each time from 0 to 12 hr, and on each of two aliquots at 24 or 25 hr. **A,** Thymidine 5×10^{-5} M, 4 mCi/mmole; toxin titer 28. **B,** Uridine 5×10^{-5} M, 10 mCi/mmole; toxin titer 67. **C,** Leucine 3×10^{-3} M, 0.32 mCi/mmole; toxin titer 67. (Toxin inhibited the rate of settled-cell volume increase by 80, 62, and 64–69% in A, B, and C, respectively.)

both respiratory pathways (Fig. 3). When added alone, KCN initially stimulated respiration (as cyanide does in some other plant cell cultures [2,9]). Within a few hours, cyanide became inhibitory. SHAM caused a delayed, transient inhibition; respiration returned to almost normal after about 8 hr. The significant finding was that neither KCN (1 mM) nor SHAM (1 mM) alone could inhibit respiration completely. Even at higher concentrations, SHAM alone (5 mM) and KCN alone (10 mM) inhibited respiration by only 10–40% at 1 hr after addition (data not shown). When KCN and SHAM (1 mM each) were added together, however, respiration was inhibited promptly and almost completely (Fig. 3). Thus, as expected when both the cytochrome and the alternative pathway are present (2,9), both pathways had to be blocked to fully inhibit respiration.

We tested the toxins' effects on respiration by adding toxin alone, toxin plus cyanide, and toxin plus SHAM (Fig. 3). Toxin alone had little or no effect on the respiration rate for at least 21 hr. Further, toxin did not significantly enhance the effects of either cyanide or SHAM. This result indicated that the toxins did not inhibit either the cytochrome or the alternative pathway.

Lipid synthesis. In preliminary experiments, midlog cultures were incubated with [3-^{14}C] pyruvate (10^{-5} M) for 4 hr, without toxin. Pyruvate uptake was linear. In some experiments, the rate of incorporation of ^{14}C into lipids also appeared roughly linear; in other experiments the incorporation rate was slower during the first hour than during the next 3 hr. In these preliminary experiments, replicate cultures sometimes showed variation in uptake, incorporation, or both; procedural changes did not eliminate this variability.

Lipid synthesis was also monitored for 8 hr after the addition of toxin to midlog cultures; this 8-hr period covers almost the entire 9-hr interval during which growth inhibition began. Aliquots of culture were removed at various times from 0 to 4 hr after the addition of toxin (titer 200), and each aliquot was incubated with ^{14}C pyruvate (1.3×10^{-5} M, 20 mCi/mmole) for 4 hr. The average 4-hr incorporation of ^{14}C into lipids in the eight aliquots of control cells (3,423 ± 792 cpm per milliliter of culture) was similar to the average incorporation in seven aliquots of toxin-treated cells (3,524 ± 1,130 cpm per milliliter of culture). The eighth aliquot of toxin-treated cells incorporated 12,227 cpm per milliliter of culture in 4 hr. We believe, however, that this high incorporation was artifactual and was due to variability problems (see above). We therefore think it likely that lipid synthesis remained unaffected for at least 8 hr after toxin addition. The toxins also appeared not to affect pyruvate uptake during this time (data not shown).

Potassium ion leakage. Some host-selective toxins cause almost immediate leakage of electrolytes, including potassium ions, from toxin-sensitive plant tissues (27;39 and references therein). We tested the AAL toxins' effects on potassium ion leakage after collecting cells from the potassium-rich culture medium (20 mM in K^+) and then resuspending them in $CaCl_2$ (Fig. 4). In control cells, the extracellular potassium ion concentration first increased as potassium leaked from the cells, then leveled off, and finally decreased again as the cells took up the potassium they had lost. If toxin was added after the potassium concentration had leveled off, the cells then took up potassium as effectively as control cells did; there was no potassium leakage for at least 5 hr after toxin addition. We also, as a positive control, irradiated cells with UV light. UV light causes efflux of potassium from suspension-cultured rose cells (26), and it did so in cultured tomato cells as well (Fig. 4).

Sensitivity to tricarballylate. Tricarballylic acid, a component of the AAL toxin molecule, was not by itself very toxic to cell cultures (data not shown). In the cell growth assay, 1 μM to 1 mM tricarballylate had no effect on 46⊗E cells. (In the culture used, the 50%-inhibitory toxin concentration was a titer of 28, or about 560 nM.) Ten millimolar tricarballylate stimulated growth slightly. At 30 and 47 mM tricarballylate the growth rate was inhibited by up to 60%. Even this inhibition, however, may not have been caused by tricarballylate but by excess potassium. Because KOH was used to adjust the pH, media with 30 and 47 mM tricarballylate contained about four and six times, respectively, as much potassium as our standard medium; such high potassium concentrations may inhibit growth (25).

Toxin sensitivity in cultures supplemented with aspartate or pyrimidines. In leaves, pyrimidine precursors such as L-aspartate and orotate afforded protection against the AAL toxins (13,24). In

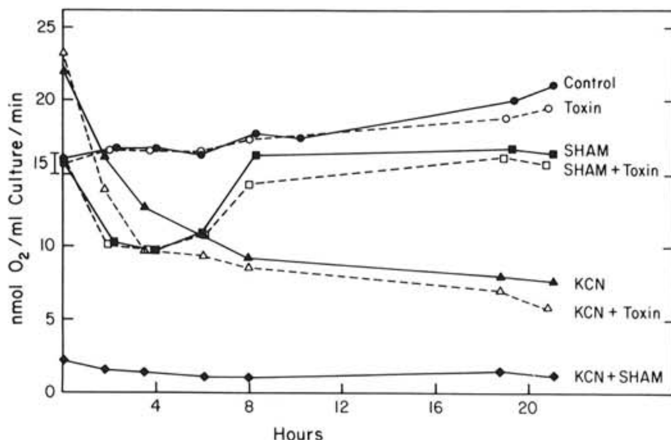

Fig. 3. Effects of toxin and respiratory inhibitors on respiration in cultures at an early stage of growth (see Methods). The bar beside the vertical axis shows the range of base-line respiration rates in seven replicate cultures. At zero time, cultures received the indicated additions (toxin, titer 67; SHAM, 1 mM; KCN, 1 mM). Aliquots of culture were removed at the indicated times for measurement of respiration rate; the first measurements were made about 4–5 min after inhibitor addition.

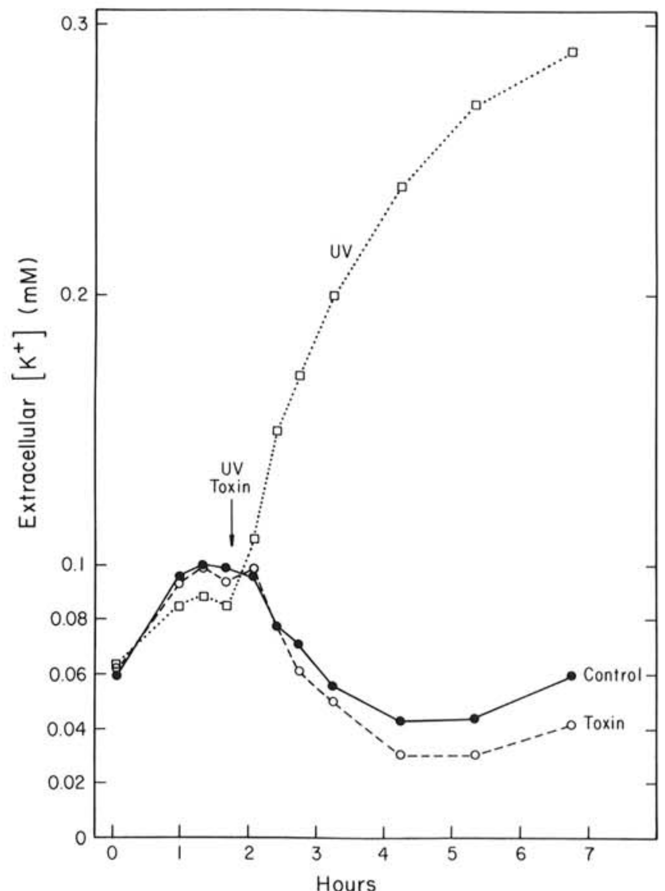

Fig. 4. Effects of toxin and UV light on potassium ion leakage in cells from a midlog culture. Cells were suspended in 10^{-2} M $CaCl_2$ at zero time. At the time indicated (arrow), toxin (titer 67) was added to one aliquot of cells, and another aliquot was irradiated with UV light (254 nm, 4.65 W/m^2 for 10 min).

cell cultures, we used the cell growth assay to test the effects of L-aspartate (10 μM to 23 mM), orotate (1 μM to 1 mM), uracil (5 μM to 5 mM), and uridine (1 and 10 mM). So that any protective effects would be evident, each experiment was done with a toxin concentration that inhibited growth only partially—by 20–80% (in Fig. 1A, the curves labeled 2.0, 6.7, and 20 show inhibition in this range). None of the pyrimidines, at any concentration tested, had any effect on toxin sensitivity. Each growth curve obtained with pyrimidine plus toxin was compared to the corresponding growth curve obtained with toxin alone, and the two curves were virtually superimposable. Toxin sensitivity was also unaffected by aspartate concentrations of $\leqslant$ 1 mM. Aspartate at 22–23 mM did seem to partially protect in an initial experiment, but in a second experiment this result did not repeat.

Rapid degradation of the added compounds could account for their failure to protect the cells. But we saw no evidence of such degradation when we monitored the extracellular aspartate concentration in cultures supplemented with 23 mM aspartate. After 6 days of incubation, the culture medium still contained 14–18 mM aspartate.

DISCUSSION

Cultured tomato cells whose growth was inhibited by the AAL toxins were, in many respects, metabolically normal. Thus, our results offer evidence *against* several mechanisms of toxin action.

The toxins did not inhibit DNA synthesis. When toxin was added to exponentially growing cells, incorporation of [^{3}H] thymidine into DNA remained normal for at least several hours after growth inhibition began. From this result we assume that both parameters involved in thymidine incorporation—the rate of DNA synthesis and the specific activity of the TTP pool—were normal. We calculated, on the basis of the amount of [^{3}H] thymidine incorporated, that the experiment measured DNA replication, not merely repair synthesis of DNA. This experiment did reveal a moderate inhibition of thymidine uptake, at a time when thymidine incorporation remained normal. Evidently the uptake inhibition, which we will discuss further below, did not affect the specific activity of the TTP pool.

The results of experiments with [^{3}H] uridine, together with other evidence (see below), suggest that growth inhibition was not caused by inhibition of RNA synthesis. Inhibition of uridine uptake and incorporation into RNA began simultaneously, about 6 hr after toxin addition. Our interpretation of these results is that the toxins inhibited uridine uptake, and that this led to decreases in the UTP and CTP pool specific activities and therefore in the incorporation rate. We would expect a reduction in uridine uptake to quickly affect the pool specific activities, because the kinetics of uridine incorporation in 4-hr experiments (see Results) suggested that the pool specific activities were very dependent on the amount of [^{3}H] uridine taken up.

An alternative interpretation of the [^{3}H] uridine labeling experiment is that the toxins inhibited transcription. The resulting accumulation of nucleotides might have caused uridine to accumulate and uridine uptake to decrease. In tobacco cells, the transcription inhibitor Actinomycin D did reduce uptake of [^{14}C] uridine, as well as its incorporation into RNA (10); the decrease in uptake, however, was always less than or equal to the decrease in incorporation. In our toxin-treated tomato cultures, on the other hand, uptake was always reduced more than incorporation was. We therefore believe that the toxins inhibited uptake and not RNA synthesis.

The experimental results cannot be accounted for by an inhibition of pyrimidine synthesis, such as might result from ACTase inhibition. Smaller pyrimidine nucleotide pools might decrease the rate of RNA synthesis and, thus, reduce uridine incorporation but should not decrease uridine uptake.

The mechanism by which the toxins inhibited uptake of thymidine and uridine is unclear, although the inhibition seems to be relatively specific; we detected little or no effect on uptake of leucine, pyruvate, or potassium. But whatever its mechanism, we doubt that the inhibition of thymidine and uridine uptake was the cause of growth inhibition. Instead, for two reasons, we believe that this uptake inhibition was a harmless secondary effect of the toxins. First, at least 6 hr elapsed between the addition of toxin and the onset of uptake inhibition. Second, because uridine and thymidine are not normally provided in the culture medium, we would not expect inhibition of their uptake to be harmful.

Growth inhibition clearly did not result from inhibition of protein synthesis. When toxin was added to exponentially growing cells, incorporation of [^{3}H] leucine into protein remained normal for many hours after growth inhibition had begun. We therefore assume that the toxins had no effect on the specific activity of the leucyl-tRNA pool, nor on the rate of protein synthesis, nor on the rate of protein degradation. The normal protein synthesis, which implies an adequate supply of rRNA, tRNA, and mRNA, is another reason we doubt that growth inhibition was caused by an inhibition of RNA synthesis.

Respiration, when tested at an early stage of cell growth (see Methods), also was unaffected by the toxins. We could not test respiration after adding toxin to exponentially growing cells, but the normal protein synthesis in these cells implied normal respiration. Our cultures are grown in the dark and rely on respiration to supply the energy that protein synthesis requires.

Finally, the toxins did not inhibit incorporation of radioactivity from [^{14}C] pyruvate into lipids. This result implies that the toxins did not inhibit lipid synthesis, although this conclusion is tentative because the experiment was of relatively short duration.

Thus, whatever the toxins' mechanism of action is, our results suggest that it does not involve inhibition of DNA, RNA, protein, or lipid synthesis, or of respiration. It follows that many related processes—such as the production of respiratory substrates and the synthesis of amino acids and nucleotides—must also be unimpaired. Our results give no direct evidence about what the mechanism of action of the toxins might be. We do have a clue, however: The toxins inhibited the rate of dry matter accumulation by about 80%. Because the bulk of the dry matter of cultured plant cells is protein and carbohydrate (22,28), and because the toxins did not inhibit protein synthesis, it follows that they must have inhibited carbohydrate synthesis. We believe that synthesis of carbohydrates, including cell wall polysaccharides, should be investigated to determine whether the inhibition is direct or is a secondary effect of the toxins.

Most of the previous research on the effects of the AAL toxins (1,13–15,24,27,32) has been done by D. G. Gilchrist and colleagues, using whole plants. Although no metabolic studies were done previously, some of our other findings in cultured cells can be compared to earlier work.

In several relatively trivial respects, our results agree with previous findings. The toxins did not induce either potassium ion leakage in our cell cultures or rapid electrolyte leakage in tomato leaves (13,24,27). The tricarballylate moiety of the toxins was not by itself responsible for toxicity to our cultures, or to leaves (B. McFarland, *personal communication*). We, like Barsel (1), found that the toxins kill cultured cells as they do leaf cells; they do not merely inhibit growth. We also agree with Barsel (1) that in most tomato genotypes tested, the toxin resistance level of the leaf is similar to that of the suspension culture. For example, we and others found the *L. esculentum* cultivar Earlypak-7 to be toxin sensitive, both as leaf (14,32) and in culture (1).

Our results and Barsel's (1) disagree about the toxin sensitivity of the *L. esculentum* cultivar Ace in culture. We found Ace cultures to be toxin sensitive based on growth inhibition. Barsel (1) classified Ace cultures as resistant based on growth inhibition and cell killing; we think, however, that her experiments do not show clear resistance to growth inhibition. We and others (1,14,32) have found Ace leaves to be toxin resistant.

Our results in cultured cells differ from previous findings in leaves in one important respect. We found that even millimolar levels of L-aspartate and orotate did not protect cultured cells from the toxins. In contrast, McFarland and Gilchrist found that Earlypak-7 leaves were protected from the toxins by L-aspartate and by orotate, at concentrations as low as 3–50 μM (13,24). This difference is significant because McFarland's and Gilchrist's results

contributed to the hypothesis (13,15,24) that the toxins inhibit ACTase. ACTase catalyzes the first reaction unique to the pyrimidine biosynthetic pathway. Aspartate is a substrate of ACTase, and orotate is a later intermediate in pyrimidine biosynthesis.

In whole plants there is additional evidence for ACTase inhibition. Carbamyl phosphate (another ACTase substrate) and carbamyl aspartate (an ACTase product) also effectively protected Earlypak-7 leaves from the toxins, and uracil gave some protection (24). Further, the toxins inhibited ACTase in vitro, and ACTase from toxin-sensitive (Earlypak-7) seedlings was roughly twice as sensitive to inhibition as ACTase from toxin-resistant (Ace) seedlings was (24).

But the toxins' effects on our cell cultures appear inconsistent with ACTase inhibition. The simplest expectation for a lethal effect of ACTase inhibition is that the cells become starved for pyrimidines and, thus, experience inhibition of DNA and RNA synthesis and eventually of protein synthesis. We observed none of these effects. Further, we found that uracil and uridine, like orotate, were unable to protect cultured cells from the toxins. We would expect any of these three compounds, unless they were degraded (6,21), to correct pyrimidine starvation. Each of the three can be converted to other essential pyrimidines (29), and each is incorporated into nucleic acids by cultured plant cells (6,21). Our cell cultures apparently did not degrade uridine extensively, since substantial amounts were incorporated into RNA. We calculated that the amount of uridine incorporated nearly equalled the cells' total pyrimidine requirement for nucleic acid synthesis.

The discrepancy between the Gilchrist group's hypothesis that the AAL toxins inhibit ACTase and our conclusion that ACTase was not inhibited could have several explanations. One is the use of different genotypes of tomato. The Gilchrist group used *L. esculentum* cultivars, whereas we used an *L. peruvianum*/*L. esculentum* hybrid. Another possibility is that ACTase is the toxins' site of action in intact plants, but not in cultured cells. There seems no obvious reason for such a difference, however, since ACTase is involved in central metabolism and not in any process specific to the intact plant.

A third possibility is that pyrimidine starvation is not the lethal consequence of ACTase inhibition as we assumed it to be. Gilchrist et al (15) have suggested that the cell can overcome pyrimidine shortage by recycling its pyrimidines, and that ACTase inhibition may have some other toxic effect—perhaps not detectable in our experiments. In that case, however, we might still have expected aspartate to overcome ACTase inhibition and thus to prevent toxicity in our cultures. It did not.

The final possibility we want to consider is that ACTase may not be the AAL toxins' site of action, even in intact plants. In vitro inhibition of tomato ACTase requires high toxin concentrations, about 0.5–10 mM (24). But a toxin concentration of about 20 nM (10 ng/ml) is sufficient to induce necrosis in a detached Earlypak-7 leaf (5,32), and concentrations similar to this are found in the leaves of plants affected by stem canker disease (32). Thus, it is not clear whether ACTase inhibition fully explains the toxins's in vivo effects. We think the possibility that the toxins have another site of action, in addition to or instead of ACTase, should be further explored.

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