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Biochemical Changes in Aging Mycelial Cultures of Alternaria solani

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BIOCHEMICAL CHANGES IN AGING MYCELIAL CULTURES OF *ALTERNARIA SOLANI*

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of the requirements for the degree of

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ABSTRACT

The cellular aging process was examined in vegetative cultures of Alternaria solani. Uniform spore inoculated, membrane-covered plates allowed each culture to be treated as a single physiological entity. As cultures of Alternaria solani aged, the DNA remained constant; RNA and protein increased and then leveled off, while carbohydrate continued to increase.

BIOCHEMICAL CHANGES IN AGING MYCELIAL CULTURES OF *ALTERNARIA SOLANI*

INTRODUCTION

Fungi serve as excellent organisms for the study of cellular aging because they lack the complexity of higher multicellular organisms and yet may be studied as cooperate entities. Unicellular organisms, on the other hand, are studied as cell populations.

Different methods have been employed for fungal aging studies. Zalokar (1959) used both liquid shake and standing cultures to observe morphological and biochemical differences between young and old vegetative cells of Neurospora crassa, but did not examine the entire mycelium. Yanagita and Kogané (1962) used mold colonies of Aspergillus niger and Penicillium urticae, produced from point inoculations of spores upon the center of cellophane membrances on semi-solid media. They studied cytochemical differentiation between non-uniform zones which were progressively developing reproductive organs from the center to the advancing edge.

Yanagita and Kogané (1963) used a spray-inoculation of A. niger spores on a "cellophane-agar medium" to produce a mycelial mat which had masses of cells of a known age (60 hr.). They measured the biosynthesis of sulfur compounds of these mats which were differentiating from

vegetative to reproductive colonies. This dense-spray inoculation method produced a two dimensional mat in which the correspondency between zones of progressive colonies (point inoculation) and different ages of the mycelial mat (spray inoculation) had "been proved cytologically by the degree of basophilia and by the prophile of fine structure of cells as revealed by electron-microscopy" (Yanagita, Kogané 1963).

Gottlieb and Van Etten (1964) used liquid standing cultures of Penicillium atrovenetum, produced by introducing a spore suspension into the medium, to study biochemical changes with time. The vegetative mycelium which first formed developed reproductive structures during the course of aging.

Gottlieb and Van Etten (1965) modified this procedure by using circular colonies of Rhizoctonia solani and Sclerotium bataticola, each begun from a point inoculum upon a liquid nutrient medium, which permitted them to investigate biochemical and physiological aspects of aging in growing surface colonies. Aerial spores were not formed, but sclerotia formation occurred in older regions of S. bataticola colonies. Gottlieb and Skowronski (1970) also used circular surface colonies, prepared as above, to determine metabolic differences in peripheral hyphae of R. solani during exponential and stationary phases of growth.

Nagasaki (1968) studied colonies of A. niger, prepared by spray inoculation upon a cellophane membrance of semi-solid medium, to determine enzyme activity in aging cultures. The colonies changed from a vegetative stage to a reproductive stage.

These various studies suggested that the spore-spray inoculated cultures of Yanagita and Kogané (1963) and Nagasaki (1968) were more effective than submerged liquid and surface cultures in reducing differentiation (Burnett, 1968) due to a more equal exposure of the surface layer to the air and nutrients (Zalokar, 1959). However, these spore-spray inoculated cultures did not eliminate reproductive differentiation since conidia were soon formed in great abundance and interfered with the normal process of vegetative cellular aging.

Nevertheless, this spore-spray method of inoculation enabled the uniform mycelial mat to be treated as an individual entity which aged as a unit. This should be more advantageous than the conventional point inoculum method. In the point inoculum method, the oldest cells at the center and the youngest cells at the growing edge are connected by cytoplasmic continuity thru hyphal pores and are directly influenced by each other. Hence, the cells at any location within the progressively differentiating circular colony represent an average effect of all the cells throughout the colony, and one is less able to

distinguish between the older and the younger cells of these radial hyphae. A fungal mat formed by the spray inoculum method uses this phenomenon of cytoplasmic continuity through poroid hyphae, in addition to anastomoses, in which hyphal interconnections allow cytoplasm to flow from one part of the mycelium to the other (Zalokar, 1959), to its greatest advantage. This extensive hyphal communication suggests that this system may be considered a "single physiological unit" (Burnett, 1968) which makes it a very convenient tool for obtaining large quantities of non-differentiating uniform cells for studying the cellular aging phenomenon.

It is apparent that the methods thus far used to study fungal aging have had one common characteristic: differentiation. Most of the studies have not separated the aspects of growth and reproduction from the normal maintainance of non-differentiating aging vegetative colonies.

There are two purposes of this present study. The first purpose is to develop within the same system a uniform fungal colony in which differentiation due to reproduction is eliminated, and progressive differentiation between different parts of the mycelium resulting from environmental influence is minimized. Alternaria solani was chosen for this study since differentiation from vegetative growth to reproductive growth could be prevented

thru photoinhibition of sporulation (Lukens, 1963).

The second purpose of this study is to examine the biochemical changes that accompany aging. Very few studies have been made on the biochemical aging phenomenon in fungi. Zalokar (1959) found in Neurospora that the rate of protein and RNA synthesis did not decrease substantially in hyphal regions that were distant from the growing tip. He found these regions to be filled with cytoplasm and dense with nuclei, whereas old hyphal regions were vacuolated and filled with fat droplets. Yanagita and Kogané (1962) found in A. niger that hyphal cells of the advancing edge of radial colonies showed a high degree of basophilia (RNA, DNA) as opposed to the central region. Yanagita and Kogané (1963) found phosphate to be most actively incorporated at the growing edge in linear surface colonies.

Gottlieb and Van Etten (1964) using P. atrovenetum, found the percentage of "active compounds" (nucleotides, RNA, amino acids, protein) was highest in lag phase, and "storage compounds," (carbohydrates, fatty acids) increased to a maximum near the termination of log phase. Gottlieb and Van Etten (1964) examined lipid changes in P. atrovenetum with age. They found a high content of nonsaponifiable lipid and ergosterol in the young mycelium, which then decreased with age, and a shift from more unsaturated to less unsaturated fatty acid with age. Gottlieb and

Van Etten (1965) found in R. solani and S. bataticola few changes with age in total amino nitrogen, RNA, and protein, (when calculated in ratio to DNA) but total lipid to DNA increased rapidly in age with S. bataticola whereas carbohydrate to DNA did not substantially change. However, the ratio of total carbohydrate to DNA in R. solani increased with age, whereas the total lipid-DNA ratio increased only slightly.

Skowronski and Gottlieb (1970) found that the protein content from peripheral hyphae from young fungal colonies of R. solani was 1.6 times greater than in peripheral hyphae from old fungal colonies. The overall rate of amino acid uptake was less in old than in young fungal colonies.

Nagasaki (1968) examined activities of enzymes related to phosphorous metabolism during aging, and observed that enzymes with higher pH optima were active in younger mycelia and that those with a lower pH optima were active in aged mycelia. He found ribonuclease (T_2-type), responsible for ribosome degradation, was most active in aged mycelia.

In this present study the gross biochemical changes that occur in aging cultures of A. solani are examined. DNA, RNA, protein and carbohydrate of different aged cultures are studied using uniform, non-reproductively differentiating fungal mats described earlier.

MATERIALS AND METHODS

Organism. Alternaria solani stock cultures were maintained on potato dextrose agar (Difco) at 30 C under white light to prevent sporulation (Lukens, 1963). In order to grow large quantities of fungus, inocula from refrigerated agar slant cultures were homogenized in a blender with 0.1 M phosphate buffer, pH 6 and then introduced into 4 liter Erlenmeyer flasks containing liquid media. The medium consisted of the following: glucose, 20 g; $(NH_4)_2SO_4$, 3 g; $MgSO_4.7H_20$, 0.25 g; glycine, 1 g; KH_2PO_4, 3 g; thiamine-HCl, 2 µg; niacine, 20 µg; biotin, 1 g; i-inositol, 200 µg; pyridoxine, 10 µg; folic acid, 10 µg; double distilled water to 1 liter; the pH was then adjusted to 6.0 before sterilization. The culture flasks were placed on a rotary shaker at room temperature for approximately one week.

Spore production. A. solani grown in liquid culture was used for spore production using a modification of the technique of Lukens (1960). The fungal mass in each flask was recovered by pouring the entire contents of each flask into a brass collecting cone under aseptic conditions. The fungus was homogenized at top speed for 1 min in a Sorvall micro-blender, sedimented 3,000 x g

for 10 min in a Sorvall RC2-B centrifuge and washed with doubly distilled water four times until the supernatant fluid was clear. Two volumes of 0.1 M phosphate buffer, pH 6 were added to one volume of mycelial homogenate and poured onto Whatman #1 filter paper of 15 cm diameter, in a large pan. The pan was rotated to permit an even distribution of the fungus on the filter paper. Saran Wrap, sterilized in ethanol, covered the pan, which was slightly tilted so that excess homogenate would drain to one end of the pan, leaving a thin layer of fungal fragments on the filter paper. The filter paper cultures were incubated at 23 C in continuous white light for 31 hr for the production of conidiophores, followed by incubation at 21 C in total darkness for 16 hr for the production of conidia (Waggoner and Horsfall, 1969).

Spore inoculation. Fungal mats used for this investigation were produced by dispersing spores upon 5.4 cm diameter petri plates containing semi-solid medium with a thickness of approximately 1 cm and overlaid with cellophane (Dupont 193-Pudo). Except where otherwise indicated the medium consisted of the following: glucose, 50 g; $(NH_4)_2SO_4$, 3 g; $MgSO_4.7H_2O$, 0.25 g; glycine, 1 g; KH_2PO_4, 3 g; thiamine-HCl, 20 µg; niacin, 20 µg; biotin, 1 µg; i-inositol, 200 µg; pyridoxine, 10 µg; folic acid, 10 µg; agar, 20 g; doubly distilled water to 1 liter; pH adjusted to 6.0.

Spore dispersal was accomplished in a UV sterilized inoculating room by brushing spores from the dried filter paper with a sterile acrylic brush into a spore chamber. The chamber consisted of two vertical copper tubes of 2.5 inch diameter and 16.5 inch length, separated by a 250 mesh brass screen. The entire chamber was fitted over a petri plate. The top of the chamber was closed with a metal cover to maintain sterility inside the chamber. The sterile agar petri plate was mounted on a motor-driven turn table. Spores were brushed into the top of the chamber and allowed 30 seconds to settle upon the wire mesh. The chamber was vibrated for 15 seconds by a vortex mixer, mounted in an inverted position above the chamber, which caused only small spore clusters (1-3 spores) which had settled on the screen to fall through, leaving behind larger aggregates. The dispersed spores were allowed to settle for 30 seconds upon the cellulose membrane of the agar plate. To correct for slight convection currents in the chamber that caused uneven settling, the petri plate was rotated rapidly at 280 rpm. The seeded petri plate was removed and covered. Spore counts were made on seeded plates using a 2.89 mm sq. occular grid to determine the relative density of the spore inoculation.

Membrane studies. The effect of glucose concentration on membrane integrity was investigated since the membrane was observed to weaken when the glucose level in

the medium was low (2 percent). Fungal mats were formed on plates overlaid with cellophane which contained the standard nutrient medium with varying glucose percentage as follows: 0; 2; 5; 15; and 25. Uninoculated plates were used as controls. All mats were grown at 30 C under constant white light for one week after which the membrane was peeled from the medium and the fungal mat separated from the membrane. Membrane integrity was measured with a force gage (Hunter Spring L-5), which measured the puncture resistance of the membrane against a 1.1 cm diameter steel piston.

Growth and drying procedures. Spores on seeded membranes were allowed to germinate and grow at 30 C under constant white light for periods up to 168 hr. Samples were taken at 24 hr intervals. Two independent experiments were combined for a total of 6 replicates for each of the 24 hr time periods examined. At 24 hr growth intervals, fungal mats were peeled and scraped from their cellulose membranes, rinsed with doubly distilled H_2O, and placed on aluminum foil trays in a drying oven at 50 C. The total dry weight for each mat was recorded and the dried mats were pulverized with mortar and pestle and stored in small vials over phosphorous pentoxide under vacuum until used.

To determine whether there was uniformity within as well as between mats, 7 small discs of 1.15 cm diameter were removed from each of 3 stationary mats (96 hr) using a cork borer; one central disc, 3 intermediate discs, 3

peripheral discs (2.7 cm and 1.35 cm from the edge and at the edge respectively). The dry weights of all discs were compared for variation within and between mats. Determination of the pH of the culture medium and microscopic examination of the cellulose membrane were made after each mat was removed.

Viability. The viability of stationary mats was determined by removing 1.15 cm diameter discs from each of the central, intermediate, and peripheral areas (2.13 cm and 1.35 cm from edge and at the edge respectively) for each plate at the 98, 124, 149 and 168 sampling hr. The discs were then placed in 50 ml of liquid medium for 24 hr to observe the relative visual increase of growth.

Biochemical determinations. Percentage of compounds per unit dry wt were made only on the vegetative mycelium; therefore, those calculations were not established for zero time. (I) Twenty mg of dried material from each timed replicate was used except for 24 hr in which 3 replicates from each independent experiment were pooled, since the dry weight of each replicate alone was not enough to measure accurately. Thus, there were only 2 measurements for the 24 hr period as opposed to 5 or 6 for all other sampling times. Dried mycelia were washed with doubly distilled H_2O and centrifuged. This washing procedure was repeated 4 times. The washed pellet was homogenized in an Eberbach homogenizer for 3 min with cold

5% trichloroacetic acid. A 10 ml suspension of the cold 5% trichloroacetic acid was prepared and kept at 4 C for one hour and then centrifuged. The supernatant fluid was analyzed for acid soluble carbohydrate using the anthrone method (Morris, 1948). (II) Step I residue was washed twice in 10 ml of 5% cold trichloroacetic acid and washed twice in 10 ml of ethyl alcohol (75%)-ether (3:1 V/V) for 1 hour at room temperature. (III) The washed residue from step II was suspended in 1 ml of 10% trichloroacetic acid and placed in a water bath at 90 C for 30 min followed by centrifugation. The supernatant was analyzed for DNA by the diphenylamine method and for RNA by the orcinol method (Dische, 1955). (IV) The residue from step III was suspended in 5 ml of 1 N NaOH, boiled in a water bath for 20 min followed by centrifugation. The supernatant was analyzed for protein by the Folin-Ciocalteau method (Lowry, 1951).

RESULTS

Technique. The chamber technique of spore application is effective in producing a uniform seeding upon the cellulose membrane. Spore density readings from 2 non-peripheral zones (0.6 mm or greater from edge) and 1 peripheral zone (at edge) for each of 21 plates are compared. No significant difference is found between any of the zones in any one plate at the 95 percent confidence level using an analysis of variance test (Table 1).

There is no correlation between the average spore density per plate and the dry weight of the mycelial mat found after germination for the 3 replicates at any one sampling hour (Table II). Thus, a plate inoculated with a high density of spores did not always result in a mycelial mat which had a higher dry weight than a mycelial mat sampled at the same time and which had been inoculated with a lower density of spores. For example, Table II reveals for the 168 hr sampling, that plate No. 1 had an average spore density of 45 and a mycelial dry weight of 97.9 mg whereas, plate No. 2 had a lower spore density of 25.3 but a higher mycelial weight of 102.0 mg. However, plates inoculated with less than 10-12 spores per 2.89 mm^2 fail to develop into complete mycelial mats.

TABLE I

Spore density measurements from selected areas of petri plates seeded with spores of Alternaria solani

Plate No.	Nonperipheral Zone 1*	Nonperipheral Zone 2*	Peripheral Zone *
1	13	14	13
2	31	16	24
3	16	22	20
4	20	15	12
5	24	28	14
6	21	24	18
8	46	38	40
9	47	39	40
10	24	42	37
11	20	23	32
12	24	42	37
13	18	15	22
14	22	33	23
15	48	64	44
16	39	31	34
17	16	14	19
18	20	20	16
19	31	52	47
20	26	23	25
21	26	24	28
$\bar{x}$	25.95	28.38	26.66
$\bar{\bar{x}}$	26.971		
F_t $[^2_{60}$ 0.05]	3.2		
F_c	0.249		

*no. of spores/2.89 mm^2

TABLE II

Spore density inoculum and mycelial dry weight at 24 hr intervals from spore seeded plates of *Alternaria solani*

Age (hr)	Plate no.	Spore density inoculum (spores/2.89 mm^2)	Mycelial dry weight (mg)
24	1	30.0	5.7
	2	40.6	9.9
	3	39.0	15.2
48	1	27.6	64.9
	2	32.0	76.9
	3	22.6	73.8
72	1	20.0	73.4
	2	31.0	82.0
	3	45.0	88.5
96	1	23.6	82.7
	2	32.0	92.9
	3	22.6	84.8
120	1	45.0	97.4
	2	39.0	78.7
	3	44.0	95.6
144	1	14.6	85.9
	2	30.3	88.4
	3	32.3	97.1
168	1	45.0	97.9
	2	25.3	102.0
	3	25.0	86.2

An F test (Woolf, 330) determination of the dry weight of 4 small discs (1.15 cm dia) removed from the non-peripheral zone (1 central, 3 intermediate; 2.7 cm and 1.35 cm respectively) and 3 small discs from the peripheral zone (at edge) for each of the 96 hr mycelial mats reveals no significant difference between the non-peripheral and peripheral zones of each mat (TABLE III). This suggests uniformity between these different zones for each individual mycelial mat of the stationary phase and therefore homogeneity within mats. An F test (Woolf, 330) also reveals no significant difference between the total dry weight of all discs of each 96 hr mycelial mat (TABLE III). This suggests there is uniformity among stationary mycelial mats of the same age.

Initial experiments indicated that within approximately 3 days after inoculation there was a reduction in membrane integrity as evidenced by the tearing of the membrane upon removal from the plate and hyphal growth through the membrane onto the medium. Enzymatic degradation was considered as one possible cause. Therefore, tests were run for a period of 7 days to determine if a higher glucose concentration would inhibit degradation of the membrane. Mycelial mats grown on medium with no glucose were difficult to remove without the cellulose membrane crumbling. The membrane overlying a 2% glucose medium was stronger than the membrane overlying a medium

TABLE III

Uniformity within and among mycelial mats of *Alternaria solani* based on dry weight of discs* from peripheral and nonperipheral zones in stationary phase

Zone	Mat A	Mat B	Mat C
Nonperipheral			
1	4.8 mg	4.5 mg	4.6 mg
2	4.4	4.5	4.2
3	4.5	4.5	4.0
4	4.4	4.0	4.3
Peripheral			
1	4.2	4.0	4.2
2	4.3	4.5	4.2
3	4.5	4.6	4.2

F test for uniformity within mats

$F_c = 2.045$ $F_c = 0.0012$ $F_c = 0.2586$

$F_t[{}^{1}_{5}0.05]=6.6$ $F_t[{}^{1}_{5}0.05]=6.6$ $F_t[{}^{1}_{5}0.05]=6.6$

F test for uniformity among mats

$F_c = 1.5991$

$F_t[{}^{2}_{18}0.05]=4.4$

*diameter 1.15cm

with no glucose, but many hyphae had penetrated into the medium. Glucose concentrations of 15% and 25% were more effective in preventing membrane degradation, but the mycelial growth appeared to be less than mats formed with 5% glucose suggesting an intolerance to such high levels of glucose. A glucose concentration of 5% was high enough to maintain cellulose membrane integrity for 7 days and to prevent hyphae from growing through into the medium (TABLE IV). Since the only intended function of the membrane was to inhibit the production of submerged hyphae in order to avoid physiological differences among hyphae of the same mat, the medium with 5% glucose was used in all further experiments.

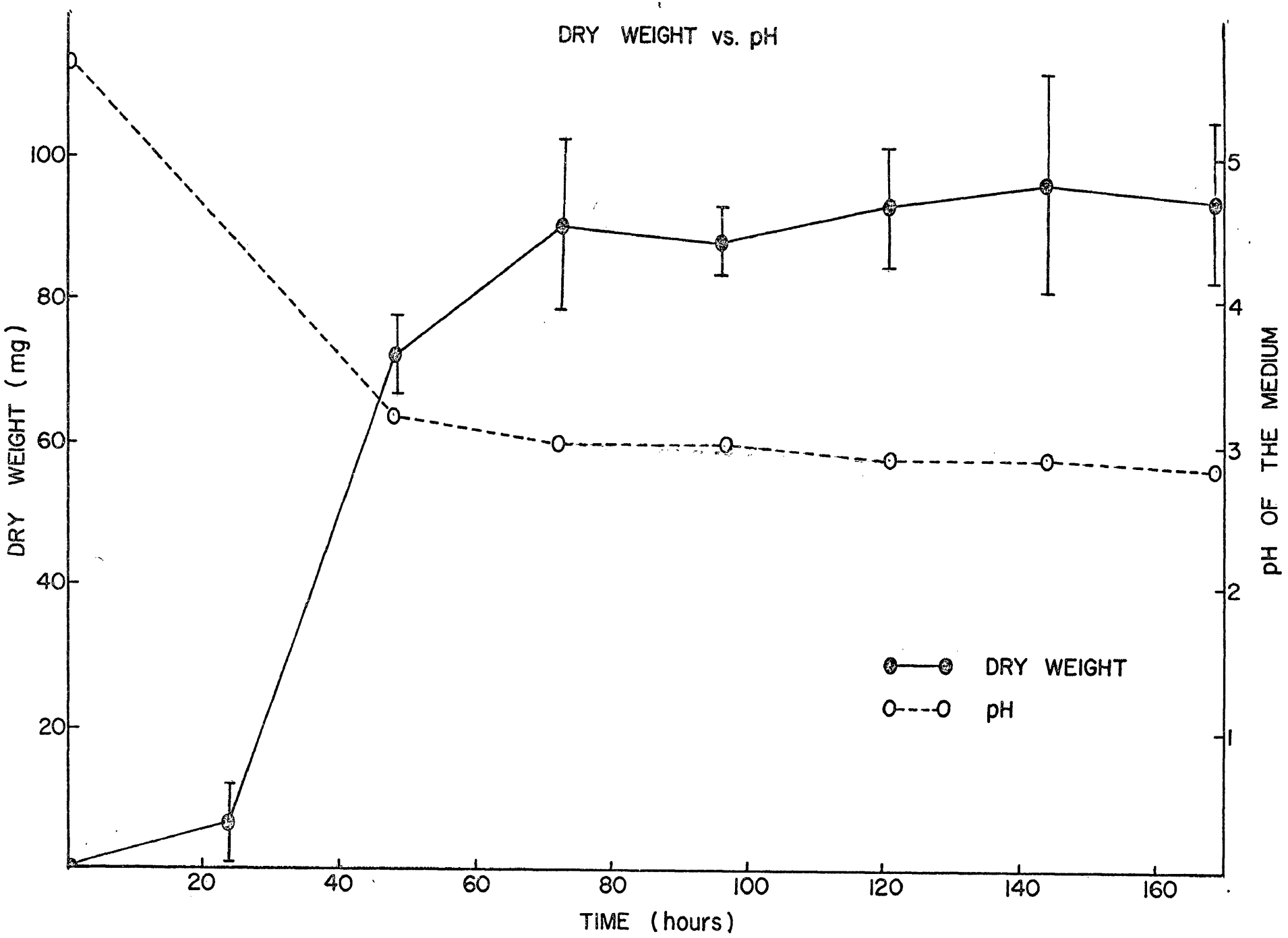
Growth curve and pH. The growth curve for A. solani, based upon dry weight (Graph I) has 3 apparent phases: a lag phase from 0 to 24 hours; a log phase from 24 to 48 hours and a stationary phase from 48 to 168 hours. A death phase is not detectable by weight loss over the 168 hr period. The contribution of the spore inoculum (0.3 mg) is considered negligible in relation to the dry weight of the mycelium. The 95% confidence limits placed about each 24 hour dry weight measurement (each based upon the average of 6 replications) show that the confidence limits about the 24 hr and 48 hr measurements do not overlap with any other confidence limits (Fig. I). However, the 95% confidence limits between the 72 hr and 168 hr all

TABLE IV

Mycelial growth and membrane integrity in *Alternaria solani* as affected by glucose concentration

% glucose	Relative mycelial growth	Membrane Integrity	
		Tensiometer reading	Qualitative description
0	++	0.0025	loss of integrity; membrane crumbles; much hyphal penetration
2	+++	0.9000	integrity poor; membrane tears; hyphal penetration
5	+++	1.4160	integrity maintained; no hyphal penetration
15	+	3.2160	integrity maintained; no hyphal penetration
25	+	4.0000	integrity maintained; no hyphal penetration
uninoculated (control)	0	6.8300	100% integrity

Figure 1.--Growth curve and pH curve of Alternaria solani. Dry weight (mg) of mycelial mats (—●—) and pH of the media (--O--) were measured at 24 hr intervals.


DRY WEIGHT vs. pH
DRY WEIGHT (mg)
100
80
60
40
20
20
40
60
80
100
120
140
160
TIME (hours)
5
4
3
2
1
pH OF THE MEDIUM
DRY WEIGHT
pH

overlap. This plateau of confidence limits apparently indicates a continuous stationary phase for this period.

The development of the mat was followed with the aid of a light microscope (25x). Immediately after inoculation, the spore seeding was almost undetectable on the cellophane membrane. At 24 hr the membrane was almost totally covered with hyphae, but a considerable proportion of the membrane still was visible. From 48 hr to 168 hr, the entire membrane was covered with a very thin mycelial layer which appeared uniform throughout.

The pH of the medium (Figure I) is seen to decline at a steady rate from 5.65 at zero time to 3.20 at 48 hr. From 48 hr, the pH decreases very gradually to 2.85 at 168 hr.

Viability results. Each of the fungal mats which were tested for viability (with growth as a parameter) showed no difference in growth among the mycelial discs taken from central, intermediate and peripheral zones. Each thin disc removed from these stationary phase fungal mats (98, 124, 149, 168 hr) all developed into thick disc-like pads after the 24 hr incubation period in nutrient media with the exception of the 168 hr sample which had only a small amount of new growth.

Biochemical determinations. Biochemical measurements made at 24 hr have the largest 95% confidence limits of all sampling intervals, since there are only 2

measurements (3 replicates pooled for each independent experiment) as opposed to 5 or 6 measurements made at all other sampling times.

Cold trichloroacetic acid soluble carbohydrate. The cold trichloroacetic acid soluble fraction includes all pure mono, di and polymer dextrins and dextrans and starches (Hassid and Abraham, 1957). As seen in Figure II, the percentage of carbohydrate per unit dry weight appears to be low during the lag and log phases of growth. There is a relatively sharp increase from 2.75% at 48 hr to 16.26% at 120 hr; however, the confidence limits for 96, 120, 144, and 168 hours overlap, suggesting that the carbohydrate may have begun to plateau at 96 hr. Total carbohydrate (Figure II) does not increase until after 24 hr, rises gradually until 48 hr and significantly increases thereafter, paralleling the percent carbohydrate per unit dry weight curve.

DNA. The percent DNA per unit dry weight (Figure III) appears to drop during the 24-48 hr period following inoculation. From this point on it remains very constant as indicated by the close 95% confidence limits from 48 hr until the termination of the experiment. There was too much variability at 24 hr for an accurate estimate of percent per unit dry weight to be ascertained. The DNA appears to peak at 2.01 percent of dry weight at 144 hr. Total DNA follows the dry weight curve during lag phase

Figure 2.--The relationship between carbohydrate and age in Alternaria solani. The percent carbohydrate per unit dry weight (--O--) and total carbohydrate (—▲—) are compared with the dry weight (mg) of the mycelium (—●—) at 24 hr intervals.

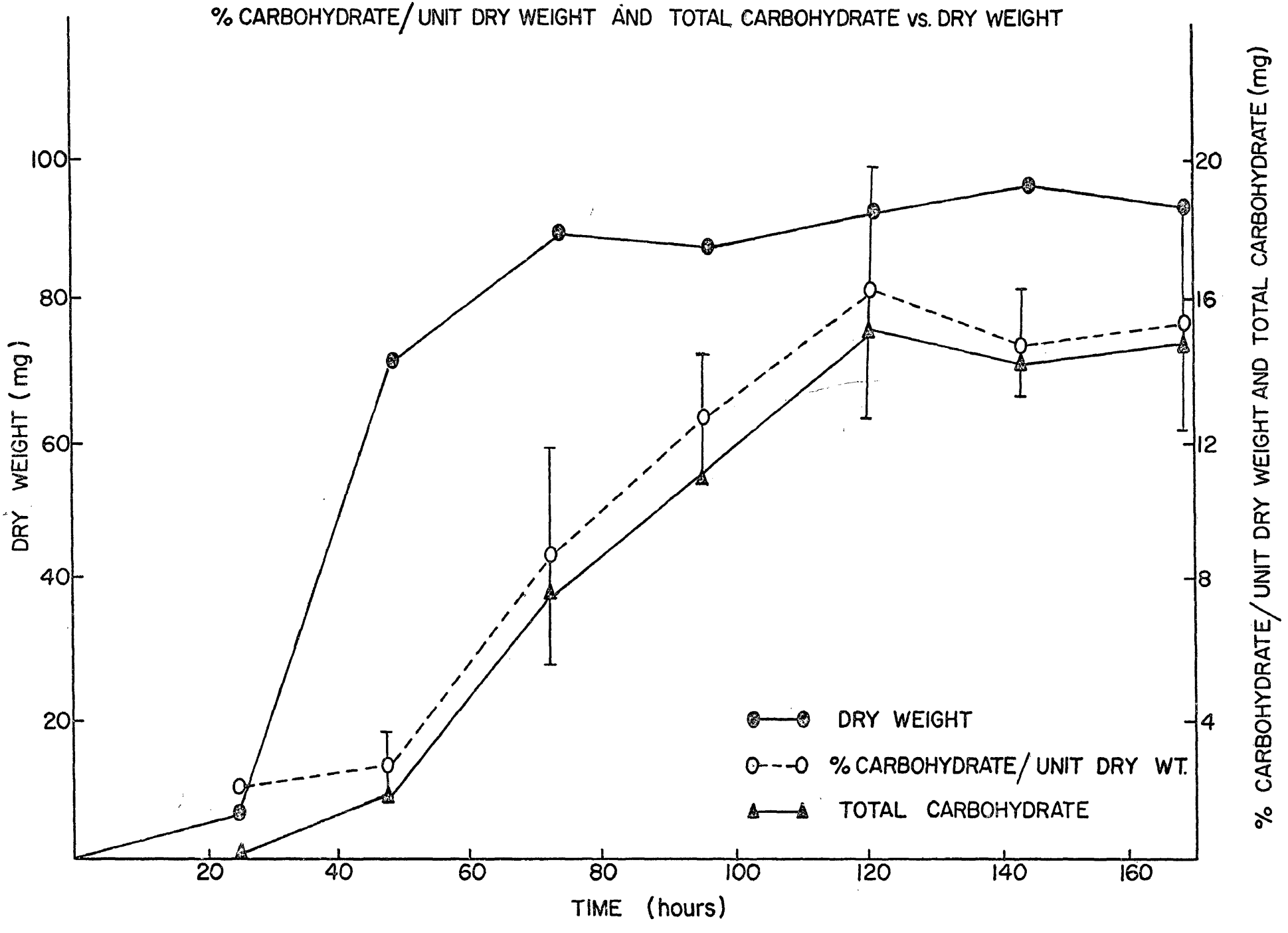
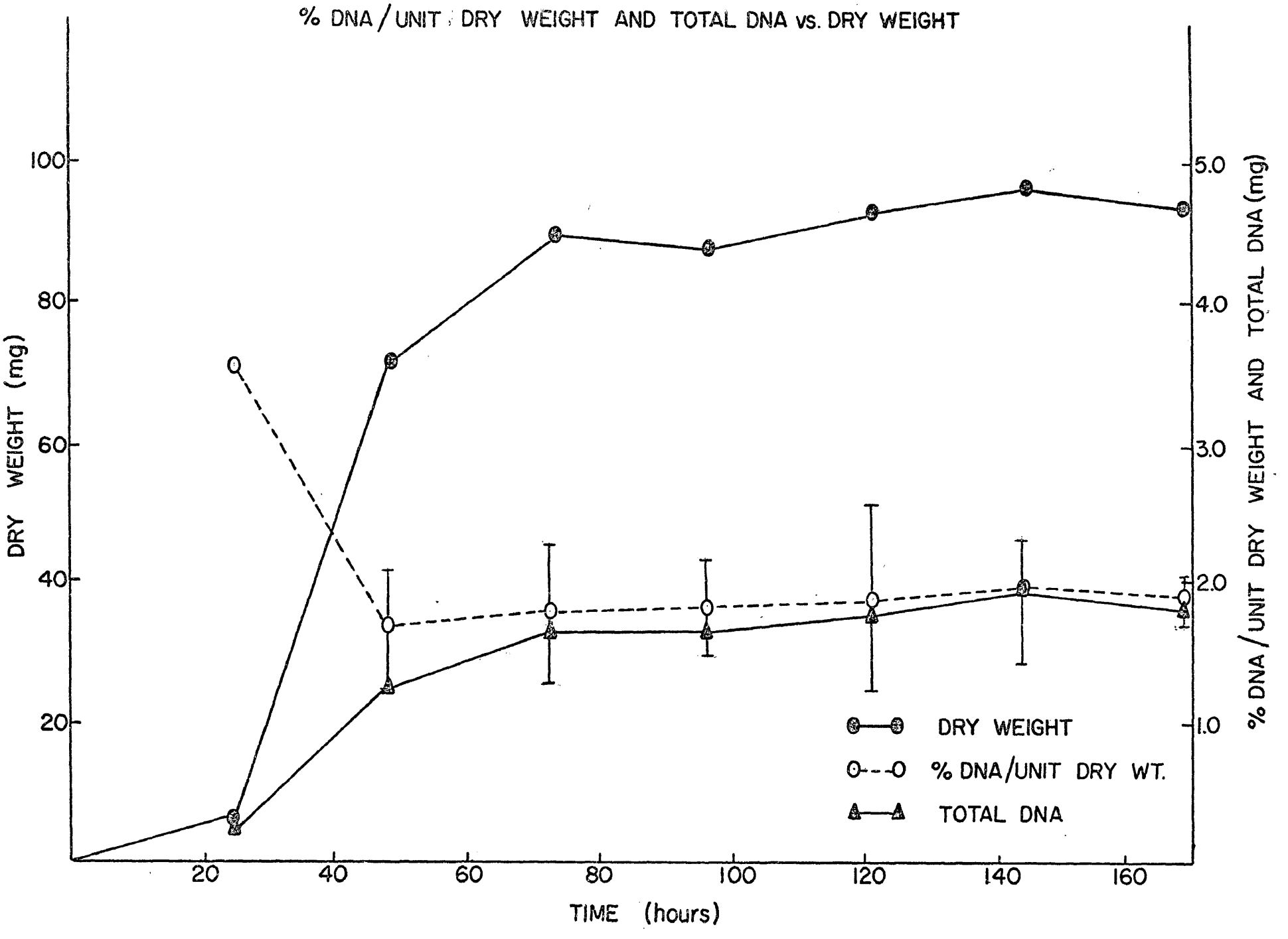

% CARBOHYDRATE/ UNIT DRY WEIGHT AND TOTAL CARBOHYDRATE vs. DRY WEIGHT
DRY WEIGHT (mg)
% CARBOHYDRATE/ UNIT DRY WEIGHT AND TOTAL CARBOHYDRATE (mg)
TIME (hours)
100
80
60
40
20
20
16
12
8
4
20
40
60
80
100
120
140
160
DRY WEIGHT
% CARBOHYDRATE/ UNIT DRY WT.
TOTAL CARBOHYDRATE

Figure 3.--The relationship between DNA and age in Alternaria Solani. The percent DNA per unit dry weight (--0--) and total DNA (—▲—) are compared with the dry weight (mg) of the mycelium (—●—) at 24 hr intervals.


% DNA / UNIT DRY WEIGHT AND TOTAL DNA vs. DRY WEIGHT
DRY WEIGHT (mg)
% DNA / UNIT DRY WEIGHT AND TOTAL DNA (mg)
TIME (hours)
100
80
60
40
20
5.0
4.0
3.0
2.0
1.0
20
40
60
80
100
120
140
160
DRY WEIGHT
% DNA/UNIT DRY WT.
TOTAL DNA

but increases less rapidly during log phase. From 72 hrs on, the total DNA per mat curve follows closely the percent DNA per unit dry weight curve.

RNA. The percent RNA per unit dry weight (Figure IV) rapidly drops during the 24-48 hr period following inoculation. After that time it increases and closely follows the dry weight curve, leveling off at approximately 72 hours. However, the total RNA follows the dry weight curve during lag phase, increases less rapidly than dry weight through the log phase, after which it once again closely follows the dry weight curve.

Protein. The percent protein per unit weight (Figure V) drops rapidly during the first 24 hr-48 hrs and then rapidly increases from 7.03 at 48 hr to 10.93 at 72 hr. It remains level after 72 hr. The total protein does not significantly increase during lag phase. From 24 to 72 hr it increases and then parallels the percent protein per unit dry weight curve.

Figure 4.--The relationship between RNA and age in *Alternaria solani*. The percent RNA per unit dry weight (--0--) and total RNA (—▲—) are compared with dry weight (mg) of the mycelium (—●—) at 24 hr intervals.

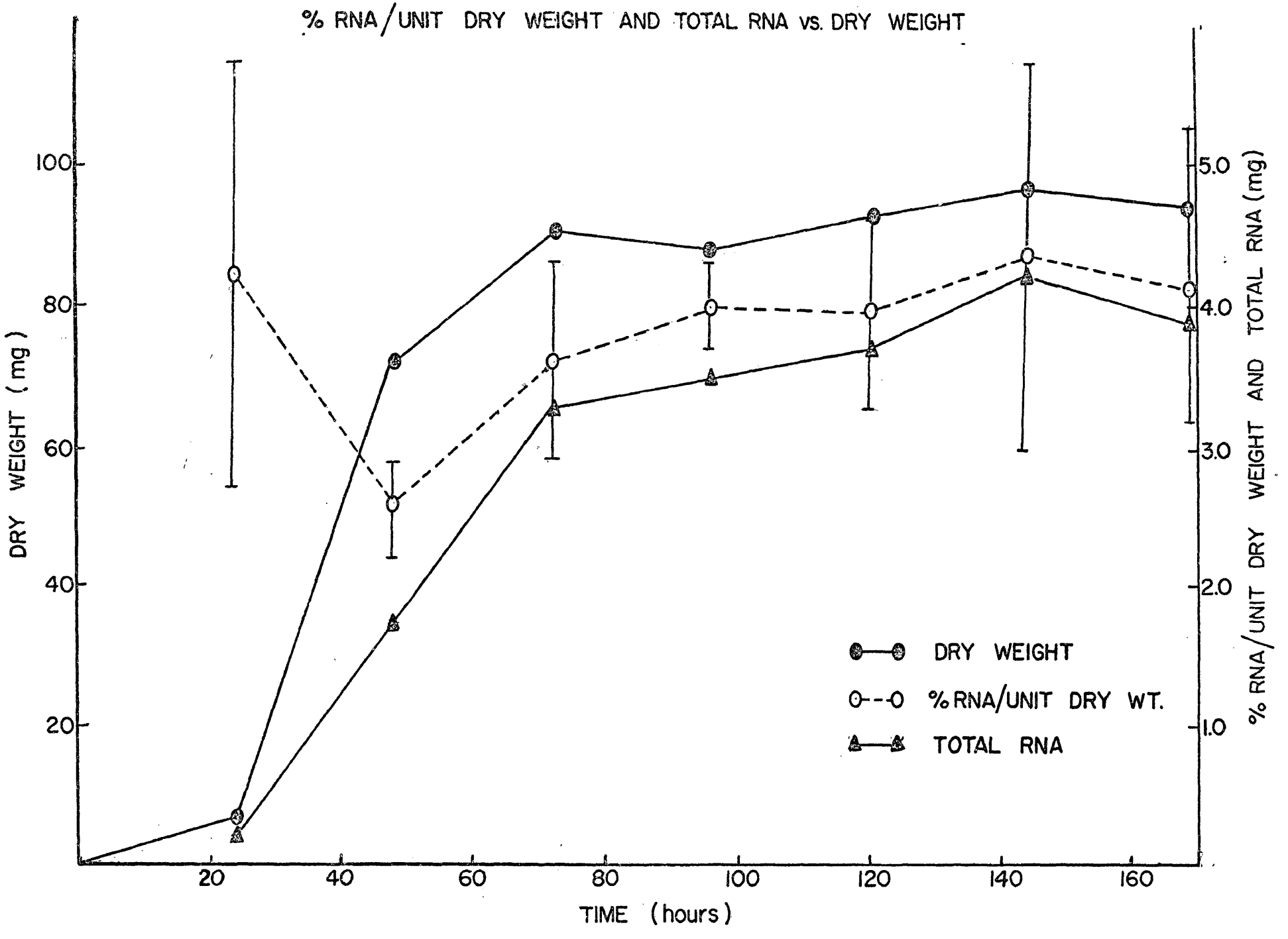
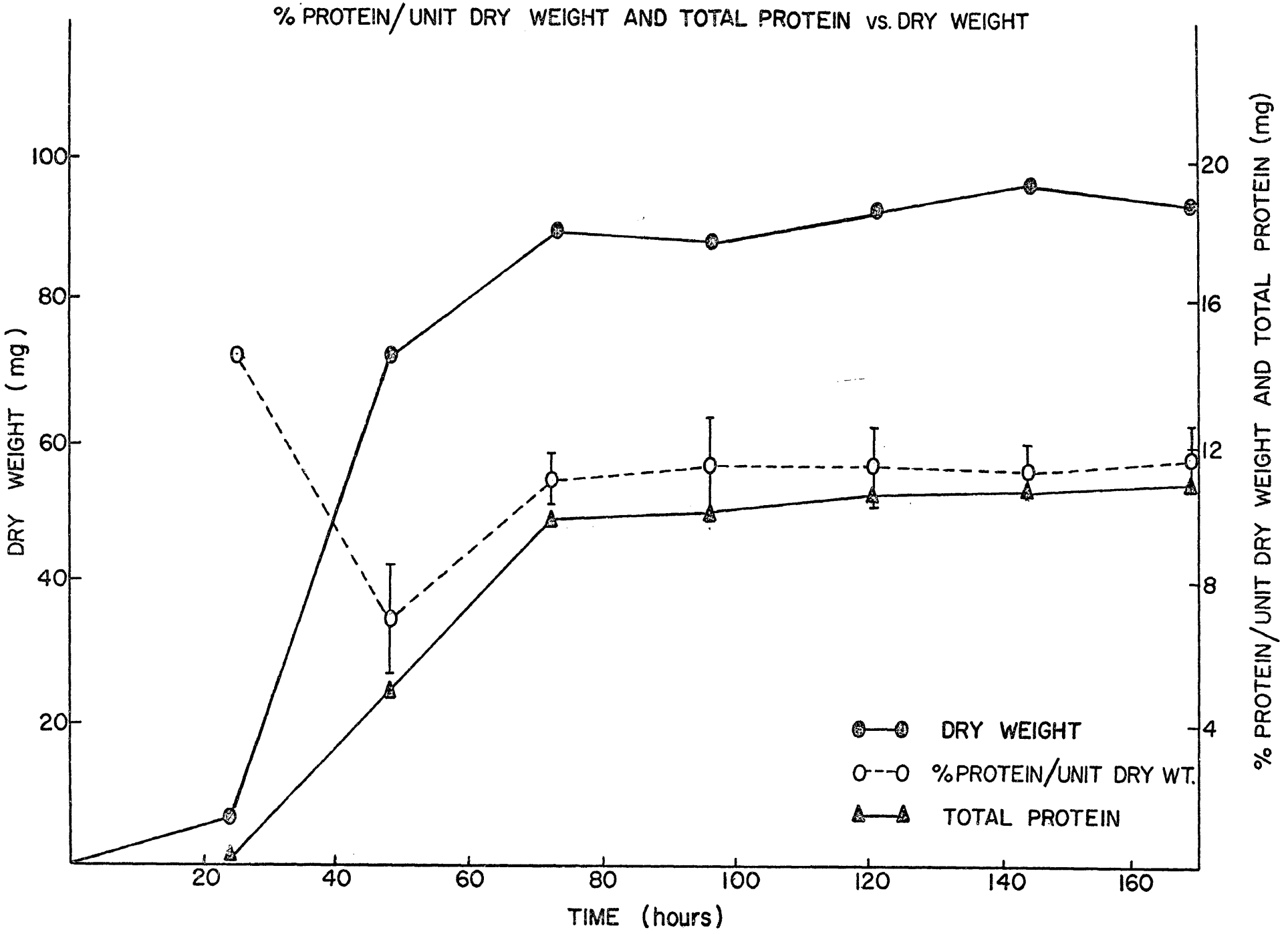

% RNA/UNIT DRY WEIGHT AND TOTAL RNA vs. DRY WEIGHT
DRY WEIGHT (mg)
% RNA/UNIT DRY WEIGHT AND TOTAL RNA (mg)
TIME (hours)
100
80
60
40
20
5.0
4.0
3.0
2.0
1.0
20
40
60
80
100
120
140
160
DRY WEIGHT
% RNA/UNIT DRY WT.
TOTAL RNA

Figure 5.--The relationship between protein and age in Alternaria solani. The percent protein per unit dry weight (--0--) and total RNA (—▲—) are compared with dry weight (mg) of the mycelium (—●—) at 24 hr intervals.


% PROTEIN/ UNIT DRY WEIGHT AND TOTAL PROTEIN vs. DRY WEIGHT
DRY WEIGHT (mg)
% PROTEIN/UNIT DRY WEIGHT AND TOTAL PROTEIN (mg)
TIME (hours)
100
80
60
40
20
20
16
12
8
4
20
40
60
80
100
120
140
160
DRY WEIGHT
% PROTEIN/UNIT DRY WT.
TOTAL PROTEIN

DISCUSSION

A. solani proved to be an excellent organism with which to work since spore production could be controlled through photo-inhibition (Lukens, 1963). Prevention of an "upper layer" of conidial reproductive structures eliminates significant differences in biological activity that might occur between a reproductive layer and a mycelial layer. Zalokar (1959) found enzyme activity differences between conidia and the surface mycelial layer of Neurospora. Yanagita and Kogané (1963) observed in A. niger, which was grown on cellophane, that there is a difference in the chemical nature between the proteins in the lower substrate mycelial layer and the upper aerial and fruiting body layer. Yanagita and Kogané (1962) also found that the upper and lower layers of A. niger are different in the base composition of the ribonucleic acids synthesized.

The uniform vegetative mycelial mat, produced by a dense, uniform inoculum of spores upon a cellophane membrane, appears to be useful for the aging study, since all parts of the mat (peripheral and nonperipheral) aged together and hence, the mat may be treated as a single aging unit. Behal (1968), also used this concept to study biochemical differentiation when he applied a heavy,

uniform distribution of A. niger spores over the surface of the medium which produced a fungal "pad of uniform age and, therefore, uniform rate of development over its entire surface." In addition to the technique of uniform inoculation which results in a uniform aging unit, the very nature of hyphal communication by means of anastomoses, observed in A. solani by Stall (1958); and septal pores through which material can be transported by protoplasmic streaming, makes it preferable that the whole network be considered a single "organism."

The absence of any correlation between the average spore density per plate and the dry weight of the mycelial mat formed after germination (for 3 replicates at any sampling hour), suggests that only a threshold number of spores (approximately 10-12 spores per 2.89 mm^2) is needed to produce a uniform mycelial mat. A greater than threshold number of spores does not necessarily produce a mycelial mat which has a greater dry weight. Plates inoculated with too few spores (below threshold) did not develop into full mycelial mats, but appeared to have spaces between the hyphal network. These spaces may have possibly been due to staling-like substances produced by germinating spores. Staling substances prevent contact among adjacent hyphae by creating zones of inhibition (Robinson and Park, 1965). However, a threshold or greater than threshold number of spores was used; the physical

density was enough to eliminate zones of inhibition from forming and hence, overcome the staling phenomena.

The cellophane membrane prevents submerged hyphal growth; it produces a two dimensional growth consisting mainly of a thin vegetative mycelial layer with few vegetative aerial hyphae. In three dimensional growth, as in standing liquid cultures, the difference in exposure to the air and nutrients between the surface layer and the submerged layer also produces a difference in biological activity (in addition to the formation of reproductive structures). Zalokar (1959) found that the surface layer of hyphae grown in standing liquid cultures contains most of the growing hyphae rich in cytoplasm, while the deeply submerged layer is heavily vacuolated and laden with fat droplets. He found differences in enzymatic activity between the layers which accompanied the morphological differences.

During the lag phase and early log phase the fungal growth consists of a developing network of anastomising vegetative hyphae which have not fully formed. Thus, prior to late log phase, the mycelial mat may not be considered to be a single unit, but rather very dense colonies which progressively lose their distinction upon continued hyphal fusion. At the end of log phase, growth slows, as all of these colonies fuse and form one entity.

Each fungal mat remains viable throughout stationary phase with the exception of the 168 hr sample which shows only a small increase in new growth after 24 hours relative to the 98 hr, 124 hr, and 149 hr samples. This poor growth increase of the 168 hr mat may be due to autolysis or to a failure of the cells of late stationary phase to enter log phase of growth as rapidly as do those cells of earlier stationary phase. Although the method of viability determination employed was not entirely satisfactory, it served temporarily as a reasonable indicator.

One criterion for autolysis is loss in dry weight (Lahoz, Reyes, Beltra, 1965; Lahoz, Beltra, Balosteros, 1969; Sekhon and Colotelo, 1968; Lahoz, Miralles, 1970). In the present experiment, the dry weight remains at a continuous stationary plateau. A. solani may not have depleted its carbon source from the medium and/or may have partially relied upon the cellophane membrane as a carbon source and hence maintained a steady state of growth.

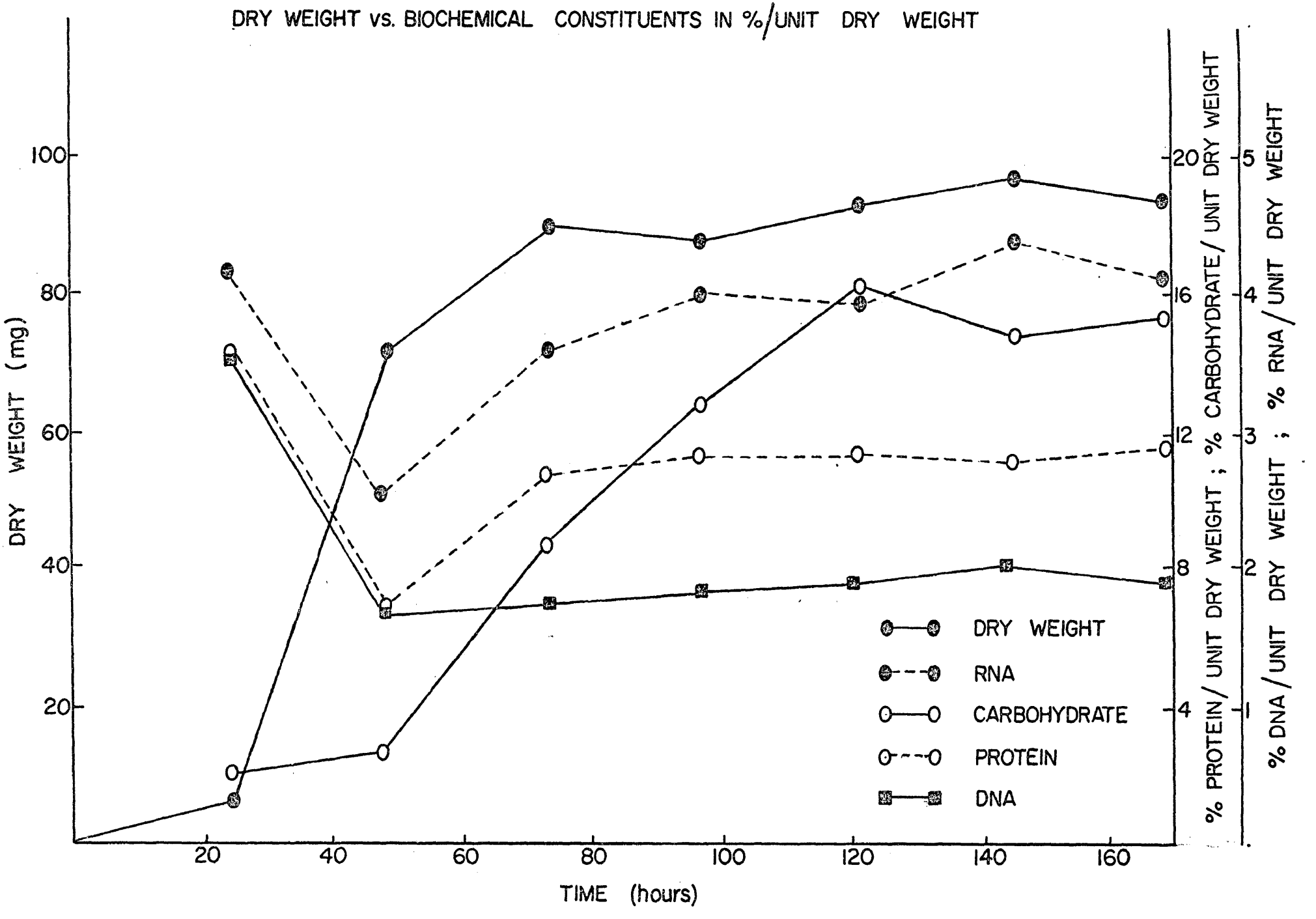
Lahoz, Reyes, and Beltra (1965) observed that autolysis (decrease in dry weight) in A. flavus is accompanied by a decrease in the free glucose. Lahoz, Beltra, and Balestros (1969) noted that in Nectria galligena the total carbohydrate (anthrone) slightly increases during the first days of autolysis and decreases thereafter. Lahoz and Miralles (1970) found carbohydrate (anthrone) declines continuously during autolysis.

In this study, confidence limits around each of the measurements for each sampling hour indicates that carbohydrate per unit dry weight continues to increase until 96 hr and then remains stationary up to 168 hr without decreasing. Since total carbohydrate does not decrease after it reaches a plateau, autolysis perhaps did not occur or was minimal, and cell viability was maintained.

It is noted (Figure I) that the pH declines rapidly during the initial stages of growth (lag phase and log phase) and then remains fairly constant throughout the stationary phase. Although not measured, alternaric acid, which is a metabolic product of A. solani (Brian, et al., 1951) may be produced at a greater rate during the initial stages of development and may possibly account for the early rapid decline in pH. When stationary phase is reached, the mycelial mat is fully developed and as the metabolic growth rate almost stops, so may the production of alternaric acid; this possibly may account for the steady pH seen throughout stationary phase.

A comparison of the biochemical changes in percent per unit dry weight that occur during the aging process of A. solani is made (Figure VI). Biochemical changes were determined during lag and log phase of growth, when the mat was developing into a single physiological unit. Biochemical changes were also noted during the stationary phase which followed, in which the mat ages as a single

Figure 6.-- DNA, RNA, Protein and Carbohydrate as related to age in Alternaria solani. Biochemical constituents are in % per unit dry weight. RNA (--●--), Carbohydrate (—0—), Protein (--0--) and DNA (—▣—) are compared with dry weight (mg) of the mycelium (—●—) at 24 hr intervals.


DRY WEIGHT vs. BIOCHEMICAL CONSTITUENTS IN %/UNIT DRY WEIGHT
DRY WEIGHT (mg)
100
80
60
40
20
20
40
60
80
100
120
140
160
TIME (hours)
% PROTEIN/UNIT DRY WEIGHT ; % CARBOHYDRATE/UNIT DRY WEIGHT
20
16
12
8
4
% DNA/UNIT DRY WEIGHT ; % RNA/UNIT DRY WEIGHT
5
4
3
2
1
DRY WEIGHT
RNA
CARBOHYDRATE
PROTEIN
DNA

entity. The percent of DNA per unit dry weight falls sharply throughout log phase and thereafter remains stationary. The DNA is much higher, relative to the dry weight, during the beginning of log phase than toward the end of log phase, and during stationary phase. Gottleib and Van Etten (1964) found that in P. atrovenetum, DNA of the mycelium decreases in the lag phase and then increases slightly to plateau for the duration of log phase; the DNA then decreases to a constant percentage during the stationary and death phases.

The higher percentage of DNA observed in the initial stages of growth (following spore germination) is correlated with the high levels of DNA in spores seen by other workers. Stall (1958) observed that in A. solani mature conidial cells are predominately multinucleate with as many as eight nuclei present in the base of the conidium, whereas one to three nuclei are observed in non-ultimate vegetative mycelial cells. Thus, the higher concentration of nuclei in the spore cells as observed by Stall (1958) may account for the higher level of DNA during the initial stages of growth. This relationship is in agreement with the finding of Gottleib and Van Etten (1964) in which the percentage of DNA is always slightly higher in the spores of P. atrovenetum than in the mycelium.

Stall (1959) observed in A. solani that any cells of the multicellular conidium may germinate and that the

germ tubes contain multinucleate cells with the same range of nuclear numbers as mycelial cells. He also noted that the ultimate cells of A. solani have higher numbers of nuclei than non-ultimate cells. King and Alexander (1969), by the use of living phase microscopy, observed that the hyphal tip cells of A. solani contain 26 to 27 nuclei, whereas the first seven basal cells behind the hyphal tip cell contain 4 to 5 nuclei per cell. This complements the finding of Zalokar (1959); that young regions of differentiating Neurospora hyphae are dense with nuclei and cytoplasm as compared to old regions, which are vacuolated and filled with fat droplets.

In this study, the greatest number of hyphal tip and ultimate cells could be seen during the lag and log phases of growth prior to hyphal fusion. This again accounts for high concentration of DNA relative to the dry weight during log phase of growth, since during this period there are many rapidly dividing young cells which have not yet accumulated storage material (carbohydrate) to contribute to full mycelial growth. As the stationary phase is approached, there are a greater number of hyphal fusions. Hence, the ultimate and non-ultimate cells are no longer distinguishable from one another as the mat becomes one entity, with a decrease in cell division and an increase in mycelial growth.

The percent RNA per unit dry weight (Figure VI) has the same downward slope as the DNA from the beginning to the end of log phase. The level of RNA then increases sharply in the 48 to 72 hr period and then shows a gradual, but constant, increase throughout stationary phase. These results are similar to those obtained by Gottleib and Van Etten (1964) using P. atrovenetum. In their study RNA increases to a maximum during the lag phase and subsequently decreases as the fungus ages. Yanagita and Kogané (1962) noted that the RNA content of conidia of A. niger decreases as germination proceeds, i.e., a high content of basophilia seems to be one of the characteristics of reproductive organs in molds. Zalokar (1959) observed, by staining methods, that there is a high concentration of RNA material in hyphal tips. In this study, the initial high level of RNA present at the beginning of log phase of growth subsequently decreases during the remainder of log phase. The phenomenon may be similar to that for DNA, i.e., spores and hyphal tips contain higher percentages of nucleic acids relative to the dry weight than do older hyphal cells; and as other constituents of the mycelium gradually contribute to dry weight, the relative levels of both DNA and RNA drop.

Percentage protein (Figure VI) parallels DNA and RNA as it decreases from the beginning to the end of log phase of growth. The slope parallels RNA as it increases

during the 48 to 72 hr period. After 72 hours, the protein curve closely follows that of both RNA and DNA, although the RNA level peaks slightly at 144 hr. Gottleib and Van Etten (1964) found that in P. atrovenetum, protein, as well as RNA, increases to a maximum during lag phase, and then decreases as the fungus ages. They also found that the changes in the amount of protein in vegetative cells are closely correlated with those occurring in the amount of RNA, "and thus agree with the concepts of protein synthesis." The high protein levels observed in A. solani during the log phase of growth are probably a result of a rapid rate of protein synthesis, as active protein synthesis is characteristic of young growing cells. Skowronski and Gottleib (1970) found that the protein content is 1.6 times greater in the peripheral hyphae of R. solani than in old fungal cells.

The percentage of carbohydrate per unit dry weight (Figure VI) is at its lowest level at the beginning of log phase; whereas DNA, RNA, and protein are all at their highest level at this time. The carbohydrate remains low during the log phase and then rapidly increases from the end of log phase until the 120 hr of stationary phase, and does not fluctuate subsequently. Zalokar (1959) observed upon staining that although glycogen is present in the hyphae of Neurospora, it is absent from the hyphal tips. This suggests that the low carbohydrate content of A.

solani during lag and log phases is due to the great concentration of rapidly growing hyphal tips which are involved with synthesis of new materials (i.e., DNA, RNA protein) as opposed to the accumulation of storage compounds. Gottleib and Van Etten (1964) observed that in P. atrovenetum, carbohydrate was present in higher concentration in the mycelium than in the spores. They also noted that the increased synthesis of carbohydrate is concomitant with a decreased synthesis of RNA, protein, and amino acids. In A. solani, the carbohydrate was observed to increase at the end of log phase, when protein and RNA are at their lowest levels. During stationary phase, when RNA and protein level off, the carbohydrate continues to increase. Thus, as the mycelial mat develops, the percentage DNA, RNA, and protein per unit dry weight are maximum at the beginning of log phase and then decrease to a minimum at the end of log phase. The percent carbohydrate per unit dry weight remains low during log phase.

Cell wall, non-extractable TCA material and lipid were not measured in this study. This accounts for the fact that the percent per unit dry weight of DNA, RNA, protein and carbohydrate add up to less than one-half the total dry weight. The relative concentrations by which these non-measured compounds change as A. solani ages can only be speculated upon at this time. It is reasonable to

assume that cell wall material as percent dry weight may possibly increase as A. solani ages, since young hyphal cell wall tips are required to be more "plastic" and thin for rapid growth, and more subapical fungal cells (typical of older cells) become structurally rigid due to the increased accumulation of microfibrillar elements in the cell wall (Burnett, 1968). It is also possible to speculate that lipids on a percent dry weight basis, may be greater in non-dividing older A. solani cells and exist as storage compounds to help maintain metabolism. Van Etten and Gottlieb (1965) found in P. atrovenetum that total fatty acids on a dry weight basis increase from a minimum in spores to a maximum near the end of log phase of growth.

In summary, as the stationary phase is approached, the mycelial mat may be considered to be a single aging entity. Throughout stationary phase, as the mat ages, the percent DNA per unit dry weight remains level. However, the percent RNA and protein per unit dry weight increase during initial stationary phase and then plateau. The percent carbohydrate per unit dry weight increases rapidly from early stationary phase and subsequently levels off.

Thus, as mycelial mats of A. solani age as single entities (stationary phase), a constant DNA level suggests that no further cell division occurs. The "metabolically

active synthesising compounds," RNA and protein, increase slightly and then remain constant. An accumulation of carbohydrate "storage material" tends to be maximum as A. solani ages.

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