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Biochemical Changes During the Growth of Fungi: I. Nitrogen Compounds and Carbohydrate Changes in *Penicillium atrovenetum*

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BIOCHEMICAL CHANGES DURING THE GROWTH OF FUNGI

I. NITROGEN COMPOUNDS AND CARBOHYDRATE CHANGES IN *PENICILLIUM ATROVENETUM*[1]

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ABSTRACT

GOTTLIEB, DAVID (University of Illinois, Urbana), AND JAMES L. VAN ETTEN. Biochemical changes during the growth of fungi. I. Nitrogen compounds and carbohydrate changes in *Penicillium atrovenetum*. J. Bacteriol. **88**:114-121. 1964.—Changes in the biochemical constituents of cells were studied during the growth and development of *Penicillium atrovenetum*. Growth of the fungus, as measured by the dry weight, could be divided into four phases: lag, log, stationary, and death. The percentages of total nitrogen, cold trichloroacetic acid-soluble nitrogen, ribonucleic acid (RNA), and protein increased to a maximum during the lag phase, and subsequently decreased as the fungus aged. The percentage of deoxyribonucleic acid (DNA) was always slightly higher in the spores than in the mycelium. The DNA in the mycelium decreased in the lag phase, and then increased slightly to a plateau for the duration of the log phase, followed by a decrease to a constant percentage during the stationary and death phases. Carbohydrates were present in higher concentration in the mycelium than in the spores. The percentage of carbohydrates in the mycelium increased continually until it reached a maximum late in the log phase, and then decreased as the fungus entered the death phase. The results reported for this fungus are, in general, in agreement with those reported for other microorganisms. Namely, the percentages of enzyme-forming compounds, such as amino acids, nucleotides, RNA, and protein, were highest in the lag phase, whereas storage compounds such as carbohydrates increased to a maximum near the end of the log phase. The definition of log phase in fungi depends on the criteria that are used. If, instead of using the linear increase in dry weight to delimit this growth period, one uses the end of net protein, RNA, and DNA synthesis, a more realistic concept of growth emerges.

[1] Part of the dissertation of James L. Van Etten, presented to the Graduate Faculty of the University of Illinois in partial fulfillment of requirements for the M.S. degree.

Investigations of changes in cellular composition, as a guide to metabolic activity of fungi during the growth cycle, have rarely been made. Most studies on the chemical composition of fungi have been concerned with relatively few components, such as fat and protein. Furthermore, changes in composition during the entire growth cycle of a fungus have not been followed. The per cent total nitrogen on a dry-weight basis was highest in young mycelium and decreased as the cells aged (Behr, 1930). Protein (Suskind and Bonner, 1960) and soluble nitrogen (Meyers and Knight, 1961; Pillai and Srinivasan, 1956) were also highest in young mycelium and decreased in older mycelium. However, the composition of the mycelium in the lag phase was not determined, and their initial observations were made after the fungus had probably reached the log phase of growth.

Suskind and Bonner (1960) and Krishnan, Damle, and Bajaj (1957) reported that the percentage of ribonucleic acid (RNA) on a dry-weight basis decreased with age in *Neurospora crassa* and *Aspergillus niger*. Krishnan et al. (1957) also reported that the percentage of deoxyribonucleic acid (DNA) on a dry-weight basis remained fairly constant as the culture aged. Fluri (1959) demonstrated that certain carbohydrate fractions, including trehalose, glycogen, and chitin, increased during the growth of *Phycomyces blakesleeanus*. Blumenthal and Roseman (1957) found an increase in percentage of chitin on a dry-weight basis during the development of many fungi, but in others no increase occurred.

This report deals with the changes in the total nitrogen, cold trichloroacetic acid-soluble nitrogen, protein, RNA, DNA, carbohydrates, and chitin during the entire growth cycle of *Penicillium atrovenetum*.

MATERIALS AND METHODS

Organism. P. atrovenetum IMI 61837 was obtained from the Commonwealth Mycological

Institute, Kew, Surrey, England. Spores for inoculum were obtained by growing the fungus for 6 days on a synthetic glucose-asparagine medium (glucose, 10 g; asparagine, 2 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 0.2 mg; $ZnSO_4 \cdot 7H_2O$, 0.2 mg; $MnCl_2 \cdot 4H_2O$, 0.1 mg; agar, 20 g; and water, to 1 liter). Sterile distilled water containing 4 ppm of Vatsol was added to the agar culture. The culture flask was shaken to obtain a suspension of spores which was diluted to give 50% transmission at 530 mμ. For growth studies and chemical analyses, 1 ml of the inoculum was pipetted into 500-ml Erlenmeyer flasks which contained 30 ml of Raulin Thom medium (Raistrick and Stösse, 1958), modified by limiting glucose to 3%. The fungus was grown as stationary surface cultures, in the dark at 26 C.

The mycelia were collected for chemical analyses at various time intervals by filtration, and were washed four times with distilled water. These samples were dried to a constant weight over phosphorus pentoxide in a vacuum desiccator at room temperature. The dried samples were ground in a Wiley mill, passed through a 60-mesh screen, and stored over phosphorus pentoxide in a desiccator.

To obtain spores for chemical analyses, 5 ml of inoculum were pipetted into 2.8-liter Fernbach flasks containing 250 ml of the glucose-asparagine medium, and the flasks were rotated to distribute the inoculum evenly on the agar surface. Spores were harvested after 14 days of growth by brushing the surface of the culture lightly with a camel's hair brush and shaking the spores into a collecting vessel. These spores were dried in the same manner as was the mycelium.

Growth measurements. The mycelia or spores were filtered in Gooch crucibles which had previously been packed with asbestos and dried at 85 C in a vacuum oven to a constant weight. They were washed four times with distilled water and dried to a constant weight. Because the mass of spores and the young mycelium for samples from 0 to 36 hr was relatively small in any one flask, many flasks were pooled and the average dry weight was determined. Three separate pools were used as replicates for each determination. The dry weights of the older samples (after 36 hr) were determined separately from each of three single flasks.

Carbohydrates and chitin. Total carbohydrates were determined on the dried mycelium by the anthrone method of Morris (1948) with glucose as a standard. Chitin was determined by the method of Roseman and Daffner (1956) with recrystallized glucosamine hydrochloride as a standard.

Fractionation of the cells. The cells were fractionated by a modification of the procedure used by Roberts et al. (1955) for the fractionation of *Escherichia coli*. (i) Dried cells (210 mg) were suspended in 28 ml of 5% trichloroacetic acid at 5 C with a Potter-Elvehjem homogenizer. After 30 min the suspension was centrifuged. The supernatant fluid (cold trichloroacetic acid-soluble fraction) was analyzed for nitrogen. (ii) The residue from step 1 was suspended in 28 ml of 75% ethanol for 30 min at 50 C. The supernatant fluid obtained after centrifugation was termed the alcohol-soluble fraction. (iii) The residue from step 2 was suspended in 28 ml of a 1:1 solution of ether and 75% ethanol for 15 min at 50 C and centrifuged. The supernatant fluid was termed the alcohol-ether soluble fraction. (iv) The residue from step 3 was suspended in 28 ml of 5% trichloroacetic acid and placed in a boiling-water bath for 30 min. After centrifugation and subsequent removal of the supernatant fluid, the process was repeated once more with fresh trichloroacetic acid. The supernatant fluids from this step were combined as the hot trichloroacetic acid-soluble fraction. This fraction was analyzed for RNA and DNA. (v) The residue from step 4, labeled the alcohol-insoluble protein fraction, was washed with 25 ml of acidified ethanol and then with 25 ml of ether. After each washing, the material was centrifuged, and the supernatant fluid was decanted to leave a solid residue, which was analyzed for chitin and protein. (vi) The supernatant fluids from steps 2 and 3 were combined and diluted with 56 ml of ether and 56 ml of water. The water phase was extracted once more with 56 ml of ether, and the two ether extracts were then combined and analyzed for sterols and fatty acids. The water phase containing alcohol-soluble protein was analyzed for nitrogen.

Nitrogen determinations. Total nitrogen, cold trichloroacetic acid-soluble nitrogen, alcohol-soluble protein nitrogen, and alcohol-insoluble protein nitrogen were determined by the micro-Kjeldahl method of Willits and Ogg (1950). The alcohol-insoluble protein nitrogen (corrected for chitin) was multiplied by 6.25 to give an approximation of the total alcohol-insoluble protein. Because the fungus contained only a small

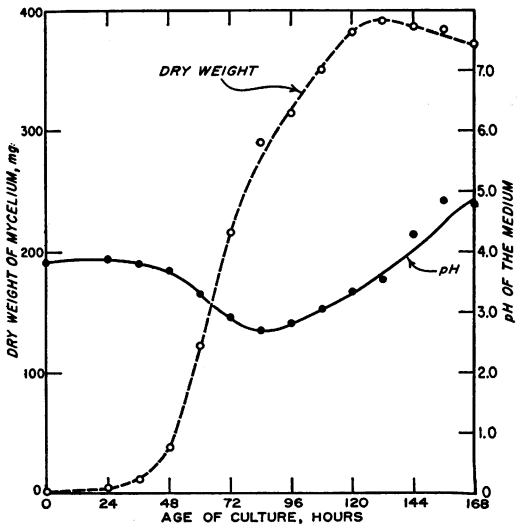

FIG. 1. *Growth curve for Penicillium atrovenetum expressed as dry weight of the mycelium per culture flask as the culture ages, and pH of the brew as the culture ages.*

amount of alcohol-soluble protein nitrogen, the alcohol-insoluble protein nitrogen was referred to as the protein nitrogen.

Nucleic acids. The hot trichloroacetic acid-soluble fraction was analyzed for RNA and DNA. Because the determination of RNA by the orcinol method (Mejbaum, 1939) gave inconsistent results for this fungus, despite repeated efforts and modifications, the following method was used to estimate the RNA present in the mycelium (Wu, 1959). Samples of the hot trichloroacetic acid extract were diluted 14-fold with water, and the optical density was measured with a Beckman DU spectrophotometer at 260 and 290 mμ. A blank was prepared by diluting 5% trichloroacetic acid in the same manner. The amount of RNA was calculated by taking the difference between the optical density at 260 and 290 mμ and comparing this with a yeast RNA standard. By this means, consistent values were obtained for replicate samples.

The diphenylamine test for DNA (Dische, 1955) could not be used on the hot trichloroacetic acid extract because of an unknown material which absorbed in the same range as did the diphenylamine. The DNA was determined on the whole cells by the method of McIntire and Sproull (1957), with salmon sperm DNA obtained from the Mann Research Laboratory, New York, N.Y., as a standard.

Lipids. The ether fraction was evaporated to dryness with a rotary evaporator. The lipids were divided into fatty acids and nonsaponifiable fractions, according to the method of Caltrider, Ramachandran, and Gottlieb (1963). Sterols were sought in the nonsaponifiable fraction by both the Liebermann-Burchard test (Stadtman, 1957) and gas chromatography, but were found to be absent. Because only a small amount of 0.01 M sodium hydroxide was needed to titrate the fatty acid fraction, it was doubtful whether any fatty acids were extracted by this method. The absence of fatty acids in this extract was also confirmed by gas chromatography.

In all values, the term "per cent" indicates the percentage of a material on a dry-weight basis. Each value is the average of at least three replicates. The total amounts of each constituent per flask were calculated from the percentage of each constituent in the fungus and the total dry weight per flask.

RESULTS

With dry weight used as the criterion, the growth of *P. atrovenetum* can be divided into four phases: lag, 0 to 36 hr; log, 36 to 120 hr; stationary, 120 to 144 hr; and death, after 144 hr (Fig. 1). Most spores were formed during the stationary phase, but a few small isolated areas within a culture were usually observed sporulating in the early log phase.

Total nitrogen. The per cent total nitrogen in the mycelium increased rapidly in the initial 24 hr, from 6.3 to 8.0 (Fig. 2). During this time, the spores germinated and formed hyphae. Subsequently, the percentage of total nitrogen rapidly decreased until it reached a minimum of 3.6 at 120 hr, and then, as maximal sporulation occurred between 132 to 144 hr, it increased to 4.15%. The total nitrogen in the mycelium per flask increased with increasing dry weight, reached a maximum at 84 hr, and leveled off while the mycelial weight was still increasing. After 120 hr, concomitant with sporulation, the total nitrogen again increased.

Cold trichloroacetic acid-soluble nitrogen and alcohol-soluble protein. The percentage of cold trichloroacetic acid-soluble nitrogen (nucleotides, amino acids, inorganic nitrogen, etc.) increased rapidly from 0.69 to 1.85 in the initial 36 hr (Fig. 3). It then decreased rapidly until 72 hr and more slowly during the remainder of the growth cycle, reaching a minimum of 0.39% at 132 hr.

The total trichloroacetic acid-soluble nitrogen per flask reached a maximum at 84 hr and subsequently decreased slightly.

The per cent alcohol-soluble protein nitrogen was determined in the spores, and mycelium after 24, 84, and 144 hr of growth. This fraction was approximately 0.2% during the entire growth cycle.

Chitin. To determine whether all of the chitin in the mycelium was present in the protein fraction, chitin was determined on the 132-hr sample in both the whole mycelium and in the protein fraction. If chitin were present in this fraction, the amount of nitrogen due to chitin should be subtracted from the total nitrogen in the protein fraction to determine protein nitrogen. The whole mycelium contained 3.7% chitin. When the chitin in the protein fraction was calculated on the basis of dry mycelial weight, the value was 3.9%. Because these values were not significantly different, all subsequent determinations of chitin were made on the whole mycelium. To obtain accurate protein nitrogen values, the amount of nitrogen due to chitin was subtracted from the total nitrogen present in the protein fraction (corrected protein nitrogen).

A rapid decrease in chitin was evident during spore germination, but, from then on, no consistent trend in the content of chitin was observed with the age of the culture. The chitin content

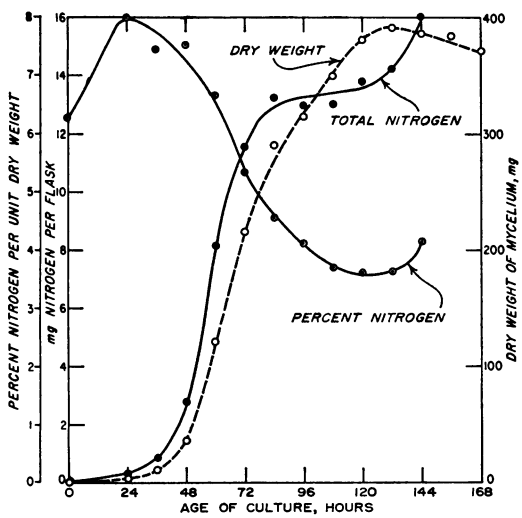

FIG. 2. *The relationship between total nitrogen and the age of Penicillium atrovenetum. The total nitrogen is expressed as per cent nitrogen per dry weight and as total nitrogen per flask.*

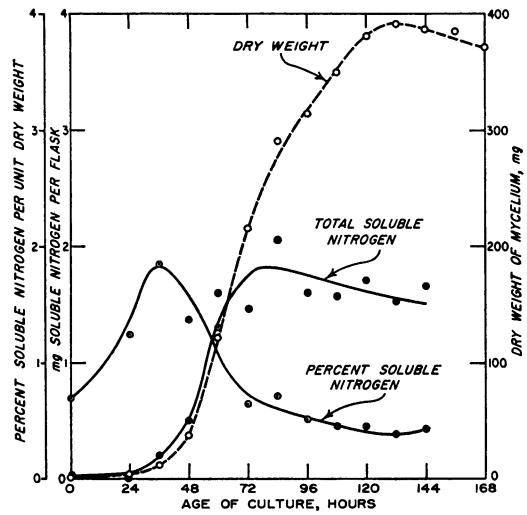

FIG. 3. *The relationship between cold trichloroacetic acid-soluble nitrogen and the age of Penicillium atrovenetum. The soluble nitrogen is expressed as per cent soluble nitrogen per dry weight and as total soluble nitrogen per flask.*

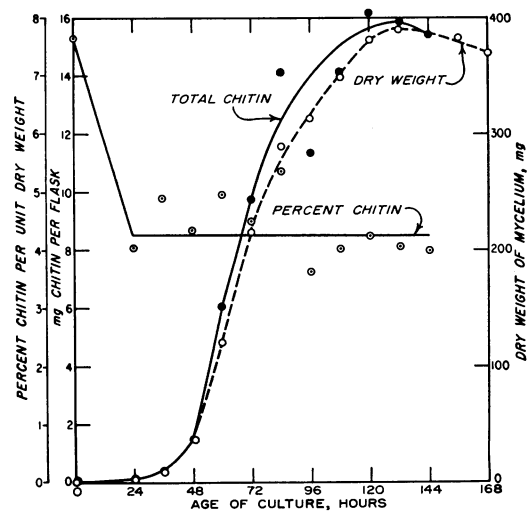

FIG. 4. *The relationship between chitin and the age of Penicillium atrovenetum. The chitin is expressed as per cent chitin per dry weight and as total chitin per flask.*

obtained from mycelia of different ages varied from 3.5 to 5.1% with an average of 4.3 (Fig. 4). However, the concentration of chitin in the spores was always markedly higher (7.7%). These two values were used for calculating the nitrogen due to chitin in the protein fractions.

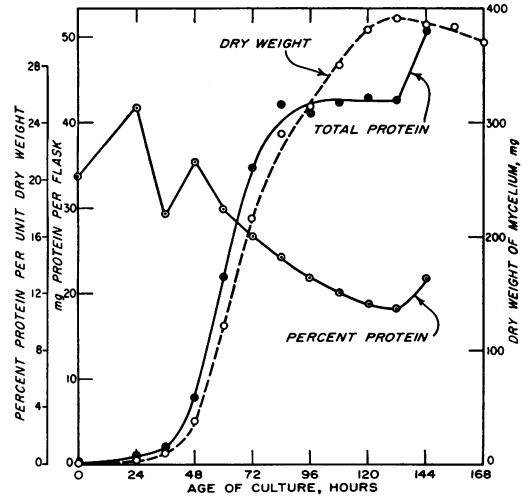

FIG. 5. *The relationship between protein and the age of Penicillium atrovenetum. The protein is expressed as per cent protein per dry weight and as total protein per flask.*

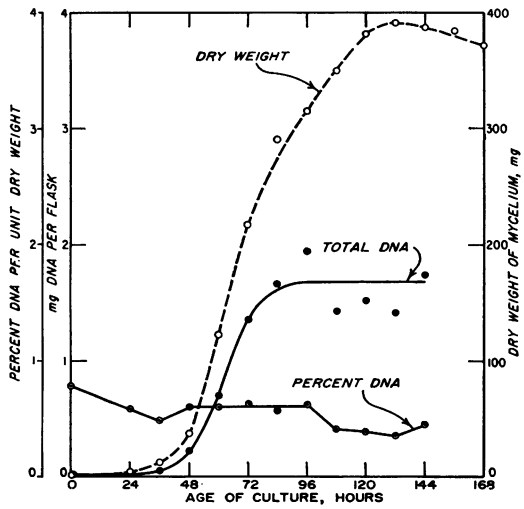

FIG. 6. *The relationship between DNA and the age of Penicillium atrovenetum. The DNA is expressed as per cent DNA per dry weight and total DNA per flask.*

Protein. The percentage of protein had two maxima during the early growth phases (Fig. 5). It increased to a peak value of 25% during the first 24 hr, and then decreased during the following 12 hr to 17.6%. A second maximum of 21.1% was reached at 48 hr. Based on consistent results obtained from three different experiments, the decrease from 24 to 36 hr was real and not due to an artifact. After 48 hr, the protein decreased gradually until it reached a low of 10.9% at 132 hr, and then slightly increased to 13.2% at 144 hr at the time of sporulation. The total protein per flask reached a peak at 84 hr, remained approximately constant until 132 hr, and then increased as the fungus sporulated.

DNA. The percentage of DNA was higher in the spores (0.78) than in any other phase of growth (Fig. 6). During the lag phase, DNA decreased to 0.48% at 36 hr. A slight increase to approximately 0.60% took place in the log phase, and the percentage remained constant through 96 hr of growth. Between 96 and 108 hr, the DNA decreased to about 0.41% and remained stationary up to 132 hr. A very slight increase in the percentage of DNA accompanied sporulation. The total DNA per culture flask increased to a maximum at 84 to 96 hr, after which it remained approximately constant.

RNA. The changes in the percentage of RNA paralleled the changes in protein. There were two maxima during the early growth phases: a major peak of 4.65% at 24 hr and a minor one of 3.43% at 48 hr of growth (Fig. 7). The decrease from 24 to 36 hr was found in four independent experiments replicated three times. After 48 hr, the percentage of RNA in the mycelium diminished continually to 1.71 at 132 hr. The total RNA per flask reached a peak at 84 hr, and then decreased slightly during the remaining phases of growth.

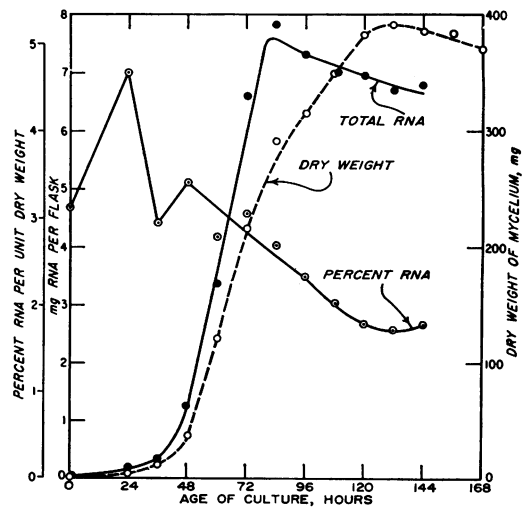

FIG. 7. *The relationship between RNA and the age of Penicillium atrovenetum. The RNA is expressed as per cent RNA per dry weight and as total RNA per flask.*

Carbohydrates. Carbohydrates, measured as total anthrone-positive material, sharply increased from 20.6 to 27.6% during the initial 24 hr and at a lower rate during the rest of the lag period (Fig. 8). They then rapidly increased to 40.3% at 96 hr, and remained fairly constant until 132 hr when they decreased slightly. The total carbohydrates per culture flask paralleled the growth curve, and both reached a maximum at 132 hr. The total carbohydrates and dry weight of the mycelium then started to decrease as the organism entered the death phase.

Lipids. Fatty acids and sterols were not extracted by the fractionation procedure used. A better extraction procedure has since been developed, and the identification of the sterols and fatty acids with the development of *P. atrovenetum* will be reported in a subsequent paper.

DISCUSSION

Changes in constitution during the initial growth phases of *P. atrovenetum* resemble those of bacteria, actinomyces, and yeasts, because the percentages of total nitrogen, protein, RNA, and cold trichloroacetic acid-soluble nitrogenous compounds per dry weight reached their maxima during the lag phase of growth. Though the percentages of each of these four substances decrease after the lag phase, this decrease is probably not due to their degradation, but appears to be brought about by the increased synthesis of other constituents of the mycelium. At the same time that these decreases occurred, the total nitrogen, total cold trichloroacetic acid-soluble nitrogen, total protein, and total RNA per flask increased to a maximum at 84 to 96 hr of growth and remained fairly stationary for the next 36 hr. The percentage of carbohydrates, on the other hand, continually increased and reached a maximum late in the log phase. Therefore, the decreases in percentage were probably caused by the syntheses of storage compounds by *P. atrovenetum*, such as carbohydrates and, as will be shown in a subsequent publication, also fatty acids.

The rate of carbohydrate synthesis was relatively uniform during the lag and most of the log phase. Nevertheless, the percentage of carbohydrates increased during this period. This increase was probably due to decreased synthesis of protein, amino acids, and RNA. Such a decreased synthesis could explain the fact that the

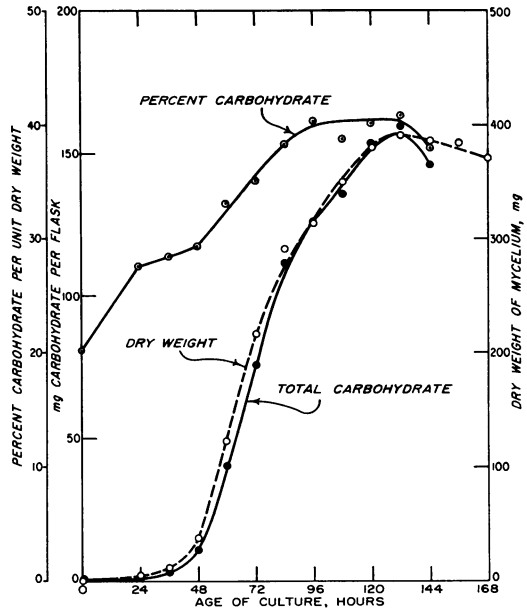

FIG. 8. *The relationship between carbohydrate and the age of Penicillium atrovenetum. The carbohydrate is expressed as per cent carbohydrate per dry weight and as total carbohydrate per flask.*

greatest percentage of cell wall and storage materials, such as chitin and glycogen, have been found late in growth of fungi (Blumenthal and Roseman, 1957; Fluri, 1959). The decrease in the percentage of carbohydrates with the onset of the death phase is reasonable. Because fungi usually deplete the medium of carbohydrates during the period of rapid growth, they could later use storage carbohydrates as an energy source to maintain themselves.

Although, in a number of fungi, the percentage of chitin per dry weight increases with the age of the culture (Behr, 1930; Blumenthal and Roseman, 1957), *P. atrovenetum* is apparently one of the minority in which this does not occur. Nevertheless, one must recognize that the chemical methods used for the determination of chitin are generally poor, and thus the difference between the results for *P. atrovenetum* and those reported for many other fungi might be caused by the inadequacies of technique.

During sporulation, both the percentages of total nitrogen and nitrogen in the protein fraction increased, but RNA did not rise significantly. Prior to sporulation of *P. atrovenetum*, changes in the amount of protein are closely correlated with changes in the amount of RNA, and thus agree

with the current concepts of protein synthesis. Therefore, the apparent increase in protein nitrogen during sporulation in the absence of a similar trend for RNA might be due to the presence of another nitrogen-containing compound, such as chitin. It is also possible that the spores on the mycelium contributed to this increase, because the percentage of chitin in the spores was higher than in the mycelium. However, the chitin analyses do not support this concept.

The percentage of DNA in *P. atrovenetum* was initially greatest in the spores, decreased in the lag phase, increased in the log phase, and finally decreased in the stationary phase. With *Neurospora crassa*, Zalokar (1959) showed that the growing hyphal tips have more nuclei than do older regions further back on the hyphae. Therefore, during the log phase of growth of a fungus, when new hyphae are formed very rapidly, one might expect a slight increase in the percentage of DNA on a dry-weight basis. The decreased percentage of DNA in *P. atrovenetum* after 96 hr is probably due to the synthesis of lipid and carbohydrate. The total DNA per flask reached a maximum at 96 to 108 hr and then leveled off. Most of the hyphal cells were probably formed by 96 to 108 hr, and the increase in weight of the mycelium after this time was probably due to the continuing synthesis of fatty acids and carbohydrates. Without an increase in new mycelium, the total amount of DNA per flask would not be expected to increase.

The double peaks in the early growth phases for the percentage of both RNA and protein are surprising. Similar results were reported for RNA in a few bacteria. Malmgren and Heden (1947) obtained a double-peaked RNA curve during the lag and early log phases of growth for several gram-positive bacteria. Wade (1952) also observed a double peak in the percentage of RNA during the lag phase of growth of *E. coli*. He believed that this initial decrease occurred between the first and second generation of cells. However, this concept probably does not explain the results with *P. atrovenetum*, because it is unlikely that the cells of this fungus were undergoing synchronous cellular division. The appearance of the double peak in *E. coli* also depended on the medium; Wade (1952) obtained two RNA peaks on one medium but only one on another. The investigators who reported a double maxima for the percentage of RNA in bacteria did not determine the percentage of protein in their studies, and one does not know whether the changes in protein paralleled the changes in RNA. The decrease in percentage of RNA and protein at 36 hr to form the double maxima with *P. atrovenetum* was probably a coincidence, and was caused by the rapid synthesis of other components at the same period. Furthermore, both the total protein and total RNA per flask did not decrease at 36 hr, but continued to increase even though their percentages per dry weight showed a decrease.

The usual method for determining RNA, such as the orcinol procedure, failed to give consistent results for replicates of the same sample. The procedure finally adopted produced only rough approximations of the RNA content in the hot trichloroacetic acid fraction. One disadvantage in using this method for the RNA determinations was that any DNA in the solution would also absorb in the same ultraviolet region. However, even if DNA were present in the extract, it would not alter the trend obtained for RNA, because the concentration of DNA in *P. atrovenetum* is very small in relation to that of RNA (about 15% or less).

The initial increase of enzyme-forming compounds in the early lag phase, when the spore germinates and begins to form hyphae, should be expected during germination. For example, spores of *Ustilago maydis* contain no active glucose-6-phosphate dehydrogenase, transketolase, transaldolase, aconitase, succinic dehydrogenase, or fumarase, but synthesize these enzymes during the germination process. The activity of other enzymes of the Embden-Meyerhof-Parnas and hexose monophosphate shunt pathways also increased during this period (Caltrider and Gottlieb, 1963).

With the continued rapid growth of hyphae (the log phase), the relative syntheses of protein, RNA, and soluble nitrogen compounds gradually decreased, but carbohydrate synthesis continued and the maximal percentage was reached late in the log phase. DNA and chitin remained constant during this period. In the stationary phase of growth, all components, with the exception of carbohydrates and chitin, were at their lowest.

An interesting feature of the growth study is that the net synthesis of most of the components stopped at about the middle of the log phase, and only carbohydrate and fatty acids continued to increase. The growth period at which the net synthesis of protein, RNA, and DNA ceases to

increase might be considered the true metabolic end of the log phase, because only storage materials and chitin continue to be formed. During this period, the fungus forms its spores in preparation for new generations.

In general, the results with *P. atrovenetum* indicate that its metabolism during growth is very similar to that of other microorganisms. The percentage of enzyme-forming compounds, such as amino acids, nucleotides, RNA, and protein, are highest in the lag phase, whereas storage compounds, such as carbohydrates and fatty acids, increase to a maximum near the end of the log phase.

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