BIOCHEMICAL CHANGES OCCURRING DURING GROWTH OF COCCIDIOIDES IMMITTIS IN A DEFINED MEDIUM

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The nutritional requirements of Coccidoides immittis strain Cash, grown in a medium consisting of glucose, ammonium lactate, and inorganic salts, were reported by Goldschmidt and Taylor (1958). The present paper considers some of the biochemical changes which take place in this medium during growth. The utilization of glucose, lactate, and ammonia was studied with respect to growth, fragmentation of hyphae, and the accumulation of extracellular peptides and polysaccharide(s) during growth.

METHODS

The strain of C. immittis, growth medium, culture conditions, and the determination of pH, protein, and viable count were the same as those previously described (Goldschmidt and Taylor, 1958).

Culture filtrates were obtained for analyses at varying intervals of time by pooling duplicate flasks and collecting the mycelium on a Seitz filter. All analytical values reported were corrected for loss of culture volume due to evaporation during incubation. The filtrates were analyzed for total nitrogen by standard techniques of Kjeldahl digestion, steam distillation, and titration. Ammonia was determined by steam distillation and titration. The nonammonia nitrogen referred to as soluble organic nitrogen was calculated by subtracting the ammonia nitrogen from the total nitrogen. Cellular nitrogen was calculated from the difference between the initial total nitrogen at zero time and the residual total nitrogen in the culture filtrates. Glucose was determined by the colorimetric method of Somogyi (1952) after treatment of the filtrate with the zinc sulfate-barium hydroxide reagent of Nelson (1944). Total carbohydrate was determined by the anthrone method of Seifter et al. (1950) using glucose as a standard. Lactic acid was determined by the procedure of Barker and Summerson (1941).

Antisera were prepared by injecting antigen into the ear veins of rabbits. The animals were injected 8 times over a period of 3 weeks with equivalent doses of formalin-killed cultures (approximately 0.9 mg cellular protein per dose) and bled 5 days later. Precipitin titers of the culture filtrates were determined by the ring test method using capillary tubing as described by Boyd (1943).

RESULTS

Substrate utilization, growth, and fragmentation. The data presented in figures 1 and 2 show the chemical changes which occurred during growth of the cultures over an 8 day period. The ammonia nitrogen, lactate, and glucose were completely utilized by the 5th day. Growth, as measured by either increase in cellular protein or cellular nitrogen, approached maximal levels between the 3rd and 4th day. The viable count, however, did not increase until the 4th day which was subsequent to the synthesis of the cellular material. These results suggest that growth occurred solely in the mycelial phase and fragmentation occurred only after maximum growth was reached.

The photomicrographs (figure 3) show the changes in morphology which occurred during growth of the culture. It was noted that there was little fragmentation of the cultures prior to the 4th day. Short chains of arthrospores were observed on the 4th day (figure 3b). The average chain length of the arthrospores decreased on subsequent incubation until the 6th day (figure 3c). After the 6th day, there were no significant changes in the microscopic appearance of the cultures. The morphological changes in the cultures also suggest that fragmentation was delayed until the 4th day when maximum growth was obtained. The correlation of the fragmentation index (figure 1) with the morphological changes in the cultures, as revealed by the photomicrographs, was very good.

Accumulation of peptide. The presence of nonammonia nitrogenous compounds in the filtrates was revealed by the difference in values for total nitrogen and ammonia nitrogen. The data of
Figure 1. Utilization of glucose and lactate during growth of Coccidioides immitis strain Cash. The fragmentation index is defined as the ratio of viable count $\times 10^{-7}$ per mg cellular protein.

Figure 2. Utilization of ammonia during growth of Coccidioides immitis strain Cash.
Figure 3. Cytological changes during growth of *Coccidioides immitis* strain Cash. All photomicrographs were taken of wet mounts stained with lactophenol blue, 358 X magnification. a. 2 day culture; b. 4 day culture; c. 6 day culture; d. 8 day culture. Note the change from hyphae to single arthrospores during growth.

Goldschmidt and Taylor (1958) indicated that the culture filtrates contained negligible amounts of protein based on the absence of turbidity after heating or treating culture filtrates with trichloracetic acid. However, the filtrate of 6-day-old cultures contained 5.2 mg per ml of peptide material as determined by the biuret procedure. The peptide can account for essentially all of the nonammonia nitrogen in the filtrate, if one assumes that the peptide contains 16 per cent
nitrogen (peptide nitrogen, 5.2 mg \times 0.16 = 0.83 mg nitrogen; nonammonia nitrogen, 58.8 \mu moles \times 14 = 0.823 mg nitrogen).

Preliminary experiments employing paper chromatography indicated only trace amounts of free amino acids in the filtrates prior to acid hydrolysis. After acid hydrolysis (6 N HCl for 20 hr at 110 °C), large amounts of many amino acids were observed on 2 dimension chromatograms employing water saturated phenol and n-butanol:acetic acid:water (4:1:5 parts by volume).

\textit{Polysaccharide}. The filtrate of 7-day-old cultures contained 3.8 mg per ml total carbohydrate and only 0.26 mg per ml of reducing sugar, thus indicating the presence of approximately 3.5 mg per ml of nonreducing carbohydrate. The nonreducing carbohydrate was precipitated by 4 volumes of ethanol and was not dialyzable, thereby indicating the polysaccharide nature of this carbohydrate. Hydrolysis of the polysaccharide for 6 hr with 1 N H\textsubscript{2}SO\textsubscript{4} at 105 °C yielded essentially equivalent values for reducing sugar and total carbohydrate. A number of different reducing sugars were observed on paper chromatograms of this hydrolyzate, three of which were identified tentatively as mannose, glucose, and galactose. The identification of the fourth sugar will be presented elsewhere.

\textit{Precipitin antigen}. Antisera from rabbits immunized with formalin-killed cells of \textit{C. immitis} gave precipitin titers of 1:100 with 7-day-old culture filtrates. There was no evidence of any increase in titers with cultures incubated for 2, 3, or 4 weeks.

**DISCUSSION**

The preceding data indicate that growth of \textit{C. immitis} occurs in two distinct processes, the synthesis of protein to a maximum level followed by fragmentation of the mycelium. The results of Roessler \textit{et al.} (1946) suggest that this delay in fragmentation was typical of their cultures also.

Fragmentation occurred after most of the ammonia nitrogen and lactate was utilized. It is possible that the concentration of ammonium lactate in the medium might have some influence on fragmentation since previous results (Goldschmidt and Taylor, 1958) had suggested that ammonium lactate might be inhibitory to fragmentation in high concentrations. Similar results were observed when deionized acid hydrolyzed casein was used at various concentrations as a source of nitrogen. These results suggest that there may be some inhibition of fragmentation by nitrogenous compounds, however more data are needed to confirm this suggestion.

Morton and Broadbent (1955) showed that many fungi accumulate large amounts of extracellular organic nitrogen. Most of this nitrogen was accounted for as an extracellular, dialyzable polypeptide. They found that the production of the extracellular peptide decreased when the trace element concentration of the medium was raised.

Preliminary observations indicate that the production of extracellular peptide by \textit{C. immitis} strain Cash decreased when the iron concentration of the medium was increased. The significance of the extracellular nitrogen material produced by \textit{C. immitis} and the cultural conditions influencing its formation require further investigation.

The relationship between the extracellular polysaccharide and precipitin antigen described in this paper requires additional investigation. Previous studies by Hirsch and D’Andrea (1927) and Hassid \textit{et al.} (1943) indicated that the precipitin antigen was a polysaccharide.

**SUMMARY**

The utilization of glucose, lactate, and ammonia by growing cultures of \textit{Coccidioides immitis} has been determined. The data indicate that growth occurred first followed by fragmentation after maximum growth was obtained. Fragmentation started after most of the ammonium lactate was utilized.

Cultures of \textit{C. immitis} strain Cash produced 5 g extracellular peptide and 3.5 g extracellular polysaccharide per L during 1 week incubation in the defined medium.

**REFERENCES**


