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Reduction of a Tetrazolium Salt in Determining Growth Activity of Yeast-Phase *Histoplasma capsulatum*

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A colorimetric method using a tetrazolium compound, 3,4,5-dimethylthiozalil-(1 or 2),2,5-diphenyltetrazolium bromide (TTBr), was developed for studying the growth activity of yeast-phase *Histoplasma capsulatum*. Materials extracted in phosphate buffer, *p*H 7.0, from cells at different stages of growth reduced TTBr. Colorimetric changes were correlated with enzymatic activity. Under standardized conditions specified herein, the optical density of the reduced tetrazole was an index of the growth activity of the organism.

Reduction of tetrazolium compounds by living cells has been used over the past two decades as an indicator of the H^+ transfer capacity of cells in several species of bacteria (3, 4, 7, 8; A. E. Stockland and G. L. San Clement, Bacteriol. Proc., p. 63, 1967), but in only one fungus, *Penicillium chrysogenum* (2).

Preliminary studies in this laboratory revealed the capacity of soluble materials extracted from yeasts of the pathogenic fungus *Histoplasma capsulatum* to reduce seven different quaternary tetrazoles (Nutritional Biochemicals Corp., Cleveland, Ohio): tetrazolium violet, tetrazolium blue, tetrazolium red, nitro-blue tetrazolium, neotetrazolium chloride, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride, and 3,4,5-dimethylthiozalil-(1 or 2),2,5-diphenyltetrazolium bromide (TTBr). Of these seven, TTBr gave the most vividly contrasting and gradual color changes between nonreduced (brilliant yellow) and reduced (deep purple) forms. It was also the most readily soluble in 0.2% aqueous concentration, at which each of the dyes was used.

The subject of this report is a description of the development of a colorimetric procedure with TTBr. Both lag and logarithmic stages of yeast-phase growth of *H. capsulatum*, with increasing H^+ transfer capacity of cells, can be rapidly and quantitatively determined as indicated by increasing reduction of TTBr. The relevance of the procedure to enzymatic and other critically comparative studies of the parasitic phase of this dimorphic mycotic pathogen is discussed.

MATERIALS AND METHODS

Strains. H. capsulatum strains G-154B, G-154D, G-154P, and G-66, all human isolates, were cultivated in the yeast phase in Trypticase Soy Broth (TSB) dialysate (6) and their respective growth curves determined as described in an earlier report (5). Sample suspensions for testing the reduction of the seven tetrazoles were removed during the midlogarithmic stage of growth, or after approximately 48 hr of incubation. Samples for developing the colorimetric procedure with TTBr were removed and tested as specified in the separate experiments.

Buffer solution. Phosphate buffer (0.067 M KH_2PO_4 and 0.067 M $Na_2HPO_4 \cdot 2H_2O$) to yield *p*H 7.0 was used throughout.

Tests for reduction of tetrazolium compounds. A 2-ml portion of suspension was centrifuged at 2,500 rev/min for 5 min in a no. 2 International centrifuge, and the broth was decanted. The cells were resuspended in 2 ml of buffer solution. Quantities of 0.5 ml were transferred to 5-ml spectrophotometric tubes in duplicate, and 0.5 ml of 0.2% aqueous tetrazolium-salt solution was added to each. After shaking, the tubes were incubated in a 37 C water bath for 1 hr. The reduced product was solubilized immediately by adding 2.5 ml of acetone, stoppering, and vigorously shaking the tubes. The cells were then sedimented by centrifugation at 2,500 rev/min for 15 min. Colorimetric determinations were made on a Coleman Junior spectrophotometer (model 6A) set at 550 mμ.

Calibration of curve for TTBr. Duplicate sets of twofold dilutions of a 0.2% aqueous solution of TTBr were made in a series of spectrophotometric tubes in 0.5-ml quantities. These were reduced by adding a few crystals of sodium thiosulfate ($Na_2S_2O_3$) and 1 drop of 1 N sodium hydroxide. After incubation at 37 C in a water bath for 1 hr, optical densities (OD)

were determined on the Coleman Junior spectrophotometer (model 6A) set at 550 mμ. Final concentrations of dye after addition of 2.5 ml of acetone varied from 28.5 to 0.05 mg/100 ml. Although readings were made immediately, the color remained stable for 24 hr.

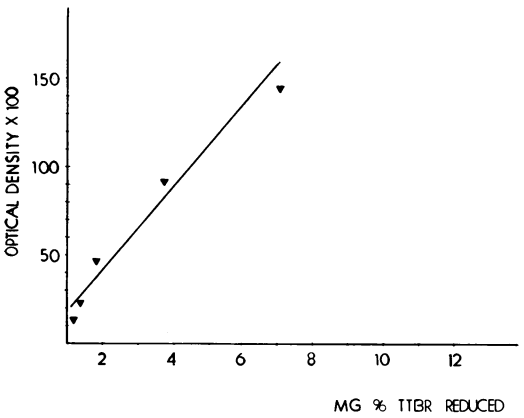

FIG. 1. *Linear relationship between the amount of reduced TTBr and optical density.*

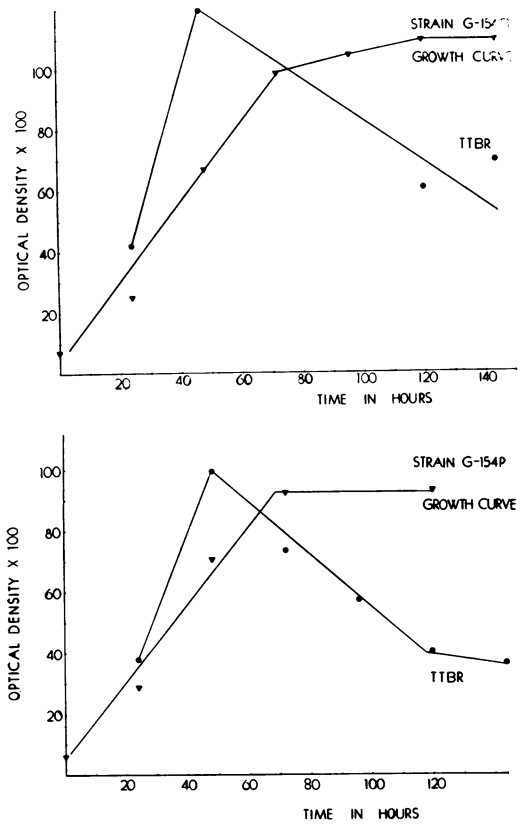

FIG. 2. *Quantities of TTBr reduced and optical density of growth (ordinate) and time in hours (abscissa), using strains G-154B and G-154 P.*

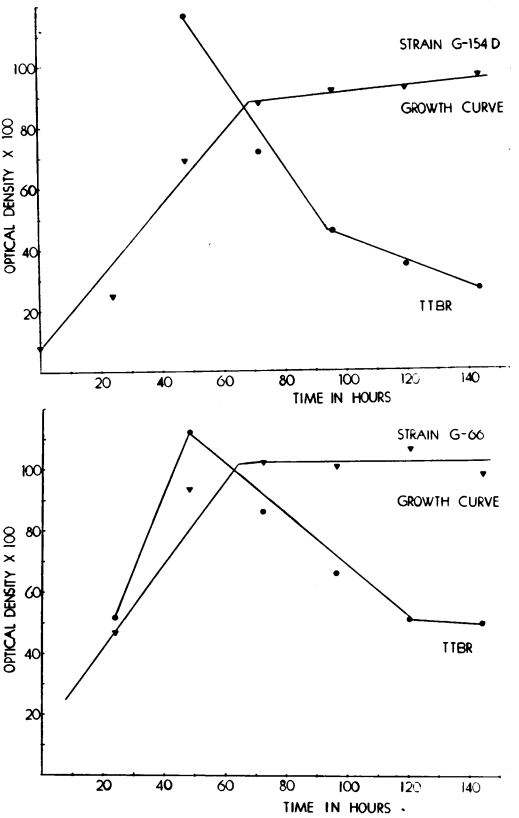

FIG. 3. *Quantities of TTBr reduced and optical density of growth (ordinate) and time in hours (abscissa), using strains G-154D and G-66.*

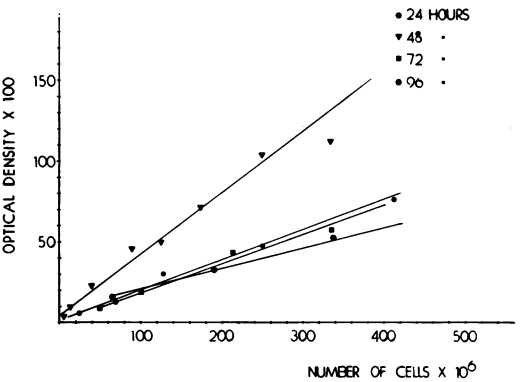

FIG. 4. *Correlation between the quantity of TTBr reduced and the number of cells per milliliter after 24, 48, 72, and 96 hr of incubation.*

RESULTS

There was a linear correlation between the OD and the quantity of TTBr reduced aerobically

within the range of 1 to 8 mg/100 ml (Fig. 1). Within these limits, OD is a linear function of the amount of dye reduced. TTBr reduction by *H. capsulatum*, within these same limits, then, may also be expressed directly as the OD of the reduced solution.

This was confirmed when the OD of cellular suspensions and TTBr reduction of four strains of *H. capsulatum* yeasts were determined over a 6-day period of incubation at intervals specified in Fig. 2 and 3. For each of the strains, maximal TTBr reduction occurred contemporaneously with the mid-logarithmic stage of growth, or at approximately 48 hr postinoculation, with cells known also to be in the logarithmic stage of growth (5). Thereafter, TTBr reduction decreased rapidly, usually prior to the beginning of the stationary stage.

This correlation held regardless of the total number of cells in the test suspensions. Samples of strain G-66 collected after 24, 48, 72, and 96 hr of incubation were serially diluted to contain 400×10^6 to 20×10^6 total cells/ml. Cells harvested at 48 hr showed highest metabolic activity in the lowest as well as the highest dilutions (Fig. 4).

DISCUSSION

A relationship demonstrable by colorimetric measurement of reduced TTBr has been established between the H^+ transfer capacity and the lag, log, and stationary stages of growth of yeast-phase *H. capsulatum*. The colorimetric measurements were found to correlate well with biological data obtained in earlier studies with growth-curve determinations, plate and cell counts, and vital staining with Janus Green B (1, 5).

The capacity of *H. capsulatum* to reduce TTBr offers a new approach to the study of this and possibly other dimorphic mycotic pathogens. As with other microorganisms with which the quaternary tetrazoles have been used, TTBr reduction provides a tool for isolating and identifying the enzymatic systems involved in oxyreduction processes of *H. capsulatum*. It also provides a rapid means of assaying the metabolic state, as well as size, of an inoculum for critically comparative studies, such as pathogenicity of strains, activity of drugs, both in vivo and in vitro, and production of reproducible crude antigens for diagnostic and analytical purposes. As noted in earlier reports, the number of viable *H. capsulatum* cells in different states of metabolic activity varies widely in cultures of the same age if the metabolic state, as well as the size, of the initiating inoculum is not rigidly controlled (1, 5). Although determination of growth curves for each strain minimizes this difficulty, the procedure requires specialized equipment. The simple colorimetric procedure of TTBr reduction reported herein may be substituted for the growth curve in determining the peak of metabolic activity, and may be carried out in any laboratory. However, as illustrated in Fig. 2–4, several serial determinations should be made between 24 and 72 hr, while H^+ transfer capacity is in the ascendancy. Single, even though high, values during this same period may result in the use of cells which already have entered the stationary phase. Under the conditions of the experiments reported herein, peak metabolic activity usually occurred at 48 hr and was associated with a TTBr reduction of 5 to 6 mg/100 ml.

The positive, although less pronounced, reactions obtained with six other tetrazoles indicate that further study of these compounds with *H. capsulatum* and other mycotic pathogens is warranted.

ACKNOWLEDGMENTS

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