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Monitored Environment System to Control Cell Growth, Morphology, and Metabolic Rate in Fungi by Oxidation-Reduction Potentials

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A device for the adjustment, maintenance, and monitoring of a desired oxidation-reduction potential in a liquid environment is described. The filamentous species, *Penicillium lilacinum* and *Trichophyton schoenleinii*, and the dimorphic species, *Histoplasma capsulatum*, developed budding yeastlike cells when grown in a liquid medium with a low oxidation-reduction potential. When the organisms were inoculated into fresh medium, they returned to the filamentous condition as the oxidation-reduction potential was restored to a more positive (oxidizing) environment.

Oxidation-reduction (O/R) potentials of biological systems have been investigated for many years. Most of these investigations have been concerned with measuring the O/R potentials achieved in media during the growth cycle of cells. Burrows and co-workers (2, 3) found that the observed final O/R in specific media could be used to separate closely related material species. J. S. Winberg (M.S. Thesis, Univ. of Illinois, Chicago, 1960) verified this observation and speculated that, although the observed O/R is a summation of several metabolic systems, it must reflect the dominant system of a particular species. McAlister (11) demonstrated a similar characteristic O/R in nutrient media after a specific time lapse for fungal species.

The effect of controlled O/R potentials on living cells was investigated by Vennesland and Hanke (25). These investigators found that certain anaerobic bacteria possessed a limiting potential above which they could not grow, and that these bacteria would grow in the presence of molecular oxygen if the O/R potential of the medium was kept below this critical level. Apparently, the metabolic pathways of the organisms were dependent on a reduced environment. Recently, Dobson (4) described a device using bursts of CO_2 or O_2 to maintain O/R levels. However, the apparatus was useful for only a few hours.

The influence of a reduced environment during the cell division of yeasts was investigated by Scherr (19, 21), and the requirement of a low O/R potential, obtained by free sulfhydryl groups, was assumed to be responsible for the conversion of the fungus *Histoplasma capsulatum* from the mycelial to the yeast phase. The relation between free sulfhydryl groups and particular events during plant cell division has been reviewed by Erickson (5).

Fungi grown on a gradient tilt plate with increasing concentrations of cysteine exhibited increased metabolic rate and decreased cell-division time (14-16). The organisms which normally grew as long mycelial threads appeared as small budding yeasts and exhibited a concomitant increase in invasive ability for the deep organs of experimental animals. The present paper is concerned with the development of an electrolytic device for the maintenance of desired O/R potentials in a liquid medium and the morphology and metabolic rate of cells in such media.

MATERIALS AND METHODS

Purpose and design. The monitored environment system (MES) is a device designed to maintain a preselected O/R potential by direct current electrolysis at a constant-*p*H. O/R potentials with a sensitivity of 0.3 mv are possible. When the medium within the chamber reaches the preselected O/R level, the MES maintains this potential within ±1 mv. Deviation from the set O/R potential by as little as 0.2 mv results in a correcting electrolysis current. The chamber design is a modification of an electrolysis system developed by Hanke (7), which was manually controlled. The MES employs a photoelectric cell-monitor and maintains a constant O/R in a poised system for at least 2 months without a change in *p*H.

The growth chamber and remote vessels appear in Fig. 1. The chamber is an 80 × 100 mm round,

wide-mouth (Pyrex) jar with a rubber stopper. Fitted into the stopper and inserted in the chamber are: (1) two platinum electrodes and a tube with medium connected by a salt bridge to remote vessel A (the measuring system); (2) a large surface-area platinum electrode and two media tubes connected by salt bridges to remote vessel B (the electrolysis system); (3) a combination glass and calomel electrode for continuous *p*H measurement; (4) a capillary tube to bubble CO_2 or air mixtures or both into the medium when needed for *p*H maintenance. Constant agitation of the medium is provided by a magnetic stirrer and the unit is housed in a 37 C incubator. The control unit lacks only remote vessel B and the platinum electrode for this vessel.

The basic plan for the MES is presented in Fig. 2. The calibration of the platinum electrodes in the monitor chamber and in the control chamber is accomplished by the asymmetry controls and electrode selector (*see* diagram of circuits in Fig. 3). The calibration circuit for the span control and zero control of the electrolysis power supply is included in Fig. 3. The circuit diagram for the electrolysis power supply, however, is presented in detail in Fig. 4. The current provides a well-regulated potential of 30 v and up to 250 ma for electrolysis.

An essential feature of the MES is that the electrolysis power supply does not affect the readings of the measuring electrodes. An electrolysis current going into or out of these electrodes causes an error in the observed O/R potentials. In the MES, the detection circuit is electronically separated from the electrolysis power supply by two stages of isolation.

The JSB-2 (West Co., Schiller Park, Ill.) provides the first isolation stage, as well as a power gain, for the measured signal from the Beckman 76 expanded-scale *p*H meter (Beckman Instruments, Inc., Fullerton, Calif.; Fig. 2). The Beckman 76 measures the O/R potential of the monitor chamber. The JSB-2 output is proportional to the deviation of the measured O/R potential from the preselected potential, and the output is applied to the lamp circuit in the electrolysis power supply and to the automatic polarity reversing circuit. A photocell in series with the power supply output is in a light-tight unit with the lamps powered by the JSB-2. When the JSB-2 output is maximal, the lamps glow intensely to produce reduced resistance of the photocell and to allow maximal electrolysis current. When the lamps are not glowing, the resistance of the photocell is very high, allowing virtually no electrolysis current. This circuit provides the second isolation stage.

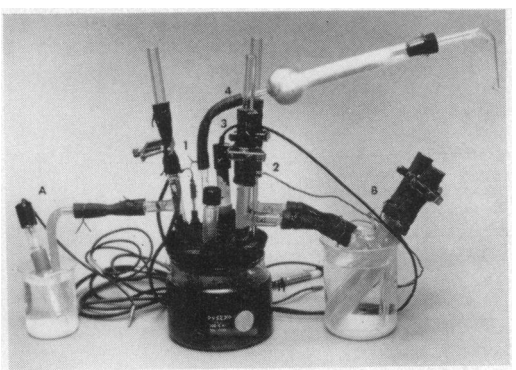

FIG. 1. *Monitored environment chamber (explanation of details in text).*

The electrolysis power supply is coupled to the electrolysis electrode by a relay; the JSB-2 output is applied to a direct current amplifier in this circuit which controls the relay. When the O/R potential observed on the Beckman meter connected to the JSB-2 exceeds the preselected O/R potential, the relay is activated to reverse the polarity of the electrolysis current. The reversed current is maintained until the actual and selected O/R potentials coincide. At this time, the amplifier deactivates the relay, thereby restoring the electrolytic current to the original polarity. The diagram for the automatic polarity reversing circuit is presented in Fig. 5.

Operation. The platinum electrodes were calibrated with a quinhydrone standard (quinhydrone-saturated phosphate buffer, provided by the Beckman Instrument Co., at *p*H 4.0). The asymmetry controls were set to give a reading of +219 mv on the Beckman 76 *p*H-meter, at the beginning and also at the end of each experiment, to detect any deviation in measurement of the platinum electrodes. At this point, the platinum electrodes were inserted into the MES chamber which was loaded with 450 ml of Brain Heart Infusion (BHI) broth (Difco).

The two salt bridges for the electrolysis circuit, prepared according to Hanke's method (7), were placed in a beaker containing 4% agar saturated with KCl. A salt bridge for the O/R potential measuring system was prepared and placed in a beaker containing 4% agar saturated with KCl (Fig. 1). The MES chamber and both sets of salt bridges in their respective beakers were autoclaved (15 psi, 15 min). During sterilization, the air was driven from the bridges which then filled with molten agar on one side and medium on the other side. After cooling, a sterile liquid junction was obtained for medium and agar. The measuring electrodes were allowed to equilibrate in the medium overnight. The combination *p*H electrodes (Beckman No. 39180), previously sterilized by immersion in 0.5% $HgCl_2$ for 1 hr and washed with sterile distilled water, were inserted into the appropriate chambers (Fig. 2).

The medium was inoculated with approximately 10^8 mycelial fragments, and the MES growth chamber, with its associated beakers, was placed in the incubator at 37 C. The beakers were then replaced with other beakers containing a saturated KCl solution. A platinum electrode was placed in the beaker for the electrolysis current (B in Fig. 1), and a calomel reference electrode was immersed in the other beaker for the measuring system-circuit (A in Fig. 1). Magnetic stirring rods in the monitor and control MES chambers were activated and allowed to agitate continuously, and the O/R potential and *p*H were recorded the following day.

An O/R potential was selected by adjusting the

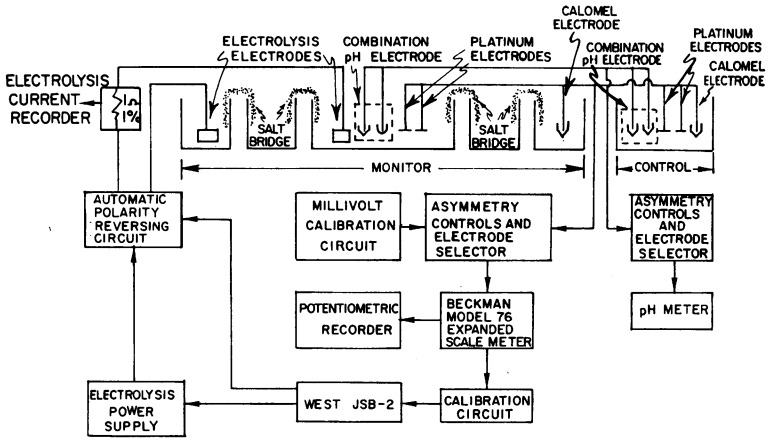

FIG. 2. *Summary diagram of Monitored Environment System.*

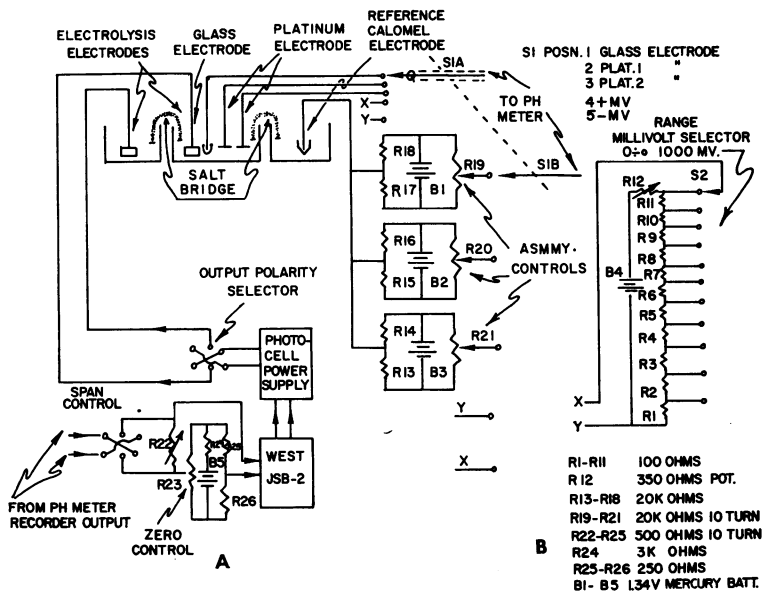

FIG. 3. *Calibration circuit diagram of the span control and zero control for the West JSB-2 and for the photocell o, the electrolysis power supply (A). An automatic polarity reversing circuit intervenes between the output polarity selector and the electrolysis electrode. The asymmetry controls and electrode selector of the measuring system is shown in (B).*

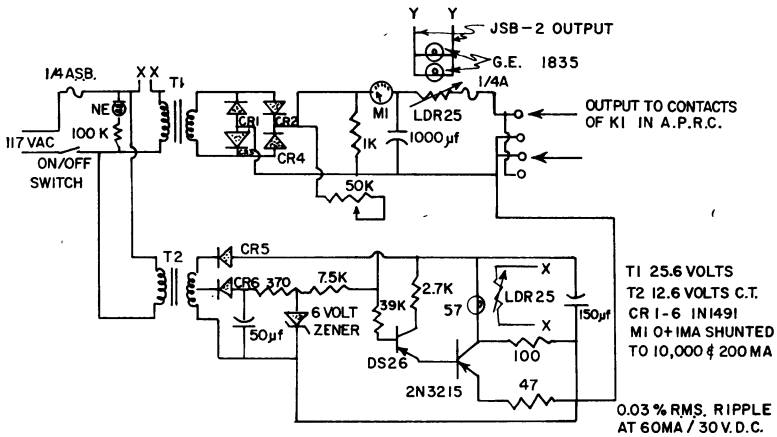

FIG. 4. *Circuit diagram of electrolysis power supply.*

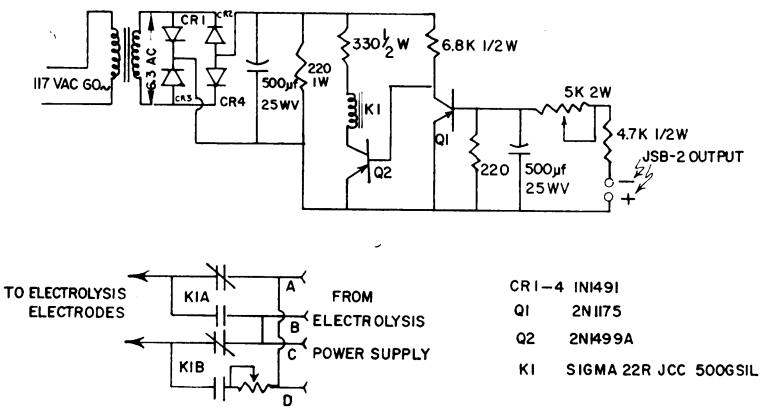

FIG. 5. *Circuit diagram of automatic polarity reversing system.*

fixed needle of the meter of the photocell power supply (Fig. 3) to the desired value on the Beckman 76 scale, and the electrolysis power supply was activated. The mobile needle of the photocell power supply moved towards the fixed needle during electrolysis, and when they coincided electrolysis stopped. The O/R potential of the control MES chamber was observed on a second Beckman 76 *p*H-meter. The O/R potential and *p*H of the MES growth and control chambers were recorded daily for the duration of the experiment.

RESULTS

Equipment evaluation. The control chamber and monitored chamber were inoculated with 2×10^5 mycelial fragments per milliliter and the monitored chamber was set at an O/R potential of +46. The *p*H and O/R potentals were recorded daily for 28 days. The O/R potential of the monitored chamber remained constant with no significant change in *p*H. A sample of performance data is given in Table 1.

Biological effects. With the use of *Candida albicans* (yeastlike), *H. capsulatum* (dimorphic), *Trichophyton schoenleinii*, and *Penicillium lilacinum* (filamentous), the MES was employed to investigate the effect of electrolysis on the medium. The MES was also used to determine the effect of controlled O/R potential on cell morphology and on metabolic rates in *T. schoenleinii*, *P. lilacinum*, and *H. capsulatum*.

Effect of electrolysis on the medium. The MES and control chambers were inoculated with approximately 2×10^5 mycelial fragments per milliliter of *T. schoenleinii* or *P. lilacinum*, and the O/R potential of the MES chamber was set at +46 mv. Although growth of the control cultures was detected within 24 hr, no growth was observed in the MES chambers. By the fourth day, the growth, as measured by turbidity, in the MES and in the control chambers was not significantly different. When the MES and control chambers were inoculated with approximately 2×10^5 mycelial fragments of *H. capsulatum*, the increase in turbidity in both chambers, however, occurred at the same rate.

TABLE 1. *Equipment evaluation by use of Trichophyton schoenleinii at O/R potential + 46 mv*[a]

Day	Control chamber		Monitored vessel	
	*p*H	O/R	*p*H	O/R
1	7.0	310	7.0	212
2	Inoculated and electrolysis started and monitored at +46			
3	6.8	345	7.2	46
5	6.9	364	7.2	46
10	6.4	370	7.4	46
28	6.8	396	7.6	46

[a] BHI broth in chambers equilibrated overnight and inoculated with 2×10^8 mycelial fragments of *T. schoenleinii*. On day two, monitored chamber was electrolyzed to O/R + 46 mv. At day 28, experiment was terminated, morphology was assessed, and all electrodes were checked for deviation.

The BHI medium was kept in the MES chamber for 30 days during constant electrolysis to maintain an O/R potential of +46 mv. The chambers were then inoculated with approximately 2×10^5 cells of *C. albicans* per milliliter. The duration of the lag phase was 6 hr compared with 18 hr for fresh medium.

Medium maintained at an O/R potential of +46 mv for 30 days was then positively electrolyzed to an O/R potential of +400 mv and inoculated with *C. albicans*, *T. schoenleinii*, or *P. lilacinum*. Fresh media were inoculated with portions of the inocula at the same time. No

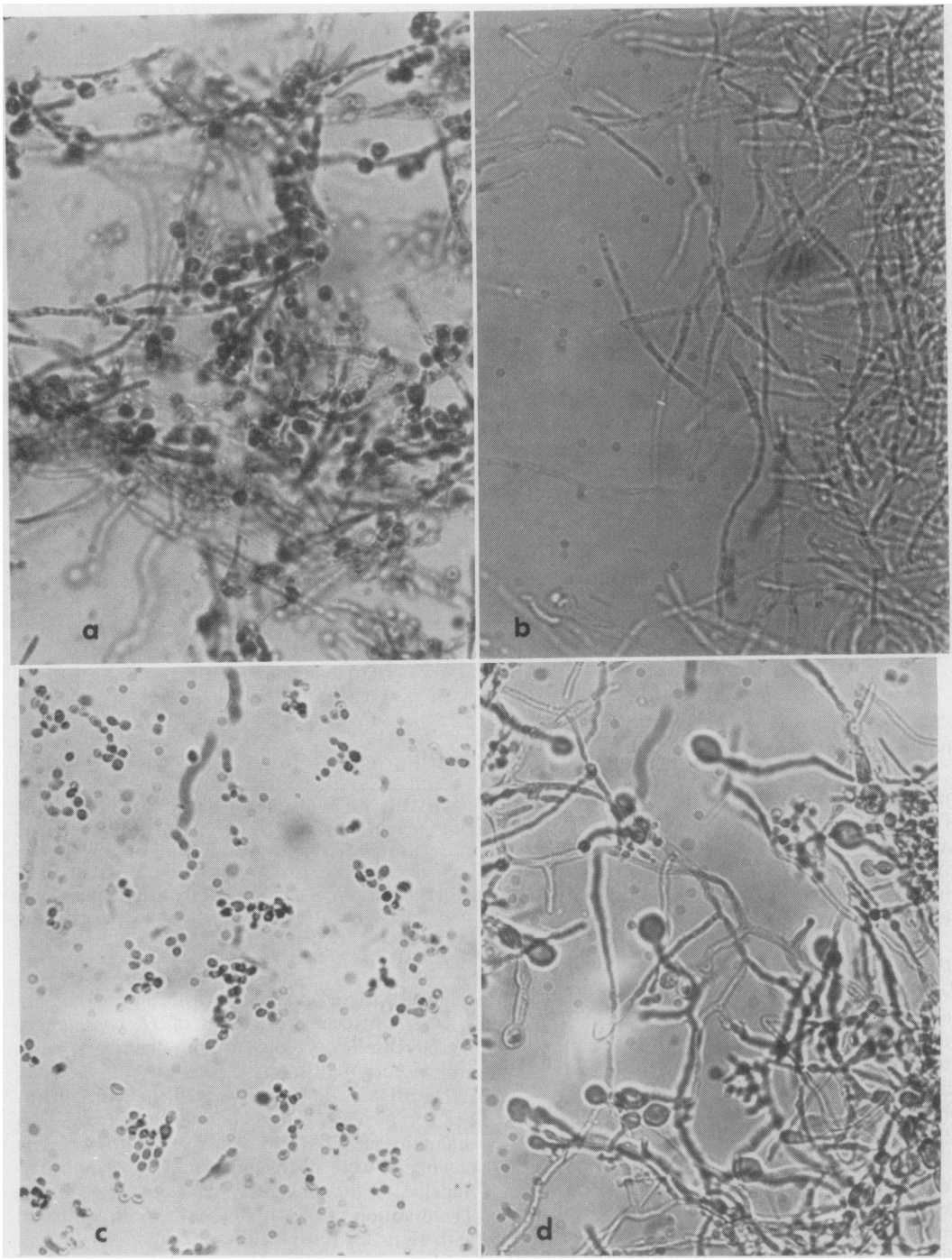

FIG. 6. *Effect of imposed oxidation-reduction potential on morphology of fungi. (a) Penicillium lilacinum, after 10 days in the monitored chamber at O/R potential of +46 mv, showing growth as budding yeastlike forms (mycelial inoculum). (b) P. lilacinum in control chamber (average O/R +395). (c) Histoplasma capsulatum after 5 days in monitored chamber, at O/R potential of +46 mv. Yeastlike cells predominate (mycelial inoculum). (d) H capsulatum in control chamber (average O/R +380).*

TABLE 2. *Metabolic rate and morphogenesis as a function of oxidation-reduction potential*[a]

Inocula (2×10^5/ml)	N(Q_{O_2})	Metabolic rate at 37C
Trichophyton schoenleinii	[b]C (O/R + 395)	6.6
	M (O/R + 46)	107.2
Histoplasma capsulatum	C (O/R + 380)	37.9
	M (O/R + 46)	221.5

[a] Both organisms were in the yeastlike stage when recovered from the monitored chamber (mycelial stage inoculum).

[b] C, control chamber; M, monitored chamber.

significant differences in the duration of the lag phase were observed.

Effect of controlled O/R potential on cell morphology. The media in the MES and control chambers were inoculated with approximately 2×10^5 mycelial fragments per milliliter of *T. schoenleinii*, *P. lilacinum*, or *H. capsulatum*, and the MES chamber was set at an O/R potential of +46 mv. The cultures of *T. schoenleinii* and *P. lilacinum* in both chambers were examined microscopically on the fifth day of incubation and daily thereafter. On the fifth day in the MES chamber, the cells of the hyphae displayed an obviously shorter and thicker appearance. By the tenth day, round, budding yeastlike cells appeared (Fig. 6a), and the number of such cells increased during the remainder of the incubation period. The inoculum in the control chamber did not produce yeastlike cells and remained mycelial in morphology (Fig. 6b). The change in cell type was transitory. When the yeastlike cells from the monitored chamber were plated on BHI agar slants, they exhibited the characteristic mycelial morphology.

The effect of the highly reduced O/R potential in the MES chamber on cell morphology for *H. capsulatum* was more striking than that for the filamentous fungi. By the fifth day of incubation, all the cells in the MES chamber were yeastlike and budding (Fig. 6c). The culture in the control chamber, however, contained mostly mycelial strands, chlamydospores, and occasional yeastlike cells (Fig. 6d).

The media in the MES and control chambers were inoculated with approximately 2×10^5 mycelial fragments per milliliter of *T. schoenleinii* or *H. capsulatum*, and the MES chamber was set at an O/R potential of +46 mv. After 10 days of incubation, samples from each chamber were added to Warburg vessels and the metabolic rates at 37 C were determined (Table 2). The yeastlike cells of *T. schoenleinii* and *H. capsulatum* had a significantly higher metabolic rate than the cells of the control chamber.

DISCUSSION

The mycelial to yeastlike (M-Y) transformation was accomplished for the dimorphic fungus *H. capsulatum* (17, 18) and for filamentous fungi (14–16) by the incorporation of cysteine in the medium. Scherr (19) speculated that a reduced O/R potental might have been responsible for the M-Y transformation. This assumption could not be tested until a suitable means to maintain a reduced O/R potential could be devised. The MES presented in this paper not only maintained a reduced O/R potential but also allowed for the selection of a desired O/R potential in the culture medium.

The filamentous species, *T. schoenleinii* and *P. lilacinum*, and the dimorphic species, *H. capsulatum*, gave yeastlike cells when grown in the BHI broth without added cysteine, maintained at +46 mv in the MES growth chamber. These observations indicated that the reduced O/R potential of the medium was responsible for the M-Y transformation.

The MES may be a useful tool in studying the effect of a maintained selected O/R potential on such biological phenomena as growth, spore germination, metabolic reactions, and dimorphism.

The growth of several anaerobic bacterial species (7) and of *Trichomonas vaginalis* (8) depended on a reduced O/R potential in the medium. The germination of spores from several species of *Bacillus* could be regulated by a wide range of O/R potentials, depending on the species (28).

Kanel (9) observed that reduced O/R potentials favored lactic acid production, and high O/R potentials favored fumaric acid production in *Rhizopus*. Tengerdy (23, 24) reported a correlation between reduced O/R potentials and the production of a number of fermentation products in *Pseudomonas*.

The literature on the transformation of species of yeasts from the yeastlike cell to a filamentous state has been reviewed by Scherr and Weaver (21). Of the several theories proposed to account for this transformation, the most attractive theory assumes that the synthesis of protoplasm and cell division are separate processes. Bacteria grown in mineral-deficient medium yield filamentous forms with the same dry weight as filamentous forms grown in complete medium (26). Yeasts grown in media containing different compounds were able to grow but had an impaired synthesis of cell-wall material, yielding fila-

mentous forms (R. P. Nelson, Thesis, Univ. of Illinois, Chicago, 1960).

A mycelial mutant of *C. albicans* which is unable to form yeastlike cells under standard growth conditions, has been investigated (12, 13). This mutant had an impaired reductase system but could produce yeastlike cells when cysteine was incorporated into the medium (27). Nickerson (12) suggested that carbohydrate metabolism (particularly available glucose), by providing an adequate supply of sulfhydryl groups within the cell, is involved in the maintenence of the yeastlike growth in wild-type *C. albicans*. Bumzehem and Scherr (Bacteriol. Proc., p. 52, 1957) determined the effect of sulfur-containing compounds on the growth and dimorphism of the mycelial mutant of *C. albicans* and explained their observations on the basis of the sulfhydryl groups produced in the medium. It would be interesting to determine the effect of O/R potential on the dimorphism of the mycelial mutant.

Bartnicki-Garcia (1) and Haidle and Storck (6) demonstated a dimorphism in *Mucor rouxii* by varying the CO_2 content of the medium. It is not clear how these findings are related to the dimorphism found with cysteine or reduced O/R potential.

The M-Y transformation in fungi associated with human, deep mycotic infections has been responsible for a number of investigations on the nature of the transformation and its relation to pathogenicity (10, 14, 16–20, 22). The MES should provide a valuable tool in investigating dimorphism and its relation to pathogenicity.

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