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Adhesion of the Clay Minerals Montmorillonite, Kaolinite, and Attapulgite Reduces Respiration of *Histoplasma capsulatum*

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The respiration of three phenotypes of *Histoplasma capsulatum*, the causal agent of histoplasmosis in humans, was markedly reduced by low concentrations of montmorillonite but was reduced less by even higher concentrations of kaolinite or attapulgite (palygorskite). The reduction in respiration followed a pattern that suggested saturation-type kinetics: an initial sharp reduction that occurred with low concentrations of clay (0.01 to 0.5% [wt/vol]), followed by a more gradual reduction with higher concentrations (1 to 8%). Increases in viscosity (which could impair the movement of O_2) caused by the clays were not responsible for the reduction in respiration, and the clays did not interfere with the availability of nutrients. Scanning electron microscopy after extensive washing showed that the clay particles were tightly bound to the hyphae, suggesting that the clays reduced the rate of respiration of *H. capsulatum* by adhering to the mycelial surface and, thereby, interfered with the movement of nutrients, metabolites, and gases across the mycelial wall.

Histoplasma capsulatum, a highly infectious mycotic pathogen of humans and a soil-borne organism (10, 26), is endemic in many areas throughout the world, including parts of the United States (1, 9), but its distribution is restricted. This discrete geographic distribution of *H. capsulatum* appears to be dependent on the clay mineral composition of the soil (19, 20, 23). The absence of montmorillonite (M), a crystalline, three-layer, hydrous aluminosilicate (14), was highly correlated with the presence of *H. capsulatum* in soil, a relation that was not apparent with other types of clay minerals. M was not present in essentially all soils from which *H. capsulatum* was isolated (23).

The assumption that the geographic distribution of *H. capsulatum* is affected by the clay mineralogy of soils has been based only on field observations. In vitro studies are therefore necessary to understand this interaction and to define the mechanisms involved. Stotzky and Rem (24) found that M, and to some extent kaolinite (K), markedly inhibited the respiration of 27 species of fungi. However, their studies did not include *H. capsulatum*. The purpose of this study was to investigate the effects of mined M, K, and attapulgite (A; palygorskite) on the respiration of *H. capsulatum* in pure culture.

MATERIALS AND METHODS

Source and maintenance of fungi. Three strains of *H. capsulatum* were obtained from Morris Gordon of the Mycology Laboratory, Department of Health of the State of New York, Albany. The strains were classified by the identification code used by the Department of Health: 210, isolated from chimney debris in New York State; 211, isolated from a human bone marrow specimen; and 230, isolated from a human lymph node specimen. The strains were maintained on Mycobiotic agar (pH 6.5; Difco Laboratories, Detroit, Mich.) at room temperature (22 ± 2°C).

Clay minerals. M (Bentonite; Fisher Scientific Co., Pittsburgh, Pa.) had a cation exchange capacity of 97.7 meq/100 g; a particle size distribution of <8 μm, 100%; <2 μm, 34%; and <1 μm, 3%; and the following concentration (in milliequivalents per 100 g of clay) of exchangeable cations: H^+, 8.5; K^+, 1.5; Na^+, 49.6; Ca^{2+}, 41.9; and Mg^{2+}, 10.3 (17).

K (Kaolin; Fisher) had a cation exchange capacity of 5.8 meq/100 g, a particle size distribution of <8 μm, 98%; <2 μm, 52%; and <0.5 μm, 3%; and the exchangeable cation content (in milliequivalents per 100 g of clay) was H^+, 3.7; K^+, 0.1; Na^+, 0.3; Ca^{2+}, 0.6; and Mg^{2+}, 0.5 (17).

A (Attaclay; Minerals and Chemicals, Philipp Corp.) had a cation exchange capacity of 34 meq/100 g and an average particle size of 18 μm, and the exchangeable cation content (in milliequivalents per 100 g of clay) was H^+, 0.5; K^+, 3.0; Na^+, 3.1; Ca^{2+}, 24.0; and Mg^{2+}, 17.0 (18).

Medium. The medium, BACTEC system culture vial 6B, used in the respiration studies was composed of tryptic soy broth (TSB) enriched with ^{14}C-labeled glucose and phenylalanine (PA) and was obtained from Johnston Laboratories, Inc., Towson, Md. The BACTEC vials contained the following ingredients (wt/vol): 2.75% tryptic soy, 0.005% hemin, 0.00005% vitamin K, 0.001% vitamin B_6, 0.0375% sodium carbonate, 0.25% sucrose, 0.025% sodium polyethanol sulfate, 2.0 μCi of ^{14}C-labeled glucose and PA, and 30 ml of distilled water.

Hemin. Hemin ($C_{34}H_{32}ClFeN_4O_4$) was purchased from Eastman Kodak Co., Rochester, N.Y.

Carboxymethyl cellulose. Sodium carboxymethyl cellulose (CMC) (cellulose gum) was purchased from Fisher Scientific Co.

^{14}C-labeled PA. [^{14}C]1-PA was purchased from New England Nuclear Corp., Medical Diagnostic Division, Boston, Mass.

Precautionary measures. Because *H. capsulatum* is a highly infectious organism, it was handled under a bacteriological safety hood (S/P L5229) that provided a combination of resistance heaters, which maintained 121°C, and a filter system and ensured that all vegetative cells or spores released within the hood were killed by heat, trapped in the filter before the air flow reached the exhaust duct, or both. After the respiratory measurements, the $^{14}CO_2$ produced was absorbed in the BACTEC 460 instrument in a CO_2 trap to prevent the release of radioactive gas into the laboratory. The trap was a disposable canister containing 1.2 kg of soda

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lime, with a color indicator, and when saturated, the trap was replaced. After the experiments were completed, the vials were autoclaved, placed into a special drum, and transferred to an area designated for radioactive waste disposal by Teledyne Isotope Co.

Description of experiments. (i) Measurement of respiration. The effect of the clays on the growth rate of *H. capsulatum* was measured by CO_2 production. The desired concentrations (0.01, 0.1, 0.5, 1, 2, 4, and 8% [wt/vol]) of each clay were autoclaved three times for 20 min at 121°C and 15 lb/in^2 in glassine envelopes and then dispensed into BACTEC vials containing 30 ml of sterile medium. The fungus was grown on Mycobiotic agar at room temperature for 3 to 4 weeks; circular plugs (12 mm in diameter), made with a sterile metal cork borer, were cut into small fragments (1 to 2 mm) with sterile teasing needles, and the fragments of fungus were added to the vials. Respiration was measured every other day for 12 days with the radiometric automatic respirator, BACTEC 460 (Johnston Laboratories). Between measurements, the vials were incubated at room temperature with agitation (180 rpm; Thomas Clinical Rotator, Arthur H. Thomas Co., Philadelphia, Pa.) for 16 h per day.

The $^{14}CO_2$ resulting from the metabolism of the ^{14}C-labeled substrates present in the vial was passed through an ionization chamber and translated into an electrical current, and the radioactivity was recorded as a growth index, which was directly related to the amount of $^{14}CO_2$ produced. A growth index reading of 100 corresponded to 25 nCi of $^{14}CO_2$. The respiration of the fungus in the absence of clay (control vial) represented 100% respiration.

Three replicate samples were used for each clay-fungus combination, and each experiment was repeated at least twice. The data are presented as the arithmetic mean ± the standard error of the means. The two-tailed Student *t* test was used to evaluate the significance of differences between the means, and $P < 0.05$ was considered to be significant.

(ii) Viscosity measurements. To determine whether the increased viscosity caused by the clays was a factor in reducing the respiration of the fungus (24), the clays were replaced by CMC at concentrations that resulted in viscosities similar to those of the various concentrations of M or K. The apparent viscosity of the clays and the CMC was measured with a falling ball viscometer (V2200; Roger Gilmont Instruments), and the viscosity was calculated by the method of Aronson and Nelson (2). The respiration of *H. capsulatum* 210 was measured in the presence of concentrations of CMC that resulted in viscosities similar to those elicited by the different concentrations of M and K. There were three replicate samples for each concentration, and respiration was monitored every second day for 12 days.

(iii) Interference by M with the availability of nutrients. To determine whether the reduction in respiration caused by M was the result of the removal of nutrients from the liquid phase of the experimental system by their adsorption on the clay, the BACTEC vial was supplemented during the respiration period with (i) 3 ml (10%) of TSB on day 4 of the experiment, which corresponded with the beginning of the stationary phase of fungal growth; (ii) three to five times the amount of hemin normally present; or (iii) 1 μCi (3.2 μg) or 2 μCi (6.4 μg) of ^{14}C-labeled PA, bringing the total radioactivity in the vials to 3 and 4 μCi, respectively. Three replicate samples were used for each variable, and respiration was measured every second day for 12 days.

(iv) Scanning electron microscopy. To determine whether the clays interfered with fungal respiration as the result of their adsorption to the mycelial surface, mycelium was removed from BACTEC vials containing 0, 0.5, or 1% M or K after 2 days and observed with an AMR 1000 scanning electron microscope. The mycelial fragments were thoroughly washed by agitation in sterile distilled water and centrifuged for 10 min at 2,000 × *g*. The supernatants were decanted, and 5% glutaraldehyde in sodium cacodylate buffer (pH 7.1 to 7.4) was added. The specimens were refrigerated overnight and centrifuged, and after the supernatants were decanted, the specimens were dehydrated in an ethanol series of 30, 50, 70, 90, 95, and 100%, with 5 min in 30 and 50% ethanol and 10 min in each of the succeeding concentrations. The last wash in 100% ethanol was repeated three times. Each wash was followed by centrifugation and decantation. After the last wash in 100% ethanol, the specimens were impacted on a membrane (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) and subjected to critical point drying (5, 8, 25) in a CPD-7 chamber. The chamber was filled four times with CO_2 (99.9%) at 5-min intervals at 16°C and 820 lb/in^2. After the fourth change of CO_2, the temperature was increased to 40°C and the pressure to 1,100 lb/in^2. The specimens were made conductive with a gold-palladium coating in a carbon evaporator (Denton Vaccum DV 502). Scanning electron microscopy was performed at a magnification of ×5,000 to ×20,000.

(v) Effect of surface area on respiration. To determine whether there was a relation between the surface area of the experimental systems and the respiration rate of the fungus, each concentration of clay was converted to its appropriate specific surface area (in square meters per gram) (24), and the functional relation was expressed as a linear regression and as the correlation coefficient.

RESULTS AND DISCUSSION

Effects of clay concentration on respiration. The respiration of *H. capsulatum* was markedly reduced in the presence of increasing concentrations of M, K, and A. The reduction followed a pattern that suggested saturation-type kinetics: an initial sharp reduction in respiration with low concentrations of the clays (0.01 to 0.5%), followed by a slight and more gradual reduction with higher concentrations. The initial sharp reduction in respiration caused by the low concentrations of clays ranged from 19 to 25% for M, 10 to 18% for K, and 8 to 15% for A. The maximum reduction in respiration of strain 210 was approximately 40% in the presence of M, approximately 18% with K, and approximately 32% with A (Fig. 1). Similar patterns were obtained with strains 211 and 230.

The increased viscosity caused by increasing concentrations of M and K was not a significant factor in reducing the respiration of *H. capsulatum* (i.e., there was no apparent inhibitory impairment in the movement of O_2) (Fig. 2).

Interference by M with the availability of nutrients. To compensate for the possible binding of nutrients on M and, hence, for a decrease in their bioavailability, extra nutrients were added to the BACTEC vials, either as TSB, which contained all the ingredients originally present, or as separate components, such as hemin or PA.

The addition of TSB on the day 4 of the experiment, when the metabolic rate of the fungus began to plateau, did not enhance respiration (Fig. 3). The addition of TSB or distilled water (as a control) apparently caused a change in the liquid-to-gas ratio in the vial and resulted in a reduction in the amount of CO_2 recovered.

The addition of hemin, even at five times the concentration present in the initial TSB, did not increase the respiration rate, indicating that the concentration of hemin (i.e., the

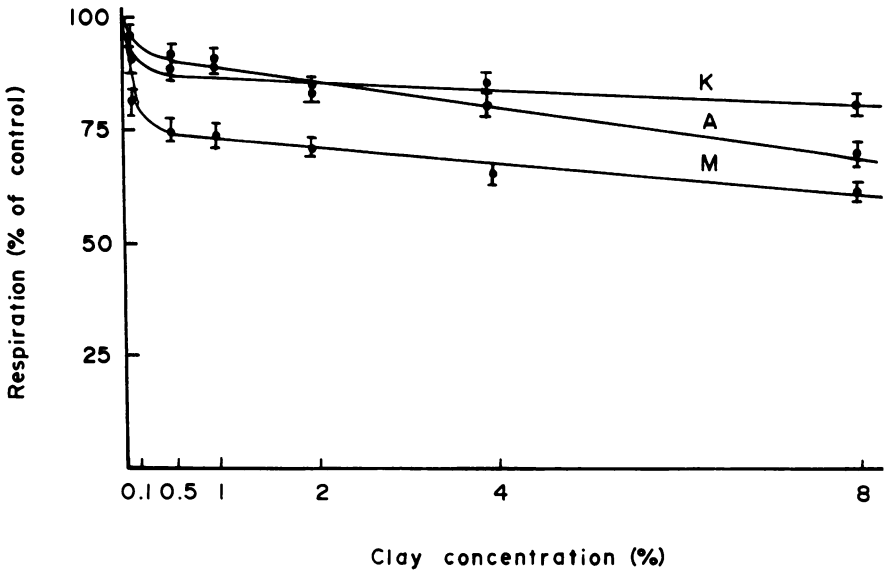

FIG. 1. Effect of various concentrations of M, K, and A on respiration of *H. capsulatum* during a 12-day incubation.

amount of available iron) originally present was adequate to sustain maximal respiration, even in the presence of M (Fig. 4).

The addition of PA caused a proportional increase in the rate of respiration in both the experimental and control vials, indicating that the bioavailability of PA was also not affected by M (Fig. 5).

The nutritional conditions in the experimental vials, even though some binding of nutrients on the clays may have occurred, were apparently adequate for maximum respiration. Hence, the reductions in respiration caused by the clays were not the result of a reduction in nutrient availability.

Scanning electron microscopy. Results of scanning electron microscopy indicated that M and K were bound to the surface of the fungal mycelium (a hyphal fragment from the control vial [i.e., no clay] is shown in Fig. 6). The coating of the mycelia appeared to be greater with 1% (Fig. 7B and 8B) than with 0.5% (Fig. 7A and 8A) K or M. Although only two concentrations of M and K were evaluated, the apparent greater coating of the hyphae with 1% than with 0.5% clay indicated that the amount of adhesion increased as the concentration of the clays was increased. The sharp reduction in respiration that occurred with clay concentrations to 0.5% indicated that the maximum effect of the adhesion of the clays was exerted by approximately 0.5% clay and that although more clay was bound as the concentration of the clays was increased, this caused only a relatively slight further reduction in respiration.

The reduction in respiration of *H. capsulatum* in the presence of the clays apparently reflected the coating of the mycelia by the clays and the effect of such coating on the

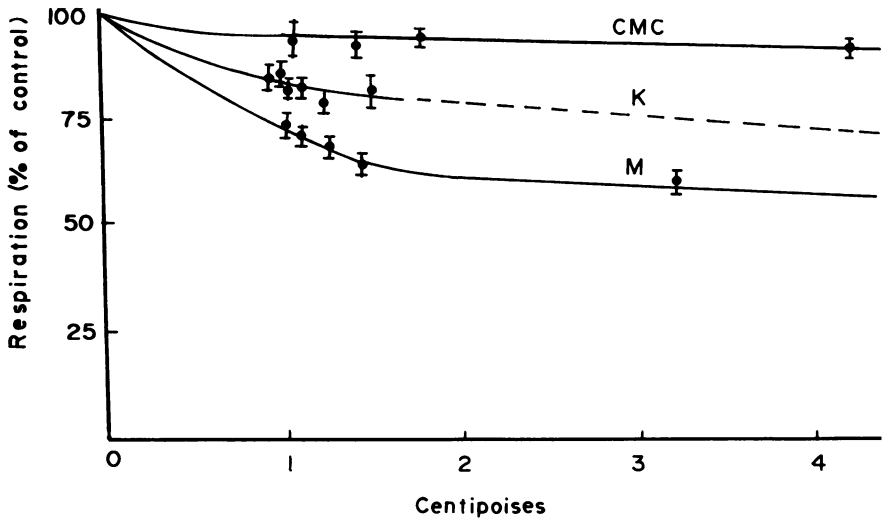

FIG. 2. Effect of viscosity, caused by various concentrations of M, K, or CMC, on the respiration of *H. capsulatum* during a 12-day incubation (see text).

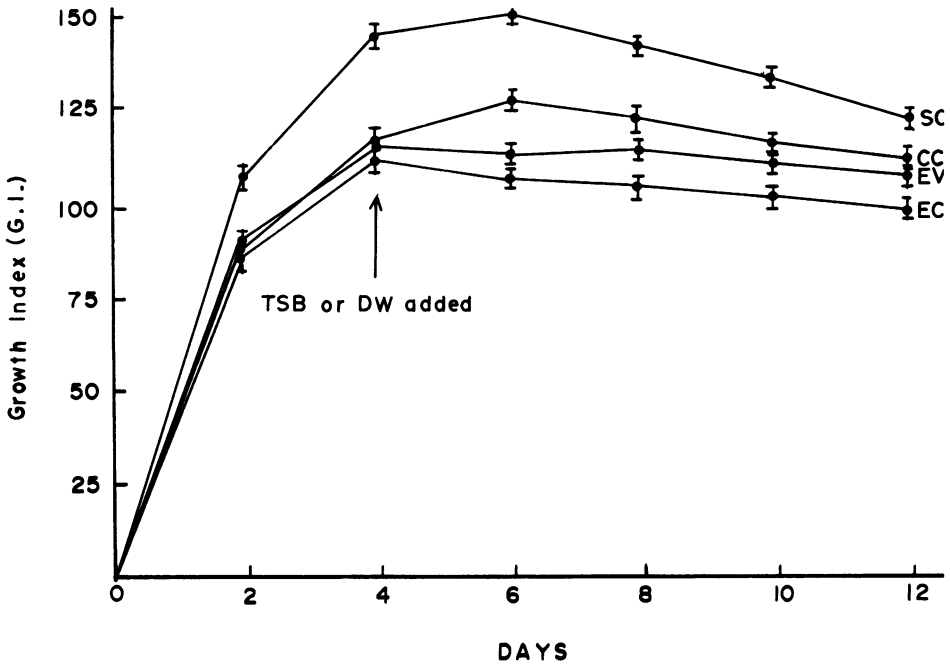

FIG. 3. Effect of nutrient broth (TSB) or distilled water (DW) added during the period of intensive growth on the respiration rate of *H. capsulatum* in the presence of 0.1% M. Abbreviations: SC, strain control, no. M; CC, clay control, 0.1% M; EC, experimental control, 0.1% M and 3 ml of DW; EV, experimental variable, 0.1% M and 3 ml of TSB.

metabolism of the fungus, e.g., interference with the movement of nutrients, gases, and waste products across the mycelial wall. There was a high direct correlation between the specific surface area of the clay-containing systems and the reduction in respiration (Fig. 9), further indicating that the coating of the mycelium by the clays was partially responsible for the reduction in respiration.

The mechanisms by which the clays adhered to the

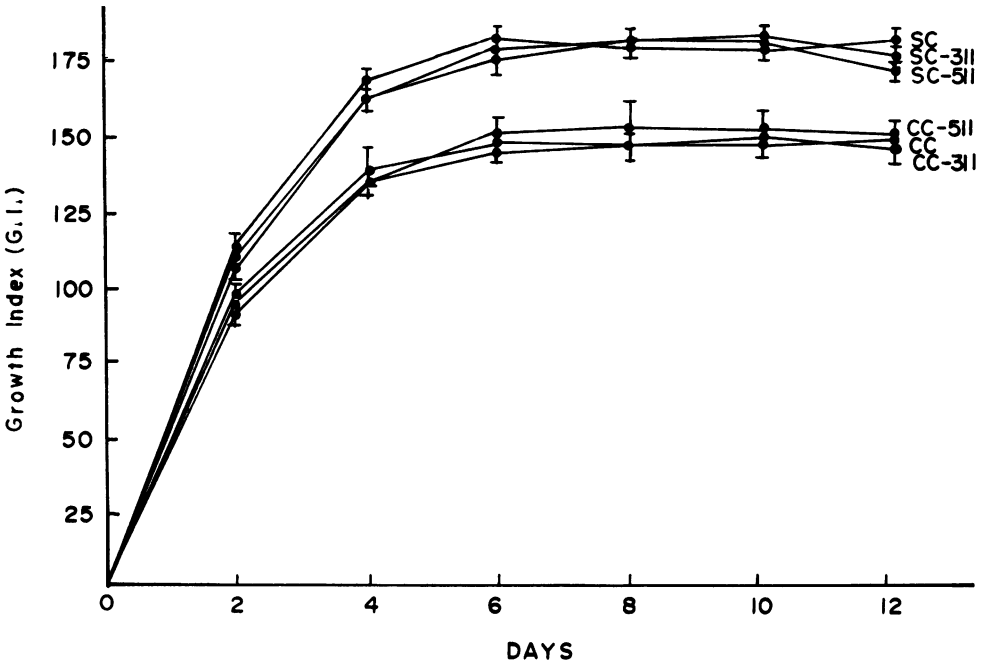

FIG. 4. Effect of various concentrations of hemin (H) on the respiration rate of *H. capsulatum* in the presence of 0.1% M during a 12-day incubation. Abbreviations: SC, strain control, no M; SC-3H, H at three times the concentration in SC; SC-5H, H at five times the concentration in SC; CC, clay control, 0.1% M; CC-3H, H at three times the concentration in CC; CC-5H, H at five times the concentration in CC.

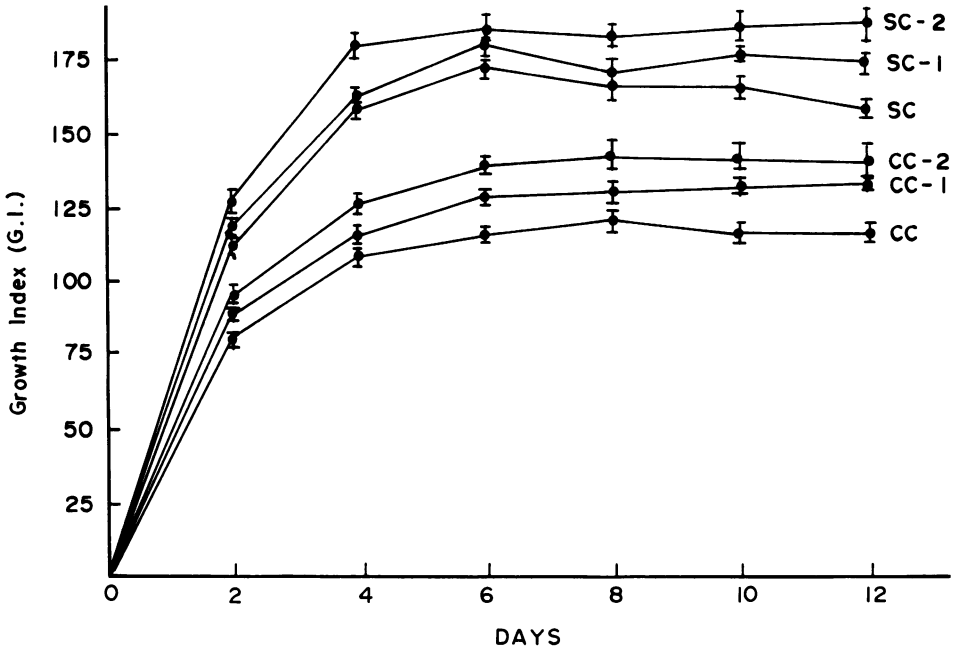

FIG. 5. Effect of various concentrations of ^{14}C-labeled PA on the respiration rate of *H. capsulatum* in the presence of 0.5% M during a 12-day incubation. Abbreviations: SC, strain control, no M; SC-1, SC plus 1 μCi of PA; SC-2, SC plus 2 μCi of PA; CC, clay control, 0.5% M; CC-1, CC plus 1 μCi of PA; CC-2, CC plus 2 μCi of PA.

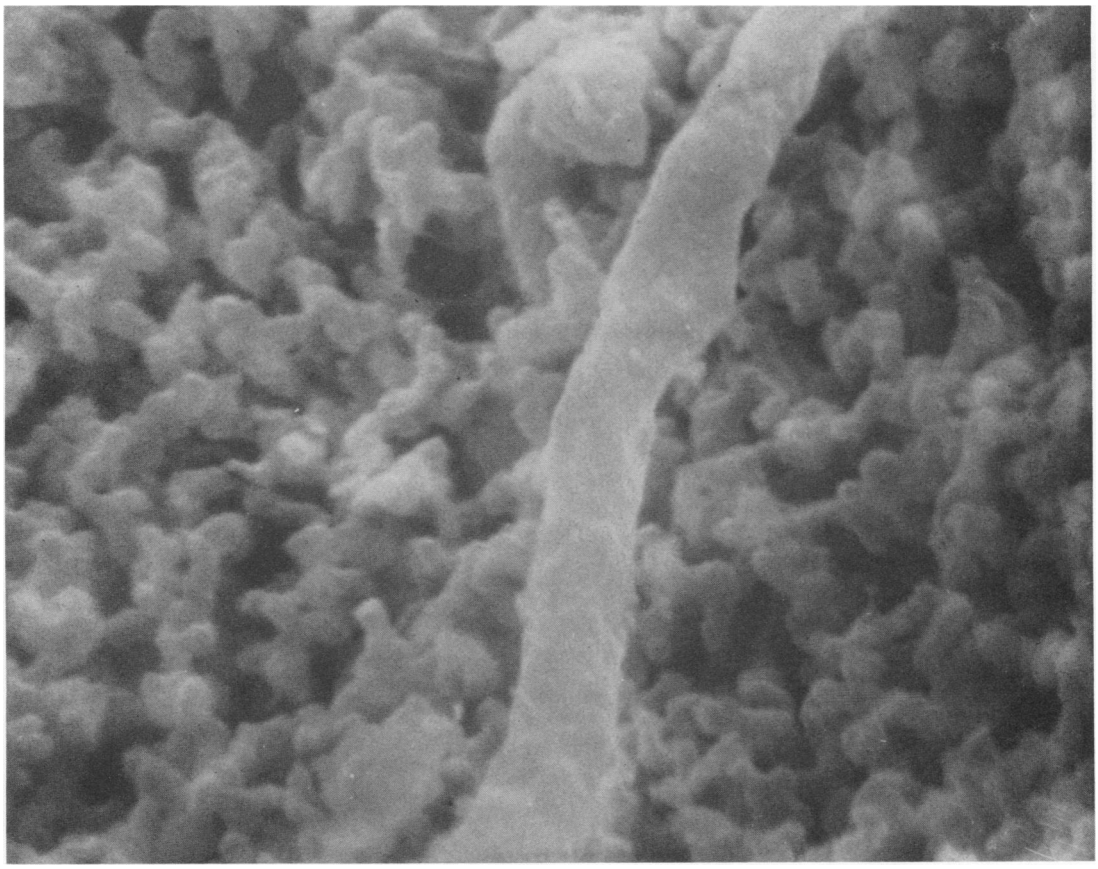

FIG. 6. Scanning electron micrograph of a mycelial fragment of *H. capsulatum* 210. Magnification, ×10,000.

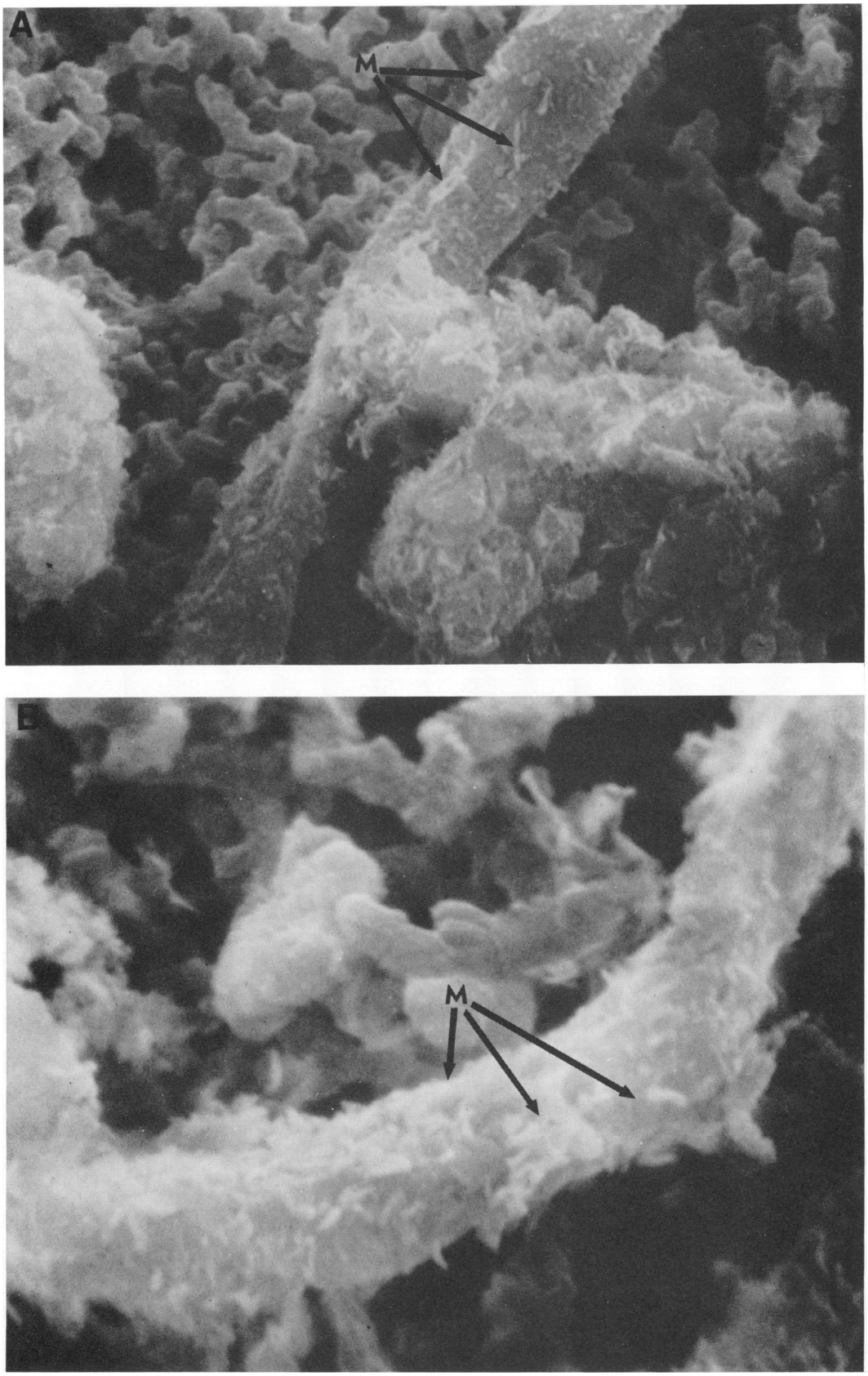

FIG. 7. Scanning electron micrograph of a mycelial fragment of *H. capsulatum* coated with flakes of M. The sample was taken after 2 days from a respiration experiment containing 0.5% (magnification, ×10,000) (A) or 1% M (magnification, ×20,000) (B).

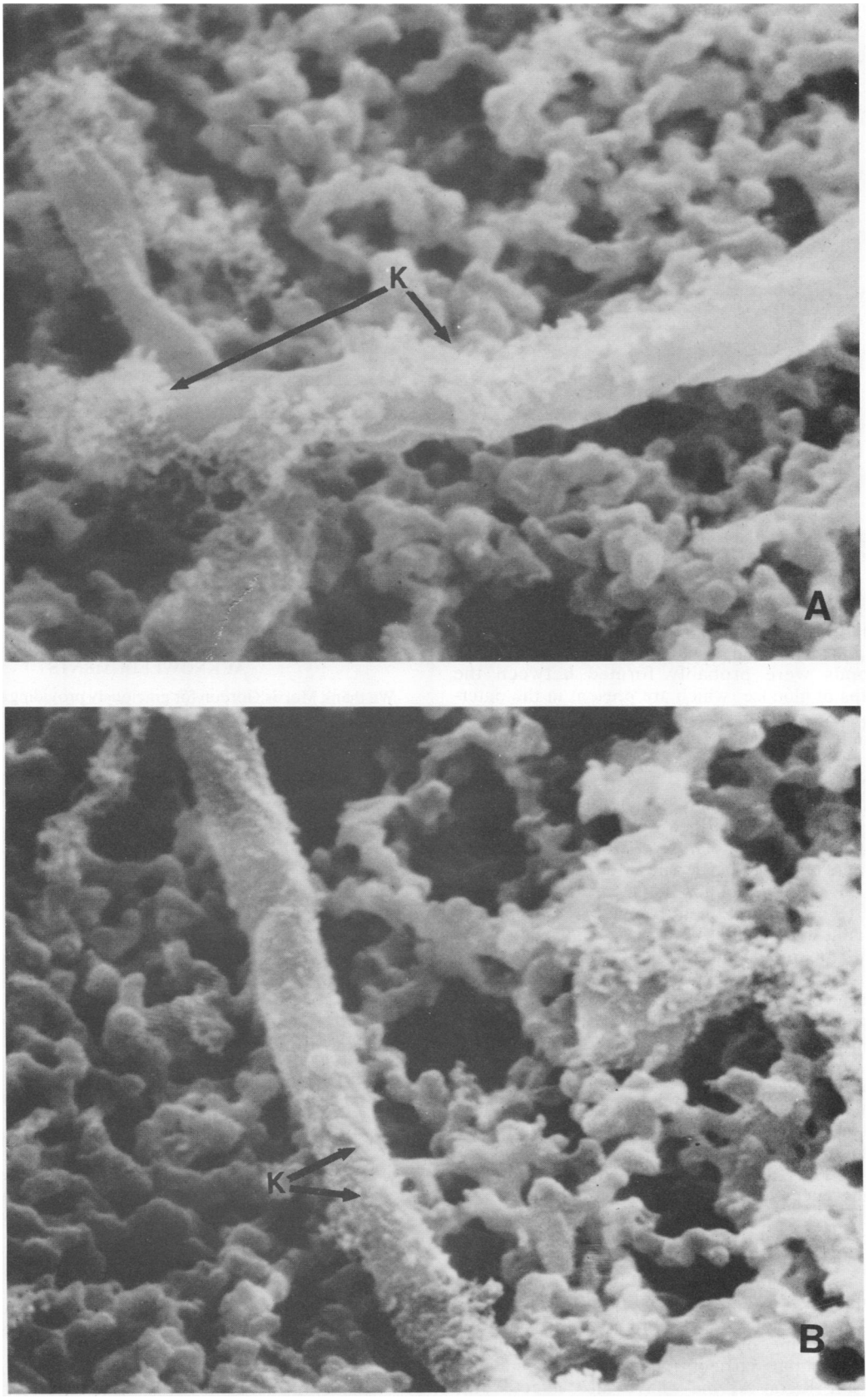

FIG. 8. Scanning electron micrograph of a mycelial fragment of *H. capsulatum* coated with flakes of K. The sample was taken after 2 days from a respiration experiment containing 0.5% (magnification, ×10,000) (A) or 1% K (magnification, ×10,000) (B).

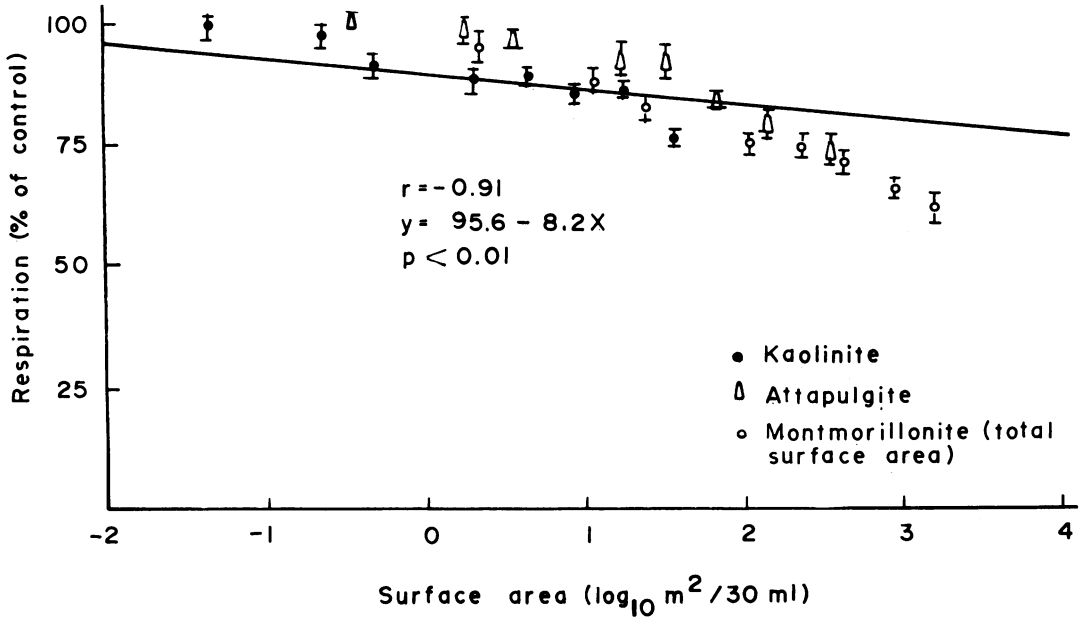

FIG. 9. Relation between the respiration of *H. capsulatum* and the specific surface area present in the broth system, as the result of the addition of different concentrations of M (total surface area), A, or K.

mycelium are not known. The interaction between the clays and the mycelial surface probably involved London-van der Waals forces and hydrogen bonding (12, 15, 16, 21, 22). Hydrogen bonds were probably formed between the hydroxyl groups of glucose, which are present in the outermost layer of the mycelium of *H. capsulatum* as β-glucans (3, 4, 11), and the water of hydration of the charge-compensating cations on the clays (e.g., water bridging) (21, 22).

Conclusions. The mechanisms by which the clays adhered to the mycelium need to be clarified. Because the outermost layer of the cell wall of *H. capsulatum* is composed of glucans (polymers of glucose), there is ample opportunity for the formation of hydrogen bonds between the hyphae and clay minerals. Furthermore, many fungi excrete polysaccharides with adhesive qualities (13) that may also have been involved in the apparent tight adhesion of the clays to the surface of *H. capsulatum*. These mechanisms may have been involved in the adhesion of M and K to the hyphae of *Gaeumanomyces graminis* (6, 7), although there may have been some hydrophobic effects (6).

In the case of *H. capsulatum*, the reduction in the respiration of the fungus in the presence of the clays was attributed, in part, to the reduction in the amount of mycelial surface available for the transmembrane movement of nutrients, waste products, and gases as the result of the adhesion of the clays. However, the adhesion of the clays did not explain the differences in the amount of reduction in respiration caused by the same maximum concentration of each clay. Even though the reduction in respiration showed a high correlation with an increase in the specific surface area of the broth system as the concentration of the clays was increased, the amount of K and M bound to hyphae appeared to be similar (the adhesion of A was not studied by electron microscopy), and none of the clays interfered with the availability of essential nutrients. These findings indicate that M, but not K or A, may have also affected the respiration of the fungus by other mechanisms, yet to be elucidated, in addition to the direct effect caused by the adhesion of M to the hyphae.

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