Biochemical Changes During Fungal Sporulation and Spore Germination

I. Phenyl Methyl Sulfonyl Fluoride Inhibition of Macroconidial Germination in *Microsporium gypseum*¹

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Macroconidia of *Microsporium gypseum* release free amino acids into the medium during germination. A single alkaline protease is also found in the germination supernatant fraction. The purified protease is capable of hydrolyzing isolated spore coats in vitro. Phenyl methyl sulfonyl fluoride (PMSF) is an effective inhibitor of the protease. Incorporation of PMSF at 10^{-4} M into the germination system inhibits spore germination and the release of free amino nitrogen. Addition of PMSF after germ tube emergence is completed has no effect on subsequent outgrowth. The addition of exogenous purified protease to quiescent spores results in more than a 2.5-fold increase in germinated spores. It is concluded that spore coat proteolysis is an essential event in the germination of dermatophyte macroconidia. A model system to explain macroconidial germination response to inhibition, temperature shift, and addition of protease is presented.

The suggestion that proteolysis is an important event in bacterial spore germination has been supported by the work of Powell and Strange (15), Strange and Dark (20), Gould and Hutchins (6), Sierra (17), and others.

The association of wall lytic enzymes and sporulation has also been documented in lower fungi (1, 3, 7, 9, 22). However, the requirement for proteolysis during fungal spore germination has not been conclusively established. The results of this investigation suggest that spore coat proteolysis is a primary event in macroconidial germination.

MATERIALS AND METHODS

Organism. A strain of *Microsporium gypseum* (Bodin) Guiart and Grigorakis, 1928, originally obtained from F. Blank, Temple University, Philadelphia, Pa., was used in all studies.

Sporulation medium and macroconidial preparations. Spore production and isolation were carried out as described previously (11).

Germination media. Macroconidia (10^6 per ml) were germinated in either pH 6.5 physiological saline or 0.1% (w/v) neopeptone (Difco) and 1.0% (w/v) glucose medium (pH 6.5).

Germination conditions. Spores were germinated at 25 or 37 C in 50-ml Erlenmeyer flasks containing 5 ml

of medium. The flasks were shaken at 125 rev/min in a R77 Metabolyte shaker water bath (New Brunswick Scientific Co., New Brunswick, N.J.).

Estimation of per cent germination. Per cent macroconidial germination was estimated microscopically as described previously (11).

Spore coat and hyphal wall preparation. Macroconidial or hyphal suspensions in pH 6.5 physiological saline were mixed with 0.6 volume fine glass beads (100 μ m, Sigma Chemical Co., St. Louis, Mo.) and blended at 4 C in a colloid mill (Gifford-Wood, Inc., Hudson, N.Y.), to 100% breakage as judged by microscopic examination. The glass beads were allowed to settle, and the supernatant fraction was centrifuged at $600 \times g$ for 1 min. The pellet obtained was resuspended in 10 volumes of cold physiological saline, pH 6.5, by mixing (Vortex Jr. Mixer, Scientific Industries, Inc., Queen's Village, N.Y.). Walls or spore coats were recovered by centrifugation at $600 \times g$ for 1 min. The pellet was washed eight times in physiological saline (pH 6.5) and eight times in distilled water by centrifugation at $600 \times g$ for 1 min at 4 C. Vigorous dispersal of the pellet was necessary after each washing. The final pellet was resuspended in 10 volumes of 10% sodium lauryl sulfate and extracted on a tube roller at 37 C for 3 days. The sodium lauryl sulfate solution was changed once each day. The extracted pellet was washed 10 times in distilled water at 4 C. Purified spore coats and hyphal walls were then dried to a constant weight in vacuo at 4 C.

High-voltage electrophoresis. Purified hyphal walls and spore coats were subjected to electrophoresis at

¹ A preliminary report covering part of this work was presented at the 69th Annual Meeting of the American Society for Microbiology, Miami Beach, Florida, May 1969.

3000 v for 45 min in a Gilson model D electrophorator by using a buffer (pH 6.5; acetic acid, 4 ml; pyridine, 100 ml; and water, 900 ml). The paper strips were stained for free amino acids by the ninhydrin method of Smith (18), for carbohydrate by the silver nitrate method of Smith (18), and for protein by nigrosin (0.002% in 2% acetic acid for 24 hr, decolorized in water).

Hydrolysis conditions. Spore coats and hyphal walls at 1 mg/ml were hydrolyzed in 6 N HCl for 12 hr at 110°C prior to amino acid analysis. Germination medium supernatant fractions were mixed with an equal volume of 12 N HCl and hydrolyzed for 12 hr at 110°C prior to amino acid analysis. Glucosamine was liberated by hydrolyzing coats or walls (1 mg/ml) in 6 N HCl for 6 hr at 110°C. Glucosamine standards were hydrolyzed under the same conditions.

Analytical determinations. All protein estimations were made by the method of Lowry et al. (12). Prior to total cellular protein estimation, the germinated spores were pelleted ($4,000 \times g$ for 1 min) three successive times through 10 ml of physiological saline (pH 6.5). The resulting pellets were resuspended in equivalent volumes of saline (pH 6.5). Total hexose was determined by the anthrone reaction (14). Free amino nitrogen analysis was carried out by the method of Yemm and Cocking (23). Ash was estimated by incinerating the walls or coats at 700°C for 6 hr. Glucosamine was determined by the method of Randle and Morgan (16). Amino acid residues were quantitated in a Beckman model 120 B amino acid analyzer by the method of Spackman, Stein, and Moore (19).

Protease activity was measured by the method of McDonald and Chen (13). Alkaline phosphatase activity was assayed as described by Garen and Levinthal (5).

Protease purification. Macroconidial suspension (50 ml, 10^8 conidia per ml), in physiological saline, (pH 6.5), was placed in a 500-ml Erlenmeyer flask, and the contents were shaken at 125 rev/min in a water bath at 37°C for 8 hr. The suspension was centrifuged at $20,000 \times g$ for 10 min at 4°C. The supernatant fluid was removed carefully and dialyzed against two changes of 200 volumes of physiological saline (pH 6.5), during a period of 24 hr at 4°C. The dialysate volume was reduced approximately 10-fold by dialysis against polyethylene glycol (20,000 molecular weight, Fisher Scientific Co.). The final yield of enzyme was 3 to 4 mg of protease per 50 ml of original spore suspension and was stored at -20°C until used.

Disc gel electrophoresis. Disc gel electrophoresis was at pH 9.1, by using the procedure suggested for the Canalco (Rockville, Md.) model 6 system. Proteolytic activity was located by placing the unstained gel on a slide covered with a mixture of casein (1% w/v) and agar (1% w/v) at pH 8.0. The gel was removed after 15 min (visual localization of clearing of area on slide) or after 1 hr (photographic localization) at 37°C (see Fig. 2). The slide was flooded with a solution of mercuric chloride (1% w/v), prior to final observation.

Preparation of cell-free extract. Samples (3 ml) of a suspension of macroconidia (10^8 /ml) in 0.2 M tris-

(hydroxymethyl)aminomethane (Tris), pH 7.4, were sonically disrupted under nitrogen at a dial setting of 70 with a Biosonic model B (Bronwill Scientific Co., Rochester, N.Y.) fitted with a microprobe attachment. The tube was held in ice during the treatment. The samples were given seven 30-sec bursts with a 30-sec interval for cooling between bursts. Debris was removed by centrifugation at $20,000 \times g$ for 30 min at 4°C.

Phenyl methyl sulfonyl fluoride. Solutions of 10^{-1} M PMSF in twice-distilled isopropanol were prepared immediately prior to use.

Chemicals. Glucose, alkaline phosphatase, glucosamine, ninhydrin, PMSF, and sodium lauryl sulfate were obtained from Calbiochem, Los Angeles, Calif. Bovine serum albumin and casein were obtained from Sigma Chemical Co., St. Louis, Mo. Hippuryl phenylalanine and benzyl arginine ethyl ester (BAEE), were purchased from Worthington Biochemical Corp., Freehold, N.J. Reagents for disc gel electrophoresis were obtained from Canalco.

RESULTS

Development of the germination system. As a preliminary to this investigation, methods were developed for the rapid purification of ungerminated macroconidia and for the germination of 80 to 90% of a given spore suspension in physiological saline, pH 6.5 (11). It was also shown that the endogenous reserves of the macroconidia were sufficient for at least 8 hr of germination and outgrowth (Leighton, Stock and Kelln, *in preparation*). Hence, we had a germination system which was independent of added nutrient materials.

Characterization of spore coats and hyphal cell walls. Table 1 lists the gross chemical composition of the spore coats and hyphal cell walls. If the fungal spore has germinated as a result of weakening the spore coat, as electron microscopic evidence certainly has indicated (21; J. Das and S. H. Black. *Bacteriol. Proc.*, p. 23, 1969), then presumably a protease, chitinase, or cellulase-like enzyme could be involved.

The question arose as to whether the protein values listed in Table 1 were actually structural protein or adsorbed amino acids and peptides released during cell disintegration. Samples of purified spore coats and hyphal walls were placed on paper strips and examined for the presence of amino acids (ninhydrin), protein (nigrosin), and carbohydrate (AgNO_3). Spore coats and hyphal walls gave no ninhydrin reaction, but were positive for carbohydrate and protein (possibly most of the free N-terminal amino acids in the cell wall protein were "blocked," i.e., ninhydrin nonreactive, owing to acetylation of the free amino groups). Ninhydrin staining after high-voltage electrophoresis did not reveal any

TABLE 1. Chemical composition of spore coats and vegetative wall of *Microsporum gypseum*

Component	Spore coats	Vegetative walls
	%	%
Hexose as glucose.....	31.2	40.4
Amino sugar as <i>N</i> -acetyl glucosamine.....	52.5	27.7
Protein.....	12.9	10.6
Ash.....	8.0	11.0

reactive material and suggested that nonstructural amino acids were absent. Staining the paper strips for protein and carbohydrate revealed three glycoprotein bands in the spore coats and one glycoprotein band in the hyphal walls. Since carbohydrate and protein always migrated with each other, we believe that the protein values truly represent structural protein.

Chemical analysis of germination supernatant fluids. If the spore coat was hydrolyzed during germination, the obvious place to look for degradation products would be in the germination supernatant fluid. Concentrated supernatant fluids were assayed for Randle-Morgan-positive material, anthrone-positive material, and ninhydrin-reactive material. Only the ninhydrin test gave a positive result.

Figure 1 shows the time course of free amino nitrogen release into the supernatant fluid. It is interesting to note that microscopic examination showed that the germination process was completed for all spores by 7 hr, i.e., those spores which were going to germinate had done so by this time.

Therefore, it was decided to characterize further the nature of this ninhydrin-reactive material by amino acid analysis. Table 2 lists the amino acid composition of hydrolyzed 2- and 8-hr supernatants. The analyses were nearly identical with respect to individual concentrations of the residues. This information suggested to us that the process producing this material may be of a rather specific nature, as opposed to general intracellular turnover products diffusing out into the supernatant fluid. We checked for spore lysis during the course of the germination process by assaying 8-hr supernatant fractions for alkaline phosphatase activity (a known constitutive intracellular enzyme; Leighton, Stock, and Kelln, *in preparation*). We were never able to detect any alkaline phosphatase activity in the supernatant fluid.

Analysis of germination supernatant fluids for proteolytic activity and protease characterization. Figure 2 presents the results of basic disc gel

FIG. 1. Release of free amino nitrogen into the supernatant fraction during spore germination. Germination system was pH 6.5 physiological saline with incubation at 37 C. Macroconidial concentration was 10^8 /ml.

TABLE 2. Amino acid composition of 2-hr and 8-hr germination medium supernatant fluid^a

Amino acid	2-Hr supernatant	8-Hr supernatant
Lysine.....	3.75	3.80
Histidine.....	1.99	1.95
Arginine.....	1.70	1.63
Aspartic acid.....	6.97	6.93
Threonine.....	2.40	2.10
Serine.....	2.11	2.21
Glutamic acid.....	10.3	11.9
Proline.....	1.70	1.75
Cysteine.....	Present	Present
Glycine.....	3.05	2.94
Alanine.....	1.85	2.18
Valine.....	1.42	1.37
Methionine.....	0.21	0.29
Isoleucine.....	2.36	2.35
Leucine.....	1.56	1.58
Tyrosine.....	0.67	0.65
Phenylalanine.....	0.46	0.52

^a Expressed as micromoles of amino acid per 40 mg of protein in the supernatant fraction.

FIG. 2. Basic disc gel electrophoresis of dialyzed, concentrated 8-hr spore germination supernatant fraction. Left and center photographs represent electrophoresis of supernatant fraction for different time periods. The origin is at top of all photographs. The right photograph shows localization of protease activity in an unstained duplicate gel of the center photograph. The transparent area, which appears dark owing to the background, is the zone of casein hydrolysis. Incubation was for 1 hr at 37 C, which caused some diffusion of the band.

electrophoresis on dialyzed, concentrated germination supernatant fluid. Only one protein band was detectable, and this band was shown to have proteolytic activity.

Figure 3 illustrates the *pH* profile of the purified protease against casein; Fig. 4 shows the thermal denaturation curve for purified protease. Both Fig. 3 and 4 suggested a single proteolytic activity and provided corroborative evidence for the disc gel electrophoresis results.

We were interested to know whether the isolated protease would attack purified spore coats. The results of this experiment are presented in Fig. 5.

Table 3 lists the amino acid composition of hyphal walls, ungerminated spore coats and 7-hr spore coats. These analyses were repeated several times to check the reproducibility of the isolation

procedures. The individual residue values never varied more than 5%, and all analyses were qualitatively identical. The hyphal wall and spore coat proteins appeared to be different types of protein quantitatively. The residue per cent decrease during 7 hr of germination indicated a preferential loss of aromatic residues. This observation was also supported by the fact that the protease was capable of attacking hippuryl phenylalanine but not BAEE. These data suggested that the protease might be chymotrypsin-like.

Effect of PMSF on proteolysis and germination. The above information established that proteolysis occurred during spore germination, but, as yet, there was no conclusive evidence that this relationship was anything more than coincidence. To establish a causal relationship between

FIG. 3. The pH profile of protease activity against casein. One unit equals the amount of enzyme which will solubilize the equivalent of 1 μ g of bovine serum albumin in 1 min. Incubation was at 37 C for 30 min.

FIG. 4. Thermal denaturation of protease activity. Portions (1 ml) of purified protease were preincubated for 20 min at the temperatures indicated. Proteolytic activity was then measured against pH 8.0 casein, with incubation at 37 C for 30 min.

proteolysis and spore germination, a selective protease inhibitor which would not disturb normal cellular functions was required. Such an inhibitor was PMSF, a less toxic substitute for diisopropylfluorophosphate (DFP). PMSF is particularly effective against chymotrypsin and to a lesser degree, trypsin.

Figure 6 illustrates the effect of various PMSF concentrations on protease activity against casein. Unfortunately, because of the limited solubility of the inhibitor and the fact that the solvent was

isopropanol, the highest usable concentration in our germination system was 10^{-4} M. Above this concentration, the isopropanol became slightly inhibitory to cell growth. The inhibitor was effective only when it was preincubated with

FIG. 5. Hydrolysis of purified spore coats by *Microsporum* protease. Reaction mixture was 50 μ g of protease/ml in pH 6.5 saline, with 1 mg/ml of spore coats. Spore coats were pelleted at $600 \times g$ for 1 min at the times indicated, and the optical density at 280 nm was determined on the supernatant fractions. Symbols: (○) reaction mixture, shaken at 150 rev/min in a New Brunswick rotary shaker at 37 C; (□) substrate control, reaction mixture minus protease; (●) enzyme control, reaction mixture minus spore coats.

FIG. 6. Effect of PMSF on protease activity against pH 8.0 casein, with preincubation time of 30 min at 37 C. Assay conditions were 30 min incubation at 37 C. NPI, no preincubation with 10^{-4} M PMSF.

the enzyme. This was the expected result, since PMSF is an irreversible type inhibitor.

The effect of 10^{-4} M PMSF addition on free amino nitrogen release into the germination supernatant fluid is depicted in Fig. 7A. The inhibition of free amino nitrogen release became maximal after the first hour of incubation. To clarify the time course of PMSF inhibition of

TABLE 3. Amino acid composition of vegetative walls, spore coats, and 7-hr spore coats of *Microsporium gypseum*^a

Amino acid	Vegetative walls	Spore coats	7-Hr spore coats	Per cent decrease in residues during 7 hr of germination
Lysine.....	2.37	9.50	8.10	14.7
Histidine.....	1.17	4.88	3.78	22.5
Arginine.....	1.30	1.58	1.43	9.5
Aspartic acid..	6.53	16.8	13.0	22.7
Threonine.....	6.29	8.30	6.30	24.1
Serine.....	5.16	5.40	4.83	11.6
Glutamic acid	5.95	9.75	8.15	16.4
Proline.....	4.99	7.50	5.52	26.4
Cysteine	Present	Present	Present	
Glycine	8.86	10.7	8.68	18.9
Alanine	5.75	4.65	4.33	6.9
Valine	3.87	5.18	4.83	6.8
Methionine	0.467	0.250	0.180	28.0
Isoleucine	2.25	2.68	1.78	33.6
Leucine	2.92	2.72	2.30	16.4
Tyrosine	5.32	2.60	0.873	63.5
Phenylalanine	1.00	2.35	0.973	58.7

^a Expressed as micromoles of amino acid per 100 mg of walls or coats.

FIG. 7. (A) Effect on free amino nitrogen release of zero time addition of 10^{-4} M PMSF to pH 6.5 saline germination system, with incubation at 37 C. (B) Replot of left side data from the 1-hr sample point. Symbols: ●, 10^{-4} M PMSF added; ○, no 10^{-4} M PMSF addition.

free amino nitrogen release, the data were re-plotted by using 1 hr as the point of origin (Fig. 7B). The inhibition of free amino nitrogen release from 1 to 8 hr was approximately 70%. This value was in good agreement with the *in vitro* data (Fig. 6). We feel that the free amino nitrogen released during the first hour was probably from spores which were already committed to germinate at the time they were harvested (see below) and, therefore, were not susceptible to inhibition.

An attempt was made to localize the proteolytic activity in the spore. Sonically disrupted pellets, supernatant fluids, and culture supernatant fluids were assayed for proteolytic activity. Most of the activity was localized in the sonically disrupted pellet and was released into the germination supernatant fluid with time (Fig. 8A). We thought that the intracellular activity was possibly just protease solubilized from the spore coat by sonic disruption. A similar experiment was done, but in the presence of PMSF. Due to its high negative charge, it would seem unlikely that PMSF would cross the membrane and, hence, should act only on surface protease. Therefore, if there were any true intracellular activity, this activity should increase relative to pellet protease activity after washing and sonicating. This was not the case (Fig. 8B), as intra- and extracellular levels of protease were similar in the presence of PMSF. Hence, we concluded that the majority of the protease activity was localized in the walls in the $20,000 \times g$ particulate fraction.

Figure 9 illustrates the effect of PMSF on germination by another approach. One must be cautious in extrapolating germination percentages

FIG. 8. Localization of proteolytic activity in germination supernatant fractions, sonically disrupted pellets and their supernatant fractions. (A) No 10^{-4} M PMSF added; (B) 10^{-4} M PMSF added at zero time. Symbols: ○, germination supernatant fraction (pH 6.5 saline); ●, sonically disrupted $20,000 \times g$ pellet; △, sonically disrupted $20,000 \times g$ supernatant.

FIG. 9. Effect of 10^{-4} M PMSF addition on 24 hr total cellular protein values. Germination system; 1% (w/v) glucose, 0.1% (w/v) neopeptone, pH 6.5. Total incubation period was 24 hr at 37 C. Symbols: $\circ$, 10^{-4} M PMSF added to 10^6 spores/ml at times indicated; $\bullet$, no 10^{-4} M PMSF added; $\triangle$, 0.001% isopropanol (solvent for PMSF) added at zero time.

to mean potential to outgrow and produce a hyphal network. Hence, a system was needed wherein we could correlate growth with germination percentage and inhibition effect. Here the germination medium was 1% (w/v) glucose and 0.1% (w/v) neopeptone (pH 6.5). One-tenth per cent neopeptone, instead of the usual 1%, was used to minimize binding of PMSF by the medium. This medium was sufficient to support growth for several days (Leighton and Stock, unpublished data).

In this medium, 100% microscopic germination was effected by 7 to 8 hr. PMSF was added at the times indicated, and the spores were allowed to germinate and grow for a total of 24 hr. Total cellular protein of washed spores (three-times washed in pH 6.5 saline) was measured and germination percentages were also determined after 24 hr (Fig. 10). Note that the addition of inhibitor after 7 hr had no effect on total protein values, i.e., growth. This information also clearly demonstrated that 10^{-4} M PMSF had no effect on vegetative growth, since addition after 7 hr resulted in the same protein values as the control system which received no PMSF.

Temperature response of protease activity and protease stimulation on germination. We had previously reported that endogenous levels of germination (30% at 25 C) could be stimulated to 90% spore germination by raising the incubation temperature to 37 C (11). Figure 11 shows

FIG. 10. Effect of 10^{-4} M PMSF addition on 24-hr germination percentages. Germination system; 1% (w/v) glucose, 0.1% (w/v) neopeptone, pH 6.5. 10^{-4} M PMSF was added to 10^6 spores/ml at times indicated. C, Control, no PMSF added; IPC, no PMSF, but 0.001% isopropanol added (solvent for PMSF).

FIG. 11. Temperature response curve for *Microsporum* protease. Assay conditions: pH 8.0 casein with incubation for 30 min at temperatures indicated.

the temperature response curve of the protease in the casein assay systems. The enzyme and germination temperature response curves appeared to be comparatively similar up to 37 C. Hence, we wondered whether increased protease activity at 37 C could explain the increased germination. Figure 12 illustrates the effect of additions of purified protease to a saline germination system at 25 C. At a purified protease concentration of 63 $\mu\text{g}/\text{ml}$, the germination percentage at 25 C was equal to that obtained by incubating untreated spores at 37 C. Heat inactivation of the protease (100 C for 10 min) completely destroyed its ability to stimulate germination.

DISCUSSION

The concept that proteolysis is an essential event in spore germination was suggested originally more than 15 years ago (15). To test this hypothesis conclusively, it was necessary to have a method for selectively interfering with the proteolytic process. One approach would be the use of a selective protease inhibitor, and PMSF was found to be such a compound. This reagent actively inhibited proteolysis *in vitro* and *in vivo*. This inhibitor also fulfilled the necessary requirement of not inhibiting vegetative growth.

Sierra (17) was the first to attempt to inhibit spore germination with a serine-alkylating agent. The germination of *Bacillus* spores was inhibited

completely by exposure to DFP. Unfortunately, 1 hr of exposure to the inhibitor was lethal to the cells. The tolerance of fungal cells to PMSF was possibly due to its high negative charge (i.e., relative impermeability) and its reduced toxicity as compared to DFP.

One question that arose was why 10^{-4} M PMSF only inhibited 70% of the proteolytic activity after 30 min of preincubation. Perhaps the inhibitor was not binding with high efficiency to the enzyme, either because of the conformation of the inhibitor or the enzyme active site. Hence, prolonged preincubation with PMSF would be necessary for complete inhibition. The prolonged preincubation of enzyme with inhibitor was not possible in this *in vivo* system. Another possibility was that serine was not the active-site residue of this protease but was very close to the active site. Hence, alkylation of the serine residue would only partially inactivate the enzyme. This would be similar to the alkylation of methionine 192 (adjacent to active serine 195) in chymotrypsin, which resulted in the loss of 80% of enzymatic activity (10).

The relationship between free amino nitrogen release (spore coat hydrolysis), the location and occurrence of the protease, and the PMSF inhibition data certainly support our contention that proteolysis was essential for macroconidial germination. It was a singular finding that the protease was the only large protein component recovered from the germination supernatant fluid. The thermal denaturation curve and the pH activity curve also supported the disc gel evidence that there was a single protease present in the supernatant fraction.

The *Microsporium* enzyme was found to be very similar in pH optimum to Strange and Dark's enzyme (20), and also to the alkaline protease of *B. subtilis* (2). It is interesting that the *B. subtilis* alkaline protease was also susceptible to PMSF and had aromatic specificity.

If a protease was partially responsible for spore germination, one should be able to stimulate quiescent spores to germinate by adding the enzyme exogenously under the proper conditions. This was the case with lysozyme and the Strange and Dark enzyme (6). This prediction was found also to be true for our system. Addition of protease at 63 $\mu\text{g}/\text{ml}$ resulted in greater than a 2.5-fold increase in spore germination at 25 C. The same effect can be achieved by raising the incubation temperature from 25 to 37 C (11). Whether this increased stimulation at 37 C in the absence of added protease can be wholly explained by increased proteolytic activity cannot be conclusively ascertained at this time.

FIG. 12. Effect of protease addition to 25 C germination system. Germination medium was pH 6.5 saline with a total incubation time of 6 hr. Protease, at concentrations indicated, was added at 0 time. 63 Control, 63 μg of protease per ml, heated at 100 C for 10 min prior to addition.

The fact that exogenous protease could stimulate quiescent spores to germinate implied that some spores may be protease-rich with respect to the population. That is, about 30% of the spores had enough protease to germinate at 25 C, whereas 60% of the spores required supplementation under these experimental conditions.

One explanation for this observation was suggested by the way in which fungal spores are formed. Macroconidia of *M. gypseum* are multi-septate structures containing a random distribution of nuclei per septated compartment (4). This type of nuclear distribution in spores has been observed also in *Phycomyces* (8). If protease production was a function of gene dosage, then it would follow that potentiality to germinate also should follow the distribution of nuclei per spore. Even under optimal conditions (37 C), one should see a gradient in the time required for the population of spores to germinate. Such a curve was observed when spores were germinated in the 0.1% neopeptone, 1.0% glucose medium, and PMSF was added sequentially during the germination period. Sequential delays in time of addition of inhibitor resulted in larger numbers of germinated spores at 37 C. This same type of time response was also seen in the saline germination system (no PMSF addition), in which the free amino nitrogen release (i.e., amount of spore coat hydrolysis) increased with time. One additional fact common to these experiments was that there was a certain percentage of spores (30%) which germinated without added protease at 25 C and were also not susceptible to inhibition. These spores presumably would have the largest amount of protease per spore in the population. If the protease concentration were sufficiently high, the spore coats could be weakened enough so that just their suspension in germination medium would be sufficient to initiate germination. Hence, these spores would not be sensitive to temperature shift or to inhibitor. If this were true, one should observe an initial increase in free amino nitrogen release, resulting from the inhibitor-insensitive germination of precommitted spores, followed by a plateau, resulting from inhibition of uncommitted spores. This is exactly the picture seen in the presence of 10^{-4} M PMSF. Also, in the absence of PMSF, one observes a fast initial rise in free amino nitrogen followed by a period of linear release.

This hypothesis is currently being examined in a more direct way by comparing nuclei counts with potentiality to germinate at 25 C and potentiality to germinate at 37 C in the presence of 10^{-4} M PMSF.

The results of this study clearly support the concept that proteolysis was an essential occurrence in dermatophyte germination. However, the question still remains as to whether the weakening of the spore coat per se or the appearance of protease hydrolysis products was the event essential to germination.

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