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ADENOSINE TRIPHOSPHATASE STAINING OF DERMATOPHYTES

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Abstract. By using an adenosine triphosphatase stain it was possible to visualize hyphae in the scales of patients with tinea corporis, tinea pedis and tinea cruris. The yield of stained hyphae was lower than that of potassium hydroxide mounts, suggesting that some fungal elements in the stratum corneum are non-viable. The hyphae (and to a lesser extent, the conidia) of cultures of five common dermatophytes revealed considerable adenosine triphosphatase activity. No identifiable species distinctions could be made on the basis of this stain.

Simple immersion of scales in 20% potassium hydroxide solution permits rapid direct microscopic visualization of superficial fungal pathogens as non-specific, unstained hyphae. Nonetheless the urge to stain or to color these hyphae has led to many auxiliary techniques. Perhaps the most popular of the strains are the periodic acid-Schiff, the Gram, the Giemsa, acridine orange and Ink Blue[PP] (1, 3, 10).

In the course of staining scales for adenosine triphosphatase activity (7), it was found that dermatophytic hyphae showed considerable activity. The concept of enzymatic staining for the demonstration of living fungi in the stratum corneum appeared novel and interesting. The only reference to staining of these superficial fungi in tissue by enzymatic means was an isolated observation of Steigleder (8) that in formalin-fixed human stratum corneum it was possible to show fungal elements by employing a non-specific esterase stain.

The present report describes our observations on the adenosine triphosphatase staining of live dermatophytes in scales as well as in culture specimens.

METHOD

Scales were collected on Scotch tape® from untreated tinea corporis, tinea cruris, tinea pedis and tinea versicolor, plus a significant number of non-fungal scaling dermatoses.

Cultures of the following dermatophytes were grown on Sabouraud's agar and sampled on Scotch tape® at regular intervals from 4 to 28 days: Microsporum canis, Epidermophyton floccosum, Trichophyton rubrum, Trichophyton mentagrophytes and Trichophyton ajelloi. Comparative staining was done on two saprophytic strains of Penicillium and Aspergillus as well as Candida albicans, and gram positive as well as gram negative bacteria.

All specimens were stained for membrane adenosine triphosphatase (magnesium activated) by the method of Wachstein & Meisel (11). The entire glass microscope slide to which the double adherent tape sample was attached was incubated in an adenosine triphosphate solution for 10 to 50 min at 37°C. After a 5-min rinse in distilled water, it was immersed in 0.5% ammonium sulfide solution for 10 min, and then in distilled water for 15 min. After mounting in glycerine jelly, the ATPase activity could be read as a brown-black deposit of lead sulfide. Unstained slides, as well as specimens incubated in the absence of ATP, served as controls.

The adenosine triphosphate medium, filtered before use, consisted of:

10 ml 125 mg/100 ml adenosine-5-triphosphate disodium salt (Sigma)
10 ml Tris buffer, pH 7.2 (5 ml 1 M Tris maleate, 5 ml 1 M maleic acid, 10 ml 0.5 N sodium hydroxide)
2.5 ml 0.1 M $MgSO_4$
1.5 ml 28% lead nitrate (add dropwise)
1.0 ml distilled water

In some specimens, calcium-activated adenosine triphosphatase activity was determined by the method of Padykula & Herman (5). Comparative visualization and staining of the hyphae was done with direct potassium hydroxide mounts, the Giemsa and acridine orange stains, and for alkaline phosphatase (5).

RESULTS

Scales from tinea pedis due to Epidermophyton floccosum, Trichophyton rubrum or Trichophyton mentagrophytes all revealed numerous black hyphal elements (Fig. 1) which were appreciably

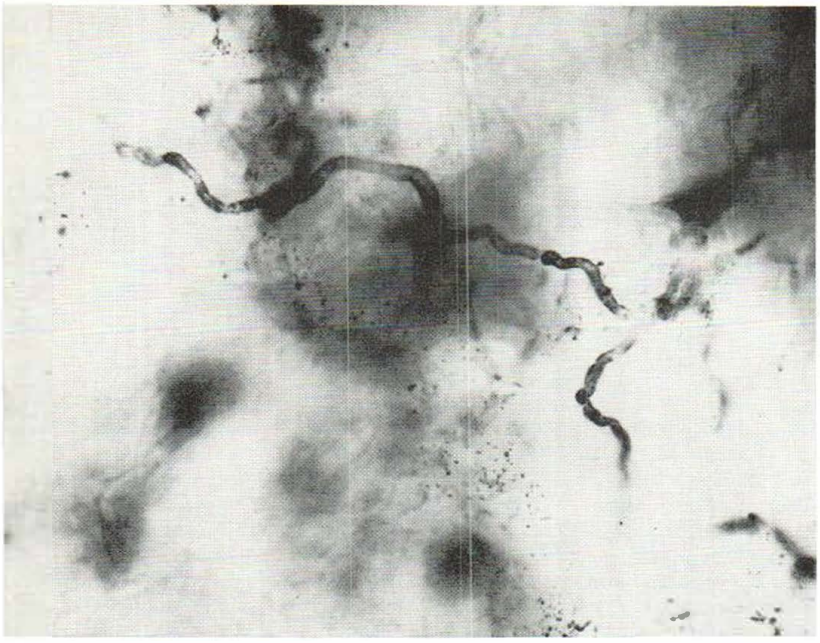

Fig. 1. Scale from tinea pedis showing brown adenosine triphosphatase staining of hyphae. Note, major stain is in hyphal wall. The width of stain probably reflects diffusion since no fixation was used. × 550.

broader than the hyphae visualized in potassium hydroxide mounts. Hyphae could be visualized at varying levels in thick scales. Scales from tinea cruris and corporis showed fewer hyphal elements. In some instances the KOH mounts revealed a greater number of hyphae than the ATPase stain. Tinea versicolor showed poor or no staining of the hyphae and the spherical cells of M. furfur. Scrapings from Candida albican lesions showed only a weak staining of the yeast capsule. In the scales of seborrheic dermatitis, pityrosporon ovale organisms did stain. Scales from non-fungal dermatoses failed to show any staining suggestive of hyphae, although the smaller dendritic staining characteristic of psoriasis (7) was seen regularly in the scales from this dis-

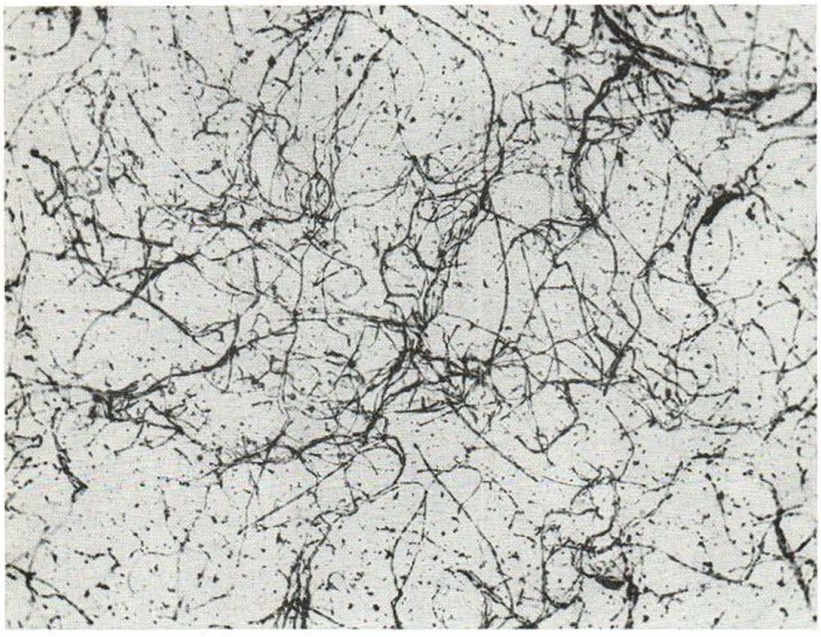

Fig. 2. Low power view of Scotch tape sample of colony of Epidermophyton floccosum. Note black staining of hyphae for adenosine triphosphatase. The same degree of staining was observed in other dermatophytes. × 115.

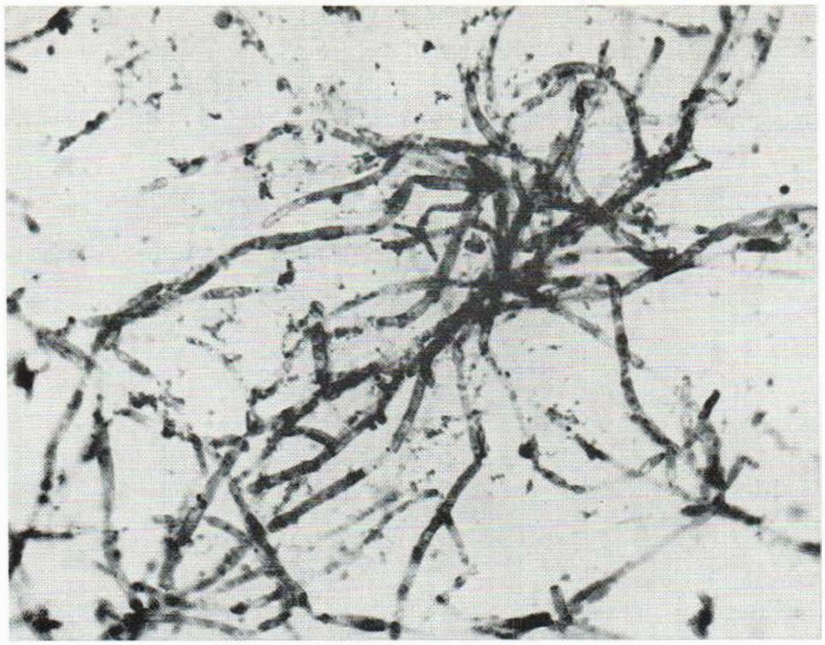

Fig. 3. Higher power view of segment of Fig. 2. × 550.

ease and occasionally from other scaling dermatoses including tinea. Small particulate staining was adjudged to represent non-specific stain precipitate bacteria on spores. At times the scale edges showed a stain precipitate artefact which could be optically identified as such.

The stain faded over a period of months. Pretreatment of the scales with formalin essentially prevented staining. The hyphae, moreover, did not stain when the technique for demonstrating calcium-activated adenosine triphosphatase was employed.

Staining of the culture samples (Figs. 2–4) revealed black deposits outlining both the hyphae and spores. The hyphae when stained for longer periods showed not only the wall darkened, but

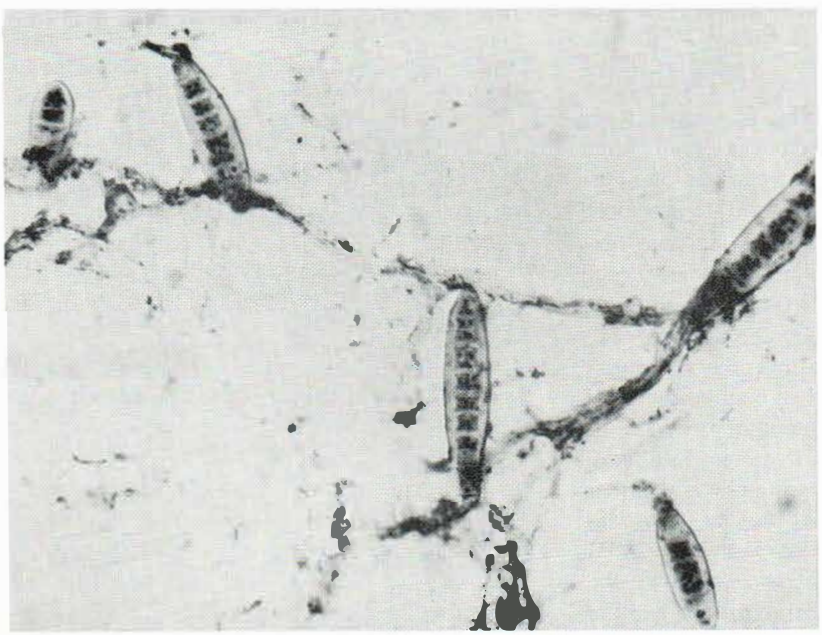

Fig. 4. Macro aleuriospores from Epidermophyton floccosum culture. Adenosine triphosphatase stain. Note limited staining of wall and intracellular mitochondrial elements. × 550.

also granular deposits which may have been extra- as well as intra-cellular. Hyphal segments varied in their degree of staining, but all were stained when ATP incubation was restricted to 50 min. The microconidia (micro-aleuriospores) showed only wall staining and the macroconidia (macro-aleuriospores) showed intra-cellular granules besides staining of the wall. No species or age differences in staining could be detected.

DISCUSSION

There are three classic studies of the enzymatic staining properties of dermatophytes in culture. In 1959, Steigleder & Röttchen (9) first demonstrated staining for esterase activity in a variety of dermatophytes. Interestingly, the active growing portions were more intensely stained. In the following year, Roth & Winklemann (6) successfully stained cultural specimens of 18 species of dermatophytes and saprophytic fungi for non-specific esterases, alkaline phosphatase, and specific as well as non-specific cholinesterase. No differences could be noted between saprophytes and pathogenic organisms. Again, the older elements stained less. Finally in 1966, Meinhof, Braun-Falco & Thianprosit (4) stained cultural samples of 12 dermatophytes for 20 different enzymes. This exhaustive study included observations on phosphorylases, succinic dehydrogenase, cytochrome oxidase, NADP tetrazolium reductase, non-specific esterases and leucine aminopeptidase.

The present study extends the enzymes histochemically demonstrated in fungi to adenosine triphosphatase and applies this enzymatic stain to a demonstration of fungi in unfixed scales from fungus infections in man. The technique of staining unfixed scale specimens is no replacement for the direct potassium hydroxide mount visualization of fungi since it is time-consuming, and, if anything, gives a lower yield of organisms. Nonetheless, it is a new tool for studying the parameter of viability of the fungi seen in stratum corneum. It appears that hyphae may be rendered non-viable by desiccation, immune processes or other mechanisms as well as treatment. Hitherto, the major clue to this supposition was the fact that cultural studies routinely fail to reveal as high a percentage of positives as does direct KOH examination (2). Previous stains as well as the KOH mount give no evidence as to whether the fungal element is dead or alive. The present procedure would seem to offer a new way of studying the biology of tinea infection since the enzyme stain is associated with active, growing hyphae.

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