A STUDY OF THE RELATIONSHIPS OF SELECTED DERMATOPHYTES USING SUBCELLULAR FRACTIONS AS ANTIGENS IN IMMUNODIFFUSION TECHNIQUES

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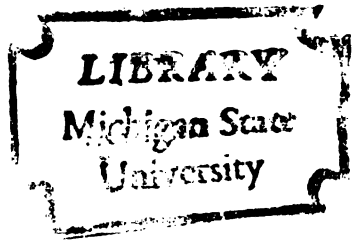

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A STUDY OF THE RELATIONSHIPS OF SELECTED DERMATOPHYTES USING SUBCELLULAR FRACTIONS AS ANTIGENS IN IMMUNODIFFUSION TECHNIQUES

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ABSTRACT

A STUDY OF THE RELATIONSHIPS OF SELECTED DERMATOPHYTES USING SUBCELLULAR FRACTIONS AS ANTIGENS IN IMMUNODIFFUSION TECHNIQUES

by Alvin Lee Rogers

The objective of these investigations was to determine the degree of relatedness between selected dermatophytes using subcellular fractions as antigens in immunodiffusion techniques.

The precipitinogenic relationships of 20 representative dermatophytes were compared using an agar-gel slide double-diffusion technique. The organisms included species from the four genera of dermatophytes, Epidermophyton, Keratinomyces, Microsporum, and Trichophyton, and strains within a species. Two subcellular fractions separated by centrifugation from each fungus were used as antigens. Antisera were produced in rabbits to the fractions from 3 Trichophyton species.

Antigenic differences existed among the fractions of an organism, species and groups, and in addition, among strains within a species. No one precipitinogen was found to be common to all dermatophytes tested.

From 4 to 9 precipitinogens were detected in homologous antigen-antibody systems, and in all tests, the homologous systems reacted to give greater numbers of precipitated bands than the heterologous systems.

The trichophytons were separated into serological groups which fall into the old colony morphological grouping. Trichophyton terrestre appears to belong to a separate group. The reaction with fractions from the strains of T. mentagrophytes indicated the possibility of more than one species.

The species M. ferrugineum was closer antigenically to M. gypseum than to any Trichophyton species. K. ajelloi also appeared closer to the Microsporum species.

Epidermophyton floccosum appeared not to be antigenically closely related to any of the organisms investigated.

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By

Alvin Lee Rogers

A THESIS

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To my parents

and

Aunt Cora Clay

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INTRODUCTION

The dermatophytes are a group of fungi which attack the keratinized tissues, viz., the epidermis, hair and nails, of humans and animals. Recently many of the organisms of this group have been isolated from the soil where they live as saprobes.

The taxonomy of this group was first based on the clinical symptoms and the morphology of the fungi in their pathogenic environment as illustrated by Sabouraud in his textbook in 1910. In 1934, Emmons based his classification on the morphology of the fungi in culture. Then, in 1957, Georg and Camp utilized physiological characteristics under cultural conditions to aid in the separation of species. There is still a certain amount of disagreement on the relationship of the organisms in this important group, for example:

1. There is the problem of considering all strains of Trichophyton mentagrophytes as one species (Emmons, 1934) or placing them into another genus, Ctenomyces Eidam 1880 with 7 species as Langeron and Vanbreuseghem did in 1952.
2. Another variable has been the placement of the species ferrugineum in either the genus Microsporum (Ajello, 1962) or Trichophyton (Langeron and Vanbreuseghem, 1952).

3. The number of genera and species is also uncertain. Langeron and Vanbreuseghem (1952) listed 5 genera with 27 species and Vanbreuseghem created a new genus (1952). Since that date 11 new species have been described, making a total of 6 genera and 38 species. Ajello (1962) considers 4 genera and 22 species as valid, and Beneke lists 4 genera and 26 species in 1966 (2 new species have been described since this publication giving a total of 28 species). The number of genera listed by the United Kingdon's Medical Mycology Committee (1967) is 3 (Keratinomyces is not listed), and the number of species is 26. All the keratinophilic saprobes not proven to be parasitic were not included along with the proven parasites. Several of the species listed are considered to be synonymous by other workers (Ajello et al., 1963).
4. Sometimes separation of species by the use of morphological and physiological characteristics is difficult. Trichophyton rubrum and T. mentagrophytes are usually easily separated, but subcultures of T. rubrum isolates may fail to produce color on the reverse side as pointed out by Weidman in 1929, while some isolates of T. mentagrophytes produce a reddish-brown color. The shape of the conidia may overlap or in some cases no conidia are produced at all and the physiological

characteristics are similar. The method of "in vitro" hair invasion is usually different. T. mentagrophytes invades by a wedge shaped penetration.

5. The asexually produced spore structures (imperfect stage) are often not stable enough to permit their use in accurate identification.

Seeliger (1960) reported that the application of serology offers a valuable additional tool in the studies of mycological phylogenetic relationships and that it could solve some difficult taxonomic problems.

The purpose of these investigations was to determine the degree of relationship between selected dermatophytes using antisera produced by rabbits injected with subcellular fractions of the dermatophytes against the subcellular fractions as antigens in immunodiffusion experiments.

REVIEW OF LITERATURE

Classification of the Dermatophytes
Variations in Systems

Dermatophytosis, ringworm in man and animals, has been known for centuries (Feuland, 1886; and Gates, 1939) but the first causative agents were not reported until the nineteenth century. Robert Remak in 1837 (Kisch, 1954) discovered hyphae in favic crusts but did not consider them as the causative agent. It was not until two years later that Schoenlein (1839) showed the relationship between the fungus and the favic crusts. Remak named the fungus Achorion schoenleinii in 1845. During the lapse of time between the discovery and the naming of the organism, Gruby (1841) was investigating ringworm. He recognized favus and by microscopic examination diagnosed the disease and successfully inoculated humans and animals with the fungal agent. In 1842 he discovered the ectothrix and endothrix trichophytons but did not name them. Microsporum audouinii, an organism that produces ringworm in children, was discovered and described by Gruby in 1843. The clinical descriptions by Gruby were not precise but the mycological portions were only erroneous in that the fungi grew downward, not upward as is described in the pilar lesion (Sabouraud, 1936). The name Trichophyton was introduced into the literature in 1845 by Malmsten when he described Trichophyton tonsurans.

Pure cultures were first obtained in 1886 by Grawitz and shortly thereafter by Duclaux (1886). In 1887 Verujsky distinguished between external and mycelial spores. Using the external spores, Sabouraud (1892) grouped the dermatophytes into divisions: (a) the large spored and (b) the small spored organisms, each group with more than one organism thus settling a dispute between the unicists and the pluralists.

The first mention of "pleomorphism" among the dermatophytes occurred in 1896 when Bodin published his work on "teignes tondantes" in the horse. Until this time Sabouraud (1910) considered this phenomenon of polymorphism as symbiosis. Sabouraud's classification, in "Les Teignes," published in 1910 is the oldest organized attempt to classify the dermatophytes. He used as a basis the morphology of the fungi in the parasitic stage as well as hair-invasion to designate the three divisions (now genera), and morphology of the fungi in the saprophytic phase to establish the species (Sabouraud, 1910). Thus, much of the fundamental work we owe to Sabouraud and his students. Reclassification cannot detract from his monumental work on these forms (Emmons, 1934).

Ota and Langeron (1923) based the classification of the dermatophytes upon cultural morphology as they grew on natural media, thus the transfer of the basis of classification was from parasitism to saprophytism. In their classification a new genus Sabouraudites was substituted for

Microsporum, using Microsporum audouinii Gruby as the type species. The new genus, Sabouraudites, includes all the species of dermatophytes that produce macroconidia which includes all the Microsporums and many of the Trichophytons, thus unnatural groups result (Emmons, 1934).

In 1924 Grigorakis used macroconidia, perithecia, microconidia, aleuria, and chlamydospores as the basis for the classifying the dermatophytes. The members of the genus Microsporum along with all other species of the dermatophytes which produced persistent macroconidia were placed into the new genus Closterosporia. According to Emmons (1934), this division did not follow natural lines as species producing thin- and thick-walled macroconidia were placed in the same genus.

Vuillemin (1925), Nannizzi (1927), and Guiart and Grigorakis (1928) all modified the classification of the dermatophytes. According to Emmons (1934) their groupings were not along natural divisions, while Dodge (1935) stated that they did not follow the rules of nomenclature.

After growing many of the known species of Trichophyton on 13 different media, Lageron and Milochevitch (1930), on the basis of colony morphology, simplified the classification of the dermatophytes by placing into synonymy many species names. They used for their basis the classification of Ota and Lageron (1923), retaining the genera: Ctenomyces Eidam, 1880; Sabouraudites Ota and Lageron, 1923; Epidermophyton Harz, 1870; and Trichophyton Malmsten, 1845.

Ota and Kawatzure (1933) enlarged the concept of the species, including the gypseum forms while placing into synonymy many species that they considered to be T. rubrum Castellani, 1909. Their reclassification was based on cultures grown on natural media as well as animal inoculations.

Dodge (1935) using Sabouraud's classification (1910) as a basis, listed 9 genera and 118 species of dermatophytes which he considered valid. Many of the species were based upon clinical instead of botanical differences.

Emmons in 1934 proposed and established botanical criteria for the classification and identification of the dermatophytes. Since at that time no perfect stages of the dermatophytes had been found and confirmed, they were considered to be members of the "form" class Deuteromycetes, in the order Moniliales and the family Moniliaceae (Ainsworth, 1961). The classification of these fungi therefore will depend on the vegetative structures, mainly conidia, which do not always show true relationships. A genus in the Fungi Imperfecti may include species of diverse origin, as illustrated in Microsporum gypseum "complex" which is composed of 3 perfect species (Stockdale, 1963). Still, a critical study of the characteristics of the spores, and their sterigmata indicates fundamental differences between groups within a form genus.

Emmons (1934) suggested that the dermatophytes should be classified into 3 natural genera: Trichophyton, Epidermophyton, and Microsporum. His natural grouping is based upon the method of reproduction of the organisms in culture, primarily considering macroconidia. He also recognized the occurrence of a wide range of morphological variations among individual isolates of a species.

Emmons described Trichophyton as having clavate macroconidia with blunt ends and broad bases which are encircled by a collar marking the attachment to the conidiophore. The walls are thin and smooth, but may be constricted where the cross-septa appear.

The Epidermophyton macroconidia are usually clavate to egg-shaped, and moderately thick-walled. The species of this genus never has microconidia.

The large thick, rough-walled macroconidia of Microsporum are typically spindle-shaped with pointed tips, and have the broad, faceted, collared base which characterizes the microconidia and macroconidia of the dermatophytes (Emmons, 1934).

The microconidia or conidia of the dermatophytes, varying in shape and size, are of little use in differentiating species.

Conant et al. (1944, 1954) placed many of the species of dermatophytes into synonymy in both editions of Manual of Clinical Mycology.

The classification of Langeron, Milochevitch, and Vanbreuseghem (Langeron and Vanbreuseghem, 1952) is a modification of Langeron and Milochevitch (1930). The only difference is the addition of the genus Langeronia Vanbreuseghem, 1950 and species discovered since 1930.

Vanbreuseghem described a new genus, Keratinomyces Vanbreuseghem 1952, which is considered a fourth genus in Emmon's classification. Langeron's and Vanbreuseghem's classification would contain 6 genera which are: Ctenomyces Eidam, 1880; Sabouraudites Ota and Langeror, 1923; Trichophyton Malmster, 1845; Langeronia Vanbreuseghem, 1950; Epidermophyton Sabouraud, 1910; and Keratinomyces Vanbreuseghem, 1952. A description of these follows.

The organisms that belong to the genus Ctenomyces Eidam, 1880 have microconidia disposed in clusters or along hyphae and have distaff-shaped macroconidia (fuseaux en quenouille). Aerial hyphae that give rise to conidia are branched at right angles and terminate with the formation of Lorraine Cross configurations. The members also have spiral- and antler-like filaments which are regarded as equivalent to the appendages of perithecia. The nodular bodies simulate the commencement of ascogonium-formation.

The genus Sabouraudites Ota and Langeron, 1923 is characterized by having microconidia of the Acladium type and numerous macroconidia with lanceolate, granulose walls. Spirals rarely appear upon ordinary media.

The Trichophyton Malmster, 1845 cultures may have Acladium type microconidia along aerial hyphae and if macroconidia are present they are cigar- or sausage-shaped, with smooth and thin walls having obtuse extremities. The base of the macroconidium is only slightly larger than the hypha from which it arises. The glabrous colonies have few or no conidia. Spirals are not seen when grown on ordinary media.

Langeronia Vanbreuseghem, 1950, is characterized by having mycelium with short articulations as lateral branches growing in opposite directions. These lateral branches produce tertiary branches. The conidia are rare but of the Acladium type. Arthrospores are formed and give the impression of false branches. No spirals nor macroconidia are found.

Epidermophyton Sabouraud 1910 contains only one species, E. floccosum Harz, 1870 with club-shaped macroconidia, often arranged like bunches of bananas. There are no spirals and no microconidia.

Keratinomyces ajelloi Vanbreuseghem, 1952, the only member of the genus Keratinomyces has numerous large cylindrofusiform, thick, smooth-walled macroconidia with 5 to 12 cells. Microconidia are usually abundant, pyriform to ovate in shape, and sessile.

Thus, of the many classifications of the dermatophytes that have been proposed, 2 are used most often: (1) Emmon's classification published in 1934 and brought up to date by

Ajello (1962), Ajello *et al*. (1963), Beneke (1966), and Ingram (1967); and (2) Langeron and Milochevitch's classification published in 1930 with a revision by Langeron and Vanbreuseghem in 1952. The classification by Emmons is widely used in the English speaking world and the latter is used by many Europeans and South Americans. Both of these classifications are based on characteristics of the dermatophytes in culture. Table 1 gives the two classifications.

The following are additional species not listed in the manuals by Beneke (1966) or Langeron and Vanbreuseghem (1952):

Trichophyton kuryangei Vanbreuseghem and Rosenthal, 1961

Trichophyton Vanbreuseghemii Rioux, Jarry and Juminer, 1964

Trichophyton gloriae Ajello and Cheng, 1967

Matruchot and Dassonville (1899a) were the first to suggest that the dermatophytes might be related to the Gymnoascaceae. They found that certain species of this family produced asexual spores similar to those of some dermatophytes. The *Trichophyton* species produce many spirals which Matruchot and Dassonville (1899b) compared to the peridial hyphal spirals of *Ctenomyces serratus*. In 1900 these two authors found an isolate of *Trichophyton* that produced clumps of hyphae which were similar to cleistothecia but only produced conidia. Similar structures which they called "fruit conidiens" were produced in *C. serratus* and in a number of species of *Trichophyton*. Based primarily

TABLE 1.--Classification of the dermatophytes.

Emmon's Classification Modified by Ajello, 1962, and Beneke, 1966	Langeron, Milochevitch, and Vanbreuseghem's Classification, 1952
Microsporum	Sabouraudites
M. audouinii Gruby, 1843	S. audouinii Gruby, 1843
	S. langeronii Vanbreuseghem, 1950
	S. ravalieri Vanbreuseghem, 1951
M. canis Bodin, 1902	S. canis Bodin, 1902
*M. cookei Ajello, 1959	
*M. distortum di Menna & Marples, 1954	
M. gypseum (Bodin) Guiart & Grigorakis, 1928	S. gypseum Bodin, 1907
*M. nanum (Fuentes, Aboulafia & Vidal) Fuentes, 1956	
*M. vanbreuseghemii Georg, Ajello, Friedman and Brinkman, 1962	
M. ferrugineum Ota, 1922	
	S. gallinae Mennin, 1881
Epidermophyton	Epidermophyton
E. floccosum (Harz) Langeron and Milochevitch, 1930	E. floccosum Harz, 1870
*Keratinomyces	
*K. ajelloi Vanbreuseghem, 1952	
Trichophyton	Ctenomyces
T. mentagrophytes (Robin) Blanchard, 1896	C. mentagrophytes Robin, 1853
	C. asteroides Sabouraud, 1909
	C. granulosus Sabouraud, 1908
	C. persicolor Sabouraud, 1910
	C. interdigitalis Priestley, 1917
	T. quickeanum Zopf, 1890
T. equinum (Matruchot & Dassonville) Gedoelst, 1902	
T. rubrum (Castellani) Sabouraud, 1911	T. rubrum Castellani, 1909
T. verrucosum Bodin, 1902	T. album Sabouraud, 1909
	T. discoides Sabouraud, 1909
T. gallinae (Megnin) Silva & Benham, 1952	
T. megninii Blanchard, 1896	T. megninii R. Blanchard, 1895
*T. simii (Pinoy) Stockdale Mackenzie & Austwick, 1965	
T. tonsurans Malmsten, 1845	T. tonsurans Malmsten, 1845
	T. sabouraudii R. Blanchard, 1895
	T. sulfureum Fox, 1908
T. schoenleinii (Lebert) Langeron and Milochevitch, 1930	T. schoenleinii Lebert, 1843
T. violaceum Sabouraud, apud Bodin, 1902	T. violaceum Bodin, 1902
T. gourvillii Catanei, 1933	
T. concentricum Blanchard, 1896	T. concentricum R. Blanchard, 1895
*T. georgiae Varsavsky and Ajello, 1964	
*T. terrestre Durie and Frey, 1957	
	T. ferrugineum Ota, 1921
	Langeronia
T. soudanense Joyeux, 1912	L. soudanensis Joyenux, 1912
T. yaoundei Cochet and Doby-Dubois, 1957	

*Indicates the species described since Langeron and Vanbreuseghem's book in 1952.

upon this evidence they transferred Trichophyton mentagrophytes to the genus Ctenomyces.

The perfect stages for a number of the dermatophytes have been discovered in recent years (Table 2). All the perfect stages of Microsporum so far discovered are members of the same natural genus Nanizzia and those of the imperfect genus Trichophyton are in the genus Arthroderma which includes the imperfect species K. ajelloi.

Once the perfect stages of all the dermatophytes are found, the comparative relationships of the sexual stages promises to provide a more sound criterion for the validation of species and the determination of phylogenetic relationships within the genus and within the dermatophytes. Until these stages are found other methods including morphological characteristics need to be used in classifying the dermatophytes difficult to separate.

In the species which produce characteristic macroconidia little difficulty exists in distinguishing species. But within the genera Microsporum and Trichophyton several species are in a controversial location. Microsporum ferrugineum Ota (1922) does not produce macroconidia and resembles the waxy form of trichophytons in culture so some mycologists place this species in the genus Trichophyton (Langeron and Vanbreuseghem, 1952; Vanbreuseghem, 1963). Many others prefer the genus Microsporum because of the way it attacks hair "in vivo", similar to other Microsporum species (Ajello, 1962; and Beneke, 1966). Microsporum

TABLE 2.--Dermatophytes with known perfect states.

Imperfect State	Perfect State
Microsporum	*Nannizzia*
M. cookei Ajello, 1959	*N. cajetani* Ajello, 1961
M. fulvum Uriburu, 1909	*N. fulva* Stockdale, 1963
M. gypseum (Bodin) Guiart & Grigorakis, 1928	*N. gypsea* (Nannizzi) Stockdale, 1963
	N. incurvata Stockdale, 1961
M. nanum (Fuentes, Aboulafia & Vidal) Fuentes, 1959	*N. obtusa* Dawson & Gentles, 1961
M. vanbreuseghemii Georg, Ajello, Friedman & Brinkman, 1962	*N. grubyia* Georg, Ajello, Friedman & Brinkman, 1962
Keratinomyces ajelloi vanbreuseghem, 1952	*Arthroderma uncinatum* Dawson & Gentles 1961
Trichophyton	
T. georgiae Varsavsky & Ajello, 1964	*A. ciferrii* Varsavsky & Ajello, 1964
T. gloriae Ajello & Cheng, 1967	*A. gloriae* Ajello & Cheng, 1967
T. mentagrophytes (Robin) Blanchard, 1896	*A. benhamiae* Ajello & Cheng, 1967
T. simii (Pinoy) Stockdale, Mackenzie & Austwick, 1965	*A. simii* Stockdale, Mackenzie & Austwick, 1965
T. terrestre Durie & Frey, 1957	*A. quadrifidum* Dawson & Gentles, 1961
	A. lenticularum Pore, Tsao & Plunkett 1965
T. Vanbreuseghemii Rioux, Jarry, & Juminer, 1964	*A. gertleri* (in press) Mykosen

langeronii Vanbreuseghem, 1950, M. rivalierii Vanbreuseghem, 1951, species isolated primarily in Africa, and M. ferrugineum are considered geographic forms of M. audouinii by Zaias et al. (1965), but Vanbreuseghem (1963) considered the first 2 valid species while the species ferrugineum was placed in a separate genus. Representatives of the genus Trichophyton have macroconidia either lacking, rare or are not characteristic enough to separate species (Emmons, 1934; Ajello and Georg, 1957).

Georg and Camp (1957) mentioned that with many atypical strains and species which produced colonies resembling each other identification on the basis of morphological criteria alone was impossible. They reported on the effects of vitamin and amino acids on the growth of the trichophytons and the use of these substances as an aid to identify species. This group of tests has been used very successfully except for the differentiation of T. rubrum and T. mentagrophytes, which can be confused with each other if T. mentagrophytes has a red undersurface in culture or if T. rubrum is pleomorphic. Several methods have been used to distinguish these two species. The growth of these two organisms on different media, corn meal glucose agar (Bocobo and Benham, 1949), and potato dextrose agar (Bohme and Friedrich, 1962) results in the development of red pigment in T. rubrum but not in T. mentagrophytes. Dyson and Landay (1963) found 3 out of 24 isolates of T. mentagrophytes produced red

undersurface pigments on corn meal glucose agar and 7 of the 24 produced red pigments on potato dextrose agar. They concluded the two media are not valid criteria for differentiation of these two species. They used the "in vitro" hair test (Vanbreuseghem, 1949; Ajello and Georg, 1957) to distinguish the two species. In this test T. mentagrophytes penetrates hair segments, while T. rubrum does not. Sudman and Schmitt in 1965 reported potato dextrose agar to be a valid means of differentiating the two species as corroborated by the "in vitro" hair test.

Philpot (1967) developed a urease test to distinguish these two species. Trichophyton mentagrophytes produces urease thus is able to utilize urea, changing the yellow color to red throughout the medium due to the presence of phenol red. She found 92.8% of the 20 isolates of T. mentagrophytes produced a red color within seven days but no isolates of T. rubrum or T. mentagrophytes variety erinacei were positive.

Since morphological, nutritional, and physiological means of separation have not resolved the placement of some of the dermatophytes, other methods need to be improved and utilized.

Seeliger (1960) in a review on the advances made in the application of double-diffusion and immuno-electrophoresis to fungi said:

> I think that the application of serology offers a valuable additional tool for investigating phylogenetic relationships among fungi. Serological test could, indeed, help in the solution of some difficult taxonomic problems. Last, but not least, further studies might be rewarding to the mycologist who tries to improve and accelerate his diagnostic work in the study of fungi as well as of pathologic conditions caused by, or associated with, these fascinating microorganisms.

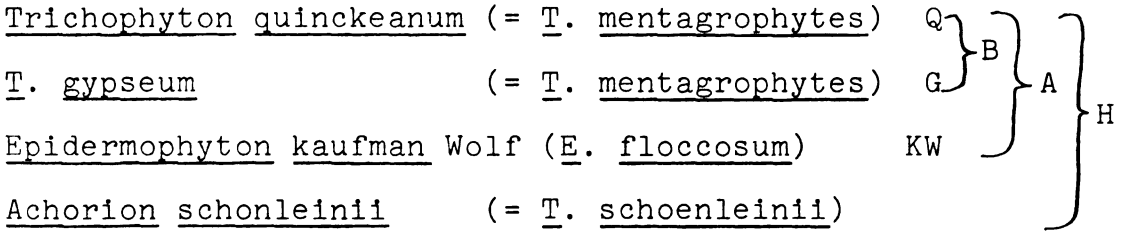
Agglutination and Precipitation Techniques Used in the Identification of Fungi

Serological investigations using fungi have been performed primarily as a diagnostic tool to detect mycotic infections (De Beurman et al., 1908; Kligman and De Lamater, 1950; Conant et al., 1954; Salvin, 1963; and Campbell, 1967). The mycologists adapted many of the serological methods used in bacteriology to the immunological study of human parasitic fungi (Seeliger, 1962). These investigators used variations in the basic methods of agglutination, precipitation and complement fixation (Salvin, 1963). The first report of diagnosis of thrush and sporotrichosis using agglutination was in the early twentieth century (Widal et al., 1910). Moses in 1916 reported complement fixing antibodies in blastomycosis in Brazil. Later, in 1925 Balls reported on the use of the precipitin tests in the identification of yeast. This was followed by a group of workers using agglutination (Benham, 1931, 1935; and Almon and Stovall, 1934), precipitin (Lamb and Lamb,

1935; and Stone and Garrod, 1931), and complement fixation (Negroni, 1934; and Martin and Jones, 1940) tests for the diagnosis, identification and the systematic study of the yeast-like organisms.

Chester (1937) emphasized the problems involved in interpreting the early serological results as applied to plant taxonomy. This concern has also been expressed by Campbell (1960) and Seeliger (1962). The crude techniques make the results unreliable and thus the taxonomic conclusions based on such data of limited value (Seeliger, 1962).

In 1937 Jadassohn et al. reported on the antigenic analysis of four species of the dermatophytes. As antigens they used desiccated trichophytins which were prepared by repeatedly freezing components of the fungi in the growth medium, removal of the mycelium, and drying the remaining material. Guinea pigs were sensitized by injecting the trichophytin for each strain of fungus. Then by the Schultz-Dale method, using the sensitized guinea pig uterus and observing the reaction "in vitro" of the uterine horns as the different trichophytins were added to the Tyrode solution, the following antigenic formulae were worked out for these four species of dermatophytes.



Sharp (1945) used the precipitin test to study the dermatophytes. He used 29 strains, from 1 to 6 strains each of the following: Microsporum felineum (= M. canis), M. audouinii, M. gypseum, Trichophyton mentagrophytes, T. rubrum, T. epilans (= T. tonsurans), and Epidermophyton floccosum. These tests revealed common antigens throughout the entire group, with T. rubrum, and T. mentagrophytes exhibiting some cross reaction and M. felineum and M. audouinii showing close relationships with clear distinction from the other dermatophytes. He reported no species specific antigens in the members tested. In addition Sharp studied 14 nonpathogenic fungi recovered from the skin. He checked these against an antiserum made with Aspergillus flavus. This antiserum did not precipitate any of the dermatophytes or any of the other saprophytes used in the precipitin tests.

Collodin particle agglutination reactions used by Huppert (1955) resulted in extensive cross-reactions between different species of dermatophytes. On the basis of absorption tests with alcohol precipitates from cell extracts, three distinct immunological groups were defined among 15 strains of T. mentagrophytes.

The slide agglutination method has been used by Tsuchiya and colleagues to produce the antigenic formulae for a number of species in the following genera: Hansenula (1957, 1964), Rhodotorula (1957), Candida (1958),

Saccharomyces (1965), Kloeckera and Hanseneaspira (1966). In these tests either monospecific or absorbed antisera were prepared.

Cross precipitation reactions were used by Tomomatsu (1961) to study 11 strains of dermatophytes belonging to the genera Trichophyton (T. mentagrophytes, T. rubrum, T. sabouraudi, T. sulfureum, T. schoenleinii, T. ferrugineum, T. megninii), and Microsporum (M. audouinii, M. canis, M. gypseum), and Epidermophyton (E. floccosum). These fungi were mechanically disintegrated and then fractionated to produce crude polysaccharides for use as antigens. He showed as in previous reports that cross reactions occurred between all species. There was an indication of serological differentiation of Epidermophyton and Microsporum species from Trichophyton species.

Ito (1965a) produced 22 purified fractions having trichophytin activity, as shown by intracutaneous reaction, isolated from the mycelium and culture filtrate of Trichophyton mentagrophytes var. asteroides by means of elution column chromatography using DEAE-Sephadex A-50. These 22 fractions were analyzed by paper chromatography after hydrolysis (1965b). The majority consisted of peptide-containing polysaccharides or sugar-containing peptides, except for two ribonucleic acid fractions from the mycelium, and five simple peptide fractions and a simple polysaccharide fraction from the culture medium. The main active antigenic

principles of trichophytins were found to be peptide-polysaccharide complexes.

Any further consideration of agglutination, precipitation, and complement fixation extends beyond the purpose of this thesis. The problems and progress entailed in the various serological procedures mentioned above as applied to the study of mycoses in man and systematic studies have been reviewed by Kligman and De Lamater (1950), Seeliger (1958, 1960, 1962), Salvin (1963), Kaufman (1966), and Campbell (1967).

Immunodiffusion

Immunodiffusion is the term used to replace the phrase "agar diffusion precipitin test" and refers to serologic tests conducted in or on semi-solid media (Crowle, 1961). Bechold (1905) was the first to use an immunodiffusion technique. He placed anti-goat rabbit serum with 1% gelatin in a test tube. After gelling, goat serum was poured onto the gel. Two heavy distinct precipitates developed that differed from inorganic precipitating substances but no mention was made that these could be caused by separate antigens in the goat serum.

Oudin (1946) employed the method of simple or single-diffusion by placing an antigen solution over a solid immune serum-agar in a thin-bore tube. Then he observed the formation of an antigen-antibody precipitate appearing as a sharp band that migrated down the tube as more antigen diffused

into the gelled mixture. The number of bands formed was equal to the number of antigen-antibody systems present.

Oudin (1948) reported that there is a straight line relationship between movement of a precipitate band and the square root of the time.

Le Beau (1952) applied the Oudin tube technique to the study of the allergenic fractions of Alternaria species.

In 1948 Ouchterlony and Elek working independently developed the double-diffusion technique while investigating toxins and antitoxins of Corynebacterium diphtheriae. Ouchterlony poured a solution of 1% agar in saline into petri dishes, then cut wells in the solidified agar, and filled them with antigen and antibody solution. The gel technique of Elek (1948), also called the "Double diffusion Gradient Test," differs from Ouchterlony's method in that no holes were cut in the agar, instead two filter paper strips impregnated with the reactants were placed on the agar at right angles. Both techniques involve the diffusion toward each other of antigens and antibodies at different rates through agar which results in the formation of precipitate in the form of a line or lines where the homologous antigens and antibodies meet. Investigators find immunodiffusion tests helpful in separating multiple precipitating systems into individual components if the concentrations of the reactants and physico-chemical factors are carefully controlled. This separation is based on the assumption that specific precipitates in semisolid media permit the

diffusion of unrelated antigens and antibodies. The number of bands that develop in a multiple precipitating system represents the minimum number of antigen-antibody systems present because a line formed by one system may mask other bands (Ouchterlony, 1958). "Reaction of Identity" is formed when two identical antigens are compared by immunodiffusion test and develop a continuous line of precipation. The diffusion of serologically related antigens against an antiserum containing specific antibodies results in a spur extending above the precipitation line and is called "Reaction of Partial Identity." When serologically unrelated antigens are diffused against an antiserum containing homologous antibodies and compared, a formation results in which the precipitation lines cross each other and is referred to as "Reaction of Non-Identity (Ouchterlony, 1958).

It is the difference in the rates of diffusion of different soluble constituents which makes this test so valuable in the study and differentiation of antigens and antibodies (Biguet, et al., 1965). Thus, the double diffusion technique makes serological tests on fungi more definitive than earlier methods (Seeliger, 1962; and Campbell, 1967).

Immunodiffusion Applied to Identification of Fungi

In his thesis at Bonn University, Seeliger (1954) applied for the first time the double diffusion technique to fungi. In 1955 he reported by use of the immunodiffusion

agar gel method the following organisms were serologically related in varying degrees: (1) Aspergillus glaucus, A. fumigatus, and A. niger; (2) strains of Madurella grisea; (3) Monosporium clapieri and Sportrichum schenckii; (4) Sporotrichum schenckii and polysaccharide fractions of Cephalosporium recifei; (5) Cryptococcus neoformans and C. diffluens; (6) Histoplasma capsulatum, Blastomyces dermatitidis and Blastomyces brasiliensis (= Paracoccidioides brasiliensis); (7) Candida albicans and Saccharomyces cerevisiae; and C. albicans and C. tropicalis. Thus immunodiffusion relationships in the above fungi are evidence of the great potential of this technique for use in studying other fungi.

Seeliger (1956) applied immunodiffusion, agglutination, precipitation, and complement fixation tests to study mycetoma in man caused by hyphomycetes. Diagnosis was as successful with double diffusion as the precipitin test. He showed that Allescheria boydii (the perfect stage of Monosporium apiospermum), Indiella americana, and Acremoniella lutzi are serologically indistinguishable. Serological specificity was illustrated in M. apiospermum-A. boydii, Madurella grisea, M. americana and Glenospora clapieri, agents of maduromycosis. In this investigation he diffused the antisera derived from humans with maduromycosis caused by M. apiospermum and from rabbits inoculated with M. apiospermum against antigens from 21 fungal organisms not causing mycetomas. Reactions occurred between the M.

apiospermum antisera and the antigens from T. rubrum, T. mentagrophytes and the others, but the reverse antigen-antibody test was negative.

A test was needed by Aschan-Aberg (1956) to show that the homothallic strain (Mi-460) of Collybia velutipes had been derived from two monokaryotic, ornithine dependent, heterothallic mutants, since fruiting could not be induced. This strain, Mi-460, also differed from the mutant strains by producing clamp connections, large spherical oidia and large amounts of cellulolytic enzymes. The morphological differences between the normal heterothallic and the Mi-460 strains were so pronounced that although the same species, they appeared to be different. Aschan-Aberg used immuno-diffusion to show the relationship of the strains and hypothesized the genesis of the homothallic strain.

Five serogroups of the genus Sporobolomyces were described by Seeliger (1957) based on the number and intensity of precipitation bands in agar gel. Sporobolomyces and Rhodotorula showed no common antigens. Seeliger has performed other serological studies with yeasts using the agglutination techniques (1957).

Kaden (1957) used immunodiffusion and precipitation tests to determine that Sporotrichum schenckii, S. beurmanni and S. asteroides were the same species.

Diagnosis of histoplasmosis using precipitin reactions in agar gel was performed by Heiner (1958) on 2026 patients; 7 with proven histoplasmosis, 257 with suspected

histoplasmosis, 13 with proven blastomycosis, 7 with coccidioidomycosis, and the others with no known mycoses. Using a modification of the Ouchterlony technique he elicited six precipitins when histoplasmin interacted with serum from patients with histoplasmosis. The designated "h" band was consistently found in the serum of patients having active histoplasmosis. The "m" precipitin band was also present in active cases, but could be elicited by histoplasmin skin tests in persons with a positive reaction. Four other precipitin bands called "x", "y", "c", and "n" were less consistently present and occurred with no definite relation to activity of the disease. Blastomycin and coccidioidin were tested in the immunodiffusion method with sera from patients having proven cases of histoplasmosis resulting in a reaction, thus demonstrating a common antigen in these 3 organisms and a possible explanation for the cross reaction between them in serological tests.

Pepys (1959), and Pepys et al. (1959) did double-diffusion experiments with some species from the following genera: Aspergillus, Cladosporium, Penicillium, Alternaria, and Candida. They found 2 precipitin bands between anti-serum produced using A. fumigatus and the extracts of A. fumigatus. One line was common with extracts from A. flavus, A. nidulans, A. niger, Cladosporium herbarium, and Penicillium notatum while no affinities were shown with extracts from A. terreus, Alternaria sp. and Candida albicans.

Aspergillus fumigatus antiserum was absorbed with extracts from each of the above fungi after which it was diffused with the same extracts in the agar-gel inhibition tests. Results from this test confirmed the previous results except for A. terreus. This test showed that A. terreus is related to the other Aspergillus species.

Sera from patients some known to have ringworm caused by Trichophyton rubrum and some with known aspergillosis were used by Pepys et al. (1959) in performing agar-gel diffusion test with extracts from Aspergillus fumigatus, Cladosporium herbarum, Penicillium notatum, mycelium and cultural filtrates of T. rubrum, and T. mentagrophytes. Common antigens were shown to be present in T. rubrum, T. mentagrophytes, Cladosporium herbarium, Penicillium notatum, and Aspergillus fumigatus. The Trichophyton antigens produced a broad usually dense precipitin band but in some tests 3 lines were produced. Common antigens were found in the culture filtrates and cell sap extracts of the trichophytons with additional bands formed between the cell sap of T. mentagrophytes and sera over the number in the reaction with the culture filtrate. The reverse was true with T. rubrum, there were more antigens in the culture filtrate.

Extracts of Candida albicans, C. brumpti, C. zeylanoides, C. tropicalis, and C. stellotoidea were reacted with C. albicans antiserum in double diffusion

and immuno-electrophoresis experiments by Biquet et al. (1959). Five lines were identifiable with C. albicans, 4 lines were shared with C. brampti, 3 lines with C. zeylanoides and C. stellatoidea, and 2 lines with C. tropicalis.

The immunodiffusion technique was applied to sera of allergic patients by Augustin and Hayward in 1959. Serum from a patient sensitive to Aspergillus fumigatus was reacted with extracts from several Deuteromycetes. One precipitation band was shared by Cladosporium herbarum, Aspergillus fumigatus, A. niger, and Penicillium notatum. A faint line for A. fumigatus was the major line for C. herbarum and vice versa. The major line of A. fumigatus was also the major line for Paecilomyces sp. and Penicillium cyclopium but a minor line for P. notatum and C. herbarum. Significant differences were found between C. herbarum and C. fulvum.

Blastomyces dermatitidis and Histoplasma capsulatum were distinguished serologically by the agar-gel method by Ball et al. (1960). They reported two precipitin bands with histoplasmin, Heiner's "h" and "m" bands, and only one line with blastomycin, serologically distinct from "m" and "h" which they called "b".

The "h" and "m" antigens apparently specific for detection of histoplasmosis have been separated from crude histoplasmin and purified by means of column chromotography on DEAE-cellulose by Greene et al. (1960). These fractions

were used in immunodiffusion tests and confirmed the results of Heiner (1958).

Later, in 1960 according to Greer and Gordon, the New York State Department of Health discontinued the use of the quantitative complement fixation test for diagnosing histoplasmosis and replaced it with a modified Ouchterlony double diffusion gel precipitin test developed by Heiner (1958).

Agar-gel diffusion studies showed that 12 isolates of Trichophyton rubrum possessed an antigen not found in 20 isolates of T. mentagrophytes and 4 strains of T. mentagrophytes variety nodular (Landay, 1961). Landay observed spur formations in 9 of the isolates of T. mentagrophytes when extracts from mycelia and culture filtrates of these organisms were placed adjacent to T. rubrum extracts which may indicate heterogeneity of the T. mentagrophytes species. Species of T. rubrum and T. mentagrophytes were separated using morphology and the hair invasion test (Ajello and Georg, 1957). Dyson and Landay (1963) concluded that the above immunodiffusion test results corroborated the validity of the hair invasion test.

Histoplasmin and 5,000 sera from patients in tuberculosis sanatoria were used by Schubert et al. (1961) in the double diffusion method. They considered the demonstration of a single band ("h" and/or "m") as the index of positivity and the results obtained correlated well with those of the

complement fixation test in which a titer of 1:8 or greater was regarded as positive.

Abernathy and Heiner (1961) demonstrated positive reaction, a specific blastomycin band, in only 14 of 22 proven cases of blastomycosis using the immunodiffusion technique. Correlation with the complement fixations test was satisfactory.

Biguet et al.(1961) used a combination of techniques, including double diffusion, starch electrophoresis, chromatography on DEAE cellulose and immunoelectrophoresis to study the antigens of macerated Candida albicans. These techniques revealed 9 antigens in C. albicans, two specific for this species. Candida albicans was shown to be related to C. stellatoidea, and C. tropicalis with C. zeylanoids. Only C. albicans antiserum was used in this investigation. In 1962 Biguet et al. used antisera for all species tested to confirm the previous work. This confirmed the work by Tsuchiya et al. (1958) who used the slide agglutination technique to study species of the genus Candida.

Tran Van Ky et al. (1963) carried out electrophoretic studies on an antigenic extract of C. albicans containing glycoproteins with a few protein bonds. Double diffusion in agar gel was used to show 7 fractions, 5 of which were identical with other Candida sp. in the antigenic analysis of the extract. The results confirmed the work of Biguet et al. (1961, 1962).

Schubert and Hampson (1962) concluded from their research that the agar gel method could not serve as an inclusive screening test for coccidioidomycosis as it is less sensitive than the complement-fixation test.

Huppert and Bailey (1963) obtained sera from patients in a hospital for immunodiffusion, complement-fixation, and precipitin tests; using as antigen a toluene lysate from pooled growths of strains of Coccidioides immitis. The results between the immunodiffusion and the complement-fixation tests correlated well in 10 proven coccidioidomycosis cases. They were the first to attempt to relate the bands formed in agar gel with antibody demonstrated in the precipitin and complement-fixation test described by Smith et al. (1950). Thus, they recommended the employment of the immunodiffusion and the precipitin methods for routine serological testing of coccidioidomycosis.

Rowe, Newcomer and Wright (1963) studied the effect of temperature, pH and enzymes on the soluble antigens derived from the culture filtrates in which different growth phases of Coccidioides immitis were grown. They were examined by the double diffusion technique against sera of patients with coccidioidomycosis. High temperature and extreme changes of pH did influence the formation of precipitin lines although the enzymes used had no apparent effect. Evidence was obtained which indicated antigenic differences between various strains and different growth phases.

Rowe, Newcomer and Landau (1963) studied the effect different cultural factors have on the development of antigens by C. immitis using the immunodiffusion technique. Factors influencing an increase in the amount of antigen were type of media used (such as brain heart infusion medium supplemented with dextrose), a rise in temperature from 30°C to 37°C, and the strain used.

The sera of asthmatics were examined in the immunodiffusion technique by Feinberg and Temple (1963) for the precipitating antibodies to 14 different fungus extracts. They found Aspergillus giganteus and A. fumigatus shared one precipitin, but A. giganteus had two lines not shared with A. fumigatus. Aspergillus giganteus and A. terreus had one common line as did A. terreus and A. nidulans.

McMillen and Devroe (1963) used the agar-gel method to study well documented cases of histoplasmosis. Data presented suggested that immunodiffusion reactions when used in conjunction with complement-fixation titers were useful in differentiating active from inactive infections. The "h" and "m" bands were demonstrable shortly after infection and throughout the active stage of the disease which parallelled the rise, peak and drop in complement-fixation titers. Busey and Hinton (1965) reported similar results.

Goldin and McMillen (1963) developed a micro-method for the agar-gel precipitation test for histoplasmosis which

simplified and gives more rapid reactions than the Petri-dish procedure used by Heiner (1958).

Seeliger and Schröter (1963) used agglutination, precipitation, agar-gel diffusion and complement fixation tests with whole cell antigens and crude polysaccharide extracts from all recognized species of the genus Trichosporon to show that they can be subdivided into 4 serological groups.

Using sera obtained from rabbits injected by three different methods with antigenic solution of Hansenula wingei, Brookbank and Heisler (1963) failed to demonstrate specificity for mating type in this yeast in the double diffusion technique. There was a common band with H. anomalie, and H. saturnus. This was the only component in common.

Kind and Meyer (1963) established species specificity for Actinomyces israelii and A. bovis using the Ouchterlony agar-gel diffusion method. Actinomyces naeslundii cross-reacted with both of these species, suggesting a transitional form with antigenic relationship to the other 2 species used in the test. These 3 actinomycetes showed no cross reactions with any anaerobic diphtheroids used in the experiment.

Employing the immunodiffusion technique Torheim (1963) studied antigens extracted from and antisera produced by 12 strains of Geotrichum and related fungi. Three distinct serological groups were discovered. The first serological group included Geotrichum candidum, G. asteroides, G.

javanense, G. matalense, G. pulmoneum, G. versiforme, Endomyces lactis, and E. magnusii. He suggested that the first 7 strains are in reality the same species since they are serologically identical. Endomyces manusii did not show identical reaction with the others but did cross react with them. The second serological group contained G. amycelicum, G. hirtum and Trichosporon cutaneum which were not serologically identical but cross-react with each other. Serogroup 3 included only Endomycopsis selenospora.

Markowitz (1964) found 3 serologically active fractions, II, IV, and V, after fractionizing on a DEAE-cellulose column, a mixture of polysaccharides obtained from culture filtrate of Histoplasma capsulatum grown in the yeast phase. With immunoelectrophoresis Markowitz showed fractions II, IV, and V each contained at least 2 antigenic components.

Taschdjian et al. (1964) suggested that immunodiffusion test may be of value as an aid in the diagnosis of chronic systemic candidiasis.

A case of mycetoma caused by Allescheria boydii was serologically identified by Baxter et al. (1965) using the immunodiffusion technique with the patient's serum and extracts of the fungus.

Franke (1965) applied immunodiffusion and immunoelectrophoresis to a study of species of myxomycetes in the order Physarales. He observed strong affinities among the following: Physarum gyrosum, P. tenerum, P. pusillum, P. oblonga, and Fuligo septica; Physarum sp. and Cienkowskia

reticulata; Diderma effusum, and P. pusillum; P. polycephalum, and P. flavicomum; Fuligo septica and many of the 22 species tested. The results supported the close relationship of Didymium nigripes and D. iridis. Fuligo cinerea and F. septica were related only in the absorbed antisera tests. Two isolates of F. septica gave identical results but the results of the 4 isolates of Physarum pusillum reacted different serologically.

La Börde and Abernathy (1965) separated the "h" and "m" antigens from crude histoplasmin by DEAE ion exchange column to study their immunological and physical-chemical characteristics. The "m" antigen produced 3 bands in the immunodiffusion test while the "h" antigen only produced one band. No common lines were formed between these two antigens in immunodiffusion or immunoelectrophoresis.

In 1965 Wiggins and Schubert disagreed with the prognostic scheme proposed by McMillen and Devroe (1963) due to no demonstrable "h" band in immunodiffusion test using sera from a number of histoplasmosis cases considered to be clinically active.

Huppert and Bailey (1965a and 1965b) described a method for preparing an antigen solution-toluene lysate which correlates in the immunodiffusion test with the results obtained in the standard tube precipitin test and complement fixation test for detecting coccidioidomycosis. This antigen solution contained the original toluene lysate coccidioidin initially described by Pappagianis et al. (1961), and a second lysate preparation which contained only the tube

precipitin component, the heat labile complement component having been deliberately destroyed by heat.

Burrell et al. (1966) studied serologically three species of the genus Phytophthora by means of gel diffusion and immunofluorescence. Species-specific sera were obtained and proved efficient for the identification of P. cactorum, P. cinnamoni, and P. erythroseptica.

Sawaki, et al. (1966) reported two antigen-antibody systems in coccidioidomycosis demonstrated by immunodiffusion and immunoelectrophoresis, through the use of I^{131}-coccidioidin. Sera which were positive in the complement-fixation test but negative in the tube precipitin test produced one line of precipitation in the immunodiffusion test. The same was true in the immunoelectrophoresis and appeared in the γ_2-globulin region. The sera positive to both precipitin and complement fixation tests yielded 2 different lines in both immunodiffusion and immunoelectrophoresis with 1 arch in the γ_2-globulin area and the other in the γ_1-globulin region. By the use of radioimmunoelectrophoresis, they demonstrated that the antibody activity in Ig-G globulin parallels the CF titer and the Ig-M antibody parallels the TP (= tube precipitin test) reactivity. There was antibody activity with the Ig-A globulin but it did not reflect either CF or TP activity.

Walters et al. (1966) studied 404 sera from 50 patients with chronic histoplasmosis in the Ouchterlony test using a concentrated histoplasmin as antigen and

observed only "h" and "m" precipitin bands. In the immunoelectrophoresis tests they found 4 precipitin bands, 1, 2, 3, and 4, in 7 combinations.

Immunodiffusion technique was used to diagnose 21 cases of paracoccidioidomycosis in a study with pooled filtrates from 3 strains of Paracoccidioides brasiliensis in the yeast and mycelial phases as antigens (Restrepo, 1966). Sixteen of these cases were positive using the agar gel method to only 15 for the complement fixation test. Two cases were negative with both tests. One of the two negative cases produced the causitive organism, P. brasiliensis when cultures were made. Both tests produced negative results in 3 clinically cured patients. The above antigenic solution was tested with sera from patients with culture-confirmed cases of sporotrichosis, cocidioidomycosis, and blastomycosis, resulting in no cross reactions.

Common antigens were shown in Trichophyton rubrum, T. mentagrophytes, Hormodendrum sp., and Penicillium sp. using the agar gel diffusion test by Reyes and Friedman (1966). These investigators found specific antibodies developed in sera of animals immunized with dermatophytes as well as an antibody directed against a common antigen found in dermatophytes, Hormodendrum sp., and Penicillium sp.

Grappel et al. (1967) produced antisera in rabbits with autoclaved mycelial suspensions of Microsporum quinckeanum (= Trichophyton mentagrophytes) which were

reacted with three neutral polysaccharides, galactomannans I, galactomannans II and glucans, isolated from five species of dermatophytes, M. quinckeanum (= T. mentagrophytes), T. granulosum (= T. mentagrophytes), T. interdigitale (= T. mentagrophytes), T. rubrum, and T. schoenleinii by the immunodiffusion method. Significant differences were found among the glucans and galactomannans II, but not among the galactomannans I of these species.

Georg et al. (1967) evaluated the immunodiffusion technique for diagnosing actinomycosis and found it to be good but cross reactions occurred between sporotrichosis, nocardiosis, tuberculosis, hydradenitis supurative leukemia, and hepatoma.

Ray (1967) used a modification of the agar-gel precipitin-inhibition technique (Ray and Kadull, 1964) to detect Coccidioides immitis antibodies in sera from human clinical cases and experimental animals infected with this organism. He had more consistent and reliable results than with complement fixation and double immunodiffusion tests.

Using the agar gel diffusion technique Landay et al. (1967) reacted antispherule and antiarthrospore from Coccidioides immitis and anti-Histoplasma capsulatum sera with extracts of spherule, arthrospores, and coccidioidin. The antispherule sera formed multiple precipitin bands with extracts of spherules and of arthrospores while the anti-arthrospore serum failed to precipitate with the spherule extract and formed a single band in the presence of an

arthrospore extract solution. When these extracts were tested with different antisera and anti-Histoplasma, it was found that the spherule preparation formed bands only in combination with antipurified spherule serum, whereas the arthrospore extract precipitated with anti-purified spherule, antiarthrospore, and anti-Histoplasma capsulatum sera, 1 band with antiserum from rabbits with coccidioidomycosis but no bands in the presence of antiarthrospore serum. Coccidioidin formed 2 bands in the presence of any of these antisera. The conclusion was made that the extract from the spherule phase of C. immitis differed from the extracts of the arthrospore and mycelial phases.

Okada and Yamamura (1964) prepared antisera in rabbits against microsomal and unsedimented fractions isolated from adult chicken livers. The reactions of these antisera with several microsomal extracts and unsedimented fractions prepared from livers in different stages of development were studied by immunodiffusion technique. Adult liver microsomes showed 5-6 components which are predominant in the deoxycholate (DOC) extract of microsomes. Three of these components were highly "liver-specific." One was detected in the 9-day old embryonic liver and the number progressively increased to 3 at the time of hatching. Common components occurred in the microsomal and unsedimented fractions and appeared as early as the 6th day in development. Two of these "common components" were associated with esterase activity. Unsedimented fraction of 6-day embryonic liver

contained 4 components specific for this fraction. These components showed complete or partial identity with those seen in the unsedimented fraction of adult liver.

A similar investigation was done by Okada and Sato (1963) with chicken kidney. They found 3 highly specific kidney components in the DOC extract of the microsomes and 2 components common with all fractions tested. The sera were absorbed with extracts of liver and lung to remove the antibodies that would cross react.

Immunodiffusion techniques have been used very little in investigating the dermatophytes. This technique was applied to the study of the relationship of these organisms to each other using some subcellular fractions. The possible use of microsomal fractions as highly specific components of the dermatophytes as was found in the chicken liver by Okada and Yamamura (1964) was investigated in this research.

Thus from a review of the literature it is apparent that investigators are using immunodiffusion primarily to diagnose mycotic infections, while only a few (Biguet et al., 1959; Seeliger, 1960; Torhiem, 1963; and Franke, 1965) have commented on the potential taxonomic value of this technique as an aid in clarification of the phylogenic relationship of fungi.

MATERIALS AND METHODS

I. Dermatophytes Studied

Twenty cultures of dermatophytes which included at least one species from each genus and 2 or more strains (isolates) of the species to be used in preparing antisera, the reference strains, were selected for these investigations. Stock cultures were grown on Sabouraud's glucose agar. The species of dermatophytes investigated and their sources are listed on the following page.

II. Verification of Trichophyton Species

Macroscopic and microscopic characteristics are used to separate the typical species of the dermatophytes in the genera Epidermophyton, Keratinomyces, and Microsporum. Some members of the genus Trichophyton are difficult to separate using these characteristics, thus other means are used, including nutritional requirements, pigment production, hair invasion and urease production.

A. Trichophyton Agars

Mycelial inocula about one-eighth of an inch in diameter were removed from each Sabouraud's glucose agar culture of Trichophyton studied. These were imbedded in seven nutritional media used for the differentiation of Trichophyton species (Bacto-Trichophyton Agars, Difco, as

1.	Epidermophyton floccosum (Harz) Langeron and M. Lochevitch, 1930	Tinea corporis, Ulbrich Dermatology Clinic, Detroit, Michigan, identified by E. S. Beneke
2.	Keratinomyces ajelloi Vanbreuseghem, 1952	Collection at the Section of Dermatology, Department of Medicine, University of Chicago, identified by T. Benedek.
3.	Microsporum ferrugineum Ota, 1922	Collection at the Department of Dermatology, School of Medicine, University of Michigan
4.	Microsporum gypseum (Bodin) Guiart and Grigorakis, 1928	Soil, Saõ Paulo, Brasil, 1961, identified by E. S. Beneke
5.	Trichophyton equinum (Matruchot and Dassonville) Gedoelst, 1902	Collection at the Department of Dermatology, School of Medicine, University of Michigan
6.	Trichophyton gallinae (Megnin) Silva and Benham, 1952	Collection data unknown
7.	Trichophyton megninii Blanchard, 1896	#X434, Collection at N.C.D.C.[1]
8.	Trichophyton mentagrophytes var. granulosum Neven-Lemaire, 1911	Tinea on a dog, Michigan State University Veterinary Clinic, identified by E. S. Beneke
9.	Trichophyton mentagrophytes var. granulosum Neven-Lemaire, 1911	#455 N.C.D.C.[1] Tinea pedis, identified by J. Fisher, Toronto, Canada
10.	Trichophyton mentagrophytes var. erinacei Smith et Marples, 1963	#X536 N.C.D.C.[1] Tinea on a hedgehog, identified by M. Marples, New Zealand.
11.	Trichophyton rubrum (Castellani) Sabouraud, 1911	Tinea corporis, isolated by G. Dardas, identified by A. L. Rogers, 1965
12.	Trichophyton rubrum (Castellani) Sabouraud, 1911	Tinea corporis, isolated by E. S. Beneke, identified by A. L. Rogers, 1965.
13.	Trichophyton schoenleinii (Lebert) Langeron and Milochevitch, 1930	Collection at Department of Dermatology, School of Medicine, University of Michigan
14.	Trichophyton soudanense Joyeux, 1912	Collection at the Department of Dermatology, School of Medicine, University of Michigan
15.	Trichophyton terrestre Durie and Frey, 1957	#414 N.C.D.C. Isolated from pig by Austwick, England.
16.	Trichophyton terrestre Durie and Frey, 1957	#X285 N.C.D.C.[1] Soil isolate, G. Orr, University of California, Los Angeles.
17.	Trichophyton terrestre Durie and Frey, 1957	Soil, Saõ Paulo, Brasil, identified by A. L. Rogers, 1961
18.	Trichophyton tonsurans Malmsten, 1845	Tinea capitis, Ulbrich Dermatology Clinic, Detroit, identified by E. S. Beneke
19.	Trichophyton verrucosum Bodin, 1902	Collection at the Section of Dermatology, Department of Medicine, University of Chicago
20.	Trichophyton violaceum Sabouraud, apud Bodin, 1902	Collection at Department of Dermatology, School of Medicine, University of Michigan

Specimens of all the above are deposited in the collection of fungi (E. S. Beneke), at Michigan State University, East Lansing, Michigan.

[1]National Communicable Disease Center, Mycology Unit, Atlanta, Georgia.

formulated by Georg and Camp, 1957). The cultures were incubated at 24°C for three weeks and checked for amount of growth on each medium.

B. Tests to Separate T. mentagrophytes and T. rubrum

1. Pigment production.--Petri dishes containing corn meal dextrose agar formulated by Bocobo and Benham (1949), potato dextrose agar (Difco) and Sabouraud's glucose agar (Difco) were inoculated with small pieces of mycelia from each isolate of T. mentagrophytes and T. rubrum. After incubation at 24°C for 3 to 4 weeks the presence or absence of undersurface pigment was noted.

2. "In vitro" hair invasion (Ajello & Georg, 1957.-- The medium contained 25 ml of sterile distilled water, 5 drops of 10% yeast extract and an assortment of human and horse hairs placed in petri dishes. Mycelial transfers were made from the isolates of T. mentagrophytes and T. rubrum into this medium and incubated at 24°C. These cultures were agitated for one minute each day for 21 days. At the end of this time hair segments were removed from the medium with sterile forceps, placed in a drop of lactophenol cotton blue mounting fluid and examined under the microscope to determine whether or not they had been perforated.

3. The Urease test (Philpot, 1967).--Urease is known to be present in T. mentagrophytes but not in T. rubrum, thus the urease test was performed on these isolates used in this investigation. Christensen's medium was modified

as suggested by Philpot (1967). The medium contained the following: peptone, 1 g; NaCl, 5 g; KH_2PO_4, 2 g; glucose, 5 g; agar, 20 g; distilled water to make 1 liter. These ingredients were dissolved by heat and 6 ml of phenol red solution (0.2% in 50% alcohol) was added. After the medium was autoclaved at 115°C for 15 minutes and cooled to 50°C, 100 ml of a 20% aqueous solution of urea, sterilized by filtration, was added. Fifteen ml of the medium was dispensed aseptically into sterile ½ oz bottles, and slanted. Small pieces of mycelium from the T. rubrum and T. mentagrophytes strains were transferred to these slants, incubated at 24°C, and observed daily for 21 days. The development of a deep red color throughout the medium was regarded as the end point of the reaction.

All of the tests to separate the Trichophytons were done in at least duplicate.

III. Media For Growth Of The Dermatophytes

Preliminary studies were performed to determine which of several media would yield the largest amount of hyphal wet weight in the shortest time in order to supply large quantities of the organisms for the microsomes to be used as an antigenic fraction. Trichophyton mentagrophytes (dog), T. terrestre (414) and T. rubrum (Dar) were the organisms selected for this test. Sabouraud's glucose broth was the medium selected for the growth of the fungi used in the major portion of the research. The formula for this medium

and those for other media used in the preliminary studies are as follows:

Sabouraud's Glucose Broth

Glucose		40.0 g
Neopeptone		10.0 g
Distilled water	q.s.	1000.0 ml

Ingredients were added to the distilled water, dissolved, and the pH adjusted to 5.6. The medium was dispensed and autoclaved at 121°C, 15 pounds pressure for 15 minutes.

Schaffer, Molomut and Center's Medium (1959)

Sucrose	40.00 g
Ammonium sulfate	2.00 g
Ammonium nitrate	1.30 g
Ammonium citrate	1.00 g
Potassium phosphate, monobasic	0.15 g
Potassium phosphate, dibasic	0.15 g
Ammonium phosphate, dibasic	0.20 g
Citric acid	1.00 g
Potassium citrate	0.40 g
Magnesium sulfate	0.24 g
Calcium carbonate	0.80 g
Trace elements, stock solution*	1.00 ml
Distilled water q.s.	1000.00 ml

*Formula for Trace Elements, Stock Solution:

Copper sulfate	4.0 g
Manganese sulfate	5.0 g
Potassium dichromate	2.0 g
Zinc sulfate	5.0 g
Ferric sulfate	1.0 g
Distilled water q.s.	1000.0 ml

The ingredients were added to the distilled water, heated to dissolve, then filtered to clarify. The pH was adjusted to 6.5, then the medium was dispensed into

containers and autoclaved at 121°C, 15 pounds pressure for 12 minutes.

Bulmer's Modification of Czapek's Medium (1965)

Sodium nitrate		2.00 g
Potassium phosphate		1.00 g
Magnesium sulfate		0.50 g
Potassium chloride		0.50 g
Ferric sulfate		0.01 g
Sucrose		30.00 g
Glucose		5.00 g
Peptone		5.00 g
Yeast extract		1.00 g
Distilled water	q.s.	1000.00 ml

The ingredients were dissolved in the distilled water, the pH adjusted to 5.6, the medium dispensed into containers and autoclaved at 121°C, 15 pounds pressure for 15 minutes.

IV. Antigens

A. Preparation of Antigens

Strains of the dermatophytes used for antigen production were grown on Sabouraud's glucose agar, the medium used for stock cultures, in 50 ml test tubes for two weeks at 25°C. The fungal mat was removed with as little medium as possible and blended for one minute in a 360 ml Monel metal semi-micro Waring Blendor with 25 ml of Sabouraud's glucose broth or the medium in which it was to be grown. Ten ml of the blended fungus, the inoculum, was pipetted into 2000 ml Erlenmeyer flask containing 500 ml of the sterilized test medium and placed on a reciprocating shaker which had a stroke of one inch and gave 90 two-inch

excursions per minute. Incubation temperature was 24°C. The flasks containing the different media were covered with pieces of aluminum foil four inches square and autoclaved before inoculations were made.

Materials and equipment used in the preparation of the antigens were sterilized and procedures were performed under as aseptic conditions as possible.

At the end of the 14 day incubation period the fungi were vacuum filtered using No. 500, 12½ cm. diameter Sargent filter papers, 142 mm diameter Buchner funnels, and filtering flasks. The mycelia were washed with sterile distilled water at least six times, until no medium color remained in the filtrate. Two additional washes were applied if the fungus produced a water soluble pigment. Washing was to remove as much medium as possible. The mass of hyphae was weighed and the weights recorded.

The 20 different organisms were processed in the following manner. Eighteen grams of a fungus, 10 grams of no. 110 Superbrite glass beads from Minnesota Mining and Manufacturing Company, and 50 ml of a homogenizing medium were blended at 0°C in a 360 ml Monel metal semi-micro Waring Blendor equipped with a jacket for 30 minutes or until only small fragments of hyphae could be detected microscopically. The temperature was kept at near 0°C by filling the jacket with crushed ice and slowly running water through the ice which was replaced as it melted.

The homogenizing medium was composed of 0.25M sucrose, 0.05M 2-amino-2 hydroxymethyl-1, 3-propanediol (TRIS), and 0.01M MgCl adjusted to pH 7.8 (Pollard, et al. 1966).

This homogenate was vacuum filtered through 4 layers of Kleenex tissue in a cold Buchner funnel into a filtering flask in crushed ice. The filtrate was centrifuged at 0°C in an International Centrifuge (Model HR-1) at 20,000 times gravity for 20 minutes to remove most organelles. The supernatant solution was decanted and the pellet was discarded. The supernatant solution was centrifuged at 104,000 times gravity for 4 hours in a Beckman Ultracentrifuge Model L-2 at 0°C. The resulting supernatant solution was decanted and used as one antigenic fraction, referred to in this thesis as "supernatant antigen." This fraction was stored at -10°C. The pellet was rinsed twice with homogenizing solution at 0°C, resuspended in 25 ml of the same cold solution and centrifuged for 1½ hours at 104,000 times gravity. At the end of this centrifugation the supernatant solution was discarded, the pellet rinsed twice with cold homogenizing solution and suspended in a small amount of the same solution. This fraction was used as the second antigenic fraction and will be referred to as "pellet antigen."

Sterility tests were conducted by placing 1 ml quantities of each fraction into test tubes containing either Sabouraud's glucose agar to detect fungus growth or nutrient agar to detect bacterial growth. If inocula in

either medium showed growth during a two week period, the antigenic fraction was discarded and the fractionation was repeated with that fungus. The antigenic fractions were dispensed into 3 ml vials and stored at -10°C. In the cases of Trichophyton mentagrophytes (Dog), T. terrestre (414), and T. rubrum (Dar.), the reference strains, several repeats of the fractionation procedure were necessary to have sufficient quantity of fractions to perform the desired studies so the like antigenic fractions for each of the isolates were pooled before any experiments were begun. After pooling, sterility tests were conducted and the fractions were dispensed and stored at -10°C.

B. Protein Determination

The amount of protein in each of the dermatophyte fractions for all organisms was determined using the method of Lowry et al. (1951). Crystalline bovine plasma albumin was used as the standard. The tests were run in duplicate, using freshly made reagents, except for the Folin phenol reagent. The determinations were performed at the beginning of the investigation.

V. Antibody Production

A. Rabbits Used

German checker rabbits, both bucks and does, approximately six months old were used for production of antisera. Three rabbits were injected with each antigenic fraction to

produce antibodies. The rabbits were watered and fed on commercial pellets. Weights were taken at the beginning and at the end of the studies. An increase in weight in each rabbit was noted.

B. Antigens Used for Production of Antisera

Supernatant and pellet antigens were used from Trichophyton mentagrophytes (dog), T. terrestre (414), and T. rubrum (Dar), reference strains, to produce the antisera used in these investigations. Supernatant antigens were injected into rabbits as follows: rabbits numbered 47, 48 and 49 received T. mentagrophytes (dog); numbers 53, 54, and 55 received T. terrestre (414), and rabbits numbered 59, 60, and 61 received T. rubrum (Dar). The pellet antigens were injected into the following rabbits, numbers 50, 51, and 52 received T. mentagrophytes (dog), numbers 56, 57, and 58 received T. terrestre (414), and numbers 62, 63, and 64 received T. rubrum (Dar).

C. Injections (Tables 3 and 4)

Two weeks before injections each rabbit was bled from the marginal vein of an ear to obtain control sera (pre-injection sera).

Antigenic solutions injected were emulsified with an equal volume of incomplete Freund's adjuvant (Difco) since this material is known to enhance antibody production (Gorzynski, 1963). Complete Freund's adjuvant (Difco) was

not used as it contains killed Mycobacteria which might have produced antibodies that would cross react with the dermatophytes.

Details of the injections of antigens are given in Tables 3 and 4. The first injection, containing 1½ ml of antigenic solution and 1½ ml of incomplete Freund's adjuvant for each rabbit, was given in the subscapular muscle. Two days later each rabbit received an intramuscular injection consisting of 1 ml of antigenic solution emulsified with 1 ml of incomplete adjuvant, in the right thigh. The third injection of the same concentration was given in the left thigh two days after the second injection. The fourth injection with the same concentration as the second was given intraperitoneally 6 weeks later and was considered the first booster. Every 2 weeks for eight more weeks similar booster injections were given in alternating sides of the abdomen. Leskovitz and Waksman (1960) reported that intramuscular and subcutaneous injections gave the highest circulating antibody titers in their experiments. When booster injections were given intraperitoneally, the level of antibodies was doubled.

Preliminary work included intravenous injections, but since these caused anaphylactic death in three rabbits the method was discontinued.

D. Bleeding and Antiserum Preparation

Rabbits were bled from the marginal ear vein. Xylol was applied to the area around the vein which encouraged

TABLE 3.--Injection and bleeding schedule for rabbits injected with supernatant fraction Trichophyton mentagrophytes (dog) was injected into rabbits numbered 47, 48, and 49; T. terrestre (414) was injected into rabbits numbered 53, 54, and 55; and T. rubrum (Dar) was injected into rabbits numbered 59, 60, and 61.

Month	Day	Injection		Bleeding from Marginal vein of an ear
		Quantity	Site	
1	1			50 cc Control sera
	14	2 x 1.5 emulsion (1.5 ml antigenic fraction/1.5 ml adjuvant)	intramuscular subscapular muscle	
	16	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intramuscular right thigh	
	18	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intramuscular left thigh	
2	46			50 cc
	60	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal right side of lower adbomen	
3	68			50 cc
	74	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal left side of lower abdomen	
	82			50 cc
	88	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal right side of lower abdomen	
4	96			50 cc
	102	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal left side of lower abdomen	
	110			50 cc
	116	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal right side of lower abdomen	
5	124			50 cc

TABLE 4.--Injection and bleeding schedule for rabbits injected with pellet fraction. Trichophyton mentagrophytes (dog) was injected into rabbits numbered 50, 51, and 52; T. terrestre (414) was injected into rabbits numbered 56, 57, and 58; and T. rubrum (Dar) was injected into rabbits numbered 62, 63, and 64.

Month	Day	Injection		Bleeding from Marginal vein of an ear
		Quantity	Site	
1	2			50 cc Control sera
	15	2 x 1.5 emulsion (1.5 ml antigenic fraction/1.5 ml adjuvant)	intramuscular subscapular muscle	
	17	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intramuscular right thigh	
	19	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intramuscular left thigh	
2	47			50 cc
	61	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal right side of lower abdomen	
3	69			50 cc
	75	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal left side of lower abdomen	
	83			50 cc
	89	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal right side of lower abdomen	
4	97			50 cc
	103	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal left side of lower abdomen	
	111			50 cc
	117	2 x 1 emulsion (1 ml antigenic fraction/1 ml adjuvant)	intraperitoneal right side of lower abdomen	
5	125			50 cc

dilation of the veins and faster bleeding. Vaseline was spread over the vein and area under the ear so the blood would not clot. A razor blade was used to cut the vein as near the tip of the ear as possible. Fifty ml of blood was collected into sterilized, 50 ml centrifuge tubes.

The collected blood was allowed to set for about 30 minutes at room temperature to encourage clotting. The clot was "ringed" from the sides of the tube and refrigerated at 4°C overnight to allow full contraction of the clot. The tube was removed, allowed to reach room temperature, and the serum decanted. The clot was then centrifuged and any additional serum was poured off. Merthiolate from a 1% stock solution was added to the serum to make a final concentration of 1:10,000. The serum was then frozen and the clot discarded. The bleeding schedule is given in Tables 3 and 4.

VI. Immunodiffusion Techniques

A. Electrolyte Solutions Used for Preparing Diffusion Media

The electrolyte solution used to prepare the medium employed for all final investigations was 0.15M phosphate-saline at pH 7.2. The formula for this buffer and those for others used in preliminary experiments follows:

Phosphate-Saline, 0.15M, pH 7.2

Monopotassium phosphate solution, 0.15M		150 ml
Disodium phosphate solution, 0.15M		350 ml
Sodium chloride solution, 0.15M		500 ml

Phosphate-Saline, 0.15M, pH 7.4

Monopotassium phosphate solution, 0.15M		150 ml
Disodium phosphate solution, 0.15M		350 ml
Sodium chloride solution, 0.15M		500 ml

Barbital, Ionicity 0.15,* pH 7.4

Sodium barbital		6.98 g
Sodium chloride		6.00 g
1 N hydrochloric acid		2.70 ml
Distilled water	q.s.	1000.00 ml

Ethylenediamine-Acetic Acid (EDTA) Ionicity 0.15,* pH 7.4

Ethylenediamine tetraacetic acid		15.80 g
Glacial acetic acid		23.80 g
Distilled water	q.s.	1000.00 ml

Tris, Ionicity 0.15,* pH 7.4

2-Amino-2-hydroxymethyl-1, 3-propanediol		9.30 g
1 N hydrochloric acid		74.00 g
Sodium chloride		7.00 g
Distilled water	q.s.	1000.00 ml

Isotonic Sodium Chloride, Ionicity 0.15,* pH variable

Sodium chloride		8.80 g
Distilled water	q.s.	1000.00 ml

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*Ionicity values (based on electric conductivity tests) taken from p. 302 in Immunodiffusion by A. J. Crowle (1961).

B. Media for Diffusion Studies

Different diffusion media were used in preliminary tests with the petri dish and glass slide methods. Oxoid Agar No. 3* was the gelling agent. To 200 ml of an electrolyte solution contained in a flask, 2 ml of a 1% solution of merthiolate, a weighed quantity of agar and a magnetic stirrer were added, and the flask placed on a hot plate. When the agar had dissolved and boiled for one minute, it was immediately put in petri dishes or placed on slides.

1. Gels for the Preliminary Studies by the Petri Dish Method.--Gels containing 1%, 1.5% and 2% agar were prepared as above in 0.15 M phosphate-saline buffer, pH 7.2 and 0.15M phosphate-saline buffer, pH 7.4 for studying antigen-antibody systems by the petri dish method.

2. Gels for the Preliminary Studies by the Agar Slide Method.--Preliminary studies to determine the best diffusion medium for the development of precipitation bands were conducted on slides covered with agar media by varying the agar concentration, pH, ionic strength, and the buffering system. Electrolyte solutions used to prepare 1%, 1.5% and 2% agar media, with ionic strength of 0.15 were: sodium chloride barbital, tris hydroxymethyl and aminomethane (TRIA), ethylenediamine tetraacetic acid

*Oxoid Agar No. 3, Consolidated Laboratories, Inc., Chicago Heights, Illinois.

(EDTA), and phosphate-saline. Other media used were 1%, 1.5% and 2.0% agar gel prepared in 0.15M phosphate-saline, pH 7.2.

C. Immunodiffusion Test Procedure

In preliminary experiments the petri dish and agar slide methods were used. The later method was employed in the final studies of precipitinogenic relationships. Sabouraud's glucose broth, incomplete Freund's adjuvant, the homogenizing medium, and the preinjection rabbit serum served as controls. The reactants were supernatant and pellet antigens from the dermatophytes and rabbit antisera.

1. Petri Dish Method.--Glass petri dishes, 100 x 15 mm, and 60 x 10 mm were washed with "7X"* cleaning liquid and put through one rinse of tap water, five rinses of distilled water and a final rnise in 95% ethanol. Twenty-five ml of diffusion medium was used for large plates and 10 ml for the smaller plates. They were placed in a portable humidity chamber at 24°C for 6-12 hours. With plexiglas templates as guides, circular reactant wells were cut with copper cylinders or with a Feinberg agar gel cutter #1803** in the small plates and the agar discs were removed with a 16-gauge needle. Wells employed were 12mm in diameter,

*Linbro Chemical Company, New Haven, Connecticut.

**Feinberg agar gel cutter #1803, Colsolidated Laboratories, Inc., Chicago Heights, Illinois.

placed 13 mm apart, or 10 mm in diameter, placed 12 mm apart in the large dishes. and in the small dishes the wells were 7 mm in diameter, placed 10 mm apart. The bottoms of the wells were sealed with 0.3% agar, prepared with the same solution used to produce the diffusion medium and the plates were placed in the chamber for one hour or longer.

The wells were filled using sterile disposable Pasteur pipettes. The dishes were incubated for 10 days in a moist chamber at a constant temperature of 24°C. The wells of the reactants were refilled the first 4 days and at no time during this period were the wells empty. Reactions were observed daily.

2. Agar Slide Method.--Lantern slide glass plates, 10.1 X 8.2 X 0.1 cm or 4 X 3¼ inches, were washed with "A-JAX", rinsed in tapwater, five distilled water rinses, and left in 95% ethanol until used. They were then wiped with a lintless soft cloth, placed on a level surface and covered with a uniform 2.0 mm thick layer formed by pipetting 16.5 ml of diffusion medium onto each slide. After solidifying these agar blanks were placed into moist chambers at 24°C for 6-12 hours.

Circular reactant wells were cut with cork borers. In preliminary studies several different well sizes and distances between wells were used. Wells used were 5 mm wells placed 6 mm apart, or 6 mm wells placed at distances of 6, 7, and 8 mm apart. The final investigations were all performed with a 6 mm central well surrounded by six 6 mm

wells with 6 mm distances between all wells. Agar discs were removed from the wells with a glass pipette attached to a rubber hose connecting to a filter and then to a vacuum line.

The reactants were added by means of sterile disposable pipettes. Antisera were added 4 hours before the antigens to allow for the difference in rates of diffusion of the two substances. Slides were incubated in moist chambers at 24°C for 48 hours after the addition of the antigens. Observations were made at 8 hour intervals for at least 48 hours.

3. Observations.--The petri dishes and the lantern slides were illuminated from the sides with light from daylight, fluorescent, and 4 watt tubes in 2 adjustable illumination lamps. They were placed into openings in cardboards cut to fit the dishes and slides. The cardboard was mounted on the rods of a Bausch and Lomb Photomicrographic camera Model L apparatus fitted with a macrostage, 8½ inches from the black base, with the lights 4¼ inches beneath the cardboard. Precipitation lines were recorded by drawing with pencil while viewing the dishes and slides under these conditions.

4. The Influence of Antigen Dilution.--Preliminary tests were conducted by the agar slide method with different dilutions of the antigens against undiluted homologous antisera. The supernatant and pellet antigens of Trichophyton mentagrophytes (dog), and T. terrestre (414)

were used as follows: undiluted, and diluted 1:2, 1:4, 1:8 and 1:16. The dilutions were made using the stock antigenic fractions and the homogenizing solutions.

5. Photography.--All petri dishes and lantern slides were photographed under the same conditions as for viewing. Pictures were taken of the dishes at the end of one week, and at 10 days, while the lantern slides were photographed at 24 and 48 hours. Sometimes additional pictures were taken of the slides after staining.

6. Staining of Precipitating Lines.--After the slides were photographed, they were washed with physiological saline, pH 7.2 for 24 hours and then placed in a bath of distilled water for 24 hours. The surface of each slide was covered with a moistened piece of Whatman #1 filter paper, which prevented the agar from curling as it dried, and left to dry overnight. After moistening the filter paper, it was gently removed. The plates were then immersed in Crowle's triple stain for proteins (1961) for one minute. The background stain was removed with a 1½% acetic acid. Then the plates were rinsed in distilled water, drained, dried, marked with a diamond point glass marker, and separated with a piece of tissue paper before storage.

PRELIMINARY RESULTS AND DISCUSSION

I. Media for Growth of Dermatophytes

The weights of the vacuum filtered, wet mycelia for the dermatophytes grown in 3 different media are given in Table 5. The weights of the mycelia grown in Sabouraud's glucose broth was approximately 3 times greater than the weights of the mycelia grown in Schaffer, Molomut, and Center's medium and 2 times greater than that grown in Bulmer's modification of Czapek's medium at the end of 14 days. The variations might be due to the amount of glucose in the different media, 40 grams in Sabouraud's, 5 grams in Bulmer's and no glucose in Schaffer *et al*. medium. The later two had sucrose, which may not be as readily utilized by the organisms.

TABLE 5.--Weight of the wet mycelium for the three *Trichophyton* sp. grown in different media at the end of 14 days.

Media	Weights of Vacuum Filtered Mycelium*		
	T.mentagrophytes (dog)	*T.terrestre* (414)	*T.rubrum* (Dar)
Sabouraud's Glucose Broth	13.9	15.2	21.1
Schaffer, Molomut and Center's Medium	4.9	5.3	7.0
Bulmer's Modification of Medium	9.0	11.8	13.8

*Weights given are averages of mycelia from three flasks of each type of medium.

Schaffer, Molomut and Center's medium is a synthetic medium with an inorganic nitrogen source compared to the organic sources in the other two media. Any residue of this inorganic source of nitrogen would have no influence on antibody formation when injected into rabbits, while the organic sources might, but since in this medium and in Bulmer's modification of Czepek's there was so much less mycelial growth by weight than in Sabouraud's glucose broth, the medium containing the greatest amount of mycelial growth was selected for the antigenic relationship studies. In the immunodiffusion studies when Sabouraud's glucose broth was used as a control reactant, no reactions occurred between the antisera and this medium.

II. Relative Sensitivities of Methods

Table 6 presents a comparison of the relative sensitivities of the petri dish and agar slide methods for studying the precipitation lines formed by homologous antigen-antibody systems of T. mentagrophytes (dog) and T. terrestre (414).

In repeated tests performed by the agar slide method 8 precipitation lines were detected (Plate I, Fig. 1) in the reaction area between the T. mentagrophytes (dog) supernatant antigens and its antiserum, 4 lines between T. mentagrophytes (dog) pellet antigens and the homologous antiserum, while between T. terrestre (414) supernatant antigen homologous system 9 lines were detected (Plate II,

TABLE 6.--The number of precipitation lines detected in double-diffusion tests using two sizes of petri dishes and the agar slide method.

Methods		Lines Formed by the Homologous Antigen-Antibody System			
		T. mentagrophytes (dog)		T. terrestre (414)	
		Supernatant fraction	Pellet	Supernatant fraction	Pellet
Petri Dish (100X15mm)	1.0% Agar*	3	2	5	2
	1.5% Agar	3	2	5	2
	2.0% Agar	3	2	5	2
Petri Dish (60X10mm)	1.0% Agar	5	4	7	3
	1.5% Agar	5	4	7	3
	2.0% Agar	5	4	7	3
Agar Slide	1.0% Agar	8	4	9	5
	1.5% Agar	8	4	9	5
	2.0% Agar	8	4	9	5

*The diffusion media used in all methods were prepared with 0.15M phosphate-saline at pH 7.2.

Fig. 1), and 5 lines in the homologous antigen-antibody system of T. terrestre (414) pellet.

In tests conducted by the petri dish method using large petri dishes, 100 X 15 mm, the homologous antigen antibody systems of T. mentagrophytes (dog) (Plate I, Fig. 2) gave the following: supernatant antigen formed 3 precipitation lines and pellet, 2 lines; while the homologous systems of T. terrestre (414) supernatant antigen produced 5 lines, and the pellet, 2 lines (Plate II, Fig. 2).

Plate I

Immunodiffusion reactions using homologous antigen-antibody systems of T. mentagrophytes (dog) fractions in agar gel method employing two sizes of petri dishes and a lantern slide.

Fig. 1.--Lantern slide

a = T. mentagrophytes (dog) supernatant fraction antiserum
b = T. mentagrophytes (dog) pellet antiserum
c = T. mentagrophytes (dog) supernatant fraction

Fig. 2.--Petri dish (100 x 15 mm)

a = T. mentagrophytes (dog) supernatant fraction antiserum
b = T. mentagrophytes (dog) pellet antiserum
c = T. mentagrophytes (dog) supernatant fraction

Fig. 3.--Petri dish (60 x 10 mm)

a = T. mentagrophytes (dog) supernatant fraction antiserum
b = T. mentagrophytes (dog) pellet antiserum
c = T. mentagrophytes (dog) supernatant fraction
d = T. mentagrophytes (dog) pellet fraction

Plate I

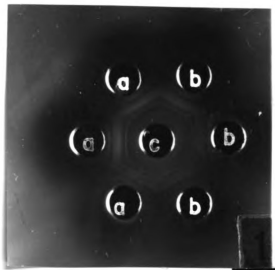


Fig. 1

Fig. 2

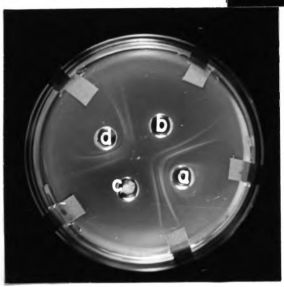


Fig. 3

Plate II

Immunodiffusion reactions using homologous antigen-antibody systems of T. terrestre (414) fractions in agar gel method employing two sizes of petri dishes and a lantern slide.

Fig. 1.--Lantern slide

a = T. terrestre (414) supernatant fraction antiserum
b = T. terrestre (414) pellet antiserum
c = T. terrestre (414) supernatant fraction

Fig. 2.--Petri dish (100 x 15 mm)

a = T. terrestre (414) supernatant fraction antiserum
b = T. terrestre (414) pellet antiserum
c = T. terrestre (414) supernatant fraction

Fig. 3.--Petri dish (60 x 10 mm)

a = T. terrestre (414) supernatant fraction antiserum
b = T. terrestre (414) pellet antiserum
c = T. terrestre (414) supernatant fraction
d = T. terrestre (414) pellet fraction

Plate II

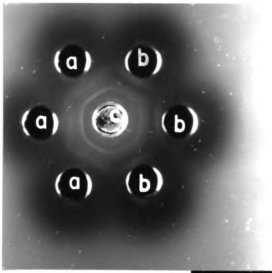


Fig. 1

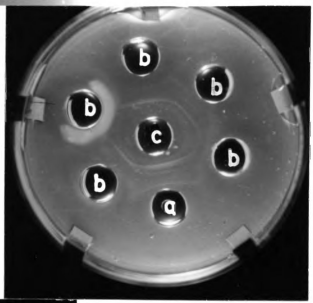


Fig. 2

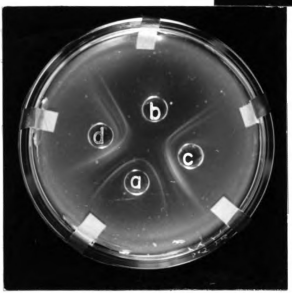


Fig. 3

The results of the reactions in small petri dishes for the homologous antigen-antibody were as follows: T. mentagrophytes (dog) (Plate 1, Fig. 3) supernatant antigen produced 5 lines, and the pellet, 4 lines, while in T. terrestre (414) (Plate II, Fig. 3) supernatant antigen developed 7 lines, but the pellet fraction, only 3 lines.

Varying the method by replenishing the antiserum reactant depots on the agar slides and petri dishes served to intensify the lines of precipitation that developed, and did not increase the number of lines.

In addition to being the most sensitive, the agar slide method was the simplest method of the three to use, the reactions were more easily observed, photographed, and the amount of antigenic fraction was less. Thus the agar slide method was employed for the relationship study.

III. Reactions in Various Diffusion Media

An identical number of lines formed in all diffusion media for a given fraction. The number of lines are as follows: 8 lines from the homologous antigen-antibody systems of T. mentagrophytes (dog) supernatant fraction, 4 lines from T. mentagrophytes (dog) pellet, 9 lines from T. terrestre (414) supernatant fraction, and 5 lines formed in T. terrestre (414) pellet.

A 1% agar gel prepared with 0.15M phosphate-saline, pH 7.2, was selected as the medium for studying the antigenic relationships of the dermatophytes.

IV. Dilution of Antigens

After selecting the diffusion medium and the method for studying the precipitinogenic relationships, tests were performed to determine the necessity of diluting the antigenic solutions.

At various dilutions of the supernatant and pellet antigens of T. mentagrophytes (dog), T. terrestre (414), and T. rubrum (Dar), with their undiluted homologous antisera, it was found that the undiluted antigen fractions produced the greatest number of lines, as indicated in Table 7. Therefore the undiluted antigenic fractions and the undiluted antisera were used in the precipitinogenic relationships study of the dermatophytes.

TABLE 7.--The number of precipitating lines detected in the antigen dilution tests using homologous antigen-antibody systems in the agar slide method.*

Dilutions of Antigens**	Lines Formed by the Homologous Antigen-Antibody System			
	T. mentagrophytes (dog)		T. terrestre (414)	
	Supernatant Fraction	Pellet	Supernatant Fraction	Pellet
Undiluted	8	4	9	5
Diluted 1:2	8	3	9	4
Diluted 1:4	6	2	8	4
Diluted 1:8	5	2	6	1
Diluted 1:16	4	1	4	1

*All tests were conducted in 1.0% agar prepared with 0.15M phosphate saline at pH 7.2 and incubated at 24°C.

**Diluted with homogenizing solution

V. Antibody Response in Individual Rabbits

Tests of the reactivity of antisera collected from rabbits showed some variation in the precipitin response of individual rabbits to injections of the same antigenic preparations. The results of these tests are presented in Table 8.

TABLE 8.--The number of precipitating lines detected in individual rabbit antiserum by homologous antigen-antibody reactions.*

Antigenic Fraction	# Rabbits Injected	Maximum Number of Precipitation Lines Detected in Individual Rabbit Antiserum		
T. mentagrophytes (dog)				
Supernatant	3	8 (47)**	7 (48)	8 (49)
Pellet	3	4 (50)	3 (51)	4 (52)
T. terrestre (414)				
Supernatant	3	9 (53)	9 (54)	9 (55)
Pellet	3	5 (56)	5 (57)	5 (58)
T. rubrum (Dar)				
Supernatant	3	7 (59)	7 (60)	6 (61)
Pellet	3	4 (62)	4 (63)	2 (64)

*All tests were conducted on agar slides in 1.0% agar prepared with 0.15M phosphate-saline, pH 7.2, incubated at 24°C.

**Number in parenthesis is the number assigned to the rabbit.

Of the 6 antigenic fractions that were each injected into 3 rabbits, only the fractions prepared from T. terrestre supernatant fraction and pellet elicited identical

precipitin responses in all rabbits. The other antigenic fractions elicited identical responses in only 2 rabbits.

The antisera employed in the study of precipitinogenic relationships were those which contained the greatest number of detectable precipitins in the preliminary tests of homologous antigen-antibody systems.

RESULTS

I. Verification of the Dermatophytes Used in This Investigation

A. Identification of Species in the Genera Epidermophyton, Keratinomyces and Microsporum

Dermatophytes are usually identified by macroscopic and microscopic characteristics. The species in the genera Epidermophyton, Keratinomyces, and Microsporum produced characteristic macroconidia except for a few species in the genus Microsporum (M. audouinii, M. ferrugineum, and M. rivalieri). The differences in the macroconidia were used to separate the species, E. floccosum, K. ajelloi, and M. gypseum.

Epidermophyton floccosum produced many large, clavate, smooth, thin-walled macroconidia in clusters and singly on hyphae. No microconidia were produced.

The thick, smooth-walled macroconidia of K. ajelloi were cylindrofusiform, and had 5 to 12 cells. Microconidia were abundant and pyriform to ovate in shape.

Thin, echinulate walls were present in the ellipsoid, 3 to 9-celled, macroconidia of M. gypseum. Sessile and stalked, clavate microconidia were numerous.

Since M. ferrugineum does not produce macroconidia it was identified by colony morphology and its reactions on the

Trichophyton Agars. The deep yellow to orange-colored colony was slow-growing, heaped, glabrous and waxy, with many deep furrows. The growth of this organism on the Trichophyton Agars showed no specific nutrient requirements. At times a non-pigmented isolate develops resembling T. verrucosum. This requires the use of the Trichophyton Agars for differentiation of the organisms.

B. Identification of Trichophyton Species

1. Use of Trichophyton Agars.--A number of the species of the genus Trichophyton may be differentiated on the basis of colony morphology, however others cannot be distinguished readily on this basis. Some of the species are more readily distinguished on a physiological basis by the use of the Trichophyton Agars.

The growth characteristics on Trichophyton Agars for the Trichophyton sp. used in this investigation are given in Table 9. All cultures of the Trichophytons used had characteristic colonies and the usual nutritional growth requirement (Table 9) for the species to which it was assigned by the individual who did the original identification.

2. Separation of T. mentagrophytes and T. rubrum.--

a. Pigment Production: Table 10 contains the data on the undersurface pigment production for T. mentagrophytes and T. rubrum. The 2 T. rubrum isolates

TABLE 9.--Growth pattern of *Trichophyton* species on Trichophyton Agars.*

Organism	Casein	Casein + Inositol	Casein + Thiamin	Casein + Thiamine + Inositol	Casine + Nicotinic Acid	Ammonium Nitrate	Ammonium Nitrate + Histidine
Microsporum ferrugineum	4+		4+				
Trichophyton equinum							
T. gallinae						4+	4+
T. megninii						0	4+
T. mentagrophytes (dog)	4+		4+		4+		
T. mentagrophytes (455)	4+		4+		4+		
T. mentagrophytes (536)	4+		4+		4+		
T. rubrum (Dar)	4+		4+				
T. rubrum (J.H.)	4+		4+				
T. schoenleinii	4+	4+	4+	4+			
T. verrucosum	0	+1	0	4+			
T. soudanense	4+		4+		4+		
T. terrestre (414)	4+		4+		4+		
T. terrestre (285)	4+		4+		4+		
T. terrestre (Brasil)	4+		4+		4+		
T. tonsurans	± to 1+				4+		
T. violaceum	±		4+				

*A 4+ indicates good growth, while a 0 indicates no growth. Spaces left blank are not critical in differentiation of species, with growth usually occurring on these media.

TABLE 10.--The differentiation of *Trichophyton rubrum* and *T. mentagrophytes*.

Organism	Undersurface Pigment Color: Sabouraud's Dextrose	Undersurface Pigment Color: Cornmeal Dextrose	Undersurface Pigment Color: Potato Dextrose	In Vitro Hair Invasion	Urease Test Days to Red Color
T. mentagrophytes (Dog)	Brown-red	White	White	+	5 days
T. mentagrophytes (455)	Brown-red	Lt. Tan	White	+	6 days
T. mentagrophytes var. *erinacei* (536)	Yellow	Yellow	Yellow	+	7 days*
T. rubrum (Dar)	Wine Red	Wine Red	Red	-	-
T. rubrum (J.H.)	Wine Red	Wine Red	Red	-	-

*The color of the medium was light red on the 7th day, deep red on the 12th day.

produced a wine red pigment in all the media used, including Sabouraud's glucose agar, corn meal agar, and potato dextrose agar. *Trichophyton mentagrophytes* (dog) and *T. mentagrophytes* (455) both produced a red pigment in Sabouraud's glucose agar but no red pigment in any other media. The third strain, *T. mentagrophytes* (536), developed a yellow undersurface color in all these media.

b. In Vitro Hair Test: Hair invasion has been used to differentiate Trichophyton rubrum and T. mentagrophytes. Microscopically, T. mentagrophytes showed a wedge shaped or irregular clear area in the hair, apparently from protsolysis. Mycelium and spores were observed clinging to the outside of the hair shaft. The three isolates of T. mentagrophytes invaded the hair in 5 replications but one strain, T. mentagrophytes (455) (var. granulosum) was always slower.

Trichophyton rubrum did not invade the hair shaft. Microscopically, mycelial elements and spores of T. rubrum could be seen clinging to the outside of the hair shaft but no wedge shaped or irregularly digested areas were observed (see Table 10).

c. The Urease Test: Trichophyton mentagrophytes isolates possess the enzyme urease (Rosenthal, 1965) and are able to split urea in a modification of Christensen's medium. Thus it was possible to differentiate T. mentagrophytes from T. rubrum. The T. mentagrophytes strains showed the presence of urease while the T. rubrum isolates gave no indication of this enzyme at the end of 21 days as indicated in Table 10.

II. The Amount of Protein in the Antigenic Fractions of the Dermatophytes

The amount of protein in the 40 different antigenic fractions was determined using Lowry method (1950). The

tests were performed before the immunodiffusion studies were begun. The results are shown in Table 11 for the supernatant and pellet antigens. The range in milligrams per milliliter for the supernatant fractions was 0.725 to 3.700, while the range for the pellet fractions was 0.350 to 1.950.

III. Results of Precipitinogenic Studies

The reactions of the homologous and heterologous antigen-antibody systems, using fractions of a dermatophyte isolate, were compared by two procedures. In the first procedure the antigenic fraction was placed in the center well and diffused against the antisera of the 2 fractions (supernatant and pellet) from an isolate placed in 6 equidistant depots (3 adjacent wells per antiserum) surrounding the antigen fraction well (Plate III). In the second procedure the antiserum was placed in the center well and diffused against the antigenic fractions from an isolate in 3 of the 6 equidistant wells, alternated with fractions from other organisms (Plate IV through IX). The latter procedure was followed in testing the relationships of antigen fractions for all organisms used in these investigations.

A. Homologous Antigen-Antibody Reactions

In immunodiffusion investigations of 6 fractions, 2 from each of 3 species of *Trichophyton*, employing homologous

TABLE 11.--The amount of protein expressed in mg/ml in the dermatophytes fractions as determined by the Lowry et al. (1951) method.

Organism	Supernatant	Pellet
T. mentagrophytes (dog)	1.850	1.000
T. mentagrophytes (455)	2.380	1.950
T. mentagrophytes (536)	1.600	0.750
T. rubrum (Dar)	1.475	0.850
T. rubrum (J.H.)	1.705	1.010
T. terrestre (414)	1.842	1.322
T. terrestre (285)	2.225	1.825
T. terrestre (Brasil)	3.050	0.890
T. equinum	2.100	1.475
T. gallinae	1.340	1.040
T. menginii	0.725	0.350
T. schoenleinii	1.275	0.625
T. soudanense	3.700	0.700
T. tonsurans	2.275	1.500
T. verrucosum	1.550	0.810
T. violaceum	2.900	0.735
E. floccosum	1.175	0.475
K. ajelloi	2.025	0.875
M. ferrugineum	2.150	0.550
M. gypseum	1.800	0.475

fraction antigens and antisera from 4 to 9 precipitation lines were observed (Tables 12, and Plates III through IX).

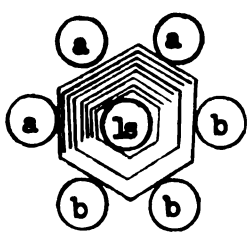
Plate III

Diagrams of immunodiffusion test precipitation lines produced by homologous and heterologous antigen-antibody systems of the fractions within the species Trichophyton mentagrophytes (dog), T. terrestre (414), and T. rubrum (Dar)

a = T. mentagrophytes (dog) supernatant fraction antiserum
b = T. mentagrophytes (dog) pellet antiserum
1s = T. mentagrophytes (dog) supernatant antigens
1p = T. mentagrophytes (dog) pellet antigens

c = T. terrestre (414) supernatant fraction antiserum
d = T. terrestre (414) pellet antiserum
6s = T. terrestre (414) supernatant antigens
6p = T. terrestre (414) pellet antigens

e = T. rubrum (Dar) supernatant fraction antiserum
f = T. rubrum (Dar) pellet antiserum
4s = T. rubrum (Dar) supernatant antigens
4p = T. rubrum (Dar) pellet antigens



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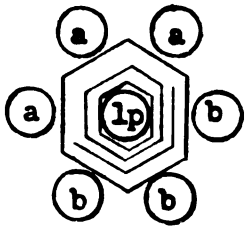

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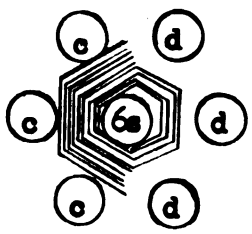

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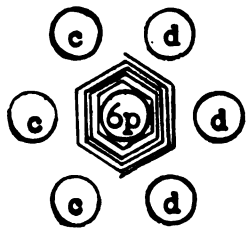

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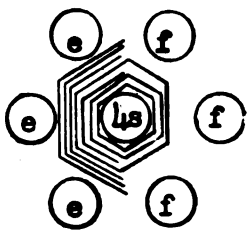

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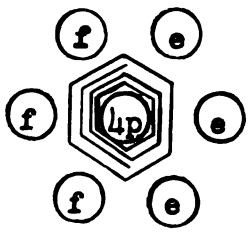

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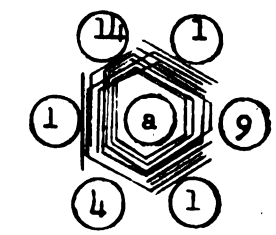
Plate IV

Diagrams of immunodiffusion test precipitation lines produced by supernatant antigen fractions from selected dermatophytes and T. mentagrophytes (dog) supernatant and pellet antisera.

a = T. mentagrophytes (dog) supernatant fraction antiserum
b = T. mentagrophytes (dog) pellet antiserum

1 = T. mentagrophytes (dog) supernatant antigen
2 = T. mentagrophytes (455) supernatant antigen
3 = T. mentagrophytes (536) supernatant antigen
4 = T. rubrum (Dar) supernatant antigen
5 = T. rubrum (JH) supernatant antigen
6 = T. terrestre (414) supernatant antigen
7 = T. terrestre (285) supernatant antigen
8 = T. terrestre (Brasil) supernatant antigen
9 = T. equinum supernatant antigen
10 = T. gallinae supernatant antigen
11 = T. megninii supernatant antigen
12 = T. schoenleinii supernatant antigen
13 = T. soudanense supernatant antigen
14 = T. tonsurans supernatant antigen
15 = T. verrucosum supernatant antigen
16 = T. violaceum supernatant antigen
17 = E. floccosum supernatant antigen
18 = K. ajelloi supernatant antigen
19 = M. ferrugineum supernatant antigen
20 = M. gypseum supernatant antigen



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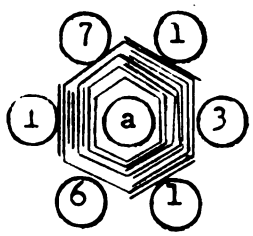

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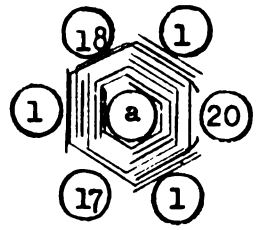

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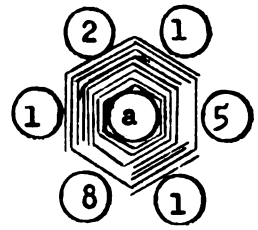

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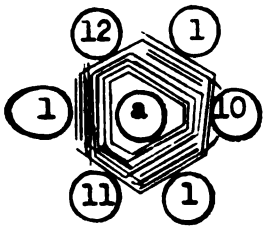

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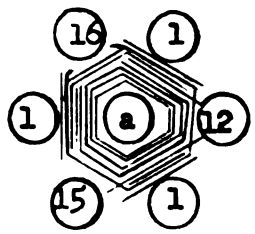

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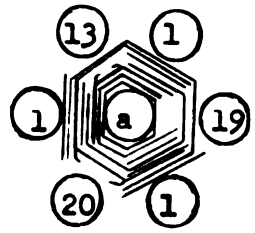

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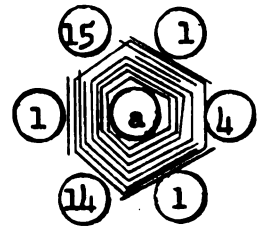

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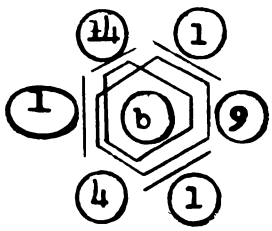

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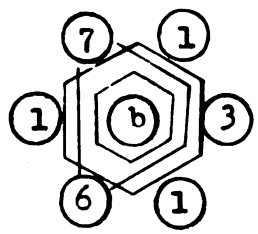

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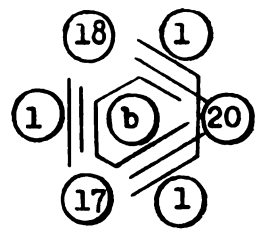

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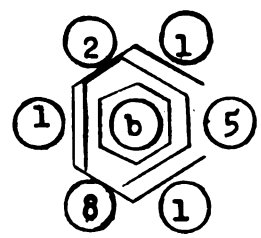

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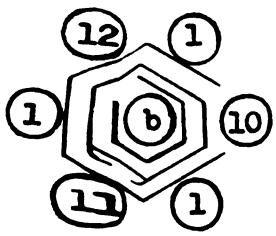

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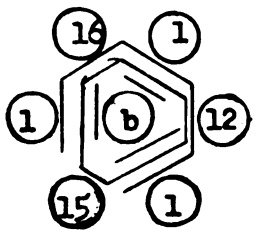

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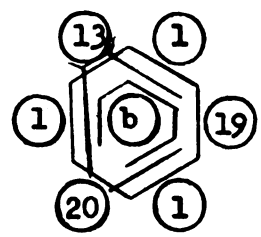

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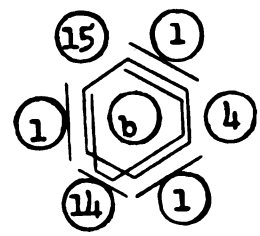

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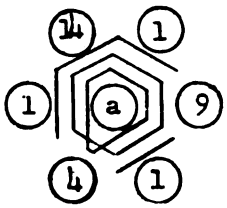
Plate V

Diagrams of immunodiffusion test precipitation lines produced by pellet antigen fractions from selected dermatophytes and T. mentagrophytes (dog) supernatant and pellet antisera.

a = T. mentagrophytes (dog) supernatant fraction antiserum.
b = T. mentagrophytes (dog) pellet antiserum.

1 = T. mentagrophytes (dog) pellet
2 = T. mentagrophytes (455) pellet
3 = T. mentagrophytes (536) pellet
4 = T. rubrum (Dar) pellet
5 = T. rubrum (JH) pellet
6 = T. terrestre (414) pellet
7 = T. terrestre (285) pellet
8 = T. terrestre (Brasil) pellet
9 = T. equinum pellet
10 = T. gallinae pellet
11 = T. megninii pellet
12 = T. schoenleinii pellet
13 = T. soudanense pellet
14 = T. tonsurans pellet
15 = T. verrucosum pellet
16 = T. violaceum pellet
17 = E. floccosum pellet
18 = K. ajelloi pellet
19 = M. ferrugineum pellet
20 = M. gypseum pellet



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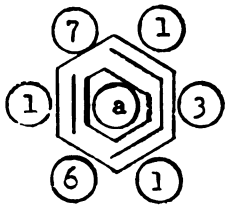

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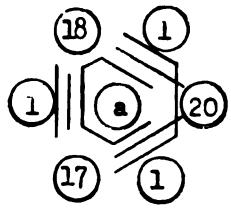

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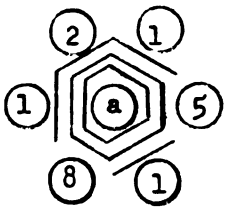

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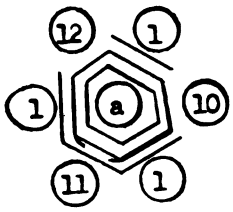

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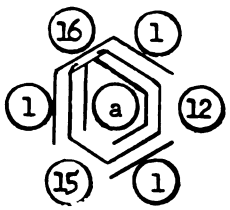

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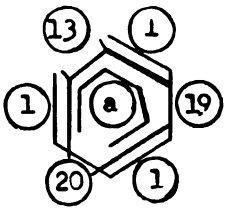

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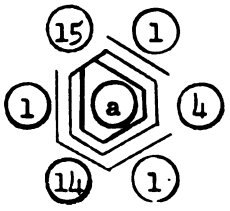

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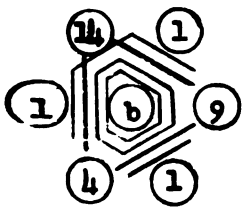

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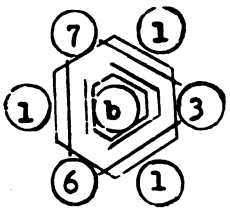

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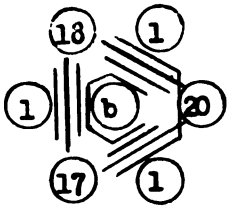

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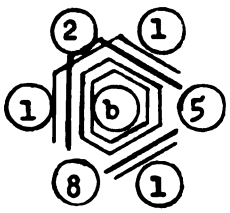

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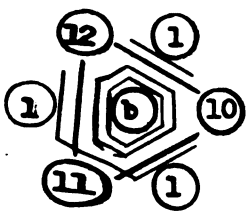

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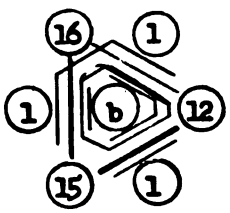

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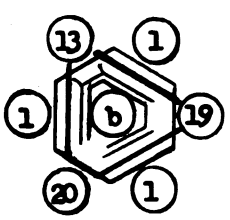

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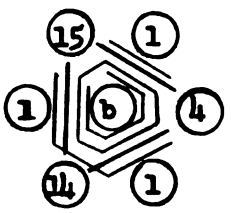


P

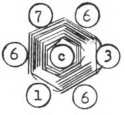
Plate VI

Diagrams of immunodiffusion test precipitation lines produced by supernatant antigen fractions from selected dermatophytes and T. terrestre (414) supernatant and pellet antisera.

c = T. terrestre (414) supernatant fraction antiserum
d = T. terrestre (414) pellet antiserum

1 = T. mentagrophytes (dog) supernatant antigen
2 = T. mentagrophytes (455) supernatant antigen
3 = T. mentagrophytes (536) supernatant antigen
4 = T. rubrum (Dar) supernatant antigen
5 = T. rubrum (JH) supernatant antigen
6 = T. terrestre (414) supernatant antigen
7 = T. terrestre (285) supernatant antigen
8 = T. terrestre (Brasil) supernatant antigen
9 = T. equinum supernatant antigen
10 = T. gallinae supernatant antigen
11 = T. megninii supernatant antigen
12 = T. schoenleinii supernatant antigen
13 = T. soudanense supernatant antigen
14 = T. tonsurans supernatant antigen
15 = T. verrucosum supernatant antigen
16 = T. violaceum supernatant antigen
17 = E. floccosum supernatant antigen
18 = K. ajelloi supernatant antigen
19 = M. ferrugineum supernatant antigen
20 = M. gypseum supernatant antigen

A



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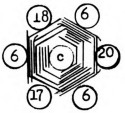


C

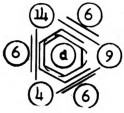
D

E

F

G

H

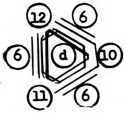


I

J

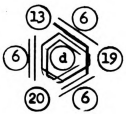
K

L



M

N



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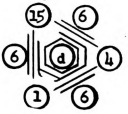


P

Plate VII

Diagrams of immunodiffusion test precipitation lines produced by pellet antigen fractions from selected dermatophytes and T. terrestre (414) supernatant and pellet antisera.

c = T. terrestre (414) supernatant fraction antiserum
d = T. terrestre (414) pellet antiserum

1 = T. mentagrophytes (dog) pellet
2 = T. mentagrophytes (455) pellet
3 = T. mentagrophytes (536) pellet
4 = T. rubrum (Dar) pellet
5 = T. rubrum (JH) pellet
6 = T. terrestre (414) pellet
7 = T. terrestre (285) pellet
8 = T. terrestre (Brasil) pellet
9 = T. equinum pellet
10 = T. gallinae pellet
11 = T. megninii pellet
12 = T. schoenleinii pellet
13 = T. soudanense pellet
14 = T. tonsurans pellet
15 = T. verrucosum pellet
16 = T. violaceum pellet
17 = E. floccosum pellet
18 = K. ajelloi pellet
19 = M. ferrugineum pellet
20 = M. gyseum pellet

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

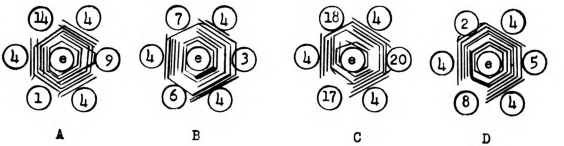
P

Plate VIII

Diagrams of immunodiffusion test precipitation lines produced by supernatant antigen fractions from selected dermatophytes and T. rubrum (Dar) supernatant and pellet antisera.

e = T. rubrum (Dar) supernatant fraction antiserum
f = T. rubrum (Dar) pellet antiserum.

1 = T. mentagrophytes (dog) supernatant antigen
2 = T. mentagrophytes (455) supernatant antigen
3 = T. mentagrophytes (536) supernatant antigen
4 = T. rubrum (Dar) supernatant antigen
5 = T. rubrum (JH) supernatant antigen
6 = T. terrestre (414) supernatant antigen
7 = T. terrestre (285) supernatant antigen
8 = T. terrestre (Brasil) supernatant antigen
9 = T. equinum supernatant antigen
10 = T. gallinae supernatant antigen
11 = T. megninii supernatant antigen
12 = T. schoenleinii supernatant antigen
13 = T. soudanense supernatant antigen
14 = T. tonsurans supernatant antigen
15 = T. verrucosum supernatant antigen
16 = T. violaceum supernatant antigen
17 = E. floccosum supernatant antigen
18 = K. ajelloi supernatant antigen
19 = M. ferrugineum supernatant antigen
20 = M. gypseum supernatant antigen


14 4 4 e 9 1 4
A
7 4 4 e 3 6 4
B
18 4 4 e 20 17 4
C
2 4 4 e 5 8 4
D
12 4 4 e 10 11 4
E
16 4 4 e 12 15 4
F
13 4 4 e 19 20 4
G
15 4 4 e 1 14 4
H

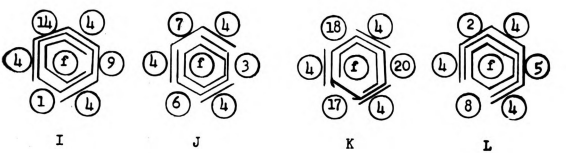
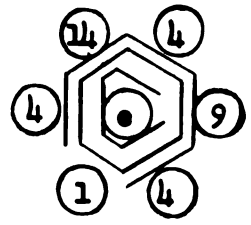

14 4 4 f 9 1 4
I
7 4 4 f 3 6 4
J
18 4 4 f 20 17 4
K
2 4 4 f 5 8 4
L
12 4 4 f 10 11 4
M
16 4 4 f 12 15 4
N
13 4 4 f 19 20 4
O
15 4 4 f 1 14 4
P

Plate IX

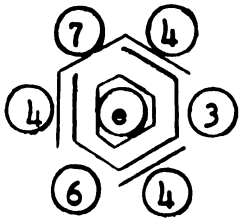
Diagrams of immunodiffusion test precipitation lines produced by pellet antigen fractions from selected dermatophytes and T. rubrum (Dar) supernatant and pellet antisera.

e = T. rubrum (Dar) supernatant fraction antiserum.
f = T. rubrum (Dar) pellet antiserum

1 = T. mentagrophytes (dog) pellet
2 = T. mentagrophytes (455) pellet
3 = T. mentagrophytes (536) pellet
4 = T. rubrum (Dar) pellet
5 = T. rubrum (JH) pellet
6 = T. terrestre (414) pellet
7 = T. terrestre (285) pellet
8 = T. terrestre (Brasil) pellet
9 = T. equinum pellet
10 = T. gallinae pellet
11 = T. megninii pellet
12 = T. schoenleinii pellet
13 = T. soudanense pellet
14 = T. tonsurans pellet
15 = T. verrucosum pellet
16 = T. violaceum pellet
17 = E. floccosum pellet
18 = K. ajelloi pellet
19 = M. ferrugineum pellet
20 = M. gypseum pellet



A



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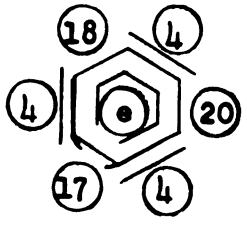

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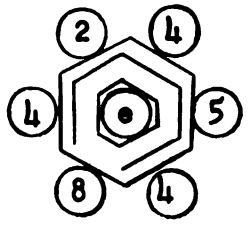

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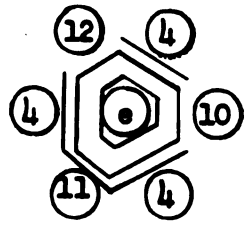

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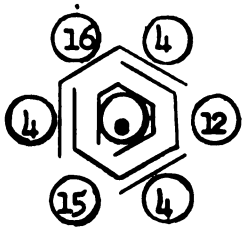

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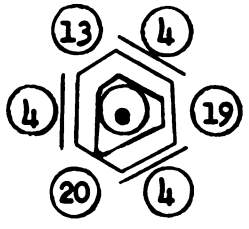

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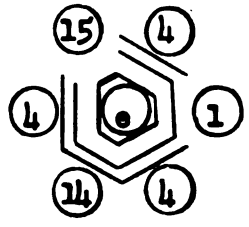

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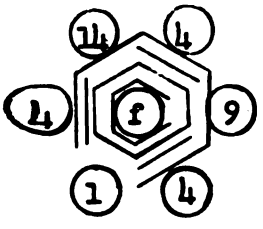

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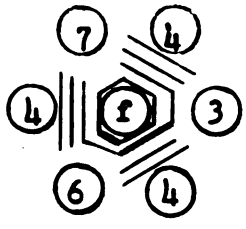

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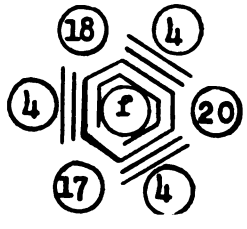
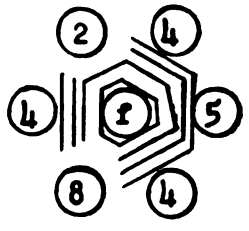


K



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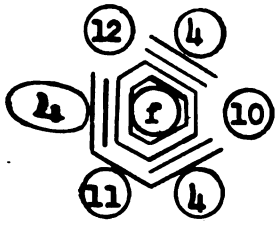

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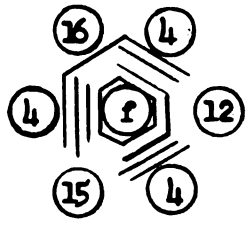

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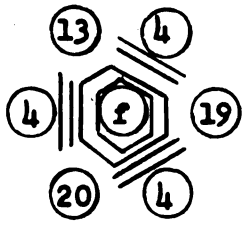
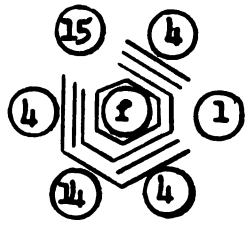


O



P

Reactions of the homologous antigen-antibody system of T. mentagrophytes (dog) supernatant developed 8 lines (Plate III,A and Plate IV, A through H) in all agar gel slides except for one that had 7 lines (Plate IV, G). This was evidence that the T. mentagrophytes (dog) fraction possessed a minimum of 8 precipitonogens.

The pellet fraction of T. mentagrophytes (dog) reacted with the homologous antiserum to produce 4 lines of precipitation (Plate III, B and Plate V, I through P).

Trichophyton terrestre (414) supernatant fraction formed 9 lines of precipitation with the homologous antiserum (Plate III, C and Plate VI, A through H). Five lines (Plate III, D and Plate VII, I through P) were produced with the homologous pellet fraction antigen-antiserum system of T. terrestre (414) which was originally isolated from the hair of a pig in England.

The least reactive homologous antigen-antibody supernatant system, T. rubrum (Dar) fraction, produced 7 lines (Plate III, E and Plate VIII, A through H). The pellet fraction of T. rubrum developed 4 precipitation lines when reacted with T. rubrum (Dar) pellet antiserum (Plate III, F and Plate IX, I through P).

Most of the lines of precipitation occurring between reactants in homologous systems of the supernatant fractions were of greater intensity than the lines in homologous systems of the pellet fractions. Replenishing reactant wells

enhanced the intensity but did not increase the number of bands observed.

The low intensity precipitation lines were not distinguishable in photographs and some of the moderate intensity lines were lost. Thus diagrams were made in place of photographs to show the precipitation lines as they appeared on the original agar gel slides (Plates III through IX).

B. Precipitinogenic Relationship of the Different Antigenic Fractions Within the Same Isolate

The number of precipitation lines formed in the heterologous fraction-antiserum systems within an isolate are given in Tables 12 and 13.

Trichophyton mentagrophytes (dog) supernatant when diffused against T. mentagrophytes (dog) pellet antiserum produced 3 lines which were identical with 3 of the 8 lines produced by the homologous antigen-antibody system (Plate III, A). The pellet fraction of T. mentagrophytes (dog) reacted with the supernatant antiserum of the same isolate to develop 3 lines which were identical with 3 of the 4 lines formed in the homologous pellet antigen-antibody system for this organism (Plate III, B).

Four precipitation bands (Plate III, C) were observed when T. terrestre (414) pellet antiserum was reacted with the supernatant fraction in the agar gel slide. These lines were common with 4 lines in the supernatant homologous

antigen-antibody system. The supernatant antiserum developed 4 lines when reacted with the pellet fraction of T. terrestre (414). These were lines of common identity with 4 of the 5 lines that were observed when the pellet homologous system was tested (Plate III, D).

The heterologous antigen-antibody systems of T. rubrum (Dar) produced 3 lines when the pellet antiserum was reacted with the supernatant fraction, and 3 lines when the supernatant antiserum diffused against the pellet antigenic fraction of this strain. The latter 3 lines appeared to be common with 3 of the 4 lines in the homologous pellet system (Plate III, E and F).

To conclude briefly, the number of lines produced in the homologous antigen-antibody systems indicated the supernatant fractions contained a greater number of antigenic substances than the pellet fractions. Except for one line, the number of lines produced by the pellet homologous systems were identical to lines in the supernatant, hence at least one antigen in the different pellet fractions was not found in the supernatant fractions.

C. Precipitinogenic Relationship of the Three Reference Strains

The number of precipitation lines formed in the homologous and heterologous reactions of the reference strains are summarized in Table 12.

TABLE 12.--The number of precipitation lines formed by homologous and heterologous antigen-antibody reactions of reference strains.

Antigenic Fractions	Antisera					
	T. mentagrophytes (dog)		*T. rubrum* (Dar)		*T. terrestre* (414)	
	supernatant	pellet	supernatant	pellet	supernatant	pellet
T. mentagrophytes (dog)						
supernatant	8 3		5 2		4 2	
pellet	3 4		2 2		3 3	
T. rubrum (Dar)						
supernatant	5 2		7 3		3 2	
pellet	2 2		3 4		2 2	
T. terrestre (414)						
supernatant	4 2		3 2		9 4	
pellet	2 2		2 2		4 5	

When *T. mentagrophytes* (dog) supernatant antiserum was reacted with the supernatant fraction of *T. terrestre* (414), 4 lines were developed which were identical with 4 precipitation lines formed with the *T. mentagrophytes* supernatant antigen-antibody system (Plate IV, B). In the other heterologous systems between antisera produced by the fractions of this organism after injection into rabbits and the fractions of *T. terrestre* (414) only 2 bands formed and these were lines of identity (Plate IV, J; V, B; and V, J).

TABLE 13.--The number of precipitation lines formed by homologous and heterologous antibody-antibody reactions.

Antigenic Fractions	Antisera: T. mentagrophytes (dog) supernatant	T. mentagrophytes (dog) pellet	T. rubrum supernatant	T. rubrum pellet	T. terrestre supernatant	T. terrestre pellet
*T. mentagrophytes (dog) supernatant	8	3	5	2	4	2
pellet	3	4	2	2	3	3
*T. mentagrophytes (455) supernatant	8	3	6	3	4	2
pellet	3	4	2	2	2	2
*T. mentagrophytes (536) supernatant	7	3	5	2	4	3
pellet	3	3	2	2	3	3
*T. rubrum (Dar.) supernatant	5	2	7	3	3	2
pellet	2	2	3	4	2	2
*T. rubrum (J. H.) supernatant	5	2	6	3	3	2
pellet	2	2	3	4	2	2
**T. terrestre (414) supernatant	4	2	3	2	9	4
pellet	2	2	2	2	4	5
**T. terrestre (285) supernatant	4	2	3	2	8	4
pellet	2	2	2	1	4	5
**T. terrestre (Brasil) supernatant	4	2	3	1	8	3
pellet	2	2	2	1	4	5
*T. equinum supernatant	4	2	3	2	3	1
pellet	2	2	2	2	1	1
*T. gallinae supernatant	6	2	5	2	4	3
pellet	2	2	2	2	3	3
*T. megninii supernatant	5(+1)+	3	5	3	3	2
pellet	3	3	3	3	2	2
*T. verrucosum supernatant	5	1	5	1	3	1
pellet	1	1	1	1	1	1
**T. schoenleinii supernatant	5	2	4	2	4	2
pellet	2	2	2	2	2	2(+1)+
**T. soudanense supernatant	5	2	6	2	4	2
pellet	2	2	2	2	2	2
**T. violaceum supernatant	5	2	5	2	3	2
pellet	2	2	2	2	1	2
**T. tonsurans supernatant	7	3	3	3	3	2
pellet	3	3	3	3	2	2
E. floccosum supernatant	3	1	2	1	3	1
pellet	1	1	1	1	1	1
K. ajelloi supernatant	4	1	3	2	3	3
pellet	1	1	2	2	3	3
M. ferrugineum supernatant	3	2	4	2	3	2
pellet	2	2	2	2	2	2
M. gypseum supernatant	4	1	4	2	4	2
pellet	1	1	2	2	2	2

* Ectothrix species of Trichophyton.
** Saprophytic species of Trichophyton.
*** Endothrix species of Trichophyton.
\+ One line more in one of the three plates used in the tests.

Five precipitation lines developed in the reaction of T. mentagrophytes (dog) supernatant with T. rubrum (Dar) supernatant. These lines were common with 5 of the 8 lines in the homologous T. mentagrophytes (dog) supernatant test (Plate IV, A) while the pellet fraction of T. rubrum (Dar) produced 2 lines when reacted with this antiserum (Plate IV, I). The pellet antiserum of T. mentagrophytes (dog) diffused against the supernatant and pellet fractions of T. rubrum produced 2 bands identical with 2 bands in the T. mentagrophytes (dog) homologous system (Plate IV, I and Plate V, A).

The supernatant fraction of T. mentagrophytes produced 4 lines, 2 common and 2 partially identical lines in the homologous system of T. terrestre (414) when double diffusion tests were performed with T. terrestre (414) supernatant antiserum (Plate VI, B), and only 3 lines (2 common lines and 1 partially common) were developed when the latter supernatant antiserum was diffused against the T. mentagrophytes (dog) pellet (Plate VII, B). Trichophyton terrestre (414) antiserum produced by the pellet reacted with the T. mentagrophytes (dog) supernatant by producing 1 common and 1 partially common line with those in the homologous T. terrestre (414) pellet system (Plate VI, J), but 3 lines (2 common and 1 partial identical line) were formed by the pellet fraction of T. mentagrophytes (dog) and this antiserum (Plate VII, J).

Trichophyton terrestre (414) supernatant antiserum diffused against T. rubrum (Dar) supernatant developed 1 common and 2 partially identical lines with the homologous T. terrestre (414) supernatant system (Plate VI, A). Two precipitation lines were produced by the reaction of T. rubrum (Dar) pellet with the latter antiserum (Plate VII, A), T. rubrum (Dar) pellet diffused against T. terrestre (414) pellet antiserum (Plate VII, I and P) and T. terrestre (414) pellet antiserum with T. rubrum (Dar) supernatant (Plate VI, I). One line in each of the last 3 reactions was of partial identity and the other 1 was common to lines in the homologous system to which each was compared.

The supernatant T. rubrum (Dar) antiserum produced 5 precipitation bands with the antigens in the supernatant fraction of T. mentagrophytes (dog). Four of these lines were continuous with 4 lines in the T. rubrum (Dar) homologous antigen-antibody system indicating that there is a minimum of 4 identical antigens in the supernatant fractions of these 2 organisms. The fifth line shows partial identity with a fifth line in the homologous system of T. rubrum (Dar) (Plate VIII, A). At least 2 antigens were common in T. rubrum pellet fraction with T. mentagrophytes (dog) supernatant and pellet fractions as indicated by continuous precipitation lines (Plate VIII, I; IX, A; and IX, I).

When T. rubrum (Dar) supernatant antiserum was diffused against T. terrestre (414) supernatant fraction, 3 precipitation lines appeared in the agar medium which were

continuous with lines in the homologous system of T. rubrum (Dar) (Plate VIII, B). Two identical antigens were evident in reactions of T. rubrum (Dar) supernatant antiserum with T. terrestre pellet (Plate IX, B), T. rubrum (Dar) pellet antiserum with T. terrestre supernatant Plate VIII, J), and the latter antiserum with T. terrestre (414) pellet (Plate IX, J).

To conclude briefly, the results after crossing the fractions of the reference strains with the reference antisera indicate a number of homologous antigens in these strains. Trichophyton rubrum and T. mentagrophytes show a closer relationship than T. terrestre.

D. Precipitinogenic Relationship of the Different Isolates Within a Species

In an effort to establish that the number of lines produced by any isolates of a species against a specific antiserum are characteristic of that species, 3 isolates (strains) of Trichophyton mentagrophytes, 3 isolates of T. terrestre, and 2 isolates of T. rubrum were used for the immunodiffusion studies.

Trichophyton mentagrophytes strains were selected from 3 widely different environments. The dog strain was from tinea on a dog in the Michigan State University Veterinary Clinic while the "455" strain was obtained from a tinea pedis case in Toronto, Canada, and the third strain "536" was from tinea on a hedgehog in New Zealand.

The number of lines produced by the homologous antigen-antibody systems of T. mentagrophytes (dog) was 8 for the supernatant and 4 for the pellet (Plate IV, A; Plate V, I; and Table 13). When the 2 antisera produced by this isolate after injection into rabbits were diffused against the fractions of the other 2 isolates, the only difference was found in the T. mentagrophytes (536) strain (Plate IV, B, D, and J, L; Plate V, J and L). The supernatant fraction of this strain produced 1 less line with antiserum of T. mentagrophytes (dog) supernatant (Plate IV, B and Table 13) as did the pellet fraction when diffused against the pellet antiserum of T. mentagrophytes (dog) (Plate V, J and Table 14). Trichophyton mentagrophytes (455) fractions produced the same number of lines as the homologous systems of T. mentagrophytes (dog) with 1 line being of partial identity in the supernatants (Plate IV, D).

The strains of T. terrestre were also from widely different locations. Trichophyton terrestre strain "414" was isolated from the skin of a pig in England, while strain "285" was isolated from the soil in California and strain "Brasil" was isolated from the Brasilian soil in 1961 by the author (1963).

Homologous antigen-antibody systems developed 9 precipitation lines with the supernatant fraction and 5 lines with the pellet fractions from T. terrestre (414). In the immunodiffusion test with T. terrestre (414) fraction antisera with fractions from strains "285" and "Brasil,"

a difference of 1 line occurred (total of 8 lines) with the supernatant fraction systems in all 3 times repeated (Table 13, Plate VI, B and D), and in all cases, the pellet systems of these isolates produced 5 lines (Table 13, Plate VII, J and L). Precipitation lines numbered 4 (Plate VI, J; Plate VII, B and D) in all of the agar gel tests in which pellet diffused against supernatant antigen-antibody systems for the strains of T. terrestre, except when the "Brasil" strain supernatant fraction was reacted with T. terrestre (414) pellet antiserum, only 3 lines were produced (Plate VI, L).

The 2 isolates of T. rubrum were form human cases of ringworm of the body, tinea corporis, isolated in Michigan. Diffusion of supernatant fraction T. rubrum (Dar) antiserum against the supernatant fraction of T. rubrum (J.H.) strain developed 6 lines, one line less than was produced by the homologous T. rubrum (Dar) supernatant antigen-antibody system (Plate VIII, D and Table 13). The heterologous systems of supernatant and pellet antigens produced 3 lines of precipitation (Plate VIII, L; IX, D; and Table 13) while the pellet antigens of T. rubrum (J.H.) and the pellet antiserum of T. rubrum (Dar) produced 4 lines (Plate IX, L; and Table 13).

The precipitation lines observed in the reactions of heterologous systems above were all common with lines in the respective reference strains antigen-antibody systems except when T. mentagrophytes (455) supernatant was diffused

against T. mentagrophytes (dog) supernatant antiserum, 1 line of partial identity was produced.

To summarize, the fractions from the isolates of T. terrestre were identical in reactions with T. terrestre (414) antisera except for 1 less line in the reactions of the supernatant fractions. The same was true for fractions from the isolates of T. rubrum. There was one more line between the reference strain supernatant reactants. However, in T. mentagrophytes reactions, strain 455 supernatant fraction reacted like the homologous dog strain with 1 line being of partial identity and T. mentagrophytes (536) differed by lacking 1 line in the supernatant reaction and 1 line in the pellet fraction.

E. Precipitinogenic Relationship of Selected Dermatophytes and the Reference Strains

Strains were selected from the various dermatophytes to compare reactions between different isolates within a species, species in different genera, and species in the different groups (based on "in vivo" hair invasion) of the Trichophyton genus when reacted with the reference strains antisera.

The reactions of fractions from strains belonging to the species used in the production of the reference strains antisera with heterologous species antisera produced some variation in the number of precipitation lines between strains of a species (Table 13).

Trichophyton mentagrophytes (455) supernatant produced 6 lines, of which 1 line showed partial identity (Plate VIII, D) while T. mentagrophytes (536) developed 5 lines (Plate VIII, B) when reacted against T. rubrum (Dar) supernatant antiserum. The supernatant fractions of these 2 strains when crossed with the pellet antiserum of T. rubrum (Dar) showed 1 line difference in the reactions. Two lines appeared with the 536 strain (Plate VIII, J) and 3 lines with the 455 strain (Plate VIII, L). The pellet fractions of these 2 strains reacted with the supernatant and pellet antiserum of T. rubrum (Dar) to produce 2 lines (Plate IX, B, J and L) except in the reaction of T. mentagrophytes (455) with T. rubrum (Dar) supernatant antiserum (Plate IX, D) in which 3 lines were produced. When T. mentagrophytes (455 and 536) were reacted with T. terrestre (414) antisera, only the supernatant reactants produced the same number of lines which was 4 (Plate VI, B and D) but the lines differed in that strain 455 had 1 line that was of partial identity and strain 536 had 2 partially identical lines. In all other reactions (supernatant against pellet antiserum, pellet against supernatant antiserum, and pellet against pellet antiserum) T. mentagrophytes (536) produced 3 lines with 1 of the 3 being of partial identity (Plates VI, J; VII, B and J) while T. mentagrophytes (455) produced 2 lines (Plates VI, L; VII, D and L).

The reactions of the fractions of T. rubrum (J.H.) with the antisera of T. terrestre (414) and T. mentagrophytes (dog) were as follows: Supernatant fraction with T. mentagrophytes (dog) supernatant antiserum, 5 lines developed (Plate IV, D)

and with T. terrestre (414) supernatant antiserum 3 lines developed (1 of partial identity) (Plate VI, D). With both reference strains, the reactions of supernatant antisera with T. rubrum (J.H.) pellet fraction (Plates V, D and VII, D), pellet antisera with supernatant fraction (Plates IV, L and VI, L), and pellet antisera with pellet fraction (Plates V, L and VII, L) developed 2 lines each.

Similar reactions were observed between the 2 strains of T. terrestre that were not used as reference strains when reacted with T. mentagrophytes (dog) antisera. Four lines appeared between the supernatant reactants (Plate IV, B and D). The reactions which remain all produced 2 lines of precipitation between reactants of each strain (Plate IV, J and L; VII, B and D, J and L). However, the reactions of T. terrestre (Brasil) differed by 1 line in the tests performed with T. rubrum (Dar) antisera. The lines that were produced are: 3 lines (1 line between T. terrestre (285) and antiserum is of partial identity) with the supernatant reactants (Plate VII, B and D), 2 lines with supernatant antisera against pellet fraction (Plate IX, B and D) 1 line with pellet reactants (Plate IX, J and L) and 1 line with supernatant T. terrestre (Brasil) diffused against T. rubrum pellet antiserum (Plate VIII, L), while T. terrestre (285) developed 2 lines (Plate VIII, J).

In the above reactions all the lines are common with the reference strain lines on either side of the non-reference strains in the agar slides unless otherwise stated.

For convenience the Trichophytons are placed into groups based on colony morphology or on the method of in vivo hair invasion. At present, the most frequently used system of classification is based on in vivo hair invasion (Emmons et al., 1963) and has been used as a basis for the organization of the results. The divisions and examples are:

1. Ectothrix species of Trichophyton invade the hair folicle, penetrate the hair, grow to the keratogenous zone, then the mycelium breaks out onto the surface of the hair and fragments into arthrospores. Examples are: T. mentagrophytes, T. equinum, T. rubrum, T. verrucosum, T. gallinae, and T. megninii.

2. Endothrix species of Trichophyton invade the hair in the same manner described for the ectothrix, however, the mycelium remains inside and breaks into chains of large arthrospores. Examples are: T. tonsurans, T. schoenleinii, T. violaceum, and T. soudanense.

3. Ectothrix-endothrix type of invasion is a combination of both. An example is: T. simii.

4. Hair not invaded. An example is: T. concentricum.

5. Saprophytic in soil. Examples are: T. terrestre, T. georgiae, and T. gloriae.

Two of the reference strains used belong to the ectothrix species were reacted with antisera produced by using the reference strains.

The number of precipitation lines formed by the fractions from the ectothrix species in reactions with reference antisera is shown in Table 13. The ectothrix species included T. mentagrophytes, T. rubrum, T. equinum, T. gallinae, and T. megninii. All of the species possessed 1 or more antigens related to those of the reference strains.

The reactions of the supernatant fractions of the ectothrix species varied from 8 to 7 in the reference ectothrix strains T. mentagrophytes (dog) and T. rubrum (Dar) homologous antigen-antibody systems to 4 lines in the heterologous system of T. equinum and T. mentagrophytes (dog) antiserum (Plate IV, A). When this antigenic fraction of T. equinum was diffused against T. rubrum (Dar) supernatant antiserum, 5 instead of 4 lines were produced (Plate VIII, A). Supernatant fractions of T. gallinae and T. megninii (Plate IV, E) produced 6 lines when diffused against T. mentagrophytes (dog) antiserum and 5 lines when diffused against T. rubrum (Dar) antiserum (VIII, E) while T. verrucosum produced 5 bands with each of these reference antisera (IV, F and H; VIII, F and H). The 2 reference ectothrix strains produced 4 lines in homologous pellet systems while the pellet fractions of the other ectothrix species produced the following when reacted with these 2 reference antisera: T. megninii produced 3 lines (Plate V, M; IX, M), T. equinum (Plate V, I; IX, I), and T. gallinae (Plate V, M; and IX, M) produced 2 lines each and T. verrucosum (Plate IV, N, P; IX,

N and P) produced only 1 line with each of the ectothrix reference strains. When the supernatant fractions of these ectothrix species were diffused against the 2 reference ectothrix species pellet antisera and when the pellet fractions were diffused with the supernatant antisera, the following number of lines resulted: T. megninii, 3 lines (Plate IV, M, V, E; IX, E), T. gallinae, 2 lines (Plate IV, M, V, and E; VIII, M; IX, E) T. equinum, 2 lines (Plate IV, I: V, A; VIII, I; IV, A), and T. verrucosum, 1 line (IV, N and P; V, E and H; VIII, N and P; IX, E and H).

Reactions of the ectothrix filtrates with the reference saprophytic strain, T. terrestre (414), antisera are as follows: T. gallinae developed 4 lines (VI, E), T. equinum (VI, A), T. megninii (VI, E), and T. verrucosum (VI, F and H), produced 3 lines when the reactants were supernatant in origin; T. gallinae (Plate VI, M; VII, E and M), developed 3 lines, T. megninii (VI, M; and VII, E and M), 2 lines, while T. equinum (Plate VI, I; VII, A and I), T. verrucosum (Plate VI, N and P; VII, F, H, N and P), produced 1 line each when the supernatant and pellet fractions diffused against T. terrestre (414) pellet antiserum, and supernatant antiserum with pellet fractions.

The endothrix species used in this investigation were T. schoenleinii, T. soudanense, T. tonsurans, and T. violaceum. Each fraction of these species produced at least 1 line when reacted with the reference antisera (Table 13). Reactions of the fractions from the different endothrix Trichophyton

species with the reference ectothrix species antisera produced the following precipitation lines: 5 lines developed for T. schoenleinii (Plate IV, E and F), T. soudanense (Plate IV, G), and T. violaceum (Plate IV, F), while T. tonsurans (IV, A and H) had 7 lines when the supernatant fractions were diffused against T. mentagrophytes (dog) supernatant antiserum; supernatant fractions against T. rubrum (Dar) supernatant antiserum T. soudanense (Plate VIII, G) produced 6 lines, T. violaceum (Plate VIII, F) developed 5 lines, T. schoenleinii (Plate VIII, E and F) exhibited 4 lines and T. tonsurans (Plate VIII, A and H) showed 3 lines. Pellet fractions with supernatant antisera, supernatant fractions against pellet antisera, and pellet fractions with pellet antisera showed the following: T. tonsurans (Plate IV, I: V, A and I: VIII, I; IX, A and I) produced 3 lines while T. schoenleinii (Plate IV, M; V, E, M and H; VIII, E and M), T. soudanense (Plate IV, O; V, G and O; VIII, O; IX, G and O), and T. violaceum (IV, N; V, F and N; and VIII, O, G and O) developed 2 lines each.

As the fractions of the endothrix species were diffused against the antisera of T. terrestre (414), 2 species, T. schoenleinii (Plate VI, E and F), and T. soudanense (Plate VI, G) developed 4 lines, and 2 species, T. tonsurans (Plate VI, A and H) and T. violaceum (Plate VI, F) produced 3 lines when the reactants were supernatant. Two precipitation lines were observed in each reaction of pellet reactants, pellet fractions with supernatant antisera, and pellet antisera with

supernatant fractions for T. schoenleinii (Plate VI, M; VII, E, M and N), T. soudanense (VI, O; VII, G and O), and T. tonsurans (VI, I; VII, A, H and I), while T. violaceum (VI, N; VII, N) produced only 1 line when the pellet fraction was diffused against the supernatant antiserum of T. terrestre (414) (Plate VII, F).

It should be pointed out that the number of lines produced by the pellet fractions of the reference strains and strains which belong to the same species produced 1 more line than any of the above Trichophyton species with the exception of T. mentagrophytes (536) strain.

The immunodiffusion results indicate that there may be a closer relationship among species of the Trichophyton as grouped by colony morphology rather than in vivo hair invasion.

Epidermophyton floccosum.--The number of precipitation lines formed by the fractions of E. floccosum and the reference strains are shown in Table 13. The supernatant fraction of E. floccosum produced 3 identical precipitation lines with T. mentagrophytes (dog) (Plate IV, C), and T. terrestre (414) (Plate VI, C) supernatant antisera but only 1 identical line and 1 of partial identity with T. rubrum (Dar) supernatant antiserum (Plate VIII, C). Of the 3 remaining reactions with fractions of this organism with each reference strain, each produced a precipitation line of identity (Plate IV, K; V, C and K; Plate VI, K; Plate VII, C and K), except with T. rubrum (Dar) antisera which developed a line of partial identity in each reaction Plate VIII, K; IX, C and K).

Keratinomyces ajelloi.--In the immunodiffusion tests of K. ajelloi supernatant fraction against the reference antisera the following precipitation bands developed: 4 with T. mentagrophytes (dog) supernatant antiserum (Plate IV, C), 3 with T. rubrum (Dar) supernatant antiserum 3 with T. terrestre (414) supernatant antiserum (Plate VI, C), 3 with T. terrestre (414) pellet antiserum (Plate VI, K), 2 with T. rubrum (Dar) pellet antiserum (Plate VIII, K) and 1 with T. mentagrophytes (dog) pellet antiserum (Plate IV, K). The pellet fraction reactions resulted in the following: 1 line with both antisera of T. mentagrophytes (dog) (Plate V, C and K), 2 lines with both antisera of T. rubrum (Dar) (Plate IX, C and K). All lines were continuous with lines resulting from reactions between reactants of reference strains except the partial identity lines in T. terrestre (414) immunodiffusion tests, the second line from the outside was always of partial identity. These results are summarized in Table 13.

The 2 species E. floccosum and K. ajelloi, from 2 different genera indicate no close relationship in these immunodiffusion tests to any other species except in 2 instances. Trichophyton mentagrophytes (dog) supernatant antiserum produced the same identical lines when diffused against the supernatants of M. ferrugineum and E. floccosum supernatants and K. ajelloi supernatant fraction produced the same number of lines but not all identical with M. gypseum. These reactions were not evident with the other antisera.

Microsporum ferrugineum.--Reactions of the 2 fractions of M. ferrugineum with the reference antisera resulted in a difference of 1 line in the 12 double diffusion reactions (Table 13). When T. rubrum (Dar) antiserum was diffused against M. ferrugineum supernatant, 4 lines developed (Plate VIII, G), while only 3 lines developed with T. mentagrophytes, and T. terrestre (414) supernatant antisera (Plate IV, G; VI, G). The lines were continuous with lines in the homologous antigen antibody systems and indicated common antigens. Two lines of identity appeared between each of the other reactions of the fractions of M. ferrugineum and the reference antisera (Plates IV, O; V, G and O; VI, O; VII, G and O; VIII, O ; IX, G and O).

Microsporum gypseum.--The supernatant fractions of M. gypseum reacted similarly with all supernatant antisera by producing 4 precipitating lines between reactants (Table 13). The outer line in each reaction was a line of partial identity while the other 3 were lines of identity (Plate IV, C and G; VI C and G; VIII, C and G). Lines of precipitation numbered 2 when the M. gypseum fractions were diffused against the antisera of T. rubrum (Dar) and T. terrestre (414) (Plates VIII, K and O; IX, C, G, K and O; VI, K and O; and VII, C, G, K and O) in the 6 possible combinations left. Reactions of these fractions with T. mentagrophytes (dog) antisera produced 1 line each (Plate IV, K, O; V, C, G, K and O). The outer precipitation line was a partial identity and the others were lines of identity between the following

reactions: M. gypseum supernatant and T. terrestre (414) pellet antisera (Plate VI, K and O) and M. gypseum pellet and T. terrestre (414) supernatant antisera (Plate VII, C and G) and T. terrestre (414) pellet antiserum (VII, K and O).

Thus not only did the fractions from the 2 Microsporum species differ in the number of precipitation lines produced when diffused against the 6 reference antisera but the lines of identity were not always the same when compared to the reference strain reactions (Plate IV, G and O; VI, G and O; VIII, G). The number and arrangement of lines indicated a closer relationship of these 2 species to each other than to any of the genera or species studied. Keratinomyces ajelloi appears to be a close relative to M. gypseum and M. ferrugineum.

DISCUSSION

Many variable factors must be considered in the system employed in immunodiffusion techniques. The utilization of fractions separated by centrifugation in diffusion reactions and for eliciting antibodies in rabbits is not ideal. Not only should one expect the various antigens present in the fraction to exist in varying concentrations, but also, the fraction preparation itself to contain a wide variety of fragments from subcellular parts, and metabolic products of the fungal cells. These substances may have varying affinities for combining with the determinant groups of different antigens, thus altering antigenic specificity or completely blocking the antigen.

In this investigation no attempt beyond centrifugation was made to purify the fractions used to produce antibodies in rabbits.

The method employed to elicit antibodies is another factor that may alter immunodiffusion results. A form of hyperimmunization was adopted due to the low antigenicity of the fungi used. Crowle (1961) reported that "hyperimmunization tends to induce production of a range of antibodies which make the antiserum likely to cross-react even with distantly related antigens."

There was no direct evidence to show that the form of hyperimmunization employed caused non-specificity of the antibodies. However, each of the 40 fractions of the 20 dermatophytes reacted with each reference serum in various degrees as illustrated by the variation in the number of precipitation lines formed between reactants. The reactions of homologous antigen-antibody systems of reference strains gave evidence of specificity in both fractions. The results suggest that the antibodies possessed at least moderate specificities.

The variation of individual animal in response to injections of antigens is another factor influencing the quality of antisera, and, consequently affecting the immunodiffusion results. In this study, there was some variation in the number of antigens that were detectable when a fraction was diffused against antisera obtained from different rabbits that had been injected with identical filtrates. The antiserum for each rabbit was tested individually and only those which developed the largest number of precipitation bands in homologous reactions were used for investigating the precipitinogenic relationships.

During incubation periods precipitation lines are formed at varying rates and have different relative intensities. A dense line developing at a rapid rate may sometimes mask the low intensity, slower developing line. This limitation was minimized with observations made at 8 hour intervals during incubation of the agar diffusion slides.

It is well to realize that the aforementioned shortcomings are inherent in immunodiffusion studies when interpreting results.

As shown earlier, the results of immunodiffusion studies of 40 fractions from 20 dermatophytes with the 6 reference antisera suggest that antigenic differences exist among various strains of dermatophytes. These variances not only exist among species but also among strains within a species. However, a definite pattern of antigenic relationships was noted within species.

The observation of identical precipitinogens in E. floccosum, K. ajelloi, Microsporum sp., and Trichophyton sp., substantiates the results reported by many authors of cross reactions among the dermatophytes in serological tests (Jadassohn et al., 1937; Huppert, 1955; Tomomatsu, 1961; Reyes and Friedman, 1966).

Jadassohn et al. (1937) established by use of the Schultz-Dale method that Achorion Quinckeanum, Trichophyton gypseum (both now synonyms of T. mentagrophytes), Epidermophyton Kaufman (= E. floccosum), and Achorion Schoenleinii (= Trichophyton schoenleinii) had 1 antigen in common, the first three had 1 in common, and the first two had 1 in common, while each had 1 specific antigen. The precipitation lines in this work showed these 3 species to have 3 common antigens when T. mentagrophytes (dog) supernatant antiserum was diffused against the supernatant fraction of the 3 species, 2 when T. rubrum (Dar) antiserum

was used, and only 1 when T. terrestre antiserum was used. This difference is probably due to the presence of a greater number of similar antigens in the fraction used to produce the T. mentagrophytes (dog) antiserum reacted with the supernatant fractions of the other species.

The number of lines observed in this investigation were greater than those previously reported in the literature when dermatophytes were used in the agar gel immunodiffusion technique. Landay (1961), and Dyson and Landay (1963) reported only 2 lines in homologous antigen-antibody reactions using extracts of T. mentagrophytes and extracts from 21 other isolates of this species as antigens. These two precipitation lines were identical or partially identical with two of the three lines produced by the T. rubrum homologous system. The one line difference, occurring with the T. rubrum system, was considered to have resulted from the action of a species specific antigen. In the present investigation the number of lines observed in supernatant homologous antigen-antibody systems for T. mentagrophytes was 8 and 7 for T. rubrum while the number of lines that were identical was five. Reyes and Friedman (1966) detected four lines when T. rubrum and T. mentagrophytes were diffused against T. rubrum antiserum, with all four lines common. No species specific line was detected.

The results of precipitation lines of the Trichophyton species are recorded, compared and discussed by groups based on in vivo hair invasion. This follows the arrangement

in current reference books (Emmons et al., 1963; Ajello et al., 1963; Rebell et al., 1964; and Beneke, 1966). Grouping the dermatophytes in this manner is not supported by evidence in this work. It must be remembered that the grouping of the species on the clinical characteristic of in vivo hair invasion was not originally intended to show a phylogenitic relationship of these Trichophyton sp.

In another method of grouping, the species of Trichophyton have been separated on colony morphology, using color, surface texture, surface profile, etc. for differentiation. Tomomatsu (1961) using the precipitation test, an antigen dilution technique, showed no major antigenic differences among the five groups of Trichophyton (Gypseum, Rubrum, Crateriform, Faviform, and Rosaceum groups), but reported a closer antigenic relationship among these fungi than with the members of the genera Microsporum and Epidermophyton.

The results of this study indicate the fractions from the species that have been grouped together on colony characteristics as the "Faviform group," T. schoenleinii, T. verrucosum and T. violaceum, show a close relationship to each other based on the number of lines, including the number of identical lines produced with the reference antisera. Trichophyton soudanense fractions also reacted in a similar manner to the fractions of the species of the "Faviform group" to the reference antisera, thus a close serological relationship exists between these four species.

Trichophyton gallinae and T. megninii fractions diffused against the reference antisera resulted in lines showing a close serological relationship to each other. The lines were not all identical lines, indicating that they are not the same organisms, but the occurrence of identical lines and partially identical lines with the total number of lines separate the two organisms apart from other species in the genus Trichophyton. These two organisms have been placed in the "Rosaceum group" of the Trichophyton genus.

Only one species has been placed in the Crateriform group," T. tonsurans. This organism displayed a close serological relationship to T. mentagrophytes but less so to T. rubrum.

The "Rubrum group" contains one species, T. rubrum. Serologically this organism appeared closely related to T. mentagrophytes while the number and arrangement of lines varied with the different reference strains.

Two species have been placed in the "Gypseum group," T. mentagrophytes and T. equinum. The results of this investigation indicates the two organisms are not very close serologically. The supernatant fraction of T. equinum produced lines closest in number and arrangements to T. rubrum fractions with the reference antisera.

Fractions of Trichophyton terrestre, a saprophytic species, reacted with reference antisera to produce a number of lines indicating a closer relationship to T. mentagrophytes (dog) than to T. rubrum (Dar). However, T.

terrestre antiserum reacted with the supernatant of M. gypseum to produce the same number of lines as the supernatant of T. mentagrophytes (dog). Thus, this species does not appear to belong to any of the designated groups.

On the basis of the present results it would appear from the serological viewpoint that the morphological groupings might have a better basis than the clinical basis for groupings of the Trichophyton sp.

Tomomatsu (1961) found that the extracts and antisera of the species in the genera Microsporum and Epidermophyton produced stronger reactions with each other than with the species of the genus Trichophyton. The three species of Microsporum showed no considerable antigenic differences. He obtained stronger reactions when homologous antigen-antibody systems were used. Observations in the present investigations indicate by the number of identical and partially identical lines formed, that the Trichophyton species are related closer to each other than with species of other genera tested. In addition, Microsporum ferrugineum fractions reacted with the reference antisera to produce fewer lines than fractions from the species of the Trichophyton genus and showed a closer resemblance to but not identical with the reactions to fractions of M. gypseum.

The relationship of M. ferrugineum to the genus Microsporum rather than Trichophyton was also shown by Biguet et al. (1964) using immunoelectrophoresis techniques. They found this species closer to three other species of this

genus (M. audouinii, M. langeroni, and M. canis) than to the species used in this investigation, M. gypseum. This would explain why the two species, M. gypseum and M. ferrugineum, were not closer in the present investigations.

Fractions of K. ajelloi and E. floccosum developed only a few, seldom identical lines when reacted with the reference antisera. Thus these two species are not closely related serologically to any of the Trichophyton species studied but K. ajelloi seems closer related to the Microsporum species.

When tests were performed with the antisera of a strain diffused against the supernatant fraction of the same strain, all the lines which appeared between the pellet antiserum and the supernatant fraction were identical with some of the lines between the supernatant reactants. Thus, the identical lines resulted from identical antibodies from the two antisera reacting with the same antigens in the fraction. Using each reference antiserum separately, only one precipitation line of the 4 or 5 formed during the diffusion of pellet antiserum against pellet fraction was not identical with the lines formed between supernatant antiserum and the pellet fraction within a reference strain. This indicates that the 2 antisera (supernatant and pellet from each reference strain) possessed 3 or 4 similar antibodies (depending on antiserum). Since both antisera formed by a reference strain contained identical antibodies,

one can suggest 3 possibilities for this. The first is that the 2 fractions contain some like antigenic make up; and second that not all of the supernatant antigenic material was removed during the separation procedure by centrifugation, thus a pellet fraction was contaminated with supernatant material, since the supernatant contained soluble substances and complete removal is hard to obtain; and third, a combination of the previous 2 possibilities may be the explanation.

Thus a certain number of identical antibodies was produced when the fractions of a strain with some identical antigens were injected into rabbits.

In the comparison of the fractions from the different strains within a species with the homologous antigen-antibody system, one precipitation line difference occurred when the supernatant fraction diffused against the reference antiserum of that species. The additional line appeared between the supernatant homologous systems in each reference strain [T. terrestre (414), T. rubrum (Dar), and T. mentagrophytes (dog)] and T. mentagrophytes (455) supernatant against T. mentagrophytes (dog) antiserum. These two T. mentagrophytes strains resembled each other morphologically even though they were isolated from different sources. The (dog) strain was from a dog ringworm, and strain (455) was from a case of human tinea pedis. However these dermatophytes are not specific on animal or human in their pathogenicity. In a comparison of pellet fractions with different isolates of

a species to the reference antiserum of the same species it was discovered that all the pellet isolates of a species produced the same number of precipitation lines, 1 detectable line more than any other type of pellet reaction except T. mentagrophytes (536). This 1 additional line between pellet reactants within a species appeared not to be identical with any precipitation lines that occurred in the reactions between supernatant antisera and the pellet fractions. Thus there is a component in the pellet that is specific to the species if T. mentagrophytes (536) is not considered or considered another species.

Okada and Yamamura (1964) detected 3 components which were indicated as "highly liver" specific in the (DOC) extracts of microsomes from chicken livers and 4 components in the unsedimented fraction. Some of the components of the unsedimented fraction (equivalent to the supernatant fraction in the present work) were common with some of the components in the microsomal fraction (equivalent to the pellet fraction in this study). Okada and Sato (1963) found somewhat the same results when working with chicken kidney fractions.

Since there was 1 less line in the supernatant reactions for each of the strains in the species to which the reference strain belongs except in T. mentagrophytes (455) which is the same variety (var. granulosum) as the T. mentagrophytes (dog), and since no supernatant fractions of other species of the dermatophytes produced as many identical or partially identical lines as the homologous supernatant systems it appears that these lines might be due

to differences produced in the strains within the species. The difference in the number of lines using the pellet reactants was the same for all the strains of a species except T. mentagrophytes (536) and never as many precipitation lines appeared to form between pellet fraction of the other species of dermatophytes and the pellet reference antisera. It is possible that this additional line might be species specific. Many more strains must be tested to determine this possibility.

If the additional line is species specific then T. mentagrophytes would be divided into at least 2 species based upon this present work, since the pellet reaction between T. mentagrophytes (dog) pellet antiserum and T. mentagrophytes (536) pellet produced 3 lines and the homologous reaction produced 4 lines as did the reaction with the strain 455. This indicates 2 immunological types of T. mentagrophytes as has been reported by Landay (1961), and Dyson and Landay (1963) in the 24 isolates that they used in agar gel immunodiffusion test.

Huppert (1955) established 3 distinct immunological groups among 15 strains of T. mentagrophytes used. Neither Landay nor Huppert worked with T. mentagrophytes var. erinacei (= 536) which was first reported in 1963 by Smith and Marples.

Grappel et al. (1967) reported significant differences in the serological reactions of the neutral polysaccharides isolated from M. quinckeanum (= T. mentagrophytes),

T. granulosum (= T. mentagrophytes), T. interdigital (= T. mentagrophytes), T. rubrum, and T. schoenleinii, and antiserum to autoclaved antigens of M. quickeanum in immunodiffussion and complement fixation tests. They worked with 3 species thought to be varieties of one species.

Ajello and Cheng (1967) reported cleistothecia were produced by crossing strains of T. mentagrophytes var. granulosum [the same as strains (455) and (dog) in the present work]. Upon back crossing the parent strains they found that the organism is heterothallic and the incompatibility is controlled by a single locus with 2 alleles "A" and "a". When 21 strains of T. mentagrophytes var. granulosum were crossed with the "A" and "a" strains only 4 produced cleistothecia. Thus, are the 17 strains that did not produce cleistothecia incompatible due to another factor or are they members of a different species?

With the 2 immunological groups of T. mentagrophytes reported by Landay (1961); the 3 distinct immunological groups established by Huppert (1955); the significant differences in reactivity of the neutral polysaccharides of 3 species reported by Grappel et al. (1967), considered to be morphologically one species, T. mentagrophytes, by most mycologists; the obtaining of only 4 cultures with cleistothecia from the many strains of T. mentagrophytes var. granulosum when crossed by Ajello and Cheng (1967); and the differences between strain 455 (T. mentagrophytes var.

granulosum) and strain 536 (T. mentagrophytes var. erinacei) in the present immunodiffusion study; there is probably sufficient evidence to indicate that the present species, T. mentagrophytes, is a complex of species as was found for the imperfect species, Microsporum gypseum.

In the present investigation the fractions from the reference strains which possessed the largest amount of protein per ml determined by the Lowery et al. (1951) method, produced the highest intensity lines. Protein is not the only type of organic compound that will elicit antibody formation and cause antigen-antibody reactions. Grappel et al. (1967) showed that neutral polysaccharides are antigenic components with significant differences in the serological reactions among the glucans and galactomannans II but not among the galactomannans I in the 5 species of Trichophytons used. The polysaccharide determination was not investigated in the present study.

Ito (1965) isolated 22 purified fractions having trichophytin activity from the mycelium and culture filtrate of T. mentagrophytes var. asteroides. The majority of these fractions consisted of peptide-containing polysaccharides. Two ribonucleic acid fractions were from the mycelium while 5 simple peptides fractions and a simple polysaccharide fraction were from the culture medium. Ito found the intensity of the antigenic activity of the fractions were of the following order; peptide-containing polysaccharide >

sugar-containing peptide $\geq$ simple peptide $\fallingdotseq$ ribonucleic acid > simple polysaccharide.

The number of precipitation lines in this study was not as great as the number of different protein fractions detected by Shechter et al. (1967) when the nondialysable proteins from culture filtrates and mycelial mats were fractionated by disc electrophoresis. The number of fractions varied from 9 to 15 and the fractions identities varied. They found a high number of homologous fractions present in M. gypseum and M. canis, and in T. mentagrophytes and T. tonsurans. Similar results were obtained in the present studies for the latter 2 organisms. Homologous fractions in T. mentagrophytes, T. tonsurans and T. rubrum were few as was the case for the lines in my results. They obtained only 2 homologous fractions in the 6 organisms when using the culture filtrates and no fraction was homologous for all 6 organisms in extracts from the different mycelia. This corresponds to my results as no 1 identical antigen was evident in all organisms studied.

Even though the number of lines differs in the present work from the number of fractions reported by Shechter et al. (1967), similar results were obtained in that species showed specific identities of protein fractions and antigens, and that homologous protein fractions and homologous antigens occurred in different species and species of different genera.

The sensitivity in detecting small amounts of protein is probably greater with disc electrophoresis than with the agar gel immunodiffusion technique. The rabbit's antibody producing mechanism might be able to detect the smaller quantities of proteins and produce antibodies in response to the antigens but the detection of the antigen-antibody reaction in the gel would be difficult if the antibodies and antigens are of very low concentrations.

Even though the perfect stages are being discovered for many of the dermatophytes, the recognition of species is still difficult as reported by Pore and Plunket (1967). They found the distinctive appendages of Arthroderma (the perfect genus for species of Keratinomyces and Trichophyton for which the perfect stages have been found) were of little value for determination of species, so they considered the concept of biological species. However, in their discussion of crosses, the possibility of incompatibility factors within a species was not discussed. Incompatibility factors are known to exist in strains within species of the fungi: Schizophyllum commune, Pleurotus ostreatus, Sordaria fimicola and others (K. Esser, 1966). When incompatibility factors are discovered some of these biological species in the dermatophytes may become only strains. Thus immunodiffusion, disc electrophoresis, immunoelectrophoresis and other techniques will still be needed to supply the necessary information of the true relationships.

SUMMARY

Twenty strains of the dermatophytes including strains within a species and species from the 4 different genera were grown in Sabouraud's-glucose broth for two weeks, filtered and rinsed. The mycelium was macerated, filtered and the filtrate centrifugred at 20,000 X g for 20 minutes. The supernatant was separated into two fractions by centrifugation at 104,000 X g for 4 hours. The supernatant and pellet fractions from the reference strains, Trichophyton mentagrophytes (dog), T. terrestre (414), and T. rubrum (Dar.) were used to produce antisera in rabbits by a series of intramuscular and intraperitoneal injections.

The precipitinogenic relationships of the 20 representatives were compared by using the 2 fractions of each organism and the reference strains antisera in the agar-gel slide immunodiffusion technique.

Antigenic similarities and dissimilarities existed between supernatant and pellet fractions within a strain, the fractions of the strains of a species, the fractions of the reference strains and the fractions of the 12 other dermatophytes species.

The supernatant homologous antigen-antibody reactions produced more lines than the pellet homologous reactions. Thus, more detectable antigenic substances were in the supernatant fractions.

The supernatant and pellet fractions of a strain possessed some homologous antigens, with all but one antigen in the pellet being identical to antigens in the supernatant.

The supernatant fractions of the strains within a species, produced one less line than the homologous antigen-antibody system except in T. mentagrophytes (455). The fractions of this strain or isolate acted identically to the fractions of the reference T. mentagrophytes (dog) strain and these two strains are of the same variety granulosum. These is the possibility of a "strain antigen" producing the additional line between homologous supernatant systems.

The pellet fractions of the different organisms within a species did not differ except in one case, the strain 536 T. mentagrophytes var. erinacei. The pellet homologous reactions produced one line more than in reactions with heterologous pellet fractions. Thus there is the possibility of a "species antigen." Since the pellet fraction of T. mentagrophytes var. erinacei did not indicate the presence of such an antigen, it could be considered a separate species.

The separation of the trichophytons into groups according to their colony morphology (a separation little used at present), Gypseum-, Crateriform-, Faviform-, Rosaceum-, and Rubrum- groups (with T. terrestre in a group for saprophytic species) was suggested by the degree of homogeneity of the reactions by the members of these groups.

Trichophyton soudanense was closest antigentically to T. violaceum, T. schoenleinii, and T. verrucosum, members of the faviform group.

Microsporum ferrugineum showed a closer relationship to M. gypseum than to any species of the Trichophytons studied, while E. floccosum and K. ajelloi differed in their reaction with each other and the other organisms in the immunodiffusion tests. Keratinomyces ajelloi appeared closer related serologically to M. gypseum.

The supernatant reference antiserum of T. mentagrophytes (dog) was the only one to demonstrate a homologous antigen in all the dermatophytes tested except K. ajelloi. While both T. mentagrophytes (dog) and T. rubrum (Dar) supernatant antisera showed one common antigen with all the trichophytons

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