THE DIFFERENTIATION OF DERMATOPHYTES BY BIOCHEMICAL AND SEROLOGICAL METHODS

by

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ABSTRACT

Classification of the dermatophytes, a group of keratinophilic fungi, depends primarily on morphological criteria, but individual identification is often difficult. Many species have been created, based on what are often minor differences in structure or unacceptable characters such as isolation from different hosts or different clinical conditions.

Biochemical techniques, used successfully in the differentiation of other micro-organisms, have had a limited application in the study of dermatophytes and were made the subject of special investigation. 22 species were grown on media containing 30 different carbon or nitrogen sources. It was found that both geophilic and zoophilic species had similar nutritional patterns, but for some, *e.g.* *Microsporum gypseum* and *Trichophyton mentagrophytes*, nutritional and biochemical tests are of little taxonomic value. Ability to hydrolyse urea allows differentiation of *T. mentagrophytes* from *T. rubrum* and *T. erinacei*, and *T. soudanense* from *M. ferrugineum*. Other nutritional differences which may be used to separate species are described. The results obtained are complementary to the classification of dermatophytes based on morphological criteria but do support the separation of certain species, *e.g.* *M. equinum*, hitherto regarded as variants of another species.

Serological investigations are described and five groups have been established, based on the presence or absence of common antigens. The results show that the genus *Microsporum*, with the exception of

M. gypseum and *M. persicolor*, is serologically distinct from the genus *Trichophyton*. *M. gypseum*, *T. ajelloi* and *M. persicolor* are serologically related, however, having common antigens. The genus *Trichophyton* was found to contain two serological sub-groups: (1) *T. mentagrophytes*-*T. rubrum*-*T. schoenleinii*, and (2) *T. quinckeanum*-*T. soudanense*-*T. erinacei*. *T. tonsurans* has close relationships with most other *Trichophyton* species. *T. violaceum* and *Epidermophyton floccosum* had few antigens in common with any other species. The two saprophytic species, *T. terrestre* and *M. cookei*, appeared to be serologically unrelated to other members of the respective genera.

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Sabouraudia, 1967, 5, 189-193.

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GENERAL INTRODUCTION

The taxonomic status of a genus or a species at any time tends to reflect the prevailing interests of workers in the field and what they accept as valid criteria for the differentiation of individual taxa. However, interests fluctuate with increase in knowledge of organisms and the development of new techniques, and as a result systems of classification may undergo radical changes with the passage of time. This is particularly well illustrated by the taxonomy of the dermatophytes. These fungi were first described and the genera *Trichophyton* and *Microsporum* established by Gruby and Malmstem in 1840 and 1845 (Tate, 1929a). For the next 60 years most of the work on dermatophytes was done by dermatologists who were primarily interested in clinical aspects of the dermatophytoses. Consequently isolates were assigned to categories mainly on the basis of the site from which they had been obtained. The first classification of these fungi, by Sabouraud (1910), was based mainly on the relationship of the parasite to the host; cultural features were used only secondarily. From the clinician's viewpoint, such a classification had, and still has, a practical value. For example, all agents of the clinical condition known as favus were brought together in the one genus *Achorion*, whereas the species causing acute inflammation were separated from those causing more chronic disease. However, the grouping together of such diverse fungi as *Trichophyton (Achorion) schoenleinii* and *Microsporum (Achorion) gypseum* is not consistent with modern mycological systems where morphological features are used as the primary basis for classification. In addition,

isolates which a mycologist would classify as a single species on the basis of colonial and morphological appearance, may be recovered from both chronic and inflammatory lesions, on the same or different patients. The publication by Sabouraud of the investigations he had made on the dermatophytes (1910) generated much enthusiasm amongst dermatologists and mycologists in the years that followed. But once interest in the dermatophytes _per se_ had been aroused, the clinical orientation of his classification was eventually recognised to be inadequate. Thus several classifications, all based upon morphological criteria, were published between 1910 and 1934 but no single scheme received general acceptance by the majority of workers in the field. Yet the ever-increasing number of described species made an acceptable mycological classification even more essential. Sabouraud himself had recognised 45 species collected into 5 genera but by 1935 the number of species had risen to nearly 200 (Gregory, 1935). Dodge (1936) listed 118 species distributed among 9 genera. The tendency to describe as a new species an isolate differing only in some minor respect from those already recognised was the main cause of this complexity. Thus, although characterisation of new species was now based on morphological criteria rather than on the clinical appearance of the lesions from which the fungi were isolated, there was still nomenclatural as well as taxonomic confusion.

In 1934 C.W. Emmons published a classification based upon morphology of the multicellular conidia (macroconidia) produced by dermatophytes on simple laboratory media. This scheme was subsequently widely adopted, and remains the classification in use today (Rebell & Taplin, 1972). Emmons recognised 3 genera, _Microsporum_, _Trichophyton_ and _Epidermophyton_ distinguished by the shape of the macroconidium, _viz._ thick-walled and fusiform; thinwalled and cylindrical; and thickwalled and

clubshaped, respectively. The shape, size and arrangement of macroconidia and microconidia, and the presence or absence of hyphal structures such as spiral hyphae, were used to delineate species. A fourth genus *Keratinomyces* was subsequently described (Vanbreuseghem, 1952) but this was later reduced to synonymy with *Trichophyton* by Ajello (1968).

Provided with a practical system of classification, into which most isolates could readily be placed, mycologists gave increasing attention to the characteristics of individual species of dermatophytes and detailed studies have been carried out on their morphological appearance and nutritional requirements (Georg, 1950, 1952; Schwartz & Georg, 1955). As a result of these studies, many of the described species have been shown to have varietal rather than specific status. In the second edition of "The Dermatophytes" Rebell and Taplin (1972) listed 15 species of *Microsporum*, 25 species in the genus *Trichophyton*, and 1 in the genus *Epidermophyton*.

One of the main purposes of any classification is to provide a means for identifying unknown isolates. The disadvantage of a classification based upon morphology alone is that morphological characteristics are particularly vulnerable to changes in the nature and conditions of cultivation. If therefore an isolate fails to produce the characteristic features its identification is impossible. As a result, there is now an increasing tendency to explore the use of criteria other than morphology in the study and classification of species.

One of the most important recent advances followed the discovery of the sexual state amongst dermatophytes. Although Nannizzi (1926) had reported the development of cleistothecia by several species, his work was subsequently discredited on the basis that he had worked with impure cultures. In 1961, however, Stockdale and Griffin, working independently,

re-discovered the sexual state of M. gypseum. Nannizzi was thus vindicated, and it was fitting that posthumous recognition should be given by establishment of the genus Nannizzia for the cleistothecial state (Stockdale, 1961). Almost immediately, sexual states were described for T. terrestre and T. ajelloi in the Ascomycete genus Arthroderma (Dawson & Gentles, 1961). Since then several more sexual forms (8 of Nannizzia, and 10 of Arthroderma) have been described as shown in Table 1. All known sexual states are heterothallic. By mating known and sexually-unclassified strains, both familial and phylogenetic relationships may be established (Padhye & Carmichael, 1969). However, even for species known to possess a sexual state, it is often extremely difficult to induce sexual reproduction in the laboratory. The nature of the medium has been shown to influence the mating process (Padhye, Sekhon & Carmichael, 1973; Kwon-Chung, 1967). Most dermatophytes known to form a sexual state are geophilic, or belong to species which are rarely pathogenic for man. The majority of species have not yet been shown able to reproduce in this way and mating studies are therefore not practicable. It may be that adaptation, particularly by the anthropophilic species, to growth on human skin has been accompanied by the loss of sexual potency.

Since both morphology and mating studies are influenced by changes in media and techniques, a considerable amount of effort has been expended in searching for characteristics which are not affected by growth conditions. In some instances, attempts have been made to detect antigenic similarities and differences between species using serological procedures such as complement fixation, haemagglutination or fluorescent antibody. Other attempts have involved chemical analyses of enzyme fractions or electrophoretic studies of fungal extracts.

TABLE 1 SEXUAL AND ASEXUAL STATES OF DERMATOPHYTES

Sexual State	Asexual State	Habitat
Nannizzia gypsea	Microsporum gypseum	Soil
N. incurvata	M. gypseum	Soil
N. fulva	M. fulvum	Soil
N. obtusa	M. nanum	Pigs
N. persicolor	M. persicolor	Voles
N. cajetana	M. cookei	Soil
N. grubyia	M. vanbreuseghemii	Soil
N. racemosa	M. racemosum	Rats
Arthroderma uncinatum	Trichophyton ajelloi	Soil
A. quadrifidum	T. terrestre	Soil
A. lenticularum	T. terrestre	Soil
A. insingulare	T. terrestre	Soil
A. gertleri	T. vanbreuseghemii	Soil
A. simii	T. simii	Monkeys
A. benhamiae	T. mentagrophytes	Animals
A. vanbreuseghemii	T. mentagrophytes	Animals
A. ciferri	T. georgiae	Soil
A. gloriae	T. gloriae	Soil

Nutritional studies have also been used, principally to distinguish individual species or small groups of species.

The superficial nature of ringworm does not normally lead to the development of detectable circulating antibody, although the presence of skin-sensitising antibody may be shown by skin testing. Some workers have reported antibodies to ringworm fungi in the sera of infected individuals using haemagglutination, complement fixation, charcoal agglutination and immunodiffusion (Reyes & Friedman, 1966; Grappel, Blank & Bishop, 1971, 1972). However, since antibody was also detected in the sera of unaffected individuals, the significance of these findings is questionable. It is also possible that reactions may be due to non-specific glycopeptide (C-substance) known to be produced by fungi including dermatophytes in old cultures (Longbottom & Pepys, 1964).

Although typing of micro-organisms by serological methods has been in use for many decades, the application of such techniques to fungi dates largely from 1960, e.g. Candida (Tsuchiya et al., 1961; Murray & Buckley, 1966); "black yeasts" (Nielsen & Conant, 1967; Buckley, 1968); pathogenic actinomycetes (Murray & Mahgoub, 1968). Three groups of workers (Sharp, 1942; Grappel et al., 1967, 1968, 1969, 1970; Biguet et al., 1961, 1965) have studied antigenic differences among dermatophytes using ring-precipitation, complement fixation and immunoelectrophoretic procedures respectively. All three groups found that cross-reactivity between species was common, and as a result it has not yet been possible to use these techniques for either identification or classification.

Another serological technique, fluorescent antibody staining, has also been used in the differentiation of dermatophytes but such studies have been limited. Miura (1963) attempted to separate five species in this way, but the strong auto-fluorescence shown by all species

made it difficult to assess the results.

Schecter *et al*. (1966, 1968a, 1968b) analysed the proteins present in culture filtrates and mycelial extracts of some common dermatophytes by disc electrophoresis in acrylamide gel, and obtained patterns which were specifically distinct. 57 fractions were analysed by means of factor and cluster analysis. Groups with similar patterns corresponded closely to the genera and species already defined on morphological criteria.

Carmichael and co-workers in Canada have used pyrolysis-gas liquid chromatography to analyse extracts of several different species of dermatophytes (Carmichael, Sekhon & Sigler, 1973; Sekhon & Carmichael, 1972, 1973). Although they were able to get different patterns for each of the species studied, the technique was not then reproducible and its full potentialities remain unknown.

Despite the importance of such studies, they have not yet been sufficiently developed to replace morphological criteria as a precise means of defining individual species, and much taxonomic confusion still persists within the group. There is thus considerable scope for workers interested in the taxonomy of the ringworm fungi, to explore and evaluate various serological, chemical and biochemical techniques.

The aim of the present work was to develop systems for the classification of dermatophytes which were based on criteria other than morphology. For any system of classification to be of help to the routine microbiological laboratory, the methods used would have to be relatively simple. Many of the serological and chemical methods reviewed in the preceding paragraphs are not easily used outside specialist laboratories because reagents are complicated to prepare, equipment is

often elaborate and expensive, and the reading and interpretation of results may require specialist training. In contrast nutritional tests and certain serological methods such as gel diffusion, do not normally require elaborate equipment and are within the scope of routine laboratories. Consequently the nutritional requirements of dermatophytes have been subjected to particular study with the intention of establishing simple methods for differentiating species; secondly, an attempt has been made to demonstrate affinities by analysing antigens detectable in agar gel. A broad survey of several species was planned in preference to a more detailed study of a few species. The principal objective was to determine whether recognisable differences exist and can be exploited as a means for differentiating species.

PART I. NUTRITIONAL INVESTIGATIONS

1. REVIEW OF THE LITERATURE

CARBON NUTRITION

Interest in nutritional characteristics of dermatophytes extends back over almost ninety years to the initial observation ot Verujski in 1887 that "*Trichophyton tonsurans*", but not "*Achorion schoenleinii*", was able to grow on media containing glucose and glycerol. Bodin in 1899 and 1902 snowed that *Microsporum equinum* and *M. gypseum* grew in the presence of glucose, maltose and dextrin, while *T. quinckeanum* assimilated glucose in preference to maltose and lactose. These workers also showed that dermatophytes possessed proteolytic enzymes and could liquefy gelatine. These observations have subsequently been amplified and extended by other workers (Table 2).

Only a few workers have tested the ability of dermatophytes to grow on compounds simpler than carbohydrates, *e.g.* acetate, but their studies have shown that these compounds cannot provide adequate sources of energy (Tate, 1929a; Giblett & Henry, 1950; Schwartz & Georg, 1955; Sullivan, Bereston & Wood, 1954). This is true of fungi in general (Cochrane, 1958).

On the basis of these published results some generalisations are possible. Of the *hexoses* studied, fructose and mannose are utilised as well as or better than glucose; galactose, however, seems to be less readily assimilated. This is not surprising since mannose and fructose are stereo-isomers of glucose and transformations take place easily; both fructose and glucose enter directly into the metabolism of the cell. Galactose can be converted to glucose-1-phosphate but 3 enzymes are necessary for this reaction. Thus if these enzymes were not synthesized

TABLE 2

ASSIMILATION OF CARBOHYDRATES BY DERMATOPHYTES

Species	Source	Glucose	Mannose	Fructose	Galactose	Arabinose	Xylose	Rhamnose	Ribose	Maltose	Sucrose	Lactose	Cellobiose	Trehalose	Raffinose	Melibiose	Mannitol	Sorbitol	Glycerol	Erythritol	Starch	Dextrin	Inositol
M. canis	Goddard 1934	+	+	+	−	+				+	±	−											
	Giblett & Henry 1950	+	+	+	+	+	+				+		+			+	+	+	+			+	
	Pinetti 1947	+			−					−	−	−									+		
	Lewis & Hopper 1941	+	+	+	−						−	−			−		+				−		
	Pena Yanez 1956	+		−	+	+				+	−	−			+		−	+					
	Hopkins & Iwamoto 1923	+			+					+							+	−				+	
M. equinum	Bodin 1899	+								+	−											+	
M. gypseum	Bodin 1899	+								+	−												
	Giblett & Henry 1950	+	+	+	+						−		+			+	+	+	+			+	
	Hopkins & Iwamoto 1923	+			−					±							+	±				+	
	Pinetti 1947	+			−					−	−	−									+		
	Pena Yanez 1956	+		−	+	−				−	−	−			−		−	−					
M. audouinii	Giblett & Henry 1950	+	+	+	+	−	−				−		+			+	+	+	+			+	
	Hopkins & Iwamoto 1923	±			−					−							+	±				−	
	Pinetti 1947	+			−					−	−	−									+		
	Pena Yanez 1956	+		−	−	−				−	−	−			+		−	+					
M. cookei	Koehne 1962	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	−		+	−	+	
M. distortum	Koehne 1962	+	+	+	+	+	−	+	+	−	+	−	−	−	+	+	+	−		−	−	+	
M. nanum	Koehne 1962	[illegible]	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−		+	−	−	
T. mentagrophytes	Bocobo & Benham 1949	+	+	+	+					+	+	+			+								−
	Pinetti 1947	+			+					+	+	±									±		
	Hopkins & Iwamoto 1923	+			−					+							+	±				+	
T. interdigitale	Goddard 1934	+	+	+	+	+				+	+	−											
	Pinetti 1947	+			+					−	−	−									−		
	Mosher et al. 1936	+	+	+	+					+	+	−											
T. persicolor	Stockdale 1953	+	+		−			−		+	−	−			−		+		−				
T. quinckeanum	Hopkins & Iwamoto 1923	+			−					−							+	±				−	
	Bodin 1902	+								±		±											
	Pereiro Miguens 1959	+																					
T. rubrum	Lewis & Hopper 1941	+	+	+	−					−	−	−			−		+				−		−
	Hopkins & Iwamoto 1923	+			−					+							+	+				+	
T. megninii	Hopkins & Iwamoto 1923	±			−					−							−	−				−	
	Pinetti 1947	+			+					+	±	±									±		
T. gallinae	Hopkins & Iwamoto 1923	+			−					−							+	+				−	
T. tonsurans	Verujsky 1887	+									−												
	Hopkins & Iwamoto 1923	+			−					±							+	+				±	
	Pinetti 1947	+			+					+	+	+									+		
	Sullivan et al. 1954	+									+	+									+		
	Schwartz & Georg 1955	+	+	+	+	−	−	−		−	−	−	+	+	−				−				
T. schoenleinii	Hopkins & Iwamoto 1923	+			−					−							±	−				−	
	Pinetti 1947	−			−					−	−	−									−		
T. violaceum	Hopkins & Iwamoto 1923	±			±					±							±	±				±	
	Pinetti 1947	−			−					−	−	−									−		
T. verrucosum	Hopkins & Iwamoto 1923	−			−					−							−	−				−	

KEY

+ = growth

± = poor or little growth

− = no growth

by the fungus, or if only one of them was absent, galactose would not be utilised.

Assimilation of pentoses has rarely been investigated and studies have been largely confined to species of *Microsporum*. Arabinose is utilised by *M. canis*, *M. cookei* and *M. distortum*, but not by *M. gypseum*, *M. nanum* and *M. audouinii*. Xylose is assimilated by *M. canis*, *M. cookei* and *M. nanum*. Rhamnose and ribose were tested only by Koehne (1962) who reported utilisation by the three species he studied. However, the results of Koehne should be regarded with some reserve for two reasons: firstly, he reported the poor utilisation of glucose by *M. nanum* although the fungus will grow on the basal medium - very few fungal species have been reported as unable to utilise glucose (Cochrane, 1958). Secondly, he used asparagine as his nitrogen source. In general, asparagine supports extremely good growth since it can act both as a carbon and as a nitrogen source.

Reports on the assimilation of disaccharides are conflicting. Most workers have found that maltose is not assimilated by *M. audouinii* or by *M. gypseum*. Utilisation of maltose by *Trichophyton* species has been demonstrated by some workers but not by others. It is difficult, however, to compare results directly since materials and methods have varied widely. It is generally agreed that dermatophytes do not assimilate lactose. Cellobiose is apparently used by most dermatophytes but few studies have been made with trehalose.

Reports on the utilisation of sucrose and melibiose, and the trisaccharide raffinose, are also conflicting. If, as has been suggested (Bealing & Bacon, 1953; Edelman, 1954), the same enzyme, β-fructofuranosidase, splits both raffinose and sucrose, while α-glucosidases split only sucrose, a fungus should assimilate sucrose if it utilises raffinose but

not vice versa. From Table 2, it can be seen that reports on the Microsporum species, e.g. utilisation of sucrose by M. canis, are inconsistent. Some efforts have been made to demonstrate the presence of sucrases in dermatophytes but without success (Tate, 1929a; Chattaway, Thompson & Barlow, 1954; Thompson, 1955). There is evidence that in yeasts both melibiase and sucrase (β-fructofuranosidase) are located in the cell wall and that the reactions actually occur outside the cell membrane (de la Fuente & Sols, 1962). If the breakdown of raffinose, melibiose and sucrose takes place outside the cell membrane of dermatophytes, only the constituent hexoses would require to be transported across it. The inability to assimilate these oligosaccharides would thus be due to absence of the appropriate enzymes. If the hexoses are not utilised, the inability of dermatophytes to utilise raffinose might be due to inability to assimilate breakdown products rather than the absence of enzymes. The evidence suggests, however, that dermatophytes lack the necessary enzymes, since they are able to assimilate both glucose and fructose.

The sugar alcohols mannitol and sorbitol are broken down to fructose and glucose by a single enzyme or by closely related enzymes. While most species utilised both compounds, a few were able to catabolise only one. This may imply that one compound is used preferentially or that permeability factors may be involved.

Of the storage polysaccharides, inulin and starch are apparently not used as sources of carbon, while dextrin (a breakdown product of starch) is used by most species.

The methods used by various workers in assessing the extent to which a given substrate is used vary widely, and some appear to be of doubtful significance. "Fermentation" methods were favoured by Hopkins

and Iwamoto (1923), Pinetti (1947) and Pena Yanez and Pallares (1956), by which they meant the development of an acid pH in the medium. A change of pH almost always occurs during growth of dermatophytes but in most investigations where it has been recorded, the change was towards alkalinity rather than acidity (Hopkins & Iwamoto, 1923; Biltris, 1929; Goddard, 1934; McVeigh & Campbell, 1950). Refai and Rieth (1964), for example, showed that most common dermatophytes changed the pH of a sugar peptone broth from 6.4 to 7.2/8.5 in one month whether the carbon source was glucose, galactose, sucrose or maltose. Fermentation in the strict sense is any energy-yielding reaction in which the ultimate electron acceptor is a compound other than oxygen. Since dermatophytes do not produce acid during growth, it is more satisfactory to use a method in which the value of a given nutrient as an energy source is determined by presence or absence of growth. The following methods for determining growth on media with different carbon sources have been employed.

Dry weight was used by Verujsky (1887), Goddard (1934) and Stockdale (1953). This method records directly the increase in hyphal material and is therefore one of the most accurate measurements. It is however demanding in time, space and labour.

Measurement of colony diameters on solid media (Sullivan, Bereston & Wood, 1954; Schwartz & Georg, 1955; Koehne, 1962) records only increase in surface area; this does not necessarily reflect the amount of growth since extensive yet thin growth would be recorded as greater than a compact but abundant growth. In such methods it is useful to note both the type and amount of surface and subsurface growth (Koehne, 1962).

Visual estimation of the amount of growth (Mosher *et al.*, 1936; Lewis & Hopper, 1941; Pinetti, 1947; Giblett & Henry, 1950; Pereiro Miguens, 1959) involves a subjective assessment of area and density of

growth; no measurements of any kind are used. It depends entirely upon the skill of the observer, but with practice it can give very accurate assessments. Pinetti (1947) investigated both dry weight and visual methods. He found that results using both methods were closely comparable and concluded that it was unnecessary to carry out the more laborious objective estimation.

The media used by many of these workers have had closely similar compositions. They consisted of a nitrogen source (ammonium sulphate or more rarely ammonium nitrate), phosphates (potassium dihydrogen phosphate or a mixture of sodium and potassium phosphates) as a buffer, and either magnesium sulphate alone or a mixture of several inorganic salts. Sugar sources were generally added to give a final concentration of 1%. Some investigators, however, have used sources of nitrogen other than the ammonium ion. Bacto- or neo-peptone or casamino acids have been used by some workers (Goddard, 1934; Stockdale, 1953; Sullivan, Bereston & Wood, 1954; Schwartz & Georg, 1955; Pena Yanez & Pallares, 1956). Peptone and casamino acids are both complex sources of nitrogen, supplying the fungus with readily available sources of peptides and amino acids. Dermatophytes will grow on a medium containing only peptone at a concentration of 0.2% and inorganic salts (see page30), or on a medium containing 0.05% casamino acids. Thus, the addition of sugar to such a medium does not necessarily account for the growth observed. If, however, the nitrogen source in the medium is only slightly utilised by the fungus, this will effectively become the limiting factor. The ability to use a particular nitrogen source depends very largely upon the source of carbon used (Cochrane, 1958). Ammonium ion supports growth only in the presence of carbohydrate, thus permitting a more accurate appraisal of an organism's

ability to use a particular carbon source than the more complex sources of nitrogen which can support growth on their own.

LIQUEFACTION OF GELATINE

Verujski (1887) demonstrated the liquefaction of gelatine by T. tonsurans and T. schoenleinii, and subsequent work has shown that all dermatophytes with the exception of M. ferrugineum hydrolyse this protein (Macfadyen, 1894; Greenbaum, 1922; Giblett & Henry, 1950; Rosenthal & Sokolsky, 1965).

HYDROLYSIS OF CASEIN

Most dermatophytes appear to possess a casein-hydrolysing enzyme; an exception is M. ferrugineum (Tate, 1929a; Goddard, 1934; Giblett & Henry, 1950; Rosenthal & Sokolsky, 1965). The enzyme responsible for this hydrolysis was shown by Tate (1929b) to be very similar to trypsin, an enzyme which acts best in an alkaline medium.

HYDROLYSIS OF UREA

The ability of 15 dermatophytes to hydrolyse urea was reported by Littman (1957). He used mycelium from 7-day cultures on glucose-peptone agar as inocula and reported positive reactions in some instances within 48 hours which he attributed to the transport of preformed urease in the inoculum. Rapid hydrolysers included T. megninii, T. sulphureum, T. tonsurans, T. verrucosum, T. mentagrophytes, T. sabouraudii, T. schoenleinii and M. gypseum (positive within 48 hours). T. rubrum, T. violaceum, M. audouinii and M. canis completed the hydrolysis within 6 - 9 days, while T. gallinae, T. rotundum and E. floccosum were positive only after 12 days. T. sulphureum, T. tonsurans, T. rotundum and T. sabouraudii are considered

to be varieties of *T. tonsurans* (Schwartz & Georg, 1955); thus there appeared to be strain differences within this species.

Rosenthal and Sokolsky (1965) demonstrated hydrolysis of urea within 14 days by 10 dermatophytes: *T. mentagrophytes*, *T. tonsurans*, *T. megninii*, *T. schoenleinii*, *T. violaceum*, *M. audouinii*, *M. gypseum*, *M. canis*, *M. ferrugineum* and *E. floccosum*. Strains of *T. verrucosum* hydrolysed urea at varying rates and 4 species (*T. rubrum*, *T. gallinae*, *T. soudanense* and *T. concentricum*) were completely unable to hydrolyse it. In a more extended study of *T. mentagrophytes* and *T. rubrum*, they found a positive reaction given by 20 isolates of the former species, while 34 out of 36 isolates of *T. rubrum* were negative. They suggested that this might be used as a method to distinguish these two species.

Although the results of these workers agree for many species studied, there are differences, particularly in the rate of hydrolysis of urea. Various factors may be involved: (1) strain variation may be important. The data presented by Littman for *T. tonsurans* and Rosenthal and Sokolsky for *T. schoenleinii* and *T. verrucosum* (*vide infra*) support this hypothesis. Unfortunately, the numbers of isolates tested were insufficient for this point to be clarified; (2) differences in materials and technique may influence the result. Littman used Christensen's medium (Christensen, 1946) modified by the addition of 0.5% glucose. His method of inoculation detects particularly the presence of *preformed* enzyme, and provides little information on the rate at which the enzyme is formed *de novo*. Rosenthal and Sokolsky used bacto-urea agar, which is unmodified Christensen's medium. Unfortunately they did not record the time taken by most species tested to hydrolyse urea. Data are available for five species only:

	Positive at weeks			Negative
	1	2	3	
T. mentagrophytes	19	1		
T. rubrum		2		34
T. schoenleinii	2	1	1	
T. verrucosum	3	2	2	4
T. concentricum				4

The results for T. mentagrophytes and T. rubrum correspond to those of Littman, but the times involved differ greatly. They would suggest that T. rubrum forms the enzyme adaptively whereas T. mentagrophytes already possesses an urease. The time taken for a positive reaction to develop on bacto-urea agar may also reflect slower growth of dermatophytes on this medium.

The detection of the enzyme in mycelial extracts was attempted by Tate (1929b). He noted the presence of urease in four species of dermatophytes (T. mentagrophytes, M. canis, M. audouinii and T. schoenleinii) but failed to detect it in T. tonsurans. Thompson (1955) and Chattaway, Thompson and Barlow (1954) were however unable to extract urease from T. mentagrophytes, M. canis, M. audouinii, T. schoenleinii or E. floccosum. They suggested that activity was lacking or present in very small amounts. Since they harvested the mycelia after four days, this may have been insufficient time for the enzyme to be produced in detectable quantities. Alternatively their method of extraction (maceration in a Hughes Press and suspension in phosphate buffer) may have led to loss of the enzyme.

NITROGEN REQUIREMENTS

Specific requirements for nitrogen have been investigated by several workers (Table 3). There is general agreement that nitrate is utilised poorly, if at all, whereas the ammonium ion supports fair to good growth. An exception is T. soudanense. Peptone, yeast extract and

TABLE 3 GROWTH OF DERMATOPHYTES ON VARIOUS NITROGENOUS COMPOUNDS

	Peptone	Casein Digest	Yeast Extract	Asparagine	Urea	NH_4^+	NO_3^-	References
M. audouinii	++		+++	++		+	−	Hazen 1951, Pereiro-Miguens 1955, Giblett & Henry 1950, Lowenthal 1950
M. ferrugineum	±		++	±	+	+	−	Pereiro-Miguens 1955
M. gypseum	++		++	++	±	+	−	Giblett & Henry 1950, Pereiro-Miguens 1955
M. canis	±		++	++	++	+	−	Lowenthal 1950, Lewis & Hopper 1941, Giblett & Henry 1950, Chattaway *et al.* 1962, Pereiro-Miguens 1955
T. persicolor	+		+	±	±	±	−	Stockdale 1953
T. mentagrophytes	+++ +++++	+ ++++		++ +++	++ ++	+ +	− −	Silva & Benham 1952, Pereiro-Miguens 1955, Benham 1953 McVeigh & Campbell 1950
T. quinckeanum	+			++	+++	+	−	Pereiro-Miguens 1955
T. rubrum	+	+	+	+++	++	+	−	Area-Leao & Cury 1950, Benham 1953, Pereiro-Miguens 1955, Lewis & Hopper 1941, Silva *et al.* 1955, Silva & Benham 1952
T. tonsurans	++	++		++	+	±	−	Sullivan *et al.* 1954, Schwartz & Georg 1955, Pereiro-Miguens 1955
T. gallinae	+			+	+	+	−	Pereiro-Miguens 1955, Silva & Benham 1952
T. megninii	+	+		−	−	−	−	Pereiro-Miguens 1955, Georg 1952, Silva & Benham 1952
T. schoenleinii	+		+	+	+	+	−	Pereiro-Miguens 1955
T. soudanense		+				−	−	Rosenthal *et al.* 1962
T. verrucosum	+	+	++	−	−	−	−	Pereiro-Miguens 1955, Georg 1950
T. violaceum	+	+		−	−	−	−	Pereiro-Miguens 1955, Georg 1951
T. concentricum	+++			−	−	−	−	Pereiro-Miguens 1955
E. floccosum	++		++	++	−	−	−	Pereiro-Miguens 1955, Benham 1953

− = no growth ± = poor growth +, ++, +++, ++++, +++++ = good - excellent growth

vitamin free casein digest supported good growth of all strains tested. These compounds provide a readily assimilable source of peptides and amino acids. All dermatophytes, with the exception of those with an absolute requirement for a specific growth factor, grew well on asparagine. The only dermatophytes unable to assimilate urea were M. audouinii and E. floccosum.

Several workers have studied the effect of adding or omitting one or several amino acids in an attempt to determine minimum nitrogen requirements. A summary of their results is presented in Table 4. As with the results of carbohydrate assimilation studies, it is difficult to compare results directly since materials and methods varied so widely. It is apparent from Table 4 that apart from the absolute requirement for histidine shown by T. megninii, no other single amino acid is essential for growth (Drouhet, 1952). Dermatophytes will not grow in the absence of amino acids, but only some are essential for growth. These include leucine, arginine, histidine, glycine, glutamic acid, tyrosine, valine, alanine, phenylalanine and proline. Phenylalanine has also been reported to be inhibitory. Most workers agree that hydroxyproline is not utilised or is definitely inhibitory.

REQUIREMENTS FOR GROWTH FACTORS

Several investigators have shown that some species of dermatophytes grow poorly or not at all on media lacking appropriate growth factors. M. audouinii, for example, grows adequately on glucose-peptone agar, but sporulates poorly or not all. However, macroconidia are produced when the fungus is grown on cereal grains or a medium enriched with natural products such as yeast or liver extract (Langeron & Milochevitch, 1930; Hazen, 1947, 1951). Yeast extract stimulates the growth of several

TABLE 4

AMINO ACID REQUIREMENTS OF DERMATOPHYTES

	Leucine	Asparagine	Arginine	Alanine	Lysine	P-alanine	Proline	Isoleucine	Valine	Glycine	Histidine	Glutamine	Methionine	Tyrosine	Cysteine	Serine	Threonine	Trytophane	Ornithine	Hy-proline	
T. mentagrophytes	+++	+++	+++	+	+++	+++	+	+	+	+	+	+	+	+	+	+		+		+	Mosher et al. 1936
	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	+++	−	+++	−	−		−	Robbins & Ma 1945
	−		+++			−						+++		+++						−	McVeigh & Campbell 1950
T. gypseum	+	−	+++	+	+	+	+	+	+	+	−	+++	−	+	+	−	−	−		−	Archibald & Reiss 195
T. persicolor	+++			−		−			−	+++	+++		−	−	+						Stockdale 1953
T. tonsurans	+		+++	+	−	+	+++	−	+	+	−		−	+	+	+++	−	+	+++	−	Schwartz & Georg 1955
T. rubrum	+		+	+	+		+		+		+	+									Silva & Benham 1952
	−	−	+++	−	−	−	+	−	+	−	−	+++	−	−	−	−	−	−		−	Archibald & Reiss 1950
T. megninii											+++										Silva & Benham 1952
T. gallinae	+		+	+	+		+		+		+	+									Silva & Benham 1952
T. schoenleinii	+++		+++																		Archibald & Reiss 1950
T. violaceum	−	+	+	−	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	Georg 1951
M. canis	+	+	+	+			+					+	+								Chattaway et al. 1962
			+	+								+		+	+			+			Giblett & Henry 1950
M. lanosum	+++	+++	+++	+	−	+++	+++	+	+	+	+	+++	−	+	−	+	−	−		−	Archibald & Reiss 1950
M. fulvum	+	+++	+++	+++	+	+	+	+++	+	+++	+++	+++	+++	+	+	+	+++	+		−	Johnson & Grimm 1951
	−	+++	+++	−	−	+++	+++	−	+	+	−	+++	−	−	+	−	−	−		−	Archibald & Reiss 1950
M. gypseum			+	+							+			+	+			+			Giblett & Henry 1950
M. audouinii			+	+							+			+	+			+			Giblett & Henry 1950
	+++	−	−	−	−	−	+++	−	−	−	−	+++	−	−	−	−	−	−		−	Archibald & Reiss 1950
Microsporum spp.	+	+++	+++	+	+++	+++	+		+++	+++	+++	−	+	−	−	+++	−	+			Bereston 1953
E. floccosum	−	−	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		−	Archibald & Reiss 1950

− = no growth + = growth but not essential +++ = essential for growth

dermatophytes (Area Leao & Cury, 1950). Benham (1953) noted that the addition of trypticase (an enzymic hydrolysate of casein) to the medium encouraged the production of macroconidia by both T. rubrum and T. mentagrophytes. T. equinum has been reported by Georg, Kaplan and Camp (1957) to have an absolute requirement for nicotinic acid, and this has been used to distinguish it from T. mentagrophytes which grows in the absence of this vitamin. Recently, however, strains of T. equinum have been isolated which are autotrophic although morphologically identical to the vitamin-dependent strains (Smith et al., 1968).

Some detailed investigations have been carried out on the growth requirements of the faviform Trichophyton species. Strains of T. verrucosum have been shown to have an absolute requirement either for thiamine or inositol; some strains require both and pyridoxine as well (Georg, 1950; Mackinnon, 1942; Mackinnon & Argataveytia-Allende, 1948; Robbins, Mackinnon & Ma, 1942). Other factors, however, may also be required since best growth occurred on natural products such as yeast or liver extracts (Georg, 1949). Thiamine stimulates the growth of T. violaceum (Burkeholder & Moyer, 1943; Georg, 1951), and the development of micro- and macroconidia by both T. verrucosum and T. violaceum is encouraged by growing the fungi on complex natural media such as wheat and barley grains (Langeron & Milochevitch, 1930; Georg, 1950). T. schoenleinii appears to be autotrophic for vitamins (Georg, 1950).

Thiamine stimulates the growth of T. tonsurans (Schwartz & Georg, 1955); the amount required is as low as 3 pica gm./5ml. of Sabouraud's glucose broth. Georg and Camp (1957) found that the growth of seven of the strains of T. tonsurans they investigated was not stimulated by thiamine; these strains required amino acids of the ornithine, citrulline and arginine group.

Table 5 summarises these results. The growth factors most often required by dermatophytes are thiamine and inositol. However, the increased growth shown by many of these strains upon natural products indicates the complexity of their nutritional requirements, and the close inter-relation of vitamin and nitrogen requirements.

TABLE 5 GROWTH REQUIREMENTS OF DERMATOPHYTES

Organism	Growth on Natural Products	Absolute Requirements	Stimulation	Autotrophic
M. audouinii	++		Inositol + Unknown Factor	
T. equinum		Nicotinic Acid (some strains)		+ (some strains)
T. mentagrophytes	++			+
T. verrucosum	++	Thiamine Inositol Pyridoxine		
T. violaceum	++		Thiamine	
T. schoenleinii	++			+
T. tonsurans			Thiamine	
T. rubrum	++			+

2. MATERIALS AND METHODS

FUNGI STUDIED

147 isolates distributed among 22 species of dermatophytes were used in these studies. The source and code numbers of each strain investigated are shown in Table 6. In every instance, the colonial and microscopical characteristics were fully consistent with those described for the species. In most instances they had been isolated during routine mycological examination of specimens submitted to the Mycological Reference Laboratory. Some isolates were selected from the National Collection of Pathogenic Fungi. They had been maintained by subculturing every 6 - 8 weeks and kept at room temperature.

MEDIA

Maintenance Media

All isolates were maintained on 2% malt extract agar and Hugh & Leifson agar, and kept at room temperature with subculturing at intervals of 6 - 8 weeks.

Malt Extract Agar:	Oxoid malt extract	20.0 g
	Agar (Davis)	15.0 g
	Distilled water to	1 litre

pH before autoclaving = 5.4

Hugh & Leifson Agar:	Bacto-peptone (Oxoid)	2.0 g
	Sodium chloride	5.0 g
	Dipotassium hydrogen phosphate	0.3 g
	Agar (Davis)	15.0 g
	Distilled water to	1 litre

pH before autoclaving = 6.5

The choice of Hugh & Leifson (H & L) agar was determined by two factors. Firstly, it was used as the base from which inoculation was

TABLE 6

Species	No. of Isolates	Source	Code No.
Microsporum audouinii	5	Scalp hair 1965	A1,A2,A3,A5,A6
	1	Scalp scales 1965	A4
M. canis	3	Scalp 1966	C3,C9,C11
	1	Chest scales 1966	C12
	1	Toe clefts 1966	C1
	1	Thigh 1966	C10
	2	Legs 1966	C2,C4
	1	Skin, unspecified 1966	C8
M. gypseum	1	*NCPF No.35 (Sabouraud's *M. fulvum*)	B7
	1	NCPF No.40 Leg, New Zealand	B2
	1	NCPF No.45 (Sabouraud's *M. gypseum*)	B4
	1	NCPF No.41 Skin, unspecified	B6
	2	Body skin 1968	B1,B8
M. equinum	3	Horses 1966	I1,I2,I3
	1	Human skin 1966	I4
M. ferrugineum	4	NCPF 270, 271, 272, 334 Scalp	Q1,Q2,Q3,Q4
M. persicolor	2	Skin 1966, 1967	U2,U3
	1	NCPF No. 386	U1
M. cookei	1	Soil	T1
	3	NCPF Nos.370, 371, 372 conidial states *N. cajetana* from soil	T2,T3,T4
Trichophyton gallinae	1	NCPF No.301 (Sabouraud's original strain)	D1
	1	NCPF No.302 (Dr. Georg's human isolate)	D2
T. megninii	1	NCPF No.303 (Sabouraud's original strain)	E1
	1	NCPF No.304 (Dr. Georg)	E4
	2	Scalp 1965, 1966	E2.E3
T. mentagrophytes	3	Toeclefts 1965, 1966	F2,F3,F6
	1	Scalp 1965	F4
	1	Hand 1967	F10
	1	Cheek 1965	F7
	1	NCPF No.260 from a dog	F5
	3	Skin 1964, 1966	F1,F8,F9
T. erinacei	2	NCPF Nos.375, 376 (Dr. Marple's original isolates)	G5,G8
	1	NCPF No.359 hedgehog	G6
	6	Skin 1965, 1966	G1,G2,G3,G4,G7,G9
T. equinum	6	Horses 1966, 1967	H4,H5,H6,H7,H8,H9
T. quinckeanum	4	NCPF Nos.310, 311, 340, 342 human	J1,J2,J3,J6
	1	NCPF No.341 mouse	J5
	1	NCPF No.153 kitten	J4

TABLE 6 continued

Species	No. of Isolates	Source	Code No.
T. rubrum	4	Hands 1965	K2,K3,K4,K5
	1	Body 1965	K1
	1	Toenail 1965	K6
	3	Skin unspecified 1965, 1966	K7,K8,K9
T. tonsurans var. sulfureum	5	Scalp 1965, 1966, 1967	M1,M3,M4,M7,M9
	1	Leg 1966	M6
	3	Face 1965, 1968	M5,M11,M12
	2	Neck 1967	M8,M10
	2	Arms 1968	M13,M14
T. tonsurans var. tonsurans	1	NCPF No.145 (Sabouraud's *T. crateriforme*)	N1
	2	NCPF Nos.143, 150 Skin	N2,N3
	1	Feet 1965	M2
T. violaceum	1	Scalp 1968	06
	1	Neck 1968	02
	3	Skin unspecified 1968	01,03,05
	1	From South Africa, site not given	04
T. verrucosum	3	Face 1968	P1,P3,P6
	1	Neck 1968	P2
	1	Thigh 1968	P5
	1	Skin unspecified	P4
T. soudanense	6	NCPF Nos. 274, 276, 321, 322, 323, 373 Scalp, from Nigeria	R2,R3,R4,R5,R6,R7
	2	NCPF Nos.275, 385 Skin, Nigeria	R1,R8
	1	NCPF No.273 Skin, Cameroons	R9
	1	Scalp 1969	R10
T. schoenleinii	4	NCPF Nos.124, 125, 126, 343 Scalp	S1,S2,S3,S5
	1	NCPF No.127 Leg	S4
	2	Scalp 1967, 1968	S6,S7
T. terrestre	2	NCPF Nos.362, 363 (original mating strains of *A. quadrifidum*)	V2,V3
	3	Saprophytic on human skin 1966, 1967	V4,V5,V6
	1	Horse as saprophyte 1967	V7
T. ajelloi	2	NCPF Nos.364, 365 (original mating strains of *A. uncinatum*)	W2,W3
	1	NCPF No.216 (Vanbreuseghem's original type species)	W1
	1	Scalp, conidial strain	W4
Epidermophyton floccosum	3	Groin 1965, 1966	L1,L2,L6
	1	Thigh 1966	L3
	2	Toes 1966	L4,L5

*NCPF = National Collection of Pathogenic Fungi

made to the auxanogram media (vide infra). Secondly, since it contains no sugar, there was less tendency for dermatophytes to become pleomorphic. As a conservation medium, it was preferred to Sabouraud's conservation medium since it contained less peptone (0.2% compared to 3%). For a similar reason, 2% malt extract agar was chosen in preference to 4% malt extract agar or glucose-peptone agar.

Basal Medium for Carbohydrate Studies

Potassium dihydrogen orthophosphate	1.0 g
Magnesium sulphate $7H_2O$	0.5 g
Ammonium sulphate	5.0 g
Ion Agar (Oxoid No. 1)	15.0 g
Distilled water to	1 litre

A vitamin supplement was added to the medium before autoclaving at 112°C for 15 minutes. This supplement contained:

Thiamine	1 mg
i-Inositol	250 mg
Nicotinic acid	10 mg
Histidine	150 mg
Distilled water	100 ml

2 ml of this solution was added to each 100 ml of basal medium.

For some carbohydrate studies (vide infra), yeast extract at a final concentration of 0.05% was added to the basal medium before autoclaving.

Carbon Sources

Monosaccharides	d+ Glucose
	d+ Fructose
	d+ Mannose
	d+ Galactose
	d- Ribose
	d- Rhamnose
	L+ Arabinose
	D+ Xylose
Disaccharides	Maltose
	Lactose
	d- Sucrose
	Cellobiose
	Trehalose
	d+ Melibiose
Trisaccharides	d- Raffinose

Polysaccharides	Dextrin
	Inulin
Sugar Alcohols	Mannitol
	Sorbitol
	Meso-Erythritol
	Adonitol
	Glycerol
	Dulcitol

Sugars and sugar alcohols were prepared as 5% solutions and sterilised by autoclaving at 112°C for 15 minutes. The basal medium was made up to 80% of final volume and autoclaved. 20 ml of the 5% sugar solution was added to each 80 ml of the basal medium, to give a final concentration of 1% carbohydrate. The medium was then dispensed aseptically into sterile plugged test tubes (6 x 5/8"; 150 x 16 mm) or sterile ½ oz (10 ml) bottles and sloped. Approximately 5 ml of medium was put into each tube or bottle.

Alternatively, the sterile medium was dispensed by pouring into 9 cm plastic petri dishes, to give approximately 20 ml of medium per plate.

Basal Medium for Nitrogen Studies

Potassium dihydrogen orthophosphate	1.0 g
Magnesium sulphate $7H_2O$	0.5 g
Glucose	10.0 g
Ion Agar (Oxoid No. 1)	15.0 g
Distilled water to	1 litre

The vitamin supplement was added to this medium before autoclaving. The medium was sterilised by autoclaving at 112°C (10 lbs/sq in) for 15 minutes.

Nitrogen Sources

The nitrogen solutions were prepared to give equivalent quantities of nitrogen, *viz.* sodium nitrate 0.2%; asparagine 0.26%; ammonium sulphate 0.24%; urea 0.0225%; and sterilised separately.

The nitrogen solution was added to the basal medium and the complete medium dispensed by pouring into 9 cm plastic petri dishes.

Media for Hydrolytic Studies

CYLG broth. The basal medium for the hydrolysis of casein, tyrosine and starch (Marshall & Kelsey, 1960) consisted of:

Casein digest	10.0 g
Marmite (Marmite Ltd)	5.0 g
Sodium glycerophosphate	10.0 g
Potassium lactate, 50% w/v	10.0 ml
Glucose	2.0 g
Inorganic salts solution	5.0 ml
Distilled water to	1 litre

The ingredients were dissolved by heating, mixed and filtered. The broth was made up at 10 times the required strength, maintained at 4°C and diluted 1/10 for use.

The inorganic salts solution consisted of:

10 N-Sulphuric acid	0.1 ml
Magnesium sulphate $7H_2O$	4.0 g
Manganese sulphate $4H_2O$	0.4 g
Ferrous sulphate $7H_2O$	0.4 g
Distilled water to	1 litre

The acid was added to the water and the salts dissolved without heating.

The following amounts of tyrosine, gelatine and starch were added to the basal medium before autoclaving: 0.5% tyrosine, 0.4% gelatine and 0.5% starch. The media were autoclaved at 112°C for 15 minutes and dispensed in 20 ml aliquots into 9 cm plastic petri dishes.

The casein medium was prepared by mixing skimmed milk sterilised by autoclaving with its own volume of cooled autoclaved double strength aqueous agar, and pouring plates.

Medium for Hydrolysis of Urea

The medium was prepared as follows (Philpot, 1967):

Oxoid bacto-peptone	1.0 g
Sodium chloride	5.0 g
Potassium dihydrogen orthophosphate	2.0 g
Glucose	5.0 g
Agar (Davis)	15.0 g
Distilled water to	1 litre

Phenol red solution (0.2% in 50% alcohol) was added at the rate of 6 ml/litre. The pH was adjusted to 5.5 before autoclaving. After sterilisation by autoclaving at 112°C for 15 minutes, and cooling to 45°C, urea solution (20% aqueous solution sterilised by filtration) was added aseptically at the rate of 100 ml/litre. The medium was dispensed into ½ oz (10 ml) bottles and sloped.

METHODS FOR ASSIMILATION STUDIES

Three methods for inoculating the carbon assimilation media were investigated:

(1) In the first instance, preparatory to carbon assimilation studies, the fungi were subcultured at 26°C on H & L agar for 7 - 8 days. This medium supports an adequate growth of dermatophytes, but since it contains no carbohydrate, the risk of carrying across such nutrients in the inoculum is minimized. It was thus considered unnecessary to wash the inoculum first. Small portions of the growth were then transferred by nichrome needle to the carbohydrate media. Media were incubated at 26°C and observed at 7, 14 and 21 days. For purposes of comparison, the reading at 21 days was used: by this time differences due to variations in inoculum size had disappeared, and there had been an opportunity for growth to occur on the more slowly utilised compounds.

Subsequently, partly to verify the results obtained with the initial method, and partly to enable more precise duplication of inocula to be made, two other methods were investigated.

(2) Fungi were grown for 7 days on H & L agar and the growth transferred aseptically to bottles containing sterile distilled water; the mycelium was then disrupted in a Waring blendor (MSE "Atomix") for approximately 1 minute. The resulting suspension was inoculated from

a pasteur pipette directly on to the surface of the plates of carbohydrate media. The plates were incubated at 30°C and read at 7 and 14 days. The reading at 14 days was taken as the final result.

(3) Fungi were grown for 7 days on plates of H & L agar. Plugs of fungus plus agar were made with a heat sterilised metal borer 2 mm diameter, and transferred aseptically by needle to the surface of the assimilation plates. All tests were performed in duplicate. The plates were incubated at 26°C and read at 7, 14 and 21 days. The reading at 21 days was taken as the final result.

Nine species (M. gypseum, M. cookei, M. persicolor, T. ajelloi, T. erinacei, T. terrestre, T. mentagrophytes, T. rubrum, T. tonsurans) were compared by all 3 methods.

For nitrogen assimilation studies, all isolates were subcultured for 7 days on the basal medium without nitrogen. Portions of mycelium were then transferred by needle to the surface of the nitrogen plates. Some isolates did not grow on the basal medium in this period of time, and were inoculated from a tube of H & L agar as for carbohydrate studies. Plates were incubated at 26°C and read at 7 and 14 days.

For both carbon and nitrogen studies, a tube or plate of the basal medium without added nutrients was always included, as a negative control.

For hydrolysis of casein, tyrosine, gelatine and starch, the plates of media were kept at 4°C overnight as this encourages dispersion of the crystals of tyrosine, and dried at 37°C before use. Small pieces of mycelial growth were transferred aseptically from tubes of 2% malt extract agar, and the plates incubated at 26°C for 7 days. Hydrolysis

of casein and tyrosine was shown by zones of clearing around the colonies. Hydrolysis of starch was shown by flooding the plates with iodine. Hydrolysis of gelatine was demonstrated by flooding the plates with a solution of mercuric chloride, to show zones of clearing around the colonies:

Mercuric chloride	12.0 g
Distilled water	80.0 ml
Concentrated HCl	16.0 ml

For hydrolysis of urea small portions of mycelial growth were transferred from tubes of 2% malt extract agar, and the time taken for the medium to turn red at 26°C was noted.

Method of Estimating Growth for Assimilation Studies

The amount of growth, viz. the area and depth of the colony together with the amount of aerial mycelium was estimated visually.

In the carbon assimilation studies the growth of each isolate on the control tubes containing glucose was regarded as 100%. Growth on all other compounds was directly compared with this. Each isolate was compared with itself and not with other isolates of the same species; this was to eliminate the effect of strain differences from the final results. Growth was recorded as follows:

++++ = equal to growth on glucose (100%)
+++ = 50% of growth on glucose
++ = 25% of growth on glucose
+ = trace amounts of growth
0 = no growth

For the purpose of comparing isolates, growth was scored as good, moderate or negative. Growth amounting to 50% or more of that on the glucose control after 21 days was regarded as good. Growth amounting to 25% of that on the glucose control was recorded as moderate and was considered to

be significant, since it showed that the isolate could utilise the compound under test to a slight extent. Anything less than 25% of the growth on the glucose control was regarded as negative.

For nitrogen assimilation studies and assimilations with yeast extract agar, the growth on the basal medium without the added nutrients was scored as "+". The growth on asparagine and glucose respectively were used as the positive controls, and growth on all other compounds compared to the growth on the basal medium and to growth on the positive controls, thus:

+	=	growth on basal medium
++	=	25% of growth on glucose or asparagine
+++	=	50% of growth on glucose or asparagine
++++	=	growth on glucose or asparagine (100%)

MATING STUDIES

Isolates of *M. gypseum*, *T. terrestre*, *T. ajelloi* and *T. mentagrophytes* whose sexual status was unknown, were mated with tester strains of the corresponding sexual forms. Sterilised soil was placed in a petri dish and moistened with sterile distilled water. Short pieces of human child's hair, sterilised by autoclaving, were placed on the surface of the soil, and the plates were inoculated with one of the tester pairs and the fungus under test. The plates were kept for up to 8 weeks, and scanned frequently for the appearance of cleistothecia containing asci and ascospores.

3. RESULTS

CARBON ASSIMILATION TESTS

Table 7 summarises the results of assimilation tests carried out on all isolates investigated.

Hexoses

Glucose, Fructose and Mannose were assimilated by most isolates. Some isolates used one sugar preferentially but this could not be systematised with respect to species and genera.

Galactose was utilised by comparatively few species.

Pentoses

Assimilation was limited or absent. Several isolates showed slight growth (up to 25% of the growth on glucose) particularly on ribose and arabinose. *T. schoenleinii* and *T. verrucosum* assimilated ribose.

Disaccharides

Cellobiose was assimilated by all isolates. Trehalose was assimilated by 20 of the 22 species, the notable exceptions being *T. schoenleinii* and *M. audouinii*.

Maltose was readily used as a source of carbon by most species; absence of assimilation was recorded only for *T. violaceum* and some isolates of *T. schoenleinii*. Four species, *M. equinum*, *M. persicolor*, *M. ferrugineum* and *M. cookei* grew poorly. Sucrose was also a good source of carbon for most species except *T. violaceum* and *T. ajelloi*. Poor growth was recorded for *M. persicolor*, *M. ferrugineum*, *M. cookei*, *T. ajelloi*, and *T. schoenleinii*. In general, if an isolate could assimilate sucrose, it could also assimilate maltose, and those strains which grew poorly on one sugar also grew poorly on the other.

Melibiose was not assimilated by any species.

TABLE 7

ASSIMILATION OF CARBOHYDRATES BY 22 SPECIES OF DERMATOPHYTES

	M. persicolor (4)	M. cookei (4)	M. audouinii (6)	M. gypseum (6)	M. canis (9)	M. equinum (4)	M. ferrugineum (4)	T. soudanense (9)	T. violaceum (6)	T. schoenleinii (6)	T. verrucosum (6)	T. terrestre (6)
Glucose												
Fructose												
Mannose												
Galactose												
Arabinose												
Xylose												
Rhamnose												
Ribose												
Maltose												
Sucrose												
Lactose												
Cellobiose												
Trehalose												
Melibiose												
Raffinose												
Sorbitol												
Mannitol												
Erythritol												
Adonitol												
Glycerol												
Inulin												
Dextrin												

	T. erinacei (9)	T. equinum (6)	T. quinckeanum (6)	T. mentagrophytes (9)	T. rubrum (9)	T. megninii (4)	T. gallinae (2)	T. tonsurans (17)	T. ajelloi (4)	E. floccosum (6)
Glucose										
Fructose										
Mannose										
Galactose										
Arabinose										
Rhamnose										
Xylose										
Ribose										
Maltose										
Sucrose										
Lactose										
Cellobiose										
Trehalose										
Melibiose										
Raffinose										
Sorbitol										
Mannitol										
Erythritol										
Adonitol										
Glycerol										
Inulin										
Dextrin										

Numbers of isolates tested in parentheses

Trisaccharides

Raffinose was not assimilated by any species.

Sugar Alcohols

Sorbitol and Mannitol were assimilated by most isolates.

Only five species did not assimilate Erythritol, viz. *M. ferrugineum*, *T. quinckeanum*, *T. violaceum*, *T. schoenleinii* and *E. floccosum*. A few isolates of some other species failed to grow on it.

Adonitol was a poor source of carbon for dermatophytes; only *T. terrestre*, *T. erinacei*, *T. ajelloi* and *T. verrucosum* grew moderately well on it.

Glycerol was used by few isolates, and only to a limited extent.

Storage Polysaccharides

Dextrin was assimilated by many isolates, particularly by *M. equinum* and *T. soudanense*.

Inulin was assimilated by many isolates but growth was always limited and it did not constitute a satisfactory carbon source.

From the results of these assimilation tests, the 22 compounds tested may be divided into 2 groups depending on their pattern of assimilation. Group I comprises those compounds either utilised or not utilised by all isolates:

Compound	Utilisation
Glucose	+
Fructose	+
Mannose	+
Rhamnose	-
Xylose	-
Lactose	-
Cellobiose	+
Mannitol	+
Inulin	-
Raffinose	-
Melibiose	-

In <u>Group II</u> are those compounds which were assimilated by some isolates but not by others and thus had potential as a means of differentiating dermatophytes:

Galactose
Arabinose
Ribose
Maltose
Sucrose
Trehalose
Sorbitol
Erythritol
Adonitol
Glycerol
Dextrin

NITROGEN ASSIMILATION TESTS

The results are summarised in Table 8.

All isolates were first inoculated on to slopes of the basal medium, *i.e.* on to a glucose-salts medium *without* a nitrogen source, so that any nitrogenous material carried over in the inoculum would be exhausted prior to inoculation on to slopes containing nitrogen. Subcultures were made on to basal medium, basal medium plus ammonium, basal medium plus nitrate and basal medium plus asparagine. Five species (*E. floccosum*, *T. soudanense*, *T. violaceum*, *T. tonsurans* and *T. verrucosum*) failed to grow on this medium and two species (*T. megninii* and *M. ferrugineum*) grew rather poorly. These were therefore subcultured from cultures grown on H & L agar. Although the basal medium in theory did not contain a nitrogen source, most isolates grew well even after a second subculture on it. Unless nitrogenous material was carried over in the inoculum, growth must be due to contamination of the basal medium. The distilled water used to make up the medium was found to give a pale yellow colour with Nessler's reagent, *i.e.* a trace of ammonium ion was

TABLE 8 ASSIMILATION BY DERMATOPHYTES OF FOUR NITROGENOUS COMPOUNDS OFFERED AS SOLE SOURCES OF NITROGEN

	Asparagine	Urea	NH_4^+	NO_3^-
M. audouinii	+	0	trace	0
M. canis	+++++	+++++	+++	0
M. gypseum	+++++	+++++	trace	0
M. equinum	+++++	+++	+++	0
M.ferrugineum	+++	0	trace	0
M. cookei	+++++	+++	trace	0
M. persicolor	+++	+++++	trace	0
T. gallinae	+++	+	trace	0
T. megninii	+++++	0	0	0
T. mentagrophytes	+++++	+++	0	0
T. erinacei	+++++	0	trace	0
T. equinum	+++++	+++++	+++	0
T. quinckeanum	+++++	+++	+++	0
T. rubrum	+++++	0	+++	0
T. sulphureum	+++	0	0	0
T. tonsurans	+++	+++	0	0
T. soudanense	+++	0	0	0
T. terrestre	+++	+++	0	0
T. ajelloi	+++	+++	trace	0
T. violaceum	+	0	0	0
T. schoenleinii	+++	+++	0	0
T. verrucosum	+	0	0	0
E. floccosum	+++	0	0	0

present, and this may have been sufficient to initiate growth. The ability of all the isolates to utilise the four nitrogen sources was tested on two separate occasions, with identical results.

Potassium nitrate was not utilised by any strain as sole source of nitrogen, i.e. growth on tubes supplemented with nitrate was not greater than on tubes of the basal medium alone.

Only four species showed increased growth in the presence of ammonium sulphate: M. canis, M. equinum, T. equinum and T. quinckeanum. A slight increase was observed for T. gallinae, T. erinacei, T. ajelloi, M. ferrugineum, M. cookei and M. persicolor. This shows that the ammonium ion on the whole is not a good source of nitrogen for dermatophytes. An increase in the amount offered to the fungi did not result in a greater amount of growth, there being little difference between growth on tubes with trace amounts, 0.2% or 0.5%.

Most isolates grew well on asparagine, the exceptions being four isolates of T. tonsurans which will be considered later. For most isolates, asparagine was by far the most easily assimilated form of nitrogen used in these tests.

UTILISATION OF UREA

The growth of 11 species was stimulated by urea and all 11 could also hydrolyse it although the time taken to do so varied (Table 9). Rapid hydrolysers (i.e. within 7 days) include T. equinum, T. terrestre and T. mentagrophytes, and some isolates of M. gypseum, M. canis, T. ajelloi and T. quinckeanum. Most isolates of M. canis hydrolyser urea completely within 10 days, as did most isolates of M. cookei and T. quinckeanum. Hydrolysis was completed in two weeks by M. equinum, M. persicolor and some isolates of T. schoenleinii.

TABLE 9

TIME TAKEN BY 21 SPECIES OF DERMATOPHYTES TO HYDROLYSE UREA

	Time in Days				
	1 - 7	8 - 10	11 - 14	Over 14	Negative
M. audouinii			1	2	3
M. cookei		3	1		
M. gypseum	3	1	2		
M. canis	2	7			
M. equinum		2	2		
M. ferrugineum			3		
M. persicolor			15*		
T. soudanense					20*
T. erinacei					39*
T. equinum	6				
T. quinckeanum	3	3			
T. mentagrophytes	7	1	1		
T.rubrum				7	2
T. megninii		4			
T. gallinae					2
T. tonsurans	9	7	1		
T. violaceum				5	1
T. schoenleinii			3	3	
T. verrucosum			5	3	
T. ajelloi	2	1	1		
E. floccosum			6		

*these figures include some additional strains tested subsequently

Three species, T. erinacei, T. soudanense and T. gallinae could neither assimilate nor hydrolyse urea.

Eight species, T. tonsurans, T. violaceum, T. verrucosum, T. rubrum, T. megninii, M. ferrugineum, M. audouinii and E. floccosum, were not stimulated by urea: most isolates however hydrolysed it. T. tonsurans was a rapid hydrolyser. Of the 9 isolates of T. rubrum tested, 7 hydrolysed it in two to three weeks but two isolates were unable to do so. The other six species required at least 15 days to complete hydrolysis. Included were some species which grew poorly on asparagine in the nitrogen assimilation tests, and required supplementary yeast extract for the carbon assimilation tests. Although other species show no such requirement, Littman (1957) has suggested that many dermatophytes require peptone to initiate growth before urea can be attacked. As a means of testing this hypothesis, certain isolates were inoculated on to urea slopes with and without peptone and growth recorded. Six species incapable of utilising urea were investigated in this way (Table 10). As might have been surmised from the results of the assimilation tests, there was little growth of T. tonsurans, M. audouinii, M. ferrugineum and E. floccosum in the absence of peptone, i.e. on a medium whose only source of nitrogen was urea. T. megninii failed to grow at all, but as no vitamin or amino acid supplement was added to the medium, this was to be expected. Although T. rubrum grew quite well on the deficient medium, this does not necessarily indicate that urea can be utilised since it is likely that sufficient ammonium ion was present to initiate growth. This species has shown no requirement for complex nitrogen sources. The other five species grew only very slightly in the presence of ammonium sulphate as the sole source of nitrogen.

The poor growth of T. mentagrophytes is surprising since this

TABLE 10 HYDROLYSIS OF UREA BY DERMATOPHYTES IN THE PRESENCE AND ABSENCE OF PEPTONE

Species & No. of Isolates	With Peptone		Without Peptone	
	Hydrolysis	Growth	Hydrolysis	Growth
T. mentagrophytes (9)	6 - 8 days	++++	9 - 17 days	+
T. rubrum (9)	9 -25 days	++++	11 - 28 days	++++
T. tonsurans (17)	6 -12 days	++++	8 - 19 days (5 isolates negative)	trace
T. megninii (4)	9 -14 days	++++	Negative	0
E. floccosum (6)	11 -16 days	++++	19 - 30 days (2 negative)	trace
M. audouinii (6)	11 -18 days	++++	25 - 27 days	trace
M. ferrugineum (4)	9 -19 days	++++	21 - 27 days	+

species grew well on urea in the nitrogen assimilation tests.

The results would suggest that what is being recorded is the rate at which dermatophytes can produce the enzyme (urease) when grown in the presence of the substrate (urea). Some strains can do so fairly quickly, other strains require a longer period to do so. Littman (1957) who recorded much faster rates of hydrolysis was presumably testing pre-formed enzyme since he used fairly large agar blocks of mycelium as inocula. The connection between the growth of the organism and the formation of the enzyme is illustrated in both Table 9 and Table 10. The slow growing fungi such as _T. violaceum_ and _T. verrucosum_ are slow to hydrolyse urea, and medium lacking peptone supported less growth and correspondingly the change in pH came later. That urease is actually formed and the change in pH is not due to other factors is supported by evidence from _T. rubrum_ and _T. mentagrophytes_. 8 isolates of each organism were inoculated on to complete medium and medium lacking urea. Anticipated results were obtained for the former, but there was no change of pH on the latter medium even after 21 days.

The number of days for the change in pH to become evident was directly related to temperature. At 20°C hydrolysis was completed 2 - 3 days later than at 30°C. The optimum temperature for growth of dermatophytes is also 30°C, and some species, notably _T. verrucosum_, grow rapidly at 37°C. Thus the increase in hydrolysis rate with increase in temperature is due to two factors, _viz._ the increased activity of the enzyme and the more rapid growth of the fungi.

Hydrolysis of Urea by T. mentagrophytes and T. rubrum

The results are shown in Table 11. Sixty-five isolates of _T. mentagrophytes_ (92.8%) were positive, _i.e._ turned the medium red

TABLE 11 HYDROLYSIS OF UREA BY TRICHOPHYTON MENTAGROPHYTES & TRICHOPHYTON RUBRUM

Days after Inoculation	Number of Isolates producing Urease	
	T. mentagrophytes	T. rubrum
3	1	
4	9	
5	13	
6	36	
7	6	
8	2	
9	2	1
10		
11		1
12	1	4
13		5
14		4
15		5
16		11
17		6
18		10
19		7
20		7
21		10
Over 21 & negative		32
Totals	70	103

throughout, within 7 days. One hundred and three isolates (99.1%) of T. rubrum were negative within this time. Five isolates of T. mentagrophytes (4 typically granular and 1 floccose isolate) were not positive in 7 days although positive reactions eventually appeared (2 in 8 days, 2 in 9 days and 1 in 12 days).

Most isolates of T. rubrum produced some rise in pH after a period which varied from 9 to over 21 days depending upon the isolate, but in some cases the indicator was not completely changed, i.e. the pH did not rise above 8.0. A few isolates had shown no pH change by 28 days when the experiment was concluded. The number of days taken by any one isolate to produce a rise in pH varied in consecutive tests; variations from the mean of up to 4 days have been recorded. T. mentagrophytes, by contrast, is a rapid producer of an alkaline pH although there is some variation between isolates. The time taken to produce a rise in pH varied slightly in consecutive tests (a difference of up to 2 days from the mean).

All isolates showed good growth upon the medium used.

RESULTS OF HYDROLYTIC STUDIES

Hydrolysis of Casein

Only 7% of isolates were unable to hydrolyse casein, and these were distributed among several different species (Table 12).

Hydrolysis of Tyrosine

Most isolates were able to hydrolyse tyrosine (Table 12). However, both T. verrucosum and T. violaceum required up to 14 days for hydrolysis whereas all other strains did so within 7 days. T. schoenleinii,

TABLE 12 HYDROLYSIS OF CASEIN, TYROSINE, GELATINE AND STARCH BY DERMATOPHYTES

	M. persicolor (4)	M. cookei (4)	M. audouinii (6)	M. gypseum (6)	M. canis (9)	M. equinum (4)	M. ferrugineum (4)
Casein							
Tyrosine							
Gelatine							
Starch							

	T. erinacei (9)	T. equinum (6)	T. quinckeanum (6)	T. mentagrophytes (9)	T. terrestre (6)
Casein					
Tyrosine					
Gelatine					
Starch					

	T. rubrum (9)	T. megninii (4)	T. gallinae (2)	T. tonsurans (17)	T. ajelloi (4)
Casein					
Tyrosine					
Gelatine					
Starch					

	T. soudanense (9)	T. violaceum (6)	T. schoenleinii (6)	T. verrucosum (6)	E. floccosum (6)
Casein					
Tyrosine					
Gelatine					
Starch					

Number of isolates tested in parentheses

in particular, hydrolyses tyrosine very rapidly.

Hydrolysis of Starch

Starch was not hydrolysed by an isolate (Table 12).

Liquefaction of Gelatine

A few isolates could not liquefy gelatine; these included most isolates of M. audouinii, a few of M. canis and all isolates of T. violaceum. The remaining isolates hydrolysed gelatine although the size of the zone of hydrolysis around the colony varied considerably from isolate to isolate (Table 12).

INVESTIGATIONS ON TRICHOPHYTON TONSURANS

Since considerable variability in assimilation patterns was found among the first 8 isolates of T. tonsurans tested, it was decided to extend the survey to include more isolates. A total of 17 isolates were tested; 14 had recently been isolated from material submitted to the Mycological Reference Laboratory and 3 were from the National Collection of Pathogenic Fungi. 13 of the recently isolated strains were identified on the basis of morphology as T. tonsurans var. sulfureum, and the 14th was identified as T. tonsurans var. tonsurans. Two of the T. sulfureum strains subsequently became pleomorphic.

Growth was compared on different sources of nitrogen, with and without glucose, and with different sources of agar. The results are shown in Table 13. They may be summarised as follows:

(a) Basal Medium lacking a source of carbon and with ammonium sulphate as the only source of nitrogen will not support growth. The addition of glucose resulted in growth. This was adequate if Davis

TABLE 13

COMPARISON OF GROWTH OF 17 ISOLATES OF TRICHOPHYTON TONSURANS ON MEDIA GELLED WITH DAVIS & ION AGARS WITH VARIOUS NITROGEN SOURCES

Strain	Basal Medium only			Basal Medium + Glucose			Basal Medium + Yeast Extract		Basal Medium + Glucose + Yeast Extract		Basal Medium + Asparagine	Basal Medium + Asparagine + Glucose	Basal Medium + *Vit.free C.D.	Basal Medium + Vit.free C.D. + Glucose
	Ion	Davis	Ion + *Thi	Ion	Davis	Ion + Thi	Ion	Davis	Ion	Davis				
M1	-	-	-	+	+++	+++	++	+	5+	6+	+	++	+++	6+
M2	-	-	-	+	++	++	++	+	7+	7+	+	4+	++	6+
M5	-	-	-	+	++	+	++	++	8+	8+	+	+	trace	+
N2	-	-	-	++	++	+++	+	+	6+	7+	+	++	+	+
N3	-	-	-	+++	+++	++	+	+	5+	5+	trace	+++	+	+
M3	-	-	-	-	+	++	+	+	8+	8+	+	+	+	+
M4	-	-	-	+	+	+++	+	+	5+	6+	+	+	+	+++
M6	-	-	-	+	++	++	+	+	6+	6+	+	+	+	+
M8	-	-	-	+	+	+	+	+	8+	8+	+	+	+	+
M10	-	-	-	+	++	++	+	+	7+	7+	++	++	+	+
M11	-	-	-	-	+	+	+	+	8+	8+	+	+	+	+
M12	-	-	-	+	+	+	+	+	4+	6+	+	+	+	+
M14	-	-	-	+	+	+	+	+	4+	4+	+	+	+	+
M7	-	-	-	-	-	+	+	+	4+	5+	-	-	+	+
M9	-	-	-	-	-	+	+	+	8+	8+	-	-	+	+
M13	-	-	-	-	-	-	+	+	3+	3+	-	-	+	+
N1	-	-	-	-	-	-	±	+	4+	4+	-	-	+	++

*Thi = Thiamine Vit.free C.D. = Vitamin free Casein Digest

- = no growth + = trace amounts of growth ++ → 8+ = increasing amounts of growth

agar was used as the gelling agent but poor if Ionagar was used.

(b) Thiamine stimulated growth only in the presence of carbohydrate. The four isolates which did not grow on the enriched basal medium (see page 54) were only slightly stimulated by thiamine. Sufficient thiamine must be present as impurity in Davis agar since growth on media gelled with it was comparable to growth on media gelled with Ionagar which contained thiamine. Thiamine was added as given in Georg & Camp (1957).

(c) All strains would grow on the basal medium without carbohydrate if yeast extract was added. The addition of glucose to the basal medium plus yeast extract gave very good growth. The type of agar used had no effect here.

(d) All isolates except the four which did not grow on the enriched basal medium grew slightly with asparagine alone, but even the addition of glucose did not increase growth very much. Asparagine does not therefore act as an adequate nitrogen source for these isolates.

(e) All isolates grew slightly with vitamin-free casein digest, and with glucose the isolates of T. tonsurans var. tonsurans grew well.

The inability of four isolates to grow on the basal medium with carbohydrate and the very slight stimulation shown with thiamine suggests that other growth factors are required. Of the 70 strains studied by Georg and Camp (1957), 7 would not grow on ammonium nitrate agar even in the presence of thiamine, and these were found to have a requirement for amino acids of the ornithine, citrulline and arginine cycle. The figures presented below do not, however, suggest that all the four isolates investigated in this study were similar in this respect.

Isolate	Supplements to Basal Medium None	Arginine	Arginine & Thiamine	Yeast Extract
M7	0	trace	trace	+
M9	0	trace	trace	+
M13	0	+	+	+
N1	0	+	+	+

The 17 isolates can be divided into 4 groups on the basis of their growth requirements:

(1) "*T. tonsurans*" *sensu stricto* and the two pleomorphic isolates were not nutritionally fastidious.

(2) Two isolates identified on the basis of morphology as "*T.sulfureum*" required thiamine to stimulate growth, and grew poorly without a complex nitrogen source.

(3) Two isolates, one with "*T. tonsurans*" and the other with "*T. sulfureum*" morphology, needed arginine but not thiamine.

(4) Two isolates, both "*T. sulfureum*" by morphological criteria, had requirements for nitrogen and growth substances not supplied by thiamine, arginine and simple nitrogen sources, but supplied by complex nitrogen sources such as yeast extract.

EFFECT OF YEAST EXTRACT

It was noted that variable amounts of growth occurred on the control media containing glucose as follows:

(a) Satisfactory

Microsporum gypseum
M. canis
M. cookei
M. equinum
M. ferrugineum
M. persicolor
Trichophyton mentagrophytes
T. rubrum
T. erinacei
T. quinckeanum
T. equinum
T. terrestre
T. gallinae
T. megninii

(b) Poor

Most of the assimilation media prepared for the investigation of *M. audouinii* were gelled with Davis agar (see page 30) and growth of the fungus was satisfactory. Additional tests using media gelled with Ionagar (page 30) showed very reduced growth on the glucose control.

13 of 17 isolates of *T. tonsurans* gave similar results.

T. soudanense and *T. schoenleinii* grew poorly on the glucose control and there was little difference between the positive and negative controls for some isolates. This made the results difficult to interpret.

(c) Negative

T. violaceum
T. verrucosum
4 isolates of *T. tonsurans*

Isolates of the 6 species showing poor or no growth on the carbon assimilation media also failed to grow or grew poorly on the nitrogen assimilation medium with ammonium sulphate as the sole source of nitrogen. In order to be able to test the ability of these species to utilise single sources of carbon, it was necessary to provide them with additional growth factors so that adequate growth was obtained on the controls. A pilot investigation of marmite (Marmite Ltd.) and yeast extract (see page 30) indicated that yeast extract at a concentration of 0.05% gave the best results compared with concentrations of 0.1 or

0.01% or marmite. When 0.05% yeast extract was added to the assimilation media, growth of all 6 species was good.

The results of the assimilation tests suggest that some dermatophytes require a more complex source of nitrogen than the ammonium ion, but it is possible that enhancement of growth in the presence of yeast extract is due to some unknown growth factor. Thiamine is known to stimulate the growth of T. tonsurans and T. violaceum (Georg, 1951; Georg & Camp, 1957), and the latter fungus grows poorly even on acid-casein if the vitamin is omitted. The results of the present investigation suggest that some unknown factor is necessary since neither fungus would grow even when thiamine was present with ammonium ion as sole nitrogen source. Little is known of the nitrogen nutrition of the other species although Rosenthal and Vanbreuseghem (1962) found that T.soudanense would not grow with ammonium ion as the sole source of nitrogen. Various workers have found that yeast extract stimulates the growth of many dermatophytes. Hazen (1951), for example, noted that M. audouinii was not stimulated by vitamins, and although it would grow when supplied with ammonium ion, growth was better on asparagine and excellent with yeast extract. The factors in yeast extract which favoured growth were reported to be inositol and a compound which was not identified.

A comparison of growth on yeast extract and vitamin-free casamino acids was therefore made for 3 isolates each of M. audouinii, T. violaceum, T. tonsurans, T. soudanense, T. schoenleinii and E. floccosum. The results are shown in Table 14.

The stimulatory factor in yeast extract appears to be related at least in part to the nitrogen nutrition of E. floccosum, T. soudanense and T. schoenleinii. These fungi grew as well on vitamin-free casamino

TABLE 14 A COMPARISON OF GROWTH OF SIX DERMATOPHYTE SPECIES IN THE PRESENCE OF YEAST EXTRACT AND VITAMIN-FREE CASAMINO ACIDS

	Vitamin-free Casamino Acids		Yeast Extract	
	Glucose present	Glucose absent	Glucose present	Glucose absent
M. audouinii	+++	+	+++++	+
T. schoenleinii	+++	+	+++	+
T. soudanense	++++	+	++++	+
E. floccosum	+++	+	+++	+
T. tonsurans	trace	trace	++++	+
T. violaceum	trace	trace	+++	+

3 isolates of each species were tested

acids as on yeast extract.

T. violaceum and *T. tonsurans* grew poorly in the absence of vitamins.

M. audouinii responded well to the addition of either vitamin-free casamino acids or yeast extract, but the difference in growth on the assimilation media containing ammonium nitrogen solidified with Davis agar or Ionagar suggested that a growth factor other than a complex nitrogen source might also be involved.

This requirement for yeast extract supplementation meant that the basal medium for all species of dermatophytes examined was not identical. It was therefore decided to investigate the effect of yeast extract on isolates of all species in order to ascertain whether or not it should be incorporated invariably in the basal medium. Consequently, 3 isolates of each species were inoculated on:

Basal medium alone

Basal medium plus 0.05% yeast extract

Basal medium plus 1% glucose

Basal medium plus 0.05% yeast extract plus 1% glucose

The results of this study confirmed the requirement of *T. verrucosum*, *T. violaceum*, *E. floccosum* and *T. soudanense* for additional growth factors. It showed that the growth of *T. mentagrophytes*, *T. tonsurans* and *M. audouinii* was greatly stimulated in the presence of yeast extract. The other species, however, grew as well on the basal medium with 1% glucose as the medium with glucose and yeast extract. In view of the findings with different sizes of inoculum (*vide infra*), it was decided not to add yeast extract routinely to the basal medium.

COMPARISON OF METHODS FOR INOCULATING ASSIMILATION MEDIA

The initial procedure for inoculation of carbon assimilation media was transfer of small pieces of mycelium by needle to the surface of assimilation media in test tubes or screw-capped bottles. Although this was satisfactory, it was time-consuming, particularly in the tests were to be carried out in duplicate. Consequently, attention was given to two other procedures (see page 33), and isolates of 9 species were compared by all three methods.

The results with the other two methods were interesting but predictable. The size of the colonies produced by duplicate drops from a pasteur pipette was almost identical, but growth occurred on compounds originally found not to be utilised. The growth on the plates inoculated with mycelial plugs was also greater than that obtained with the original method although the differences were less marked. Confirmation of the original results was attempted using the original method of inoculation. Although inoculations were made using plates instead of tubes, the results corresponded most closely to the initial results. From these findings, it is obvious that size of inoculum is all-important. Too much inoculum was deposited over a very small area from the pipette, and therefore significant growth was able to occur even with minimum utilisation of the carbon compound. The use of an inoculum in the form of a plug also introduced a large amount of material but the fungus first had to grow down the plug before it could grow on the assimilation medium. If the compound was not well utilised, growth of the fungus did not advance very far from the plug. The initial method therefore appears to give the widest range of growth measurements. It is nevertheless tedious to perform and unsuitable for large numbers of isolates. However, if only a few tests are to be done, this method would be satisfactory. When a large number of

comparisons are to be made, the use of some kind of replicating device to transfer mycelial plugs to a large number of plates would be preferable.

RESULTS OF MATING EXPERIMENTS

Certain species of dermatophytes, in addition to propagation by asexual conidia, also reproduce sexually. The sexual structures, which can only be induced to develop *in vitro* under certain well-defined conditions,are morphologically similar to those found in a family of the Ascomycetes, the Gymnoascaceae. The asci are grouped together in globular masses enclosed by a loosely woven mesh of specialised peridial hyphae. The fungi are heterothallic, *i.e.* mating strains of opposite genetic type are necessary for sexual reproduction to take place. All the dermatophytes which have so far been shown to reproduce in this way are classified in 2 genera, *Nannizzia* and *Arthroderma*, which are distinguished primarily by the structure of the peridial hyphae. Four species known to have a sexual stage were included in this investigation: *M. gypseum* (sexual stages: *N. gypsea*, *N. fulva*, *N. incurvata*), *T. mentagrophytes* (*A. benhamiae*), *T. terrestre* (*A. quadrifidum*) and *T. ajelloi* (*A. uncinatum*). At the start of the investigation, the mating behaviour was known for only 5 isolates, *viz.* 1 isolate of *M. gypseum* (*N. gypsea*) and one mating pair each of *A. quadrifidum* and *A. uncinatum*. Since the pattern of assimilation of sugars shown by the 6 isolates of *M. gypseum* was variable, the mating behaviour of these isolates was determined in order to see whether this could be correlated with the assimilation pattern. The mating behaviour of the isolates of the other 3 species were determined at the same time. The results are shown in Table 15.

There was no difference in nutritional pattern shown by strains of different mating type. This is in contrast to the results of Rippon

TABLE 15

MATING BEHAVIOUR OF FOUR SPECIES OF DERMATOPHYTES

Species	Code No.	Tester Strains of												Results
		N. gypsea		N. incurvata		N. fulva		A. benhamiae		A. quadrifidum		A. uncinatum		
		"–"	"+"	"–"	"+"	"–"	"+"	"–"	"+"	"–"	"+"	"–"	"+"	
M. gypseum	B1	–	–	–	–	–	–							Untypable
	B3,B4, B5,B6	–	–	+	–									Mating type "minus"
T. mentagrophytes	F1,F3,F5, F6,F8,F9							–	+					Mating type "plus"
	F2							+	–					Mating type "minus"
	F4,F7,F10							–	–					Untypable
T. terrestre	V3,V4, V5,V6									–	+			Mating type "plus"
T. ajelloi	W1,W4											–	–	Untypable

– = no cleistothecia + = fertile cleistothecia

and Garber (1963) who found differences in the production of certain enzymes by strains of opposite mating type for *M. gypseum* and *T. mentagrophytes*. However, as the two investigations were testing different characteristics, the results are not necessarily incompatible.

4. DISCUSSION

The overall similarity in the pattern of assimilation shown by the isolates of dermatophytes used in this study invalidates any attempt to separate species on biochemical criteria alone. It is not possible, for example, to distinguish by such criteria the genera already defined on morphological grounds. On the contrary, some species of Microsporum and Trichophyton have virtually identical patterns, e.g. M. gypseum and T. mentagrophytes. It is possible that the ability to assimilate some compounds preferentially is determined by the degree of parasitism of the individual isolates rather than the morphological features. In this context it is notable that almost all species of animal and soil origin are able to hydrolyse and assimilate urea. The exceptions are T. erinacei and T. gallinae. The results nevertheless support the separation of certain species originally regarded as synynomous, e.g. M. persicolor and T. mentagrophytes.

Six species of Microsporum were investigated. Three had very similar nutritional patterns. M. canis and M. gypseum were very similar and could not readily be differentiated but M. audouinii could be distinguished by its inability to utilise trehalose, its slow hydrolysis of urea and its requirement for additional growth factors. The remaining three species, however, differed not only from the first three but also from each other. The most interesting is M. equinum. The exact taxonomic status of this species is at present in some doubt. A review of standard texts reveals that Ciferri (1960) and Conant et al. (1973) regard it as con-specific with M. canis. Lewis and Hopper separated these two species in the 3rd edition of their book (1948) but failed to mention M. equinum in the 4th edition. It is also not considered as a separate species by

Rebell and Taplin (1970). Morphologically there are some minor points of difference between the two species, particularly in the appearance of the colony (M.equinum is more glabrous) and the shape of the macroconidium (smaller, less spindle-shaped and spiny in M. equinum). The four isolates investigated failed to assimilate maltose, and were able to use sorbitol and sucrose only to a limited extent. Unlike M. canis, they grew in the presence of dextrin. These results suggest that M. equinum is sufficiently dissimilar from M. canis to warrant its retention as a separate species.

M. ferrugineum failed to assimilate galactose and erythritol and grew only slightly in the presence of maltose and sucrose. On morphological grounds this species has been considered to resemble T. soudanense although Vanbreuseghem (1963) separated them by their growth on certain media (Dubos, Lowenstein, rice cream and malt agars). The separation of the two species is certainly supported by the results of this investigation. Firstly, M. ferrugineum is able to utilise ammonium ion whereas an additional source of nitrogen is required for the growth of T. soudanense. Secondly, T. soudanense uses maltose, sucrose and erythritol whereas M. ferrugineum does not. Finally M. ferrugineum hydrolyses urea whereas T. soudanense is unable to do so.

Microsporum (Trichophyton) persicolor and T. mentagrophytes were originally regarded as synonymous although Stockdale (1953) in her detailed study of the former species preferred to regard them as distinct. However, the perfect states of both species have now been described, viz. N. persicolor (Stockdale, 1967) and A. benhamiae (Ajello & Cheng, 1967) respectively, and the conidial states are no longer regarded as belonging to the same genera. T. persicolor was renamed M. persicolor (Stockdale, 1967) since its perfect state is a Nannizzia and the macroconidia are rough walled. However, these two species are often difficult to distinguish in the

laboratory in the conidial stage, so that the differences in assimilation behaviour could be very helpful. The three isolates of M. persicolor investigated were unable to utilise galactose and only grew poorly on sucrose and maltose. T. mentagrophytes utilised all three sugars well. T. mentagrophytes also hydrolysed urea rapidly whereas M. persicolor hydrolysed urea slowly. On H & L agar, M. persicolor had a pink-buff coloration whereas T. mentagrophytes did not.

Support for the separation of T. erinacei from T. mentagrophytes is also given by the nutritional results. This species was first described by Smith and Marples in 1963 as a variety of T. mentagrophytes although several differences were recognised including pigmentation of the colony, the shape of the microconidia and the absence of spiral hyphae so characteristic a feature of granular strains of T. mentagrophytes. The main point of difference detected in the present work was the inability of T. erinacei to assimilate and hydrolyse urea, whereas T. mentagrophytes did both. In addition T. erinacei was unable to assimilate galactose.

The agent of favus in mice, T. quinckeanum (a rare pathogen of humans) has been regarded by some authors as identical to T. mentagrophytes (Ajello, Bostick & Shu-lan Cheng, 1968). Mating experiments (Ajello, Bostick & Shu-lan Cheng, 1968), using known tester strains of T. mentagrophytes, showed complete identity for one strain out of 11 strains tested. From this they argued in favour of close affinity between these two species. There has, however, been no published confirmation of this finding and Stockdale (pers.comm.) has been unable to repeat this experiment. The nutritional tests revealed certain differences which may be significant, viz. inability of T. quinckeanum to assimilate galactose and erythritol, and slight stimulation by sorbitol. The six isolates tested also responded slightly to the presence of the ammonium ion whereas the isolates of

T. mentagrophytes did not.

Although the isolates of T. megninii tested failed to grow in the absence of histidine, they were in all other respects very similar to T. rubrum. Unfortunately only two isolates of T. gallinae were available for study, but these differed from T. rubrum and T. megninii notably in the assimilation of dextrin and sucrose and complete lack of urease.

The three glabrous species tested, T. schoenleinii, T. violaceum and T. verrucosum, are alike in their relatively slow rates of growth and reduced morphology on ordinary laboratory media. Nutritional and biochemical differences were nevertheless found among the isolates tested as follows:

Test	T. schoenleinii	T. violaceum	T. verrucosum
Ribose assimilation	Growth	No growth	Growth
Sucrose assimilation	Slight growth	No growth	Growth
Trehalose assimilation	No growth	Growth	Growth
Tyrosine hydrolysis in seven days	Positive	Negative	Negative
Gelatine liquefaction	Positive	Negative	Negative

T. schoenleinii hydrolysed tyrosine and gelatine rapidly in contrast to the other species which possess very little in the way of proteolytic activity. T. verrucosum differed from T. violaceum in being able to assimilate ribose and sucrose.

The ability of isolates to mate is normally regarded as an indication of th eir complete genetic identity. The fact that nutritional differences were found between T. quinckeanum and T. mentagrophytes is of considerable interest in view of the results obtained with T. terrestre, T. ajelloi and M. gypseum. Four isolates of T. ajelloi were tested; one pair mated to form the sexual stage (A. uncinatum) whereas the other two isolates were unable to take part in the sexual process, i.e. they were

conidial strains. However, no nutritional differences could be detected between the sexual and conidial isolates. Similarly, although a compatible mating pair was included among the strains of T. terrestre investigated, and the remaining four isolates mated with one of the tester strains, no nutritional differences could be detected between them. The six isolates of M. gypseum investigated showed some variation in their pattern of assimilation but this could not be correlated with mating behaviour. "M. gypseum" is the conidial state of two sexual fungi: N. gypsea and N. incurvata, and a morphologically similar fungus M. fulvum is the conidial state of N. fulva (Stockdale, 1963). Although the conidial forms vary slightly, in practice it is difficult to tell them apart, and the term "M. gypseum" is used by many medical mycologists to refer to any one of the three. Mating studies showed that four of the six isolates were N. incurvata, one was N. gypsea and the sixth failed to produce a sexual stage. Although there were nutritional differences, particularly in the utilisation of trehalose, and the hydrolysis of casein and urea, none of these differences correlated with the mating behaviour. The ability to assimilate particular compounds is likely to be influenced by the natural habitat of the isolates and since all three species of Nannizzia are soil inhabitants, this lack of correlation may not occasion surprise.

The results of the present study do not always correspond with those of other workers (Table 16). It is difficult to compare results directly since materials and methods have varied widely, and some of the discrepancies may be explained if this is borne in mind. For example, the results obtained by Hopkins and Iwamoto for T. megninii are totally at variance with the results of the present investigation and may be explained by their failure to add histidine to the basal medium. They recorded poor growth of their isolates of M. audouinii and T. tonsurans

TABLE 16 ASSIMILATION OF CARBON COMPOUNDS BY DERMATOPHYTES: COMPARISON OF RESULTS OF VARIOUS WORKERS

Organism	Compound	TI	B	G&H	H&I	S&G	M	ST	G	P	L&H	P-Y
M. canis	Galactose	+		+	+				−	−	−	−
	Arabinose	−		+	−				+			+
	Xylose	−		+	−							
	Maltose	+		+	+				+	−	−	+
	Sucrose	+		+	−				±	−	−	−
	Melibiose	−		+								
	Sorbitol	+		+	−							+
	Glycerol	−		+	−							
	Dextrin	−		+	+							
M. equinum	Maltose	−	+									
	Sucrose	+	−									
	Dextrin	+	+									
M. audouinii	Galactose	−		+	−					−		−
	Maltose	+		+	−					−		−
	Sucrose	+		−	−					−		−
	Mannitol	+		+	+							−
	Sorbitol	+		+	±							+
	Glycerol	−		+	−							
	Dextrin	−		+	−							
M. gypseum	Galactose	+		+	±					−		−
	Maltose	+	+	+	+					−		−
	Sucrose	+	−	−	−							
	Melibiose	−		+								
	Mannitol	+		+	+							−
	Sorbitol	+		+	±							+
	Glycerol	±		+	+							
M. persicolor	Galactose	−						−				
	Maltose	±						+				
	Sucrose	±						−				
	Glycerol	±						−				
T. mentagrophytes	Galactose	+			−		+		+	+		
	Raffinose	−			−							
	Dextrin	−			−							
T. quinckeanum	Galactose	−			−							
	Maltose	+	+		−							
T. rubrum	Galactose	+			−						−	
	Maltose	+			+						−	
	Sucrose	+			−						−	
	Raffinose	−			−						−	
	Dextrin	−			+						−	
T. megninii	Galactose	+			−					+		
	Maltose	+			−					+		
	Sucrose	+			−					+		
T. tonsurans	Galactose	+			±	+				+		
	Maltose	+			±	−				+		
	Sucrose	+			±	−				+		
	Raffinose	−			−	−						

Workers cited

TI	This investigation
B	Bodin, 1899
G&H	Giblett & Henry, 1950
H&I	Hopkins & Iwamoto, 1923
S&G	Schwartz & Georg, 1955
M	Mosher et al., 1936
ST	Stockdale, 1953
G	Goddard, 1934
P	Pinetti, 1947-9
L&H	Lewish & Hopper, 1941
P-Y	Pena Yanez & Pallares, 1956

− No growth

± Poor growth

\+ Good growth

and this may be the reason why they did not detect growth of these species on galactose, maltose and sucrose. Similarly, Giblett and Henry noted that M. canis and M. audouinii grew on glycerol and dextrin and M. gypseum on melibiose, without however recording the amount of growth obtained. They state that growth was variable so the discrepancy could arise from their method of recording growth. The results of hydrolytic tests with casein and gelatine show a large measure of agreement. Almost all workers found that their isolates hydrolysed casein and gelatine. Only about 7% of isolates in the present investigation failed to hydrolyse casein, and it was also found that only T. violaceum, T. verrucosum and a few isolates of M. canis failed to hydrolyse gelatine when incorporated at 1% in a nutrient agar base. With reference to the assimilation of nitrogen compounds, all investigations including the present one have found that asparagine acted as a good source of nitrogen, that nitrate was not utilised, and that ammonium ion promoted fairly good growth except for T. tonsurans, T. soudanense, T. verrucosum, T. violaceum and E. floccosum. Urea was not utilised as sole source of nitrogen by M. audouinii, T. violaceum, T. verrucosum or E. floccosum. The present investigator would add to this list T. soudanense, some isolates of T. tonsurans and T. rubrum. Some workers have found that both T. tonsurans and T. rubrum can utilise urea (Schwartz & Georg, 1955). Since the present investigator found differences between strains regarded morphologically as T. tonsurans and those with a T. sulfureum type of colony, strain differences are obviously important. Thus, the overall coincidence of results is reasonable if due allowance is made for differences in technique and materials used.

The aim of the present investigation was the development of simple nutritional tests which could be used in the differentiation and

classification of the dermatophytes. Based upon the results obtained in the present study, a practical scheme allowing differentiation of the 22 species investigated follows. It illustrates how recognition of unknown isolates of dermatophytes may be achieved by a sequential 3-stage evaluative process. Species are divided into six groups based primarily on the assimilation of nitrogen compounds and the hydrolysis of gelatine. These groups are further sub-divided on the pattern of assimilation of, firstly, galactose, trehalose and ribose, and secondly, erythritol, maltose, sucrose, adonitol and sorbitol. It was not found, possible, however, to separate *T. mentagrophytes* from *M. gypseum* and *M. cookei* from *M. persicolor*.

These groups do not correspond with the morphological genera (*Microsporum*, *Trichophyton*, *Epidermophyton*). In Group I are found most of the geophilic and zoophilic species, in Groups II to VI most of the human parasites. Since similarity in structure is not correlated with similarity in nutritional requirements, it is possible to use nutritional tests to distinguish between species, for example, *T. mentagrophytes* and *M. persicolor*; *T. soudanense* and *M. ferrugineum*; *T. mentagrophytes* and *T. rubrum*; *T. violaceum*, *T. verrucosum* and *T. schoenleinii*. The most useful test devised so far is the urease test which can be used to distinguish *T. rubrum* from *T. mentagrophytes* and *M. ferrugineum* from *T. soudanense*. It is also helpful as a criterion for the identification of *T.erinacei* (Rebell & Taplin, 1970). The 3-stage breakdown suggests further ways in which nutritional differences may be used to differentiate the majority of species.

The aim of this investigation has therefore been achieved. The tests described are within the competence of a routine microbiological laboratory and may easily be added to the laboratory diagnostic procedures. They will not replace traditional methods of identification, based primarily on morphology, but will provide useful supplementary methods when morphology fails to provide an answer.

THREE STAGE BREAKDOWN FOR IDENTIFICATION OF DERMATOPHYTES:

STAGE 1

GROUP	UREA HYDROLYSIS	UREA ASSIMILATION	NH_4^+ as SOURCE of NITROGEN	GELATINE HYDROLYSIS	SPECIES
I	+	+	+	+	Tsc,Mc,Mg, Tm,Tq,Mco, Meq,Teq,Tt Mp,Ta
II	+	-	+	+	Tto,Tr,Tmg, Mf
III	+	-	-	+	Ef,Tto
IV	+	-	-	-	Tvi,Tve,Ma
V	-	-	-	-	*T. erinacei*
VI	-	-	-	+	*T.soudanense*

Tsc *Trichophyton schoenleinii*
Tm *T. mentagrophytes*
Tq *T. quinckeanum*
Teq *T. equinum*
Tt *T. terrestre*
Ta *T. ajelloi*
Tto *T. tonsurans*
Tr *T. rubrum*
Tmg *T. megninii*
Tvi *T. violaceum*
Tve *T. verrucosum*
Mc *Microsporum canis*
Mg *M. gypseum*
Mco *M. cookei*
Meq *M. equinum*
Mp *M. persicolor*
Mf *M. ferrugineum*
Ma *M. audouinii*
Ef *Epidermophyton floccosum*

STAGE 2

GROUP	SUBGROUP	GALACTOSE	RIBOSE	TREHALOSE	SPECIES
I	A	+	+	-	*T. schoenleinii*
	B	-	-	+	Ta,Mco,Meq,Teq,Tt,Mg, Mc,Tq
	C	+	-	+	*Microsporum gypseum* *T. mentagrophytes*
II	D	+	-	+	Tto, Tr, Tmg
	E	-	-	+	*M. ferrugineum*
III	F	-	-	-	*E. floccosum*
	G	-	-	+	*T. tonsurans*
IV	H	+	+	+	*T. verrucosum*
	I	-	-	+	*T. violaceum*
	J	-	-	-	*M. audouinii*

STAGE 3A FOR GROUP IB

SUBGROUP	ERYTHRITOL	SUCROSE	MALTOSE	ADONITOL	SORBITOL	SPECIES
1	+	+	+	-	+	M. canis T. equinum*
2	+	+	+	+	+	T. terrestre
3	+	-	+	+	+	T. ajelloi
4	+	-	-	-	+	M. cookei M. persicolor
5	-	+	+	-	-	T. quinckeanum
6	+	+	-	-	+	M. equinum
	*can be distinguished by omitting nicotinic acid					

STAGE 3B FOR GROUP IID

	UREA HYDROLYSED WITHIN 8 DAYS	UREA HYDROLYSED SLOWLY	
7	+	-	T. tonsurans
8	-	+	T. rubrum T. megninii**
	**can be distinguished by omitting histidine		

PART II. SEROLOGICAL INVESTIGATIONS

1. REVIEW OF THE LITERATURE

Very limited use has been made of serological techniques to explore relationships among the ringworm fungi. Most of the published work relates to studies of their cell wall polysaccharides by Bishop and co-workers (Alfes, Bishop & Blank, 1963; Bishop, Blank & Hranisavljevic-Jakovljevic, 1962; Bishop, Perry & Blank, 1966; Bishop *et al.*, 1965, 1966; Blank & Perry, 1964), and to a comparison between extracts of the whole organisms, basically glycoprotein in nature, made by Biguet *et al.* (1961, 1965, 1968). Bishop and his co-workers extracted polysaccharides from the cell walls of several dermatophytes, after removing all the nitrogenous material. Each polysaccharide could be broken down into 3 main components: 2 galactomannans, designated I and II, and a glucan. The galactomannans differed in the linkage of the linear manno-pyranose chains and the galacto-furanose/manno-pyranose end groups and side chains.

The detailed chemical analyses were followed by attempts to relate structure of polysaccharides to serological affinities of the species from which they were derived. Antisera were prepared by injecting the extracts into rabbits. The antigenic nature and inter-relationships of 27 polysaccharides isolated from single isolates of 9 species of dermatophytes were compared using double diffusion in agar gel, immuno-electrophoresis, and complement fixation. A summary of their findings is shown in Table 17.

Bishop *et al.* concluded that Galactomannans I are the most antigenic, yet chemically most similar. The main difference between galactomannans from the species examined is in the amount of galactose present. Galactomannans II are less antigenic but chemically more

TABLE 17 SEROLOGICAL REACTIVITY OF POLYSACCHARIDES FROM DERMATOPHYTES

ANTISERA TO:	Tqu	Tsc	Tin	Tgr	Tru	Mpr	Mfe	Tsa	Tto
Galactomannans I									
T. quinckeanum	++	++	++	++	++				
T. schoenleinii	++	+++	++	++	++				
T. interdigitale	-	++	++	-	++				
T. granulosum	++	++	++	++	++				
T. rubrum	++	++	++	++	++				
M. praecox						+++	+	++	++
M. ferrugineum						++	+++	++	++
T. sabouraudii						++	+	+++	++
T. tonsurans						++	++	++	++
Galactomannans II									
T. quinckeanum	+++	+++	+	+	+				
T. schoenleinii	+	++	++	++	+				
T. interdigitale	+	++	++	-	-				
T. granulosum	++	++	+	++	+				
T. rubrum	+	++	++	++	+				
M. praecox						+++	+	++	++
M. ferrugineum						+	-	+	+
T. sabouraudii						+++	+	++	++
T. tonsurans						+++	+	++	++
Glucans									
T. quinckeanum	++	+	+	+	-				
T. schoenleinii	+++	++	+++	++	+				
T. interdigitale	-	-	-	-	-				
T. granulosum	-	-	-	++	-				
T. rubrum	-	-	-	-	-				
M. praecox						++	-	+	-
M. ferrugineum						++	-	++	++
T. sabouraudii						++	+	+	+
T. tonsurans						-	-	-	-

- = no reactivity; + = poor reactivity; ++,+++ = good reactivity

Tqu	T. quinckeanum	Tgr	T. granulosum	Mfe	M. ferrugineum
Tsc	T. schoenleinii	Tru	T. rubrum	Tsa	T. sabouraudii
Tin	T. interdigitale	Mpr	M. praecox	Tto	T. tonsurans

variable. Bishop et al. found it difficult to decide which structural feature accounted for the serological reactivity, particularly since there were differences between the two groups of species in the types of chemical linkage and the percentage of chemical groups, but suggested that the amount of galactose was again the most important factor. Support for this hypothesis is provided by evidence from the mannans which were found to be much less reactive than their parent galactomannans. Glucans showed such variability that Bishop et al. considered them to be of little value in determining serological relationships.

The serological results obtained, however, do not support the interpretation put on them by Bishop et al. Almost all extracts showed reactions of identity with each other and there were very few specific reactions. It seems that cellular polysaccharides from dermatophytes are both structurally and serologically alike. Examination of Table 17 shows that most extracts reacted in the same way to the individual antisera. In some instances, and particularly with M. ferrugineum, the antiserum reacted less well with its own galactomannan II and glucan than with those from other species. Since M. ferrugineum reacted very poorly with all antisera it would seem that the polysaccharides from this species at least are poor immunising agents, and this is probably true for some of the other polysaccharides as well. There is little correlation evident between chemical content and serological reactivity. The highest percentage of galactose occurs in the galactomannans I of T. schoenleinii and T. interdigitale but they have different serological reactivity. M. ferrugineum and M. praecox do not react similarly although they have almost the same percentages of galactose, while T. sabouraudii and T. tonsurans react similarly yet differ by a factor of almost 2 in the amount of galactose present. Bishop et al. draw few taxonomic conclusions from their work,

but do suggest that these results support the separation of T. granulosum from T. interdigitale and T. quinckeanum, and T. sabouraudii from T. tonsurans. They themselves make little reference to the work of Basarab, How and Cruickshank (1968) but their results show more consistency if interpreted in the same way as these workers. Basarab, How and Cruickshank found that after removal of the protein from mycelial extracts of dermatophytes, a mixture of specific and non-specific skin reactions was obtained in guinea-pigs sensitised by prior injections of the original extracts. The non-specificity could however be eliminated by suitable extraction procedures. The carbohydrate fraction gave rise only to "immediate" (Type I) skin reactions; the protein fraction was essential for the production of precipitating antibody. It was also established that chemical variability had little influence upon specificity, at least as far as the type I component of the skin test was concerned. Similar reactions were given by extracts that differed in their chemical structure. If such an interpretation is made of Bishop et al. work, the results appear more consistent. Galactomannans I, which are present in greatest amount, are chemically variable but give very similar reactions, and these extracts correspond closely to those studied by Basarab, How and Cruickshank. Barker, Cruickshank and Holder (1963) found that polysaccharides extracted from T. interdigitale and T. granulosum differed, particularly in the types of linkage and the amount of branching, yet these fungi are at present considered to be varieties of a single species.

Antigenic studies of dermatophytes have also been made by Sharp (1945), Dyson & Landay (1963), Kielstein (1966), Sagara (1968), and Biguet et al. (Biguet, Andrieu & Tran Van Ky, 1961; Biguet, Andrieu & Laloux, 1965; Andrieu, Biguet & Laloux, 1968). Sharp used ring precipitation tests to study 7 dermatophyte species and one strain each

of 14 non-pathogenic fungi recovered from the skin around ringworm lesions. The antigens consisted of saline extracts of mycelium, and the same material was used to prepare antisera in rabbits. There were crossreactions between species of dermatophytes, but not between dermatophytes and non-dermatophytes. This indicated that common antigens existed within the dermatophytes, but that this group was sharply differentiated from the saprophytic moulds tested. However, there was considerable difference between species in the amount of cross reactivity, as the following table shows; the figures represent the amount of precipitation obtained.

Antigens	Antisera to					
	Ef	Mc	Ma	Tm	Tr	Tt
M. canis (Mc)	3+	4+	2/4+	0/1+	2+	0/1+
M. audouinii (Ma)	2+	3+	3+	0/1+	2+	0/1+
M. gypseum	2+	1+	0	0	2+	0
T. mentagrophytes (Tm)	2/3+	0/1+	0	4+	3+	1/2+
T. rubrum (Tr)	2+	1+	0	2+	4+	1+
T. tonsurans (Tt)	3+	1+	0	2+	2+	3+
E. floccosum (Ef)	4+	0	0	1+	2+	0

M. canis and M. audouinii are obviously closely related, and can be distinguished from the other genera. The single isolate of M. gypseum tested reacted better with antisera to T. rubrum and E. floccosum than to the other Microsporum species which suggests that it is not closely related to them. The 3 Trichophyton species reacted closely with one another and also with E. floccosum and M. gypseum.

Dyson and Landay (1963) used 4-6 week old culture filtrates and saline suspensions of mycelium in gel diffusion tests to distinguish

T. mentagrophytes from T. rubrum. Although there were at least 2 common antigens, all their isolates of T. rubrum had an additional specific antigen which distinguished them from T. mentagrophytes.

Sagara (1968) also compared mycelial extracts of these two species by gel diffusion, and found that differentiation was possible but extracts of 2 varieties of T. mentagrophytes (var. interdigitale and var. asteroides) were very similar to each other.

Kielstein (1966) used crude polysaccharide extracts of 12 dermatophyte species in gel diffusion tests, and found several antigens in common and other which showed partial identity. In that study it was not found possible to separate the species on the basis of their serological affinities.

Biguet et al. (1961) also used mycelial extracts to produce antisera in rabbits, by means of subcutaneous injection in Freund's adjuvant. In a later paper (1965) the authors suggested that better results were obtained if a more purified antigen was used and injected intramuscularly. Injection was continued until there was no further increase in the number of bands obtained by immunoelectrophoresis. Two types of antigen were prepared. Firstly, the culture medium was concentrated and freeze-dried. Secondly, the mycelium was alternately frozen and thawed several times and then ground with salt to extract the protein.

The first results (1961) were most encouraging. An antiserum to T. mentagrophytes was prepared, which gave 15 lines on immuno-electrophoresis with its homologous antigen. Four antigens prepared in the same way and tested against this antiserum gave fewer lines, viz. T. schoenleinii 7, M. canis 5, E. floccosum 2 and T. soudanense 2, but all these lines were also given by T. mentagrophytes. Two lines were

given by all 5 species, and 3 were common only to T. mentagrophytes, T. schoenleinii and M. canis.

The survey was then extended to further species of dermatophytes. In particular, they compared (1965) the reaction of M. ferrugineum and M. canis in an endeavour to establish the precise taxonomic affinities of the former species. M. ferrugineum, first described from Manchuria in 1922 (Ota, 1922), was transferred to the genus Trichophyton by Langeron and Milochevitch (1930) but much argument has raged over its allocation to either genus. Biguet found that M. ferrugineum and M. canis gave almost identical reactions to heterologous antisera, and M. audouinii antigen also reacted to both antisera. The number of lines given by antigens of 5 Trichophyton species was much smaller, indicating that these species are not so closely allied to the genus Microsporum.

This work was then extended (Andrieu, Biguet & Laloux, 1968) until a total of 16 species of dermatophytes were studied (Table 18). All these species showed considerable cross-reactivity but the authors drew the following conclusions:

(a) M. ferrugineum is closest immunologically to M. canis and M. audouinii, and very different from T. soudanense with which it may be confused on morphological grounds.

(b) M. canis, M. audouinii and M. langeronii are very close serologically.

(c) M. gypseum has affinities with all genera but is not particularly close to Microsporum species.

(d) T. mentagrophytes has affinities with all genera.

(e) T. schoenleinii is serologically related to all genera.

(f) T. rubrum is closer to T. violaceum than to T. mentagrophytes.

(g) T. violaceum is close to T. yaoundei, and T. soudanense

TABLE 18

CROSS REACTIONS AMONG THE DERMATOPHYTES
Andrieu, Biguet & Laloux 1968

ANTIGENS - SYMBOLS	NUMBER OF ELECTROPHORETIC BANDS														
	20	19	17	16	15	14	13	12	11	10	9	8	7	6	5	4
K. ajelloi - KA				KA						Mg	Tm,Mc,Mf Tsc,Ml,Ef	Tvi,Ma Tg		Tr	Ty	Tve,Tt Tso
T.mentagrophytes - TM			TM					Mc,Tsc,Ty Ef,Tvi,Tr	Mg	Mc,Mf,Tt Ka,Ma,Tve	Tg Tso					
M. gypseum - MG				MG						Ma,Tm,Ef	Mc,Ka	Ml,Tsc Tvi,Tr,Tg	Mf,Tso Ef	Tve	Ty	
M. canis - MC	MC			Mf	Ma Ml				Mg,Tm		Tsc,Ef	Tvi,Ka,Tg	Tt,Tr	Ty,Tso	Tve	
M. langeronii - ML		ML		Mc	Ma Mf					Mg	Tm,Tsc	Ka,Ef	Tvi,Tr Tg	Tso	Ty,Tve,Tt	
M. audouinii - MA				MA		Ml Mf	Mc		Mg			Tm,Tvi,Ka Tsc,Ef	Tg,Tr	Ty,Tt Tso	Tve	
M. ferrugineum - MF				MF			Ma Mc Ml				Mg	Tm,Ka	Tvi,Tg	Tsc,Tso Ef,Tr	Tve,Ty Tt	
T. schoenleinii - TSc					TSc			Tm	Tvi,Tg	Mg,Tve,Tr Ty,Tso,Tt		Ma,Mc,Ka Ml		Mf		
T. verrucosum - TVe										TVe	Tm,Tvi Tso,Tg	Tsc,Tt,Tr	Ty	Ef	Mg,Ma,Mc Ml,Mf	Ka
T. violaceum - TVi				TVi	Tg	Ty		Tr	Tm,Tso		Tt	Tsc	Tve,Mg Mc,Ef	Ml,Mf	Ma,Ka	
T. yaoundei - TY					Tg	TY Tvi			Tm,Tso Tr	Tsc,Tt			Tve,Mg	Mc,Ef Ka,Mf	Ma,Ml	
T. gourvilii - TG						Tvi	TG	Tso		Tr, Tt	Tm	Ty	Tsc,Mg Mc,Ef	Tve,Ma Ka	Ml,Mf	
T. soudanense - TSo				Tg		Tvi	TSo		Tr	Tt	Tm,Tve Ty	Tsc,Mg Ef		Ma,Mc Ml	Mf,Ka	
T. tonsurans - Tt								TT Tm	Tsc,Tg Tr,Tso		Tvi	Tve,Ty		Ma,Mc Ml	Mg,Ef	Mf,Ka
T. rubrum - TR					TR			Tvi,Tg	Tm	Tsc,Tso Ty	Tt	Tve	Mc,Ef	Ma,Mg Mf	Ml,Ka	
E. floccosum - EF				EF						Tm,Mg	Ma,Tr Mc	Ml,Tg,Ka Tvi,Tso	Mf,Tsc Tt	Tve,Ty		

to T. gourvilii but all 4 species show many other mutual similarities.

However, examination of these results shows that although there is much cross-reactivity, the antigenic extracts of Microsporum species react poorly with Trichophyton antisera. Similarly, antigenic extracts of Trichophyton species, with the exception of T. mentagrophytes whose antigenic extract appears to react equally well with antisera from all species and genera, react poorly with Microsporum antisera. E. floccosum extract reacts with all antisera but that from K. ajelloi shows more affinity with Microsporum and Epidermophyton than with Trichophyton. Four antigens and antisera were poorly reactive (T. verrucosum, T. yaoundei, T. soudanense, T. gourvilii); they also sometimes reacted more strongly with a heterologous reactant than with the homologous one.

The disadvantage of immunoelectrophoresis is that, although the lines are revealed plainly, it is difficult to compare antigens and/or antisera directly. Such a comparison can more readily be achieved by using double diffusion in agar gel. Biguet et al. do not report any results by this method. It would be of interest and value to know, for instance, how many of the lines were common to all species and how many were group or species-specific. The work to be reported in the following sections was undertaken to demonstrate, by means of gel diffusion tests, the presence of common and/or specific antigenic components within different species of dermatophytes and isolates of the same species. If differences were found, it was intended then to explore the possibility of using them to determine taxonomic affinities, and also as an additional method of identifying unknown isolates. The work is therefore an extension to, and a corollary of, the work of the investigators reported above.

2. MATERIALS AND METHODS

CULTURE MEDIA AND SEROLOGICAL REAGENTS

Culture Media

1.	Glucose	20.0 g
	Peptone (Mycological) (Difco)	10.0 g
	Distilled water to	1 litre

The unadjusted pH of this medium was 5.6.

2.	Glucose	20.0 g
	Peptone (Bacteriological)	10.0 g
	Distilled water to	1 litre

The unadjusted pH of this medium was 6.7.

Both media were sterilised by autoclaving at 112°C (10 lbs/sq.in) for 15 minutes.

The difference in pH of the two media and the type of peptone used had no effect upon the rate of growth of the test fungi.

Agar for Precipitin Tests

1% Ionagar (Oxoid) was used for precipitin (double diffusion) tests. It was prepared by autoclaving the agar in half the final volume of distilled water for 10 minutes, allowing the agar to cool to 45°C in a water bath, and adding an equal volume of borate buffer.

Borate Buffer

Borax	20.0 g
Boric acid	10.0 g
Ethylene diaminetetra-acetic acid disodium salt (EDTA)	5.0 g
Distilled water to	1 litre

The pH of this buffer was 8.4.

Borate Buffer for reconstituting Freeze-dried Antigens

Boric acid	6.7 g
Borax	13.4 g
Sodium merthiolate	0.1 g
Distilled water to	1 litre

The pH of this buffer was 8.4.

This solution was used to reconsitute the freeze-dried antigens.

Washing Fluid for Precipitin Plates

Sodium chloride	4.0 g
Sodium tetraborate	4.0 g
Distilled water to	1 litre

Protein Stain

Naphthalene black	0.05 g
Methanol	40.00 ml
Acetic Acid	10.00 ml
Distilled water	50.00 ml

The same fluid, without the naphthalene black, was used to differentiate the stained slides. The slides were stained for 10 minutes, rinsed briefly in methylated spirit to remove excess stain, and then differentiated by rinsing twice in acetic acid-methanol-water for 10 minutes each. Precipitin lines were dark blue.

PREPARATION OF ANTIGENS

Mycelium, from which antigens were prepared, was obtained from fungi grown in the following ways:

(a) in 250 ml glucose-peptone broth (page 80) in 1 litre flasks (still culture). The fungus was allowed to grow for not more than 3 weeks; the culture fluid was then decanted and discarded, and the mycelium stored at -20°C for at least 24 hours.

(b) in 5 litre flasks containing 3 litres of medium and agitated

continuously at room temperature on a magnetic stirrer (Baird & Tatlock) (Stirred culture). The fungus was first grown in 150 ml medium in a 500 ml flask for up to 5 days to provide a large inoculum. The contents of the small flask were then transferred to the large flask. The fungus was allowed to grow for 5 - 7 days. After this time autolysis of the fungus occurred and the antigens prepared from such material were found to be less reactive than those prepared from the log phase culture. The yield of mycelium varied depending upon the rate of growth of the fungus. For the more slowly growing species it was considered preferable to pool the low yields of several flasks rather than obtain a greater yield of autolysed material. In all instances the contents of the flasks were filtered and the mats stored at -20°C for at least 24 hours. The frozen mycelial mats were then thawed and disintegrated. In the early stages of the work an M.S.E. macerator was used, in which the mycelium is broken up by a four-bladed rotating rod. Subsequently a high-speed blender ("Alto-Mix") was used in place of the macerator. The blender disintegrated the mycelium more rapidly than the macerator (1 minute compared to 10 - 15 minutes) but the product was not otherwise influenced by the change.

The macerated material was further disrupted by ultrasonic disintegration (M.S.E. Ultrasonic Disintegrator 100 Watt Model). The cylinder in which the mycelium was placed during sonication stood in a beaker of ice to minimise heating and denaturing during disintegration. The sonicated material was allowed to stand overnight at 4°C, centrifuged at 2000 G and the precipitate discarded. The supernatant fluid was treated in one of three ways:

(1) 1.5% sodium acetate was dissolved in the supernatant fluid, and then twice its volume of acetone at -20°C was added; the mixture was left at -20°C for 2-3 hours, centrifuged at 2000 G and the supernatant

fluid discarded. The precipitate was dissolved in approximately 10 ml of distilled water and freeze-dried. These antigens are designated "A".

(2) the supernatant fluid was treated with acetone as above, but the precipitate was redissolved in approximately 20 ml distilled water. This solution was then purified and concentrated by dialysis in semi-permeable Visking tubing immersed in polyethylene glycol 6000. When the volume had been reduced to 10 ml, the solution was removed from the tubing and freeze-dried (Antigens "A2").

(3) the supernatant fluid was concentrated and purified using polyethylene glycol 6000 as in (2) above, until its volume was reduced to approximately 20 ml. It was then centrifuged at 15,000 G for 1 hour, the precipitate discarded and the supernatant fluid freeze-dried (Antigens "APG").

Freeze-dried antigens were reconstituted in merthiolate borate buffer to give final concentrations of 25 mg/ml of solid material (antigens "A" and "A2") or 50 mg/ml (antigens "APG").

The antigens prepared, and their methods of preparation, are shown in Table 19.

PREPARATION OF ANTISERA

Antisera were prepared in white rabbits using two schedules of injection. The rabbits were female, of varying ages and weights.

(a) 20% w/v mycelial suspensions of sonically disrupted material (page 82) were killed by the addition of 0.5% formalin in sodium chloride. Twice-weekly injections of 1 ml of this suspension were given intra-peritoneally to each of a pair of rabbits. After 20 injections, the animals were bled from an ear-vein, and the serum tested for the presence of precipitating antibody by double diffusion in agar gel (see page 85)

TABLE 19 ANTIGENS AND ANTISERA

Antigens	Code No. of Isolate	Method of Preparation	Antisera
M. audouinii	A4	A	A5
	A5	A	
	A6	A	
M. gypseum	B2	A	B2
	B4	A	B4
	B8	A	
M. canis	C10	A	C10
	C11	A	
	C12	A	
M. cookei	T1	A2	
	T2	A2	
	T4	A2	
M. equinum	I1	A2	
	I2	A2	
	I3	A2	
M. ferrugineum	Q2	APG	Q2
	Q3	APG	
M. persicolor	U1	A2	U1
	U2	A2	
	U3	A2	
T. mentagrophytes	F1	A	
	F4	A	F4
	F5	A	
T. rubrum	K5	A	K5
	K6	A	
	K7	A	
T. tonsurans	M1	A	M6
	M3	A	
	M6	A, APG	
T. megninii	E2	A2	
	E3	A2	
	E4	A2	
T. erinacei	G3	A2	G3
	G7	A2	
	G9	A2	
T. equinum	H7	A2	H7
	H8	A2	
	H9	A2	

TABLE 19 continued

Antigens	Code No. of Isolate	Method of Preparation	Antisera
T. quinckeanum	J1	A2	J1
	J2	A2	
	J3	A2	
T. violaceum	02	APG	05
	05	APG	
T. verrucosum	P1	APG	P1
	P4	APG	
T. soudanense	R7	A2	
	R8	A2	
	R9	A2	
	R10	A2	R10
T. schoenleinii	S6	APG	
	S6	APG	S7
T. terrestre	V3	A2	
	V4	A2	
	V5	A2	
T. ajelloi	W1	A2	
	W2	A2	W2
	W3	A2	
E. floccosum	L1	A	L1
	L2	A	
	L3	A	

For full details and code numbers of isolates, see Table 6.

For method of preparation, see "Materials and Methods", page 81.

against the homologous antigen. If at least 2 sharp lines were present, the rabbit was exsanguinated. If no lines were seen, or were weak, a further series of 10 injections was given.

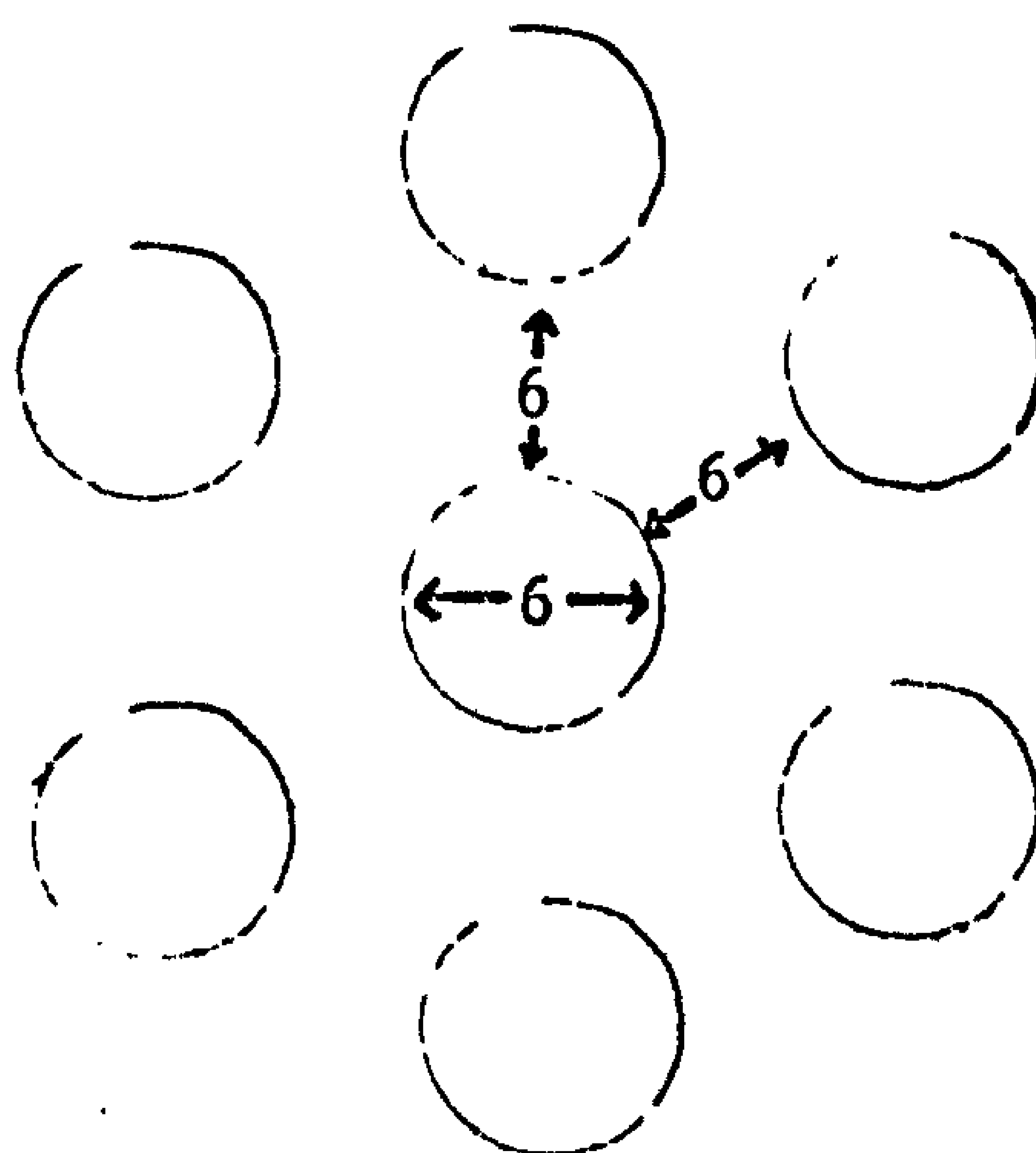
(b) 0.25 ml antigen containing approximately 5 mg of freeze-dried material in the same volume of incomplete adjuvant (1 part lanolin to 3 parts paraffin) was injected into the footpad of each rabbit. Four weeks after the injection, rabbits were bled from an ear-vein and the presence of precipitating antibody tested as above. If a further course of immunisation was required, a second injection of 0.25 ml antigen in incomplete adjuvant was given subcutaneously in the back of the rabbit. After a further four weeks, rabbits were bled and tested again for precipitating antibody. Occasionally a second "booster" injection was necessary.

When at least 2 sharp precipitin lines were present in the gel diffusion test, rabbits were anaesthetised with "nembutal" (o.25 ml/ 1 kg body weight) and exsanguinated by cardiac puncture. An equal volume of heparin was injected at the same time as the nembutal to prevent clotting of the blood. The blood was left at 4°C overnight to allow the red cells to separate; the serum was then decanted and stored at -20°C.

One antiserum was prepared against one isolate of each species with the exception of _M. equinum_, _M. cookei_, _T. megninii_ and _T. terrestre_. (Table 19).

PRECIPITIN TESTS

2.5 ml borate buffered agar (page 80) was put into a 5 cm plastic petri dish, giving a depth of medium of approximately 2 mm, and the pattern of wells cast by a metal jig with a series of pegs; the pattern and size and distance apart of wells are illustrated below.



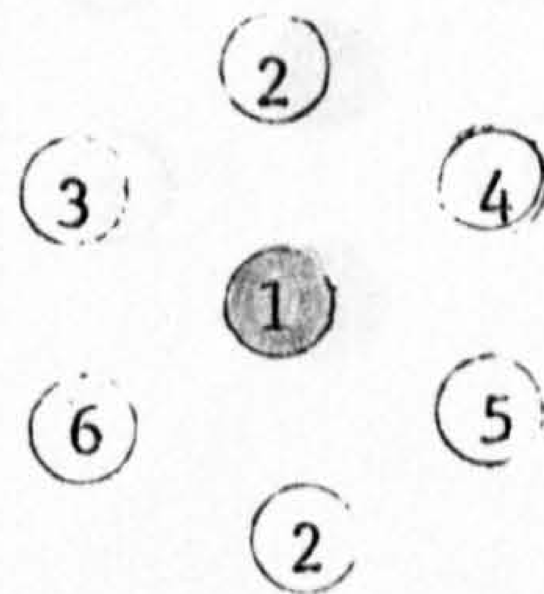
Distances are in millimetres

The agar rose slightly up the sides of the metal pegs, so that each well was surrounded by a raised "lip": this permitted a greater volume of reagent to be placed in each well for the given depth of agar, by comparison with wells which are cut by metal borers after the agar has set. Measured 50 μl quantities of antigen and antiserum were placed in the appropriate wells, and the plates kept at 26°C for 48 or 72 hours to allow diffusion and precipitation to take place. The plates were then placed vertically and washed in a saline bath for 48 hours (page 81), allowing approximately 150 ml washing fluid for each plate. After a rinse in distilled water, the agar was lifted out and placed on a glass slide 2.5 x 7.5 cm: excess agar was trimmed off with a scalpel blade, and the slides were dried in a warm air oven at 40°C. The dried films were stained by the method given on page 81. Staining of the agar gel revealed more lines than could be seen in the unstained plates.

TITRATION OF ANTIGENS

Titration of antigens against antisera were always made at 3 concentrations of antigen, viz. 12 mg/ml, 6 mg/ml and 3 mg/ml. A standard pattern of wells was used, but the arrangement of antigens and antisera varied. 3 basic arrangements were used: shaded circles represent antisera, open circles represent antigen.

(1) Standard pattern

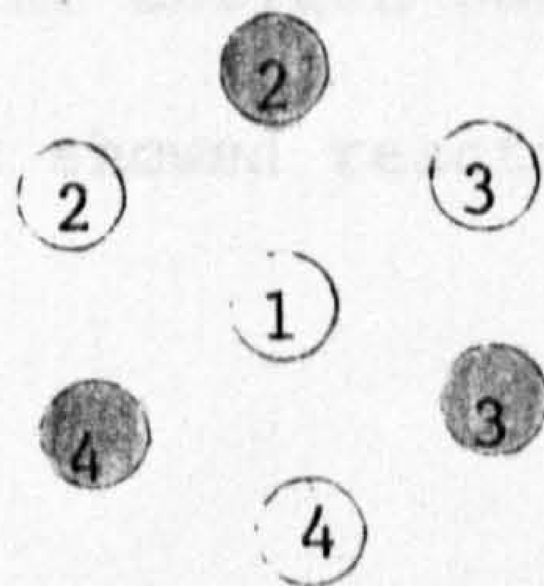


1	Control antiserum
2	Control antigen
3-6	Test antigens

Control antiserum in central well, homologous antigen in polar wells, test antigens in lateral wells.

A variation of this pattern is shown below, which helped to supplement the information upon serological systems and antigenic relationships obtained from the standard pattern.

Pattern 1A



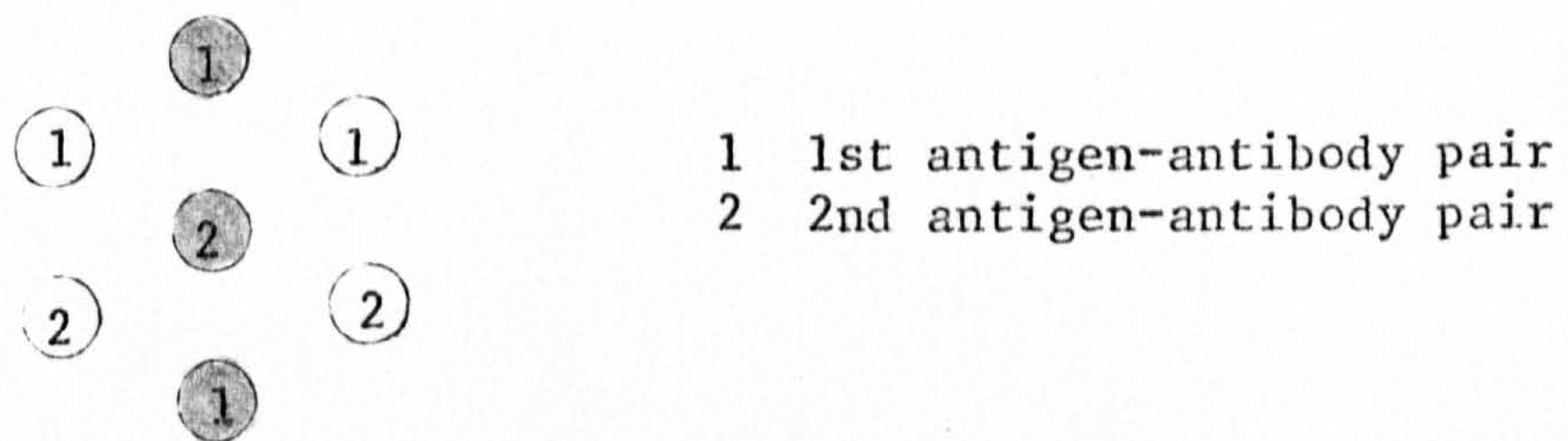
1	Test antigen
2A,2S) 3A,3S) 4A,4S)	3 different antigen-antibody pairs

Test antigen in central well, and 3 different antigen-antibody pairs in the peripheral wells (2A, 2S; 3A, 3S; 4A, 4S).

This pattern allowed the comparison of the test antigen with 3 pairs of antigens with their corresponding antisera.

(2) Antigenic relationships were also studied by varying the position of antigen and antibody as shown in the next diagram. Two antigens, with their corresponding antisera, could be compared by this pattern, and reactions of identity (representing common antigenic components) and reactions of non-identity (different antigenic components) determined.

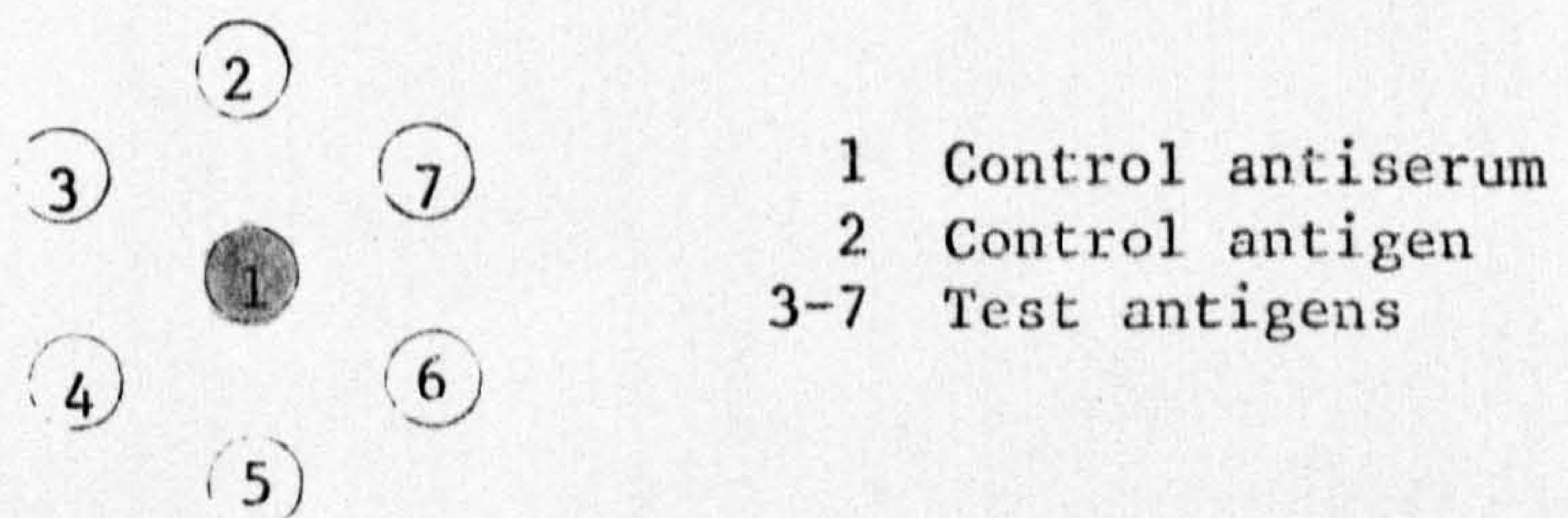
Pattern 2



Use of these immunodiffusion patterns made it possible to compare different antigenic preparations and identify both specific and common components.

(3) Several different antigens were tested against a single antiserum to show the presence of common components, where these were suspected from the results obtained using patterns 1 and 1A. One of the wells always contained the antigen corresponding to the antiserum, with which the other antigens showed reactions of non-identity.

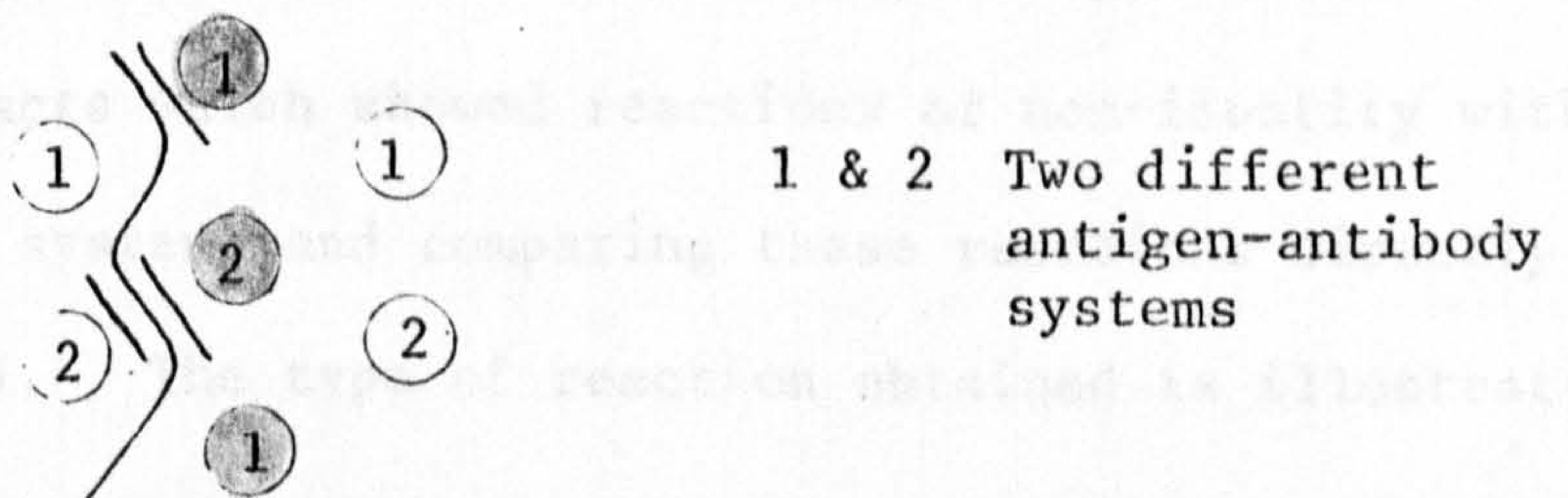
Pattern 3



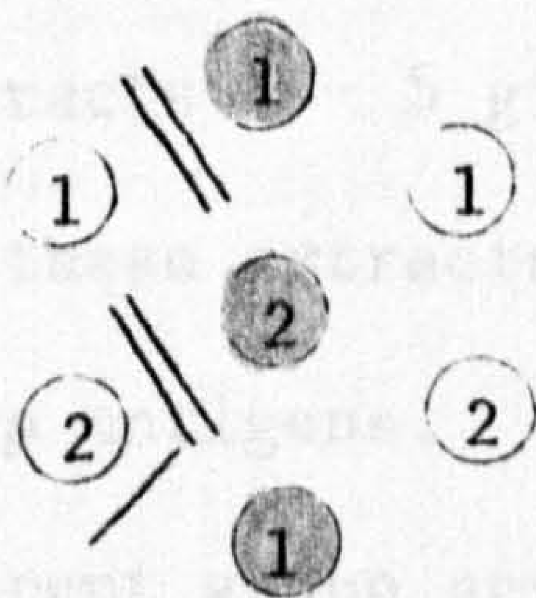
The terms used to describe the precipitin reactions obtained were in accordance with the definitions and nomenclature of Ouchterlony (1967),

3. RESULTS

From the results of precipitin tests with pattern 1 and 1A, the total number of antigen-antibody reactions among the dermatophytes tested was determined. To establish the relationship between the reactions of any two species, two different antigen-antibody systems were compared on the same plate with pattern 2 (page 88). If there was a reaction of identity, a continuous precipitin line was seen, thus:



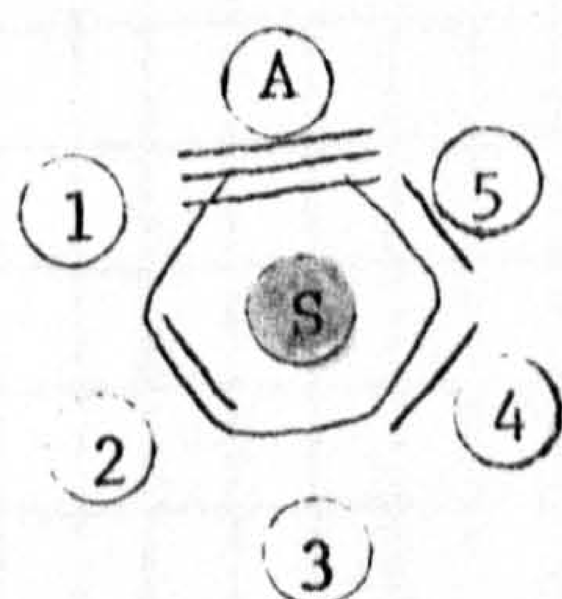
Line 'a' of system 1A-1S is identical with line 'b' of system 2A-2S, and indicates the presence of a common antigen in species 1 and 2. If there was no fusion of precipitin lines, the extracts did not possess the same antigens:



In enumerating individual antigens, attention was first paid to those obtained from M. audouinii; 4 were found and numbered 1 - 4 from the serum well. The extract of M. canis was then compared with M. audouinii using pattern 2, and found to have two lines of identity (numbers 1 and 2).

There were additional lines of non-identity and these were numbered 5 and 6. This process of enumeration and comparison was extended to more and more species until the patterns and relationships shown in Table 20 emerged. A total of 48 distinct antigens was recognised by the procedures used. Each number in Table 20 corresponds to one antigen. Several antigens are widely distributed among the dermatophytes (e.g. 19, 20, 21); the distribution of others is more limited (e.g. 3, 5), while a few are quite specific (e.g. 4, 9, 10,11).

Additional information on the relationship of species was obtained by taking antigenic extracts which showed reactions of non-identity with a given antigen-antibody system, and comparing these reactions directly, using pattern 3 (page 88). The type of reaction obtained is illustrated below:



A Antigen and
S its corresponding antiserum

1-5 Other antigenic extracts

Each of the antigenic extracts 1 - 5 give one line of identity to the test antiserum, and thus these extracts possess a common antigen. These are defined as group antigens. Table 21 is a summary of the results obtained. Different group antigens are distinguished by the suffix 1, 2, 3 or 4. More than one group antigen may be detectable by any one antiserum.

From Tables 20 and 21, it is apparent that two types of group antigens are present among the dermatophytes. For example, in Table 20, antigen 1 is a shared antigen among all the isolates of Microsporum

TABLE 20

COMMON ANTIGENS AMONG THE DERMATOPHYTES

	ANTIGENS																																															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
M. audouinii	+	+	+	+	+																																											
M. canis	+	+	+		+	+																																										
M. equinum	+	+	+		+																																											
M. ferrugineum	+	+	+				+	+																																								
T. ajelloi	+	+						+	+	+	+					+																																
M. persicolor U1	+											+	+	+		+			+	+																				+		+						
M. persicolor U2,U3	+												+						+		+					+																						
N. gypsea							+	+							+	+			+		+																											
N. incurvata							+								+	+	+		+		+																					+						
E. floccosum	+																	+			+																								+	+		
T. tonsurans																		+	+	+	+	+	+	+	+	+	+	+	+											+		+						
T. mentagrophytes																		+	+	+	+	+	+		+	+														+								
T. equinum																		+	+	+		+	+	+	+			+					+															
T. erinacei																			+	+	+			+	+	+	+			+	+	+					+											
T. soudanense																			+	+	+			+	+												+	+	+									
T. rubrum																		+	+	+	+		+	+		+	+								+	+				+								
T. quinckeanum																			+	+	+			+	•	+	+							+														
T. megninii																			+	+			+				+																					
T. schoenleinii																		+	+			+	+		+																+							
T. verrucosum																			+	+	+																			+		+	+	+				
T. violaceum																									+			+														+					+	+

TABLE 21

GROUP ANTIGENS AMONG DERMATOPHYTES

ANTIGENS - Symbol	ANTISERA																
	M. audouinii	M. canis	M. ferrugineum	T. ajelloi	M. persicolor	N. gypsea	N. incurvata	E. floccosum	T. tonsurans	T. mentagrophytes	T. equinum	T. erinacei	T. soudanense	T. rubrum	T. quinckeanum	T. schoenleinii	T. verrucosum
M. audouinii - Au				Ta1	Pe1	Gy1		Ef1	To1	Me1	Eq1	Er1	So1	Ru1	Qu1	Sc1	Ve1
M. canis - Ca				Ta1	Pe1			Ef1	To1	Me1	Eq1	Er1	So1	Ru1	Qu1	Sc1	Ve1
T. ajelloi - Ta		Ca1			Pe1			Ef2	To1	Me1	Eq1	Er1	So1	Ru2	Qu2	Sc2	Ve3
M. persicolor - Pe		Ca1		Ta2				Ef2	To2	Me1	Eq1	Er1	So1	Ru2	Qu1	Sc1	Ve3
N. gypsea - Gy		Ca1		Ta1	Pe1			Ef2	To2	Me1	Eq1	Er1	So1	Ru2	Qu1	Sc1	Ve3
N. incurvata - In		Ca1		TA3					To3				So1			Sc1	
E. floccosum - Ef		Ca1															
T. tonsurans - To	Au1	Ca1	Fe1	Ta4		Gy1	In1	Ef3									
T. mentagrophytes - Me		Ca1	Fe2			Gy1	In1	Ef4									
T. equinum - Eq		Ca2	Fe2	Ta4		Gy1	In2	Ef3									
T. erinacei - Er	Au1	Ca1	Fe2	Ta4	Pe2	Gy1	In1	Ef3									
T. soudanense - So		Ca1	Fe1					Ef3									
T. rubrum - Ru		Ca1			Pe2	Gy1	In1										
T. quinckeanum - Qu		Ca1	Fe2	Ta4	Pe2	Gy1	In2	Ef3									
T. megninii		Ca2															
T. schoenleinii - Sc			Fe3	Ta4		Gy2	In1	Ef4									
T. verrucosum - Ve				Ta4				Ef3									
T. violaceum			Fe3			Gy2											
M. ferrugineum - Fe																	

investigated, T. ajelloi and E. floccosum. The other species of Trichophyton do not possess this antigen; however, many of them do react with the antisera to these isolates, with lines of non-identity, but showing a reaction of identity with each other. For example, in Table 21, 8 species of Trichophyton give an identical reaction to antiserum against M. canis, and the same reaction is also shown by E. floccosum and the two Nannizzia species (M. gypseum).

M. audouinii has antigens in common with all the Microsporum species tested, and a single antigen is shared also with E. floccosum, T. ajelloi and M. persicolor. It is antigenically similar to M. canis and M. equinum, but less closely related to M. ferrugineum and M. gypseum. None of its 5 antigens are found in the other Trichophyton species, although the existence of common antigens is nevertheless indicated by the observation that M. audouinii extract gave lines of non-identity when tested against sera of the other Trichophyton species.

The reactions of M. canis are almost identical with those of M. audouinii, but it also has a single specific antigen (No. 6). It does not have any demonstrable antigens in common with Trichophyton species.

No antiserum was prepared for M. equinum. The antigenic extracts give lines of identity with all the Microsporum species tested, T. ajelloi, and E. floccosum.

M. ferrugineum shows reactions similar to those of M. canis but it also shares antigens with T. ajelloi and the Nannizzia species, which are not shared by the other Microsporum species. It does not share any

antigens with the other *Trichophyton* species.

Antigens and antisera were prepared against both perfect states of *M. gypseum*, viz. *N. gypsea* ('+' strain) and *N. incurvata* ('+'). A third antigenic extract was prepared from a second isolate of *N. incurvata* '+'.

N. gypsea has 6 antigens.

- 5 are shared with *N. incurvata*.
- 2 are shared with *M. ferrugineum*.
- 2 are shared with *M. persicolor*.
- 2 are shared with *T. ajelloi*.
- 2 are shared with most *Trichophyton* species and *E. floccosum*.

N. incurvata has 7 antigens.

- 1 is specific.
- 5 are shared with *N. gypsea*.
- 1 is shared with *M. ferrugineum*.
- 1 is shared with *M. persicolor* and *T. ajelloi*.
- 2 are shared with most *Trichophyton* species and *E. floccosum*.

Both *Nannizzia* species thus are closely related to each other antigenically, although they do not share the same antigens with other species. Both also give reactions of non-identity with *Trichophyton* and *Epidermophyton*. They do not have common antigens with *M. canis*.

No antiserum was prepared for *M. cookei*. The antigenic extracts reacted weakly with *M. audouinii*, *M. canis*, *E. floccosum* and a few *Trichophyton* antisera, but there was no reaction of identity with the antigenic extracts from these species.

M. persicolor has 9 antigens.

3 are specific.

1 is shared with Microsporum, E. floccosum, T. ajelloi.

1 is shared with T. ajelloi and Nannizzia species.

4 are shared with Trichophyton species.

Antigenically, M. persicolor thus forms a link between the genus Microsporum and the genus Trichophyton, in a manner similar to that shown by T. ajelloi and the two Nannizzia species. Moreover, from Table 21 it can be seen that M. persicolor, T. ajelloi and N. gypsea react identically in many instances to M. audouinii and M. canis when reacted against Trichophyton antisera; this indicates that all these species possess common antigens in addition to those tabulated in Table 20.

Five antigens were detected for E. floccosum, of which 2 were specific, 2 were shared with Trichophyton species and Nannizzia species and 1 is shared with Microsporum species and T. ajelloi. E. floccosum also gives reactions of non-identity with these antisera, indicating that although it is related to all other genera, it possesses other, more specific, antigens as well.

T. ajelloi has 3 specific antigens. It also shares antigens with the Microsporum species and E. floccosum but there was no reaction of identity with other Trichophyton species. However, T. ajelloi reacts to most Trichophyton antisera with the same line of identity given by M. persicolor, M. canis, M. audouinii and N. gypsea (Table 21).

T. violaceum has 5 antigens, of which 2 are specific, 2 are shared with a few Trichophyton species and N. incurvata, and 1 is shared with

several Trichophyton species. The antigenic extracts gave reactions of non-identity with most other antisera as well.

No antiserum was prepared for T. terrestre. The antigenic extracts reacted weakly to a few antisera but in all cases gave reactions of non-identity to the control antigens.

There was much cross-reactivity among the other 10 Trichophyton species, as Table 22 shows. All species had additional antigens to those revealed in Table 20 because they gave reactions of non-identity to Microsporum species when tested with the corresponding antisera. From Table 21 it can be seen that these reactions of non-identity are caused by group antigens.

TABLE 22 COMMON ANTIGENS WITHIN THE GENUS TRICHOPHYTON

	No.of antigens	Specific	Shared with		
			2 Species	Upto 6 Species	More than 6 Species
T. tonsurans	14	1	2	8	3
T. mentagrophytes	9	0	1	5	3
T. equinum	9	1	2	4	2
T. erinacei	11	3	1	4	3
T. soudanense	8	2	1	2	3
T. quinckeanum	7	1	0	3	3
T. rubrum	11	2	0	6	3
T. schoenleinii	6	1	1	3	1
T. verrucosum	7	2	0	2	3
T. megninii	4	-*	0	2	2

*No antiserum prepared for this species

Comparison of 3 Methods of Antigen Production

3 methods of antigen production were used in this study (pages 82 -83). The sequence in which they are described is temporal, in that method 1 was used initially, and subsequently replaced by method 2 and finally by method 3, as methods of antigen production were modified in the Mycological Reference Laboratory. Two types of culture - still and stirred - were used (page 81). Material from still cultures was processed by method 1 only, material from stirred cultures by methods 2 and 3. In spite of the differences in method of growth and extraction, there was remarkably little variation in the final products from the same isolate. Fresh batches were always compared with previous batches against the single homologous antiserum, and any that were not completely comparable were discarded: this occurred very infrequently. Since only 3 - 5 lines were present in the precipitin test, it is possible that only the dominant antigenic components were produced and detected each time; if growth had been allowed to continue beyond the times given (pages 81 & 82), it is probable that more precipitin lines would have been present but their quantities and rates of appearance would have been more variable.

4. DISCUSSION

The use of double diffusion in agar (Ouchterlony, 1948) as a technique for the serological study of dermatophytes has been limited to a few groups of workers (Dyson & Landay, 1963; Kielstein, 1966, Sagara, 1968). Moreover their studies were confined to comparisons between a small number of species. Related studies on serological relationships of these fungi (Sharp, 1945; Biguet, Andrieu, & Laloux, 1961, 1965; Andrieu, Biguet & Laloux, 1968) were undertaken with other methods. Sharp used a ring precipitin test and Biguet and co-workers favoured immunoelectrophoresis (Grabar & Williams, 1953). Apart from being a relatively simple technique, the Ouchterlony method can yield information of the number of antigen-antibody reactions present, and, by varying the size and shape, and pattern of wells, data on the inter-relationships of antigens can be obtained. Immunoelectrophoresis, an immunodiffusion technique in which one of the reactants is fractionated electrophoretically before it is allowed to react with the other, can be used to separate antigenic mixtures if the components have different electrophoretic mobilities. It is particularly useful for antigens that appear to be identical by the simple double diffusion method. However, it is difficult to show lines of identity (common antigens) by this method, whereas this can be shown easily by the type of double diffusion test used in this work. For these reasons, double diffusion was the technique chosen for the present investigation which has been d irected towards a study of antigenic affinities and differences among the dermatophytes.

Biguet and his colleagues (1968) hyperimmunised their rabbits to the point at which they could obtain up to 20 arcs of precipitation when the antisera were tested against antigens by immunoelectrophoresis.

Although the number of reactions obtained with this method is higher than for double diffusion, so also is the number of reactions common to two or more species. Sharp (1945) had earlier shown that the specificity of the responses decreased as the titre rose. It was decided at the onset of the present investigation, therefore, that rabbits should be immunised for as short a period as was commensurate with the production of a "reasonable" antigen-antibody reaction, bearing in mind the fact that antisera tend to lose potency on storage. The number of precipitin lines considered to be "reasonable" varied from antiserum to antiserum, but six lines were obtained with *T. equinum* antiserum and four with several others. With the exception of the isolate of *T. verrucosum* which proved to be a poor immunising agent for the pair of rabbits used, rabbits were not immunised for more than two months: thus the antisera to *T. violaceum* and *M. ferrugineum*, both of which were also poor immunising agents, were "weak" containing only 2 and 3 lines respectively, but they were more specific than some of the antisera producing a greater number of precipitin lines in double diffusion. A balance had thus to be maintained between reactivity and specificity of antisera. The change from twice-weekly intra-peritoneal injections of sonically-disrupted mycelium to a combination of one footpad injection followed by one or two subcutaneous injections of the same material in adjuvant did not seem to affect the rate at which antibodies were produced. Unfortunately the change in technique came only towards the end of the experimental work described here, and only three antisera were prepared by both methods (to *T. tonsurans*, *T. mentagrophytes* and *E. floccosum*). It may be significant that four or five lines were obtained in the precipitin test with both methods; the differences between the two batches of antisera were evidently no greater than that between batches of antisera produced on separate occasions by

the same method.

In view of the results of workers such as Biguet and Sharp, cross reactivity amongst the dermatophyte antigens was not unexpected. A group of organisms closely related on the basis of morphology, biochemical behaviour, and histopathogenesis, are not likely to be separated easily by serological affinities. It is however obvious from the results obtained that in spite of the considerable number of common reactions, there are in fact significant differences between species and groups of species. The results described in this attempt to separate species on the basis of their serological affinities are encouraging, but much work remains to be done. Nevertheless the general picture that has been evolved is clear. This is most readily demonstrated by Table 20 which shows the number and distribution of common and specific antigens amongst the species of dermatophytes tested.

Species of Microsporum, with the exception of M. gypseum (N. gypsea and N. incurvata) and M. persicolor, form a coherent group distinct from Epidermophyton and Trichophyton. This is apparent not only from the considerable number of shared antigens within the group, but also by the way in which antigens from these species react to antisera prepared against Epidermophyton and Trichophyton: in most instances they gave a line of identity, indicating the presence of common antigens. M. gypseum is, however, less closely related to the other Microsporum species; indeed, it is linked equally well with Epidermophyton and Trichophyton. The same is true of M. persicolor. Apart from 2 antigens shared with M. ferrugineum, M. gypseum has no antigens in common with the other species of Microsporum tested, and M. persicolor possesses only the common group antigen No. 1 (Table 20). But both species share antigens with Trichophyton species and E. floccosum. Sharp (1945) also found that M. gypseum was not closely

related to M. canis and M. audouinii, and this is corroborated by the work of Andrieu, Biguet and Laloux (1968). There are detectable differences between the two perfect states of M. gypseum, N. gypsea and N. incurvata, although they are obviously closer to each other than to other species, having 5 antigens in common. It is interesting that there are serological differences between the 2 perfect states since nutritionally they appeared to be identical. Rippon and Garber (1969) have shown that not only do the two perfect states differ from each other with respect to particular enzymes such as elastase, but that strains of opposite mating types also differ in this way.

Trichophyton (Keratinomyces) ajelloi is serologically related most closely to the genus Microsporum. In the system of detection used it shares 2 antigens with M. canis, M. audouinii and M. ferrugineum, and a third one with M. ferrugineum alone, but has no antigens in common with Trichophyton species. Antigenic extracts from only two species, M. ferrugineum and N. gypsea, gave lines of identity with T. ajelloi when tested with T. ajelloi antiserum; all other extracts gave reactions of non-identity. The pattern of reaction of T. ajelloi to most Trichophyton antisera was similar to Microsporum species showing that it shares antigens with these species (Table 21). These results do not support Ajello's transfer of this species from the genus Keratinomyces to the genus Trichophyton. He was influenced by the fact that the perfect state of this species was shown to be a member of the genus Arthroderma; so far, all dermatophytes with a perfect state in this genus have been species of Trichophyton. Such evidence is not wholly conclusive for it is not unknown for a fungus with a single sexual form to reproduce asexually by several different conidial methods. For example, the Ascomycete Ceratocystis may reproduce asexually as species of the genera Cephalosporium

Cladosporium, Theilavia and Leptgraphium; the Ascomycete Nectria has 14 different types of conidial apparatus.

M. persicolor also shares antigens with Microsporum as well as Trichophyton. It possesses one of the Microsporum antigens (No. 1 in Table 20), shares one with T. ajelloi and M. gypseum (No. 16), and has four of the Trichophyton antigens (Nos. 19, 20, 40, 42); two of these (Nos. 19 and 20) are the most widely distributed antigens found. However, M. persicolor also gives reactions of non-identity to Trichophyton antisera and this component (or components) is identical to that possessed by M. gypseum, T. ajelloi and other Microsporum species (see Table 21). Thus, with regard to serological affinities, M. persicolor like M. gypseum and T. ajelloi, forms a link between the genus Microsporum and the genus Trichophyton. Most interestingly, and in parallel with the nutritional results, it does not give the same reactions as T. mentagrophytes, with which species in the past it has been equated. Antigenic extracts from these species give reactions of non-identity with each other's extracts when tested with the corresponding antisera, and react to other antigen-antibody systems in dissimilar ways. This evidence supports the transfer by Stockdale (1967) of this organism from the genus Trichophyton to the genus Microsporum. It could be argued, however, that M. persicolor, T. ajelloi and M. gypseum should be grouped together in a separate group from the other species on the basis of their serological affinities (see Final Discussion and Conclusions, page 112). Another interesting finding with respect to M. persicolor was that the antigenic extracts prepared from three isolates of this species were not serologically identical. Extract No. 1 from the isolate used to prepare the antiserum had only 3 antigens in common with extracts Nos. 2 and 3, out of total of 8 antigens. There seem thus to be differences between morphologically and nutritionally

similar isolates. However, although great care was taken to ensure that variation in production of antigenic extracts was kept to a minimum, it is possible that these differences may be quantitative rather than qualitative. Antigens were standardised by weight alone, and the amount of active material present may have varied sufficiently for these differences to occur.

Almost all the Trichophyton species investigated form a closely related group, with many antigens in common. At least 9 fairly widely distributed antigens have been identified. It is thus difficult to separate the species simply by the way in which they cross-react. T. mentagrophytes, for example, gives 3 precipitin lines with its own antiserum, but these are given either wholly or in part with extracts from other Trichophyton species. It is apparent, however, that the differences established on morphological criteria are not annulled by the serological results. In certain cases, indeed, the serological data provide additional evidence of the separation of species which until recently were regarded as the same, e.g. T. mentagrophytes and T. erinacei.

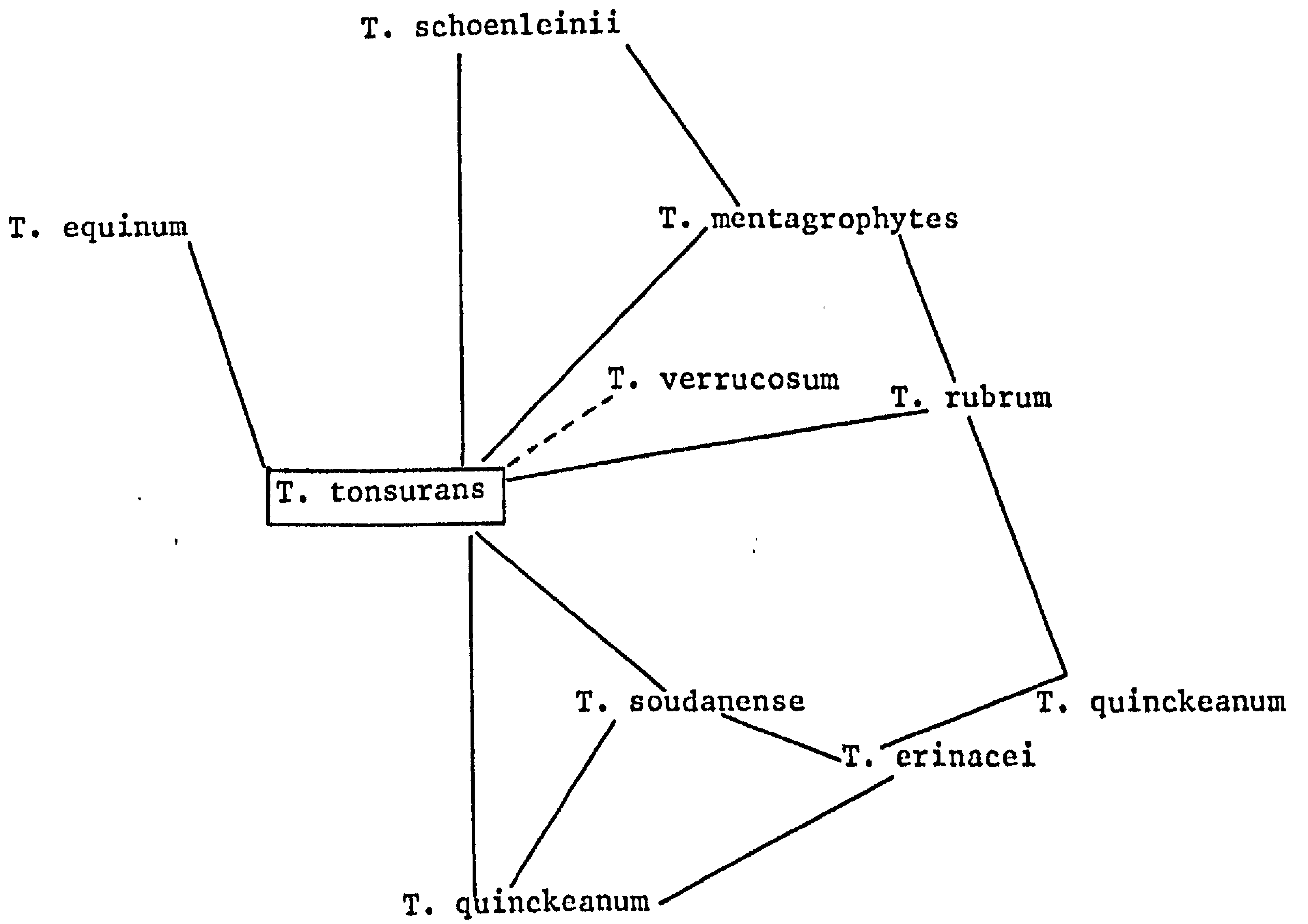
If the number of common antigens are analysed, it is possible to draw some conclusions regarding the interrelationship of species within the group.

ANTIGENS	19	20	21	26	27	25	22	23	24	40	18	28	44	37	49
T. mentagrophytes	+	+	+	+		+	+	+		+	+		+		
T. tonsurans	+	+	+	+	+	+	+	+	+	+	+	+	+		+
T. equinum	+	+				+	+	+	+		+	+			
T. rubrum	+	+	+	+	+			+	+	+	+				
T. quinckeanum	+	+	+	+	+				+						
T. erinacei	+	+	+	+	+	+			+					+	
T. soudanense	+	+	+			+			+					+	
T. schoenleini	+					+	+	+			+				
T. megninii	+	+			+			+							
T. verrucosum	+	+	+							+					+

T. mentagrophytes and T. tonsurans are almost identical serologically

since they have 10 out of 14 detectable antigens in common. T. rubrum is also close to T. tonsurans, sharing 9 antigens, but has 2 specific antigens in addition. T. mentagrophytes and T. rubrum share 8 antigens, T. equinum and T. mentagrophytes 6, and T. rubrum and T. equinum 5, while T. equinum and T. tonsurans have 8 common antigens. Thus T. equinum is close to T. tonsurans but not so closely related to T. rubrum and T. mentagrophytes. Similarly, T. quinckeanum, T. soudanense and T. erinacei are fairly closely linked to each other and to T. tonsurans, but have fewer antigens in common with T. mentagrophytes and T. equinum. T. schoenleinii is most closely related to T. mentagrophytes, T. tonsurans and T. equinum, T. verrucosum shares 5 antigens with these species. T. tonsurans appears to be the "key" species, and it links two serological sub-groups: one comprising T. mentagrophytes, T. rubrum and T. equinum and possibly T. schoenleinii, and the other T. quinckeanum, T. soudanense and T. erinacei. T. mengninii shares 4 antigens with the other Trichophyton species.

The diagram below is an attempt to summarise in a visual manner the relationships described above. The length of the lines joining species is inversely proportional to the number of antigens shared by the species. The line joining T. verrucosum to T. tonsurans is discontinuous to indicate that the position of T. verrucosum is at right angles to the plane of the paper in order to put it in a correct position vis-a-vis the other species.


T. schoenleinii
T. equinum
T. mentagrophytes
T. verrucosum
T. rubrum
T. tonsurans
T. soudanense
T. quinckeanum
T. erinacei
T. quinckeanum

T. violaceum does not appear to fit into this series at all. The reactions of its antiserum and antigenic extracts suggest that it is only remotely related to other Trichophyton species. Only T. verrucosum shows any reaction of identity with T. violaceum; the other antigenic extracts gave either weak reactions of non-identity or failed to react with the antiserum. T. violaceum shares two antigens with T. verrucosum, T. equinum and T. tonsurans, and a single one with a few other species as well.

The saprophytic species, T. terrestre and M. cookei, gave only weak reactions with antisera to both Trichophyton and Microsporum species, and are obviously much less closely related to the pathogenic species.

The results of this investigation conform essentially with those of Sharp and Biguet. Sharp (1945, 1951) noted the close relationship of M. audouinii and M. canis but found that M.gypseum reacted just as closely with T. mentagrophytes, T. rubrum and T. tonsurans. E. floccosum also reacted closely with Trichophyton species. From the results of the present investigation, E. floccosum shares 2 antigens with Trichophyton, one with Microsporum and has 2 specific antigens. This suggests that this species occupies a position similar to that of M. gypseum, linking Microsporum and Trichophyton. However, E. floccosum and M. gypseum have only one antigen in common, and thus they are not closely related.

Andrieu, Biguet and Laloux (1968) studied several of the dermatophyte species used in this work (M. audouinii, M. canis, M. ferrugineum, M. gypseum, E. floccosum, T. tonsurans, T. mentagrophytes, T. rubrum, T. soudanense, T. schoenleinii, T. verrucosum, T. violaceum and T. ajelloi). They used immunoelectrophoresis rather than gel diffusion, their antisera were much more reactive, and they demonstrated many more reactions of identity. Their results with the species of Microsporum tested are, however, essentially the same. Analysis of their results with the

Trichophyton species shows the following points of agreement between the two investigations:

(a) the close relationship shown by T. mentagrophytes with T. tonsurans, T. schoenleinii and T. rubrum.

(b) T. tonsurans reacts well with all Trichophyton species.

(c) T. ajelloi shows more reactions in common with Microsporum species than with Trichophyton species.

They also noted that M. gypseum was less closely related to the other Microsporum species.

However, Biguet and his colleagues found that T. soudanense had closer affinities with T. gourvilii and T. violaceum than with T. tonsurans and the other Trichophyton species, which was not true for the present investigation. The results reported here show that T. soudanense shares many antigens with T. tonsurans, T. mentagrophytes and the other common Trichophyton species. Biguet also suggested that T. violaceum and T. rubrum were more closely related than T. rubrum and T. mentagrophytes. Even allowing for the great difference in reactivity of the antisera in the two investigations, this is in total contrast to the results reported here. Biguet noted the close relationship between the Microsporum species other than M. gypseum, but his results show in addition that M. canis and T. mentagrophytes have several antigens in common. The results reported here are completely contrary to this: M. canis and T. mentagrophytes do not have lines of identity (Table 20). It is likely that the longer period of immunisation used by Biguet and his colleagues has resulted in more reactive, less specific antisera, and that had the present investigator immunised the rabbits for a longer period, the specificity of the antisera would also have decreased.

From the results of this investigation, therefore, the dermatophytes can be divided into 5 principal groups, and possibly a sixth, thus:

Group A: _Microsporum_ species other than _M. gypseum_

Group B: _M. gypseum_, _T. ajelloi_, _M. persicolor_

Group C: _E. floccosum_

Group D: _Trichophyton_ species

(Group E;) _T. violaceum_, if not included in Group D.

Group F: _T. terrestre_, _M. cookei_

It is obvious that, with the simple immunodiffusion system used in this investigation, it was not possible to use serological procedures to differentiate dermatophytes. Nevertheless, the demonstration of both specific and group antigens has given a new dimension to the interrelationship of these fungi. The serological groups do not correspond entirely to those based on morphology, and therefore provide an alternative way to regard the relationship between species.

FINAL DISCUSSION AND CONCLUSIONS

The objects of this survey of nutritional patterns and serological relationships among the dermatophytes were three-fold: firstly, did the data obtained support the current classification of species based upon morphological criteria; secondly, was it possible to obtain new concepts on interrelationships; and thirdly was it possible to devise simple tests assisting the identification of species. These objects have been realised at least in part.

From the results of the nutritional studies, it has been established that ability to assimilate growth substances is related more closely to the degree of parasitism shown by a given isolate than to its morphological structure. This has permitted the development of a scheme whereby isolates of similar life style may be differentiated by their patterns of nutrition. However, if a comparison of morphological groups with nutritional patterns is made (Table 23), further light is shed upon the interrelationships of species. Seven morphological groups are shown, based upon the classification of Emmons (1934) as modified from Rebell & Taplin (1970) in their key to the classification of the dermatophytes. The groups are:

(a) E. floccosum

(b) M. canis, M. audouinii, M. ferrugineum, M. cookei

(c) M. gypseum, M. persicolor

(d) T. ajelloi

(e) T. terrestre

(f) Trichophyton species which produce microconidia upon common laboratory media

(g) Trichophyton species which do not produce microconidia upon common laboratory media

TABLE 23

COMPARISON OF MORPHOLOGICAL & NUTRITIONAL GROUPS WITHIN THE DERMATOPHYTES

MORPHOLOGICAL GROUPS	NUTRITIONAL GROUPS					
	I	II	III	IV	V	VI
a			E.floccosum			
b	M. canis M. equinum M. cookei			M.audouinii		
c	M. gypseum M. persicolor					
d	T. ajelloi					
e	T. terrestre					
f	T. equinum T.mentagrophytes T. quinckeanum	T. megninii T. rubrum T. tonsurans	T.tonsurans		T.erinacei	T.soudanense
g	T. schoenleinii	M.ferrugineum		T.violaceum T.verrucosum		

Table 24 provides a similar comparison with patterns of antigenicity. Although it has not proved possible to develop a scheme of differentiation of isolates based solely upon their reactivity in an immunodiffusion system, analysis of antigenic relationships has shown the existence of six antigenic groups (page 109) which do not correspond completely with the form-genera established by Emmons. When however they are compared with the seven morphological groups given above, the correspondence is close. Both Tables 23 and 24 emphasize the distinction between M. gypseum and M. persicolor and the other Microsporum species, and also the separation of T. ajelloi and T. terrestre from the other species of Trichophyton.

Although only 4 species of Nannizzia (N. gypsea, N. incurvata, N. persicolor, N. cajetana - conidial states respectively M. gypseum, M. persicolor, M. cookei) and 3 of Arthroderma (A. uncinatum - T. ajelloi, A. quadrifidum - T. terrestre, A. benhamiae - T. mentagrophytes) were included in this investigation, the division into these genera does not correspond with the groupings obtained by either the nutritional or the serological analyses. All 7 species fall into nutritional group I in Table 23, while in Table 24 they are divided among 3 serological groups.

Only one comprehensive scheme for classification of the dermatophytes based upon criteria other than morphology is available at present. Shecter et al. (1968) analysed 57 protein extracts from 17 species of dermatophytes by disc electrophoresis. Using factor analysis, they classified their isolates, without regard to the established morphological genera, into 4 groups:

(1) T. ajelloi

(2) E. floccosum

(3) Microsporum species including M. gypseum

(4) Trichophyton species, which could be subdivided into:

TABLE 24

COMPARISON OF MORPHOLOGICAL & SEROLOGICAL GROUPS WITHIN THE DERMATOPHYTES

MORPHOLOGICAL GROUPS	SEROLOGICAL GROUPS					
	I	II	III	IV	V	VI
a			E.floccosum			
b	M. canis M. equinum					M. cookei
c		M. gypseum M.persicolor				
d		T. ajelloi				
e						T.terrestre
f				T.mentagrophytes T. equinum T. quinckeanum T. tonsurans T. rubrum T. megninii		
g	M.audouinii M.ferrugineum			T. schoenleinii T. verrucosum T. soudanense	T.violaceum	

(a) T. verrucosum, T. violaceum, T. schoenleinii, T. rubrum
T. gallinae

(b) T. tonsurans, T. terrestre, T. mentagrophytes, T. megninii

T. soudanense and T. yaoundei had affinities with both subgroups. Unfortunately they did not include M. persicolor, T. quinckeanum and T. erinacei, so that the position of these species in this scheme is unknown.

The divisions correspond almost exactly with the four morphological genera. They do not, therefore, correlate closely with the nutritional and serological groups established in this investigation.

It is thus obvious that mating behaviour, morphology, patterns of nutrition, chemical groups and serological affinities do not correspond sufficiently to enable parameters to be used in classifying the dermatophytes. In practice, the classification of a species will thus depend partly on the relative value attached to each criterion, and partly on what use will be made of the classification. The interests of the taxonomist concerned with true relationships do not necessarily coincide with those of the dermatologist who is concerned with the effect of the parasite on the host. For example, the taxonomist will regard Trichophyton mentagrophytes var. granulare and T. mentagrophytes var. interdigitale as the same organism, but these two organisms cause different lesions in the human host which may be treated in different ways. Four species, in particular, illustrate the conflict between various criteria and are considered separately: T. ajelloi, T. erinacei, T. quinckeanum and M. persicolor. This has been discussed in the conclusions to the two main sections of the work. How should all these species be regarded? T. erinacei has now been raised to specific status (Rebell & Taplin, 1970)

and this is supported by the data obtained in this thesis. It might be legitimate to reduce T. quinckeanum to synonomy with T. mentagrophytes although they cause different clinical conditions in their respective hosts. Since T. quinckeanum is almost invariably identified on the basis of its isolation from the natural host (the mouse) or from patients who are known to have been in contact with mice, it is interesting to speculate whether it would in fact be identified under any other circumstances. But is it legitimate to transfer T. ajelloi and M. persicolor when the criteria are at variance? To accommodate their range of characteristics satisfactorily, the genera Microsporum and Trichophyton would have to be extended and redefined to the point where generic criteria would cease to have much practical value. It may be legitimate to create a new genus to accommodate M. gypseum and M. persicolor and retain the genus Keratinomyces unchanged. However, the aim of any classification is to enlighten. Therefore, since no adequate alternative scheme has yet been devised, it would seem preferable to retain the accepted classification at the present time, not attempting to equate it with the division into Nannizzia and Arthroderma, while realising that these genera do not fully express the relationships within the dermatophytes. Lines of division are imposed, for convenience, upon an infinitely varying collection of organisms, and minor modifications in the position of these lines seems unneccessary when species concepts are in general well-defined and understood. It is likely that asexual morphology will for some time to come be the primary means of identifying species, with the nutritional tests providing additional information. Serological methods and chemical analyses are not yet at a stage where they can be used practically in the identification of dermatophytes, although the investigations described in this work have shed some new light upon affinities among genera and

species of this variable and complex group of fungi.

Table 25 is an attempt to summarize the results of both nutritional and serological investigations in relation to the currently recognized morphological divisions. It shows that the classification into four morphological genera masks a considerable degree of variability among species, not only in their structure but also in their nutritional patterns and serological affinities. The incomplete correlation between these criteria has been exploited in the scheme of differentiation presented on page 70. To this extent the objects of this work have been fulfilled. No investigation of this type can ever be said to be concluded. A system has been established, and the extension of both nutritional and serological tests to more dermatophyte species is essential, and could well prove interesting and rewarding.

TABLE 25 THE RELATIONSHIP OF MORPHOLOGICAL, NUTRITIONAL & SEROLOGICAL GROUPS AMONG THE DERMATOPHYTES

MORPHOLOGICAL GROUPS	SEROLOGICAL GROUPS	NUTRITIONAL GROUPS	SPECIES
b	VI	IB4	Microsporum cookei
b	I	IB6	M. equinum
	I	IB1	M. canis
g	I	IIE	M. ferrugineum
g	I	IVJ	M. audouinii
c	II	IC	M. gypseum
c	II	IB4	M. persicolor
d	II	IB3	Trichophyton ajelloi
f	IV	IC	T. mentagrophytes
f	IV	II8	T. rubrum
	IV	II6, IIIF	T. tonsurans
	IV	IB1	T. equinum
	IV	V	T. erinacei
	IV	IB5	T. quinckeanum
	IV	IID8	T. megninii
g	IV	IA	T. schoenleinii
g	IV	IVH	T. verrucosum
g	IV	VI	T. soudanense
g	V	IVI	T. violaceum
e	VI	IB2	T. terrestre
a	III	IIIF	Epidermophyton floccosum

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THE DIFFERENTIATION OF *TRICHOPHYTON MENTAGROPHYTES* FROM *T. RUBRUM* BY A SIMPLE UREASE TEST

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Seventy isolates of *Trichophyton mentagrophytes*, 104 isolates of *T. rubrum*, and 8 isolates of *T. mentagrophytes* var. *erinacei* were tested on a modified Christensen's medium for their ability to split urea. 92·8% of the isolates of *T. mentagrophytes* were positive within 7 days but no isolates of *T. rubrum* and *T. mentagrophytes* var. *erinacei*. A comparison of the urease and hair perforation tests was made. The urease test offers a rapid and reliable method of separating these two species.

The lack of agreement on the merits of existing tests for distinguishing *Trichophyton rubrum* from *T. mentagrophytes* justifies the introduction of another method. Even the hair penetration test of Ajello & Georg (1957) has been reported to be less than 100% reliable (Blaschke-Hellmessen, 1963). There is much disagreement among investigators recommending special media for pigment production (Bocobo & Benham, 1949; Boehme & Friedrich, 1961; Dyson & Landay, 1963; Blaschke-Hellmessen, 1963; Baxter, 1963; Sudman & Schmitt, 1965). Dyson & Landay (1963) claimed to be able to differentiate the two species by precipitin reactions in agar-gel but admitted that this method was too slow and complicated for routine use.

Studies by the author of some enzyme systems in the dermatophytes pointed to a distinct difference in the rates of urea hydrolysis. There were differences between strains of most dermatophytes, but a consistent difference was noted between *T. rubrum* and *T. mentagrophytes*, and also between *T. soudanense* and *Microsporum ferrugineum*. *T. soudanense* does not appear to possess an urease whereas *M. ferrugineum* does, although hydrolysis is slow. As only 7 strains of *T. soudanense* and 4 of *M. ferrugineum* were available for study, this point requires further elaboration. In a preliminary study 6 isolates of *T. mentagrophytes* hydrolysed urea rapidly (within 8 days) whereas 6 isolates of *T. rubrum* did not in 28 days. Together these two dermatophytes accounted for 62% of all dermatophytes isolated in the Mycological Reference Laboratory between 1961 and 1965 (Murray, 1966). Since these two species may sometimes be difficult to distinguish by morphological criteria or pigments produced, it seemed worthwhile to make an extended study of their capacities to hydrolyse urea.

Tate (1929) noted the presence of urease in 4 species of dermatophytes (including *T. mentagrophytes*) but did not study *T. rubrum*. Littman (1957) reported the presence of urease in both *T. mentagrophytes* and *T. rubrum*. He observed that the rate of hydrolysis was rapid (1-2 days) in *T. mentagrophytes* and less rapid (8-9 days) in *T. rubrum*. Rosenthal (1965)* reported the presence

* Because of a delay in publication, this information was not available until the last quarter of 1966 and the present work was therefore undertaken independently and without knowledge of Dr. Rosenthal's findings.

of urease in *T. mentagrophytes* and its absence in *T. rubrum*, and suggested that this might be used as an additional method to distinguish these two species.

Materials and Methods

A modification of Christensen's medium (Christensen, 1946) was used incorporating a higher percentage of glucose. The composition of the medium was:

Peptone ("Mycological" Oxoid)	1 g.
NaCl	5 g.
KH_2PO_4	2 g.
Glucose	5 g.
Agar	20 g.
Distilled water	to 1 litre

These were dissolved by heat; 6 ml. of phenol red solution (0·2% in 50% alcohol) was then added. The medium was sterilised by autoclaving at 115° C. for 15 minutes, cooled to 50° C. and 100 ml. urea (20% aqueous solution, sterilised by filtration) added. The medium was distributed aseptically in sterile ½ oz. (15 ml.) bottles and sloped.

A small amount of fungal growth was transferred from the culture under investigation to the urea slope and incubated at 26° C. The development of a deep red colour throughout the medium was regarded as the end point of the reaction.

Seventy isolates of *T. mentagrophytes* (including 20 isolates of *T. mentagrophytes* var. *interdigitale*), 104 of *T. rubrum*, and 8 of *T. mentagrophytes* var. *erinacei* were investigated. The identifications were based on the usual morphological characteristics of these species. Fifteen isolates of *T. mentagrophytes*, 7 of *T. rubrum* and 3 of *T. mentagrophytes* var. *erinacei* were obtained from the culture collection of the Mycological Reference Laboratory, where they had been maintained for periods of 3-21 years. The remaining 55 isolates of *T. mentagrophytes*, 97 of *T. rubrum* and 5 of *T. mentagrophytes* var. *erinacei* were isolated from morbid material received in the Mycological Reference Laboratory between May 1965 and May 1966.

Results

The results are shown in Table 1. Sixty-five isolates of *T. mentagrophytes* (92·8%) were positive, *i.e.* turned the medium red throughout, within 7 days. One hundred and three isolates (99·1%) of *T. rubrum* and all 8 isolates of *T. mentagrophytes* var. *erinacei* were negative within this time.

Five isolates of *T. mentagrophytes* (4 typically granular and 1 downy isolate) were not positive in 7 days, although positive reactions eventually appeared (2 in 8 days, 2 in 9 days and 1 in 12 days).

One isolate of *T. rubrum* (from the Culture Collection of the Mycological Reference Laboratory where it has been maintained in culture for 18 years) was positive within 7 days. Morphologically this isolate is granular, produces a pink pigment on 4% malt agar and a yellow brown pigment on Sabouraud's dextrose agar, and has elongated microconidia. It also perforates hair. This isolate thus partakes of the characteristics of both *T. mentagrophytes* and *T. rubrum*, and its reference to one or other species must depend on the relative importance attached to the criteria used for identification.

Most isolates of *T. rubrum* produced some rise in pH after a period which varied from 9 to over 21 days depending on the isolate, but in some cases the

Days after inoculation	*Number of isolates urease positive*		
	T. mentagrophytes	*T. rubrum*	*T. mentagrophytes* var. *erinacei*
3	1		
4	9		
5	13		
6	36		
7	6	1*	
8	2		
9	2	1	
10			
11		1	
12	1	4	
13		5	
14		4	
15		5	
16		11	
17		6	
18		10	
19		7	
20		7	
21		10	
Over 21 and negative		32	8
Totals	70	104	8

* The isolate of *T. rubrum* intermediate in character between *T. mentagrophytes* and *T. rubrum*.

TABLE 1.—TIME TAKEN TO PRODUCE ALKALINE pH. FIGURES REPRESENT MEAN VALUES OF ALL TESTS

indicator was not completely changed, *i.e.* the pH did not rise above 8·0. A few isolates had shown no pH change by 28 days when the experiment was concluded. The number of days taken by any one isolate to produce a rise in pH varied in consecutive tests; variations from the mean of up to 4 days have been recorded. *T. mentagrophytes*, by contrast, is a rapid producer of an alkaline pH, although there is some variation between isolates. The time taken to produce a rise in pH varied slightly in consecutive tests (a difference of up to 2 days from the mean).

Six isolates of *T. mentagrophytes* var. *erinacei* were still negative after 28 days. The other 2 isolates produced a rise in pH in 24 and 26 days respectively. The significance of this in relation to the taxonomic position of this fungus is discussed later.

All isolates tested showed good growth upon the medium used.

	Urease positive		*Hair perforated*	
	T. mentagrophytes	*T. rubrum*	*T. mentagrophytes*	*T. rubrum*
Within 7 days	23	0	25	4
Within 14 days	2	3		5
Over 14 days		22		N.I.

Note: N.I. = Not investigated further.

TABLE 2.—COMPARISON OF UREASE AND HAIR PERFORATION TESTS

Table 2 gives the results of the urease and hair perforation tests carried out on 25 isolates of *T. mentagrophytes* and 25 isolates of *T. rubrum*. A modification of the method of Ajello & Georg (1957) was used for the hair perforation test. Sterilised human hair was placed on the surface of 4% malt agar (incorporating 0·05% cycloheximide) in petri dishes; this was then inoculated with the isolate under investigation. Twenty-three of the 25 isolates (92%) of *T. mentagrophytes* were urease positive within 7 days and all isolates perforated hair. None of the 25 isolates of *T. rubrum* were urease positive within 7 days but 4 (16%) perforated hair.

Discussion

It is considered that sufficient isolates of both *T. rubrum* and *T. mentagrophytes* have now been tested to assure the validity of the urease test for the differentiation of these two species. Urease is present in most isolates of *T. mentagrophytes*. In contrast *T. rubrum* lacks it or produces it slowly; reddening of the medium by *T. rubrum* may be due to exhaustion of the carbon source and subsequent utilisation of the nitrogen source (urea) resulting in a rise in pH. Since over 90% of *T. mentagrophytes* isolates gave positive reactions within 7 days while *T. rubrum* was negative for a much longer period, this would appear to be a valid criterion for distinguishing the two species.

Although only 8 isolates of *T. mentagrophytes* var. *erinacei* were investigated, the results are of considerable significance. Doubts have already been cast upon the validity of its present taxonomic position. Smith and Marples, who first described this fungus in 1963, listed some considerable differences between it and *T. mentagrophytes* var. *mentagrophytes* not only in growth requirements and ability to attack keratin, but also in the pigmentation and microscopic morphology. Spiral hyphae, so characteristic of granular *T. mentagrophytes*, are rarely produced; this was considered by Murray (1964) as "particularly disquietening", a view endorsed by Quaife (1966). The absence or very slow development of urease in the 8 isolates tested as compared to the granular variety gives added support to the view that it is a species in its own right.

The variability of dermatophytes is the nightmare of taxonomy in medical mycology. It has led in the past to a plethora of species, which have only with difficulty been reduced to synonomy. Not only can dermatophytes differ considerably from type cultures on primary isolation, but minute variations in the composition of "standard" media can have a profound effect upon morphology and pigmentation. It is for the latter reason that any test which relies upon the development of a characteristic pigment upon a given medium can be misleading. Slight differences in the constituents of the medium can give conflicting results.

As can be seen from Table 2, 100% of isolates of *Trichophyton mentagrophytes* perforated hair; however 16% of isolates of *T. rubrum* also perforated hair. This degree of accuracy is comparable to that of the urease test, but the two tests combined give a degree of positive separation higher than either test alone.

The urease test is simple to perform, easy to read, and gives relatively rapid and consistent results in the case of a positive reaction. Although the

degree of accuracy given by this test is no greater than that shown by the hair perforation test, the latter cannot be read until at least 7 days after inoculation, and the hair must be examined for perforating organs under the microscope. For laboratories engaged in routine diagnosis, where time is at a premium, the urease test offers a reliable and quick means of distinguishing *T. rubrum* from *T. mentagrophytes.*

Sumário

Estudou-se capacidade de fracionar ureia em 182 estipes de *T. mentagrophytes, T. rubrum* e *T. mentagrophytes* var. *erinacei* utilizando meio Christensen's modificado. 92·8% das estipes de *T. mentagrophytes* deram resultato positivo ao fim de 7 dias, mas nemhuma estipe de *T. rubrum* e *T. mentagrophytes* var. *erinacei* deu prova positivo. Comperou-se a prova de urease com a de perfuração do cabelo e a prímera parece ser um método rápído e seguro para diferenciar ambas especies.

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