Some Aspects of the Biochemical Differentiation of Pathogenic Fungi: a Review

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SUMMARY

Biochemical procedures for the differentiation of fungi in general and pathogenic fungi in particular have been slow to develop because many such organisms possess a sufficiently varied morphology to allow of considerable subdivision by that means alone. When, however, one considers organisms with a very reduced morphology, such as yeasts, the picture is very different and biochemical procedures have long been standard. Dependence on morphology for the identification of filamentous fungi has several disadvantages, of which slowness and lack of precision are the two most important. It is not desirable in medicine to have to wait several months till a particular identifiable structure is produced and there are a few pathogenic fungi which remain permanently nondescript. The lack of precision is best shown by the plethora of synonyms and ill described 'species' that have long plagued mycology.

The capacity of fungi to utilize various compounds and the chemical nature of their structure have both been used as methods of differentiation. The former method has been successfully used to show differences between morphologically similar organisms in several genera such as *Microsporium*, *Trichophyton* and *Madurella*. This method has also proved of the utmost value in distinguishing those aerobic Actinomycetes which are responsible for mycetoma from one another. Tests commonly used are the ability to degrade compounds such as casein, tyrosine, xanthine and urea or the lack of ability to grow in the absence of certain amino acids or vitamins or the ability to assimilate a particular compound such as dulcitol, erythritol, lactose or sucrose. Chemical analysis of fungi has not yet progressed so far but differences have been demonstrated, and the detection of arabinose and diaminopimelic acid in the cell walls of Actinomycetes appears to be on the verge of becoming standard technique.

INTRODUCTION

In a sense biochemical differentiation of pathogenic fungi has been with us since the beginning of medical mycology. Sabouraud (1910), in *Les Teignes*, noted the effects of certain variations of culture medium on the growth and appearance of dermatophytes. Today, in the absence of the crude ingredients then used, it is difficult or impossible accurately to reproduce those early results. That the appearances of fungi differ when grown on different substrates is well known and the importance of the quality of peptone was forcibly brought home to us by the import restrictions of 1946 (Carlier, 1948). But even before the publication of *Les Teignes* some work involving the nutrition of pathogenic fungi had been done by Verujsky (1887) and Bodin (1899, 1902)

The pressure to develop techniques of identification for filamentous pathogenic fungi has not been great because these organisms do possess a sufficient variety of structure to allow of a fair degree of separation by this method alone. Morphology rightly still plays an important part in the identification of pathogens but there are occasions when it proves inadequate. Increasingly, what the eye can see is being supplemented by other tests, biochemical and serological, which often allow an earlier or more precise diagnosis to be made. Because of the lack of pressure to develop alternatives to identification by form alone, research has been rather patchy and sporadic. There is not yet a coherent pattern of biochemical tests applicable to pathogenic fungi. Nevertheless, the many isolated ventures have at least shown that fungi do exhibit biochemical differences and there is no fundamental objection to the use of biochemical methods as supplements to other methods.

Biochemical tests currently in use to assist in the differentiation of pathogenic fungi can broadly be divided into two categories:

- (1) Tests to assess what particular compounds fungi assimilate or degrade in their growth processes. Alternatively, the identification of metabolites produced under controlled conditions may serve as a test.
- (2) Tests to assess what fungi are actually made of.

Assimilation and degradation tests

Assimilation tests are extremely important in the taxonomy of yeasts. This is an example of how a group of organisms of reduced morphology has given rise to a coherent pattern of biochemical tests; but such tests are of much less value in the study of filamentous fungi. This is no doubt in part due to lack of fundamental work but, also, such fungi are such extraordinarily catholic feeders that it is difficult to find compounds of sufficient selectivity. Nevertheless, several valuable tests have been described.

The nutrition of dermatophytes was reviewed by Stockdale (1953). Nutritional factors capable of differentiating dermatophytes were observed by Goddard (1934) who noted that *Trichophyton interdigitale* assimilated galactose but that *Microsporum canis* did not; neither of these fungi assimilated lactose. Earlier, Tate (1929) failed to detect invertase or lactase in *T. tonsurans*, *M. audouinii*, *M. canis* and *T. schoenleinii* but all contained maltase and diastase. Mallinckrodt-Haupt (1927), working with *T. gypsum*, observed that animal fats, but not cod liver oil, supported growth whereas vegetable oils other than olive oil, almond oil and cocoa butter did not. These observations are interesting in themselves but do not further the cause of differentiation much.

Georg (1949, 1950, 1951, 1952) studied the nutritional requirements of several dermatophytes and Georg & Camp (1956) described methods for differentiating some rather difficult fungi from one another. Simple basal media to which single nutrients (vitamins, amino acids) could be added were used as the bases of these tests. Table 1 shows some of their results; it is of particular interest to note that their findings tend to validate *Trichophyton equinum* and *T. megninii* as genuine species. These organisms had been fighting a losing battle to avoid being classed as *T. mentagrophytes* and *T. rubrum*, respectively, but the readily demonstrable absolute requirement for nicotinic acid or histidine clearly separates them. Other useful differences are shown in Table 1; *T. verrucosum* and *T. schoenleinii* are morphologically similar but one grows well in the absence of thiamine and inositol and the other demands them. *T. tonsurans* can also be readily differentiated from *T. rubrum* and *T. mentagrophytes*.

The requirements of single amino acids by dermatophytes were studied by Pinetti, Bonomi & Lostia (1959). In general, any amino acid serves dermatophytes well enough as a sole source of nitrogen, but there are exceptions. As examples one can quote *Epidermophyton floccosum* which cannot utilize lysine, and *Trichophyton megninii*, which is really happy only in the presence of histidine. The latter fungus may also show some growth in the presence of threonine, alanine and lysine but apparently cannot utilize glycine, leucine, asparagine, cystine, valine or phenylalanine. Rosenthal & Vanbreuseghem (1962) studied the nutritional requirements of several dermatophytes, and Table 2 shows that *T. soudanense* cannot utilize ammonium nitrate or histidine as nitrogen sources whereas *T. yaoundei* and *Microsporum langeronii* can.

Table 1. Nutritional requirements of several *Trichophyton* species

Recommended for routine use to assist in identification (Georg & Camp, 1956).

Species	Growth on basal medium alone	Growth on basal medium with added				
		Inositol	Thiamine	Thiamine and Inositol	Histidine	Nicotinic acid
<i>T. verrucosum</i>	o	±	±	+	.	.
<i>T. schoenleinii</i>	+	+	+	+	.	.
<i>T. concentricum</i>	+	+	+	+	.	.
<i>T. tonsurans</i>	±	.	+	.	.	.
<i>T. mentagrophytes</i>	+	.	+	.	.	+
<i>T. equinum</i>	o	.	.	.	.	+
<i>T. rubrum</i>	+	.	+	.	+	.
<i>T. megninii</i>	o	.	.	.	+	.
<i>T. gallinae</i>	+	.	.	.	+	.
<i>M. ferrugineum</i>	+	.	+	.	.	.
<i>T. violaceum</i>	±	.	+	.	.	.

o = no growth; ± = weak growth; + = good growth; . = no test.

Table 2. Histidine or Ammonium nitrate as sole nitrogen sources for three dermatophytes

Rosenthal & Vanbreuseghem (1962).

Species	Nitrogen source	
	NH ₄ NO ₃	Histidine
<i>Trichophyton soudanense</i>	o	o
<i>T. yaoundei</i>	+	+
<i>Microsporum langeronii</i>	+	+

o = no growth; + = growth.

Koehne (1962) studied the nutrition of three species of the genus *Microsporum* and showed that while a very large number of compounds provided adequate carbon sources there were several small differences. He noted that *Microsporum cookei*, *M. distortum* and *M. nanum* differed in their abilities to utilize starch, erythritol and dulcitol (Table 3). These species are as a rule perfectly easy to distinguish morphologically, but the fact that differences exist shows that an extension of the findings might easily lead to knowledge of taxonomic significance. For example, can the different members of the *M. gypseum* complex be so distinguished or is there a true and acceptable difference between *M. audouinii* and *M. langeronii*?

A problem that may arise in any laboratory is to decide whether an isolated fungus is a dermatophyte or not, and a neat presumptive test has been suggested by Baxter (1965) and amplified by Quaife (1967); the test depends on the fact that dermatophytes, unlike many other fungi, tend to increase the pH value of the supporting medium. Ink blue changes from blue to colourless between pH 6.5 and 7.2 and can safely be incorporated in glucose peptone agar to a concentration of 0.05 %. Dermatophytes and other keratinophilic fungi such as *Trichophyton terrestre* discharge the blue colour, whereas the wide range of other fungi which Baxter and Quaife tested had no such effect.

Table 3. *Differences between three Microsporum species with respect to dulcitol, erythritol and starch*

Koehne (1962).

<i>Microsporum</i> spp.	Carbon source		
	Dulcitol	Erythritol	Starch
	Growth		
<i>M. cookei</i>	o	+++	++
<i>M. distortum</i>	+	o	+
<i>M. nanum</i>	+	++	o

o = no growth; + = weak growth; ++ = moderate growth; +++ = vigorous growth.

Table 4. *Hydrolysis of casein, tyrosine, gelatine and urea by dermatophytes*

Philpot (1967), Rosenthal & Sokolsky (1965) and unpublished observations at the Mycological Reference Laboratory.

Organism	Hydrolysis of			
	Casein	Tyrosine	Gelatine	Urea
<i>Trichophyton mentagrophytes</i>	6/6*	6/6	6/6	6/6
<i>T. rubrum</i>	7/7	7/7	7/7	0/7
<i>T. gallinae</i>	1/1	1/1	1/1	0/1
<i>T. megninii</i>	2/2	2/2	2/2	2/2
<i>T. sulphureum</i>	6/6	6/6	6/6	6/6
<i>T. tonsurans</i>	4/4	4/4	4/4	4/4
<i>T. soudanense</i>	6/6	6/6	6/6	0/6
<i>Microsporum ferrugineum</i>	1/4	4/4	1/4	0/4
<i>T. schoenleinii</i>	6/6	6/6	6/6	1/6
<i>T. verrucosum</i>	6/6	0/6	6/6	5/5

* Number of strains positive/number of strains tested.

We and others have made a few observations about the ability of certain dermatophytes to hydrolyse casein, tyrosine, gelatine and urea, and some quite useful differences have come to light (Philpot, 1967; Rosenthal & Sokolsky, 1965); see Table 4. Urease production seems to be a useful way of distinguishing *Trichophyton rubrum* and *T. gallinae* from *T. megninii* and *T. mentagrophytes*, as well as *T. tonsurans* from *T. soudanense*. At first we thought that *T. soudanense* and *Microsporum ferrugineum* could be readily distinguished from one another by their proteolytic activities but, unless one of our strains of *M. ferrugineum* was incorrectly identified, this is only partly true.

Another group of filamentous fungi in which there has been some progress in biochemical differentiation is the group responsible for mycetoma. This is true of both fungi, agents of maduromycetoma, and aerobic actinomycetes, agents of the clinically similar actinomycetoma. It is again to be noted that the reduced morphology of actinomycetes has led to the use of a better array of biochemical tests for their differentiation. Table 5 lists some of the fungi known or thought to be capable of causing mycetoma, together with some details of their chemical behaviour. These studies have been largely made by Mackinnon, Ferrada & Montemayor (1949*a, b*), Mackinnon (1951*a, b*, 1954), Segretain (1957) and Segretain & Segretain (1960). It is perhaps of interest to note that *Madurella mycetomi* has a long list of synonyms including *M. ikedae* and probably also *M. americana*, and it is largely the work of Mackinnon which has brought this difficult group into a single species. Mackinnon and his colleagues, together with Segretain, found simple nitrogen compounds of no value for differentiation of mycetoma-causing organisms, but an examination of Table 5 at once reveals that a fair degree of separation can be made by maltose, sucrose and lactose. Opinions on the proteolytic powers of these fungi vary but the results of sugar assimilations have been constant.

Table 5. *The action of mycetoma-causing fungi on carbohydrates and proteins*

This table is compiled from data published by: Mackinnon *et al.* (1949*a, b*); Mackinnon (1951*a, b*, 1954); Segretain (1957); Segretain & Segretain (1960).

Organism	Assimilation of				Hydrolysis of		
	Maltose	Sucrose	Galactose	Lactose	Coagulated serum	Milk	Gelatine
<i>Madurella mycetomi</i>	+	o	+	+	+	+	+
<i>M. ikedae</i>	+	o	+	+	+	+	+
<i>M. americana</i>	+	o	+	+	+	+	+
<i>M. grisea</i>	+	+	+	o	±	+	+
<i>Allescheria boydii</i>	o	±	±	o	±	+	+
<i>Cephalosporium recifei</i>	+	+	+	o	o	+	o
<i>C. falciforme</i>	+	+	+	o	±	±	+
<i>C. acremonium</i>	+	+	+	+	+	+	+
<i>P. jeanselmei</i>	+	+	+	o	o	o	o

o = no growth; ± = variable growth; + = good growth.

The organisms so far considered possess a fair variety of structures which make differentiation by morphology a not impossible task. Those aerobic actinomycetes which cause mycetoma possess very little morphological variation and the laboratory becomes almost totally dependent on biochemical features. The organisms most often encountered in human pathology are *Nocardia asteroides*, *N. brasiliensis*, *N. caviae*, *Streptomyces madurae*, *S. pelletierii* and *S. somaliensis*. The tests for them have become fairly standard; some are outlined in Table 6. Many workers have contributed the data in Table 6, notably Gordon & Mihm (1957, 1959, 1962*a, b*), Mariat (1957, 1958, 1962, 1963), Georg, Ajello, McDurmont & Hosty (1961) and Gonzalez-Mendoza & Mariat (1964). The use of this table of biochemical features, together

with morphology, especially of parasitic forms in the tissues when available, not only makes identification of the pathogenic species relatively easy but usually permits the numerous saprobic but similar organisms to be recognized.

Tests based on the chemical analysis of fungi

A good deal of work has been done on the chemical constitution of dermatophytes, though in a manner unlikely to help the taxonomist much. In general all dermatophytes seem to be composed of the same lipids, carbohydrates and amino acids but some differences are known to exist. Chattaway, Toothill & Barlow (1961) showed that strains of *Trichophyton mentagrophytes* did not contain aspartic acid, serine and proline in the free state; in contrast, *T. rubrum*, *Microsporum canis* and *Epidermophyton floccosum* did. There are also differences in sterol content, ergosterol being characteristic of most and brassicasterol of others. There are some contradictions in the

Table 6. *Biochemical differentiation of pathogenic Nocardia and Streptomyces species*

No single test is completely reliable but the use of several gives excellent results. These tests are used routinely in many laboratories and are based on the works of Mariat (1957, 1958, 1962, 1963) and Gordon & Mihm (1957, 1959, 1962 a, b).

Species	Acid fastness	Hydrolysis of					Assimilation of		Production of urease
		Gelatin	Casein	Tyrosine	Xanthine	Egg Albumin	Maltose	Mannitol	
<i>Nocardia asteroides</i>	±	±	o	o	o	o	o	o	+
<i>N. brasiliensis</i>	±	+	+	+	o	o	.	+	+
<i>N. caviae</i>	±	o	o	o	+	.	±	+	±
<i>Streptomyces madurae</i>	—	+	+	.	.	o	.	+	o
<i>S. pelletierii</i>	—	+	+	.	.	+	o	o	o
<i>S. somaliensis</i>	—	+	+	.	.	+	+	o	o

o = no response; + = positive response; ± = variable response; . = uncertain or untried.

literature. According to Wirth, Beesley & Miller (1961) and Wirth & Anand (1964), *T. rubrum* contains both ergosterol and brassicasterol, the latter predominating in most strains; but Blank & Shortland (1962) affirmed that brassicasterol was dominant in *T. violaceum*, *T. verrucosum* and *T. megninii* and that ergosterol was dominant in *T. tonsurans*, *T. rubrum*, *T. mentagrophytes* (and its varieties), *M. audouinii*, *M. canis* and *M. gypseum*. Were Wirth and colleagues perhaps mistakenly working with the rather similar *T. megninii*? Audette, Baxter & Walker (1961) reported that ergosterol was the more abundant sterol in *T. mentagrophytes*.

Analysis of cell walls has proved of particular value in the separation of species of the genus *Nocardia* from saprobic species of the genus *Streptomyces*. Theoretically this can be done by a simple stain for acid-fastness but this is far from reliable. Two suitable methods, both involving paper chromatography, have been described. Cummins & Harris (1956, 1958) showed that the cell walls of species of the genera *Mycobacterium* and *Nocardia* were rich in arabinose but species of the genera *Actinomyces* and *Streptomyces* were deficient. Murray & Proctor (1965) described a simple method of paper chromatography that allows this criterion to be used as a laboratory routine. By this means, certain pathogenic, aerobic actinomycetes were grouped as follows.

(1) Those containing a great deal of arabinose: *Nocardia asteroides*, *N. brasiliensis*, *N. caviae*, several *Mycobacterium* species.

(2) Those containing less but a still detectable amount of arabinose: *Streptomyces madurae*, *S. pelletierii*.

(3) Those containing no arabinose: *Streptomyces somaliensis*, saprobic *Streptomyces* species.

It is interesting to compare this separation based on arabinose content with that found by Gonzalez-Ochoa & Vazquez-Hoyos (1953) who used serological methods. Their groups were as follows.

(1) 'Bovis' group: anaerobic *Actinomyces* species, *Nocardia asteroides*, *N. brasiliensis*.

(2) 'Madurae' group: *Streptomyces madurae*, *S. pelletierii*.

(3) 'Somaliensis' group: only *Streptomyces somaliensis*.

(4) 'Paraguayensis' group: saprobic *Streptomyces* species.

The agreement of the two methods is of a high order, the main differences being that anaerobic *Actinomyces* species lack arabinose in the cell wall and *S. somaliensis* goes with the saprobic species by the chromatographic method.

A somewhat similar method which uses diaminopimelic acid as a marker was described by Becker, Lechevalier, Gordon & Lechevalier (1964). This method gives results in accord with those given by the arabinose method but simple sugars are easier to handle than amino acids and related compounds. Extended studies are likely to show that this type of analysis for arabinose, diaminopimelic acid and other compounds is of great taxonomic value.

General considerations

How valuable is biochemistry in differentiating pathogenic fungi? In clinical practice a mixture of cultural morphology, histology and serology seldom leave the diagnostician in doubt. However, at a more fundamental level the degree of precision hitherto available has certainly not been sufficient. The fact that fungi exhibit great variety of form has probably retarded the development of alternative differential criteria. But it is well known that wide form variations occur in any given species from time to time, and mycology has always been plagued by a great plenitude of 'species', many of which have been created on quite insufficient morphological data. Considerable space has been given here to those fungi which cause ringworm and mycetoma because in these two groups traditional methods of classification have created a great array of 'species' whose true status in many cases has still to be correctly assessed. One feels that the study of form is reaching its limit and that future differentiations must include other criteria; biochemical and serological methods are the obvious candidates in this field and both have already proved useful.

It would be too much to say that biochemical methods have now been developed to the point where they can serve as routine tools. The published results are patchy but they are sufficient to show the earnest seeker that biochemical differences do exist and that diligent research ought in time to create a useful and usable pattern of methods. Assimilation tests should be sought before tests involving chemical analysis of the fungus for the very simple reason that they are easier to perform and generally require no specialized apparatus.

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