

# ROUTINE NUTRITIONAL TESTS FOR THE IDENTIFICATION OF DERMATOPHYTES

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Dermatophytes are usually identified on the basis of the gross and microscopic cultural characteristics that develop on Sabouraud's dextrose agar. Most isolates develop colony forms, pigmentation, and spores which are diagnostic for the species. However, many atypical strains are encountered. In addition, there are a number of species which seldom produce spores and whose colonies resemble each other so closely that they can not be identified solely on the basis of morphological criteria. As an aid in the identification of the dermatophytes, particularly various *Trichophyton* species, a number of practical physiological tests have been developed.

## MATERIALS AND METHODS

The methods used are based essentially on the ones developed during the study of the nutritional requirements of various dermatophyte species (Georg, 1950, 1951, 1952; Swartz and Georg, 1955).

*The basal media.* The two basal media used in the tests, casein and  $\text{NH}_4\text{NO}_3$  agar, were made with (1) a "washed agar" that had been prepared in the laboratory by washing lots of agar (Difco) 15 times in distilled water, immersion in boiling 95 per cent ethyl alcohol, and drying, (2) three different samples of "purified agar" prepared by Difco Laboratories, and (3) untreated agar (Difco).

Experiments were also carried out to determine the effect of trace elements in the growth of the fungi. The two basal media were prepared with and without the addition of a trace mineral solution (Georg, 1952). The only salts added to the media were  $\text{MgSO}_4$  and  $\text{KH}_2\text{PO}_4$ .

The media prepared with the different nitrogen sources and with and without the trace mineral solution were used in nutritional tests with several typical strains of *Trichophyton verrucosum*, *Trichophyton tonsurans*, and *Trichophyton megninii*. These species produce characteristic growth patterns in the presence of certain vitamins and amino

acids. The media with the variously prepared agars were assayed for traces of thiamin by using spores of *Phycomyces blakesleeanus*.

*Vitamins and amino acids.* Tests were performed using vitamin and amino acid solutions prepared in acidified water and sterilized by filtration through Seitz filters. Parallel tests were run using similar solutions sterilized by autoclaving at 120 C for 10 min.

## RESULTS

It was found that various types of "purified agars" contained thiamin, although in quantities less than in the agar routinely used in mycological or bacteriological studies. However the test media prepared with agar (Difco) were satisfactory for use in the nutritional tests. It was found that the addition of trace mineral elements to the media had no visible effect on the growth of the dermatophytes. Autoclaving of the vitamin and amino acid solutions did not diminish their activity. On the other hand, it was found essential to acid clean glassware in order to remove traces of thiamin which would vitiate the tests.

*Procedure for routine nutritional tests.* Stock media consisted of (1) casein agar, and (2) ammonium nitrate agar.

### *Casein agar.*

Casein, 10 per cent acid hydrolyzed, vitamin free (Nutritional Biochemicals Co.) or Casamino Acid (Difco), vitamin free 2.5 g. . . . . 25.0 ml  
Glucose . . . . . 40.0 g  
 $\text{MgSO}_4$  . . . . . 0.1 g  
 $\text{KH}_2\text{PO}_4$  . . . . . 1.8 g  
Agar (Difco) . . . . . 20.0 g  
Water (distilled) . . . . . q.s. 1,000.0 ml

Dissolve by heating and distribute in 100 ml quantities into flasks and autoclave at 120 C for 15 min. The contents of several of the flasks may

be distributed into test tubes and slanted for use as vitamin free controls. Other flasks may be kept for preparing the different media containing vitamins as needed.

*Ammonium nitrate agar.* The preparation of this medium is similar to that of the casein agar except that 1.5 g  $\text{NH}_4\text{NO}_3$  is substituted for the casein.

*Test media.* The test media are prepared by adding vitamins to the casein medium and histidine to the  $\text{NH}_4\text{NO}_3$  agar to give the media the following final concentrations: (1) Thiamin-casein agar, thiamin hydrochloride 0.2  $\mu\text{g}/\text{ml}$ , (2) Inositol-casein agar, *i*-inositol 50  $\mu\text{g}/\text{ml}$ , (3) Thiamin-inositol casein agar, thiamin 0.2  $\mu\text{g}/\text{ml}$  and inositol 50  $\mu\text{g}/\text{ml}$ , and (4) Histidine-ammonium nitrate agar, *L*-histidine 30  $\mu\text{g}/\text{ml}$ .

The growth factors are added to melted stock media. These are then distributed into tubes, autoclaved at 120 C for 10 min, slanted and stored at 5 C for use as needed.

*Inoculation of nutrition tubes.* The inoculum may be taken from the growth obtained on any of the usual isolation media. Care must be taken, however, to take only a small fragment of the growth and thus avoid carrying any significant quantity of the medium to the nutrition tube.

*Nutritional patterns for the dermatophytes.* The nutritional patterns were found to fall into 4 groups.

*Group I.* The *Trichophyton* species in this group are those which seldom produce spores or distinctive pigments, and whose colonies resemble one another so closely that they can not be identified by morphological criteria. These species are: (a) *T. verrucosum*, (b) *T. schoenleinii*, and (c) *T. concentricum*. They are usually identified on the basis of clinical source as well as geographical origin.

(a) *T. verrucosum*:—Nutritional studies of *T. verrucosum* have been reported previously (Georg, 1950). Since this publication, 100 strains have been identified and studied by this laboratory. Seventy-four had been isolated from humans, 23 from cattle, 2 from donkeys, and 1 from a horse.

None of the strains grew on either  $\text{NH}_4\text{NO}_3$  or casein vitamin free agar media. Eighty-four strains were shown to require thiamin and inositol for growth. Sixteen strains required only thiamin. Additions of other water soluble vita-

TABLE 1  
*Growth patterns of Trichophyton verrucosum, Trichophyton schoenleinii, and Trichophyton concentricum. (This is a four tube test incubated at 37 C and read after 7 and 14 days)*

Dermatophyte	Test Media			
	Casein	Casein + inositol	Casein + thiamine	Casein + thiamin and inositol
<i>Trichophyton verrucosum</i> (100 strains studied)				
84%.....	0	±*	0	4+†
16%.....	0	0	4+	4+
<i>Trichophyton schoenleinii</i> (50 strains studied)				
2 exceptions—see text.....	4+	4+	4+	4+
<i>Trichophyton concentricum</i> (19 strains studied)				
50%.....	4+	4+	4+	4+
50%.....	2+	2+	4+	4+

\* ± Indicates a trace of submerged growth about the inoculum.

† 4+ Indicates maximum growth for that series of tubes, growth in other tubes being judged by comparison.

mins,<sup>1</sup> singly or in combination with the required vitamins, produced no effect on the growth.

Further tests showed that molecular thiamin was required by all strains studied. Thiamin could not be substituted by either pyrimidine or thiazole compounds or a combination of these. These findings are similar to those obtained by other workers (Robbins *et al.*, 1942, Burkholder and Moyer, 1943; Schopfer and Blumer, 1943; Mackinnon and Artagaveytia-Allende, 1948). The strain studied by Robbins *et al.* was shown to require 3 vitamins for growth: thiamin, inositol, and pyridoxine. Biotin appeared to be an additional factor for a strain studied by Schopfer and Blumer. Such requirements were not found among the 100 strains included in this study.

The diagnostic nutritional patterns of *T. ver-*

<sup>1</sup> Calcium pantothenate 100  $\mu\text{g}/\text{ml}$ , riboflavin 100  $\mu\text{g}/\text{ml}$ , *p*-aminobenzoic acid 100  $\mu\text{g}/\text{ml}$ , nicotinic acid or nicotinamide 100  $\mu\text{g}/\text{ml}$ , biotin 0.05  $\mu\text{g}/\text{ml}$ , choline chloride 100  $\mu\text{g}/\text{ml}$ , and folic acid 100  $\mu\text{g}/\text{ml}$ .

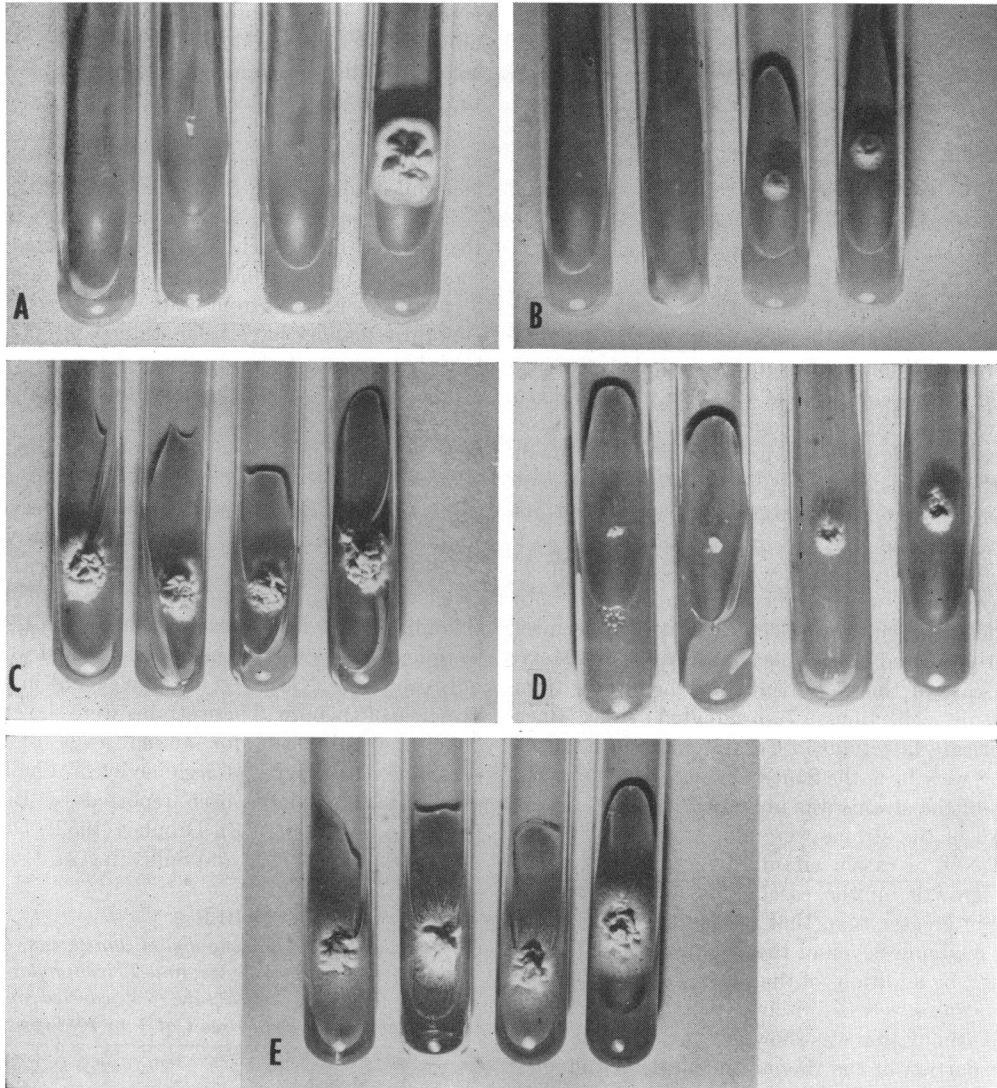


Figure 1. Four tube nutritional test: Left to right, (1) casein, (2) casein + inositol, (3) casein + thiamin, (4) casein + thiamin and inositol. A. *Trichophyton verrucosum* strains which require both thiamin and inositol. B. *Trichophyton verrucosum* strains which require thiamin only. C. *Trichophyton concentricum* strains which are autotrophic for the vitamins. D. *Trichophyton concentricum* strains which are stimulated by thiamin. E. *Trichophyton schoenleinii* strains which are autotrophic for the vitamins.

*rucosum* may be demonstrated by a four tube test as indicated in table 1.

(b) *T. schoenleinii*.—Preliminary studies of the nutritional requirements of *T. schoenleinii*, utilizing 6 strains, have been reported previously (Georg, 1950). Since then, 50 additional strains have been studied. Thirty-one were recent isolates from typical favic-type hairs, and 19 were obtained from culture collections. Of these 50

strains, 48 or 96 per cent presented a similar nutritional pattern. Characteristically *T. schoenleinii* was found to be autotrophic for the vitamins and developed on both  $\text{NH}_4\text{NO}_3$  and casein vitamin-free agar media. Although several of the isolates grew very slowly and poorly on the vitamin-free media, definite growth could be easily observed. Additions of water soluble vitamins, singly and in combinations, had no effect on the

growth of any of the 48 strains. Similar results have been reported by Drouhet and Mariat (1952).

*T. schoenleinii* can be readily differentiated from *T. verrucosum* by the four-tube test as illustrated in table 1. Since *T. schoenleinii* is autotrophic for the vitamins, equal amounts of growth occur on all 4 tubes.

Two strains of *T. schoenleinii* were encountered which presented different nutritional patterns. One strain, isolated from a typical case of favus, was exceptional in that its growth was stimulated by the addition of thiamin. A second strain, isolated from a chronic case of favus, was unable to grow on the vitamin free media. Additions of the water soluble vitamins did not allow the growth of this fungus, however, addition of 10 per cent yeast extract (0.05 ml/100 ml) permitted its growth on the  $\text{NH}_4\text{NO}_3$  or casein media.

(c) *T. concentricum*.—Eighteen strains of *T. concentricum* were studied. Seventeen were obtained from culture collections. One had been recently isolated from clinical material from a Mexican case of tinea imbricata. Of the strains from culture collections, 7 originated from tinea imbricata infections in Central and South America, 8 were from the South Pacific area. The origin of 3 of the strains was unknown.

All of the strains were able to grow on either  $\text{NH}_4\text{NO}_3$  or casein vitamin free media, however, the growth on the casein medium was considerably greater than that on the inorganic nitrogen medium. Seven of the isolates were not stimulated by additions of the water soluble vitamins. Ten strains were definitely stimulated by thiamin. This stimulation was shown to be due to the thiazole portion of the thiamin molecule. Equimolar amounts of thiazole, 4 methyl 5 *b*-hydroxyethyl thiazole (Merck and Co.), produced the same effect as the intact thiamin molecule. The various pyrimidines tested were inactive. A similar requirement for thiazole has been reported by Drouhet and Mariat (1952) for a strain of *T. concentricum* which they studied.

There was no correlation between the geographic source of the isolates and their nutritional requirements. The two nutritional patterns of *T. concentricum* as shown by a four-tube test are indicated in table 1.

Group II. In this group are included: (a) *Trichophyton tonsurans*, (b) *T. mentagrophytes*, and (c) *T. rubrum*. These are *Trichophyton* spe-

cies which usually produce microconidia, but only occasionally produce macroconidia. Their colonial forms and pigments are so variable that these fungi are frequently difficult to differentiate. A two-tube test with casein vitamin free agar and a similar medium with added thiamin is useful in identifying these species.

(a) *T. tonsurans*.—Nutritional studies with this fungus have been previously reported by Swartz and Georg (1955). To date 60 strains of *T. tonsurans* isolated from hairs parasitized in an endothrix manner have been studied. All strains have been shown to be autotrophic for the water soluble vitamins with the exception of thiamin for which they showed a partial requirement. Two or three times as much growth occurred on casein agar with added thiamin as on control casein tubes. Equimolar amounts of several pyrimidine compounds, such as 2 methyl-4-amino, 5-aminoethyl pyrimidine (Nutritional Biochemicals Co.) produced the same effect as thiamin. The various thiazole compounds tested were inactive. Seven of the strains studied would not grow on  $\text{NH}_4\text{NO}_3$  medium even in the presence of thiamin. These strains were shown to have a requirement for amino acids of the ornithine, citrulline, and arginine cycle. Findings similar to these have been reported by Burkholder and Moyer (1943), Drouhet (1952), Drouhet and Mariat (1952), and Sullivan *et al.* (1954).

TABLE 2

*Growth patterns of Trichophyton tonsurans, Trichophyton mentagrophytes, and Trichophyton rubrum. (This is a two tube test carried out at room temperature and read at 7 to 10 days)*

Dermatophyte	Test Media	
	Casein	Casein + thiamin
<i>Trichophyton tonsurans</i> (70 strains studied) . . . . .	± to 1+	4+
<i>Trichophyton mentagrophytes</i> (50 strains studied) . . . . .	4+	4+
<i>Trichophyton rubrum</i> (50 strains studied) . . . . .	4+	4+

*T. mentagrophytes* may be differentiated from *T. rubrum* by *in vitro* hair cultures (Ajello and Georg, 1957). *T. mentagrophytes* forms perforating organs in human hair samples suspended in water within 2 to 3 weeks. *T. rubrum* does not perforate human hair.

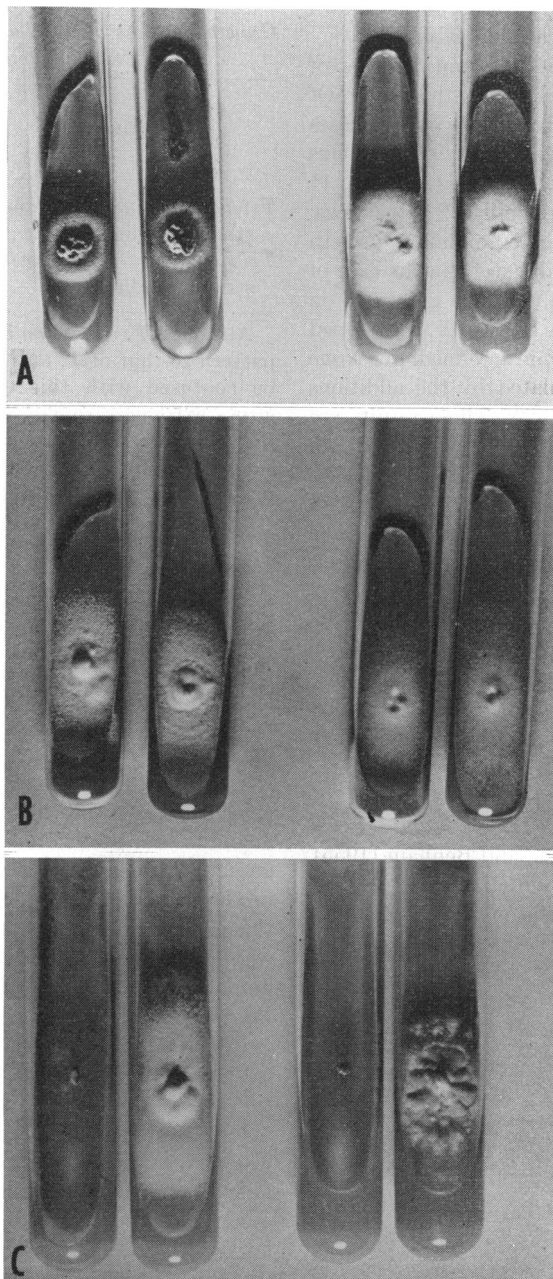


Figure 2. Two tube nutritional test: Tube at left of each pair, casein; tube at right of each pair, casein + thiamin. A. *Trichophyton rubrum*: granular strain (pair at left), fluffy strain (pair at right). Both autotrophic for thiamin. B. *Trichophyton mentagrophytes*: granular strain (pair at left), downy strain (pair at right). Both autotrophic for thiamin. C. *Trichophyton tonsurans*: Two strains. Both show stimulation by thiamin.

The nutritional pattern of *T. tonsurans* as shown by a two-tube test is indicated in table 2.

(b) *T. mentagrophytes*:—Sixty-nine strains of *T. mentagrophytes* were included in this study. Of these, 38 strains were derived from human cases of ringworm and 31 strains from spontaneous ringworm of animals which included: 6 dogs, 11 wild mice, 7 wild rats, 3 chinchillas, 2 guinea pigs, 1 kangaroo, and 1 horse. The amount of growth on  $\text{NH}_4\text{NO}_3$  agar was small except in the case of partially pleomorphic strains which grew well on this medium. All strains, however, developed rapidly on the casein vitamin free medium. None of the strains were stimulated by the additions of the water soluble vitamins. These results are similar to those reported by Robbins and Ma (1945), and McVeigh and Campbell (1950), Drouhet and Mariat (1952), and Silva and Benham (1954). The lack of stimulation by thiamin is in contrast to that observed with *T. tonsurans* and is illustrated in table 2.

(c) *T. rubrum*:—Forty-four morphologically typical strains isolated from human ringworm were studied. All of these strains were able to grow on vitamin free media. However, considerably greater growth occurred on the casein than on the  $\text{NH}_4\text{NO}_3$ . No stimulation by any of the water soluble vitamins was found. This corresponds to the findings of Drouhet and Mariat (1952) and of Silva, Keston, and Benham (1955). The lack of stimulation by thiamin is in contrast to that observed in *T. tonsurans*. The nutritional pattern as shown by the two-tube test is illustrated in table 2.

Thus *T. rubrum* and *T. mentagrophytes* can not be differentiated from each other by these nutritional tests. Another type of physiological test can be used, however, for their differentiation. This test is based on the fact that *T. mentagrophytes* perforates hairs in *in vitro* hair cultures while *T. rubrum* does not. Details of this test have been described by Ajello and Georg (1957).

*Group III. Trichophyton* species which seldom produce microconidia but which usually develop characteristically pigmented colonies are placed in this group. Occasionally, however, atypical strains are encountered and physiological tests may be useful in distinguishing them from other dermatophyte species. The dermatophytes in this group are: (a) *Trichophyton violaceum*, and (b) *Trichophyton ferrugineum*.

TABLE 3  
*Growth patterns of Trichophyton ferrugineum and Trichophyton violaceum*

Dermatophyte	Test Media	
	Casein	Casein + thiamin
<i>Trichophyton ferrugineum</i> (14 strains studied) . . . . .	4+	4+
<i>Trichophyton violaceum</i> (13 strains studied) . . . . .	±	4+

Although *T. violaceum* has a similar nutritional pattern to that of *T. tonsurans*, it is not likely to be confused with this organism. *T. violaceum* grows extremely slowly, even in the presence of thiamin, and usually produces a small glabrous colony with no spores. *T. tonsurans* grows rapidly in the presence of thiamin and shows many microconidia in the surface growth.

(a) *T. violaceum*:—Nutritional studies on this fungus have been previously reported by Georg (1951). Thirteen strains, 9 from culture collections and 4 recent isolates from endothrix parasitized hairs, have been included in this study. All strains grew very poorly on both vitamin free media. All strains, however, were greatly stimulated by thiamin. This was particularly apparent on the thiamin enriched casein medium. Equal amounts of pyrimidine, such as 2-methyl-4-amino, 5-aminoethyl pyrimidine hydrochloride, produced the same effect as thiamin. Various thiazole compounds tested were inactive, as were the other water soluble vitamins. These findings correspond to those of Drouhet and Mariat (1952). They are indicated in table 3.

(b) *T. ferrugineum*:—Fourteen morphologically typical strains obtained from culture collections were studied. All strains except one were able to grow on the  $\text{NH}_4\text{NO}_3$  vitamin free agar. The exceptional isolate was found to require L-leucine for growth. All strains grew well on casein vitamin free media and were not stimulated by additions of any of the water soluble vitamins as described above. The nutritional patterns of *T. violaceum* and *T. ferrugineum* are illustrated in table 3.

*Group IV.* This group includes those *Trichophyton* species which can be identified solely on the basis of specific nutritional requirements which serve to distinguish them from all other dermatophyte species. These species are: (a) *T. megninii* and (b) *T. equinum*.

(a) *T. megninii*.—Nutritional studies on this fungus have been previously reported by Georg (1952). Thirteen strains, 9 from culture collections and 4 recently isolated strains from Israel and Sardinia, were studied. None of the strains grew on the  $\text{NH}_4\text{NO}_3$  vitamin free medium, however, all grew well on the casein vitamin free medium. Further studies indicated that this organism requires L-histidine for growth. This nutritional pattern is illustrated in table 4. No other dermatophyte species shows this regular requirement for histidine.

In the past *T. megninii* has been confused with *T. gallinae*. However, *T. gallinae* possesses both morphological as well as physiological differences which serve to differentiate it.

Nutritional studies on *T. gallinae* have been reported previously by Georg (1952). Seven strains, 6 isolated from ringworm of chickens and 1 strain from a human infection described by Torres and Georg (1956), have been studied. All of the strains grew well on both  $\text{NH}_4\text{NO}_3$  and casein vitamin free agar. None of the strains were stimulated by additions of amino acids or the water soluble vitamins. These findings are similar to those reported by Silva and Benham (1952), and by Drouhet (1953). The nutritional pattern of *T. gallinae* is included in table 4 for comparison with *T. megninii*.

(b) *T. equinum*.—Nutritional studies on this fungus have been reported previously by Georg (1949) and Georg *et al.* (1957). Thirteen strains of *T. equinum* were observed. All strains except one have been isolated from cases of equine ringworm. The lone exception had been isolated from a dog.

TABLE 4

Growth patterns of *Trichophyton megninii* and *Trichophyton gallinae*

Dermatophyte	Test Media	
	$\text{NH}_4\text{NO}_3$	$\text{NH}_4\text{NO}_3$ + histidine
<i>Trichophyton megninii</i> * (13 strains studied) . . . . .	0	4+
<i>Trichophyton gallinae</i> † (7 strains studied) . . . . .	4+	4+

\* No other dermatophyte shows this regular requirement for histidine.

† *T. gallinae* is included on this chart, as this species has been confused with *T. megninii* in the past.

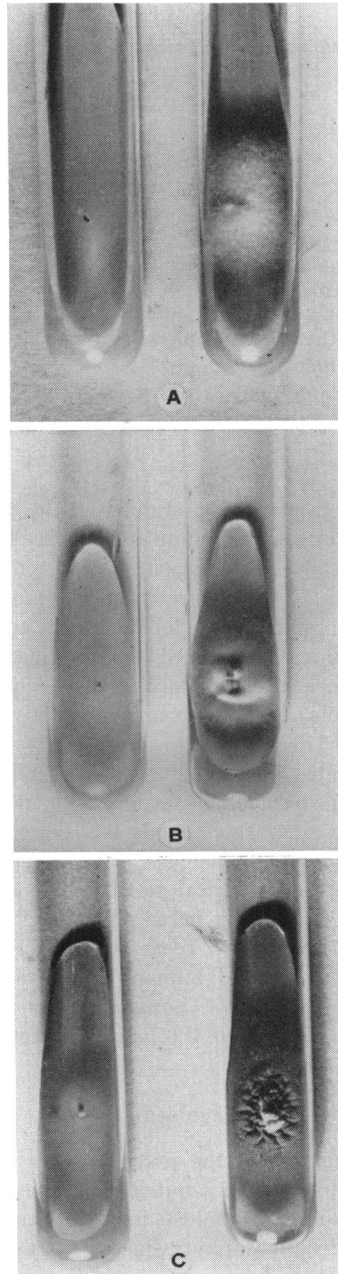


Figure 3. Two tube nutritional test: A. *Trichophyton megninii*. Tube at left  $\text{NH}_4\text{NO}_3$ , tube at right  $\text{NH}_4\text{NO}_3$  + histidine. Shows complete requirement for histidine. B. *Trichophyton equinum*. Tube at left casein, tube at right casein + nicotinic acid. Shows complete requirement for nicotinic acid. C. *Trichophyton violaceum*. Tube at left casein, tube at right casein + thiamin. Shows stimulation by thiamin.

TABLE 5  
Growth pattern of *Trichophyton equinum*\*

Dermatophyte	Test Media	
	Casein	Casein + nicotinic acid
<i>Trichophyton equinum</i> (13 strains studied) . . . . .	0	4+
<i>Trichophyton mentagrophytes</i> † (50 strains studied) . . . . .	4+	4+

\* No other dermatophyte shows this requirement for nicotinic acid.

† *T. mentagrophytes* has been included on this chart, as this species has been confused with *T. equinum*.

None of the strains studied could grow on  $\text{NH}_4\text{NO}_3$  or casein vitamin free media. The addition of various vitamins, singly or in combination as indicated above, showed that *T. equinum* has a complete requirement for nicotinic acid. This compound may be replaced by nicotinic amide or the amino acid, L-tryptophan. The requirement for nicotinic acid appears to be distinctive for this particular dermatophyte species. It may be demonstrated by a two-tube test as indicated in table 5. *T. mentagrophytes* has been included in this table since this fungus has been confused with *T. equinum* in the past.

Another physiological test for the identification of *T. equinum* is based on the fact that this fungus, in contrast to *T. mentagrophytes*, will not grow on autoclaved human hair suspended in sterile water. It will, however, grow on autoclaved horse hair, or the hairs of other equines (Georg *et al.*, 1957).

#### DISCUSSION

Simplification of the procedures for studying dermatophytes from a nutritional point of view, allows the use of such tests for the routine identification of dermatophyte species. Certain of the dermatophytes have very distinctive nutritional requirements which set them apart from all other dermatophytes. For example, *T. verrucosum* has a complete requirement for thiamin, or thiamin and inositol; *T. equinum* requires nicotinic acid; and *T. megninii* requires L-histidine for growth. One species, *T. tonsurans*, does not have complete requirements for any vitamin, but its growth is greatly stimulated by additions of thiamin. Other

species such as *T. mentagrophytes* and *T. rubrum* appear to be completely autotrophic for the vitamins.

In the process of studying groups of strains from culture collections by these nutritional tests, several errors in classification have been uncovered. A culture listed in the American Type Culture Collection as *T. equinum* (ATCC 9870) was found to be completely deficient for thiamin and inositol and as a result was identified as *T. verrucosum*. In another case, a culture from the London School of Hygiene and Tropical Medicine listed as *T. concentricum* (L. S. H. T. M., D793) was found to require both thiamin and inositol for growth and thus was identified as *T. verrucosum*. This finding was of especial significance because the culture had not been isolated from a case of tinea imbricata. It had been recovered in South Africa from a meat inspector, who had had a deep folliculitis of six weeks' duration on the cheek and intra-mandibular region. The geographic location of this case, as well as its clinical picture, gives weight to the re-identification of this fungus as *T. verrucosum*. There is one published report of the isolation of *T. concentricum* from South Africa (Pijper, 1918), but the description of the fungus isolated does not conform in any way to the characteristics of this species. Thus there is no proof that *T. concentricum* occurs in Africa.

It was interesting to find that the *T. concentricum* strains from South America and the South Pacific had similar nutritional requirements. This gives further weight to the belief that the causative organisms of "tokelau" in the South Pacific and of "chimbêrê" in South America are identical as suggested by Fonseca (1930) and Figueroa and Conant (1940).

#### SUMMARY

Practical nutritional tests have been described as an aid in the identification of certain dermatophytes.

Although the tests require the use of acid cleaned glassware, the preparation of the media and the procedures for their use are quite simple.

These procedures are particularly valuable in the identification of those dermatophytes which seldom produce spores and whose colonies resemble each other so closely that they can not be identified on morphological criteria alone. It is also an aid in the identification of morphologically atypical strains.



Diagnostic nutritional patterns for the following dermatophytes are presented: *Trichophyton concentricum*, *Trichophyton equinum*, *Trichophyton ferrugineum*, *Trichophyton gallinae*, *Trichophyton megninii*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton schoenleinii*, *Trichophyton tonsurans*, *Trichophyton verrucosum*, and *Trichophyton violaceum*.

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