BENTLEY, M. L. (1953). *J. gen. Microbiol.* 8, 365–377.

Enzymes of Pathogenic Fungi

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SUMMARY: Submerged growth of four dermatophytes, *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum* and *T. mentagrophytes* has been obtained by shaking during growth. The respiratory rate (μl. O_2 consumed/hr./mg. dry weight) of washed cells from 4–6-day cultures was between 7 and 12. As commonly observed with other fungi, addition of substrate to washed respiring cells did not much increase the respiratory rate. Acetone powders were prepared from cells grown in shaken culture. Such preparations oxidatively deaminated amino-acids slightly, about as much as similar preparations of *Penicillium chrysogenum*. A preparation of the enzyme asparaginase was extracted from *Microsporum gypseum* and some of its characteristics studied.

The metabolic processes of the dermatophytes have only recently received much attention. Vitamin requirements have been demonstrated, for example, for *Trichophyton faviforme* (Georg, 1950), *T. discoides* (Robbins, Mackinnon & Ma, 1942) and *T. album* (Schopfer & Blumer, 1943) and the amino-acid requirements of *T. mentagrophytes* were investigated by Robbins & Ma (1945). Nickerson & Chadwick (1946) studied respiration and assimilation in several species of dermatophytes and the effect of various compounds upon them. Melton (1951) extended this work to shaken cultures of *Microsporum canis*, and Stahl, McQue, Mandels & Siu (1949) studied the sulphur metabolism of *M. gypseum*.

The enzyme systems of these organisms have not been well characterized, although as early as 1895, Macfadyen (1895–6) found an extracellular proteolytic enzyme in the culture fluid of *T. tonsurans*. Bodin & Lenormand (1901) found various extracellular enzymes in cultures of *M. equinum*: one that clotted milk, another which dissolved the clot, and enzymes which liquefied gelatin, hydrolysed egg albumin and coagulated serum. Tate (1929) reported a survey of the distribution of several respiratory, proteolytic and lipolytic enzymes, carbohydrases and urease in acetone powders of species that were representative of the chief groups of dermatophytes.

It has been observed frequently that cultures of the dermatophytes become alkaline during growth, the change mainly being due to the production of ammonia (Nickerson, 1947). These observations suggested that enzymic mechanisms for ammonia production already known in other fungi, e.g. oxidative deamination of amino-acids by L- and D-amino-acid oxidases (Knight, 1948; Horowitz, 1944) and amidases such as urease, asparaginase (Shibata, 1904; Lang, 1904), may operate in the dermatophytes. It also seemed possible

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that specific amino-acid dehydrogenases, deaminases or amino-acid decarboxylases (Gale, 1946) present in bacterial and animal tissues might be found in the dermatophytes.

This paper reports an attempt to investigate enzyme systems responsible for ammonia formation in *T. mentagrophytes*, *T. rubrum*, *M. canis* and *M. gypseum*. Preliminary experiments to detect amino-acid decarboxylases were all negative. Oxidative deamination of amino-acids was observed, but the activity of the systems was low. Attention was next turned to asparaginase; and since this enzyme proved somewhat easier to work with, it was characterized further.

METHODS

Maintenance and cultivation of the dermatophytes. The cultures used in these experiments were obtained from the M.R.C. Medical Mycology Unit at the London School of Hygiene and Tropical Medicine. They were *M. gypseum*, *M. canis*, *T. mentagrophytes* and *T. rubrum* and had been isolated in 1950. Subcultures were maintained on malt agar slopes except for a short time at the beginning of the work when beer-wort slopes were used. Stock cultures were transferred about every 6 weeks. For experimental purposes a series of several tubes was inoculated from stock cultures and incubated for 2 weeks at 22°. Succeeding series were inoculated from a 2-week culture of the preceding series. Under these conditions of rapid transfer, *M. gypseum*, and particularly *M. canis*, tended to form rapidly growing non-sporing mycelia instead of the desired sporulating forms. Thereupon the organisms were transferred to barley grains (Langeron & Milochevitch, 1930); growth then reverted to the sporulating form after 6–8 weeks' incubation at 22°. Upon subsequent subculture on malt agar *M. gypseum* was kept sporulating during about 6 months. *M. canis*, on the other hand, very quickly became non-sporing again on malt agar. Eventually the use of the latter organism was abandoned.

The medium used for experimental work contained glucose (anhydrous; 2 %, w/v), mycological peptone (Oxoid; 2 %, w/v) and bacteriological yeast extract (Difco; 1 %, w/v), made up with distilled water and autoclaved at 15 lb./sq.in. for 15 min. before use. For the preliminary studies on the oxidation and deamination of amino-acids, 100 ml. lots of this medium were placed in 500 ml. Erlenmeyer flasks loosely plugged with cotton-wool. In later experiments in which asparaginase was studied, 75 ml. volumes were used in 250 ml. Erlenmeyer flasks. To inoculate the medium, sterile water or saline (5 ml.) was added aseptically to a 2-week sporulating subculture of the organism. The mycelium and spores were scraped off the agar and, after shaking, the suspension was poured into the culture flask, leaving the agar behind. Initially the washings from two agar slopes were used to inoculate each flask; but it was later found advisable to use four slopes/flask in order to obtain a more uniformly sized mycelial pellet. This procedure did not give a standard inoculum, since the mycelium on the agar slope was not readily wetted and the spores tended to form a film on the sides of the tubes, thus resulting in loss on transfer.

After inoculation the flasks were placed on a shaker which moved at

95–100 cyc./min. with a 9 cm. stroke. Under these conditions the fungi grew in balls or pellets of mycelium throughout the culture fluid. After 4–6 days at *c.* 18°, growth was sufficient, and a few pellets were then removed aseptically from the medium and cultivated on malt agar slopes to test for contamination. Although a few pellets did not represent the entire culture, this method would have detected gross contamination. The few experiments in which contamination was detected were discarded.

Enzyme preparations. The balls of mycelium were separated from the culture medium by filtration through muslin and were then washed with successive portions of distilled water (total about 2 l.) until the washings were colourless. In early experiments the balls of mycelium were used without further treatment; but for the study of asparaginase they were washed with running tap water for 90–180 min. This procedure, suggested by Dr S. G. Knight, had been found helpful in lowering the endogenous respiration of mycelium of certain *Penicillium* spp.

Acetone powders were prepared from washed mycelium by squeezing out as much water as possible, transferring the mycelial balls to acetone cooled to $-10°$ (no lower) and stirring for 6 min. The mycelial balls were then rapidly filtered-off on a large Buchner funnel, washed twice with acetone at 5° and then dried by sucking air through them. When the cake became whitish buff it was crumbled on to filter-paper and allowed to stand until the acetone vapour had disappeared. The powder was stored at 3° over P_2O_5 in an evacuated desiccator. The yield of acetone powder from six flasks of culture medium was about 1·5 g. In a few experiments dried mycelium was prepared by washing the mycelial balls with running tap water, filtering-off and sucking as dry as possible on a Buchner funnel, and then drying *in vacuo* over P_2O_5 for 18 hr. at room temperature; this material was then stored at 3°.

Chemical determinations. All chemical determinations were carried out on trichloroacetic acid (final concentration, 5 %) filtrates of the reaction mixtures. Free ammonia was distilled-off by a modification of the method of Pucher, Vickery & Leavenworth (1935). Saturated potassium carbonate was added to neutralize the acid of the filtrates and liberate ammonia, which was estimated in the distillate by nesslerization. Aspartic acid and asparagine were determined enzymatically by the method of Krebs (1950); for asparagine determinations asparaginase prepared from guinea-pig serum by ethanol precipitation was added to the system. Asparagine was also determined by estimation of ammonia liberated after 3 hr. hydrolysis in N-hydrochloric acid at 100°. Protein was determined colorimetrically with the phenol reagent of Folin & Ciocalteau using tyrosine as a standard (Minot & Keller, 1936). Oxygen uptake was measured manometrically with a Barcroft apparatus or with a Warburg apparatus; CO_2 was determined with the latter equipment.

RESULTS

An immediate difficulty in studying the four dermatophytes was their high respiration in the absence of added substrate (Table 1). The Q_{O_2} (μl. O_2 consumed/mg. dry wt./hr.) of the mycelial balls alone was usually *c.* 10–12,

never less than 5. Addition of glucose, amino-acids or organic acids did not produce a much higher value. In some cases the Q_{O_2} in the presence of a substrate was lower than the value in its absence, but such results were frequently not reproducible.

Table 1. *Respiratory quotients of certain dermatophytes in the absence and presence of substrate*

Phosphate buffer and substrate were both 0·016 M; volume 3 ml.; gas-phase air; at 38°.

Organism	Treatment	Substrate	pH	Q_{O_2}
T. rubrum	Washed	—	7	10·7
		glucose	7	11·3
M. gypseum	Aerated for 3 hr. at *c.* 18°, then placed for 24 hr. at 3°	—	7	7·6
		glucose	7	12·3
		glucose and glycine	7	8·7
		glucose and succinate	7	10·1
M. canis	As for *M. gypseum*	—	8	6·8
		L-glutamic acid	8	4·6

Aeration of the mycelial balls for a few hours in phosphate buffer (pH 7) or distilled water, at room temperature, either lowered the respiratory rate in the presence and absence of substrate alike or, when prolonged, abolished activity. Similar aeration followed by storage overnight in the refrigerator did not lower the respiration of the mycelium in buffer alone more than that with substrate. Melton (1951) was able to lower the endogenous respiration of *M. canis* grown in shaken culture by starving the organisms for a day or more. He probably succeeded with the technique because his organisms were grown for 2–3 weeks.

The values for respiratory rate of the mycelial balls shown in Table 1 are considerably higher than those reported by Nickerson & Chadwick (1946), who found values from 0·2 to 2·0 for the Q_{O_2} of *T. rubrum* and *T. gypseum*, but are similar to those reported by Wolf (1948) for shaken cultures of *Penicillium chrysogenum* Q176. The differences are probably due in the main to the fact that Nickerson & Chadwick used 2–4-week surface cultures. Darby & Goddard (1950) showed that the respiratory rate of shaken cultures of *Myrothecium verrucaria* was four times greater than that of static cultures. Differences in age of culture also cause considerable variation in respiratory rate as Wolf (1947) showed with cultures of *P. notatum*.

Acetone powders

To avoid the difficulty of pronounced respiration of the mycelial balls without added substrate, acetone powders of the mycelium from shaken cultures were made. A rough Q_{O_2} computed from results with *M. canis* as μ l. O_2 consumed/hr./mg. acetone powder was compared with that of intact mycelium. Values of this $Q_{O_2}=0·1–0·2$ were found for the respiration of the powder in buffer (see Table 2), suggesting damage to the respiratory systems during the acetone treatment. However, this residual respiration, though slight, continued for several hours at a gradually diminishing rate. By addition of yeast

extract or of substrate (glucose or amino-acids) the respiratory rate was doubled. Addition of yeast extract and substrate together produced a Q_{O_2} of 1·0. However, the activity was so low that the significance of these results is not certain.

Table 2. *Oxygen uptake of acetone powders of* Microsporum canis *with and without substrate*

Experiments (1) and (2): 0·02 M-phosphate, 20 mg. acetone powder.
Experiment (3): 0·03 M-acetate, 40 mg. acetone powder in each vessel.
Concentration of substrates given in brackets. Yeast extract: 0·33 %; volume 3 ml.; gas-phase, air; at 38°.

Exp. no.	pH	Substrate	Yeast extract	Q_{O_2}
1	6	None	–	0·16
	6	None	+	0·43
	6	Glucose (0·016 M)	+	0·96
2	8	L-glutamic acid (0·033 M)	–	0·55
	8	L-glutamic acid (0·033 M)	+	1·08
3	5	None	–	0·16
	5	Glucose (0·008 M)	–	0·27
	5	L-aspartic acid (0·005 M)	–	0·37
	5	Succinic acid (0·008 M)	–	0·20
	5	L-leucine (0·016 M)	–	0·33

Ammonia formation accompanied the oxygen uptake by the acetone powder respiring in buffer alone, about 0·8–1·5 μmole NH_3/μmole O_2 consumed. In the presence of each of several amino-acids increased ammonia formation was observed, which suggested oxidative deamination of the added amino-acid. Oxidative deamination of amino-acids by L-amino-acid oxidase of animal tissues and bacteria has been shown to proceed as indicated in equation (1):

$$H_2O + R\text{CHNH}_2\text{COOH} + O_2 \rightarrow R\text{COCOOH} + H_2O_2 + NH_3, \quad (1)$$

$$H_2O_2 \rightarrow H_2O + [O]. \quad (2)$$

In the presence of catalase, which Tate (1929) showed to be present in all the dermatophytes he tested, the hydrogen peroxide formed in the oxidation is decomposed (equation (2)), so that the overall reaction observed is

$$R\text{CHNH}_2\text{COOH} + \tfrac{1}{2}O_2 \rightarrow R\text{COCOOH} + NH_3. \quad (3)$$

One μatom oxygen is consumed/μmole ammonia formed. Fairly good correspondence with these quantitative relations was observed with aspartic acid as substrate for *M. gypseum*. With the other amino-acids, the activity was so low that agreement with the equations seemed fortuitous (see Table 3). In view of the low O_2 uptake and possible variations of catalase content of the acetone powders, deamination was subsequently followed by direct estimation of ammonia which seemed more sensitive and more reliable than measurement of O_2 uptake.

An L-amino-acid oxidase has been described in acetone powders of *P. chrysogenum* NRRL-1951-B25 and Q176 (Knight, 1948), and in Table 4 these results are compared with those obtained with acetone powders of *M. canis* and *M. gypseum*. Knight established that for L-alanine and L-methionine

1 μmole ammonia was liberated by the two strains of Penicillium for each μatom O_2 consumed. Presumably the relation held for the other amino-acids tested. Therefore, in Table 4, his results, originally in terms of mm.^{3} oxygen consumed/50 mg. acetone powder/80 min., have been expressed as μmole ammonia/50 mg. acetone powder/80 min. The stimulation of ammonia formation by amino-acids observed in the present study of the dermatophytes has

Table 3. *Oxygen uptake and ammonia formation by acetone powders of* Microsporum gypseum

Amino-acids and phosphate buffer (pH 8) were both 0·016 M. 50 mg. acetone powder/vessel. The vessels were incubated 3 hr. at 38° in air.

	Ammonia formed		Oxygen consumed	
Addition	Not corrected (μmole)	Corrected for blank (μmole)	Not corrected (μatom)	Corrected for blank (μatom)
None	3·94*	—	2·16	—
L-leucine	4·59	0·65	2·85	0·64
L-aspartic acid	9·50	5·56	6·92	4·76
L-phenylalanine	4·75	0·81	2·64	0·48
L-glutamic acid	7·59	3·65	2·78	0·62
Glycine	5·43	1·49	—	—

* The zero time value was 2·93 μmole ammonia: therefore, 1·01 μmole were formed during the incubation.

Table 4. *Comparison of activity in deaminating amino-acids of certain dermatophytes and* Penicillium chrysogenum NRRL-1951-B-25.

The experiments with the dermatophytes were done in 0·02 M-phosphate (pH 8). With *Microsporum gypseum*, values designated *a*. 40 mg. acetone powder/tube; remaining tubes, 50 mg. acetone powder/tube. The amino-acids were 0·03 M. Tubes were shaken in air at *c*. 18°. With *M. canis* 40 mg. acetone powder/tube and amino-acids as follows: DL-asparagine, L-leucine and L-alanine, 0·03 M; L-glutamic acid, L-aspartic acid and L-lysine, 0·01 M. The tubes were incubated 5 hr. in air at 25°.

		μmole NH_3/50 mg. acetone powder/80 min.*	
Organism	Amino-acid	Dermatophytes	*P. chrysogenum* (Knight, 1948)
M. gypseum	L-leucine	0·29 (16 %)	0·37
	L-leucine	0·36 (12 %)*a*	—
	L-aspartic acid	2·47 (116 %)	0·62
	DL-aspartic acid	0·75 (26 %)*a*	—
	L-phenylalanine	0·36 (24 %)	0·18
	L-glutamic acid	1·69 (94 %)	0·94
	L-glutamic acid	0·0*a*	—
	Glycine	0·66 (28 %)	0·22
	L-alanine	(−) 0·60 (−20 %)*a*	5·1
	DL-asparagine	0·43 (14 %)*a*	—
M. canis	DL-asparagine	1·98	—
	L-glutamic acid	0·55 (23 %)	0·94
	L-leucine	1·48 (62 %)	0·37
	L-alanine	(−) 0·48 (−20 %)	5·1
	L-aspartic acid	1·19 (50 %)	0·62
	L-lysine	0·0	—

* All values corrected for the blank.

been converted to the same units. Both sets of experiments were carried out at pH 8 in 0·02 or 0·016 M-phosphate buffer (see Table 4). Knight's experiments were carried out at 30°.

L-Alanine was most rapidly attacked by the penicillium preparations and L-aspartic acid by the two dermatophyte preparations; but with the latter the rate was about half that of the former. The activity of the penicillium and *M. canis* and *M. gypseum* toward the other amino-acids was slight and of the same order of magnitude. To give a clearer idea of the quantitative relationship between ammonia formation by the blank (acetone powder and buffer alone) and in the presence of amino-acids, the original increment over the blank expressed as percentage of the blank has been placed in parentheses beside the values in column 3 of Table 4.

The smaller amount of ammonia formed in the presence of L-alanine relative to the blank has been observed several times with *M. canis* and *M. gypseum*. Without further investigation its significance cannot be assessed. However, Schade & Thimann (1940) found much the same in their study of the metabolism of the water mould *Leptomitus lacteus*. Leucine was vigorously oxidized by the mould, but the ratio of ammonia produced to O_2 taken up was lower than that found when the mould respired in buffer alone, indicating that some ammonia was absorbed. Moreover, the R.Q. was lower than that required for complete oxidation of leucine. They explained the observation by assuming that leucine was oxidized to a compound with the formula of alanine and that this product was assimilated.

Dialysed aqueous extracts of acetone powders of *T. mentagrophytes*, *M. gypseum* and *M. canis* deaminated L-aspartic acid; the activity of these extracts toward other amino-acids was very low or negligible. A typical experiment showing the activity of aqueous extracts in deaminating amino-acids is shown in Table 5. Seven ml. of water were added to 500 mg. acetone powder of *M. gypseum*; after stirring for 15 min. and centrifuging, the supernatant was dialysed for 80 min. against running water. One ml. of the dialysed solution was used in each of the five tubes indicated in Table 5. To the residue left after centrifugation, 6 ml. of water were added, and 1 ml. of this suspension was then used in each of the four tubes shown in Table 5. Under these conditions of extraction the activity for deaminating L-leucine and DL-asparagine remained in the residue and only activity toward DL-aspartic acid was extracted, although not completely. Similar treatment of acetone powder of *T. mentagrophytes* also extracted the activity toward aspartic acid and probably that toward phenylalanine.

The evidence thus suggests oxidative deamination of the amino-acids by enzymes in *M. canis*, *M. gypseum* and *T. mentagrophytes*, such as an L-amino-acid oxidase or specific amino-acid deaminases. In this connexion, a study of the metabolism of *Blastomyces dermatitidis* is of interest (Bernheim, 1942). Bernheim found that in the presence of amino-acids, more ammonia was produced than in the control; but, although the oxygen uptakes caused by the amino-acids were great enough to account for their complete oxidative deamination, in no case was the theoretical amount of ammonia recovered.

Furthermore, analyses showed that in the case of L-phenylalanine, no α-amino-nitrogen disappeared, and in the case of D-alanine more α-amino-nitrogen was found at the end of the experiment than had been added as D-alanine.

Table 5. *Activity of extracts of acetone powders of certain dermatophytes in deaminating amino-acids*

Microsporum gypseum. One ml. dialysed extract of acetone powder or 1 ml. of aqueous suspension of the residue after extraction (see text) was incubated in 0·02 M-phosphate buffer (pH 8) with 0·002 M-$MgCl_2$ and as indicated, 0·016 M-DL-asparagine, 0·03 M-L-leucine or 0·03 M-DL-aspartic acid. Marmite (0·16 %) was added to all the tubes containing the extract (except to the first control) and to all tubes containing suspended residue. Total vol. 3·6 ml. The tubes were incubated in air for 300 min. at *c.* 18°.

EXTRACT

Substrate	Ammonia-N evolved in 5 hr. (μg.)
None	50
None	120
L-asparagine	121
L-leucine	125
DL-aspartic acid	182

SUSPENDED RESIDUE

Substrate	Ammonia-N evolved in 5 hr. (μg.)
DL-asparagine	146
L-leucine	153
DL-aspartic acid	180
None	121

Trichophyton mentagrophytes. One ml. of dialysed extract of acetone powder was placed in each tube. The amino-acids were present in a final concentration of 0·03 M and phosphate buffer (pH 8), 0·02 M. The tubes were shaken in air for 214 min. at 22°.

EXTRACT

Substrate	Ammonia-N evolved in 214 min. (μg.)
None	57·5
L-glutamic acid	61·7
L-leucine	65·5
DL-aspartic acid	106·0
Glycine	62·7
DL-phenylalanine	80·0
DL-proline	57·5

Asparaginase

Ammonia was produced when acetone powders of *M. canis* or *M. gypseum* were incubated with asparagine (Table 1), suggesting the presence of an asparaginase. So little ammonia was formed when acetone powders of *T. mentagrophytes* and asparagine were incubated together that it was doubtful whether an asparaginase was present in them.

The yield of 1 mole NH_3 and 1 mole L-aspartic acid from 1 mole L-asparagine was established by chemical analyses for ammonia and asparagine and the enzymic analysis for L-aspartic acid and L-asparagine (Krebs, 1950) (Table 6). By virtue of the specificity of the enzymic method for L-aspartic acid, the

recovery of an equivalent of aspartic acid for each mole of L-asparagine disappearing indicated that the natural isomer of aspartic acid was the product of the above reaction. It is also clear from the analytical results that the α-amino nitrogen of asparagine was not attacked. The results of an experiment (no. 3) with acetone powder suspended in phosphate buffer at pH 8 present difficulties. Under these conditions, as shown previously, aspartic acid added to acetone powder stimulated ammonia formation, presumably by deamination of aspartic acid. The most likely reason why the aspartic acid formed upon hydrolysis of asparagine is not deaminated is that the aspartic acid concentration is very far below optimal for its deamination.

Table 6. *Chemical balance of hydrolysis of* L-β-*asparagine by asparaginase in* Microsporum gypseum

Exp. 1. Veronal extract of acetone powder was dialysed for 1 hr. against running tap water. Experimental tubes contained 1 ml. of dialysed veronal extract, 0·5 ml. of 0·1 M-L-β-asparagine made up in phosphate buffer (pH 8); 1 ml. of 0·067 M-phosphate buffer (pH 8) and water to 3 ml. A duplicate mixture was made and immediately precipitated with trichloroacetic acid. Controls were included in which water replaced asparagine or extract. The tubes were incubated for 5 hr. in air at *c.* 18°.

Exp. 2. Veronal extract was prepared from dried cells and dialysed for 2 hr. against running tap water. Conditions and controls as in exp. 1, except incubation was continued for only 60 min.

Exp. 3. Acetone powder (350 mg.) was suspended in 8 ml. of 0·067 M-phosphate (pH 8) and 1 ml. of this suspension was then added to each tube along with 1 ml. of 0·067 M-phosphate (pH 8), 0·5 ml. of 0·1 M-L-β-asparagine and water to 3 ml. The tubes were shaken in air at *c.* 18°.

Exp. no.	L-β-asparagine disappearing (μmole)	Ammonia formed (μmole)	L-aspartic acid formed (μmole)
1	−33·9	+32·5	Not tested
2	−8·3	+8·1	Not tested
3	−9·3	+8·6	+8·6

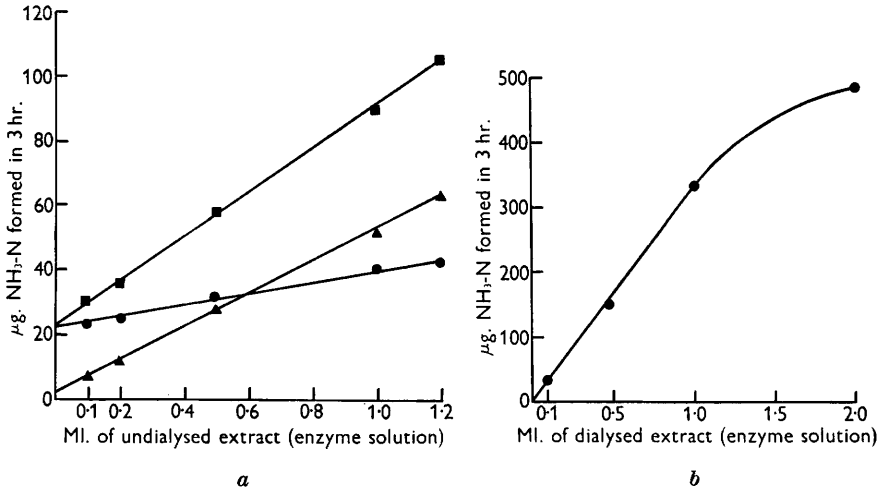
The activity of the asparaginase was extremely variable (cf. Figs. 1*a*, *b*). The probable explanation is that the organisms were grown at temperatures ranging from 15 to 30°. The activity was consistently low during the summer and in winter about 5–6 times higher. Both temperature and age of culture have a pronounced effect upon the activity of asparaginase in *Aspergillus niger* (Bach, 1929*a*; Schmalfuss & Mothes, 1930).

Characteristics of asparaginase in Microsporum gypseum

An asparaginase preparation was obtained from acetone powders of *M. gypseum* by extracting them with 0·1 M-veronal, pH 9. Tenth-molar veronal solution was added to the acetone powder in the proportion of 1 ml./44 mg. powder, the mixture was stirred by hand for 30 min., centrifuged at *c.* 4000 r.p.m. and used directly or after dialysis for a few hours against running tap water. The protein concentration of such extracts was about 10–12 %. Asparaginase could not be extracted with water (Table 5). The requirement of a slightly alkaline reaction for optimal extraction of asparaginase has also been observed with yeast (Grassmann & Mayr, 1933); Geddes & Hunter (1928) found simple

aqueous extracts of yeast were inactive, but they were able to extract the enzyme with 50% (v/v) glycerol at pH 7 in 3 days.

Figs. 1*a* and *b* show graphically the results of two experiments in which the rate of deamidation was proportional to the amount of enzyme present. Diminution of rate shown in curve *b* (Fig. 1) at the highest concentration of enzyme was probably due to suboptimal concentration of substrate. Fig. 2 shows that initially the reaction rate was constant, but after 3 hr. it decreased. No decisive explanation can be given for this decrease. Attempts to reverse the reaction have so far given equivocal results, so that inactivation of the enzyme seems probable.



Figs. 1*a*, *b*. Proportionality between rate of hydrolysis of asparagine and asparaginase concentration as shown by the ammonia formed from asparagine in the presence of varying amounts of a veronal extract of acetone powder.

Fig. 1*a*. The tubes contained 0·067 M-phosphate buffer (pH 8; 1 ml.), indicated portions of a veronal extract which contained 11·3 mg. protein/ml., 50 μmole L-β-asparagine in water (0·5 ml.) or water (0·5 ml.) in a final volume of 3 ml. The tubes were incubated for 3 hr. in air at 22°. Extract without asparagine (●——●); extract with asparagine (■——■); difference curve (▲——▲).

Fig. 1*b*. The tubes contained the indicated portions of veronal extract dialysed 2 hr. against running tap water. Conditions as in Fig. 1*a*. The values on the curve have been corrected for the ammonia nitrogen formed in the absence of asparagine.

Study of the effect of pH value on the activity of asparaginase in dialysed veronal extracts showed there is a broad optimal pH range between 7·8 and 9 and a maximum close to pH 8·5. Busch (1942) and Geddes & Hunter (1928) found the optimal pH for yeast asparaginase very close to 8. Bach (1929*b*) found it at 8·6–8·4 for *A. niger*, while Schmalfuss & Mothes (1930) found it at pH 7·7–7·8 for the same organism.

A typical curve relating activity of the enzyme to concentration of L-β-asparagine is given in Fig. 3. The enzyme has a high affinity for its substrate,

half maximum velocity of the enzyme in a veronal extract with 12·8 mg. protein/ml. being at *c.* 0·001 M-asparagine.

As might be expected, since the reaction appears to involve only simple hydrolysis of the amide bond of asparagine, the reaction occurred as well anaerobically as aerobically. For example, using 35 mg. of acetone powder suspended in phosphate buffer pH 8, 38 μg. of ammonia nitrogen were produced aerobically from asparagine and 44 μg. anaerobically.

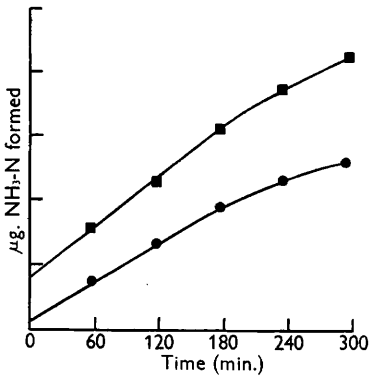


Fig. 2

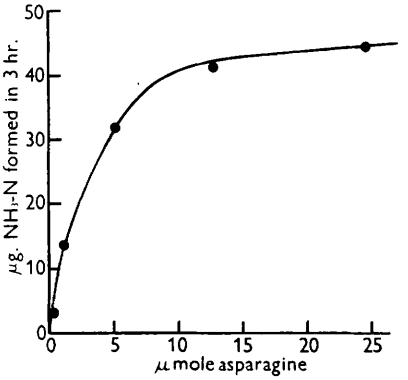


Fig. 3

Fig. 2. Time course of asparaginase action. Conditions as in Table 6, Exp. 3. The tubes were incubated for the indicated periods of time in air at 22°. Corrected (●——●) and not corrected (■——■) for ammonia nitrogen formed in the absence of asparagine.

Fig. 3. Proportionality between the rate of hydrolysis of asparagine by asparaginase in a veronal extract of acetone powder and substrate concentration. The tubes contained 1 ml. of veronal extract (12·8 mg. protein/ml.), 0·02 M-phosphate (pH 8), and indicated portions of L-β-asparagine in a final volume of 3 ml.; the tubes were incubated at 22° for 3 hr.

DISCUSSION

Since the dermatophytes can grow on a mixture of amino-acids or even keratin as sole carbon source (Nickerson, 1947), it seems likely that oxidative deamination of amino-acids is an important step in their utilization. The rate of oxidative deamination observed in acetone powders seems to be too low for the reaction to be essential in the metabolism of the amino-acids. However, as pointed out, the measurement of activity has not been observed under optimal conditions. The relation of asparaginase to the metabolism of *M. gypseum* is similarly difficult to assess. The variability of its activity suggests that its importance to the fungus may change with the age of the culture and the environmental conditions.

The author wishes to express her appreciation to Dr R. Riddell for his encouragement and interest in this investigation.

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(*Received* 2 *September* 1952)