The Action of Inhibitors on Dermatophytes

By F. W. CHATTAWAY AND C. C. THOMPSON

Department of Biochemistry, University of Leeds

AND A. J. E. BARLOW

Department of Dermatology, The Royal Infirmary, Huddersfield

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During recent years there has been a considerable increase in the incidence of infections due to the dermatophytes, and a correspondingly increased interest in the treatment of such infections with fungicides (Goldsmith & Hellier, 1954). Little is known, however, of the mechanism of action of fungicides upon these organisms nor of their detailed metabolism upon which a rational study of fungicide action might be based. Respiratory activity of agar cultures of a number of dermatophytes was studied by Nickerson & Chadwick (1946), and inhibitory activity of sodium propionate and undecylenate demonstrated. Melton (1951) studied the respiration of submerged cultures of *Microsporum canis* showing the inhibiting activity of propionic, butyric and undecylenic acids and sodium cyanide, and Bentley (1953) determined Q_{O_2} values of submerged cultures of *M. canis*, *Microsporum gypseum* and *Trichophyton rubrum*. All these studies were of endogenous respiration, and only very small or negligible responses were obtained with added substrates. Cell-free enzymic activities have been reported by Tate (1929), who showed qualitatively the presence of a number of enzymes in acetone-dried powders of several dermatophytes; by Bentley (1953) who studied quantitatively the amino acid oxidase activity of the three fungi mentioned, and by Chattaway, Barlow & Thompson (1954), who reported the presence of a number of enzymes associated with carbohydrate metabolism as well as amino acid oxidase in crushed mycelium of *M. canis*. In this paper a detailed study is reported of the effect of inhibitors of carbohydrate metabolism in other organisms, and also of fatty acids and various detergents, upon the respiration of a number of dermatophyte species.

Fatty acids and detergents are known to have a fungistatic and fungicidal action on the dermatophytes (e.g. Peck & Rosenfeld, 1939; Wyss, Ludwig & Joiner, 1945; Dunn, 1937; Quisno & Foter, 1946; Walker, Porter & DeKay, 1952). This activity depends upon pH and, for the fatty acids, upon carbon-chain length, but little is known of the mechanism of action. In this paper this problem is approached by a quantitative study, first of the

effect of fatty acids and detergents on endogenous respiration under different conditions, and secondly of the cell constituents released from washed mycelium in the presence of these materials.

A preliminary report has been given of some of this work (Barlow, Chattaway & Thompson, 1953).

METHODS AND MATERIALS

Organisms. The organisms used were representative strains of *M. canis* Bodin, *Microsporum audouinii* Gruby, *T. rubrum*, *Trichophyton schoenleinii* (LEB) Langer & Milochev, and *Epidermophyton floccosum* (Harz) Langer. All were freshly isolated from cases of dermatophyte infection. Stock cultures were subcultured at monthly intervals.

Media. Organisms were maintained on a 4% maltose-1% Difco proteose-peptone-agar medium adjusted to pH 5.6. The organisms were grown on the same medium, made up as broth, for experimental work.

Inoculum. All cultures for experimental work were grown as stationary cultures in 100 or 250 ml. conical flasks containing 20 or 30 ml. of medium, respectively, and incubated at 20°. The inoculum was a small piece of mycelium cut aseptically from a previously grown pad.

Replacement cultures. The required medium (20 ml.) was prepared in 100 ml. flasks, each constituent being autoclaved separately and added aseptically. Mycelial pads of approx. 24 mg. dry wt., and 13-17 days old for *M. audouinii*, *M. canis* and *T. rubrum*, and 20 days old for *T. schoenleinii* and *E. floccosum*, were washed six times in tubes of sterile distilled water before floating on the medium. Incubation was at 20° in stationary culture.

Buffers. For pH 5.0-8.2, 0.1M Sørensen phosphate buffers were used; for pH 3-5, McIlvaine citrate-phosphate buffers, and for pH 8-11, glycine-NaOH buffers according to Miller & Golder (1950).

Reagents. Undecylic and undecylenic acids, sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) were obtained from L. Light and Co., Ltd. Phenoxetol (2-phenoxyethanol) was supplied by Nipa Laboratories Ltd., Cardiff. Other fatty acids and reagents were B.D.H. laboratory reagents or AnalaR grade where available.

Respiratory determinations. These were made by conventional Warburg techniques in air at 30°. The mycelium was a piece of a surface culture of about 7 mg. dry wt. and of the same age as that used for replacement culture experiments.

α -Keto acids. These were estimated by the method of Friedemann & Haugen (1943) and total keto acid values

expressed as $\mu\text{g.}$ of pyruvic acid. Identification and isolation of the 2:4-dinitrophenylhydrazones was by paper chromatography by the method of Cavallini, Frontali & Toschi (1949) as modified by Dagley, Fewster & Happold (1952). All melting points are uncorrected.

Amino acids. α -Amino N was measured by the method of Danielson (1933). Amino acids released from fungal cells were identified by paper chromatography after desalting the solutions by adsorption on Zeocarb 215 (Permutit Co., Ltd.) and elution with 0.2 N- NH_3 according to Partridge & Brimley (1952). Solvents used for chromatography were *sec.*-butanol-3% (v/v) NH_3 (sp.gr. 0.880) (15:6, v/v) and *sec.*-butanol-90% formic acid (3:1, v/v).

Pentoses were estimated by the method of Mejbaum (1939), orthophosphate by the method of Fiske & Subbarow (1925) and total N by the micro-Kjeldahl method in a Markham still (Markham, 1942). Dry wt. was determined by heating mycelium at 100° to constant weight. Absorption spectra and colorimetric readings were determined on a Unicam spectrophotometer SP. 500.

RESULTS

It is characteristic of the dermatophytes, as of many other fungi, that they show a high rate of endogenous respiration which is very little affected by starvation of the mycelial mat. After numerous unsuccessful attempts to reduce this rate by starvation with aeration or shaking, or to stimulate the respiration significantly by the addition of substrates, it was decided to study the effect of certain inhibitors on the endogenous respiration. This respiration rate was found to be constant for pieces of mycelium from the same mycelial mat, but to vary appreciably from one experiment to another. The range of values found over a considerable

number of experiments is shown in Table 1; the values are expressed as $q_{O_2} = \mu\text{l. of } O_2/\text{mg. of N/hr.}$ As some indication of the type of material being oxidized during endogenous respiration R.Q. determinations were made, and typical results are also shown in Table 1.

Effect of inhibitors on endogenous respiration

Enzyme inhibitors. Inhibitors were added after the mycelium had been respiring for 1 hr. The percentage inhibition of respiration was calculated over the 2-3 hr. period after the commencement of the experiment, i.e. from 1 to 2 hr. after addition of the inhibitor. Each value recorded was the average of duplicate determinations; control flasks without added inhibitor were included in each experiment. Compounds known to inhibit glycolysis or terminal respiratory processes in other organisms were used. A range of concentrations of each inhibitor was employed, and typical results at two levels are recorded in Table 2.

Table 1. *Endogenous respiration of mycelial mats of dermatophytes*

Each vessel contained 3 ml. of 0.1 M Sørensen phosphate buffer, pH 6.4, with 0.2 ml. of 20% KOH and filter paper (Whatman no. 531) in the centre well. Gas phase: air; bath temp., 30°. $q_{O_2} = \mu\text{l. of } O_2/\text{mg. of N/hr.}$

Organism	Range of q_{O_2}	R.Q.
<i>M. audouini</i>	104-145	0.99
<i>M. canis</i>	159-192	0.99
<i>T. schoenleini</i>	157-190	0.88
<i>T. rubrum</i>	114-152	0.98
<i>E. floccosum</i>	143-172	0.92

Table 2. *Inhibition of endogenous respiration of dermatophytes*

Each Warburg vessel contained 2.5 ml. of 0.1 M Sørensen phosphate buffer, pH 6.4, with 0.2 ml. of 20% KOH and filter paper (Whatman no. 531) in the centre well. Inhibitors were placed in the side arm of the vessel. Gas phase: air; bath temp., 30°. Percentage inhibition determined as described in text. + Indicates percentage stimulation of respiration; — indicates the substance was not tested at that concn.

Inhibitor	Concn. (M)	Inhibition or stimulation of respiration (%)				
		<i>M. audouini</i>	<i>M. canis</i>	<i>T. schoenleini</i>	<i>T. rubrum</i>	<i>E. floccosum</i>
KAsO_2	0.01	82	75	71	87	65
	0.001	21	21	47	—	42
NaF	0.1	81	61	65	84	50
	0.01	27	—	16	59	29
Iodoacetate	0.016	70	65	66	77	54
	0.001	11	32	27	38	13
Fluoroacetate	0.02	6	4	49	40	31
	0.005	—	—	29	10	13
Azide	0.01	84	82	52	73	86
	0.0001	+23	+8	+11	+7	0
2:4-Dinitrophenol	0.0017	66	52	52	49	30
	0.0001	+23	+10	+17	+29	+15
Malonate	0.055	12	10	8	12	17
	0.055*	14	17	12	14	15

* 0.1 M citrate-0.13 M phosphate buffer, pH 4.0.

Arsenite, fluoride and iodoacetate show a high degree of inhibition with all the organisms, although a high concentration of fluoride was required. Fluoroacetate and malonate show a poor degree of inhibition, which may be due in part to difficulties of penetration. The oxidation of succinate by crushed mycelium, prepared in a Hughes (1951) press, is inhibited by malonate (Chattaway *et al.* 1954). Employment of a lower pH did not increase the degree of inhibition, although in other organisms this is found to increase the degree of penetration of malonate through cell walls (Barron, Ardao & Hearon, 1950). Azide and 2,4-dinitrophenol show effects similar to those recorded in other species, with stimulation of respiration at low concentrations and inhibition at higher levels.

Fatty acids. (a) *Effect of chain length.* The effect on endogenous respiration of the homologous series of saturated fatty acids from C_2 to C_{14} was studied. Oxygen uptake/mg. of N was determined for a piece of mycelium suspended in buffer over a 3 hr. period in the presence of the fatty acid, and compared with that for a control flask without added fatty acid. Typical results at varying concentrations of fatty acids are shown in Fig. 1 for *M. canis*, and analogous results were obtained with *M. audouini*. A considerable inhibiting effect, which increases with increasing chain length, is found. The figure for undecylenic acid is included since this substance has been much used in therapeutic preparations for dermatophyte infections; it was not found to be any more effective

Fig. 1. Effect of carbon-chain length of saturated fatty acids on the inhibition of endogenous respiration of *M. canis* suspended in 0.08M phosphate buffer, pH 7.4. x indicates percentage inhibition by 0.004M undecylenic acid at pH 7.4.

as an inhibitor under these conditions than the saturated acid of the same carbon-chain length.

(b) *Effect of pH.* The effect of the fatty acids of carbon-chain length C_1 – C_{10} on endogenous respiration at pH values 5.4, 6.4 and 7.4 was examined for *M. canis* and *M. audouini*, and typical results are shown in Fig. 2. Determination of percentage inhibition was as described above. In all cases an increasing inhibitory power was found with decreasing pH. The effects of chain length of the fatty acids and pH are closely analogous with those found by Wyss *et al.* (1945) for the fungicidal and fungistatic activity of fatty acids on *Trichophyton interdigitale* and *Trichophyton purpureum* in agar culture.

(c) *Permanence of the respiratory inhibition.* Nickerson & Chadwick (1946) showed for *E. floccosum* that removal of the mycelial mat from the fatty acid solution led to a return of the respiration rate to its original value. This point has been further investigated, particularly with reference to the possible recovery of respiration after 100% inhibition had occurred. It was determined that, in the presence of 0.02M octanoic acid at pH 5.4, the endogenous respiration of *M. canis* suspended in 0.08M phosphate buffer at 30° was completely inhibited after 2 min. Therefore, mycelial pads grown

Fig. 2. Effect of pH on the inhibition by fatty acids of the endogenous respiration of *M. canis*. Fatty acid concn. 0.02M. x, Octanoic acid; ●, hexanoic acid; ○, butyric acid.

under the standard conditions were washed six times with distilled water and floated on to 0.02M octanoic acid under the above conditions. Pieces of mycelium were then removed at intervals, washed six times in distilled water and floated on to 0.08M phosphate buffer, pH 5.4, and the endogenous respiration rate was determined over a 3 hr. period and the mean Q_{O_2} value determined. This was then compared with the value for mycelial mats treated similarly but in the absence of the fatty acid, and the percentage recovery of respiration determined.

Fig. 3. Recovery of endogenous respiration of *M. canis* after contact with 0.02M octanoic acid at pH 5.4.

Fig. 4. Effect of pH on the inhibition of endogenous respiration of *M. canis* by detergents. ●, 4.6×10^{-4} M CTAB; ○, 5.8×10^{-4} M SDS; ×, 3.5×10^{-2} M phenoxetol.

The results are shown in Fig. 3. Thus although inhibition is complete after 2 min. contact with the inhibitor, it can be reversed to the extent of 60 % by rapid washing after this time. After longer times of exposure the degree of recovery falls rapidly and after 30 min. the inhibition is almost irreversible under the conditions employed.

Detergents. Representatives of cationic, anionic and neutral detergents were all found to inhibit endogenous respiration of *M. canis*, the percentage inhibition being determined as described for the enzyme inhibitors. Inhibition of 50 % was given by 3.67×10^{-4} M CTAB at pH 8.2, 6.94×10^{-4} M SDS at pH 6.4 and 3.15×10^{-2} M phenoxetol at pH 6.4, all measurements being in 0.1M phosphate buffer. The effect of pH on the inhibition is shown in Fig. 4. The results show an increased degree of inhibition at alkaline pH values for the cationic detergent, at acid pH values for the anionic detergent and little change in activity with pH for the neutral detergent. The permanence of the inhibition produced by the three detergents was studied under the same conditions as those described for octanoic acid inhibition, and the results were virtually identical with those illustrated in Fig. 3, the inhibition being irreversible after approx. 30 min. exposure to the detergent. Thus the effects of fatty acids and the three types of detergent on the endogenous respiration appear to be parallel, although the effective concentrations vary appreciably.

Release of cell constituents during inhibition of respiration by fatty acids and detergents

It is well established for a number of bacteria that detergents are capable of causing a release of soluble cell constituents (Hotchkiss, 1946; Salton, 1951), but this phenomenon does not appear to have been demonstrated for fungi. The effect of detergents and fatty acids in promoting the release of such materials from *M. canis* has been shown, and an attempt made to determine whether such release can be correlated with the inhibitory action of these materials on endogenous respiration. Octanoic acid and the detergents used in the studies of respiration were employed, and the release of amino nitrogen, pentoses, inorganic phosphate and material absorbing at 260 mμ. was measured. The last was measured since the absorption spectrum of the culture filtrates showed a maximum value at 260 mμ., as was also shown by Salton (1951) for the supernatants after the action of CTAB on *Staphylococcus aureus*. Washed mycelial pads were floated aseptically on to a solution of the fatty acid or detergent in phosphate buffer (except when phosphate release was studied, when no buffer was used) and the released materials estimated after the required time interval. Four pads were employed in each determination, the samples were pooled and

the mycelial dry weights averaged. The release of constituents by octanoic acid over a period of 10 hr. is illustrated in Fig. 5. A similar result was obtained with the detergents, both in relation to the amount of materials released and to the shape of the curves. It is seen that there is a small spontaneous release of materials in the control flasks. The release of amino nitrogen is the most rapid; chromatographic examination of the concentrated filtrate obtained in the presence of octanoic acid showed the presence of serine, glycine, threonine, glutamic acid, proline,

alanine, leucine and isoleucine, and several materials which were not identified. The same pattern of amino acid release was seen in the control solution. Chromatographic examination for carbohydrates, with 3 % of *p*-anisidine hydrochloride in *n*-butanol as a spray reagent, revealed the presence of spots corresponding to glucose and ribose.

Effect of concentration. The release of cell constituents was studied at concentrations of octanoic acid and detergents under conditions known to give over 85 % inhibition of endogenous respiration as well as at levels which had little or no effect on respiration. Values were determined over a 20 hr. period and typical results after 4 hr. are shown in Table 3. The lower concentrations of inhibitors are seen not to release constituents to an appreciably greater extent than in the controls.

Effect of pH. The inhibitory activity of the fatty acids and detergents on endogenous respiration was shown earlier to be considerably affected by pH, and the effect of changing pH on release of cell constituents was also studied. A concentration of inhibitor known to be strongly active on respiration at the optimum pH for that substance was used. Material absorbing at 260 m μ . was determined for octanoic acid, CTAB and SDS, but pentose determinations were made for phenoxetol treatment as this substance itself absorbs strongly at 260 m μ . Typical results are shown in Table 4. It is apparent that release of constituents by the detergents is relatively unaffected by pH change, but with octanoic acid release virtually does not occur at pH values where capacity to inhibit respiration is low.

Accumulation of α -keto acids in replacement cultures

The marked inhibition of endogenous respiration by arsenite suggested that there might be an accumulation in the culture medium of α -keto acids,

Fig. 5. Release of soluble cell constituents from *M. canis* by octanoic acid. Phosphate buffer, 0.1M, pH 5.4, except curves 4 and 8. Curves 1-4, 0.02M octanoic acid; curves 5-8, controls. Curves 1, 5 (○), α -amino N; curves 2, 6 (×), material absorbing at 260 m μ .; curves 3, 7 (●), pentose; curves 4, 8 (○), inorganic phosphate.

Table 3. *Effect of concentration of inhibitor on release of cell constituents from M. canis*

Mycelial pads of about 24 mg. dry wt. in stationary culture. In all flasks there was 20 ml. of 0.1M phosphate buffer, except for inorganic phosphate determinations, when no buffer was used. Time, 4 hr. at 20°.

Inhibitor	Concn. (M)	pH	NH ₃ -N (μ g./mg. dry wt.)	Pentose (μ g./mg. dry wt.)	PO ₄ ³⁻ (μ g./mg. dry wt.)	10 ³ (log I ₀ /I) at 260 m μ ./ mg. dry wt.
Octanoic acid	2.0 × 10 ⁻²	5.4	—	—	—	—
	4.0 × 10 ⁻³	5.4	—	—	—	2.5
Control	—	5.4	—	—	—	1.25
CTAB	9.2 × 10 ⁻⁴	8.2	23.6	17.8	12.4	2.63
	4.6 × 10 ⁻⁵	8.2	16.4	5.6	6.6	0.87
Control	—	8.2	10.2	4.2	2.8	0.80
SDS	1.2 × 10 ⁻³	6.4	31.6	19.8	8.4	5.88
	5.8 × 10 ⁻⁵	6.4	11.8	5.6	3.2	0.56
Control	—	6.4	11.0	5.0	2.8	0.81
Phenoxetol	5.8 × 10 ⁻²	6.4	30.4	11.6	18.0	—
	5.8 × 10 ⁻³	6.4	11.2	6.0	4.6	—
Control	—	6.4	11.0	5.0	2.8	—

Table 4. *Effect of pH on the release of cell constituents from M. canis by octanoic acid and detergents*

Mycelial pads of approx. 24 mg. dry wt. were used in stationary culture. For all pH values 20 ml. of 0.1 M phosphate buffer was used, except pH 8.6 when 20 ml. of glycine-NaOH buffer was used. Figures in parentheses indicate release in absence of inhibitor. Temp., 20°.

pH	10 ² (log I ₀ /I)/mg. dry wt.			Pentose (μg./mg. dry wt.) Phenoxetol† (3.5 × 10 ⁻² M)
	Octanoic acid* (1.5 × 10 ⁻² M)	CTAB† (1.6 × 10 ⁻³ M)	SDS† (3.5 × 10 ⁻⁴ M)	
5.4	3.05 (0.565)	1.275 (0.500)	1.645 (0.500)	8.6 (2.9)
6.2	2.45 (0.610)	1.395 (0.695)	1.500 (0.695)	10.9 (3.4)
7.0	1.50 (0.640)	1.550 (0.640)	1.595 (0.640)	11.1 (3.2)
7.8	0.455 (0.430)	1.925 (0.580)	1.435 (0.580)	8.6 (3.0)
8.6	0.465 (0.560)	1.740 (0.785)	1.430 (0.785)	9.2 (3.0)

* Release after 3 hr.

† Release after 2 hr.

if these fungi were incubated in the presence of arsenite for longer periods. Such accumulation has been shown for strains of *Aspergillus niger* (Walker, Hall & Hopton, 1951) and *Penicillium chrysogenum* (Hockenhull, Wilkin & Winder, 1951), and can readily be demonstrated in replacement cultures of the dermatophytes used in this work. The conditions under which this occurs and the nature of the acids produced have been studied in detail. To be able to compare the amounts of α-keto acids formed in different groups of experimental flasks it was essential to determine the constancy of the acid formation related to fungal dry weight in each individual flask, under the same experimental conditions. In a typical experiment in which seven flasks containing 0.1 M phosphate buffer, pH 6.4, and 0.005 M potassium arsenite were incubated for 6 days at 20°, the mean value found was 2.34 μg. of total α-keto acids/ml./mg. dry wt. of mycelium (range 2.29–2.45 μg.). Thus acid production is relatively constant from flask to flask, although there were variations in thickness of mycelial pads and in consequence probably metabolic variability.

In subsequent experiments groups of five flasks were used, each containing one pad. At the required times 1 ml. samples were removed aseptically from each flask, and bulked for α-keto acid determination. Fungal dry weights were determined at the end of the experiment, after careful washing, and a mean value was determined. Accumulation of α-keto acids in the presence of arsenite proceeded over several days before reaching a steady value and was notably enhanced by the presence of glucose. Production was negligible in the absence of arsenite. These findings are illustrated in Fig. 6. The endogenous formation of α-keto acid in the absence of added substrate was always found. This value could be reduced by about 50 % by vigorous aeration for 12 hr. before the addition of the arsenite, but subsequent aeration for prolonged periods did not result in any further substantial reduction.

Effect of pH. Changes of pH in the range pH 5.4–8.0 were found to have little effect on the α-keto

Fig. 6. Accumulation of α-keto acids in replacement cultures of *M. audouinii*. Total keto acids expressed as μg. of pyruvic acid/ml. Phosphate buffer (0.1 M), pH 6.4, in all flasks. Other flask contents: curve 1, 0.1 M glucose, 0.005 M-KAsO₃; curve 2, 0.005 M-KAsO₃; curve 3, 0.1 M glucose. After incubation for 8 days the values for μg. of pyruvic acid/ml./mg. dry wt. were 6.9, 3.0, 0.1 for curves 1, 2 and 3 respectively.

acid formation. Hockenhull *et al.* (1951) found that *Penicillium chrysogenum* required arsenite for α-keto acid accumulation at pH 7.0 but not at pH 5.4, but in the present work it was found that arsenite was required at all the pH values tried for such accumulation to occur with *M. canis*.

Effect of concentration of arsenite and glucose. The effect of arsenite concentration on the total and endogenous accumulation of α-keto acids in the presence of 0.1 M glucose by *M. canis* and that of glucose concentration in the presence of 0.01 M arsenite is shown in Table 5.

Effect of other substrates. A variety of other carbohydrates and related substances were tested as possible substrates for α-keto acid formation, and the results are recorded in Table 6. It is of interest that the mycelium had been grown on

maltose-containing broth, but that maltose was considerably poorer than glucose as a source of α -keto acids. The latter was the only substance found which gave any appreciable yield of the α -keto acids.

Table 5. *Effect of glucose and arsenite concn. on α -keto acid formation by M. canis*

Basal medium-0.1M phosphate buffer, pH 6.4. Values are corrected for endogenous α -keto acid formation.

KAsO ₂ (M)	Glucose (M)	Total α -keto acids as μ g. of pyruvic acid/mg. dry wt.		
		5 days	9 days	12 days
0.01	0.2	57.6	77.6	—
0.01	0.1	48.8	70.0	—
0.01	0.05	45.0	67.6	—
0.01	0.01	20.0	22.6	—
0.01	0.004	15.0	15.0	—
0.01	0.001	0.0	0.0	—
0.02	0.1	—	—	89
0.012	0.1	—	—	87.5
0.008	0.1	—	—	80
0.004	0.1	—	—	42.3
0.001	0.1	—	—	0.7
Nil	0.1	—	—	0.0

Table 6. *Effect of substrate variation on α -keto acid formation by M. canis*

Basal medium 0.1M phosphate buffer, pH 6.4; 0.005M-KAsO₂. Incubated for 6 days. Values are corrected for endogenous α -keto acid formation.

Substrate (0.1M)	Total α -keto acids as μ g. of pyruvic acid/mg. dry wt.
Glucose	78.4
Fructose	20.0
Mannose	24.2
Maltose	11.2
Lactose	5.4
Sucrose	0.0
Galactose	2.8
Arabinose	0.0
Sodium lactate	0.0
Sodium succinate	0.0
Sodium citrate	0.0

Identification of α -keto acids. The α -keto acids formed by *M. canis* in replacement culture in the presence of glucose and arsenite have been separated and identified by paper chromatography of the 2:4-dinitrophenylhydrazones prepared as in the method of Friedemann & Haugen (1943). The solution of 2:4-dinitrophenylhydrazones obtained by this method was acidified and extracted with ethyl acetate, the resulting solution being used for chromatography. Preliminary chromatographic examination, running time 4 hr. on Whatman no. 4 paper, showed the presence of three spots (Table 7, spots 1-3), with a fourth spot (Table 7, spot 4) separating from spot 2 when chromatograms were developed for 24 hr. Separation of larger quantities of these derivatives for spectroscopic and chemical analysis was achieved with Whatman no. 1 paper, eluting with 10% Na₂CO₃, acidifying with cold concentrated H₂SO₄ and extracting into ethyl acetate. By comparison with authentic derivatives spots 2 and 3 have been identified as the isomeric 2:4-dinitrophenylhydrazones of pyruvic acid (Isherwood & Jones, 1955), and there is strong evidence that spot 1 is due to the corresponding derivative of α -oxoglutaric acid, insufficient material being obtained for chemical analysis. Spot 4 remains unidentified, only sufficient material being obtained for spectroscopic examination. The relevant data are summarized in Table 7.

R_p values relative to the 2:4-dinitrophenylhydrazone of pyruvic acid were determined, and spectroscopic data obtained, for the endogenous α -keto acids produced in the absence of glucose by *M. canis*, and identical values were obtained. In addition, similar determinations have been made with replacement cultures of *M. audouinii*, *T. schoenleini*, *T. rubrum* and *E. floccosum* in the presence of arsenite and in the presence and absence of glucose. Similar results were obtained for these organisms. It is concluded that all these fungi, in the presence of arsenite, accumu-

Table 7. *Properties of the 2:4-dinitrophenylhydrazones of α -keto acids produced by M. canis in replacement culture in the presence of arsenite*

Medium: 0.1M glucose; 0.1M phosphate buffer, pH 6.4; 0.01M-KAsO₂. *R* values are relative to the *trans* isomer of pyruvic 2:4-dinitrophenylhydrazone; A: *n*-butanol-ethanol-(NH₄)₂CO₃ buffer; B: *n*-butanol-NH₃ soln. $\lambda_{\max}$ values are recorded for (1) derivatives dissolved in 10% Na₂CO₃ and diluted with an equal vol. of 1.5N-NaOH; (2) derivative dissolved in ethyl acetate.

2:4-Dinitrophenylhydrazone	<i>R</i> values		M.p.	$\lambda_{\max}$		Chemical analysis
	A	B		(1)	(2)	
Spot 1	0.18	0.118	222°	418	361	—
α -Oxoglutaric acid	0.20	0.147	220°	418	361	—
Spot 2	1.0	1.0	216°	446	349	C, 40.5; H, 2.9; N, 20.9%
Spot 3	1.28	1.47	—	—	—	—
Pyruvic acid (2 spots)	1.0	1.0	216°	445	349	Calc. C, 40.3; H, 3.0; N, 20.8%
	1.28	1.44	—	—	—	—
Spot 4	1.03	—	—	365	332	—

late pyruvic and α -oxoglutaric acids together with very small quantities of an unidentified α -keto acid.

DISCUSSION

The results obtained with the enzyme inhibitors provide preliminary evidence that, in all the fungi studied, pyruvate and α -oxoglutarate oxidase systems are present, this being strengthened by the accumulation of these acids in the presence of arsenite. This accumulation is enhanced by the presence of glucose in the medium and to a lesser extent by certain other sugars, but materials such as lactate or succinate are without effect; this is contrary to the findings of Hockenhull *et al.* (1951) for *P. chrysogenum*, who showed the formation of considerable amounts of α -oxoglutarate from lactate. The inhibition by fluoroacetate indicates the probability of citrate synthesis, and inhibitions by malonate were of a low order, but, together with evidence obtained from mycelial dispersions (Chattaway *et al.* 1954), the probable presence of succinic dehydrogenase is indicated. By analogy with other organisms the results with azide and 2:4-dinitrophenol indicate that phosphorylation mechanisms are probably functioning in the respiratory process, and the fluoride and iodoacetate inhibitions indicate the presence of enzymes sensitive to these materials. The more precise evaluation of the sites of action of all these materials and the metabolic pathways concerned must await the preparation and purification of cell-free systems.

The effects of the fatty acids and detergents upon endogenous respiration and the release of cell constituents show a similarity both quantitatively and qualitatively. The increasing toxicity of fatty acids with increasing chain length up to C_{14} parallels their fungistatic and fungicidal activity for a number of dermatophytes and other fungi (Wyss *et al.* 1945), their antisporeulation activity on *Bacillus mycoides* (Hardwick, Guirard & Foster, 1951), their bactericidal effects on *Mycobacterium leprae* (Stanley, Coleman, Greer, Sacks & Adams, 1932) and their inhibition of fermentation and respiration of yeast (Samson, Katz & Harris, 1955). The toxic action of a homologous series of fatty acids and of detergents is usually explained in terms of either an action on the cell membrane, probably by combination with protein, or alternatively by inhibition of specific enzymes. It is possible that the effect is a combination of these two processes with increased cell permeability allowing penetration of the inhibitor to enzymes within the cell. The inhibition of respiration of the fungi was found to be a rapid process, being complete within 2 min. with both fatty acids and all three types of detergent. The release of cell constituents was not appreciable within this time, suggesting that the effect producing the respiratory

inhibition may not be associated with the permeability of the cell membrane but that the increased permeability may be a separate result of the inhibitor's activity, or an indirect result of the respiratory inhibition. The rate of release of cell constituents with these organisms is in contrast with those found by Salton (1951) for a number of bacteria, where release was virtually complete in 30 min. or less in the presence of bactericidal concentrations of CTAB. The observation that levels of fatty acids and detergents which do not inhibit respiration also do not lead to release of cell constituents does not necessarily mean that the processes are related, since the lower concentration may be equally incapable of producing either of two distinct effects. The effects produced by the fatty acids and detergents appear to be similar in many respects (e.g. pattern of respiratory inhibition and release of cell constituents) but the effect of pH on these processes reveals a dissimilarity. The effect of pH on respiration is a progressive inhibition with increased concentration of undissociated inhibitor for the fatty acids and cationic and anionic detergents, with little effect so far as phenoxetol is concerned. A similar result was found for the effect of octanoic acid on release of cell constituents, little release being evident at pH values where respiratory inhibition was low. However, variation of pH did not influence the release of constituents by the detergents, suggesting that the sites of action of the two groups of materials are not necessarily identical.

SUMMARY

1. The endogenous respiration of strains of *Microsporium audouinii*, *M. canis*, *Trichophyton schoenleinii*, *T. rubrum* and *Epidermophyton floccosum* is inhibited by arsenite, fluoride, iodoacetate, fluoroacetate and malonate. Azide and 2:4-dinitrophenol also inhibit respiration at higher concentrations but show a stimulatory effect at lower levels.

2. Saturated straight-chain fatty acids strongly inhibit endogenous respiration, the effect increasing with carbon-chain length up to C_{14} and being greatest at acid pH values. In the presence of optimum levels of fatty acid the inhibition is complete in 2 min., and partially reversible by washing the mycelium up to 30 min., but irreversible after this time.

3. Representative cationic, anionic and neutral detergents are also inhibitory and also show their highest activity in the presence of the undissociated form.

4. The release of amino acids, pentose, inorganic phosphate and material absorbing at 260 m μ . from mycelium exposed to fatty acids and detergents has been followed under varying conditions of time, concentration of inhibitor and pH.

5. Release of these materials from mycelium treated with fatty acid was greatest at acid pH values, but change of pH had no significant effect upon the release by detergents.

6. Incubation of mycelial pads of these fungi in the presence of arsenite leads to the accumulation of pyruvate and α -oxoglutarate together with traces of an unidentified α -keto acid. The yield of α -keto acids is increased by the presence of glucose in the medium.

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The Mechanism of the Reaction of Chymotrypsin with *p*-Nitrophenyl Acetate

BY H. GUTFREUND AND J. M. STURTEVANT*

Department of Colloid Science, University of Cambridge

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Hammond & Gutfreund (1955) have concluded from studies of the hydrolysis of acetyl-L-phenylalanine ethyl ester catalysed by chymotrypsin that at least two steps occur during this reaction: first, an initial rapid adsorption of the substrate, and, secondly, a reaction involving an imidazole group of the enzyme and the carbonyl group of the substrate. Kinetic evidence showed that this latter reaction is rate-determining. Schaffer, May & Summerson (1953) have shown that during the inactivation of chymotrypsin with alkyl phosphates the hydroxyl group of one serine of the enzyme is phosphorylated. It remained to be decided whether

the participation of the serine residue is restricted to the reactions involving irreversible inhibition of the enzyme or whether this group is also involved in a relatively rapid reaction step preceding or succeeding the rate-determining reaction with the imidazole group.

Hartley & Kilby (1952, 1954) found that chymotrypsin catalyses the hydrolysis of *p*-nitrophenyl acetate and that during the course of this reaction there occurs initially a rapid liberation of one mole of *p*-nitrophenol/mole of chymotrypsin followed by a slow hydrolysis. It has recently been shown by one of us (Gutfreund, 1955) that the initial rapid reaction can be followed by a stopped-flow method, and it was suggested that a detailed investigation of this

* Permanent address: Department of Chemistry, Yale University, New Haven, Conn., U.S.A.