The Oxidation of Fatty Acids by Mycelium of *Penicillium roqueforti*

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SUMMARY

Low concentrations of fatty acids with less than 14 carbon atoms were oxidized without a lag phase over a wide range of pH values by mycelium of *Penicillium roqueforti*. The effect of the fatty acids upon oxygen uptake by a given weight of mycelium, and the nature of the products of oxidation, were dependent upon the concentration and chain length of the fatty acid and the pH value of the system. The C₉–C₁₂ fatty acids which showed the greatest inhibitory effect were not oxidized to the corresponding methyl ketone with one less carbon atom in such high yields as the less toxic C₆–C₈ acids. The C₆–C₈ fatty acids markedly inhibited endogenous respiration at low pH values but this inhibition was reversed by increasing the pH value. The toxic effect associated with some fatty acids was less pronounced against mycelium which had been previously shaken over an extended period in phosphate buffer. It is suggested that the cellular regulation of fatty acid oxidation and methyl ketone formation involves deacylation of β -oxo acyl thiolester which provides an alternative means of recycling coenzyme A when oxidation of acetyl CoA is impaired.

INTRODUCTION

The ability of growing cultures of fungi to oxidize fatty acids of medium chain length to methyl ketones was first noted by Stärkle (1924), who showed that *Penicillium roqueforti* and two species of the genus *Aspergillus* produced methyl ketones when grown for several weeks on fatty acids of intermediate chain length. Moreover, when single fatty acids were used as substrates (Stärkle, 1924; Stokoe, 1928; Hammer & Bryant, 1937) these and certain other species of fungi only synthesized methyl ketones with one less carbon atom. It is not clear however whether the oxidation of fatty acids in the above investigations was the result of spore or mycelial activity. A decline in methyl ketone formation as spores germinate to mycelium has been reported (Thaler & Geist, 1939; Franke & Heinen, 1958). Gehrig & Knight (1963) concluded that the capacity of spores of *P. roqueforti* to form methyl ketones disappeared rapidly and progressively as the spores germinated. On the other hand Lawrence (1965*b*, 1966) found that the slow rate of formation of heptan-2-one from octanoic acid by resting spore suspensions of *P. roqueforti* was markedly increased by the addition of those amino acids and sugars that stimulate germination of the spores. Reports that low concentrations of fatty acids were oxidized by washed mycelium of various *Penicillium* species (Rolinson, 1954; Girolami & Knight, 1955; Vinze & Ghosh, 1962) would also appear to be in disagreement with the conclusions reached by Gehrig & Knight (1958, 1961, 1963) that hyphal cells of *P. roqueforti* were unable to oxidize fatty acids, regard-

less of the type of medium in which they were grown. It was not clear whether the uncertainty concerning the ability of fungal mycelium to oxidize fatty acids had arisen from the inhibitory effect of the fatty acids on respiration under certain conditions (Rolinson, 1954). The present work was undertaken in an attempt to clarify the situation.

METHODS

Gehrig & Knight (1963) grew mycelium of *Penicillium roqueforti* on a chemically defined medium developed by Meyers & Knight (1958), which contains both acetate and oleate initially at pH 4.0. Preliminary work in the present investigation, however, indicated that mycelium grown at pH 4.0 was about 5 times less active in oxidizing fatty acids than that grown at pH 6.5. As acetate at pH 4.0 has also been reported to be inhibitory towards respiration by yeasts (Neal, Weinstock & Lampen, 1965), a new chemically defined medium without fatty acids was developed.

Growth medium. After preliminary trials the following defined medium was used: ammonium nitrate, 2 g.; magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.15 g.; potassium chloride, 0.25 g.; ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 0.0125 g. These four components were dissolved in 400 ml. water and autoclaved for 20 min. at 12 pounds/sq.in. (118°). The brown precipitate which formed was allowed to settle and after 24 hr the supernatant fluid was decanted. A Seitz-filtered solution of 10 g. glucose, 0.6 g. potassium dihydrogen phosphate and 0.4 g. dipotassium hydrogen phosphate in 100 ml. water, supplemented with 1 ml. of a trace element solution (mg./l.) — zinc sulphate, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 180; copper nitrate, $\text{Cu}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 15; manganese nitrate, $\text{Mn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 80; ammonium molybdate, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 10 — was then added. This growth medium was initially at pH 6.5.

Growth of mycelium. Spores of *Penicillium roqueforti*, ATCC 6989 (American Type Culture Collection, Washington) were grown on slopes of Czapek-Dox agar (Oxoid) at 22°. After 5–6 days the spores were transferred to 100 ml. of the medium in 500 ml Erlenmeyer flasks. The suspensions were incubated overnight, without shaking, to allow spore germination and then shaken at 20° for 24 hr. The mycelium was harvested, washed and gently blended with a 'Polytron' homogenizer (Mobil Aaran A.G., Luzern, Switzerland) to free trapped ungerminated spores. The suspension was reinoculated in fresh medium and incubated for a further 24–48 hr. This resulted in a uniform and homogenous suspension, providing all organisms with comparable nutritional conditions and equal access to oxygen. The growth was harvested by filtration on a Buchner funnel, resuspended several times in about 2 l. distilled water, refiltered and finally suspended in phosphate buffer. The concentration of blended mycelium was adjusted to approximately 10 mg./ml. dry wt, the exact concentration being determined by dry weight measurement. The mycelium was finally examined microscopically to ensure that it was free of spores.

Oxygen uptake. Measurements were made manometrically at 27° in a standard Warburg apparatus (Braun, Model 'V8'). 0.2 ml. 20% (w/v) KOH was used in the inner compartment of the Warburg flasks unless otherwise stated.

Radioactive measurements. These were done by liquid scintillation techniques as previously reported (Lawrence, 1966).

Estimation of methyl ketones and methyl carbinols. Methyl ketones were estimated by pipetting 1 ml. of the contents of the Warburg flasks into 2 ml. of 2,4-DNP hydra-

zine (2 g./l. 2N-HCl) in a stoppered test-tube and the hydrazones estimated as detailed previously (Lawrence, 1965*a*). For the estimation of methyl carbinols the pH value of 10 ml. medium was adjusted until red to phenolphthalein and then extracted with diethyl ether. This extract was concentrated to about 0.5 ml. by carefully removing the ether with a stream of air. Samples (1 μ l.) of the concentrate were analysed in a gas chromatograph (Aerograph Hy-Fi, Model A-600-B, Wilkins Instrument and Research, Inc.). Columns normally used were either 20 % UCON 50 HB 2000 or 20 % Carbowax 1540 on Chromosorb W (Mesh 30–60), although initially 20 % diethylene glycol succinate (DEGS) on firebrick was used.

Identification of the peaks was accomplished by co-chromatography with authentic samples of heptan-2-ol and heptan-2-one. The retention times of heptan-2-ol relative to heptan-2-one were 1.45 on the DEGS column (115°), 1.78 with Carbowax (90°) and 2.02 with UCON (100°) columns. Quantitative measurements were made by measuring the peak areas on the chromatograms with a planimeter. Since the concentration of heptan-2-one was known accurately, that of heptan-2-ol could also be determined. Recovery trials of known amounts of heptan-2-one and heptan-2-ol carried out using the procedure above were quantitative ($\pm 5\%$) over a range of concentrations of the alcohol and ketone from 0.25–10 μ moles/ml.

Chemicals. Solutions of the salts of the fatty acids (Applied Science Laboratories, Inc., State College, Pa., U.S.A.) were prepared by adding potassium hydroxide and adjusting to the required concentration. [$1-^{14}\text{C}$] octanoic acid was obtained from The Radiochemical Centre, Amersham, Bucks. [$2-^{14}\text{C}$] octanoic acid was synthesized by a micro adaptation of the method of Reid & Ruhoff (1944). The purity of the acid was checked directly by co-chromatography with a pure sample of octanoic acid on a gas chromatograph using a column packing of 20 % (w/w) DEGS and 2 % H_3PO_4 on 60–80 mesh, acid-washed 'Celite 545' (Metcalf, 1960).

Heptan-2-ol was synthesized by reducing heptan-2-one with sodium and ethanol (Whitmore & Otterbacher, 1944). It was purified from unchanged heptan-2-one by shaking with 2,4-DNP hydrazine + HCl reagent and fractionally distilling under reduced pressure until the alcohol was completely free of ketone. The purity was checked by gas chromatography using a column of 20 % DEGS on chromosorb W (30–60 mesh).

RESULTS

Effect of growth medium on ability of mycelium to oxidize fatty acids

Lower yields of methyl ketones from fatty acids were obtained with mycelium grown in Casamino acids (3 g./500 ml.) than in the defined medium described above. The latter medium was therefore used in all subsequent work. The addition of C_{12} acid (2 μ moles/ml.) to the growth media increased mycelial growth but the mycelium subsequently showed no enhanced ability to oxidize C_{12} acid.

Effect of age of mycelium

The age at which the mycelium was harvested markedly affected the ability of the mycelium to oxidize fatty acids. Maximum oxygen uptake and methyl ketone formation in the presence of fatty acids was obtained with mycelium harvested after 48–60 hr. Oxidation of fatty acids was slower with mycelium grown for less than 30 hr, in agreement with similar observations by Vinze & Ghosh (1962).

Endogenous respiration of mycelium

In the course of most experiments a change in the rate of oxygen uptake could be clearly shown and unambiguously interpreted as a transition from endogenous respiration to the oxygen of the substrate. Moreover the basal rates of oxygen uptake before the addition of substrate and after its complete utilization were not significantly different. When allowance was made for the amounts of methyl ketone formed, almost quantitative balances ($\pm 10\%$) were usually obtained by assuming that endogenous respiration was proceeding simultaneously and subtracting its value from the oxygen uptake in the presence of substrate. It was therefore concluded that endogenous respiration was not suppressed by the addition of non-toxic concentrations of fatty acids, which supports similar findings by Blumenthal (1963) with other *Penicillium* spp. The validity of this assumption was supported by the finding that concentrations of 2,4-dinitrophenol up to $10^{-3}M$ had no effect upon oxygen uptake, indicating that no significant oxidative assimilation was taking place. (Throughout this communication the term 'toxic' acid is defined as one that suppresses the oxygen uptake of mycelium relative to endogenous. It does not necessarily mean complete inhibition. Similarly a 'non-toxic' acid is, in this context, one whose presence does not inhibit oxygen uptake by mycelium.)

Different batches of mycelium grown under apparently identical experimental conditions sometimes showed markedly different rates of endogenous respiration and also oxygen uptake in the presence of the same amount of substrate. The rates were, however, similar in duplicate experiments using the same batch of mycelium.

Metabolism of heptan-2-one

As long as octanoic acid was available, it appeared that any heptan-2-one formed was not oxidized by the mycelium. Thereafter, however, the ketone was metabolized at a rate which apparently depended upon the age of the mycelium. A typical experiment showed that mycelium harvested after 72 hr almost completely metabolised $0.4\ \mu\text{moles}$ of heptan-2-one in 4 hr, whereas the same batch of mycelium harvested and tested 24 hr earlier was only one tenth as active.

It was thought possible that heptan-2-one might be reduced to heptan-2-ol but it was found that less than 5% of the heptan-2-one was reduced to the methyl carbinol after 32 hr. On the other hand 90% of heptan-2-ol, at the same concentration, was oxidized to heptan-2-one in the same time.

The effect of pre-shaking mycelium in buffer for extended periods

Although endogenous respiration of mycelium was not usually markedly decreased, pre-shaking in buffer enabled the mycelium to oxidize fatty acid at concentrations which inhibited the oxygen uptake of freshly harvested mycelium (Table 1). The oxygen uptake by pre-shaken mycelium in the presence of non-toxic concentrations of octanoic acid ($1\ \mu\text{mole}$) was also higher than with fresh mycelium but the yield of heptan-2-one was lower.

Effect of phosphate on fatty acid oxidation

Both oxidation of substrate and endogenous respiration were inhibited in the absence of phosphate during incubation of mycelium with fatty acids. Optimum phosphate concentration for oxygen uptake in the presence of fatty acids was about

0.01 M with fresh mycelium and about 0.03 M for mycelium shaken in phosphate buffer for 36 hr. This difference may be associated with the observed decrease in toxicity of fatty acids towards preshaken mycelium.

Effect of metabolic carbon dioxide on fatty acid oxidation

Decreases of up to 50 % in the yield of methyl ketone were obtained by the removal of metabolic carbon dioxide as it was formed during incubation. The absorption of respiratory CO₂ by alkali was not therefore a satisfactory measure of fatty acid oxidation since such methods of determining respiration are valid only if the cells follow the same pathways in the presence or absence of CO₂ (Dixon, 1951).

Table 1. *The effect of preshaking the mycelium of Penicillium roqueforti on its ability to oxidize octanoic acid at pH 2.5*

Each Warburg flask contained 10 mg. dry wt equiv. mycelium, harvested after 65 hr, 50 μ moles phosphate, (pH 2.5) and distilled water to 3 ml. Time of incubation 120 min. The mycelium was either used immediately after harvesting or after being shaken in phosphate buffer, pH 6.0, for 15 or 20 hr.

	Oxygen Uptake (μ l.)			Heptan-2-one (μ moles)	
	Endogenous	1 μ mole acid	3 μ moles acid	1 μ mole acid	3 μ moles acid
Mycelium					
Fresh	155	285	22	0.45	0.32
Pre-shaken 15 hr	140	351	104	0.19	0.50
Pre-shaken 20 hr	145	350	150	0.14	0.70

Effect of concentration of fatty acid

At any one pH value and for a given weight of mycelium it was found that there was an optimum concentration of acid for maximum oxygen uptake and optimum conversion to methyl ketone. Thus at pH 6.5 and with 4 mg. dry wt mycelium maximum rates of oxidation were obtained with 3.5 μ moles octanoic acid (Fig. 1). Lower concentrations of this acid (1 μ mole) were completely oxidized with almost no trace of heptan-2-one formation and in general increased oxygen uptake was found to occur at the expense of methyl ketone formation. Increasing the octanoic acid concentration above 3.5 μ moles at this pH value decreased the rate of oxidation. A similar inhibitory effect was also obtained by using less mycelium, showing that the ratio of the number of hyphal cells to the fatty acid concentration determined the rate of oxidation. For the following studies, therefore, the weight of mycelium (10 mg.) used was that which would oxidize 3 μ moles of octanoic acid completely at pH values between 5.0 and 7.0 in 2–3 hr.

Effect of pH value

The relative amounts of methyl ketone and respiratory carbon dioxide formed during fatty acid metabolism were dependent upon the pH of the incubation medium. At pH 2.5 oxygen uptake was inhibited by 3 μ moles octanoic acid, whereas at pH 6.0 the same concentration of acid was rapidly oxidized with a lower yield of heptan-2-one. With increasing concentration of octanoic acid, the pH value at which oxygen uptake was a maximum also increased. Optimum pH values for the oxidation of 1, 3 and 25 μ moles of octanoic acid by 10 mg. dry wt mycelium were pH 5.0, 5.5 and 6.0, respectively.

Oxidation of fatty acids with an even number of carbon atoms

The rate of oxygen uptake by the mycelium increased immediately on the addition of C_4 – C_{12} fatty acids but formation of the corresponding methyl ketone was detected only after about 30 min. (Fig. 2). The rate and pattern of oxidation was markedly dependent upon the pH value (Figs. 3*a–d*). At pH 2.5 the rate of respiration by the mycelium in the presence of the C_6 – C_{12} fatty acids was less than its endogenous respiration, the maximum inhibitory effect being shown by decanoic acid (Fig. 3*a*); high yields

Fig. 1

Fig. 2

Fig. 1. The effect of concentration of octanoic acid on the rate of oxygen uptake by mycelium of *Penicillium roqueforti*. Reaction conditions: 4 mg. dry wt equiv. mycelium harvested after 48 hr, 50 μmoles phosphate buffer (pH 6.5).

Fig. 2. The rate of oxygen uptake (○—○) and methyl ketone formation (●—●) by mycelium of *Penicillium roqueforti* incubated with 3 μmoles octanoic acid. □—□, endogenous oxygen uptake. Reaction conditions: 10 mg. dry wt equiv. mycelium harvested after 48 hr, 50 μmoles phosphate buffer (pH 5.2).

of the corresponding methyl ketone with one less carbon atom were nevertheless obtained with the C_6 – C_8 acids. At pH 5.2 the pattern of oxygen uptake and methyl ketone formation was essentially the same as at pH 2.5, except that C_6 and C_8 no longer partially inhibited endogenous respiration and that C_{12} acid was most toxic (Fig. 3*b*).

At pH 6.8 rates of oxygen uptake in the presence of all fatty acids were greater than that of endogenous respiration, maximum uptakes being obtained with C_8 and C_{10} acids as substrates. The yield of pentan-2-one from hexanoic acid was considerably less than at pH 5.2 (Fig. 3*c*). At pH 8.0 oxygen uptake in excess of endogenous respiration and the yields of the corresponding methyl ketones were relatively low in the presence of all fatty acids. The rate of oxidation was greatest with C_{10} acid as substrate (Fig. 3*d*) and least with C_{14} fatty acid.

Shaking the mycelium in buffer for 48 hr considerably increased the subsequent rate at which the mycelium oxidized the C_6 – C_{12} fatty acids at all pH values between 2.5 and 6.8, although the relative yields of methyl ketone from each acid were not significantly different. The most toxic acid towards fresh mycelium at any one pH value was also the most inhibitory towards pre-shaken mycelium. The C_{14} – C_{18} fatty acids were not oxidized to any significant extent by either fresh or pre-starved mycelium at any pH.

Fig. 3. The influence of chain length of fatty acids on the oxygen uptake (unshaded bars) and formation of methyl ketones (shaded bars) by mycelium of *Penicillium roqueforti* incubated with even numbered fatty acids in the range C_4 – C_{18} at (a) pH 2.5, (b) pH 5.2, (c) pH 6.8, (d) pH 8.0. Reaction conditions: equiv. 10 mg. dry wt mycelium harvested after 66 hr, 3 μmoles of fatty acids, 50 μmoles phosphate and distilled water to 3 ml. Endogenous respiration of the different batches of mycelium is indicated by the dotted horizontal lines. Even carbon numbers refer to fatty acid substrates and odd carbon numbers to the corresponding methyl ketone with one less carbon atom.

Oxidation of fatty acids with an odd number of carbon atoms

The pattern of oxygen uptake and of methyl ketone formation was very similar for fatty acids containing both an even and an odd number of carbon atoms from C_3 to C_{11} (Fig. 4), suggesting that the oxidative pathway for all fatty acids is similar.

Mechanism of toxicity

The mechanism by which the C_8 – C_{12} fatty acids inhibited oxygen uptake was examined with particular reference to a possible relationship between this toxic effect and the production from each acid of only the corresponding methyl ketone with one less carbon atom. The addition of toxic concentrations of C_{10} or C_{11} acid markedly suppressed the oxidation of non-toxic concentrations of C_8 acid by freshly harvested mycelium, whether added at the same time or after oxidation of the non-toxic acid was proceeding normally (Fig. 5). In the latter case C_{11} acid was more immediately effective in inhibiting oxidation of the C_8 acid than the less toxic C_{10} acid but in both cases the formation of butan-2-one from the C_8 acid was markedly increased, showing that oxidation proceeded as far as the β -oxo acid (Table 2).

A considerably higher yield of methyl ketone, however, was obtained from a non-

Table 2. *The effect of toxic acids on the formation of methyl ketones from a non-toxic acid by mycelium of Penicillium roqueforti*

Each Warburg flask contained 10 mg. dry equiv. wt mycelium harvested after 60 hr, 50 μ moles phosphate buffer (pH 5.2) and 3 μ moles of each acid. Where indicated a second acid was added 30 min. later and incubation continued for a further 160 min.

Fatty acid	Methyl ketone (μ moles)		
	C_4	C_6	C_{10}
C_8	0.19	—	—
C_{10}	—	0.40	—
C_{11}	—	—	0.16
$C_8 + C_{10}$	0.39	0.37	—
C_8 followed by C_{10}	0.39	0.35	—
C_8 followed by C_{11}	0.90	—	0.20

Table 3. *The effect of pretreating mycelium of Penicillium roqueforti with different amounts of C_{10} acid for 10 or 40 min. on its ability to oxidize C_8 acid to heptan-2-one*

Each Warburg flask contained 10 mg. dry wt equiv. mycelium (pretreated with 2–10 μ moles C_{10} acid for 10 or 40 min. and then washed with phosphate buffer and distilled water) harvested after 48 hr, 2 μ moles C_8 acid, 50 μ moles phosphate buffer (pH 5.2). Period of incubation 120 min.

Pretreatment (min.)	C_{10} acid (μ moles)	C_8 acid (μ moles)	Heptan-2-one (μ moles)	Oxygen uptake (μ l.)
10	—	—	Nil	179
	—	2	0.27	414
	2	2	1.53	68
	4	2	0.50	33
	6	2	0.21	32
	8	2	0.10	30
	10	2	0.09	36
40	—	—	Nil	202
	2	—	Nil	10
	4	—	Nil	3
	—	2	0.80	438
	2	2	1.30	75
	4	2	0.24	15
	6	2	0.12	15

toxic acid by pretreatment of the mycelium with low concentrations of C_{10} acid for short periods. This pretreated mycelium was collected on a Millipore filter and washed with 0.05 M-phosphate buffer (pH 7.0) and distilled water. The mycelium was re-suspended in 3 ml. phosphate buffer (pH 5.2), 2 μ moles C_8 acid were then added and oxidation allowed to proceed for 2 hr. The use of mycelium pretreated with 2 μ moles C_{10} for 10 min. resulted in a 6-fold increase in yield of heptan-2-one (Table 3). Pre-incubation of the mycelium with 10 μ moles C_{10} acid for 10 min., however, almost completely inhibited its subsequent ability to oxidize octanoic acid to heptan-2-one.

Fig. 4

Fig. 5

Fig. 4. The oxygen uptake at pH 5.2 by mycelium of *Penicillium roqueforti* incubated with fatty acids with an odd number of carbon atoms in the range C_3 – C_{11} , compared with rates for C_8 and C_{10} fatty acids. Reaction conditions: 10 mg. dry wt equiv. mycelium, harvested after 66 hr., 3 μ moles of acid and 50 μ moles phosphate buffer. E=endogenous respiration (O—O).

Fig. 5. The inhibition of oxygen uptake by the addition of C_{10} or C_{11} fatty acids to mycelium of *Penicillium roqueforti* incubated with C_8 fatty acid. Reaction conditions: 10 mg dry wt equiv. mycelium harvested after 48 hr., 3 μ moles of each acid, 50 μ moles of phosphate buffer and distilled water to 3 ml. Oxygen uptake in the presence of C_8 acid (O—O), C_{10} acid (Δ — Δ), C_{11} acid ($\blacktriangle$ — $\blacktriangle$), C_8 and C_{10} acid added simultaneously ($\blacksquare$ — $\blacksquare$), C_8 acid followed after 30 min. by C_{10} acid ($\square$ — $\square$) or C_{11} acid ($\bullet$ — $\bullet$); endogenous respiration ($\times$ — $\times$).

The extent of damage to the enzyme systems of the mycelium was also dependent, to a lesser extent, on the length of time the cells were subjected to the toxic effect of the C_{10} acid. Preincubation of the mycelium with C_{10} acid for 40 min. resulted in a slightly lower rate of oxidation than mycelium pretreated with the same concentration of acid for 10 min. (Table 3.)

No $^{14}\text{CO}_2$ was detected when mycelium that had been pretreated with a toxic concentration of unlabelled C_{10} acid for 20 min. was subsequently incubated with $[2\text{-}^{14}\text{C}]$ octanoic acid for 2 hr, whereas about 7 % of the radioactivity was converted to $^{14}\text{CO}_2$ by untreated mycelium. The yield of $[1\text{-}^{14}\text{C}]$ heptan-2-one was, however, three times greater with mycelium that had been pre-treated with C_{10} acid.

Fig. 6. The annulment of the inhibitory effect of octanoic acid at low pH value on the oxygen uptake of mycelium of *Penicillium roqueforti* by an increase in pH value. Reaction conditions: 10mg. dry wt equiv. mycelium harvested after 48 hr, 50 μ moles phosphate buffer and distilled water to 3 ml. Oxygen uptake of mycelium pretreated at pH 2.5 for 1 hr with 4 μ moles (●—●), 6 μ moles (Δ — Δ) and 8 μ moles C_8 acid ($\blacktriangle$ — $\blacktriangle$) after addition of sufficient KOH to raise to pH 5.5; Oxygen uptake in presence of 4 μ moles C_8 acid at pH 2.5 ($\square$ — $\square$) and pH 5.5 (○—○) and of 6 μ moles C_8 acid at pH 2.5 ($\blacksquare$ — $\blacksquare$) also given; endogenous respiration ($\times$ — $\times$).

Reversibility of toxic effect of fatty acids at low pH values

Inhibition of respiration was almost instantaneous at pH 2.5 when toxic concentrations of C_6 – C_{12} fatty acids were added to freshly harvested mycelium. This effect could be annulled, after a lag phase, by increasing the pH value of the reaction mixture to 5.5 for C_6 and C_8 acids and to pH 6.8 for C_{10} and C_{12} acids. The length of the lag phase before oxidation proceeded depended on the concentration of fatty acid added (Fig. 6) and to a lesser extent upon the length of time the cell was in contact with the acid at pH 2.5.

Rates of formation of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ and $[2-^{14}\text{C}]$ octanoic acids

The rate of appearance of ^{14}C in respiratory CO_2 was 1.7–3.1 times faster from $[1-^{14}\text{C}]$ octanoic acid than from $[2-^{14}\text{C}]$ octanoic acid, over 21 % of the former and 7 % of the latter being oxidized to $^{14}\text{CO}_2$ in 150 min. at pH 6.0 (Table 4). No heptan-2-one was detected in the first 120 min. and only 0.05 μmoles after 150 min. A correction for the 0.05 μmoles $^{14}\text{CO}_2$ formed in the decarboxylation of β -oxo octanoic acid had therefore to be applied in the latter instance.

Table 4. *A comparison of the rate of appearance of ^{14}C in respiratory carbon dioxide during the oxidation of $[1-^{14}\text{C}]$ - and $[2-^{14}\text{C}]$ octanoic acids by mycelium of *Penicillium roqueforti*.*

Each flask contained equiv. 10 mg. dry wt mycelium harvested after 48 hr, 50 μmoles phosphate buffer (pH 6.0), 1.5 μmoles of either $[1-^{14}\text{C}]$ octanoic acid (43,500 counts/min.) or $[2-^{14}\text{C}]$ octanoic acid (44,750 counts/min.).

Time (min.)	$[1-^{14}\text{C}]$ octanoic acid: $^{14}\text{CO}_2$ formed		$[2-^{14}\text{C}]$ octanoic acid: $^{14}\text{CO}_2$ formed	
	Counts/ min.	Total activity (%)	Counts/ min.	Total activity (%)
30	1150	2.7	500	1.1
60	1955	4.5	1495	3.3
90	4380	10.1	2234	5.0
120	6365	14.7	2575	5.7
150	9430	21.7	3185	7.0

Effect of malonate

It was expected that the addition of malonate would inhibit the oxidation of fatty acids to carbon dioxide and thus possibly lead to an increase in methyl ketone formation. Various concentrations of malonate up to 0.1 M, however, did not inhibit respiration of mycelium either in the presence or absence of fatty acids between pH 2.5 and 6.0. Low concentrations of malonate at pH values less than 5.0 actually increased the rate of oxygen uptake, indicating ability of the hyphal cells to metabolize malonic acid. Malonate was, however, an effective inhibitor of the high succinic dehydrogenase activity in cell-free extracts obtained by disintegrating the mycelium in a Hughes press. Similar findings have been reported with preparations from higher plants (Young & Shannon, 1959; Hatch & Stumpf, 1962).

DISCUSSION

The rate of oxygen uptake by mycelium of *Penicillium roqueforti* increased immediately in the presence of low concentrations of C_4 – C_{12} fatty acids. The length of time for which the mycelium had been grown, however, greatly affected its ability to oxidize fatty acids to respiratory carbon dioxide. This is in agreement with the finding that the oxidation of acetate by the mycelium of *P. urticae* was markedly dependent upon the phase of growth at which it was harvested (Bu'Lock *et al.* 1965).

The rate of appearance of ^{14}C in respiratory carbon dioxide was 1.7–3.1 times greater for $[1-^{14}\text{C}]$ than for $[2-^{14}\text{C}]$ octanoic acid, indicating the existence of a β -oxidative

pathway in mycelial respiration (Katz & Chaikoff, 1955), although carbon dioxide production from both acids was slow. The thiolase and deacylase reactions involved in the metabolism of fatty acids to respiratory carbon dioxide and methyl ketones respectively have a common intermediate, namely the β -oxoacyl ester. By analogy with other regulatory mechanisms it would appear that this first divergent step in fatty acid oxidation is the most likely point of cellular regulation. It is possible that the lag phase which occurs in methyl ketone formation, and the observed slow formation of $^{14}\text{CO}_2$ from $[2-^{14}\text{C}]$ octanoate, may result from a product inhibition of thiolase activity and consequent induction of either deacylases or β -oxo decarboxylases. In this event deacylation of the β -oxo acyl ester and subsequent decarboxylation of the β -oxo acid to methyl ketone would provide an alternative source of coenzyme A for further fatty acid activation when the oxidation of acetyl CoA formed in the thiolase reaction is impaired. Such a mechanism would explain why only a single methyl ketone with one less carbon atom was detected in the oxidation of a single fatty acid as substrate and would support the hypothesis that secondary metabolites are formed as a result of induced enzyme mechanisms (Bu'Lock & Powell, 1965).

The lowest yields of methyl ketone were obtained with the C_9 – C_{12} fatty acids which were the most inhibitory to oxygen uptake by the mycelium. A possible explanation for this finding is that the β -oxo acyl esters of the C_9 – C_{12} acids are much less readily deacylated than those of the C_4 – C_8 acids and that less coenzyme A is therefore available in the system. This is supported by the marked decrease in formation of carbon dioxide and increase in production of heptan-2-one when octanoic acid was added to cells which had been shaken with low concentrations of the C_9 – C_{12} acids. It may also be significant that the C_{14} – C_{18} fatty acids were not oxidized by the mycelium to any significant extent at any pH and that no corresponding methyl ketones were detected from these acids.

The present investigation has emphasized the importance of pH value on the toxic effects of the homologous series of fatty acids upon the mycelium of *Penicillium roqueforti*. C_{10} and C_{12} acids were the most toxic acids at pH 2.5 and 5.2 respectively, whereas at pH 6.8 none of the acids from C_4 to C_{18} inhibited oxygen uptake. The most toxic acid at pH 8.0 was C_{14} acid but it is possible that at alkaline pH values the toxicity is a result of a surface effect as distinct from the toxicity observed at pH values less than 6.0. The inter-relationship between the toxic action of fatty acids and pH allows a ready explanation of the apparent contradiction between the results of Rolinson (1954), who reported C_8 and C_{10} fatty acids to be toxic to *P. chrysogenum* at pH 6.3, and those of Girolami & Knight (1955), who found that at pH 7.3 these two acids stimulated oxygen uptake by *P. roqueforti* to a greater extent than other acids. Similarly in this investigation the C_9 – C_{12} acids more actively inhibited the respiration of mycelium than other fatty acids at pH values below 6.0 but were the most rapidly oxidized at pH 8.0. Conversely the C_3 – C_5 acids were most readily oxidized at pH 2.5 but least rapidly at pH 8.0.

The present work supports the hypothesis that the primary inhibitory effect of toxic compounds on fungal cells occurs after they have penetrated the cell wall (Cochrane, 1958). Cells pretreated with a toxic concentration of fatty acids and then thoroughly washed with buffer were capable of oxidizing non-toxic concentrations of fatty acids to the corresponding methyl ketone but not to carbon dioxide. Similarly the C_9 – C_{12} fatty acids inhibited endogenous respiration of the mycelium markedly at pH values below

6·0 but were nevertheless oxidized to methyl ketones in high yield. This suggests that the toxic effect was not due to adsorption of the fatty acids on the cell wall but that the acids actually entered the hyphal cell. The observed variation with pH of the inhibitory effect of fatty acids on oxygen uptake by mycelium is most easily explained by assuming that only undissociated molecules of fatty acids are transported into the cell and that, at a certain concentration, they or their oxidation products are toxic.

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