Humason, G. L., Lushbaugh, C. C., *Stain Technol.* **35**, 209 (1960).
Lansing, A. I., Rosenthal, T. B., Alex, M., Dempsey, E. Q., *Anat. Rec.* **114**, 555 (1952).
McClain, P. E., *Nature (London)* **221**, 181 (1969).
McClain, P. E., Mullins, A. M., Hansard, S. L., Fox, H. D., Boulware, R. F., *J. Anim. Sci.* **24**, 1107 (1965).
Miller, E. J., Martin, G. R., Piez, K. A., *Biochem. Biophys. Res. Commun.* **17**, 248 (1964).
Neuman, R. E., Logan, M. A., *J. Biol. Chem.* **186**, 549 (1950).
Partridge, S. M., *Advan. Protein Chem.* **17**, 227 (1962).
Partridge, S. M., "Elastin," University of Wisconsin Press, Madison, Wis., 1966, pp 327-339.
Partridge, S. M., Davis, H. R., Adair, G. S., *Biochem. J.* **61**, 11 (1955).
Partridge, S. M., Elsden, D. F., Thomas, J., *Nature (London)* **197**, 297 (1963).
Partridge, S. M., Elsden, D. F., Thomas, J., *Nature (London)* **209**, 399 (1966).
Romeis, B., "Animal tissue techniques," W. H. Freeman and Co., San Francisco, Calif., 1948, p 169.
Wilson, G. D., Bray, R. W., Phillips, P. H., *J. Anim. Sci.* **13**, 826 (1954).
Woessner, J. F., *Arch. Biochem. Biophys.* **93**, 440 (1961).

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Oxidation of Sodium [U-^{14}C]Palmitate into Carbonyl Compounds by *Penicillium roqueforti* Spores

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Spore suspensions of *P. roqueforti* metabolized sodium palmitate into carbon dioxide and a variety of carbonyl compounds, including a homologous series of methyl ketones. Both D-glucose and L-proline suppressed the catabolism of palmitate to CO_2 but stimulated the rate of formation of carbonyl compounds, including methyl ketones. Spore concentrations of 6.3×10^8 spores/ml produced the optimum yield of (32.4%) carbonyl compounds from the incubation of 5 m*M* of palmitate in the presence of 20 m*M* of D-glucose under optimum conditions of pH and temperature; *i.e.*, pH 6.5 (0.1 *M* phosphate buffer) and 30°. Analyses of carbonyl compounds formed from [U-^{14}C]palmitate revealed that a homologous series of labeled methyl ketones, C3 to C15 inclusive, was produced. Pentadecanone contained the highest radioactivity, followed by tridecanone and undecanone, with the lower methyl ketones containing varying amounts of radioactivity. Appreciable amounts of carbonyl compounds other than methyl ketones (*i.e.*, labeled, saturated, and unsaturated aldehydes) were also produced from [^{14}C]palmitic acid.

Growing cultures of *Penicillium roqueforti* oxidize fatty acids into their corresponding methyl ketones with one less carbon atom (Franke and Heinen, 1958; Hammer and Bryant, 1937; Lawrence, 1966; Stärkle, 1924; Stokoe, 1928). Gehrig and Knight (1958) reported that only the spores of *P. roqueforti* oxidize fatty acids; however, Rolinson (1954), Vinze and Ghosh (1962), and Lawrence and Hawke (1968) demonstrated the oxidation of fatty acids into methyl ketones by the mycelium of *P. roqueforti*.

The addition of simple sugars (including D-glucose) and amino acids (including L-proline) stimulates oxygen uptake and formation of 2-heptanone from octanoic acid by the spores of *P. roqueforti* (Lawrence, 1965a). The formation of methyl ketones increased when metabolic carbon dioxide was retained in the incubation medium, but nonaeration of the incubation system decreased fatty acid oxidation (Lawrence, 1966). Enrichment of air with carbon dioxide increased the growth of *P. roqueforti*; however, these increases showed variation with both temperature and strain of the organism (Golding, 1940).

In contrast to mammalian, plant, and bacterial systems, a fatty acid oxidation system has not been isolated from fungi because of difficulty in disrupting the mycelia and obtaining cell-free active enzymes. However, a number of investigators showed that *P. roqueforti* oxidizes fatty acids *via* β-oxidation (Gehrig and Knight, 1963; Hammer and Bryant, 1937; Katz and Chaikoff, 1955; Lawrence, 1966; Lawrence and Hawke, 1968; Stärkle, 1924). These investigators agreed that both the β-oxidation reaction and ketone formation proceed simultaneously. The β-oxoacyl-CoA formed by β-oxidation reaction is deacylated and subsequently decarboxylated to a methyl ketone. The acetyl-CoA formed by complete β-oxidation is further oxidized *via* the tricarboxylic acid cycle to CO_2 and H_2O.

Fatty acids are actually toxic to *P. roqueforti* and the degree of toxicity depends upon chain length, concentration of acids, and the pH of the incubation medium (Franke *et al.*, 1962; Lawrence, 1966; Lawrence and Hawke, 1968). Pressman and Lardy (1956) reported that the saturated fatty acids uncouple phosphorylation and myristic acid exhibited the greatest effect, which decreased progressively from myristic acid as the fatty acid carbon chain was lengthened or shortened. Thus, it has been suggested that conversion of fatty acids to methyl ketones may be a detoxifying mechanism.

P. roqueforti is important in the manufacture of Blue and Roquefort type cheeses. Methyl ketones are the principal compounds responsible for the unique flavor of Blue cheese (Anderson, 1966; Day, 1965; Dartey and Kinsella, 1971; Hawke, 1966; Patton, 1950). Metabolism of milk fat by *P. roqueforti* and the production of methyl ketones is important in the development of Blue cheese flavor. Although palmitic acid is the major fatty acid of milk fat, little is known of its metabolism by *P. roqueforti* during cheese ripening. Palmitic acid may serve as a precursor for some of the methyl ketones found in the cheese. While the major methyl ketones are presumably derived directly from the corresponding fatty acids by oxidation, the concentrations of C7 and C9 methyl ketones exceed the molar ratios of the corresponding C8 and C10 fatty acids in milk fat, indicating their possible derivation from longer chain fatty acids.

In this study the factors affecting the metabolism of palmitic acid by the spores of *P. roqueforti* were deter-

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mined and the ability of palmitic acid to serve as a precursor of the methyl ketones commonly found in Blue cheese was examined.

MATERIALS AND METHODS

Solvents and Chemicals. Practical grade *n*-hexane (Eastman Kodak Co., Rochester, N. Y.) and analytical grade chloroform (Mallinckrodt Chemical Works, St. Louis, Mo.) were purified as reported previously (Dartey and Kinsella, 1971). Nitromethane and benzene, both spectral grade (Eastman), polyethylene glycol 400 (Fisher Scientific Co., Rochester, N. Y.), analytical grade acetone (Mallinckrodt), sodium palmitate (Pfaltz and Bauer, Inc., Flushing, N. Y.), and [U-^{14}C]palmitic acid (radiochemical purity 99%, New England Nuclear, Boston, Mass.) were used as purchased. 2,4-Dinitrophenylhydrazine (DNPH) (Eastman), hydrochloric acid, and monobasic and dibasic potassium phosphate (Baker Chemical Co., St. Louis, Mo.) were also used as purchased. The scintillation fluid contained 10 g of 2,5-diphenyloxazole (PPO) and 125 mg of *p*-bis-2-(5-phenyloxazolyl) benzene (POPOP) (Amersham/Searle, Chicago, Ill.) dissolved in 2.37 l. of toluene (Mallinckrodt).

Adsorbents. Celite (Johns-Manville analytical filter-aid) and Sea Sorb 43 (Fisher Scientific Co.) were treated as reported previously (Dartey and Kinsella, 1971). Analytical grade magnesium oxide (Mallinckrodt) and Kieselguhr G (E. Merck, Darmstadt, Brinkmann Instruments, Inc., Westbury, N. Y.) were used as purchased.

Stock suspensions of sodium palmitate (100 and 200 m*M*) were prepared in sterilized distilled water containing 5% ethanol and stirred vigorously at 50° to obtain a uniform suspension.

Spores of *P. roqueforti* (Midwest Blue Mold Company, Stillwater, Minn.) were grown at 25° by surface culture method on a medium composed of 5% malt extract broth, 2% D-glucose, and 0.5% yeast extract (Jackson and Hussong, 1958). After 6 days of undisturbed surface growth, the spent liquid medium was drained carefully from the incubation flask. The spores were suspended in sterilized distilled water and shaken vigorously to separate the spores, and the mixture was filtered through two layers of cheese cloth into a sterile Buchner funnel with Whatman No. 1 filter paper. The spores retained on the filter paper were washed several times with sterilized distilled water. Portions of the washed spores were stored at −15° in sterilized 0.1 *M* phosphate buffer solutions (pH 6.0, 6.5, and 7.0) and used within 8 weeks. Spore concentration (*i.e.*, number of spores/ml of solution) was determined by the method of Lawrence (1967).

Preparation of Incubation System. To determine factors influencing palmitate metabolism by spores of *P. roqueforti*, the effects of pH, temperature, metabolic CO_2, D-glucose, L-proline, and the relative concentrations of both spores and substrate on metabolism and the nature of products were studied. Appropriate quantities of [U-^{14}C]palmitic acid were used and the experimental details are included with the appropriate tables. The incubation experiments were performed in 125-ml culture flasks with center wells and air-tight Teflon screw caps. The center well contained 1.0 ml of 20% KOH solution, except in those experiments where the effect of metabolic CO_2 was examined.

Incubations were carried out in a constant temperature reciprocating water bath. Duplicate incubations were performed. Experiments were terminated by the addition of DNPH reagent (2 g of DNPH/l. of 2 *N* HCl; Lawrence, 1965b) to form DNPH derivatives of carbonyl compounds. The tightly closed flask was swirled gently at 10-min intervals for 1 hr. After 3 hr, duplicate samples (0.1 ml) of the KOH solutions containing radioactive CO_2 were transferred to scintillation vials and 4 ml of alcohol was added. Ten milliliters of scintillation fluid was added and the mixture was shaken vigorously. The radioactivity in the $^{14}CO_2$ (KOH solution) was determined in a Packard Tri-Carb liquid scintillation spectrometer (Model 314E). Quenching by the alcohol and the KOH solution was corrected in calculation of the results.

The contents of the incubation flask (incubation medium) were transferred into a 250-ml separatory funnel and then extracted with 120 ml of *n*-hexane. The concentration of the total carbonyl DNPH derivatives in the *n*-hexane was computed from the absorbance measurements using the molar extinction coefficient, E = 22,500 (Schwartz *et al.*, 1963).

The neutral lipids were separated from the total carbonyl DNPH derivatives by column chromatography (Schwartz *et al.*, 1963). The chloroform–nitromethane eluent was evaporated and the residue containing the monocarbonyl DNPH derivatives was dissolved in 1.0 ml of $CHCl_3$ and 4 ml of *n*-hexane. Radioactivity was determined on aliquots of the sample. The concentration of the total monocarbonyl DNP fraction was determined by the method of Schwartz *et al.* (1963).

The methyl ketone DNP class was separated from the total monocarbonyl DNPH mixture containing methyl ketones, saturated and unsaturated aldehydes by a modified thin-layer chromatographic (tlc) method (Schwartz *et al.*, 1968). Five tlc plates of 0.25-mm coating thickness were prepared from a uniform slurry of 6 g of MgO, 12 g of Celite, and 70 ml of distilled water. The plates were dried in air for about 1 hr, preceding heat treatment at 110° for 5 min, and cooled at room temperature for about 1 hr before use. The monocarbonyl DNP fraction was concentrated in 0.5 ml of $CHCl_3$. One-hundred-microliter portions of this sample were spotted on two of the MgO–Celite plates. The plates were developed in a tlc tank containing 95 ml of *n*-hexane and 25 ml of $CHCl_3$. A complete separation of the methyl ketone DNP from the saturated aldehyde DNP was achieved by developing the chromatograms three times in the above solvent system. The first time the solvent front migrated 5 cm from the origin and then 10 and 15 cm, respectively, for the second and third runs. Standard methyl ketone and monocarbonyl DNP compounds were used for identification of the separated bands (Figure 1).

The methyl ketone DNPH band from the tlc plates was scraped into a chromatographic tube with a filter disk. The methyl ketone fraction was eluted into a 200-ml round-bottomed flask with $CHCl_3$. The volume of the eluent was measured and the absorbance was determined at 363 nm wavelength. The concentration of the total methyl ketone DNP mixture was calculated as above. The sample was quantitatively transferred into a simple vial and the solvent was evaporated. The methyl ketone DNP residue was redissolved in 0.2 ml of benzene and the radioactivity was determined from aliquots of this sample.

Reverse-phase tlc was used to separate the individual methyl ketone derivatives. The method employed was a modification of the methods of Urbach (1963) and Schwartz *et al.* (1968). Five tlc plates of 0.25-mm coating thickness were prepared from a uniform slurry of 30 g of Kieselguhr G and 65 ml of distilled H_2O. The plates were dried in air, heated in an oven at 110° for 30 min, and then cooled to room temperature (25°) in a desiccating tlc plate rack. Each plate was impregnated with a 10% solution of 2-phenoxyethanol in acetone and dried in air at room temperature. An aliquot of the methyl ketone DNP mixture in benzene was spotted on the plate, along with a standard mixture of homologous methyl ketone DNPH derivatives (C3 to C15) for identification of the methyl ketones in the sample. The plate was developed three times in *n*-hexane saturated with polyethylene glycol 400. Good separations of the individual methyl ketone DNPH derivatives were obtained (Figure 2). A spot on the tlc plate

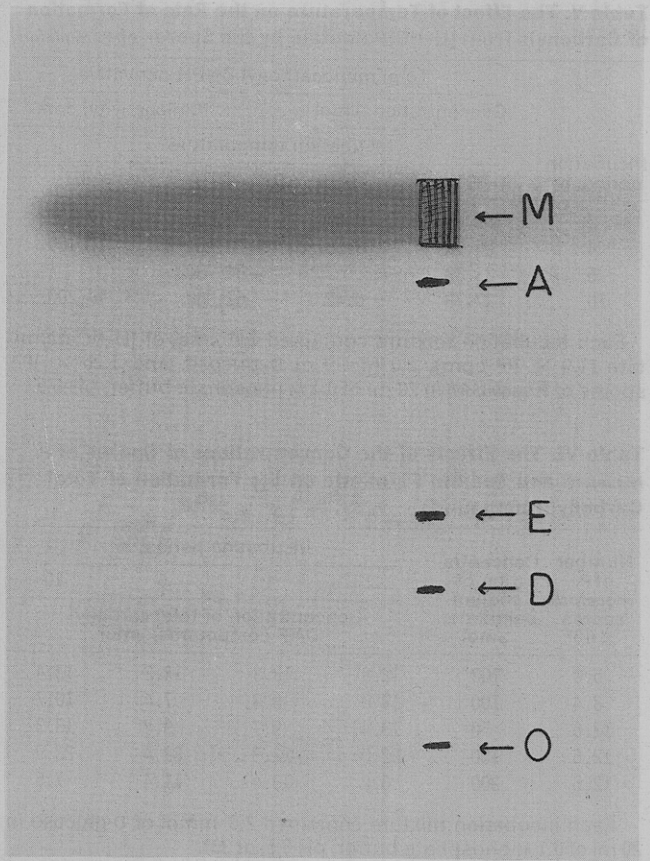

Figure 1. Thin-layer chromatogram showing the separation of monocarbonyl DNPH derivatives. M through D represent DNPH derivatives of methyl ketones, alkanals, alkenals, and alkadienals which had R_f values of 0.84, 0.74, and 0.2, respectively.

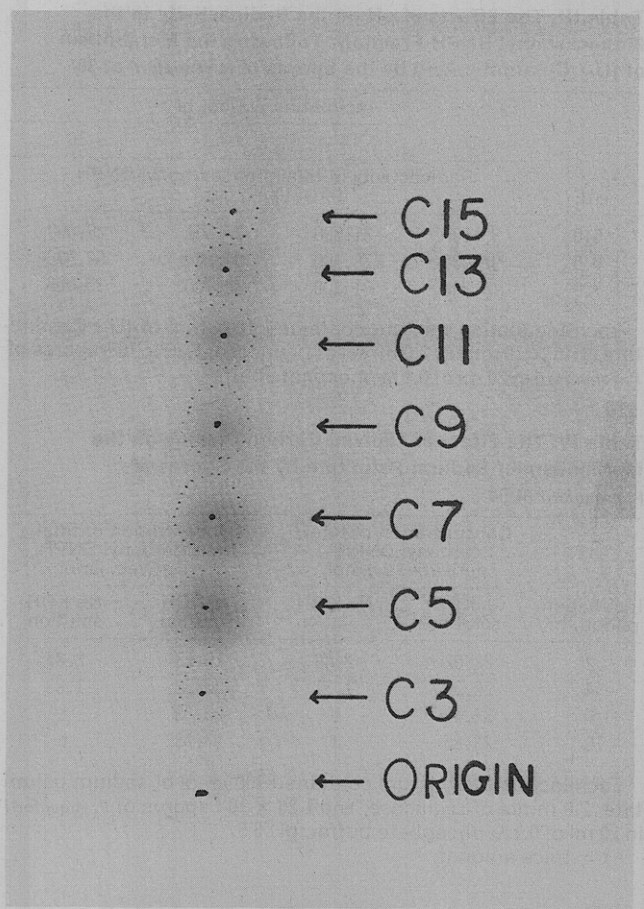

Figure 2. Thin-layer chromatogram showing the separation of homologous methyl ketone DNPH derivatives by reverse phase chromatography. The individual ketones are designated by their aggregate carbon number and have R_f values of 0.11, 0.27, 0.43, 0.55, 0.71, 0.80, and 0.89 for C3 through C15, respectively.

Table I. The Effects of D-Glucose and L-Proline on the Rate of Evolution of $^{14}CO_2$ from the Metabolism of [U-^{14}C]Palmitic Acid by the Spores of *P. roqueforti*

Substrate composition	Incubation period, hr: 10	20	30	45
	Radioactivity in $^{14}CO_2$, cpm			
Control[a]	598	1377	7923	27,990
Control + D-glucose (400 μmol)	2354		2704	2686
Control + L-proline (400 μmol)	765	1756	5385	7610
Control + D-glucose (200 μmol) + L-proline (200 μmol)	386	462	478	729

[a] The control contained 500 μmol of [U-^{14}C]palmitate (1.10×10^6 cpm), 5×10^9 spores of *P. roqueforti* in 20 ml of 0.1 *M* phosphate buffer, pH 6.5, at 30°.

Table II. The Effects of Spore Concentration, Temperature, and pH on the Rate of Degradation of [U-^{14}C]Palmitic Acid by *P. roqueforti* into $^{14}CO_2$

Spore number × 10^9	pH	Temp, °C	Incubation period, hr: 2	4	6	10
			Radioactivity in evolved $^{14}CO_2$, cpm			
5.0	6.5	30	270	240	280	350
8.4	6.5	30	460	330	630	530
12.6	6.5	30	1510	2040	2250	1700
12.6	6.5	37	410	470	600	490
12.6	7.0	30	440	1650	1610	1490
12.6	6.0	30	180	370	280	330

Each incubation mixture contained 100 μmol of [U-^{14}C]palmitate (1.10×10^6 cpm), 2.0 mmol of D-glucose, in 20 ml 0.1 *M* phosphate buffer.

corresponding to each individual standard methyl ketone DNP was scraped into a scintillation vial. Ten milliliters of scintillation fluid was added and the radioactivity in the samples determined. Quenching was determined and used in calculating radioactivity in the individual methyl ketone DNPH derivatives.

RESULTS

D-Glucose and L-proline influenced the rate of oxidation of [U-^{14}C]palmitic acid into $^{14}CO_2$ by the spores of *P. roqueforti* (Table I). When palmitate was the sole source of carbon in the incubation medium, a relatively greater amount of $^{14}CO_2$ was liberated than when either D-glucose or L-proline or both were present. Apparently, D-glucose was a better source of metabolic energy to the mold than L-proline. The presence of both D-glucose and L-proline produced the maximum effect in reducing the catabolism of palmitic acid.

The pH and temperature affected the rate of oxidation of [U-^{14}C]palmitic acid into $^{14}CO_2$ by the spores of *P. roqueforti* (Table II). Spore concentrations of approximately 6.3×10^8/ml at pH 6.5 and 30° caused the maximum production of $^{14}CO_2$. The same spore concentration was less active at pH 6.0 and 7.0 and 30°.

The production of carbonyl compounds was affected by pH of incubation, with maximum yield occurring at pH 6.5; *i.e.*, 25 to 30 μmol of carbonyls was produced from sodium palmitate (5.0 m*M*) by 12.6×10^9 spores after 10-hr incubation at 30°. Approximately 90% of these were monocarbonyls.

Table III. The Effects of pH on the Radioactivity in the Monocarbonyl DNPH Fractions Following the Metabolism of [U-^{14}C]Palmitic Acid by the Spores of *P. roqueforti* at 30°

	Incubation period, hr			
	2	4	6	10
pH	Radioactivity in total monocarbonyl DNPH derivatives, cpm			
6.0	33,300	31,500	37,600	28,000
6.5	92,400	78,400	64,200	62,300
7.0	4,800	31,100	32,700	35,600

Each incubation mixture contained 100 μmol of [U-^{14}C]palmitate (1.10×10^6 cpm), 2.0 mmol of D-glucose, 1.26×10^{10} spores of *P. roqueforti* in 20 ml of 0.1 *M* phosphate buffer.

Table IV. The Effect of Evolved Carbon Dioxide on the Metabolism of Sodium Palmitate by the Spores of *P. roqueforti* at 30°

	Concentration of total carbonyl DNPH derivatives, μmol		Concentration of total monocarbonyl DNPH derivatives, μmol	
Incubation period, hr	+ KOH solution	No KOH solution	+ KOH solution	No KOH solution
2	22.02	2.48	19.13	2.85
4	22.26	1.11	20.53	1.83
6	32.42	t[a]	22.33	t
10	23.85	t	17.73	t

Each incubation mixture contained 100 μmol of sodium palmitate, 2.0 mmol of D-glucose, and 1.26×10^{10} spores of *P. roqueforti* in 20 ml of 0.1 *M* phosphate buffer, pH 6.5.

[a] t = trace amount.

Table V. The Effect of Temperature on the Rate of Formation of Carbonyls from [U-^{14}C]Palmitate by the Spores of *P. roqueforti*

	Total monocarbonyl DNPH derivative			
	Concentration, μmol		Radioactivity, cpm	
	Incubation temperature			
Incubation period, hr	30°	37°	30°	37°
2	19.13	0.83	92,400	17,400
4	20.53	0.59	78,420	17,100
6	23.33		64,700	
10	17.73	0.42	62,300	17,200

Each incubation mixture contained 100 μmol of [U-^{14}C]palmitate (1.0×10^8 cpm), 2.0 mmol of D-glucose, and 1.26×10^{10} spores of *P. roqueforti* in 20 ml of 0.1 *M* phosphate buffer, pH 6.5.

Table VI. The Effects of the Concentrations of Spores of *P. roqueforti* and Sodium Palmitate on the Formation of Total Carbonyl Compounds

Number of *P. roqueforti* spores $\times 10^9$	Concentration of sodium palmitate, μmol	Incubation period, hr			
		2	4	6	10
		Concentration of total carbonyl DNP compounds, μmol			
5.0	100	12.8	13.8	14.2	13.4
8.4	100	13.0	8.3	7.4	10.7
12.6	50	13.4	9.7	9.8	13.3
12.6	100	22.0	22.3	32.4	23.9
12.6	200	6.2	13.4	11.4	7.5

Each incubation mixture contained 2.0 mmol of D-glucose in 20 ml of 0.1 *M* phosphate buffer, pH 6.5, at 30°.

This pattern was confirmed when the metabolism of radioactive fatty acid was studied under identical conditions (Table III). Maximum yield of radioactive monocarbonyls occurred at pH 6.5. However, radioactivity decreased with incubation period at pH 6.5, in contrast to the values obtained at pH 6.0 and 7.0, respectively.

Accumulating metabolic CO_2 inhibited the formation of carbonyl compounds (Table IV). The concentration of carbonyl compounds decreased with incubation time. Buffering was adequate in these incubations as the pH remained constant.

Incubation of spores at 37° resulted in much lower concentrations of carbonyl compounds, including methyl ketones in the media (Table V). Less marked differences, however, were noted in the radioactivity associated with the monocarbonyls produced at 30 and 37°.

A spore concentration of 12.6×10^9 spores and 5.0 m*M* of palmitate produced the highest yields of carbonyl compounds (Table VI).

The spores of *P. roqueforti* formed carbonyl compounds other than methyl ketones during the metabolism of the palmitate because significant differences were observed in the concentrations of the total carbonyl, monocarbonyl, and methyl ketone DNPH derivatives from each experiment (Table VII). The nature of these unidentified carbonyl compounds is being investigated. The concentrations of the total carbonyl, mononocarbonyl, and methyl ketone DNPH derivatives generally increased with time. The presence of 20 m*M* of D-glucose in the incubation mixture produced the highest yield of methyl ketones. In the presence of both D-glucose and L-proline, the concentrations of these products decreased with time, presumably because of further oxidation.

Radioactivity in the monocarbonyl and methyl ketone DNP fractions increased with time in the absence of either D-glucose or L-proline when [U-^{14}C]palmitic acid was the source of metabolizable substrate (Table VIII). D-Glucose (20 m*M*) increased the radioactivity in both fractions up to 30 hr of incubation. L-Proline produced more marked effects. In the presence of both D-glucose and L-proline, the radioactivity in the methyl ketone DNP fractions decreased with duration of incubation. The monocarbonyl DNP fractions showed relatively constant radioactivity after 10 hr of the reaction. Marked differences between the quantity of radioactivity in the monocarbonyls and the corresponding methyl ketones occurred in all experiments, as observed for the mass data in Table VII.

Comparison of data in Tables I and VII indicates that CO_2 evolution from the metabolism of palmitate by the spores of *P. roqueforti* is not a valid index for the extent of carbonyl formation.

Distribution of radioactivity in the individual methyl ketone DNPH derivatives formed from [U-^{14}C]palmitate is presented in Table IX. In most cases the homologous series of methyl ketones, 2-propanone through 2-pentadecanone (C3–C15), was isolated from the incubation medium. When the palmitate was the sole source of carbon, 2-propanone was not detected in the mixture. In the presence of D-glucose, radioactive methyl ketones (*i.e.*, C3, C5, C7, C9, C11, C13, and C15 inclusive) were identified. Propanone was not detected at 30 and 45 hr of incubation. All of the above methyl ketones were detected when L-proline or a mixture of D-glucose and L-proline was present in the incubation media. The distributions of radioactivity in the individual methyl ketones showed definite patterns; *i.e.*, the C15 methyl ketones possessed the greatest radioactivity in all samples, followed by C13 and C11 in that order. The radioactivity patterns in the other methyl ketones were irregular.

DISCUSSION

These results reveal that spore suspensions of *P. roqueforti* convert palmitate into carbonyl compounds, including methyl ketones. The rate of formation of carbonyls was influenced by experimental conditions such as D-glucose, L-proline, carbon dioxide, concentrations of both spores of *P. roqueforti* and palmitate, pH of medium, and temperature of incubation reaction.

Table VII. The Concentrations of Total Carbonyl, Monocarbonyl, and Methyl Ketone DNPH Derivatives Formed from the Metabolism of Palmitate by the Spores of *P. roqueforti*

Substrate composition	Incubation period, hr: 10			20			30			45		
	Concentrations of DNPH derivatives, μmol											
	Total carbonyls	Monocarbonyls	Methyl ketones	Total carbonyls	Monocarbonyls	Methyl ketones	Total carbonyls	Monocarbonlys	Methyl ketones	Total carbonyls	Monocarbonyls	Methyl ketones
Control	26.56	13.42	6.32	30.15	20.75	8.90	40.14	34.98	11.20	52.71	38.87	10.69
Control + 400 μmol of D-glucose	74.29	45.23	31.17	86.04	56.80	38.17	96.98	69.10	19.42	104.40	78.90	56.98
Control + 400 μmol of L-proline	74.40	38.62	28.67	70.71	40.20	36.66	75.79	53.90	40.67	69.91	54.40	40.33
Control + 200 μmol of D-glucose + 200 μmol of L-proline	87.27	47.36	20.59	87.45	56.33	20.45	72.63	44.67	14.06	71.55	34.33	10.53

The control contained 100 μmol of palmitate, 5 × 10^9 spores of *P. roqueforti* in 20 ml of 0.1 M phosphate buffer, pH 6.65, at 30°.

Table VIII. The Distribution of Radioactivity in the Monocarbonyl and Methyl Ketone DNPH Derivatives During the Metabolism of [U-^{14}C]Palmitic Acid by the Spores of *P. roqueforti*

Substrate composition	Incubation period, hr: 10		20		30		45	
	Radioactivity in DNPH derivative fractions, cpm							
	Monocarbonyls	Methyl ketones	Monocarbonyls	Methyl ketones	Mono carbonyls	Methyl ketones	Monocarbonyls	Methyl ketones
Control	16,000	3,630	19,500	5,640	27,000	6,330	68,500	22,680
Control + 400 μmol of D-glucose	51,300	10,500	72,900	14,000	98,100	25,000	57,600	18,500
Control + 400 μmol of L-proline	65,000	37,000	82,500	45,000	106,250	39,000	70,000	44,500
Control + 200 μmol of D-glucose + 200 μmol of L-proline	48,300	25,100	40,200	23,100	42,100	17,200	40,100	11,600

The control contained 100 μmol of [U-^{14}C]palmitate (1.10 × 10^6 cpm), 5 × 10^9 spores of *P. roqueforti* in 20 ml of 0.1 M phosphate buffer, pH 6.5, at 30°.

Table IX. Distribution of Radioactivity in Individual Methyl Ketone Derivatives Following Incubation of [U-^{14}C]Palmitate with Spores of *P. roqueforti*

Substrate composition	Incubation period, hr	Radioactivity, cpm: Methyl ketone DNPH derivatives (carbon chain length)						
		C3	C5	C7	C9	C11	C13	C15
Control	10		480			780	820	1550
	20		420		420	1200	860	2740
	30		420	420	690		860	3940
	45				7200		6120	9360
Control + 400 μmol of D-glucose	10	300	700	1200	1900	1400	2900	2100
	20	500	800	1100	1400	1600	3000	5600
	30		1200	1000	1300	2000	9200	10,900
	45		1100	800	900	2000	6200	6400
Control + 400 μmol of L-proline	10	2400	1300	1500	1000	2800	3700	9900
	20	1600	1200	1400	1300	1800	4600	14,900
	30	1200	1600	900	1500	2100	3900	10,600
	45	6400	900	600	2100	3100	3200	10,000
Control + 200 μmol of D-glucose + 200 μmol of L-proline	10	650	490	890	1730	5110	5560	9950
	20	400	390	510	680	4210	3770	6320
	30	250	230	310	680	2670	4150	4850
	45	450	210	220	440	800	3560	4730

The control contained 100 μmol of [U-^{14}C]palmitate (1.10 × 10^6 cpm) and 5 × 10^9 spores of *P. roqueforti* in 20 ml of 0.1 M phosphate buffer, pH 6.5, at 30°.

Fatty acids are metabolized by *P. roqueforti via* β-oxidation into the corresponding methyl ketones with one less carbon atom (Gehrig and Knight, 1963; Hammer and Bryant, 1937; Lawrence, 1966; Lawrence and Hawke, 1968; Stärkle, 1924). Gehrig and Knight (1958) attributed this ability only to the spores of *P. roqueforti*. However, Lawrence and Hawke (1968) observed that the mycelium of *P. roqueforti* formed methyl ketones from fatty acids. Long

chain (C14 to C18) fatty acids were not oxidized to any appreciable extent and no methyl ketones were detected from their metabolism. Thus, unlike mycelium, the spores of *P. roqueforti* oxidize palmitic acid into methyl ketones. Of the homologous series of methyl ketones (C3 to C15 inclusive) generated from the metabolism of palmitate, 2-pentadecanone contained most radioactivity compared to the other methyl ketones, indicating initial β-oxidation of the substrate by the spores of *P. roqueforti*. The occurrence of short-chain methyl ketones reflected more extensive β-oxidation during metabolism of the palmitate by the spores of *P. roqueforti*.

Significant quantities of other carbonyls were formed during the metabolism of palmitic acid. These included alkanals, alkenals, alkadienals, and some dicarbonyl compounds which are being studied further.

D-Glucose or L-proline in the incubation mixture depressed the evolution of ^{14}CO from the metabolism of [U-^{14}C]palmitic acid and caused marked increases in the concentrations of total carbonyls, including methyl ketones. It appeared that D-glucose and L-proline were preferred to palmitate for energy metabolism by spores of *P. roqueforti*, and in their presence only partial oxidation of palmitic acid occurred. These observations agree with earlier reports of Lawrence (1965a) which showed that simple sugars and amino acids, including D-glucose and L-proline, stimulated both oxygen uptake and formation of 2-heptanone from octanoic acid by spore suspensions of *P. roqueforti*. Lawrence (1967) demonstrated that both D-glucose and L-proline stimulated the formation of methyl ketones from triglycerides by the spores of *P. roqueforti*.

Maximum activity of the spores of *P. roqueforti* occurred at pH 6.5 and 30°; however, the spores showed activity at pH 6.0 and 7.0 and also at 37°. These observations are in general agreement with those of Lawrence (1966). Gehrig and Knight (1963) reported that the production of 2-heptanone from octanoic acid by the spores of *P. roqueforti* was practically abolished at 37°.

The spores of *P. roqueforti* were biologically active even after storage at −15° for a period of 8 weeks. Gehrig and Knight (1963) reported that cold storage of spores of *P. roqueforti* at −5° resulted in the production of almost double the amount of ketones from fatty acids. They suggested that a permeability factor was involved and that frozen storage induced the cell walls of the spores to become more permeable to the passage of the fatty acids into the spores for the oxidation reaction.

Metabolic CO_2 inhibited the formation of carbonyl compounds from palmitate by the spores of *P. roqueforti*. Lawrence (1966), on the other hand, reported that the formation of methyl ketones from fatty acids by the spores of *P. roqueforti* increased considerably in the presence of metabolic CO_2. Low concentrations of carbon dioxide have been reported to stimulate the germination of fungal spores (Cochrane *et al.*, 1963; Farkas and Ledingham, 1959; Golding, 1940; Grover, 1964; Vakil *et al.*, 1961; Yanagita, 1957). Thaler and Geist (1939) and Franke and Heinen (1958) reported that a decline in the formation of methyl ketones occurred when the spores of fungi germinate into the mycelium. Lawrence and Hawke (1968) reported that C14 to C18 fatty acids were not oxidized by the mycelium of *P. roqueforti* to any appreciable extent. It is conceivable that the metabolic CO_2 which was not removed from the incubation system stimulated the germination of the spores into the mycelium, resulting in the inhibition of the formation of the carbonyl compounds from palmitate, as observed in this study.

The decrease in the concentrations of the carbonyl compounds at the later stages of the incubation of palmitate with spores of *P. roqueforti* may be attributable to more complete oxidation of the acyl chains and/or the conversion of methyl ketones into secondary alcohols. Franke *et al.* (1962) and Anderson and Day (1966) demonstrated the interconversion of methyl ketones and their corresponding secondary alcohols in fungi. Anaerobic conditions favoring the production of secondary alcohols from methyl ketones obviously resulted from oxygen depletion by the spores of *P. roqueforti* during incubation. This became more pronounced with duration of each experiment.

The generation of the homologous series of methyl ketones, C3 to C15 inclusive, from palmitate was significant. Schwartz and Parks (1963), Anderson and Day (1966), Niki *et al.* (1966), and Dartey and Kinsella (1971) found that 2-heptanone and 2-nonanone were the preponderant methyl ketones in Blue cheese. Anderson and Day (1966) showed that there is an inverse relationship between the quantity of specific methyl ketones in Blue cheese and the corresponding precursor fatty acids in milk fat. The apparent immediate precursor fatty acids for 2-heptanone and 2-nonanone, *i.e.*, octanoic and decanoic acid, respectively, occur in low concentrations in milk fat when compared to palmitic acid and theoretically could not account for all the 2-heptanone and 2-nonanone formed in Blue cheese. The present findings that the homologous series of methyl ketones, C3 to C15 inclusive, can be formed from the metabolism of palmitic acid by the spores of *P. roqueforti* indicate that some of the major methyl ketones in Blue cheese, *i.e.*, 2-heptanone and 2-nonanone, may originate from longer chain fatty acids through β-oxidation as well as from the corresponding fatty acids with one more carbon atom. Additional experiments using [U-^{14}C]lauric acid have confirmed this (Dartey and Kinsella, 1973).

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LITERATURE CITED

Anderson, D. F., Ph.D. Thesis, Oregon State University, 1966.
Anderson, D. F., Day, E. A., *J. Agr. Food Chem.* **14**, 241 (1966).
Cochrane, J. C., Cochrane, V. W., Simon, F. G., Spaeth, J., *Phytopathology* **53**, 1155 (1963).
Dartey, C. K., Kinsella, J. E., *J. Agr. Food Chem.* **19**(4), 771 (1971).
Dartey, C. K., Kinsella, J. E., unpublished data, 1973.
Day, E. A., *Food Technol.* **1585**, 129 (1965).
Farkas, G. L., Ledingham, G. A., *Can. J. Microbiol.* **5**, 141 (1959).
Franke, W., Heinen, W., *Arch. Mikrobiol.* **31**, 50 (1958).
Franke, W., Platzeck, A., Eichhorn, G., *Arch. Mikrobiol.* **41**, 154 (1962).
Gehrig, R. F., Knight, S. G., *Appl. Microbiol.* **11**, 166 (1963).
Gehrig, R. F., Knight, S. G., *Nature (London)* **182**, 1237 (1958).
Golding, N. S., *J. Dairy Sci.* **23**, 891 (1940).
Grover, R. K., *New Phytol.* **63**, 12 (1964).
Hammer, B. W., Bryant, H. W., *Iowa State Coll. J. Sci.* **11**, 281 (1937).
Hawke, J. C., *J. Dairy Res.* **33**, 225 (1966).
Jackson, H. W., Hussong, R. V., *J. Dairy Sci.* **41**, 920 (1958).
Katz, J., Chaikoff, I. L., *Biochim. Biophys. Acta* **18**, 27 (1955).
Lawrence, R. C., *J. Gen. Microbiol.* **44**, 393 (1966).
Lawrence, R. C., *J. Gen. Microbiol.* **46**, 65 (1967).
Lawrence, R. C., *Nature (London)* **208**, 801 (1965a).
Lawrence, R. C., *Nature (London)* **205**, 1313 (1965b).
Lawrence, R. C., Hawke, J. C., *J. Gen. Microbiol.* **51**, 289 (1968).
Niki, T., Yoshioka, Y., Ahiko, K., International Dairy Congress, Section D: 2, Munich, Germany, 1966, pp 531–537.
Patton, S., *J. Dairy Sci.* **33**, 680 (1950).
Pressman, B. C., Lardy, H. A., *Biochem. Biophys. Acta* **21**, 458 (1956).
Rolinson, G. N., *J. Appl. Bacteriol.* **17**, 190 (1954).
Schwartz, D. P., Haller, H. S., Keeney, M., *Anal. Chem.* **35**(13), 1291 (1963).
Schwartz, D. P., Parks, O. W., *J. Dairy Sci.* **46**, 1421 (1963).
Schwartz, D. P., Shamey, J., Brewington, C. R., Parks, O. W., *Microchem. J.* **13**, 407 (1968).
Stärkle, M., *Biochem. Z.* **151**, 371 (1924).
Stokoe, W. N., *Biochem. J.* **22**, 80 (1928).
Thaler, H., Geist, G., *Biochem. Z.* **302**, 121 (1939).
Urbach, G., *J. Chromatogr.* **12**, 196 (1963).
Vakil, J. R., Raghavendra Rao, M. R., Bhattacharyya, P. K., *Arch. Mikrobiol.* **39**, 53 (1961).
Vinze, V. L., Ghosh, D., *Hindustan Antibiot. Bull.* **4**, 119 (1962).
Yanagita, T., *Arch. Mikrobiol.* **26**, 329 (1957).

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