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Oxidation of fatty acids by Candida lipolytica and Pseudomonas fragi

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OXIDATION OF FATTY ACIDS BY *CANDIDA LIPOLYTICA* AND *PSEUDOMONAS FRAGI*

by

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INTRODUCTION

The ability of microorganisms to break down fats and utilize the resulting products in their metabolism has been recognized for some time. However, very little work has been done to give a proper understanding of the details of the various processes involved.

Microbial lipases have been studied in greater detail than have the microbial oxidases. One of the areas in which the oxidative processes of microorganisms assume importance in dairy products is in the production of methyl-n-amyl ketone, one of the flavor constituents of blue cheese, by incomplete β-oxidation of caprylic acid which has been liberated by lipolytic activity. The action which other oxidase positive organisms may have in dairy products is relatively unknown, although microbial utilization of free fatty acids is known to take place in certain products when conditions are satisfactory.

The present study is concerned with some of the factors which may affect the oxidation of fatty acids by two organisms, Candida lipolytica and Pseudomonas fragi, known to be very active in the degradation of fats in dairy products.

STATEMENT OF PROBLEM

This investigation was undertaken to study the ability of *C. lipolytica* and *Ps. fragi* to oxidize sodium salts of the fatty acids.

The work is concerned with some of the nutritional and environmental factors which may affect the oxidation of fatty acids by these microorganisms.

REVIEW OF LITERATURE

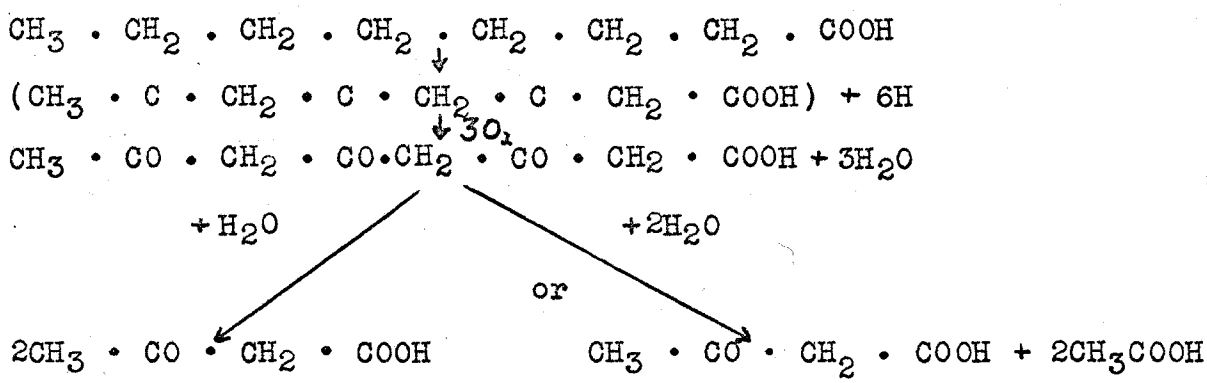
Concepts of Fatty Acid Oxidation

The work on fatty acid metabolism has not progressed as rapidly as the study of the metabolic fate of other nutritional components. However, in recent years several hypotheses to explain fatty acid oxidation have been advanced by different workers.

The theory of β-oxidation of fatty acids in the animal body first was proposed by von Knoop (1905) and further substantiated by Dakin (1908). The scheme of this oxidation is one in which the fatty acid is oxidized to a β-hydroxy acid, which in turn is oxidized to a β-keto acid. By decarboxylation the β-keto acid can be converted to a ketone, which in turn is oxidized to an acid.

From results obtained by feeding cod-liver oil, margarine and cocoa butter to animals, Leathes and Meyer-Wedell (1909) proposed the desaturation of the fatty acid to follow the scheme: $R.CH_2.CH_2.COOH \longrightarrow R.CH = CH.COOH + H_2O \longrightarrow R.CHOH.CH_2.COOH$ These investigators maintained that this desaturation process occurs prior to oxidation in the other tissues.

Because no intermediate acids could be found, Jowett and Quastel (1935) proposed the theory of "multiple alternate oxidation" which may be expressed as:



Formation of acetoacetate was shown to increase with the chain length of even numbered acids up to caprylic acid, and then decrease with the higher acids, due to their lower solubility. More acetoacetate is formed from caproic and caprylic acids than would be expected, leading these authors to suggest that more than one four-carbon fragment arises from each fatty acid molecule.

In a study in which the feeding of C_9 and C_{11} fatty acids resulted in the elimination of the corresponding dicarboxylic acids, Verkade and Lee (1934) proposed the theory of <u>omega</u> oxidation. In this scheme the terminal methyl group of the fatty acid is oxidized to a carboxyl group. The resulting dicarboxylic acid then can be oxidized at both ends to give rise finally to succinic acid.

A scheme of auto-condensation during β-oxidation was suggested by MacKay, Wick, Carne and Barnes (1941). The two terminal carbon atoms are split off as acetic acid by β-oxidation and then are recombined by auto-condensation to form acetoacetic acid and acetone as follows:

$$2CH_3\ COOH \xrightarrow[-H_2O]{} CH_3 \cdot CO \cdot CH_2 \cdot COOH \xrightarrow[-CO_2]{} CH_3 \cdot CO \cdot CH_3$$

By using isotopic carbon in the carboxyl group of n-octonoic acid, Weinhouse, Medes and Floyd (1944a, 1944b) found that the istopic carbon was present in both the carboxyl and carbonyl groups of acetoacetic acid. This indicated that the acetoacetic acid was formed by condensation of the two-carbon components resulting from β-oxidation of the fatty acid. The intermediate substance probably was acetic acid. This work gave further support to the work of MacKay, Wick, Carne and Barnes (1941).

A further study by Medes, Weinhouse and Floyd (1945), using carboxyl-labeled butyric acid, indicated a fission of the butyric acid and subsequent recombination, rather than the direct formation of acetoacetic acid from butyric acid. By using carboxyl-labeled acetic acid, Weinhouse, Medes and Floyd (1945) found that the distribution of C^{13} in the acetoacetic acid indicated that the reaction proceeds by coupling two acetyl groups.

In a recent article, Leloir (1948) reviewed the newer literature on fatty acid oxidation and reported that the experimental facts indicated the fatty acids were β-oxidized to a reactive two-carbon compound. With acetic acid this two-carbon compound would form acetoacetic acid, and with oxalacetic acid or related compound it would enter the tricarboxylic acid cycle

leading to its complete oxidation. There is some indication that the mechanism may be of a similar nature for micro-organisms.

Oxidation of Fats and Fat Components by Microorganisms

The ability of bacteria, yeasts and molds to oxidize natural fats and fatty acids has been recognized for some time. With the advent of oxygen uptake studies with the Warburg respirometer, more detailed studies of the oxidase enzymes in microorganisms have become possible.

The results of Jensen and Grettie (1933, 1937) indicate that oxidases specific for both saturated and unsaturated fatty acids are present in pure cultures of bacteria. The presence of oxidase-positive organisms was determined by the use of cocoanut oil emulsion with the p-phenylene diamine series of dyes. The use of peroxide values, Kreis test and Schiff reagent demonstrated that certain of these cultures were capable of causing oxidation of leaf lard and hydrogenated cottonseed oil. The changes noted were significantly larger than in the sterile controls, to rule out any purely chemical oxidative processes. In a similar study, Castell and Garrard (1941), using the Kreis and Schiff tests in conjunction with the oxidase test of the p-phenylene diamine dyes, showed a number of pure culture of gram-negative organisms to be capable of oxidizing triolein. They found a good correlation between

the oxidase test and the ability of the cultures to oxidize triolein.

The ability of selected strains of a variety of cultures of microorganisms to oxidize pure fats and their components, as determined by growth on a mineral salts-agar medium contained these materials as carbon sources, was reported by Peppler (1941). He found Pseudomonas aeruginosa, Serratia marcescens, Alcaligenes viscosus, Alcaligenes lipolyticus, Achromobacter lipolyticum, Bacillus atterimus, Proteus vulgaris, Corynebacterium simplex, Mycotorula lipolytica and Staphylococcus aureus to be capable of hydrolyzing triacetin, tripropionin, tributyrin and butterfat. These organisms were capable of obtaining energy from the components of pure fats in the presence of either NH_4^+ or NO_3^- as a nitrogen source. B. atterimus and S. aureus failed to attack triglycerides above tributyrin. Many species showed lipolytic ability in the presence of tricaprylin and tricaprin, but the liberated fatty acids were not attacked. Glycerol was not attacked by Alc. viscosus or Coryn. simplex, but was utilized by an S. aureus strain which would not hydrolyze esters of fatty acids of low molecular weight. The greatest number of substrates was decomposed by Ps. aeruginosa, S. marcescens, Achrom. lipolyticum and Prot. vulgaris, with resting cells of Ps. aeruginosa showing more rapid oxygen uptake on fatty acids than on glycerol. Glycerol was shown to be oxidized at a rapid rate by a selected strain of Streptococcus faecalis, as had been shown by Gunsalus

and Umbreit (1945). The over-all reaction was Glycerol + O_2 ⟶ Lactate + H_2O_2. The oxidation was inhibited by the accumulation of H_2O_2 to a final concentration of 0.002M.

Mundt and Fabian (1944) employed a more fundamental approach to the subject, using Warburg techniques in conjunction with Thunberg techniques in testing the ability of pure cultures of soil and water types of bacteria to oxidize and dehydrogenate corn oil. Of the 32 cultures studied, 25 showed utilization of oxygen in excess of endogenous respiration when using corn oil, while only six showed the ability to dehydrogenate corn oil. Even though a natural fat was used, the results indicated the presence of both a saturated fatty acid oxidase and an unsaturated fatty acid oxidase in the strains which showed oxidizing ability. Sodium chloride in a concentration of 2.5 percent in the oil was inhibitory to all strains except Pseudomonas fluorescens. Two antioxidants, hydroquinone and Avenex (oat flour), were found to be ineffective in preventing the bacterial oxidation of the corn oil.

The ability of species of the genus Brucella to oxidize fatty acids has been reported by Attimonelli (1942). The oxidation of fatty acids from formic to stearic by Brucella melitence (B. melitensis), Brucella abortus and the rough colony variants of both species showed that formic acid is not oxidized, but the other fatty acids are oxidized easily, especially by B. abortus. The acids most readily attacked were acetic and those from caproic through stearic.

A number of strains of Streptococcus mitis were shown by Niven, Evans and White (1945) to have the ability to oxidize butyric acid under aerobic conditions to form H_2O_2. At pH 7.2 some cell suspensions produced as much as 0.01M H_2O_2 from 0.011M butyrate within one hour at 25°C. Streptococci of the Lancefield Group F and the "minute" variety of Lancefield Group G oxidized the butyrate, while other species of streptococci and pneumococci did not possess this oxidizing ability.

Mazza and Cimmino (1933) reported that Bacillus coli var. communis (Escherichia coli) was capable of oxidizing stearic, palmitic and oleic acids when the Warburg apparatus was used with 24 hour cultures at pH 7.5 with air as the gas phase. The results indicate that oxygen uptake was greatest for stearic acid, followed by oleic acid and palmitic acid to a lesser degree. This work later was confirmed by Singer and Barron (1945). These workers found by using enzyme poisons that the stearate and oleate oxidases of B. coli var. communis require the presence of sulfhydryl groups in the activating protein. Since the oleic acid oxidase from peanuts was not affected by the inhibitors, the peanut enzyme was considered to be of a different nature.

In a study with anaerobes, Neave and Buswell (1930) proposed that the lower fatty acids are converted almost quantitatively to methane and carbon dioxide according to the equation $4\ C_nH_{2n}O_2 + 2(n-2)\ H_2O \longrightarrow (n+2)CO_2 + (3n-2)\ CH_4$,

where n is the number of carbon atoms in the acid. The reaction $CH_3\ COOH + 2H_2O \longrightarrow 2CO_2 + 4H_2$ also may take place. Water was shown to act as an oxidizing agent in this degradation. Their results should be interpreted with caution, since the work was conducted with a mixture of two organisms.

Using Thunberg techniques with cresyl blue as a hydrogen acceptor, Guggenheim (1944) found that formic, acetic, propionic and butyric acids were not attacked by clostridia. Lactic and pyruvic acids were actively attacked, while succinic, malic, glutaric, fumaric and maleic acids were weakly attacked.

Perhaps the most complete study to date on the fat-oxidizing enzymes of bacteria was conducted by Jezeski (1947b, 1948). Using the conventional Warburg techniques, he found that 30 of 44 pure cultures of bacteria were capable of taking up significantly greater amounts of oxygen in the presence of fat. It was pointed out that these 30 cultures did not necessarily oxidize the fatty acids, but the glycerol may have been the substrate responsible for the oxygen uptake. Four cultures (Mycobacterium phlei, a Micrococcus sp. and two Pseudomonas sp.) which were capable of oxidizing fatty acids, were used for a more detailed study. The nutritional level of the medium on which the cells were grown seemed to have no effect on the fat oxidizing power of the organisms and the enzyme appeared to have no adaptive enzyme component. Inorganic phosphate appeared to have no effect on the oxidative ability

of the enzyme. Of the natural fats used, cocoanut oil was oxidized at a greater rate than either corn oil or butter oil. By using both methyl esters and sodium soaps of the fatty acids, the data indicated that possibly there are two enzymes which are responsible for the oxidation of members of the saturated series. The studies of the pH optima gave further indication of the presence of two enzymes. The rate of oxidation increased with the increase in unsaturation of the fatty acids, as indicated by experiments with methyl esters of stearic, oleic and linoleic acid. This investigator could find no correlation between the oxidase test and the oxidizing ability.

Winzler (1940) reported that acetate is incompletely oxidized by Saccharomyces cerevisiae. Sixty-seven percent of the acetate was oxidized to carbon dioxide and water, with the remainder accounted for as carbohydrate in the yeast cell. The use of 2,4-dinitrophenol, NaCN and NaN_3 did not prevent all of the acetate from being oxidized to carbon dioxide and water, but did prevent assimilation of any of the acetate to carbohydrate in the cell.

Several workers have stated that the oxidation of saturated fatty acids by molds follows the concept of β-oxidation. Derx (1924) stated that the formation of methyl ketones proved that oxidation takes place at the β-carbon atom. The fatty acids up to and including myristic acid are absorbed by the mycelium and hinder respiratory functions. For this reason the

oxidation stops at the β-keto acid, which then splits into the methyl ketone and carbon dioxide. Stokoe (1928) found that Oidium (Oospora) lactis will completely oxidize fatty acids to lower fatty acids by β-oxidation by the following reactions:

$$R.CH_2.CH_2COOH \longrightarrow R.CO.CH_2.COOH \longrightarrow RCOOH + CH_3COOH$$

Incomplete oxidation may be shown by the presence of alcohols with the same chain length as the ketones. Hammer and Bryant (1937) suggested that one of the flavor constituents of blue cheese, methyl-n-amyl ketone, probably is formed by the β-oxidation of caprylic acid to the ketone, and the elimination of carbon dioxide by the cheese mold growing under unfavorable conditions.

While working with the yeast-like fungus Blastomyces dermatitidis, Levine and Novak (1949a) found that acids from acetic through caprylic stimulated oxygen uptake and those with longer carbon chains than caprylic inhibited oxygen uptake. Increasing the number of double bonds of an acid had no effect on the inhibition of oxygen uptake, and branching of the carbon chain, as in isovaleric acid, resulted in a pronounced decrease in the ability to stimulate respiration.

Little has been reported on the actual effect of oxidase-positive, lipolytic organisms upon dairy products. Castell and Garrard (1940) reported that the oxidase-positve organisms were absent or very few in number in salted cream and in cheese. They classified various genera and groups of microorganisms on

the basis of their oxidative ability. Members of the genera Pseudomonas and Achromobacter were strongly oxidative, Alcaligenes and Brucella possessed weaker oxidizing ability and Aerobacter, Escherichia and Proteus were weakly positive. The bacilli were very weakly positive, with the cocci and anaerobes being negative.

It should be pointed out at this time that a large amount of work has been done on fatty acid oxidation by oxidases of both plant and animal origin. Since this aspect is beyond the scope of this study, it will not be considered in detail here. An outstanding review dealing with the intermediary metabolism of fatty acids in mammalian tissue has been presented by Stadie (1945). A review by Jezeski (1947a) deals with the enzymatic oxidation of fatty acids, including a section concerning the oxidases from vegetative sources.

EXPERIMENTAL

Methods

Cultures

All cultures used were selected from the stock culture collection of the Dairy Industry Department, Iowa State College. Those chosen for study possessed typical characteristics of the species used, with the exception that the Ps. fragi culture was non-lipolytic, but strongly oxidative. Tryptone-glucose-extract agar slants were used for carrying the stock cultures.

Determination of lipolytic properties

A modification of the procedure of Jensen and Grettie (1937) was used for the study of the lipolytic properties of the microorganisms. Butterfat was used to prepare the emulsion (100 ml. butter oil, 2 gm. gum tragacanth, 200 ml. hot distilled water), and the mixture homogenized four times with a hand homogenizer to obtain a stable emulsion. Nile-blue sulfate (1:100) was added at the rate of 1 ml. per 100 ml. of melted agar, and the butterfat emulsion was added at the rate of 5 ml. per 100 ml. of melted agar. After thorough mixing, plates were poured and the agar allowed to solidify.

The cultures to be tested were streaked on the agar surface and the plates incubated at 21°C. for four days. Fat hydrolysis was indicated by the presence of blue fat globules below and around the colonies.

Determination of oxidative properties

The oxidative properties of the microorganisms were studied with a modification of the procedure of Jensen and Grettie (1937). The butterfat emulsion was used for this study, but the nile-blue sulfate was omitted. After incubation at 21°C. for four days, the plates were flooded with a 0.4 percent aqueous solution of tetramethylparaphenylenediamine hydrochloride. The presence of a deep purple colony was considered a positive test for oxidative action.

Determination of cell count

Tryptone-glucose-extract agar was used to pour plates for the standard plate count (American Public Health Association, 1948). The plates used for enumeration of C. lipolytica were counted after 48 hours incubation at 30°C., while those of Ps. fragi were incubated at 21°C. for 48 hours.

Determination of pH

The pH determinations were made either with a Leeds and Northrup glass-electrode potentiometer or with a Leeds and Northrup quinhydrone electrode and saturated calomel half-cell.

Preparation of cell suspensions

The cells used were propagated on agar slants in 6 oz. or 32 oz. medicinal ovals for 24 hours, at 30°C. for C. lipolytica and at 21°C. for Ps. fragi. To harvest the cells, they were washed from the agar surface with 20 ml. of chilled saline at pH 7.8 prepared according to Landy and Dicken (1942). The cells were packed by centrifugation in an International Type SB angle head centrifuge at 4000 rpm for 5 minutes in the case of C. lipolytica and for 15 minutes for Ps. fragi. The supernatant liquid was decanted off, the cells resuspended in 20 ml. of saline and the centrifugation and washing repeated two additional times. The final suspension was prepared by diluting the cells with saline to a constant reading of 250 on the Klett-Summerson photoelectric colorimeter, according to the method of Price (1947), using a No. 54 filter. This reading gave a suspension containing approximately 100,000,000 C. lipolytica cells per ml., or approximately 1,000,000,000 Ps. fragi cells per ml.

Selection of buffers

The selection of buffers presented a problem because a buffer had to be used which covered a wide pH range, yet was not toxic to the organisms in the concentrations used, and one which would maintain the pH constant over a 2.5 hour period. Since a wide pH range was desired for the pH optima

studies, 0.05M McIlvaine citrate-phosphate buffer, as reported by Hodgman (1948), was used for C. lipolytica and 0.07M Palitzsch borate buffer, as reported by Clark (1928), was used for Ps. fragi.

Selection of substrates

The substrates used in the flasks in all cases were fatty acids which had been neutralized with an equivalent amount of NaOH to a pH of 7.0 to 7.2, using a Leeds and Northrup glass electrode potentiometer. The stock solutions were prepared as 0.10M solutions and diluted to the desired concentration, with the exception that lauric, myristic, palmitic and stearic acids had to be prepared as 0.025M solutions due to the lesser solubility of these acids. With the longer chain acids, it was necessary to neutralize them while hot, in order to convert all of the acid to the sodium soap. In all cases the substrate was used as a 0.01M solution, giving a final molarity of 0.0017 when diluted 1:6 in the reaction flask with the cell suspension, buffer and water.

Respiration measurements

The respiration measurements were made with Warburg constant volume respirometers, using the conventional methods of Dixon (1943) and Umbreit, Burris and Stauffer (1945). The respirometer flasks, having a volume of approximately 16 ml., were calibrated with mercury following the method of Umbreit,

Burris and Stauffer (1945). The flask contents were as follows:

Buffer	1.0 ml.
Bacterial Cell Suspension	1.0 ml.
Substrate	0.5 ml.
Distilled H_2O	0.5 ml.
	3.0 ml.

Filter paper was used in the center well to provide more surface for the KOH (0.2 ml. of 20 percent) to facilitate CO_2 absorption. To measure endogenous respiration, 0.5 ml. of distilled water was substituted for the substrate in the flasks. The shaker was operated at 120 strokes a minute through a distance of 4 cm. Atmospheric air was the gas phase in the flasks. The temperature of the water bath was maintained at 30 ± 0.2°C.

Selection of synthetic media

The media used for C. lipolytica consisted of:

No. 1		No. 2	
Glucose	10 g.	Glucose	10 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.	KH_2PO_4	2 g.
$K_2HPO_4 \cdot 3H_2O$	0.8 g.	$MgSO_4 \cdot 7H_2O$	2 g.
NH_4Cl	2.0 g.	$FeSO_4 \cdot 7H_2O$	0.015 g.
Distilled Water	1000 ml.	$(NH_4)_2SO_4$	0.3 g.
		Distilled Water	1000 ml.

When agar slants were desired, 25 g. of agar was added per liter of the medium. The media were dispensed in 100 ml. quantities and sterilized in an autoclave at 250°F. for 13 to

15 minutes.

The media used for Ps. fragi consisted of medium No. 1 as listed previously and the medium for enterococci proposed by Niven and Sherman (1944). Niven's medium was modified, as sodium thioglycolate was omitted because Ps. fragi is more aerobic than the enterococci. Agar was added at the rate of 25 g. per liter when a solid medium was desired. The media were dispensed in 100 ml. quantities and autoclaved at 250°F. for 13 to 15 minutes.

Preparation of fatty acids and monoglycerides

The fatty acids and monoglycerides to be added to the synthetic growth media were prepared as 1M stock solutions for C. lipolytica and as 0.5M stock solutions for Ps. fragi. These materials were sterilized separately and added to the media immediately before inoculation. The final concentration of the fatty acids and monoglycerides in the synthetic growth media was 0.01M for C. lipolytica and 0.005M for Ps. fragi.

Preparation of protein sources and cream

The various proteins were prepared as 20 percent solutions which gave a final concentration of 0.5 percent in the media used. The stock solutions were sterilized separately and added to the media immediately before inoculation.

The cream used was a 20 percent fat product, which was hand homogenized four times before sterilization. Both the

sterile cream and the sterile skimmilk were added to give a final concentration of 5 percent in the media.

Adjustment of pH of the growth media

Tryptone-glucose-extract broth was prepared, and the pH adjusted in increments of 0.5 pH unit from pH 3.5 to 8.5, using N/1 HCl and N/1 NaOH. Agar was added at the rate of 2.5 percent and the mixture heated to dissolve the agar. After sterilization at 250°F. for 22 to 25 minutes, the pH again was checked. The pH of the agar shifted during sterilization so the values obtained on the sterilized media are reported in the tables. These media were used to check the pH at which the oxidizing ability was greatest.

Results

A comparison was made of 11 strains of C. lipolytica and and 13 strains of Ps. fragi to determine their ability to hydrolyze and oxidize butterfat. The hydrolytic ability was tested by the nile blue sulphate technique and the oxidative ability tested by the use of tetramethylparaphenylenediamine hydrochloride. The most oxidative strain of each genus was chosen for the remainder of this study. The C. lipolytica strain chosen was moderately lipolytic while the strain of Ps. fragi showed a negative reaction to nile blue sulphate.

Effect of age of cells and length of run on oxygen uptake

In a preliminary trial with C. lipolytica, the cells were grown on tryptone-glucose-extract agar slants for 24, 48, 72 and 96 hours at 30°C. The harvesting of the cells was conducted as outlined under "Methods". The packed cells were weighed, chilled saline added at the rate of 30 times their weight and the suspension prepared. This suspension was used in the Warburg apparatus to test the oxidation of sodium acetate during a four hour run. The data obtained are presented in Table 1.

The results show that the culture incubated for 24 hours gave higher oxygen uptake values, over the entire four hour run, than any of the cultures incubated for longer periods of time. Since the oxidizing ability was greatest for the 24 hour culture, all cultures for subsequent trials were incubated this length of time. Although a similar trial was not conducted on Ps. fragi, this organism also was grown for 24 hours before being used. A nearly linear relationship is noted in Table 1 in the oxygen uptake values for the first 2.5 hours, using the 24 hour culture. Since adequate oxygen uptake values were obtained in 2.5 hours, it was unnecessary to make the following runs of longer duration.

The high oxygen uptake values noted in Table 1 made it appear that the cell suspension used was more concentrated than necessary. Furthermore, better interpretation of the

Table 1

The effect of the age of cells and length of run upon the oxidizing ability of Candida lipolytica. (0.07M Clark and Lubs phosphate buffer at pH 7.0 used in reaction flasks)

Age of cells (hours)	Oxygen uptake ($mm.^3$)* Length of run (hours)							
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
24	127	308	457	554	622	679	715	746
48	88	214	342	427	466	515	530	540
72	65	171	300	421	479	520	540	550
96	17	50	83	119	154	194	239	295

*These values are corrected for endogenous respiration.

results could be obtained if the cell suspension was adjusted to a constant value each time. Plate counts were made, together with readings on a Klett-Summerson photoelectric colorimeter, to determine which readings on the colorimeter would give a sufficient cell concentration to produce sufficient oxygen uptake values. A reading of 250 on the colorimeter, using a No. 54 filter, was used for C. lipolytica and Ps. fragi in all subsequent trials. This reading was within the recommended effective range of the colorimeter and gave concentrations of 100,000,000 C. lipolytica or 1,000,000,000 Ps. fragi cells per ml. of suspension. These concentrations of cells gave adequate oxygen uptake values, and all values hereafter reported were obtained using suspensions adjusted to a reading of 250.

Effect of suspending medium on oxygen uptake

To determine the effect which the suspending medium might have on oxygen uptake values, three tryptone-glucose-extract agar slants were inoculated with C. lipolytica. After incubation at 30°C. for 24 hours, the cells were harvested and resuspended, using 0.85 percent saline, one-quarter strength Ringers solution and distilled water as the suspending media. The resulting suspensions were prepared as in "Methods" and subsequently were used to test the oxidation of the sodium salts of acetic, propionic and butyric acids. The data obtained for the endogenous respiration and the oxidation of these fatty acid salts are presented in Table 2.

Table 2

The effect of the suspending medium upon the oxidizing ability of Candida lipolytica cells. (0.05M McIlvaine citrate-phosphate buffer at pH 4.0 used in reaction flasks)

Suspending media	Oxygen uptake ($mm.^3$)											
	Endogenous			Na acetate*			Na propionate*			Na butyrate*		
	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.
Saline solution (0.85 percent)	42	67	55	156	99	128	127	106	117	230	177	204
Ringers solution (1/4 strength)	34	76	55	170	85	128	144	106	125	234	172	203
Distilled water	41	71	56	181	112	147	141	130	136	252	219	236

*These values are corrected for endogenous respiration.

The endogenous respiration was lower in trial 1 than in trial 2. With acetate, propionate and butyrate, the values in trial 1 were higher than those in trial 2 for all substrata and all suspending media. The average oxygen uptake values for the three substrata were greatest when distilled water was used as the suspending medium. The saline solution and Ringers solution gave nearly the same values, with the exception that the uptake value for cells in Ringers solution was slightly higher in trial 1 when used with propionate. The measurement of pH both before and after the 2.5 hour run showed that the saline solution maintained the pH at a more constant level than either of the other suspending media. This fact, together with the fact that the saline solution was isotonic with the cell contents, provided the basis for its selection as the suspending medium to use for C. lipolytica. Saline also was used for the Ps. fragi suspensions, although no similar trial comparing the three materials was conducted using this organism.

Effect of buffer composition on oxygen uptake

The effect which the buffer composition might have upon the oxidation of fatty acids was studied next. Work by Peters (1947) on the characteristics of the lipase produced by Mycotorula (Candida) lipolytica indicated that high lipase activity was evident from pH 4.0 to pH 8.0, with pH 6.2 to

6.5 optimum for the reaction. If hydrolytic action took place in this pH range, optimum oxidative action also might take place in this pH range. Accordingly, three buffers which covered this range were used to determine their effect on oxidation of fatty acids by C. lipolytica.

The C. lipolytica culture was grown on tryptone-glucose-extract agar slants for 24 hours at 30°C., and harvested as outlined in "Methods". The buffers used were 0.05M McIlvaine citrate-phosphate buffer (Hodgman, 1948), 0.07M Clark and Lubs phthalate buffer (Clark, 1928) and 0.07M Clark and Lubs phosphate buffer (Clark, 1928) at pH values of 5.8 and 6.0. In trial two, the citrate-phosphate and phosphate buffers were used at pH values of 5.8, 6.0 and 6.2. Sodium propionate was the substrate used in both trials for a 2.5 hour run. The data obtained are presented in Table 3.

The endogenous oxygen uptake values in trial 1 at both pH levels were highest with the citrate-phosphate buffer, followed by nearly equal lower values for the phosphate and phthalate buffers. The oxygen uptake values for sodium propionate were nearly the same for the citrate-phosphate and the phosphate buffers at pH 5.8, with the phthalate buffer giving lower uptake values. At pH 6.0 the phosphate buffer gave the highest values, followed by the phthalate and citrate-phosphate buffers in that order. A comparison of the initial and the final pH levels showed the citrate-

Table 3

The effect of buffer composition upon pH maintenance and oxidizing ability of Candida lipolytica cells

(Trial 1)

Buffer used	pH of buffer	Oxygen uptake ($mm.^3$)		pH	
		Endogenous	Na propionate*	Initial	Final
0.05M McIlvaine citrate-phosphate	5.8	34	50	5.95	6.00
0.07M Clark and Lubs phthatate	5.8	26	42	5.80	5.93
0.07M Clark and Lubs phosphate	5.8	27	51	5.80	5.93
0.05M McIlvaine citrate-phosphate	6.0	39	37	6.18	6.23
0.07M Clark and Lubs phthalate	6.0	25	56	6.00	6.18
0.07M Clark and Lubs phosphate	6.0	25	63	5.93	6.05

(Trial 2)

0.05M McIlvaine citrate-phosphate	5.8	54	86	5.98	6.05
0.07M Clark and Lubs phosphate	5.8	35	98	5.83	6.00
0.05M McIlvaine citrate-phosphate	6.0	55	74	6.18	6.23
0.07M Clark and Lubs phosphate	6.0	38	100	5.95	6.13
0.05M McIlvaine citrate-phosphate	6.2	60	72	6.35	6.41
0.07M Clark and Lubs phosphate	6.2	38	105	6.11	6.27

*These values are corrected for endogenous respiration.

phosphate buffer to maintain the pH most constant for the 2.5 hour run, followed by the phosphate buffer and the phthalate buffer. Since the citrate-phosphate buffer maintained the pH the most nearly constant, and because the phosphate buffer gave the highest oxygen uptake values, these buffers were selected for a second trial at pH levels of 5.8, 6.0 and 6.2.

The high endogenous uptake values again are noted in trial 2, when the citrate-phosphate buffer is used. The uptake values obtained with sodium propionate as the substrate again show the phosphate buffer to give the highest values. In both trials, the uptake values obtained using the citrate-phosphate buffer with propionate decreased as the pH increased, while the uptake values increased slightly as the pH increased when the phosphate buffer was used. This may indicate a slight difference in the optimum pH between the two buffers, but the magnitude of the differences makes such an interpretation hazardous. This low optimum pH for sodium propionate is also noted in Table 9, where the optimum is at pH 3.8.

The high endogenous uptake values when the citrate-phosphate buffer is used indicate that some component of this buffer may be utilized by the cells. This component undoubtedly is the citrate radical. Use of this buffer could rightfully be criticized, since this radical may be

utilized to give high values. However, the endogenous uptake values are subtracted from the total uptake values in all cases to give the corrected uptake values. These corrected values should be satisfactory if the same buffer is used throughout the entire investigation.

The maintenance of pH for a 2.5 hour run, in both trials, was best when the citrate-phosphate buffer was used. Using the citrate-phosphate buffer there was an immediate shift in pH in the magnitude of 0.2 pH unit when the reactants were mixed, but no appreciable pH shift occurred during the 2.5 hour run. When phosphate or phthalate buffers were used, there was no appreciable change in the initial pH, but a shift of approximately 0.15 pH unit occurred during the 2.5 hour run. Since the shift in pH during the 2.5 hour run was the least for the citrate-phosphate buffer, this buffer was chosen for the remainder of this investigation. A second reason for choosing the citrate-phosphate buffer is that this buffer covers a pH range of 2.0 to 8.0, which is particularly valuable when making a study of the optimum pH for the enzyme activity.

Preliminary trials with Ps. fragi had indicated that the optimum pH for fatty acid oxidation was beyond pH 8.0. Three buffers were chosen for comparative results with Ps. fragi, to determine the one best suited for the remainder of the investigations. The Ps. fragi cells were grown on

tryptone-glucose-extract agar slants at 21°C. for 24 hours, and harvested by the procedure outlined in "Methods". The buffers used were 0.05M McIlvaine citrate-phosphate buffer (Hodgman, 1948), 0.07M Clark and Lubs phosphate buffer (Clark, 1928) and 0.07M Palitzsch borate buffer (Clark, 1928) at pH 7.8 and 8.0. Sodium propionate was the substrate used, and the data are presented in Table 4.

The endogenous oxygen uptake values at both pH levels were highest for the citrate-phosphate buffer, followed by lower values for the phosphate buffer and the borate buffer. The uptake values obtained with sodium propionate at pH 7.8 were highest with the phosphate buffer followed by lower values when the citrate-phosphate and borate buffers were used. At pH 8.0 the citrate-phosphate buffer gave the highest readings, followed by the phosphate buffer, with the borate buffer again giving the lowest values. Since both the endogenous uptake values and the substrate uptake values were lowest for the borate buffer at both pH levels, there is some indication of an inhibition by this buffer. Jezeski (1947b) also reported an inhibitory effect when a borate buffer was used in studies of fatty acid oxidation by microorganisms. The maintenance of pH was equally as good for one buffer as another, with 0.05 of a pH unit as the maximum pH change in 2.5 hours.

Even though the borate buffer appeared to be somewhat inhibitory to fatty acid oxidation by Ps. fragi, it was

Table 4

The effect of buffer composition upon pH maintenance and oxidizing ability of Pseudomonas fragi cells. (Trial 1)

Buffer used	pH of buffer	Oxygen uptake (mm.3)		pH	
		Endogenous	Na propionate*	Initial	Final
0.05M McIlvaine citrate-phosphate	7.8	14	46	8.07	8.04
0.07M Clark and Lubs phosphate	7.8	12	57	7.78	7.80
0.07M Palitzsch borate	7.78	9	31	7.92	7.88
0.05M McIlvaine citrate-phosphate	8.0	18	54	8.25	8.25
0.07M Clark and Lubs phosphate	8.0	14	50	8.02	8.00
0.07M Palitzsch borate	7.95	9	26	8.01	8.06

*These values are corrected for endogenous respiration.

desirable to use it for the investigations of pH optimum, because the buffer range from pH 7.1 to 9.1 covered the range of optimum fatty acid oxidation for Ps. fragi. As the readings all were corrected for the endogenous respiration, this buffer was used for the remainder of the investigation, to keep the conditions as constant as possible.

Effect of pH on endogenous respiration

After selection of the buffers, it was necessary to determine if the endogenous uptake values would be significantly different over the pH range of 3.2 to 8.0 for C. lipolytica. The cells were grown on tryptone-glucose-extract agar slants at 30°C. for 24 hours, harvested and the suspension prepared as outlined in "Methods". The pH was measured initially and at the end of the 2.5 hour run. The total oxygen uptake is reported for a 0 to 2 hour run and for a 0.5 to 2.5 hour run. The data obtained are presented in Table 5.

It is noticeable in these data, as it was in Table 3, that there usually is an immediate increase of approximately 0.2 of a pH unit when the reactants are mixed and before the reaction takes place. Since the substrate is missing in this case, it appears that this increase must be a property of the buffer itself, since the cell suspension and water are the only other reactants. After this shift of 0.2 of a pH unit above the original pH of the buffer, there was usually little

Table 5

Endogenous respiration of *Candida lipolytica* cells.
(0.05M McIlvaine citrate-phosphate buffer used in reaction flasks)
(Average of two trials)

Desired pH	pH of reaction		Oxygen uptake ($mm.^3$)	
	Initial	Final	0-2 Hr.	0.5-2.5 Hr.
3.2	3.39	3.43	40	36
3.4	3.49	3.54	38	35
3.6	3.72	3.75	41	37
3.8	3.92	3.97	43	38
4.0	4.13	4.19	45	40
4.2	4.33	4.39	43	40
4.4	4.58	4.60	48	45
4.6	4.75	4.84	45	45
4.8	5.00	5.07	48	44
5.0	5.27	5.33	48	50
5.2	5.44	5.50	47	44
5.4	5.76	5.74	50	47
5.6	5.82	5.96	51	56
5.8	6.02	6.10	50	44
6.0	6.25	6.33	47	44
6.2	6.43	6.48	56	49
6.4	6.57	6.62	56	48
6.6	6.77	6.79	53	49
6.8	6.94	6.98	54	44
7.0	7.15	7.20	53	43
7.2	7.31	7.43	50	40
7.4	7.63	7.62	49	39
7.6	7.66	7.84	46	36
7.8	7.92	8.13	48	36
8.0	7.95	8.30	36	28

change in pH for the remainder of the 2.5 hour run. Only at a pH of7.6 and above was there a noticeable pH drift during the 2.5 hour run. When the oxygen uptake readings were reported for a 0.5 to 2.5 hour run, the oxygen consumption values for the first half hour were not used. At all levels except pH 5.0 and 5.6 the readings for the 0 to 2 hour run were higher than the readings for the 0.5 to 2.5 hour run. The maximum variation in the endogenous uptake values was 20 $mm.^3$ oxygen for the 0 to 2 hour run and 28 $mm.^3$ oxygen for the 0.5 to 2.5 hour run. The most rapid decrease in the uptake values occurred at pH levels above pH 8.0, where the buffer capacity evidently was somewhat lacking. In general, the endogenous uptake values increased to maximum readings in the pH range of 6.0 to 6.6, and then the values decreased as the pH values increased.

The variations in endogenous uptake values over the entire pH range studied were small in comparison to the total oxygen uptake values obtained when the sodium salts of fatty acids were acted upon. Accordingly, in the subsequent runs involving various pH levels the endogenous uptake value was obtained at one intermediate pH level and this value used for correction for endogenous respiration over the entire pH range studied since the error introduced by the procedure undoubtedly was smaller than that which would have resulted had the values for the different pH levels been obtained in several runs, as would have been necessary because the large number of

simultaneous runs necessary to obtain simultaneous blanks at each pH could not have been made on the equipment available.

To make a run on the endogenous uptake values for Ps. fragi, the cells were grown on tryptone-glucose-extract agar slants for 24 hours at 21°C., harvested and the suspension prepared as outlined in "Methods". The endogenous uptake values were measured over a pH range of 7.20 to 9.10 for a 2.5 hour period. The pH was determined initially and at the end of a 2.5 hour run, and the uptake values reported for a 0 to 2 hour run and a 0.5 to 2.5 hour run. The data are presented in Table 6.

In the case of the borate buffer there was a maximum pH shift of only 0.06 pH unit between the initial pH and the final pH, and most values were much more constant. It should be pointed out, however, that it was impossible to check the pH values initially and finally beyond pH 8.5 with the quinhydrone potentiometer. Since only 3 ml. of reactants were available to be checked, the other available potentiometer could not be used. There was an increase in pH, as the reactants were mixed, which was more variable than for the citrate-phosphate buffer used with C. lipolytica. The endogenous uptake values were higher for the 0 to 2 hour run than for the 0.5 to 2.5 hour run at all pH levels. The maximum variation in the endogenous values from pH 7.20 to 9.10 was 8 mm^3 oxygen

Table 6

Endogenous respiration of Pseudomonas fragi cells.
(0.07M Palitzsch borate buffer
used in reaction flasks)
(Average of two trials)

Desired pH	pH of reaction		Oxygen uptake ($mm.^3$)	
	Initial	Final	0-2 Hr.	0.5-2.5 Hr.
6.77	7.14	7.20	19	12
7.09	7.47	7.45	20	13
7.36	7.69	7.67	20	14
7.60	7.88	7.88	20	15
7.78	8.03	8.03	19	13
8.08	8.23	8.23	21	16
8.31	8.38	8.40	20	14
8.41	8.44	8.47	22	16
8.51	- *	- *	17	12
8.60	- *	- *	17	13
8.84	- *	- *	15	11
9.11	- *	- *	13	10

*Beyond the effective pH range of the quinhydrone potentiometer.

for the 0 to 2 hour run and 6 mm^3 oxygen for the 0.5 to 2.5 hour run. For both runs, the uptake values were nearly constant from pH 7.2 to 8.4, with some decrease in values as the reaction went to pH 8.5 and beyond.

The endogenous uptake values for Ps. fragi were similar to the endogenous uptake values for C. lipolytica in that the variations over the entire pH range were small in comparison to the total oxygen uptake values when the sodium salts of fatty acids were oxidized. In the subsecuent runs involving various pH levels the endogenous uptake value was obtained at one intermediate pH level and this value used for correction for endogenous respiration over the entire pH range studied. The error introduced by this procedure undoubtedly was smaller than that which would have resulted had the values for each pH level been obtained in several runs. The equipment available made it impossible to make simultaneous runs at each pH level.

Effect of time interval selected on corrected oxygen uptake values

The previous trials have shown that the endogenous respiration was usually less for the 0.5 to 2.5 hour run than for the 0 to 2 hour run. The following runs were made to determine if these lower uptake values for the 0.5 to 2.5 hour run were reflected in the uptake values when the fatty acid

substrata were used. The cells of C. lipolytica and Ps. fragi were grown on separate tryptone-glucose-extract agar slants at 30°C. and 21°C., respectively, for 24 hours. The cells were harvested in the usual manner, and the cell suspensions then were used to determine the oxygen uptake values for the oxidation of all sodium salts of fatty acids from acetic through linoleic. The oxygen uptake values are reported for a 0 to 2 hour run and a 0.5 to 2.5 hour run. The data are presented in Table 7. The results indicate that the lower endogenous uptake values for the 0.5 to 2.5 hour run are reflected in higher corrected uptake values in most cases. When C. lipolytica cells were used, the corrected values were higher for the 0.5 to 2.5 hour run for every substrate used. When Ps. fragi cells were used the corrected values were higher for the 0.5 to 2.5 hour run for 8 of the 12 substrata used. Since the oxygen uptake values were higher for the 0.5 to 2.5 hour run in the majority of the cases, all subsequent trials will be reported on the basis of a 0.5 to 2.5 hour run.

Effect of incubation temperature on enzyme production by Ps. fragi

The temperature of incubation which was optimum for lipase production by C. lipolytica was 30°C., as reported by Peters (1947). This incubation temperature was arbitrarily set for

Table 7

The effect of the length of run upon the oxidizing ability of Candida lipolytica* and Pseudomonas fragi** cells.
(Average of two trials)

Substrate	Oxygen uptake ($mm.^3$)***			
	Candida Lipolytica		Pseudomonas fragi	
	0-2 Hr.	0.5-2.5 Hr.	0-2 Hr.	0.5-2.5 Hr.
Sodium acetate	67	79	10	13
Sodium propionate	72	86	6	11
Sodium butyrate	110	122	4	11
Sodium caproate	116	128	14	19
Sodium caprylate	127	134	34	37
Sodium caprate	181	198	38	35
Sodium laurate	57	85	37	33
Sodium myristate	182	197	47	45
Sodium palmitate	79	86	40	42
Sodium stearate	72	84	33	34
Sodium oleate	173	178	24	26
Sodium linoleate	164	174	37	34

*0.05M McIlvaine citrate-phosphate buffer at pH 6.2 used in the reaction flasks with Candida lipolytica.

**0.07M Palitzsch borate buffer at pH 8.4 used in the reaction flasks with Pseudomonas fragi.

***These values are corrected for endogenous respiration.

C. lipolytica when propagating cells for testing their oxidase activity. Since the optimum growth temperature of Ps. fragi is below 30°C., the arbitrary temperature used for cell growth was 21°C. However, since the temperature of the Warburg water bath was 30°C., it was desirable to determine whether cell growth at 21°C. or at 30°C. gave the maximum oxygen uptake values. The Ps. fragi were grown on tryptone-glucose-extract agar slants at 21°C. and at 30°C. for 24 hours. The cells were harvested and the cell suspensions prepared as outlined in "Methods". These suspensions were used to determine the amount of oxygen taken up when the sodium salts of the fatty acids from acetic through stearic were oxidized. The data are presented in Table 8.

In all cases the uptake values obtained from cells grown at 21°C. were higher than the values obtained from cells grown at 30°C. In most cases, the uptake values obtained from the 21°C. cells were approximately twice the values obtained from the 30°C. cells. For all subsequent runs with Ps. fragi, the cells were incubated at 21°C. for 24 hours before harvesting.

Effect of pH of reaction on the oxidation of fatty acid salts

After establishing the methods to be used, the optimum pH for oxidation by C. lipolytica of each of the sodium salts of the 12 fatty acids was determined. The 0.05M McIlvaine

Table 8

The effect of incubation at 21 and 30°C. during growth upon the oxidizing ability of Pseudomas fragi cells.
(0.07M Palitzsch borate buffer at pH 8.4 used in the reaction flasks)
(Average of two trials)

Substrate	Oxygen uptake ($mm.^3$)*	
	Cells grown at 21°C.	Cells grown at 30°C.
Sodium acetate	23	14
Sodium propionate	21	12
Sodium butyrate	15	4
Sodium caproate	23	10
Sodium caprylate	39	7
Sodium caprate	44	21
Sodium laurate	33	10
Sodium myristate	41	16
Sodium palmitate	41	14
Sodium stearate	34	31

*These values are corrected for endogenous respiration.

citrate-phosphate buffer was used over a pH range of 3.4 to 8.2. The pH values reported in Table 9 are the values of the actual reaction, rather than the rated pH values of the buffer.

The C. lipolytica cells were grown on tryptone-glucose-extract agar slants for 24 hours at 30°C., harvested and the suspensions prepared as outlined in "Methods". Each run was made with the same cell suspension, but using 12 different pH levels for each substrate tested. All oxygen uptake values were converted to percent of maximum figures, to aid in interpretation of results. The data obtained are presented in Table 9.

The maximum uptake values listed in the last column of this table show a gradual increase in uptake values to a maximum for the C_6 to the C_{14} fatty acid salts, followed by a decrease when C_{16} and C_{18} saturated fatty acid salts were used. The salt of the unsaturated fatty acid, oleic acid, gave the highest values obtained, while the salt of linoleic acid gave somewhat lower values.

The insolubility of the higher fatty acid salts at the pH used for the reaction undoubtedly accounted for the decreasing uptake values when palmitate and stearate were used. In a study of the effect of fatty acids on the oxygen uptake of Blastomyces dermatitidis, Bernheim (1942) also noted the effect of insolubility upon oxygen uptake, but also stated that

Table 9

The effect of the pH of the reaction system upon the oxidizing ability of Candida lipolytica cells. (0.05M McIlvaine citrate-phosphate buffer used in reaction flasks) (Average of two trials)

Substrate	Percent of maximum oxygen uptake at pH:													Max. oxygen uptake ($mm.^3$)*
	3.4	3.8	4.2	4.6	5.0	5.4	5.8	6.2	6.6	7.0	7.4	7.8	8.2	
Sodium acetate	95	93	100	91	76	83	76	74	75	65	48	25		110
Sodium propionate	73	100	85	83	81	74	87	71	76	66	51	57		114
Sodium butyrate	74	91	98	100	93	83	79	81	69	59	56	49		174
Sodium caproate	1	2	1	7	36	96	100	96	80	63	58	46		203
Sodium caprylate		-18	-15	-15	-12	-15	69	85	100	77	63	64	45	236
Sodium caprate		-24	-21	-20	-20	-16	16	43	100	96	85	72	64	204
Sodium laurate	73	82	92	100	90	93	52	12	45	45	30	33		187
Sodium myristate	66	73	83	86	84	87	96	100	100	98	96	58		218
Sodium palmitate	16	36	26	38	51	65	100	83	82	78	78	82		115
Sodium stearate	2	4	5	11	12	20	26	52	52	58	74	100		97
Sodium oleate	58	58	61	61	67	71	94	91	100	96	86	76		244
Sodium linoleate	62	72	75	77	75	86	95	100	83	81	73	73		190

*These values are corrected for endogenous respiration.

the soaps of higher fatty acids may cover the surface of the cells and thus decrease penetration of oxygen and the substrata into the cells.

When considering the optimum pH of the reaction all values above 95 percent of maximum were considered as the optimum pH range. The range of the optimum pH of reaction increased step-wise from acetate at pH 3.4 to 4.2 to caprylate at pH 6.6. In the case of the remaining higher fatty acid salts the optimum pH range was 5.8 to 6.6, with the exception that the oxidation of laurate was greatest at pH 4.6 and stearate at pH 7.8. The over-all range in pH optima for the 12 substrata was from 3.4 to 7.8. Inhibition of oxygen uptake by sodium caprylate and sodium caprate was noted by negative values below pH 5.8, whereas some oxidation of other substrata, except caproate and stearate, occurred at pH levels of 3.4 and above.

The low uptake values for the oxidation of sodium caproate and sodium stearate at the lower pH levels indicate a very low level of enzyme activity, or possibly no activity at all at these pH levels. The values are so low up to pH 4.6, that they might be within the limit of error of the method used. In most cases the values decreased somewhat more rapidly as the pH levels dropped progressively lower from the optimum pH than they did as the pH levels rose above the optimum pH level.

Levine and Novak (1949b), studying the effect of pH on the respiration of Blastomyces dermatitidis, found a similar trend for more rapid decline of values at pH levels below the optimum pH, than was observed with the values obtained at pH levels above the optimum pH. They found that when using sodium caprylate, respiration was inhibited below pH 6.0 but was stimulated at pH 6.0 and 8.0. This agrees quite closely with the present study, when inhibition occurred at pH 5.4 and below but definitely positive values were obtained in the pH range from 5.8 to 8.2. Levine and Novak stated that the stimulation by the longer chained fatty acids with an increase in pH was best explained as resulting from the accompanying decrease in the concentration of the undissociated fatty acid.

The stepwise pattern of the pH optima for the substrate up to and including caprate, and the relatively constant range of the pH optima for other substrata possibly may be explained partially by a decrease in the concentration of the undissociated fatty acid as the pH increases. However, no one factor probably is entirely responsible for this pattern, although the decreased solubility of the higher fatty acids may also be a factor. The decline in values observed at high pH levels when sodium laurate was used as the test substrate appears to be a peculiarity of the compound itself, which makes it inhibitory at the higher pH levels. Repeated trials

with sodium laurate always gave similar results when C. lipolytica cells were used.

Two test substrata were selected for subsequent runs that had an optimum pH of reaction approximately the same, had a rather wide range in the molecular weights, and gave relatively high oxygen uptake values. Sodium caproate and sodium myristate were chosen to be used at pH 6.2.

To determine the effect of the pH on oxygen uptake in the presence of the sodium salts of the 12 test fatty acids by Ps. fragi, a 0.07M Palitzsch borate buffer covering a pH range of 7.2 to 9.1 was used. The pH values reported are the values of the actual reaction, rather than the rated pH values of the buffer. The Ps. fragi cells were grown on tryptone-glucose-extract agar slants at 21°C. for 24 hours, harvested and the suspension prepared as outlined in "Methods". For each substrate tested the same cell suspension was used at 12 different pH levels. To aid in interpretation of results, all oxygen uptake values were converted to percent of maximum figures. The data obtained are presented in Table 10.

The maximum uptake values obtained by using Ps. fragi cells showed relatively constant values for all substrata, with the exception that the values were lower when caproate, oleate and linoleate were used. All values of 95 percent of maximum or over are considered to be the range of optimum pH for the

Table 10

The effect of the pH of the reaction system upon the oxidizing ability of Pseudomonas fragi cells.
(0.07M Palitzsch borate buffer used in reaction flasks)
(Average of two trials)

Substrate	Percent of maximum oxygen uptake at pH:												Max. oxygen uptake (mm.3)*
	7.20	7.45	7.70	7.90	8.00	8.25	8.40	8.45	8.50	8.60	8.80	9.10	
Sodium acetate	64	86	88	88	100	88	83	71	69	69	26	- 2	42
Sodium propionate	70	96	100	100	93	96	76	62	56	62	42	13	45
Sodium butyrate	48	54	56	98	100	100	67	65	50	31	-13	4	48
Sodium caproate	70	83	90	93	97	100	79	70	60	60	38	17	29
Sodium caprylate	40	53	60	66	72	86	88	100	81	70	70	57	58
Sodium caprate	51	69	76	80	79	91	98	98	100	89	69	24	45
Sodium laurate	71	84	86	88	88	96	91	95	95	100	93	82	56
Sodium myristate	70	70	73	88	88	88	88	100	93	90	92	90	60
Sodium palmitate	46	84	87	92	97	100	97	95	93	90	87	67	61
Sodium stearate	61	82	77	84	90	100	100	100	93	93	91	90	44
Sodium oleate	58	62	73	81	81	92	92	100	92	85	73	62	26
Sodium linoleate	47	62	65	74	74	100	97	91	88	91	82	60	34

*These values are corrected for endogenous respiration.

substrata tested. The optimum pH values obtained when using Ps. fragi cells followed a pattern similar to the values obtained from C. lipolytica cells, as seen in Table 9. However, the optimum pH range is displaced upward from pH 3.4 to 7.8 in the case of C. lipolytica cells, to pH 8.00 to 8.45 for Ps. fragi cells. The lower oxygen uptake values for acetate, propionate, butyrate and caproate at the high pH levels probably are associated with the fact that the pH optima for these substrata are reached at lower levels than the pH optima for caprylate and the higher fatty acids.

With the exception of sodium acetate with an optimum at pH 8.00, the optimum range for all other substrata is from pH 8.25 to 8.45. The step-wise increase is not as noticeable with Ps. fragi cells, because the pH range over which the enzyme is active is not as great as in the case of the C. lipolytica cells. This pattern may be explained by the fact that at the pH levels used with Ps. fragi the amount of undissociated fatty acids would be very small. At the pH levels used, solubility should not play any appreciable part in affecting oxygen uptake. This appears to be borne out because the higher molecular weight fatty acids are quite readily oxidized.

Sodium caproate and sodium myristate also were selected as test substrata for subsequent runs at pH 8.4.

Effect of pH of cell growth on enzyme production

The effect which the pH of the growth medium might have upon the oxidizing ability of the microorganisms used, was next studied. Tryptone-glucose-extract agar was prepared as outlined in "Methods", and adjusted to a pH range from 3.8 to 7.8. After the agar slants were prepared, they were inoculated with C. lipolytica cells and incubated at 30°C. for 24 hours. The cells were harvested and the cell suspensions prepared as outlined in "Methods". The resulting suspensions then were tested, using sodium caproate and sodium myristate as substrata. The data obtained are presented in Table 11.

The endogenous uptake values and the values obtained from sodium myristate were somewhat higher in trial 2 than in trial 1, in most cases. The same pattern of values for both trials prevailed, except that all values from the cells grown at pH 6.4 in trial 1 were rather low.

The average results indicate that if the growth is at pH 3.8 to 4.4, optimum production by C. lipolytica of oxidases active on both the test substrata is attained. The decrease in the uptake values as the pH increased, in trial 1, was more rapid with sodium caproate than with sodium myristate, although the differences are comparatively slight up to approximately pH 6.9 and the decline in values is not always consistent.

Table 11

The effect of the pH of the growth medium upon the oxidizing ability of Candida lipolytica cells. (0.05M McIlvaine citrate-phosphate buffer at pH 6.2 used in reaction flasks)

pH of growth medium**	Oxygen uptake ($mm.^3$)								
	Endogenous			Na caproate*			Na myristate*		
	1	2	Av.	1	2	Av.	1	2	Av.
3.8	-	38	-	-	151	-	-	220	-
4.4	29	37	33	133	156	145	243	202	223
5.0	30	43	37	115	118	117	199	222	211
5.6	30	33	32	111	113	112	173	243	208
5.9	32	29	31	97	92	95	163	203	183
6.4	24	27	26	54	113	84	124	216	170
6.7	30	25	28	76	95	86	168	199	184
6.9	31	26	29	85	71	78	148	189	169
7.1	25	-	-	47	-	-	141	-	-

*These values are corrected for endogenous respiration.

**Tryptone-glucose-extract agar medium used.

Since this portion of the investigation was conducted at a late date, media adjusted to pH 6.8 had been used for growth of organisms in all other portions of this study. Conceivably, the adjustment of the pH of the growth medium would have given higher oxygen uptake values, if it had been done for all phases of this study.

To test the effect of the pH of the growth medium on fatty acid oxidase production by Ps. fragi, tryptone-glucose-extract agar slants were prepared as outlined in "Methods". The slants were inoculated with the Ps. fragi cells, incubated at 21°C. for 24 hours, harvested and the cell suspension prepared as outlined in "Methods". The resulting suspensions were tested on sodium caproate and sodium myristate. The data are presented in Table 12.

The uptake values for both trials 1 and 2 seemed to be nearly the same in all cases. Cells grown at pH 4.4 did not possess as strong oxidizing ability as the cells grown at the higher pH levels. All uptake values from cells grown at pH 5.0 to pH 7.8 for both test substrata showed that there was no sharp optimum pH for growth or production of maximum oxidizing ability. The Ps. fragi cells appeared to be able to grow well within the pH range of 5.0 to 7.8, and possessed nearly equal oxidizing abilities in all cases. This work might conceivably be criticized because the agar was not buffered more adequately at the various pH levels. There

Table 12

The effect of the pH of the growth medium upon the oxidizing ability of Pseudomonas fragi cells. (0.07M Palitzsch borate buffer at pH 8.4 used in reaction flasks)

pH of growth medium **	Oxygen uptake ($mm.^3$)								
	Endogenous			Na caproate *			Na myristate *		
	1	2	Av.	1	2	Av.	1	2	Av.
4.4	15	17	16	10	8	9	18	20	19
5.0	-	16	-	-	19	-	-	34	-
5.6	17	17	17	22	28	25	31	36	34
5.9	-	15	-	-	19	-	-	36	-
6.4	15	20	18	25	14	20	40	33	37
6.7	17	19	18	17	14	16	36	31	34
6.9	13	-	-	19	-	-	32	-	-
7.1	14	19	17	20	19	20	36	33	35
7.5	15	-	-	22	-	-	36	-	-
7.8	15	17	16	17	19	18	32	37	35

*These values are corrected for endogenous respiration.

**Tryptone-glucose-extract agar medium used.

may have been a slight localized shift in pH at the lower pH levels, which permitted the scant growth to occur in media adjusted to pH 4.4. Since there was no apparent optimum pH for production of this enzyme during the growth of Ps. fragi cells, it would not have been advantageous to adjust the pH of the standard growth medium to other than pH 6.8 - 7.2, the reaction employed whenever Ps. fragi cells were grown.

Enzyme production on synthetic media

After the study of the pH optima for C. lipolytica and Ps. fragi, it was desirable to study the effect of added nutrients upon the oxidizing ability of these two micro-organisms. The selection of a suitable defined synthetic basal medium for both C. lipolytica and Ps. fragi was necessary before this portion of the investigation could proceed. Two synthetic media, No. 1 and No. 2 as listed in "Methods", were used to determine their suitability for further use. These liquid media were prepared in 200 ml. quantities, sterilized at 250°F. for 15 minutes and separate flasks inoculated with C. lipolytica and Ps. fragi cells. After incubation at 30°C. and 21°C., respectively, for 24, 48, 72 and 96 hours, plate counts of the growth of the two cultures were made at each 24 hour interval. The data are presented in Table 13.

Table 13

Plate counts of cultures grown on synthetic medium No. 1 and synthetic medium No. 2. (Trial 1)

Length of incubation (hours)	Plate count (per ml.)			
	Candida lipolytica		Pseudomonas fragi	
	Medium 1	Medium 2	Medium 1	Medium 2
24	3,700,000	4,100,000	12,000,000	2,400,000
48	4,000,000	3,100,000	13,000,000	5,300,000
72	5,800,000	3,900,000	14,000,000	6,800,000
96	8,100,000	5,700,000	12,000,000	8,000,000

The plate counts indicate that medium No. 1 gave higher counts than medium No. 2 for both C. lipolytica and Ps. fragi, with the exception that the count was slightly higher after 24 hours in medium No. 2 with C. lipolytica. However, this advantage was very slight, and was within the limit of error of the plate counting procedure. Since medium No. 2 contained a precipitate after sterilization, it was undesirable to use because the precipitate would be removed with the cells when centrifuged. The presence of this precipitate might give faulty Klett-Summerson colorimeter readings or conceivably could influence endogenous respiration if the precipitate was of nutritive character. Since medium No. 1 gave the higher counts and contained no precipitate, it was selected for use in subsequent trials.

Peters and Nelson (1948) reported in a study of the nutritional requirements for the production of lipase of C. lipolytica that thiamin was the only growth factor of those studied which stimulated lipase production. Other growth factors studied were biotin, nicotinic acid and pantothenic acid. Amounts of these growth factors up to 1,200 μg. per liter did not increase the lipase production linearly, and amounts of 200 μg. per liter gave satisfactory results, as reported by Peters (1949). To study the effect which the growth factors may have on oxidase production, thiamin and riboflavin were added to medium No. 1 in varying amounts.

Medium No. 1 was prepared and the growth factors added to separate flasks at the following rates: (a) 200 μg. thiamin per liter, (b) 200 μg. riboflavin per liter, (c) 150 μg. thiamin, 50 μg. riboflavin per liter and (d) 50 μg. thiamin, 150 μg. riboflavin per liter. A flask containing medium 1 with no added growth factors was used as a control.

A set of flasks containing the above media was inoculated with C. lipolytica, incubated at 30°C. for 24 hours, and plate counts made. The cells were harvested and the cell suspensions prepared as outlined in "Methods". The resulting suspensions were used with sodium caprate as the substrate. The data are presented in Table 14.

The plate counts of trial 1 and trial 2 showed quite close agreement, with the exception that the count on the synthetic medium alone was lower on trial 2 than on trial 1 and the count on the synthetic medium plus 150 μg. thiamin, 50 μg. riboflavin per liter was lowest on trial 1. The same general pattern existed for both trials with the addition of thiamin to the medium giving the higher counts, the synthetic medium with no added growth factors giving very low counts and the counts for all other media used being quite close and intermediate between the extremes.

The oxygen uptake values for trial 1 were somewhat higher than those obtained in trial 2. The values for all five media tested showed rather close agreement, when each trial was compared separately. One would expect rather close agreement

Table 14

The effect of growth factors added to a synthetic basal medium upon the plate counts and oxidizing ability of Candida lipolytica. (0.05M McIlvaine citrate-phosphate buffer at pH 6.2 used in reaction flasks)

Growth media	Plate count (per ml.)		Oxygen uptake ($mm.^3$)*	
	Trial 1	Trial 2	Trial 1	Trial 2
Medium 1	790,000	290,000	233	154
Medium 1 + thiamin (200 µg./l.)	5,300,000	6,000,000	209	135
Medium 1 + riboflavin (200 µg./l.)	4,000,000	4,000,000	240	151
Medium 1 + thiamin (150 µg./l.) + riboflavin (50 µg./l.)	2,300,000	5,000,000	215	181
Medium 1 + thiamin (50 µg./l.) + riboflavin (150 µg./l.)	3,200,000	3,900,000	182	164

*These values are corrected for endogenous respiration.

since all suspensions were adjusted to the same cell concentration with the photoelectric colorimeter. Since the uptake values showed little advantage for any specific medium, but the plate count was highest when 200 µg. of thiamin per liter was added to medium 1, the use of thiamin in this concentration was continued for all subsequent trials when the synthetic medium was used.

A similar run was conducted using Ps. fragi in the synthetic media (No. 1) just described. All flasks were inoculated with Ps. fragi, incubated at 21°C. for 24 hours and plate counts made. The cells were harvested, the cell suspensions prepared as outlined in "Methods" and the oxidizing ability determined using sodium caprate as the substrate. The data are presented in Table 15.

The plate counts for trials 1 and 2 checked quite well, although there was some slight variation in the pattern of counts within each individual trial. The counts in both trials were highest when medium No. 1 was used with no added growth factors, the other four media used having lower counts in all cases. The oxygen uptake values showed good agreement between trials 1 and 2, except that when thiamin and riboflavin were used separately the values were somewhat erratic. In four of five cases the uptake values were higher for trial 2 than for trial 1. Since medium No. 1, with no added growth factors, gave the highest plate count and gave uptake values nearly the same as all other media, it was selected for subsequent work.

Table 15

The effect of growth factors added to a synthetic basal medium upon the plate counts and oxidizing ability of *Pseudomonas fragi*. (0.07M Palitzsch borate buffer at pH 8.4 used in reaction flasks)

Growth media	Plate count (per ml.)		Oxygen uptake (mm.3)*	
	Trial 1	Trial 2	Trial 1	Trial 2
Medium 1	15,000,000	17,000,000	11	15
Medium 1 + thiamin (200 µg./l.)	10,000,000	11,000,000	6	26
Medium 1 + riboflavin (200 µg./l.)	12,000,000	12,000,000	10	21
Medium 1 + thiamin (150 µg./l.) + riboflavin (50 µg./l/)	9,000,000	15,000,000	7	10
Medium 1 + thiamin (50 µg./l.) + riboflavin (150 µg./l.)	9,300,000	6,100,000	14	14

*These values are corrected for endogenous respiration.

Additional runs using synthetic medium No. 1 for Ps. fragi showed that the oxygen uptake values obtained were so nearly the same as the endogenous uptake values, that they did not show large enough values to give satisfactory results. Therefore, a more complete synthetic medium had to be found which would provide cells of Ps. fragi with adequate oxidizing ability. The synthetic medium proposed for the enterococci by Niven and Sherman (1944) proved to be suitable for this microorganism. A series of runs was made comparing the oxidizing ability of cells grown on synthetic medium No. 1, Niven's synthetic medium and tryptone-glucose-extract medium. All media were prepared as agar slants, inoculated with Ps. fragi cells and incubated at 21°C. for 24 hours. After incubation the cells were harvested and the cell suspensions prepared according to "Methods". The resulting cell suspensions were used with sodium caproate and sodium myristate as test substrata. The data obtained are reported in Table 16.

The uptake values obtained from trials 1 and 2 compared favorably for endogenous respiration and the oxidation of both test substrata. Endogenous uptake values were nearly the same for all media used. Oxygen uptake values obtained from synthetic medium No. 1 were very low for either substrate. In fact, the values obtained with sodium myristate were smaller than endogenous values, giving negative corrected values. Niven's medium proved to be satisfactory since cells grown on it gave

Table 16

The effect of growth in different media upon the oxidizing ability of Pseudomonas fragi cells. (0.07M Palitzsch borate buffer at pH 8.4 used in reaction flasks)

Growth media	Oxygen uptake ($mm.^3$)								
	Endogenous			Na caproate*			Na myristate*		
	1	2	Av.	1	2	Av.	1	2	Av.
Synthetic medium 1 + agar (2.5%)	17	13	15	6	2	4	-11	-7	-9
Nivens medium + agar (2.5%)	21	17	19	14	14	14	37	38	38
Tryptone-glucose-extract agar	17	17	17	25	32	29	45	60	53

*These values are corrected for endogenous respiration.

quite high uptake values with both test substrata. The uptake values with caproate and myristate which were obtained from cells grown on tryptone-glucose-extract agar were higher than the values from either of the other two media. Since Niven's medium yielded satisfactory uptake values it was used in all subsequent trials when a synthetic medium was needed for Ps. fragi.

Effect of complex nutrients on enzyme production

With suitable synthetic media chosen, trials were run to determine the effects of various complex nutrients, fatty acids and monoglycerides in the growth media upon the oxygen uptake values in the presence of the sodium salts of fatty acids.

The effect which various addenda to the synthetic medium may have upon the oxidizing ability of C. lipolytica next was determined. Synthetic medium No. 1 was prepared, thiamin (200 µg./l.) and agar (2.5%) were added and the mixture sterilized at 250°F. for 13 to 15 minutes. After sterilization and partial cooling of the medium, measured portions of the sterile stock solutions of the complex nutrients were added, using aseptic technics. The agar and added nutrients were mixed well and the agar allowed to harden to form large slants. A tryptone-glucose-extract agar slant was used for comparison.

The prepared agar slants were inoculated with C. lipolytica cells, incubated at 30°C. for 24 hours, the cells harvested and the cell suspensions prepared as outlined in "Methods". The

resulting suspensions were used with sodium caproate and sodium myristate as substrata. The data obtained are presented in Table 17.

In general, the uptake values for trials 1 and 2 compared quite favorably for the endogenous respiration and the oxidation of the test substrata. The endogenous uptake values were low in trial 2 for the cells in the presence of added peptone and casein hydrolysate, and these low values were reflected in low values on the test substrata. These low values for both endogenous respiration and the oxidation of the substrata would indicate that the cells possessed lesser oxidizing ability, rather than faulty operation of the Warburg apparatus. The results on the two test substrata indicate that the uptake values were higher when the test addenda were added to the medium. This increase was not great in some cases, as shown by values obtained when yeast extract was used. However, when skimmilk, casein hydrolysate or beef extract was added, the oxygen uptake values were appreciably higher than for the control medium (A). A comparison of cells grown on individual lots of agar containing cream and skimmilk showed appreciably higher uptake values when tested on caproate and myristate, indicating that butterfat in the medium causes increased fat-oxidizing activity of the resultant cells of C. lipolytica. Cells grown on tryptone-glucose-extract agar yielded the highest uptake values in all

Table 17

The effect of some added nutrients in the growth medium upon the oxidizing ability of Candida lipolytica cells. (0.05M McIlvaine citrate-phosphate buffer at pH 6.2 used in reaction flasks)

Growth media	Oxygen uptake ($mm.^3$)								
	Endogenous			Na caproate*			Na myristate*		
	1	2	Av.	1	2	Av.	1	2	Av.
A. Synthetic medium 1 + thiamin (200 μg./l.) + agar (2.5%)	72	41	57	43	29	36	132	101	117
B. Medium A + skimmilk (5%)	61	56	59	64	58	61	140	156	148
C. Medium A + cream (5%)	69	54	62	92	88	90	189	194	192
D. Medium A + peptone (0.5%)	74	17	46	69	47	58	179	111	145
E. Medium A + casein hydrolysate (0.5%)	99	33	66	87	41	64	163	108	136
F. Medium A + beef extract (0.5%)	74	49	62	52	64	58	139	164	152
G. Medium A + yeast extract (0.5%)	52	40	46	43	45	44	112	161	137
H. Tryptone - glucose - extract agar	69	33	51	112	100	106	183	226	205

*These values are corrected for endogenous respiration.

cases, with the exception that in trial 1 the value obtained from cells grown on medium C gave slightly higher uptake values with sodium myristate.

Jezeski (1947b, 1948) reported that the addition of yeast extract to the medium had no significant effect upon the oxidizing ability of microorganism, an observation which was substantiated in the present investigation. Results in the present study differ from those obtained by Jezeski because he used nutrient agar plus yeast extract, while in the present investigation a simple synthetic basal medium to which yeast extract had been added was used. If Jezeski had used a simpler basal medium and added nutrients other than yeast extract in his study, he might have obtained increases in uptake values in the presence of addenda, and thus would not have reached the conclusion that the addition of other nutrients had no effect upon oxygen uptake.

In a similar run using Ps. fragi the media were prepared as in the preceding run, with the exception that Niven's medium was substituted for medium No. 1 as the control and basal medium, and no thiamin was added. The prepared slants were inoculated with Ps. fragi cells, incubated at 21°C. for 24 hours, harvested and the cell suspensions prepared as outlined in "Methods". The cell suspensions were tested on sodium caproate and sodium myristate. The data are presented in Table 18.

The uptake values for trials 1 and 2 were slightly erratic

Table 18

The effect of some added nutrients in the growth medium upon the oxidizing ability of Pseudomonas fragi cells. (0.07M Palitzsch borate buffer at pH 8.4 used in reaction flasks)

Growth media	Oxygen uptake ($mm.^3$)								
	Endogenous			Na caproate*			Na myristate*		
	1	2	Av.	1	2	Av.	1	2	Av.
A. Nivens medium + agar (2.5%)	17	29	23	14	21	18	38	45	42
B. Medium A + skimmilk (5%)	17	28	23	12	21	17	39	39	39
C. Medium A + cream (5%)	17	30	24	11	17	14	33	34	34
D. Medium A + peptone (0.5%)	16	21	19	13	20	17	36	10	23
E. Medium A + casein hydrolysate (0.5%)	20	14	17	6	14	10	24	19	22
F. Medium A + beef extract (0.5%)	13	15	14	15	3	9	41	43	42
G. Medium A + yeast extract (0.5%)	17	17	17	12	11	12	32	32	32
H. Tryptone-glucose-extract agar	17	7	12	32	6	19	60	46	53

*These values are corrected for endogenous respiration.

for endogenous respiration as well as for the oxidation of caproate and myristate. Low values were obtained with cells grown on casein hydrolysate medium when used with caproate in trial 1, and from cells grown on beef extract and tryptone-glucose-extract agar media and used with caproate in trial 2. Endogenous uptake values for cells grown on tryptone-glucose-extract agar were low in trial 2.

The uptake values obtained from cells grown on media containing the addenda usually were less than those obtained from cells grown on Niven's synthetic medium. This might be expected, since Niven's synthetic medium contains 13 amino acids and eight growth factors which should make it a rather complete medium for an organism with basically very simple growth requirements. The values obtained in this run seem to substantiate this fact, because cells grown on Niven's medium give results rather close to the values obtained with cells grown on tryptone-glucose-extract agar.

Cells grown on a medium containing cream gave uptake values which were lower than those obtained from cells grown on a medium containing skimmilk. Since the Ps. fragi strain used was non-lipolytic when tested by the nile blue sulphate technic, the lower uptake values probably are attributable to the failure to free fatty acids and thus the absence of fatty acids to stimulate production of enzymes to oxidize such acids.

Effect of fatty acids on enzyme production

To test the effect of added free fatty acids to the growth medium, the synthetic medium No. 1 was prepared, thiamin (200 μg./l.) and agar (2.5%) added, the medium sterilized and the test fatty acids added just prior to solidification of the agar. The C. lipolytica cells were inoculated onto the agar slants, incubated at 30°C. for 24 hours, harvested and the cell suspensions prepared as listed in "Methods". In addition to the usual test substrata, caproate and myristate, the substrata homologous to the acids added to the growth medium also were used, including acetate, propionate and butyrate. The data obtained are presented in Table 19.

In most cases the uptake values obtained in trials 1 and 2 were in close agreement. In trial 2 the C. lipolytica cells failed to grow in the presence of caproic acid so the values from trial 1 are all that are available for comparison. The results indicate that in all cases when the cells were grown in the presence of added fatty acids, the uptake values obtained with homologous substrata showed a considerable increase over the values for cells grown on the control medium (A). Furthermore the uptake values from cells grown in the presence of added fatty acids were higher than the values obtained from cells grown on tryptone-glucose-extract agar when the cells were tested against the homologous substrata, with the exception that the values from cells grown in the presence of acetic acid

Table 19

The effect of fatty acids in the growth medium upon the oxidizing ability of Candida lipolytica cells. (0.05M McIlvaine citrate-phosphate buffer at pH 6.2 used in reaction flasks)

Growth media	Oxygen uptake(mm.^{3})																	
	Endogenous			Na acetate*			Na propionate *			Na butyrate*			Na caproate*			Na myristate*		
	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.
A. Synthetic medium 1 + thiamin (200µg./l.)+ agar (2.5%)	29	52	56	73	47	60	60	47	54	56	54	55	62	62	62	165	171	168
B. Medium A + acetic acid (0.01M)	71	53	62	84	71	78							85	59	72	200	202	201
C. Medium A + propionic acid (0.01M)	69	57	63				90	101	96				119	91	105	264	306	285
D. Medium A + butyric acid (0.01M)	54	39	47							170	73	122	138	100	119	330	353	342
E. Medium A + caproic acid (0.01M)	53	**	-										149	**	-	319	**	-
F. Tryptone-glucose-extract agar	54	37	46	104	65	85	92	60	76	83	70	77	139	56	98	234	168	201

*These values are corrected for endogenous respiration.

**Caproic acid in the growth medium was inhibitory to C. lipolytica in trial 2.

tended to be slightly lower. With both the caproate and myristate test substrata there was a progressive increase in uptake values as the chain length of the added fatty acid increased from acetic to butyric, with indications of a levelling-off when caproic acid was used. Using sodium caproate as the test substrate, cells grown on media containing added propionic, butyric or caproic acid gave higher uptake values in one of the two series than the values obtained from cells grown on tryptone-glucose-extract agar slants. With sodium myristate as the test substrate, the uptake values obtained from cells grown on media containing added propionic, butyric or caproic acid were higher than those obtained from cells grown on tryptone-glucose-extract agar slants; the results when acetic acid was added are inconclusive in this respect.

To test the effect of added fatty acids to a defined growth medium for Ps. fragi the media were prepared in a manner similar to the preceding run; Niven's medium without added thiamin was used. The fatty acids were added to the sterilized medium just prior to solidification of the agar. The slants were inoculated with Ps. fragi cells, incubated at 21°C. for 24 hours, the cells harvested and the cell suspensions prepared as outlined in "Methods". The resulting cell suspensions were used on the usual test substrata, caproate and myristate, as well as the substrata homologous to the added fatty acids, including acetate, butyrate and propionate. The data obtained are presented in Table 20.

Table 20

The effect of fatty acids in the growth medium upon the oxidizing ability of Pseudomonas fragi cells. (0.07M Palitzsch borate buffer at pH 8.4 used in reaction flasks)

Growth media	Oxygen uptake (mm.3)																	
	Endogenous			Na acetate*			Na propionate*			Na butyrate*			Na caproate*			Na myristate*		
	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.
A. Nivens medium + agar (2.5%)	20	17	19	33	12	23	15	8	12	10	6	8	12	12	12	40	26	33
B. Medium A + acetic acid (0.005M)	22	19	21	37	24	31							12	15	14	34	31	33
C. Medium A + propionic acid (0.005M)	20	20	20				58	20	39				13	14	14	43	16	30
D. Medium A + butyric acid (0.005M)	21	18	20							41	5	23	13	0	7	45	12	29
E. Medium A + caproic acid (0.005M)	18	13	16										17	8	13	43	26	35
F. Tryptone-glucose-extract agar	18	11	15	16	8	12	12	1	7	4	-3	1	28	15	22	36	14	25

*These values are corrected for endogenous respiration.

The results from trials 1 and 2 indicate that in most cases the uptake values for trial 2 were lower than those obtained in trial 1. Some of the uptake values in trial 2 may be of questionable value since they are so nearly the same as the endogenous uptake values. The results show that cells grown on media containing acetic, propionic and butyric acids give higher uptake values, when tested with the homologous substrata, than cells grown on the synthetic basal control medium (A). In all cases, these values also were higher than the uptake values obtained when cells grown on tryptone-glucose-extract agar were tested against the same substrata. Using the usual test substrata, caproate and myristate, no appreciable increase in uptake values was noted as the result of the addition of fatty acids to the basal synthetic medium.

Effect of monoglycerides on enzyme production

To determine the effect which growth in the presence of fatty acid monoglycerides might have on fatty acid oxidation, the following runs were made using acetic and butyric acids and their corresponding monoglycerides. For C. lipolytica, synthetic medium No. 1 was prepared, thiamin (200 µg./l) and agar (2.5%) added, the medium sterilized and the sterile test fatty acids and monoglycerides added just prior to solidification of the agar. The C. lipolytica cells were inoculated onto the slants, incubated at 30°C. for 24 hours, harvested and the

cell suspensions prepared as listed in "Methods". The cell suspensions were tested on sodium acetate and sodium butyrate, these fatty acid radicals being components of the monoglycerides which were used. Sodium caproate and sodium myristate, the usual test substrata, also were used as substrata for oxidation by the cell suspensions. The data obtained are presented in Table 21.

The results indicate that the uptake values for trials 1 and 2 agreed quite favorably, although the values for trial 2 were somewhat lower than those of trial 1 in most cases. The uptake values were higher for both trials when the cells grown on the media containing added fatty acids and tested against the homologous substrata were compared with the values obtained from cells grown on the control medium (A) and the same test substrata used. This relationship compares with the results presented in Table 19. In all cases the values obtained from cells grown in the presence of added fatty acids gave higher uptake values for all substrata tested. The advantage was slight when the acetic acid was present in the growth medium, but a marked increase in activity on all substrata tested was observed when butyric acid was added to the growth medium.

The uptake values obtained from cells grown in the presence of monoacetin were lower than the values obtained from cells grown on the control medium (A) when tested on acetate in trial 1 but were slightly higher in trial 2. Growth in the presence

Table 21

The effect of fatty acids and monoglycerides in the growth medium upon the oxidizing ability of Candida lipolytica cells. (0.05M McIlvaine citrate-phosphate buffer at pH 6.2 used in reaction flasks)

Growth media	Oxygen uptake ($mm.^3$)														
	Endogenous			Na acetate*			Na butyrate*			Na caproate*			Na myristate*		
	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.
A. Synthetic medium 1+ thiamin (200 μg./l.) + agar (2.5%)	52	43	48	48	37	43	65	63	64	53	46	50	120	112	116
B. Medium A + acetic acid (0.01M)	52	46	49	60	44	52				56	49	53	125	116	121
C. Medium A + monoacetin (0.01M)	60	39	50	32	45	39				45	69	57	74	133	104
D. Medium A + butyric acid (0.01M)	54	38	46				208	130	169	147	83	115	327	252	290
E. Medium A + monobutyrin (0.01M)	55	45	50				63	55	59	56	58	57	187	177	182
F. Tryptone-glucose-extract agar	62	36	49	77	91	84	101	76	89	131	141	136	222	187	205

*These values are corrected for endogenous respiration.

of monoacetin had no effect on ability to oxidize caproate and myristate.

When monobutyrin was present in the medium, the cells gave lower values than the control values with butyrate, but stimulation of action on caproate and myristate was obtained in all cases. The increase was very slight when caproate was used, but a rather marked increase was noted when myristate was used.

The only uptake values which exceeded the values from cells grown on tryptone-glucose-extract agar were those values obtained from cells grown in the presence of butyric acid, using butyrate and myristate as test substrata in both trials, and in trial 1 with caproate as the test substrate.

For Ps. fragi, Niven's medium was prepared, the agar (2.5%) added and the medium sterilized. After sterilization and partial cooling, the sterile fatty acid and monoglyceride solutions were added and the agar allowed to solidify. The slants were inoculated with Ps. fragi cells, incubated at 21°C. for 24 hours, the cells harvested and the suspensions prepared as listed in "Methods". The cell suspensions were tested with sodium acetate, sodium butyrate, sodium caproate and sodium myristate. The data obtained are presented in Table 22.

The uptake values obtained in trials 1 and 2 compared favorably although the values were slightly lower in most cases for trial 2, with the values obtained from cells grown on

Table 22

The effect of fatty acids and monoglycerides in the growth medium upon the oxidizing ability of Pseudomonas fragi cells.
(0.07M Palitzsch borate buffer at pH 8.4 used in reaction flasks)

Growth media	Oxygen uptake (mm.3)														
	Endogenous			Na acetate*			Na butyrate*			Na caproate*			Na myristate*		
	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.
A. Nivens medium + agar (2.5%)	24	14	19	32	18	25	9	9	9	10	13	12	37	31	34
B. Medium A + acetic acid (0.005M)	17	14	16	27	33	30				13	17	15	37	38	38
C. Medium A + monoacetin (0.005M)	15	14	15	24	27	26				16	15	16	38	26	32
D. Medium A + butyric acid (0.005M)	18	19	19				39	18	29	14	9	12	47	29	38
E. Medium A + monobutyrin (0.005M)	18	23	21				10	8	9	15	11	13	41	33	37
F. Tryptone-glucose-extract agar	17	12	15	38	13	26	8	1	5	36	22	29	67	63	65

*These values are corrected for endogenous respiration.

tryptone-glucose-extract agar being considerably lower in trial 2 when acetate and butyrate were used as test substrata. Using the homologous substrata with cells grown in the presence of added fatty acids, the uptake values were higher than the values obtained from cells grown on the control medium (A), with the exception that in trial 1 the cells grown in the presence of acetic acid gave slightly lower values than the control cells. In most cases when caproate and myristate were used as substrata, the cells grown in the presence of fatty acids gave uptake values in excess of the control values. An exception was the lower values in trial 2, when cells grown in the presence of butyric acid were tested with these substrata.

Cells grown in the presence of monoacetin gave values lower than the control values in trial 1, but values in excess of the control values in trial 2, when tested on sodium acetate. Testing on caproate and myristate, cells grown in the presence of monoacetin gave values probably not sufficiently different from the control values to establish any real differences in activity. When monobutyrin was used in the growth medium the cells grown thereon gave results similar to those obtained with the control cells when sodium butyrate was the test substrate. When caproate and myristate were the test substrata used, the uptake values were so nearly the same as those for the controls that no real differences can be considered to exist.

The presence of monoglycerides in the growth medium

appeared to have little or no effect on the oxidase production by Ps. fragi. By adding butyric acid to the growth medium the uptake values obtained on the homologous substrate were increased approximately three-fold when compared to the uptake values from the cells grown on control medium (A). This enhancement by cells grown in the presence of butyric acid was also noted in the results listed in Table 20.

DISCUSSION

Selected strains of C. lipolytica and Ps. fragi were used to test the oxidation of the sodium salts of acetic, propionic, butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, and linoleic acids, using the Warburg respirometer to measure oxygen uptake in the presence of resting cells. The use of pure fatty acids was preferred to the use of natural fats or triglycerides as substrata because the presence of a glycerol component might yield misleading results due to its oxidation. Furthermore, if fats or monoglycerides were used, the lipolytic ability of the organisms tested would be the limiting factor, in some instances, in making the fatty acids available for oxidation.

The buffers selected for use in the reaction flasks of the Warburg apparatus were chosen on the basis that they would cover a wide pH range, that they were not highly toxic to the micro-organisms used and that they would maintain the pH relatively constant at each pH level tested for a 2.5 hour run.

When using a 0.05M McIlvaine citrate-phosphate buffer, the action of C. lipolytica cells upon the 12 test substrata previously listed, indicated the optimum range of reaction to be pH 3.4 to pH 7.8, with the optima for the majority of the substrata falling within the range pH 5.8 to 6.6, as seen

in Table 9. Peters (1947) found high lipase activity of Mycotorula (Candida) lipolytica on butterfat was obtained between pH 4.0 and pH 8.0, with pH 6.2 to 6.5 as optimum. Thus the two enzymes concerned with fat utilization by this organism both show optima slightly on the acid side of neutrality.

When using a 0.07M Palitzsch borate buffer, the action of Ps. fragi cells upon the 12 test substrata indicated the range of greatest activity to be pH 8.0 to pH 8.45, with the majority of the substrata being oxidized most rapidly within the range of pH 8.25 to 8.45, as seen in Table 10. When working with the lipase of hemolytic streptococci, Stevens and West (1922), found the optimum activity of the enzyme to be at pH 7.8. This same pH value was also determined as the point of optimum activity for a pneumococcus lipase by Avery and Cullen (1920). The optimum for Ps. fragi fatty acid oxidase thus falls within the pH range previously found to be optimum for bacterial enzymes of this type.

Cells of C. lipolytica grown on tryptone-glucose-extract agar adjusted to pH 3.8 to 4.4 gave higher oxygen uptake values than cells grown on agar adjusted to higher pH levels, as seen in Table 11. The maximum lipase production by Mycotorula (Candida) lipolytica, as reported by Peters (1947), was evident within the pH range of 4.5 to 5.5, indicating that

production optima of the two enzyme systems associated with fat utilization are within the same general pH range. A pH range of 5.0 to 7.8 for cell growth proved to be most suitable for oxidase production by Ps. fragi, as evidenced by data presented in Table 12.

In a review article on the adaptive enzyme production by bacteria, Dubos (1940) reported that the production of adaptive enzymes is greatly enhanced when the substrate which it attacks is a constituent of the culture medium. This ability to produce an adaptive enzyme manifests itself on the first transfer, and the adaptive enzymes exhibit marked specificity toward the substrata which have stimulated their production.

In the present study there is some evidence of an enhancement of oxidase production by C. lipolytica and Ps. fragi when grown in the presence of certain added nutrients. It should be pointed out that this is not a true adaptive enzyme production, in the usual sense because the cells grown in the absence of the fatty acids possessed oxidizing ability to a considerable degree.

The presence of such added complex nutrients as skimmilk, peptone, casein hydrolysate, yeast extract or beef extract caused no appreciable enhancement of oxidase production by either C. lipolytica or Ps. fragi, as seen in Tables 17 and 18. C. lipolytica cells grown in the presence of 20 percent cream gave higher uptake values than did cells grown in the

presence of skimmilk, indicating an enhancement of oxidase production in the presence of butterfat. The failure of cream added to the defined growth medium to enhance the production of oxidase enzymes by Ps. fragi undoubtedly is due to the inability of this test culture to hydrolyze butterfat to a detectable degree. In the absence of free fatty acids the stimulus for increased production of enzymes oxidizing these acids would be lacking.

The addition to the growth medium of fatty acids such as acetic, propionic, butyric or caproic, resulted in a definite enhancement in the ability of both C. lipolytica and Ps. fragi to produce oxidase enzymes, as noted in Tables 19 and 20. Some tendency toward specificity of the enzymes responsible for oxygen uptake seems apparent since the stimulation for utilization of one acid sometimes was not apparent to the same degree for all other acids in the homologous series. Acetic acid seemed to behave quite differently from butyric acid in that the latter stimulated to a much greater degree the production of oxidases active against the fatty acids which usually are found as components of natural fats; propionic acid also had some tendency to be less active than butyric acid in stimulating production of fatty acid oxidases.

The addition of monoacetin and monobutyrin to the growth media appeared to have little effect in enhancing the oxidizing ability of either C. lipolytica or Ps. fragi when tested against the sodium salt of the fatty acid present in the

monoglyceride. Cells grown in the presence of monobutyrin showed uptake values appreciably higher than the control values when myristate was the test substrate. When caproate was used as the test substrate for cells grown in the presence of either of these addenda, no detectable increase over the control values was noted.

Jezeski (1947b, 1948) found no evidence of an adaptive enzyme component when Mycobacterium phlei, two Pseudomonas sp. and a Micrococcus sp. were grown on nutrient agar containing butterfat. In the present study the evidence of an enhanced oxidizing ability when C. lipolytica and Ps. fragi cells were grown in the presence of fatty acids may be explained by the basal media used, because the synthetic media were not of as high nutritive value as the medium which Jezeski used; the more complex medium employed by him may have given higher values on the unsupplemented medium.

In dairy products containing butterfat both C. lipolytica and the normally-lipolytic forms of Ps. fragi would be stimulated to produce considerable quantities of oxidases active on fatty acids, whether these fatty acids resulted from the lipolytic activity of the organisms, synthesis by other microorganisms or by the action of milk lipases. Oxidation of the liberated fatty acids by the oxidase produced may lead to oxidative rancidity in some instances.

SUMMARY AND CONCLUSIONS

A strain of Candida lipolytica and one of Pseudomonas fragi were chosen for this study upon the basis of maximum production of oxidases for fatty acids. The Ps. fragi strain selected upon this basis was atypical in that it was non-lipolytic. The C. lipolytica strain was much more oxidative than the strain of Ps. fragi. The ability of these microorganisms to oxidize the sodium salts of acetic, propionic, butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic and linoleic acids at various pH levels was determined using Warburg respirometer technics. Selected fatty acids were used as test substrata in other phases of this study.

When incubation was at 30°C., a 24-hour culture of C. lipolytica gave higher oxygen uptake values than did cultures incubated for 48, 72 or 96 hours. Incubation of Ps. fragi cultures for 24 hours at 21°C. gave higher uptake values than the cultures incubated for 24 hours at 30°C. In subsequent determinations the C. lipolytica cultures were incubated at 30°C. for 24 hours, and the Ps. fragi cultures incubated at 21°C. for 24 hours before the cells were harvested for use for manometric determinations of oxygen uptake, using sodium salts of fatty acids as test substrata.

The buffers selected for use in the reaction flasks were a 0.05M McIlvaine citrate-phosphate buffer for C. lipolytica cells, and a 0.07M Palitzsch borate buffer for Ps. fragi cells.

A run covering the period from 0.5 to 2.5 hours gave higher oxygen uptake values than a run of 0 to 2.0 hours; accordingly, the 0.5 to 2.5 hour interval was used in this investigation.

The optimum pH for the reaction of C. lipolytica cells upon the 12 test substrata previously listed varied from pH 3.4 to pH 7.8, with the majority of the substrata giving maximum oxygen uptake within the pH range of 5.8 to 6.6. The optimum pH for the reaction of Ps. fragi cells upon the sodium salts of the 12 fatty acids varied from pH 8.0 to pH 8.45, with the oxidation for the majority of the fatty acid salts being optimum within the pH range of 8.25 to 8.45.

Cells of C. lipolytica grown on tryptone-glucose-extract agar adjusted to pH 3.8 to 4.4 gave the highest uptake values, while a pH range of 5.0 to 7.8 during growth gave the highest oxygen uptake values for Ps. fragi cells.

The addition of skimmilk, peptone, casein hydrolysate, yeast extract or beef extract to the synthetic basal medium caused little increase in the production of the oxidative enzyme of either C. lipolytica or Ps. fragi. The addition of cream to the basal synthetic medium gave higher values with

C. lipolytica cells, indicating an enhancement in fatty acid utilization by cells grown in the presence of butterfat. This enhancement was not obtained when the non-lipolytic *Ps. fragi* cells were grown in the presence of butterfat, probably because the test culture could not free the fatty acids so they could exert a stimulative effect.

The addition of acetic, propionic, butyric or caproic acids to the synthetic basal medium caused an appreciable enhancement of the oxidizing ability of the *C. lipolytica* and *Ps. fragi* cells, when tested against the salts of the homologous fatty acids. This enhanced oxidizing ability was manifest, even when non-homologous test substrata were used, in the case of *C. lipolytica* cells grown in the presence of acetic, propionic or butyric acids.

The addition of monoacetin or monobutyrin to the basal synthetic medium gave no uptake values appreciably greater than the control values for either *C. lipolytica* or *Ps. fragi*, with the exception that *C. lipolytica* cells grown in the presence of monobutyrin gave greater oxygen uptake than did the controls from the unsupplemented medium when sodium myristate was the test substrate.

An enhancement, on the first transfer, of the oxidizing ability of cells of both *C. lipolytica* and *Ps. fragi* when grown in the presence of fatty acids and natural fat gives proof that these cells react to the stimulus of the presence of a

fatty acid by increasing the amount of enzyme available for the utilization of the substrate. Natural fat added to the growth medium gave no increase with Ps. fragi cells, probably because the strain of Ps. fragi chosen for this investigation was non-lipolytic.

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