A COMPARISON OF THE ACTIVITY OF PSYCHROPHILIC AND MESOPHILIC BACTERIA ON SATURATED FATTY ACIDS

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Barnet M. Sultzer

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By

Barnet M. Sultzer

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

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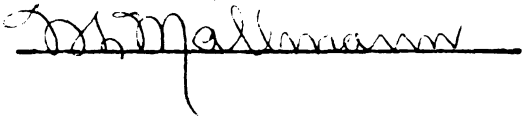
Approved 

ABSTRACT

The problem of bacterial growth and metabolism at low temperatures was investigated from the standpoint of comparing an oxidative enzyme system common to both psychrophilic and mesophilic bacteria. The ability to oxidize saturated fatty acids was employed to measure the response of such organisms to changes in temperature.

Psychrophilic species of Pseudomonas, Alcaligenes, and Achromobacter were found to be active against the sodium salts of acetic, butyric, hexanoic, and octanoic acids. Certain species failed to oxidize butyric acid, and species of Flavobacterium were active on acetic acid only. The mesophilic Sarcina flava oxidized all of the acids tested, while Serratia marcescens degraded all of the acids except butyric.

A comparison of the activity of selected psychrophilic pseudomonads and the aforementioned mesophilic cultures on octanoate revealed a similar response by the psychrophilic cells to the influence of temperature. However, the oxidative rates of a Pseudomonas species and Ps. geniculata appeared to be less affected by decreasing temperatures than the mesophiles tested. This difference was detected by means of an Arrhenius plot of the log QO_2 (N) values at temperatures between 7.5 C and 40 C, and was expressed in two ways: (1) by lower temperature characteristic (μ) values throughout the entire temperature range, and (2) by a lower temperature

at which changes in μ values occurred.

A study of cell-free extracts obtained from these same organisms demonstrated that the cofactor requirements for activating octanoate oxidation was similar for both the psychrophilic pseudomonads and the mesophilic Serratia marcescens and Sarcina flava. The cell-free preparations were obtained by either sonic oscillation or extraction of acetone powders in tris buffer. The activity obtained with each organism approximated 1/2 micromole of oxygen uptake per micromole of substrate. The preparations from all of the organisms either required or were stimulated by the addition of adenosine triphosphate and coenzyme A.

VITA

Barnet M. Sultzer

Candidate for the degree of

DOCTOR OF PHILOSOPHY

Oral Examination: August 13, 1958, 10:00 A.M., Room 101 Giltner Hall

Dissertation: A comparison of the activity of psychrophilic and mesophilic bacteria on saturated fatty acids.

Outline of Studies:

Major subject: Microbiology

Minor subject: Mycology, Chemistry

Biographical Items:

Born: March 24, 1929, Union City, New Jersey

Undergraduate Studies: Rutgers University, 1946-1950, B.S. in Biology

Graduate Studies: Michigan State College, 1950-1951, M.S. in Bacteriology

Experience: Graduate Teaching Assistant at Michigan State University, 1956
Graduate Research Assistant at Michigan State University, 1956-1958

Affiliations:

Society of American Bacteriologists
Society of the Sigma Xi
The American Association for the Advancement of Science

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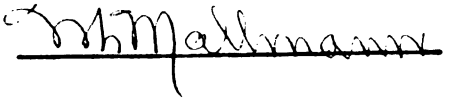
Approved 

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INTRODUCTION

The effect of low temperature in reducing enzymatic activity and inhibiting most bacteria from reproducing is a principle that has long had its application, both in the laboratory and in the preservation of perishable foods. While many microorganisms, if held for a sufficiently long time, may show some evidence of multiplication at, for example 5 C, others will die off, and still others will actively proliferate. The organisms capable of relatively rapid growth at temperatures near the freezing point of water are a biological exception. They have been, and still are, a significant problem to those concerned with the preservation of foods at refrigerator temperatures. Present marketing practices dictate lengthy storage periods at low temperatures. As a result, the psychrophilic organism has been, so to speak, rediscovered.

In the past, research has dealt primarily with observing the occurrence of these organisms, their various sources, their cardinal temperatures and some of their biochemical activities. Of late, research interest has centered about more rapid methods for detecting these organisms, and methods of inhibiting their growth in the cold. (52, 3). However, one of the most intriguing questions immediately posed by the existence of such forms of life has been neglected. Namely, why are the so-called psychrophiles capable of

relatively rapid growth at low temperatures, while closely related species and strains, as well as more distant ones, do not possess this ability?

In part, bacterial growth or proliferation is dependent upon the energy furnished by metabolic activity. Being poikilothermic, the bacterial cell and its metabolic activity can be directly influenced by changes in the environmental temperature. In essence, the cell is at the mercy of temperature changes. The questions then arise; does this activity at low temperatures reflect any differences in the metabolic systems of psychrophiles as compared to mesophiles, and is there a key enzyme system in psychrophiles which can more efficiently function at low temperatures or demonstrate a smaller apparent activation energy? Such questions are broad in their scope, imply numerous problems, and certainly may be approached from many directions.

One approach might be the comparison of specific enzyme systems common to both types of organisms. Oxidative metabolism makes available considerable energy to the cell for the functioning of endergonic synthetic reactions, as well as the many physical manifestations of life itself. One general characteristic gleaned from the literature is that those bacteria commonly found to be psychrophilic are highly aerobic. There may be a connection between the ability to grow and metabolize at low temperatures in a relatively vigorous manner, and the oxidative capacities of an organism. One

such capacity is reflected in the ability of a variety of aerobic bacteria to attack saturated fatty acids.

In the last few years, this phenomenon has received increased attention. The few reported studies have been attempts to determine the degradative mechanisms involved, forearmed with the knowledge of the fatty acid oxidase system in animals. While it would be presumptuous to think that the fatty acid oxidase system may be the key enzyme system which delineates the psychrophile from the mesophile, a study of a typical oxidative mechanism in both psychrophiles and mesophiles may reveal characteristic differences. An understanding of such differences may lead to an explanation for the biological functioning of psychrophiles at low temperatures.

With these thoughts in mind, this study was undertaken with the following purposes:

a. to determine the activity of representative psychrophilic bacteria on saturated fatty acids,

b. to compare the activity of whole cells of both psychrophiles and mesophiles on saturated fatty acids over a relatively wide range of biological temperatures, and

c. to study the requirements of cell-free extracts of both types of bacteria for the oxidation of saturated fatty acids.

LITERATURE REVIEW

Bacterial Growth at Low Temperatures:

The phenomenon of bacteria living and multiplying at temperatures approximating the freezing point of water was recorded over 70 years ago. Since then, numerous reports have appeared in the literature redescribing the occurrence of this phenomenon. The types of organisms involved, the various growth conditions imposed upon them, and the responses illicited have been dutifully observed. Generally, certain saprophytic bacteria common to soil, water and the sea have been found capable of growth at low temperatures. These same organisms have been reported inducing spoilage of a variety of perishable foods stored at refrigerator temperatures.

Probably the first to report bacterial growth at freezing temperatures was Forster in 1887, who showed that phosphorescent organisms isolated from fish preserved by cold grew well at 0 C on various media. (23). In 1892, he reported a number of organisms isolated from natural waters, foods, wastes, rubbish and soils that were able to grow at this temperature. (24). Such organisms were also present in the sea and on salt water as well as fresh water fish.

Fischer (22) reported the isolation of 14 different kinds of microorganisms in 1888 that were found to grow at 0 C. Again, these organisms included phosphorescent and non-luminous species recovered from water and soil.

Schmidt-Nielson (71) reported the growth of seven distinct types of bacteria which grew at 0 C.

Müller(54) isolated 36 cultures from sausage meat,fish, the intestinal contents of fish, milk, vegetables, meat, garden soil and muck, all of which grew at 0 C. Four strains of Bacillus fluorescens liquefaciens, one of B. fluorescens non-liquefaciens, one of Micrococcus flavus tardigradus, and one of M. carneus, as well as yeasts and fungi, were identified. Müller reported that not only were the microorganisms capable of developing at 0 C widely distributed, but their vital manifestations were the same at 0 C as at the higher temperatures, the difference being merely one of intensity.

Gazert(28), in a German expedition to the South Pole at the turn of the century, found bacteria in the bottom ooze of the South Polar seas where the temperature ranged from -0.2 C to -2.0 C. Most of the bacteria isolated grew at 0.0 C, but the optimum temperature was about 20 C.

Several years later, McLean(53) described four species of bacteria which he isolated from the ice and snow of Antarctica in locations where the possibilities of air borne contaminations were most remote. There, the mean annual temperature is about -20 C, and temperatures as low as -60 C are not uncommon. McLean believes that some bacteria actually"learn"to live in the liquid sludge of cryohydrates

which circulate between the crystals of ice.

Pennington (60) in 1908, in a study of the so-called "clean" and "market" milks held at -1.67 C to 0.55 C, found a very marked increase in the numbers of bacteria, even though the milk was semi-solid with ice. Certain species of bacteria, such as Bacillus formosus, B. solitarius, and B. ravenali, were especially resistant to the cold and frequently were the predominating species.

Ravenel, Hastings and Hammer (67) conducted studies on the effect of storage at 0 C and -9 C on the bacterial flora of milk. While no increase in numbers was obtained at -9 C for periods of 160-230 days, there was a marked increase in bacterial numbers with milk held at 0 C.

Species of Urobacteria were also found to be capable of proliferation at 1.25 C to -2.5 C in bouillon containing 3% urea. Rubentschik (70) reported an increase in count from about 3×10^3 cells per ml to about 3×10^9 cells per ml in 25 days.

Hesse (36) in 1914 isolated motile rods and vibrios from Norwegian waters which grew better at refrigeration temperatures than at 37 C. Room temperature was optimum for their growth. Mud from the Barents Sea was discovered by Butkevich (11) to contain bacteria capable of reproducing at -3 C to -7 C. Ellison, Hackler and Buice (19) observed

that bacteria continue to multiply in iced water samples. Similar studies conducted by Zobell and Feltham (95) showed that samples of sea water held at near 0 C for a few hours exhibited an increase in the total number of bacteria present; although there was a decrease in the number of predominating species, probably due to the selective action of the colder temperatures. These observers also noted a two-fold increase in the bacterial population of mud samples after storage at near 0 C for three weeks.

Zobell (92) in 1934 reported that the majority of the bacteria isolated from the sea reproduce freely at 0 C to -4 C. A total of 88 species of marine bacteria isolated from bottom deposits or sea water were observed, and all except 12 grew at these temperatures after three month's incubation. An etiological agent of a fish disease was found to produce perceptible turbidity in sea water broth at -2 C after nine days. (92). In investigations on the temperature range of growth of marine bacteria, Bedford (5) found 65 species which grew at 0 C or below in 136 days. Ten had multiplied at -7.5 C and 12 others at -5 C. The majority of these marine organisms had a temperature range of growth from -5 C to 30 C or 37 C.

Hess (35) studied several bacteria of marine origin in 1934 and observed maximum crops at 5 C and higher total yields at 0 C and -3 C than at 20 C and 37 C. Hess

considered the crop yield as a better criterion for determining the optimum temperature than the growth rate during the logarithmic phase. Practically all the cultural characteristics of these organisms were evident at -3 C. Fermentation of dextrose, sucrose, maltose, proteolysis of fish muscle protein, peptonization of milk, indol production, gelatin liquefaction, nitrate reduction, growth and fluorescence all took place at -3 C. Zobell reports similar results with marine bacteria incubated at 0 C to -2 C. In a discussion of his own results and those reported previously, Zobell (92) concluded that a large proportion of the bacteria isolated from the sea can grow at the lowest temperatures found in the depths of the ocean. Furthermore, most marine bacteria are capable of growing over a broad range of temperatures, from 30 C down to such a temperature that the physical constitution of the substrata becomes unfit for their continued metabolism. Zobell also points out that for most marine bacteria, on which information is available, their optimum temperature is very near their maximum and considerably higher than their minimum. For non-spore formers, the lethal temperature is only a few degrees higher than the maximum at which they grow. This is illustrated by Achromobacter ichthyodermis, which following primary isolation grew at -2 C; its optimum is about 25 C, its maximum is 30 C and 32 C is lethal.

These observations appear significant, for the characteristics described also apply to those psychrophilic spoilage bacteria that are currently being studied in various laboratories.

Further characterization of marine bacteria as to the effect of incubation temperatures on plate counts of sea water was reported by Zobell and Conn (94). Results with water and mud samples were almost the same, regardless of whether they were collected from great depths where the ocean temperature was less than 5 C or from shallow water at higher temperatures. Greater counts were obtained on plates incubated at 12 C to 22 C after seven to ten days than plates incubated at 25 C to 30 C, although the latter colonies appeared earlier and were higher in number at first. Very few colonies developed on plates incubated at 37 C. In fact, according to the authors, many marine bacteria are killed by ten minutes exposure to temperatures no higher than 30 C. These results likewise serve to describe the growth characteristics and thermal sensitivity of those psychrophilic organisms involved in low temperature food spoilage.

The problem of food spoilage at refrigerator temperatures has been long recognized as being directly associated with the growth and metabolic activities of the so-called psychrophilic microorganisms. For example, in 1931

Prescott, Hale and White (64) reported the odors and slimy coatings on beef in cold storage were produced by microorganisms which grow readily at temperatures only slightly above the freezing point. Berry and Magoon (7) in 1934 studied Pseudomonas fluorescens and lactobacilli which were isolated from packed peas held at -4 C. While these organisms did not result in spoilage of this specific food at -4 C, the Pseudomonas cultures reached a population of 5×10^6 cells per ml after four weeks on broth and gelatin supplemented with 3% sodium chloride to depress the freezing point. In 1922, Hunter (38) concluded that the organisms responsible for decomposition of marine fish at low temperatures, were those whose normal habitat is sea water or fish slime. Of 316 cultures, 313 were asporogenous rods. The greenish-yellow discoloration of halibut was attributed primarily to Pseudomonas fluorescens by Harrison (34) in 1929. Species of Flavobacterium and Achromobacter were also isolated. In 1933, Bedford (5) reported the discoloration and subsequent souring of halibut was due to various marine bacteria as well as to the fresh water Ps. fluorescens. All of the bacteria were active at 0 C.

Various workers have reported, in the last 10 to 15 years, psychrophilic bacteria associated with spoilage in milk and dairy products. These include Kennedy and

and Weiser (45), Greene, (30), Rogick and Burgewald (69), Thomas and Sekhar (83), Jezeski and Macy (42), Parker, Smith and Elliker (58), Erdman and Thornton (20), Davis and Babel (16), Weber (89) and Olson, Parker and Mueller (57). Generally, these workers agree on a practical definition of the term psychrophile as including those bacteria capable of relatively rapid growth at temperatures which range between 1 C and 7 C. Furthermore, the organisms most often implicated are gram negative, non-spore forming rods including members of the genera Pseudomonas, Alcaligenes, Achromobacter, Flavobacterium, Proteus, and certain coliforms. Similar organisms have also been associated with the spoilage of dressed poultry at refrigerator temperatures as reported by Walker and Ayres (87) and Ayres, et al. (3), in 1956.

The evidence presented leaves no doubt that a variety of bacteria possess minimum growth temperatures of 0 C or below. However, confusion arose in the past and still exists as to what characteristics define a psychrophile. The term itself means "cold-loving", and as such implies the organisms have higher growth rates or reach greater total populations at cold temperatures. Most often, these two attributes do not coincide; more rapid growth rates usually occur at a higher temperature than do the maximum crop yield. Thus, the first source of confusion is immediately

apparent in defining what is optimum. The second source of confusion is that many of the organisms which exhibit low minimum temperatures possess an optimum growth range at about room temperature. In fact, Zobell (92) states that while most marine bacteria are physiologically active at 0 C to 4 C, they cannot be described as psychrophiles because their optimum temperatures (as determined by plate counts of sea water) are between 18 C and 22 C. Campbell and Reed (12) described Pseudomonas, Proteus, and lactobacilli cultures which grew at 0 C to 5 C, but had optimum temperatures between 20 C and 27 C and maximum temperatures of as high as 35 C to 40 C. They concluded these organisms were products of variation and acclimitization from more mesophilic types to low temperature forms. Van der Zant and Moore (86) reported a Ps. fluorescens culture which appeared to obey the strict definition of a psychrophile in that its minimum generation time existed at 10 C; it grew better at 5 C and 10 C and not at all at 32 C. Porter (62) lists the cardinal temperatures for psychrophiles as follows: minimum 0-5 C; optimum 10-20 C; and the maximum 25-30 C. Smith, et al. (77) list a minimum of 0 to -7 C, an optimum of 15-20 C and a maximum of 30 C.

On the other hand, Haines (33) divided commonly occurring bacteria into four groups on the basis of their behavior at various temperatures:

1. Staphylococci, with an optimum of 37 C and not growing below 10 C.

2. *E. coli*, "*B. proteus*," *B. subtilis*, with an optimum at 37 C and micrococci with an optimum of 20 C to 30 C that grow very slowly at 0 C to 5 C.

3. Some strains of "*B. proteus*" capable of growth at 0 C.

4. Most strains of *Pseudomonas* and *Achromobacter* which show comparatively rapid growth at 0 C (five days) and grow down to about -5 C on supercooled media.

Gubitz (32) offered another classification, proposing the name "Kaltebakterien":

1. Typical Kaltebakterien are those which grow at 0 C in a relatively short period of time.

2. Psychrotolerant types:

   a. Those organisms which grow at 5 C and can be adapted to grow at 0 C.

   b. Those organisms which grow at 5 C and cannot be adapted to grow at 0 C.

Recently, authors including Lamanna and Mallette (47) and Thimann (82), broadly classify psychrophiles as those organisms which grow best at temperatures below 20 C. The significant criterion which should be reemphasized is the striking ability of some bacteria to grow at relatively rapid rates at temperatures from 0 C to 5 C. This characteristic in itself sets off a group of organisms which may

be rightly called psychrophiles, regardless of the fact that they may show faster growth rates or maximum growth temperatures in a range considered to be median.

Biochemical Activities of Psychrophilic Bacteria

In certain papers discussed above, some work on the biochemical activities of bacteria growing in the cold has been reported. The preponderance of the literature deals with the recording of the types of microorganisms that can grow at low temperatures as well as their various sources. Relatively few workers have attempted to analyze growth rates or metabolic activities at low temperatures or over the temperature growth range of the so-called psychrophiles.

In 1931, Prescott and Bates(63) isolated organisms from foods undergoing spoilage and observed their action in pure culture and on the foods themselves. At lower temperatures the relative rates of growth in pure culture were proportional to the temperature increments only for brief periods. The authors concluded that certain types of spoilage organisms adapt themselves to temperatures assumed to be inhibitory to decomposition processes. Hess (35) studied marine bacteria, using the Q_{10} as a measure of growth rates, and found varying responses at different temperature ranges. When the temperature was increased from -3 C to 0 C, the growth rate was accelerated 9.3 times. Raising

the temperature from 0 C to 5 C accelerated the same reaction 8.4 times, while the final rise from 5 C to 20 C resulted in a Q_{10} of only 3.7.

Foter and Rahn (25) studied the growth and fermentation of species of streptococci and lactobacilli near their minimum temperature. Streptococcus fecalis and S. gycerinaceus grew at 0 C, while S. lactis and Lactobacillus acidophilus died at this temperature and could hardly multiply at 5 C. While no explanation is given for this difference, several interesting facts can be noted. The fermentation rates were more greatly reduced at 0 C with the species that could not grow at 0 C, than with those that could. By holding S. lactis at 0 C for four to eight weeks, the cells required a number of generations before they recovered their original fermenting capacity. This was not the case with S. fecalis. S. lactis required twice as much lactose as S. fecalis, and L. acidophilus needed ten times as much to double its cell.

Kiser (46) studied the effect of low temperature on the biochemical activities of some marine bacteria. This worker found that some of the strains of Pseudomonas and Achromobacter that grew at 7 C and -4 C did not liquefy gelatin or reduce litmus milk, while at higher temperatures this did occur. Streptococci cultures responded in a similar manner. Kiser also reported that temperature coefficients

for the growth of Achromobacter sp. as 1.86 to 2.84 between 7 C and 25 C, and 4.58 to 5.82 between -4 C and 7 C. Kiser claims that while an increase in the temperature coefficient is apparent, this is much less than figures usually reported for biological phenomena occurring in that range of temperature.

Recently, Greene and Jezeski (31) reported on the influence of temperature on the development of several psychrophilic bacteria of dairy origin. Temperature coefficients were calculated for growth and biochemical activity for two Pseudomonas cultures and a strain of Aerobacter aerogenes. In general, the primary influence of temperature was upon the rate at which a reaction proceeded, but uniform changes in temperature did not result in uniform temperature coefficients. The temperature coefficients for growth calculated for ten degree intervals from 0 C to 30 C decreased as the temperature range increased. The same relation held for acid production and proteolysis; however, lipolysis exhibited by one of the Pseudomonas cultures showed the smallest coefficient between 10 C and 20 C. Evidence was also presented to show that qualitative differences in biochemical activity did occur with the different psychrophiles at certain temperatures. For example, one Pseudomonas culture liquefied gelatin below 20 C but not at higher temperatures. This same organism fermented glucose only at 30 C but not

below. The A. aerogenes strain produced acid and gas from sugars at 30 C or below but not at 37 C. Thus, variability in the biochemical activities of these psychrophiles is a function of the incubation temperature.

In a study designed to obtain information about the physiology of low temperature bacteria, Brown (10) compared the respiration of a psychrophilic pseudomonad with Ps. aeruginosa over a temperature range of 0 C to 40 C. Both organisms oxidized glucose to gluconic and 2-ketogluconic acids. However, under the experimental conditions, the Q_{O_2} values for the psychrophile were higher than those for Ps. aeruginosa at all temperatures, including those above 30 C at which the psychrophile does not grow. Also, when Ps. aeruginosa was grown at 20 C instead of 37 C, the Q_{O_2} values were increased at the lower incubation temperatures of the suspensions, 0 C and 30 C. In the range of 0 C to 30 C, the temperature coefficients for the oxidation of glucose were about twice as large for the mesophile as for the psychrophile. This result seems to have been overlooked by the author, since he concludes that the systems involved in the oxidation of glucose by the two organisms may not be greatly different in those physical properties which determine their temperature relations.

Ingraham (40) recently reported on a comparative study of the effect of temperature on the growth and metabolism of

psychrophilic and mesophilic bacteria. Using certain psychrophilic pseudomonads and a mesophilic E. coli, Ingraham observed that the psychrophiles were characterized by an 8 C to 10 C lower minimum temperature and a markedly lower temperature coefficient for growth (based on logarithmic phase generation times). Using Warburg and Thunberg techniques, the rate of respiration of the psychrophiles was found to be much less affected by temperature than that of the mesophile and was independent of the temperature at which the cells were grown. However, extracted enzyme preparations demonstrated no differences between the two types of organisms with respect to the effect of temperature on the rate of malic, isocitric, and glucose -6- phosphate dehydrogenases. Thus, the difference in the effect of temperature on the respiration of psychrophiles and mesophiles is apparently not due to a difference in the temperature coefficient of corresponding enzymes. According to Ingraham (41), the difference exhibited with the whole cells may possibly reside in permeability differences at lower temperatures.

Bacterial Oxidation of Fatty Acids:

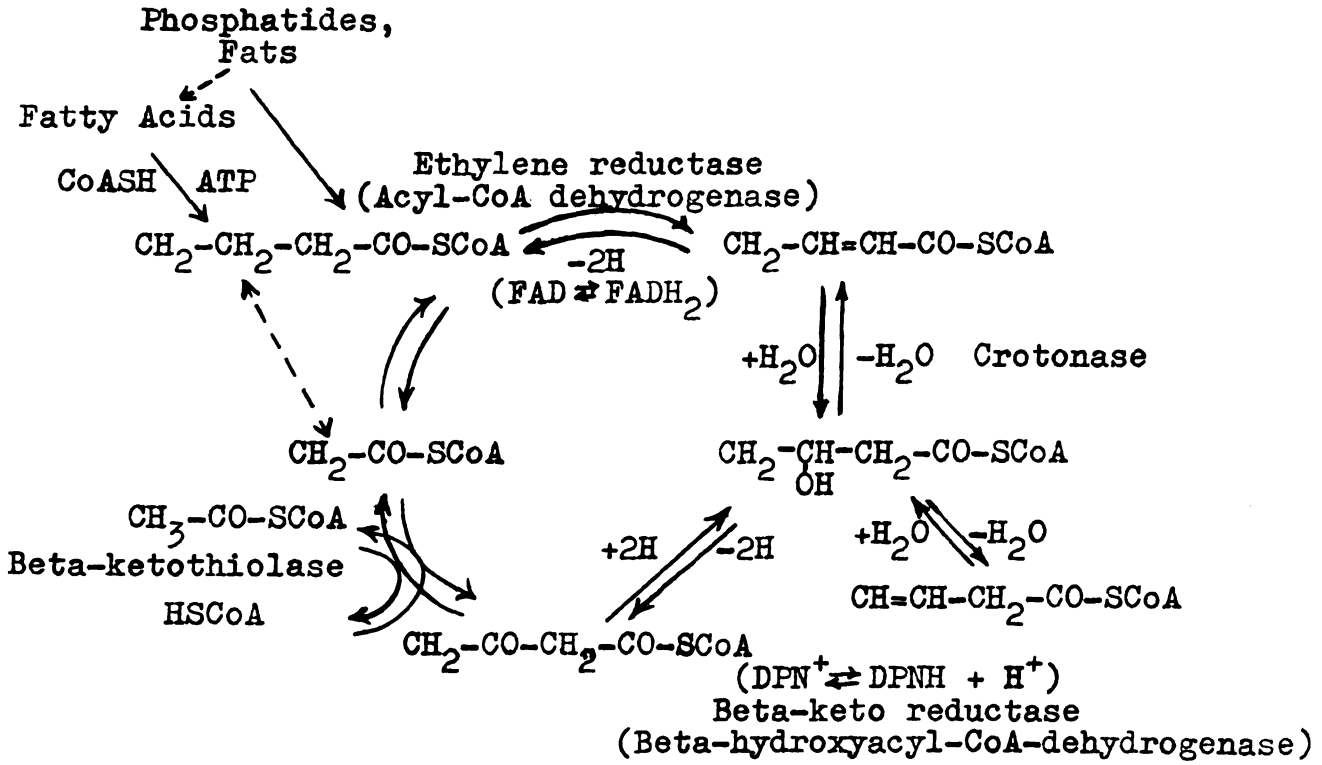
At present, the mechanism of oxidizing saturated fatty acids in mammalian systems appears to be well described and documented (51). Since the pioneer work of Leloir and Muñoz with rat and guinea pig liver extracts, the last 15 years has witnessed intensive investigations into the details of

the basic mechanism. The Knoop-Dakin classical theory of beta oxidation, proposed some 50 years ago, has been finally confirmed. The theory was based on deductions from the nature of the end products of the metabolism of phenyl substituted fatty acids. Direct confirmation was not forthcoming because of the non-accumulation of any intermediates (29).

However, the study of fatty acid oxidation at the mitochondrial level led to the recognition that the fatty acids were not oxidized as such, but only in the form of some derivative. Furthermore, the formation of this derivative is connected with oxidative phosporylation and the production of adenosine triphosphate (ATP). Two discoveries led to the ultimate explanation of fatty acid oxidation by a process of beta-oxidation, whereby the stepwise conversion of fatty acid molecules into C_2 units is accomplished. The first was the discovery of Coenzyme A (CoA), and the second was the finding of energy rich acyl mercaptan or thioester compounds (50,51). Thus, the intermediate products in the biological synthesis and degradation of the fatty acid chain which had been sought for a long time proved to be the thioesters of CoA.

According to Lynen (51), the fatty acid metabolism may be represented as a cyclical process. By conversion into the CoA derivative, the fatty acid is "activated" and

yields acetyl CoA and an acyl-CoA with two carbons less than the starting substance. This latter intermediate is immediately degraded in the same sequence, liberating acetyl CoA and another acyl-CoA compound. This cycle of events may be represented as shown below:



Except for the investigations of butyrate oxidation by Clostridium kluyveri, the study of fatty acid oxidation by bacteria has, in a sense, awaited the developments in the elucidation of the animal fatty acid oxidase system. In fact, the ability of bacteria to use saturated fatty acids has been found not as universally distributed as the capacity to use sugars. Den Dooren de Jong(18) studied extensively

the utilization of carbon sources by 14 representative bacteria in 1926. He found that carbohydrates and related compounds are the most generally used; next come malic, citric, succinic, and lactic acids followed by the fatty acids and lastly by monhydric alcohols. However, only four organisms including Serratia marcescens, Mycobacterium phlei, Sarcina lutea and Pseudomonas fluorescens could use the C_6 to C_{10} acids as a sole source of carbon. M. phlei and S. lutea could use the C_2 to C_6 acids, while P. fluorescens could use the C_2 through C_6 acids but not valerate.

Earlier, Ayers, Rupp and Johnson (2) reported 68 bactèrial strains capable of producing an alkaline reaction in milk, which could utilize fatty acids from C_2 through C_6 as carbon sources in a synthetic medium. The majority of the strains produced an alkaline reaction when grown on acetic or propionic acid. However, most strains produced an acid reaction when grown on butyrate, valerate or caproate. The alkaline reactions were explained as due to the formation of carbonates or bicarbonates. The acid reactions were thought to be the result of the degradation of the higher chain acids to smaller chain length counterparts.

In 1925, Quastel and Wheltham (65), using the Thunberg technique, demonstrated that certain fatty acids acted as hydrogen donors in the presence of resting E. coli cells as the activating source, and methylene blue as the hydrogen

acceptor. Caproic acid and acids of greater chain length were not oxidized, while acids of shorter chain lengths were oxidized.

In studies on oxidations by microorganisms which do not ferment glucose, Barron and Friedemann (4) found the following bacteria to be active on butyrate, propionate, acetate and formate using the Warburg technique: Ps. aeruginosa, Sarcina lutea, Phytomonas campestria, Gaffkya tetragena, Alcaligenes faecalis and Micrococcus freudenreichii.

In 1944, Franke and Schillinger (26) reported studies on fatty acid oxidation by different bacteria and confirmed, in part, some of the growth studies of den Dooren de Jong. That is, most of the bacteria studied were able to oxidize succinic, fumaric, malic, lactic, pyruvic, acetic and formic acids, but the oxidation of saturated fatty acids from C_4 to C_{18} was more limited.

By use of sulfhydryl group inhibitors, Singer and Barron (72) reported in 1945 that the "stearate and oleate oxidase" systems of E. coli were inhibited in oxidizing the respective substrates. The authors stated that this implied the role of sulfhydryl enzymes in the metabolism of fat by bacteria.

The oxidation of a complex lipid substrate was studied by Mundt and Fabian (55) using both the Warburg and Thunberg techniques. Some species of Alcaligenes, Sarcina, Micrococcus, Serratia, Achromobacter and various pseudomonads

were effective in oxidizing corn oil. The highest changes were found with S. lutea, M. flavescens, Serratia indica, Ps. fluorescens and Ps. aeruginosa.

Jezeski, Halvorson and Macy (43) conducted studies on oxygen uptake by certain bacteria in the presence of lipid substrates. The substrates included butter oil, cottonseed oil, corn oil, triglycerides, and the sodium salts of saturated fatty acids. Of the four organisms studied, including two Pseudomonas cultures, a Micrococcus and Myco. phlei, all showed a pH optimum in the range of 7.4 to 7.9 with most of the substrates tested. Phosphate buffer concentrations were also found to affect the oxygen uptake with the Pseudomonas cultures.

A non-sulfur purple bacterium, Rhodobacillus palustris, was found by Tsukamota (84) to be capable of using fatty acids as substrates of respiration. The maximum initial rate of respiration can be achieved by very low concentrations of the acids. Also, the author noted that the minimum concentration of substrate required to achieve maximum respiration decreases as the length of the fatty acid increases. Furthermore, Tsukamota states the oxidation appears to take place without the liberation of carbon dioxide.

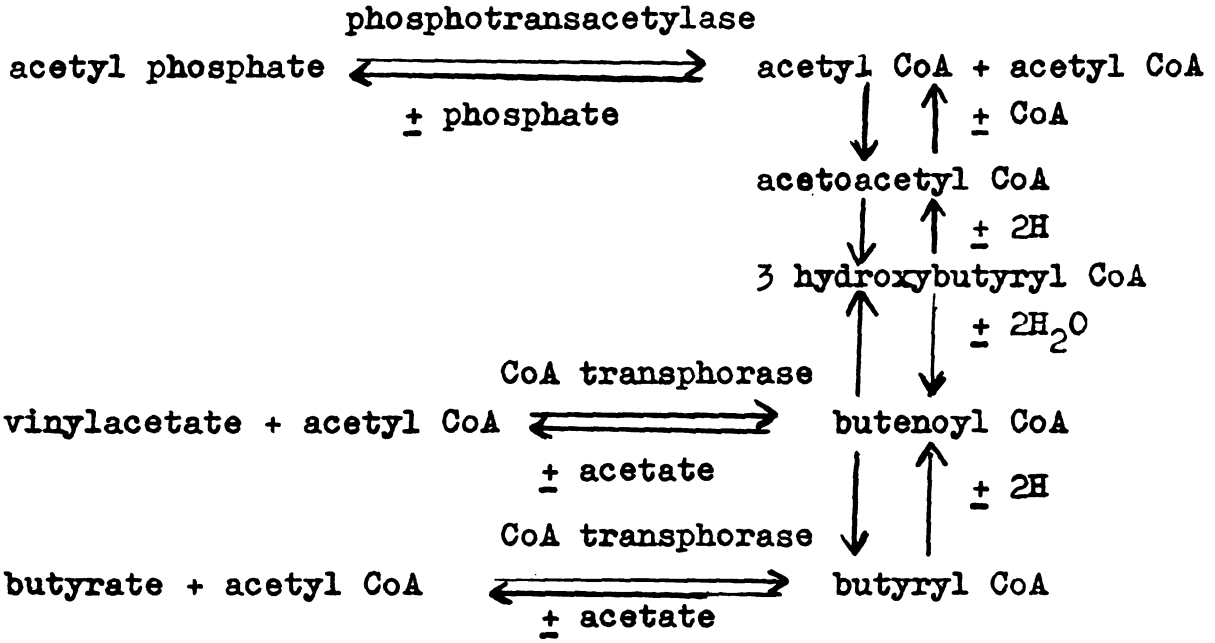
Further survey work in characterizing the ability of various bacteria to attack fatty acids was reported by Silliker and Rittenberg (73) in the first of a series of papers. The specific purpose of the study was to determine the

pattern of fatty acid oxidation, that is whether the enzymes catalyzing aerobic oxidation of fatty acids were constitutive or adaptive. Of the various organisms observed, only Serratia marcescens and Bacillus brevis showed lag periods of close to an hour in actively metabolizing various fatty acids. These results suggested the involvement of adaptive enzymes, a conclusion strengthened by the elimination of the lag when the cells were grown on a medium with the specific fatty acid in question as the sole carbon source.

The investigations of butyrate oxidation by bacteria on a cell-free extract level were first performed with Clostridium kluyveri (79). Two discoveries elucidated the mechanism of both the degradation and synthesis of butyrate by this organism. Stadtman and Barker (80) found the enzyme phosphotransacetylase in cell-free extracts which catalyzed the reaction of acetyl phosphate and CoA, thereby yielding acetyl CoA and inorganic phosphate. Acetyl phosphate is generated by an enzyme widely distributed in bacteria, that catalyzes the reaction of ATP and acetate to form acetyl phosphate and adenosine diphosphate (ADP). The enzyme CoA transphorase, the second discovery by Stadtman (78), was found to catalyze the transfer of CoA from the previously generated acetyl CoA to butyrate according to the following:

$$\text{acetyl CoA} + \text{butyrate} \rightleftharpoons \text{butyryl CoA} + \text{acetate}$$

When synthetic butyryl CoA was found to be readily oxidized by dialyzed enzymes, the possibility that butyryl CoA is the intermediate substrate for butyrate oxidation was confirmed. According to Peel and Barker (59), the current scheme of reaction mechanisms would be as follows:



Cl. kluyveri preparations also have been found to be active on valeric and caproic acids. Lieberman and Barker (49) have shown that in the absence of ortho phosphate, the beta-keto derivatives are formed. Like acetoacetate, these keto acids are decomposed to fatty acids and acyl-phosphates. Beta-keto valerate yields equimolar quantities of acetyl and propionyl phosphates and the corresponding fatty acids, under conditions precluding a rapid exchange of the phosphate group between acyl phosphates and free fatty acids. Beta-keto caproate yields only acetylphosphate

and presumably butyric acid.

Some eight years ago, research into the mechanism of fatty acid oxidation by aerobic bacteria was initiated by Randles (66), and also Silliker and Rittenberg (73,74). Randles showed that Neisseria catarrhalis could rapidly oxidize the saturated fatty acids from acetic to lauric except pelargonic and undecanoic. Propionic acid was oxidized at a slower rate than the other acids, and formate was not oxidized at all. Randles claimed that the similarity in the rate and extent of oxidation of the even-numbered fatty acids indicates similarity in the mechanism involved. With the odd-numbered fatty acids, the latter portion of the oxidation curves of valeric acid and heptanoic acids resembled the rates for propionic acid. This was interpreted as evidence for the production of propionic acid during oxidation of the higher analogs. Since the rate of propionic acid oxidation was also stimulated by small quantities of acetic acid, acetate or a product of acetate was thought to be involved in propionate oxidation. Randles (66) claimed this type of evidence supported the concept of beta-oxidation in bacteria.

Another specific attempt to elucidate the mechanism of aerobic oxidation of fatty acids by bacteria was reported by Silliker and Rittenberg (74). Using the technique of simultaneous adaptation, whole cells of Serratia marcescens

were found to oxidize homologous as well as other acids; with no or a greatly reduced lag period after growth on, or exposure to one of the straight chain saturated fatty acids. Derivatives of capric acid, including alpha-beta unsaturated, beta-hydroxy, and beta-keto acids were also oxidized without a lag period after exposure to capric acid itself. However, butyric acid was found not to be oxidized by either whole or dried cells. In the light of this evidence, the authors postulate a single enzyme system in which the individual enzymes function in a repeating sequence catalyzing the degradation of all of the fatty acids oxidized by S. marcescens, excluding acetic acid.

Further studies reported by Silliker and Rittenberg (76) indicate that 2, 4 dinitrophenol (DNP) selectively inhibits the oxidation of certain of the fatty acids. Fatty acids of 2, 6 and 7 carbon chain length were directly blocked in their oxidation by adapted and unadapted cells. Adaptation to the oxidation of 8, 9, 11 and 13 carbon acids was also prevented by DNP, but not adaptation to the oxidation of the 10, 12 and 14 carbon acids. Where oxidation proceeds in the presence of DNP, oxidative assimilation is partially or completely blocked; and the fatty acid oxidation approaches or goes to completion. The authors state this evidence shows that the short chain fatty acids are not direct intermediates in the oxidation of higher acids, since DNP inhibited the

oxidation of the short chain acids but not the higher ones. However, one could also reason, in view of the existence of different enzymes specific for activating the different chain length fatty acids in animals, that the action of the DNP was actually differential inhibition for different activating enzymes. While admitting the possibility of "active" forms of the lower acids as intermediates, the authors conclude that apparently beta-oxidation is not the mechanism used by S. marcescens.

Later work by Rittenberg and Ivler (68) tends to support the above conclusions. Using phenyl substituted fatty acids, both Pseudomonas fluorescens and S. marcescens adaptively oxidized benzoate through phenyl butyrate. On the other hand, growth on or oxidation of a higher odd or even-substituted acid did not result in adaptation to benzoate or phenyl acetate respectively, while adaptation did occur with S. marcescens. The authors conclude that the data rule out benzoate and phenylacetate as intermediates in the oxidation of the higher substituted odd and even-numbered fatty acids, thereby eliminating beta-oxidation as a possible mechanism. However, since S. marcescens does not oxidize butyric acid, quite possibly the reported oxidation of at least the phenyl substituted butyrate was a result of the degradation of the aromatic moiety. Thus, the adaptation phenomenon in this case may have been more apparent than real.

Further studies with S. marcescens using the approach of specific inhibitors to reveal mechanism were conducted by Waltman and Rittenberg (88). The compound 2, 4, 6 trichlorophenol was found to prevent the oxidation of alpha-beta unsaturated, beta-hydroxy, and beta-keto derivatives of capric acid. Shorter chain length fatty acids were also inhibited, but the oxidation of capric acid and longer chain acids was unaffected. The data suggested that this specific compound acted by blocking the activating enzymes involved in the initial stages of oxidation.

Subsequently, other bacteria have been investigated by various workers to reveal fatty acid oxidation mechanisms. Wolin, Evans and Niven (91) reported in 1952 that a strain of Streptococcus mitis possessed a constitutive enzyme system that mediated the oxidation of butyric acid to two molecules of acetic acid and two molecules of hydrogen peroxide. The general equation for the reaction is:

$$\text{butyrate} + 2O_2 + 2H_2O \longrightarrow 2 \text{ acetate} + 2H_2O_2$$

If excess catalase is present, only one mole of O_2 is needed to oxidize one mole of butyrate to two moles of acetate. The organism was also found not to oxidize propionic, isovaleric or caproic acids, but it did oxidize valeric at a slower rate than butyric acid.

Fiore and Messore (21) studied the catabolic activity of H. pertussis suspensions in the presence of fatty acids.

Esterification of the fatty acids was found to improve the oxidation of the even-numbered acids and also resulted in a slight oxidation of the odd-numbered acids. The authors state, in view of this evidence, that probably H. pertussis oxidizes fatty acids by omega-oxidation.

Webley, Duff and Farmer (90) reported evidence for the support of beta-oxidation as the mechanism for the breakdown of omega-phenyl substituted fatty acids by Nocardia opaca. When phenylacetic, phenylbutyric, phenylcaproic and phenylcaprylic acids were used as substrates, chromatographic analysis showed a common product, o-hydroxyphenylacetic acid. Also, N. opaca showed an adaptive lag in its oxidation of phenylacetic acid which could be eliminated by exposure of the cells to phenylbutyric, phenylcaproic and phenylcaprylic acids, but the odd-numbered acids were ineffective.

All of the preceding work with aerobic bacteria was accomplished with whole cells. The first reported study with cell-free extracts from Ps. fluorescens was by Ivler, Wolfe and Rittenberg (39) in 1955. A requirement for ATP and CoA existed for the oxidation of capric acid. Acetate was also found at the end of the reaction by means of chromatography of the hydroxamate derivatives. Ammonium sulfate purification of the crude extract produced a fraction (40-60% saturation) which consumed one-half micromole of

oxygen per micromole of substrate. The authors therefore conclude that the mechanism of capric acid oxidation by Ps. fluorescens is a beta-oxidation of an activated caprate.

In 1956, Murray and Dawes (56) claimed to have demonstrated that Sarcina lutea extracts required CoA and ATP for the oxidation of acetate, butyrate, valerate, caproate, heptoate and caprylate. The lability of the system was emphasized by the fact that while protoplasts gave activity equal to whole cells, the supernatant from the lysed preparations gave no activity with the addition of cofactors. The activity detected with the supernatant from extracts prepared by homogenizing with fine glass beads was evident but small.

Dagley (14) made cell-free extracts from a vibrio and showed that caprylic acid is oxidized by molecular oxygen to two carbon units. While no mention was made of a need for ATP with these extracts, CoA, diphosphopyridine nucleotide (DPN), and magnesium ions were required as cofactors. Complete oxidation was approached when fumarate was added. Volatile acid was produced in the reaction and identified by vapor-phase chromatography as acetic acid.

In an interesting study of the bacterial metabolism of unsaturated fatty acids, Peterson (61) reported that heat-killed suspensions of several species containing cytochrome systems were able to catalyze the oxidation of the unsaturated linkage of methyl linoleate. Heat-killed Myco. phlei

cells were found to be leached of their cytochrome pigments, and the soluble pigments were found to have prooxidant activity. Also, purified bacterial cytochromes actively accelerated oxygen uptake when incubated with methyl linoleate with the concomitant reduction of the cytochrome pigments. This evidence, while not discounting the role of beta-oxidation, emphasizes the importance of the cytochrome system in fatty acid oxidation.

EXPERIMENTAL STUDIES

A. Survey of the Activity of Psychrophilic Bacteria on Saturated Fatty Acids

An experimental survey was initiated to determine the oxidative activity of psychrophilic bacteria on fatty acids. A most convenient and accurate method for measuring such activity would be by means of recording oxygen uptake according to manometric techniques. Several workers have used this method for determining the oxidative capacities of various bacteria on both complex lipids and fatty acids (43,55,66,68,73).

1. Selection of Cultures:

Isolations were made from various fresh foods that had shown obvious signs of spoilage after storage at 5 C. Eleven isolates were obtained from milk, ten from beef and seven from dressed poultry. Five known cultures of representative psychrophiles, obtained from the laboratory stock culture collection, were also included in this study. These organisms included Pseudomonas fluorescens, Achromobacter sp., Alcaligenes sp., Flavobacterium rhenanum and Pseudomonas geniculata.

The isolates obtained from the spoiled foods were purified by the usual methods and then characterized as to their response to various incubation temperatures. The known psychrophiles were likewise characterized. All cultures used

in this part of the work were grown and stocked on Brain Heart Infusion (BHI) agar (Difco). Inocula were prepared from 24 hour cultures grown at 20 C by suspension in sterile distilled water. Agar slants were streaked with a single stroke, using a sterile 3mm loop. Replicate slants were incubated at 1 C, 5 C, 10 C, 20 C, 30 C, 35 C and 45 C. Observations of the degree of visible growth were made daily for a period of two weeks.

On the basis of these temperature studies, representative isolates were selected for subsequent manometric studies and identified as to the genus. Three mesophilic cultures, including Sarcina flava, Serratia marcescens and Aerobacter aerogenes were also obtained from the laboratory stock culture collection. These organisms were characterized as described above; however, the cultures to be used for inocula

2. Manometric Methods:

The organisms to be tested were transferred daily for three days prior to harvesting. Twenty-four hour cultures were suspended in sterile distilled water and centrifuged at about 4000 rpm in a Servall clinical centrifuge. The cells were washed in this manner a total of three times and the final suspension made in M/20 phosphate buffer at pH 7.4. All suspensions were diluted with buffer, so that a 1/10 dilution of the suspension would give an optical density

reading of 0.6 on the Bausch & Lomb Spectronic 20 Colorimeter at a wavelength of 540mμ. The final suspension was aerated for 30 minutes at room temperature prior to using in the Warburg apparatus.

Conventional manometric techniques were employed to determine the oxygen uptake of cell suspensions in the presence of the short chain saturated fatty acids. (85) The acids included in this study were acetic, butyric, hexanoic and octanoic. All of these substrates were made up as the sodium salts and used in a final concentration of 10 micromoles. The contents of the reaction flasks included 1 ml. of the adjusted cell suspension, 1 ml of M/20 phosphate buffer at pH 7.4, 1 ml of the substrate solution distributed in the side cups and 0.2 ml of 20% KOH in the center well, so that the final volume in each cup was 3.2 ml. The flasks were equilibrated for 15 minutes, and the uptake of oxygen was followed for a period of three hours at 30 C. All results reported represent values after subtracting the endogenous rates.

3. Results:

a. Temperature Characterization:

From the brief study of the various isolates and stock cultures as to their growth responses at various incubation temperatures, three arbitrary categories may be designated which summarize the results. While these categories, and

the criteria upon which they are based, are relative to the experimental methods employed; such groupings serve a useful function in delineating the general growth responses of the organisms to different temperatures. In this way, selection of representative cultures for further study was made feasible. The categories are as follows:

1. Psychrophiles: those organisms demonstrating good growth at 5 C or below in one week or less, and no growth at 35 C in two weeks.

2. Facultative Psychrophiles: those organisms capable of good growth in less than one week at 5 C and 35 C.

3. Mesophiles: those organisms demonstrating no growth at 5 C in two weeks and good growth at 35 C in one week or less.

Of the 28 isolates obtained from the various spoiled foods, 25 were grouped as psychrophiles. The remaining were classified as facultative psychrophiles. The stock cultures considered to be psychrophilic were Ps. fluorescens, Ps. geniculata, Achromobacter sp. and Alcaligenes sp. The culture of F. rhenanum was classified as a facultative psychrophile. S. marcescens, S. flava and A. aerogenes were classified as mesophiles.

b. Identification:

All of the 28 isolates were found to be gram negative rods. Three representative cultures from each spoiled food

product were subjected to various identification tests. The results are summarized in Table 1. Among those organisms studied, two genera were found to exist. Cultures 1M, 4M, 1B, 7B, 9B, 17C and 19C are species of Pseudomonas. Cultures 2M and 4C are species of Flavobacterium. While no more detailed examination was made of these cultures to determine the species, the cultures were checked in their reactions against the stock cultures referred to above to confirm the genus.

The Pseudomonas cultures were characteristically weakly saccharolytic, but all those tested were lipolytic as determined by the use of nile blue sulfate indicator in a natural lipid medium. The Flavobacterium cultures were diametrically opposed. The ability of both types of organisms to oxidize saturated fatty acid, as shown later, appears to correlate with their behavior on fat and carbohydrates; i.e. the lipolytic cultures oxidized the saturated fatty acids while the saccharolytic forms did not.

c. Fatty Acid Oxidation:

The results of the survey of fatty acid oxidation by various bacteria are summarized in Table 2. Several interesting observations may be noted from these data. Of the eight Pseudomonas cultures examined, seven oxidized the sodium salts of the four even-numbered saturated fatty acids. Excluding acetate, the rate of oxidation increased with

the increasing chain length of the fatty acids. One Pseudomonas isolate, as well as Achromobacter sp. and S. marcescens, did not oxidize sodium butyrate to any appreciable extent. Furthermore, the Flavobacterium species oxidized only sodium acetate, and the A. aerogenes culture demonstrated only slight oxidation of sodium butyrate, sodium hexanoate and sodium octanoate.

The failure to oxidize materially sodium butyrate by several different organisms suggests at least two possibilities. Either a divergent pathway exists in the catabolism of the higher analogs, or the organisms in some way fail to synthesize the specific enzyme needed for butyrate oxidation. Silliker and Rittenberg (74) have previously reported that dried cell preparations of S. marcescens likewise fail to oxidize butyrate, apparently ruling out a permeability barrier as an explanation. In view of its occurrence in several different genera, this particular anomaly seems to have significance; and any theory of fatty acid oxidation mechanisms in aerobic bacteria should take it into account.

The pattern of oxidation, while not revealed in the summary of the results, must also be cited. All of the organisms except S. marcescens showed an immediate response when exposed to the fatty acids they could metabolize. In other words, oxidation proceeded at an immediate and steady rate, indicative of the presence of constitutive enzymes.

However, S. marcescens did not respond in this manner. When previously grown on BHI agar, these cells showed a characteristic lag period of about 30 to 50 minutes before oxidation proceeded at a steady rate. This lag period existed with hexanoate and octanoate but not acetate. Silliker and Rittenberg (73) first reported this phenomenon with S. marcescens. When the organism was previously grown on a medium containing the particular fatty acid or a closely related one as the primary carbon source, the lag period disappeared. From these results, the authors concluded that this organism possessed an adaptive fatty acid oxidase system which mediated the catabolism of fatty acids greater in chain length than butyrate.

Customarily, when reporting oxidation rates, the endogenous rates are considered as control values and subtracted from the rates obtained with the particular substrate tested. However, when activity rates are small or endogenous rates are high, such endogenous rates become quite significant. As a result, the ever present problem of whether endogenous respiration does or does not proceed when the cells are exposed to the substrate takes on added significance. When the cells used in this study were grown on a rich nutrient medium such as BHI agar, the endogenous rates were at a high level. While the aeration of the cell suspension for 30 minutes or longer at room temperature reduces the endogenous

activity, a more satisfactory method for reducing such activity might be the use of a mineral salts medium for growth purposes. Such a medium, when including a specific fatty acid under study, would eliminate the lag periods in the oxidation of the fatty acid by S. marcescens.

B. Temperature Characteristic (μ) Studies

1. Application of the Arrhenius equation:

The temperature coefficient (Q_{10}) is being commonly used in biological studies to describe the rates of a physiological reaction as influenced by temperature changes.

A more quantitative relation, proposed by Arrhenius, is given by the following equation: (1)

$$\frac{d\ln k}{dT} = \frac{\mu}{RT^2} \qquad (1.0)$$

where k is the reaction velocity constant, R is the gas constant (1.987 cal per degree per mole). T is the absolute temperature, and μ is a constant. Integration of this equation would be:

$$\int d\ln k = \int \frac{\mu T^{-2}}{R} \cdot dT$$

$$\ln k = \frac{-\mu}{R} \cdot \left(\frac{1}{T} + C\right)$$

or

$$\log k = \frac{-\mu}{2.303R} \cdot \left(\frac{1}{T} + C\right) \qquad (1.1)$$

Furthermore, integration between limits would result in the following:

$$\log \frac{k_2}{k_1} = \frac{\mu}{2.303R} \cdot \left(\frac{1}{T_1} - \frac{1}{T_2}\right) \qquad (1.2)$$

The values of μ can be calculated from the rates at two different temperatures or from the slope of the straight

line when log k is plotted against $\frac{1}{T}$.

In the terminology of chemical kinetics, the symbol is replaced by ΔH_a which is now interpreted as the heat of activation or the energy of activation. (15). This constant has the dimensions of calories per mole and is considered to be the number of calories required to activate a mole of the reactant to the energy state required for participation in the reaction concerned. Whether this same interpretation can be justifiably applied to heterogenous enzyme reactions occurring within, or apart from, the living cell is a moot point. However, the constant μ, frequently designated as the temperature characteristic, serves a definite purpose in comparing quantitatively the influence of temperature on various biological processes. Temperature effects on various in vivo and in vitro enzyme systems derived from plants, animals and microbes have been analyzed in this manner (76). Generally, the enzyme reactions follow the Arrhenius equation over a relatively wide biokinetic temperature range up to temperatures where heat inactivation of the enzyme becomes apparent.

A quantitative approach to the comparison of the metabolic activity of psychrophilic and mesophilic bacteria might be the employment of the Arrhenius equation. A plot of the reaction velocity constants of fatty acid oxidation at various temperatures would yield temperature characteristic

values that may characterize the behavior of organisms that differ in their ability to metabolize these acids at low temperatures. Also, as an incidental consideration, fatty acid oxidase systems in bacteria have not been subjected to this method of study and μ values not reported. With these considerations in mind, this method of approach was initiated.

In view of the results obtained from the survey of the activity of psychrophilic and mesophilic bacteria on saturated fatty acids, octanoic acid was chosen as the substrate for study. The organisms that were selected, while having a common ability to degrade octanoic acid, differed in their morphological and biochemical characteristics. Two typical psychrophilic bacteria, Pseudomonas sp. 4M and Ps. geniculata were chosen. Both organisms grew relatively rapidly at 0 C and not at 35 C under the experimental conditions employed. The Pseudomonas sp. 4M differed from Ps. fluorescens only in its failure to produce acid from glucose. S. marcescens is a mesophile but exhibits a fairly wide range of growth temperatures. This culture demonstrated a minimum temperature of growth at 5 C and a maximum temperature of growth at about 40 C. It is a facultative aerobe and possesses an adaptive octanoate enzyme system. Another mesophile chosen for this study, but possessing a narrower range of growth temperatures, was S. flava. This particular strain showed no growth at 5 C for over two weeks, but grew rapidly at

35 C and exhibited abundant growth after one week at 45 C. It is an obligate aerobe and constitutively oxidizes all those short chain saturated fatty acids tested.

2. Culture and Manometric Methods:

As previously mentioned, the problem of high endogenous rates and adaptive enzyme systems necessitated the development of a suitable medium which would eliminate these difficulties. Such a medium, which also produces abundant crop yields in 24 hours, has the following formula:

$(NH_4)_2SO_4$	3.0 gms.
$MgSO_4 \cdot 7H_2O$	0.1 gm.
Octanoic Acid	10^{-2} Molar
K_2HPO_4	8.0 gms.
KH_2PO_4	2.0 gms.
Yeast Extract (Difco)	3.0 gms.
Agar	20.0 gms.
Distilled Water	1000 ml

Prior to sterilization, the medium was adjusted to pH 7.4 with sodium hydroxide.

The test organisms were carried on this medium throughout the remaining part of this work. The psychrophilic cultures were grown at 20 C and transferred weekly. The mesophiles were grown at 35 C and likewise transferred weekly. Those organisms to be tested in the Warburg were transferred daily for three days prior to harvesting. Twenty-four hour

cultures were used as resting cell suspensions in all experiments. The cells were harvested from agar slants and washed as already described. However, no aeration was necessary.

In all of the manometric experiments, a Bronwill Warburg Apparatus Model UV was employed. This model allows for accurate temperature control between several degrees above 0 C and 45 C. By using a Revco "Zero-Max" Model 142X circulating pump, iced water was circulated through the cooling coil of the Warburg apparatus; so that temperatures between 7.5 C and 12.5 C could be accurately maintained.

Because octanoate gives maximal rates of oxidation compared to butyrate and hexanoate, this acid in the form of its sodium salt was used throughout this part of the work. Cell suspensions were made up in M/20 phosphate buffer at pH 7.4. The suspensions were adjusted with this buffer, so that a 1/10 dilution would give a reading on the Bausch & Lomb Spectronic 20 Colorimeter at 540mμ which in turn would correspond to a cell nitrogen content of approximately 1 mg. per milliliter. Nitrogen determinations were made using the microkjeldahl method. Duplicate samples of each cell suspension were made and the results averaged. These results are presented in Table 3.

The reaction flasks contained the following: 1 ml of the adjusted cell suspension, 1 ml of M/20 phosphate buffer at pH 7.4, 0.8 ml of distilled water, 0.2 ml of 0.1 molar sodium

octanoate in the side cup and 0.2 ml of 20% KOH in the center well. The excess concentration of substrate was used to insure a zero order reaction. The flasks were shaken at 120 oscillations per minute. Endogenous control flasks were also included. Duplicate, and in some cases triplicate, reaction flasks were run at each temperature. The temperature range of study was from 7.5 C to 40.0 C at 2.5 C or 5 C intervals. Fresh cell suspensions, obtained from the original stock suspensions held at 1 C, were used at each temperature, and the flasks were equilibrated for 15 minutes prior to recording oxidation rates.

The rates of oxidation were followed for 60 minutes with readings every ten minutes. The endogenous rates were subtracted from the average values of the replicate reaction flasks. The results were plotted as oxygen uptake in microliters against time in minutes, and all cases found to be linear. The slopes of the straight lines, drawn through the plotted points, were determined. An example of such plots are presented in Graph 5. QO_2 (N) values were calculated from these slopes as the microliters of oxygen taken up per mg. cell nitrogen per hour. The logarithms of the QO_2 (N) values were plotted against the reciprocals of the absolute temperatures. The best straight line was drawn between the plotted points according to the least square method. The representative temperature response of each organism is

shown in Graphs 1 - 4. From the slopes of these lines, temperature characteristic (μ) values were determined according to equation 1.1 and are summarized in Table 4.

Several runs were made for each organism over the cited temperature range. Though the conditions of culture, cell density and chemical environment of the reaction flasks were kept as uniform as possible, the oxidation rates could not be exactly reproduced from one run to another. However, the different oxidative responses of each organism to changes in temperature were within the limits of deviation as shown by the comparison of the two psychrophilic pseudomonads. (Graph 7)

3. Results:

The plots of the QO_2 (N) values obtained at each temperature in the presence of sodium octanoate reveal the influence of temperature changes on the measured reaction rates for each organism tested. Both of the psychrophilic pseudomonads were found to oxidize octanoate at greater rates throughout the temperature range of 7.5 C to 35 C, compared to either S. marcescens or S. flava. For example, in the temperature range of 15 C to 35 C, the log QO_2 (N) values for Pseudomonas sp. 4M and Ps. geniculata were between approximately 2.0 and 2.4. However, for the same temperature range, S. flava demonstrated log QO_2 (N) values of between about 1.35 to 2.2. The values exhibited by S. marcescens over the same temperature

range were between about 1.6 to 2.2. At temperatures below 15 C, the higher activity rates for the psychrophilic pseudomonads were as equally pronounced. Between 7.5 C and 15 C, the log values for these organisms were about 1.65 to 2.0. S. marcescens exhibited log values of about 1.3 to 1.6. The rates for S. flava were not measured below 15 C, but extrapolation of the line as shown in Graph 3 yields values of less than 1.0 at 7.5 C to about 1.35 at 15 C.

While the rates of oxidation of octanoate by the psychrophilic bacteria are generally of a higher order as compared to the mesophilic bacteria, the maximum rates achieved by all of the organisms occurred at nearly the same temperature. The oxidative activity dropped precipitously at 37.5 C in the case of Pseudomonas sp. 4M and S. flava. The same response occurred at 35 C with Ps. geniculata and S. marcescens. Thus, no real correlation appears to exist between the maximum growth temperature of these organisms and the maximum rate of oxidative activity with octanoate. In fact, under the experimental conditions employed, the enzyme system in vivo functioned at temperatures somewhat higher than the maximum growth temperature for the psychrophilic pseudomonads. Conversely, the oxidative activity of the mesophilic cultures was maximal at temperatures somewhat below their maximum temperature of growth. Nevertheless, the similarities in the temperature of maximal oxidative response for all of the

organisms tested suggests a possible similarity in the enzyme systems of these diverse bacterial types.

Differences in the temperature response of the psychrophilic and mesophilic cells can be discerned from inspection of the various plots (Graphs 1-4) The plot of the reaction velocity constants did not obey the Arrhenius equation over the entire temperature range studied. Changes in the slopes of the lines occurred with each organism. Since the μ values are sensitive to slight changes in slope, considerable variation in these values are apparent due to the changes in the reaction velocity constants in the lower temperature range. A comparison of the two psychrophilic species shows a rather similar overall response in the oxidation rates (Graph 7) The μ values are recorded in Table 4. The change in slope is particularly noticeable with each psychrophile at about 15 C. The ratio of the μ values below and above 15 C is about 2.10' for Pseudomonas sp. 4M and 2.13 for Ps. geniculata. In the case of the mesophilic species, the μ values above 25 C are comparable. However, the changes in the slopes are somewhat different and occur at different temperatures. The response of S. marcescens shifted at about 20 C, and the ratio of the μ values below and above this temperature is about 2.00. S. flava responded with a decided change in slope at about 25 C, and the ratio of the μ values below and above this temperature approximates 2.50.

Thus, the influence of temperature on the reaction velocity constants derived from the oxidation of sodium octanoate, by selected psychrophilic and mesophilic bacteria, has been demonstrated by temperature characteristic plots. From the data presented, the oxidative rates of the psychrophilic pseudomonads are apparently less affected by a decrease in temperature than the mesophilic S. flava. While the ratio of the μvalues for S. marcescens approximates the ratios obtained with both of the psychrophiles, the μvalues above 25 C equals the μvalue of S. flava. The temperature characteristic plots also appear to be correlated with the growth response of each organism to changes in the incubation temperature. The psychrophiles show a decided change in slope at 15 C. S. flava, with a minimum temperature of growth at about 10 C, demonstrates a change in slope at 25 C. On the other hand, S. marcescens which can grow at 5 C if held for longer than two weeks, shows a change in slope at about 20 C. Whether this correlation would exist for other substrates remains for future investigation.

With these results in mind, two paths of investigation were possible. Further studies on whole cells could be initiated to determine various factors that may influence the oxidative rates and the temperature characteristics. In other words, would changes in the chemical environment affect the μvalues? The use of specific inhibitors may provide

clues as to what rate limiting reactions might govern the overall oxidative rates. Other fatty acids, tricarboxylic acid cycle intermediates, or carbohydrates could also be tested to determine whether the apparent pattern of response for octanoate by the psychrophiles and mesophiles could be duplicated.

The other choice of study would be investigations with cell-free systems extracted from the test organisms. If the temperature characteristic plots could be duplicated on a crude enzyme extract level, the possibility would exist that the rate limiting reactions might be defined. However, the primary obstacle to such an investigation is obtaining active preparations. Furthermore, the activity must be sufficiently comparable to whole cells in order to study reaction rates at low temperatures. In view of the inherent difficulties, several different methods of preparing extracts were attempted. The methods and results of these experiments will be described next.

C. Cell-Free Extract Studies:

1. Methods:

The octanoate medium previously described was used for growth purposes. Four liter quantities were prepared and sterilized by autoclaving. The sterile medium was distributed in 25mm. x 150mm. sterile petri dishes, about 100 ml per dish. The plates were allowed to harden and then were dried overnight at room temperature. Twenty-four hour cultures grown on the same medium in the form of agar slants were suspended in sterile water to give a heavy suspension of cells. About 0.3 ml of the suspension was inoculated on each plate and spread over the entire surface by means of a sterile glass rod. The plates were allowed to dry for about 30 minutes at room temperature and then incubated at the temperature appropriate for the particular organism under study. S. marcescens and S. flava were incubated at 35 C for 24 hours. Ps. geniculata and Pseudomonas sp. 4M were incubated at 20 C for 24 hours.

The cell crop was harvested using glass spreaders and suspended in chilled distilled water. The suspension was centrifuged in the cold, and this washing procedure repeated twice. This method of culture resulted in crop yields of one gram or more per liter of medium on a dry weight basis.

The following procedures were attempted in order to obtain active cell-free preparations:

a. Sonic oscillation of fresh cell suspensions - (13)

The washed cell pellet was suspended in M/20 phosphate buffer at pH 7.4 to bring the suspension to a density of about 200 mg. per ml on a wet weight basis. Generally, this brought the volume to about 30 ml. This buffered suspension was oscillated for 15 minutes in a 250 watt 10KC Raytheon Sonic Oscillator at a maximum obtainable output current of about 1.0 amp. The oscillator cup was kept at less than 5 C by rapidly circulating ice water through it using a Revco "Zero Max" circulating pump. The resulting suspension was then centrifuged in the cold at about 20,000 x g for 30 minutes. The clear, opalescent supernatant was used immediately as the crude cell-free extract in manometric studies.

Certain modifications or further treatment of the crude extract were also instituted. The extract was diluted either 1/3 or 1/5 with phosphate buffer. Also, the undiluted extract was dialyzed overnight in the cold against two liters of distilled water in order to reduce the high endogenous activity.

b. Slow drying in vacuum with manual grinding - (13,39)

The packed cells obtained by centrifugation were transferred to an evaporating dish and spread with a spatula in a thin layer. The dish was placed in a desiccator over anhydrous calcium sulfate (Drierite), evacuated and sealed. The preparation was allowed to stand in this manner either

overnight in the cold, two hours at room temperature or 15 minutes at room temperature. After drying, the preparation had a hard, glassy consistency. The dried cells were then transferred to a mortar that had previously been held at -15 C for several hours. Two to three parts of Alcoa A-303 "levigated alumina" were added and the preparation moistened with M/20 phosphate buffer at pH 7.4. The alumina powder was supplied through the courtesy of Dr. H.L. Sadoff of this department. The preparation was ground manually for five minutes in the cold, applying maximum pressure. The thick paste by this time had become quite tacky and of a creamy consistency. The paste was transferred with washings to a centrifuge cup and made up to a volume which gave a cell concentration of about 200 mg. per ml on a wet weight basis. The preparation was then centrifuged in the cold for 30 minutes at about 20,000 x g. The clear, opalescent supernatant was used as the cell-free extract for manometric studies.

c. Acetone dried preparations - (13,85)

The washed cell pellet was diluted with sufficient distilled water to give a creamy suspension. Ten to 15 volumes of dry acetone were cooled to -20 C or lower in a 500 ml flask surrounded by dry ice. The acetone was vigorously stirred by means of a magnetic stirrer, and the cell suspension was slowly added to it. The acetone solution was stirred for several minutes after all of the cells had been added,

and the flocculant precipitate was then allowed to settle. The supernatant was poured off and the precipitate collected in a large Buchner filter using Whatman #50 filter paper. The precipitate was washed by pouring about two to five volumes of -20 C acetone on the filter and then dried by continuing aspiration for several minutes. When the atmosphere was humid, difficulty was encountered in removing all of the solvent. In such cases, the precipitate was scraped from the filter paper and transferred to an evaporating dish. The dish was placed in a desiccator over a large block of paraffin, evacuated and allowed to set overnight at room temperature.

The acetone powders obtained in this manner were stored at -15 C. The powders were tested for activity by preparing a suspension of 25 mg. per ml of M/20 tris (hydroxymethyl) amino methane buffer at pH 7.4. Extraction of the powders for the purpose of obtaining active cell-free enzyme extracts was also accomplished. Sonic oscillation of the powders was attempted using suspensions of 50 mg. of powder per ml of tris buffer. The organisms differed in their susceptibility to sonic oscillation. While the gram negative organisms were disrupted in 15 minutes, S. flava was quite refractory. Both the fresh cells and powder took as long as 60 minutes of oscillation before disruption was evident. The supernatant was collected after centrifugation, as described above, and used as an enzyme extract. Also, suspensions of the same

concentration were stirred at 5 C with a magnetic stirrer for varying lengths of time from two to eight hours. The suspension was centrifuged and the supernatant collected for testing.

Determinations of the amount of protein per ml of extract were made by means of turbidity measurements of trichloroacetic acid (TCA) precipitates at 600mμ. The cell-free extract in 0.1 to 0.4 ml quantities was pipetted into a 10 ml cuvette of 1 cm. diameter and made up to a final volume of 1 ml. Four ml of 1.25% TCA were added, and the cuvette was allowed to stand for ten minutes until maximum turbidity developed. A blank was prepared using water instead of TCA. Comparisons were made with standard solutions of purified albumin which were treated in the same manner. A linear function existed between albumin concentrations of 0.5 and 1.5 mg.

Manometric methods were used to test the activity of the various cell-free extracts and acetone powders. Several cofactors known to be necessary for enzyme activation in animal fatty acid oxidase systems were used to determine the requirements, if any, of these bacterial extracts. In this manner, differences or similarities between the psychrophilic and mesophilic bacteria might be demonstrated, as well as between bacterial and animal mechanisms of fatty acid oxidation. The cofactors tested included Coenzyme A (CoA), Mg^{++}, adenosine

triphosphate (ATP), cytochrome c, diphosphopyridine nucleotide (DPN), flavinadenine dinucleotide (FAD), and yeast concentrate. All of these compounds were obtained from the Sigma Chemical Company. The solution of CoA was made up according to methods described by Colowick and Kaplan (13). Reduced glutathione (GSH) was employed to maintain the CoA in a stable state. The GSH, from the Nutritional Biochemical Company, was supplied through the courtesy of Dr. H.L. Sadoff. Cytochrome c was used in a concentration of .0003 M and DPN in a concentration of .005 M. FAD was added in quantities of 0.2 mg. and yeast concentrate in quantities of 0.1 mg. per 3.0 ml of the reaction mixture.

2. Results:

Sonic oscillation of fresh cells of Pseudomonas sp. 4M produced extracts which exhibited high endogenous activity. In the absence or presence of CoA, ATP, Mg^{++} and phosphate, no activity with octanoate was demonstrated. This endogenous activity was reduced by diluting the extract with phosphate buffer 1/5. However, fatty acid oxidative activity was still not evident. The possibility existed that not only were the accumulated intermediates diluted out, but also the enzymes under study. A 1/3 dilution of the extract was tried in the presence of the previously mentioned cofactors and also DPN. The endogenous activity was again reduced to zero, and slight oxidative activity with octanoate was detected. Undiluted

dialyzed preparations also showed slight activity in the presence of added cofactors. However, because of the extremely low level of activity and since the preparations were not stable either in the frozen state or when kept at 1 C, this method was abandoned.

The second technique consisted of drying fresh cells of Pseudomonas sp. 4M for short periods and subjecting the material to hand grinding with alumina powder. Regardless of the drying time employed, no activity was obtained in the presence or absence of added cofactors.

The preparation of acetone powders, on the other hand, was found to be successful with all of the test organisms, although this success was limited. Endogenous rates were reduced about 60% as compared to the extracts obtained from sonic oscillation. Activity in the presence of octanoate was small as compared to whole cells, and unfortunately, this limited activity precluded temperature characteristic studies. Nevertheless, the activity was quite sufficient for determining cofactor requirements. From this standpoint, the psychrophilic and mesophilic organisms could be compared. Both acetone powders and cell-free extracts prepared from these powders generally responded in a similar manner. The extraction procedure, found to yield active preparations for all of the gram negative organisms, was simply stirring the powder suspended in M/20 tris buffer at pH 7.4 for four hours

at 5 C. Sonic oscillation of the powders of the gram negative organisms was uniformly unsuccessful. Evidently, the octanoate oxidase system of these organisms is labile to sonic oscillation. S. flava powders were as refractory as fresh cells and required 60 minutes of sonic oscillation before disruption was evident. Extracts obtained in this manner from S. flava varied in their activity, i.e., three out of five preparations exhibited typical activity against octanoate. The remaining extracts were inactive.

The acetone powders of Pseudomonas sp. 4M gave results similar to those represented in Table 5. Activity existed only in the presence of both CoA and ATP, and then to the extent of about 1/2 micromole of O_2 per 1 micromole of substrate. These powders, as well as all of the subsequent ones, were stored at -15 C, but as much as 90% of the activity was lost after two weeks. Therefore, all powders were routinely tested immediately after preparation and the activity of older preparations compared to the original values as a control. In the case of Ps. geniculata, a residual amount of CoA was present in the powder preparations, since either ATP alone or CoA and ATP stimulated activity to about the same extent. (Table 6) The addition of cytochrome c, DPN or yeast concentrate had no stimulatory effect. The acetone powders of S. flava varied in their cofactor requirements for the oxidation of octanoate. The first batch showed no initial

activity unless supplemented with ATP and CoA. However, two succeeding batches were found to be active without the addition of any cofactors, and the oxidation of two micromoles of octanoate went to completion. These powders were pooled and extracted by sonic oscillation. Cofactor requirements could then be assessed.

The powder preparations of S. marcescens offered some difficulty. Of six batches, three demonstrated activity when supplemented with the appropriate cofactors. The remaining three were completely inactive. According to Colowick and Kaplan (13), such variability is not uncommon because of the extreme lability of adaptive systems.

The cell-free extracts for each organism exhibited comparable results as shown in Tables 7-9 and Graph 6. In each case, CoA and ATP was required. The addition of FAD, cytochrome c or DPN had no stimulatory effect over the levels achieved with CoA and ATP. In one instance only, α-ketoglutarate stimulated oxygen uptake without the addition of added cofactors. This phenomenon occurred with the preparation of Ps. geniculata, which contained residual amounts of both ATP and CoA. (Table 7).

As in the case of the acetone powders, the cell-free extracts took up about 1/2 micromole of oxygen per 1 micromole of substrate. While the rates of oxygen uptake varied somewhat with each preparation, the overall uptake was always the

same. In view of the amount of oxygen uptake and the fact that CoA and ATP stimulated activity but DPN did not, quite probably the methods of obtaining cell-free extracts from acetone powders resulted in the recovery of the activating enzymes of the octanoate oxidase system.

DISCUSSION

The results of the studies of temperature characteristics draw attention to differences between the respiration response of psychrophilic and mesophilic bacteria to saturated fatty acids. The psychrophilic pseudomonads not only oxidized octanoate at higher rates at temperatures below 15 C, but also throughout the higher range of temperatures studied. Whether these higher oxidative rates may be construed as characteristic of psychrophilic bacteria as compared to mesophilic forms, has not been proven by these studies. However, the data from the survey of the activity of various psychrophiles and mesophiles on saturated fatty acids lend support to this characterization. Psychrophilic species of Alcaligenes, Achromobacter and Pseudomonas were generally more active against those acids degraded than the mesophilic S. marcescens, A. aerogenes and S. flava. Other supporting evidence may be found in a report by Brown (10). Over a temperature range of 0 - 40 C, greater oxidative activity was exhibited by a psychrophilic pseudomonad on glucose than that demonstrated by a strain of Ps. aeruginosa. Nevertheless, the possibility remains that these higher rates of activity may be merely a function of the particular species. Further rate studies on a variety of oxidizable substrates attacked by both forms would be necessary to lend more credence to this hypothesis.

Oxidation rates also differed in the manner in which they

were influenced by a decrease in the incubation temperature of the suspension. While the μ values were not constant over the entire temperature range, a striking similarity existed between Pseudomonas sp. 4M and Ps. geniculata. The most obvious shift in the μ values occurred at about the same temperature, 15 C. The mesophiles responded with changes in values at higher temperatures which, as mentioned previously, appear to correlate with the general growth response of these organisms to temperature. From inspection of all of the values, the psychrophiles seem to be less influenced by a decrease in temperature than those mesophiles tested. The work recently reported by Ingraham tends to support these data.(40). Rates of metabolism for whole cells in terms of glucose oxidation were also plotted using the Arrhenius equation. Temperature coefficients for the psychrophilic pseudomonads were significantly less than for the mesophile Escherichia coli.

A consideration of possible reasons for these differences would now be appropriate. Two simultaneous effects are known to occur with specific enzyme reactions when the temperature of the environment is increased. The rate of the catalyzed reaction is enhanced, as well as the rate of inactivation of the enzyme due to thermal denaturation. In fact, the existence of the so-called optimum reaction rate depends on the very functioning of both effects. The obvious manifestation

of the thermal denaturation of an enzyme is the usually precipitous decline in the observed reaction rate at temperatures slightly above the optimum. Consequently, the μ values change radically immediately above and below the optimum temperature. Changes in μ values that occur with a rise in temperature below the optimum have been frequently recorded. This non-linearity of response has been the subject of much controversy and discussion. (44)

Many of the early reports describing non-linear responses resulted from variable environmental conditions and plots drawn by visual inspection that are inherently subject to bias(76). Also, as Bodansky(8)pointed out, many of the measures of velocity employed were not true reaction velocity constants, and variable μvalues resulted. On the other hand, more controlled experimental conditions to insure a constant chemical environment still result in μ values which appear to be a function of temperature.

One possible mechanism through which the μ value of both individual enzyme reactions and physiological processes is influenced with an increase in temperature, is an equilibrium between the native and denatured forms of the catalyst. (44) The extent of this equilibrium towards the inactive or denatured form of the enzyme would be increased with a rise in temperature, and become obviously manifest above the optimum temperature. According to Johnson, et al. (44) the

influence of this equilibrium on the observed rate may also occur at temperatures well below the normal optimum. If this occurs, the changes in the μ values at different temperatures observed with the various psychrophiles and mesophiles may be a function of a differential sensitivity of the corresponding enzyme systems to a rise in temperature. Such a differential sensitivity for psychrophiles might be expressed by a characteristically lower temperature where the μ values change, as did occur in this study.

Another possibile explanation of non-linearity over the entire temperature range is the concept of rate-limiting reactions differentially sensitive to temperature. The fact that so many physiological and enzyme processes show a close conformity to the Arrhenius relation over a significant biokinetic temperature range, is at least consistent with the postulate of a single rate-limiting step that may govern the overall rate of a caternary series of reactions (76). In particular, metabolic schemes so far discovered in animals, plants and microbes are typical chain-like reactions. For example, the beta-oxidation of saturated fatty acids is a mechanism involving five basic steps which can be repeated after the cleavage of an activated C_2 unit. However, concomitantly, other chain reactions occur which are coupled to the basic degradative mechanism, i.e., the oxidation of FAD and DPN by the cytochrome system. Thus, any one step

in such a complex series may be rate-limiting. However, when measuring a reaction *in vivo*, other biological factors may themselves become rate-limiting. The accumulation of intermediates due to a block in the catabolic mechanism, or differences in cell membrane densities with altered permeability may be examples. Unfortunately, the nature of the reaction rates with cell-free systems was precluded in this work. An *in vitro* study is necessary if rate-limiting reactions are to be defined in terms of specific enzymes.

While the whole cell studies revealed certain differences in the response of psychrophiles and mesophiles to octanoate, the results of the cell-free extract studies emphasized certain basic similarities. In the preparations of the psychrophilic *Ps. geniculata*, residual amounts of CoA and ATP were present, but the addition of these cofactors stimulated activity against octanoate. In cell-free extracts of *S. flava*, *S. marcescens* and *Pseudomonas* sp. 4M, absolute requirements for these cofactors existed. In view of the report that *Ps. fluorescens* requires CoA and ATP for activity against decanoic acid and since evidence exists for the same requirements by *S. lutea* with other acids, these results are not unexpected (39,56). Evidently, as in animal systems, the degradation of saturated fatty acids in aerobic bacteria proceeds by means of CoA derivatives. While the detailed pathways have not been elucidated, the fact that such

activation is necessary and acetate has been isolated, strongly suggests beta-oxidation as the metabolic mechanism.

An anomaly exists in the case of certain organisms which must be explained if the mechanism of oxidizing fatty acids is to be fully understood. The Achromobacter sp., Pseudomonas sp. 1M, as well as S. marcescens did not oxidize butyrate to any appreciable extent. The question arises as to whether this inability is due to a lack of the necessary activating enzyme, a specific block in the beta-oxidation mechanism or a different pathway. Also, as the chain length of the fatty acid increases, excluding acetate, there appears to be a corresponding increase in oxidation. Thus, butyrate is oxidized at a slower rate than its higher analogs. An understanding of this phenomenon would also contribute to a clearer comprehension of the actual mechanisms involved.

In this work, the results obtained with S. marcescens do not support the conclusions of Silliker and Rittenberg (75) or Rittenberg and Ivler (68). These workers employed the technique of simultaneous adaptation on the cellular level. As mentioned earlier, their results with phenyl substituted fatty acids and the selective inhibition of 2, 4 dinitrophenol led these workers to conclude that beta-oxidation is not the mechanism in S. marcescens. These authors did postulate a repeating enzyme system and did not rule out the possibility of "active" intermediates. The cell-free extracts of

S. marcescens that demonstrated activity against octanoate in this study, did so only when supplemented with CoA and ATP. Therefore, it seems probable that the octanoate was converted to the CoA derivative form and so was "activated". For absolute verification, typical beta-oxidation intermediates should be isolated by means of reconstructed systems of soluble enzymes. However, the results so far reported suggest this mechanism as highly probable.

In the early study of the mechanisms involved in the metabolic oxidation of fatty acids in animal tissue such as liver, a major difficulty was the failure to obtain enzymatically active cell-free preparations. The liver homogenate technique was the first method that produced active preparations when supplemented with the appropriate cofactors, but they were extremely unstable. (27). Later work at the mitochondrial level initiated more successful experiments which eventually elucidated the details of beta-oxidation. The same problem of labile enzymes exists at present with the aerobic bacterial systems. The various methods of isolating cell-free systems reported to date, and the experiments reported here, emphasize the ease with which the fatty acid oxidase system becomes denatured. As mentioned earlier, the quantitative data and the failure of DPN to stimulate oxidation indicate that the acetone powder extraction technique appears to have resulted in the isolation of the thiokinase

and acyl dehydrogenase enzymes.

Attempts were made to "spark" fatty acid oxidation by employing a small amount of α-ketoglutarate. Trace amounts of any one of several components of the citric acid cycle have been repeatedly found to be required for complete oxidation of fatty acids by liver homogenates. As shown in the data, Ps. geniculata extracts could be stimulated by α-ketoglutarate, but only to the same extent as with added cofactors. However, this stimulation did not proceed regularly with the other organisms tested. Again, such results serve to emphasize the lability of the enzyme system, since such stimulation could not proceed with inactive enzymes.

SUMMARY

1. A survey of the oxidative activity on certain saturated fatty acids by psychrophilic bacteria has been made. Psychrophilic species of Pseudomonas, Alcaligenes and Achromobacter were found to be active against the sodium salts of acetic, butyric, hexanoic and octanoic acids. Certain species failed to oxidize butyric acid. Species of Flavobacterium were active on acetic acid only.

2. A comparison of the activity of selected psychrophiles and mesophiles on octanoic acid revealed a similar response of the psychrophilic cells to the influence of temperature. The oxidative rates of these organisms appeared to be less affected by decreasing temperatures than the mesophiles tested. This difference was expressed in two ways: (1) by lower μ values throughout the entire temperature range, and (2) by a lower temperature at which changes in μ values occurred.

3. A study of cell-free extracts obtained from these same organisms demonstrated that the cofactor requirements for activating fatty acid oxidation was similar in both the psychrophilic pseudomonads and the mesophilic Serratia marcescens and Sarcina flava. All the organisms tested required adenosine triphosphate and Coenzyme A.

APPENDIX

TABLE 1.

Physiological Reactions of Selected Psychrophile Isolates

Characteristic	Isolate								
	1M	2M	4M	1B	7B	9B	4C	17C	19C
Gelatin Liquefaction	+	-	+	-	-	+	-	+	+
Indol	-	-	-	-	-	-	-	-	-
Methyl Red	-	+	-	-	-	-	+	-	-
Acetyl Methyl Carbinol	-	+	-	-	-	-	+	-	-
Citrate	+	+	+	+	+	+	+	+	+
Nitrate Reduction	-	+	+	-	-	-	+	+	+
Litmus Milk	Ak Rdn	Ak	Ak Pep	Ak	Ak	Ak	Ak SC	Ak	Ak
H_2S	-	-	-	-	-	-	-	-	-
Chromogenesis on Agar	YG	Y	YG	YG	YG	YG	Y	YG	YG
Lactose	-	-	-	-	-	-	A	-	-
Maltose	-	A	-	-	-	-	A	-	-
Sucrose	-	A	-	-	A	-	A	-	-
Glucose	A	A	A	A	A	A	A	A	A
Fructose	-	A	-	-	-	-	A	-	-
Galactose	-	A	-	-	-	-	A	-	-

TABLE 1. (cont.)

Characteristic	Isolate								
	1M	2M	4M	1B	7B	9B	4C	17C	19C
Xylose	-	A	-	-	-	-	A	-	-
Arabinose	-	A	-	-	A	A	A	-	-
Glycerol	-	A	-	-	-	-	A	-	-
Mannitol	-	A	-	-	-	-	A	-	-
Motility	+	+	+	+	+	+	+	+	+
Flagella	p	per	p	p	p	p	per	p	p
Aerobiosis	a	fa	a	a	a	a	fa	a	a
Lipolysis	+	-	+	+	+	+	-	+	+

Key: A = acid, Ak = alkaline, YG = yellow-green diffusable pigment, Y = yellow non-diffusable pigment, per = peritrichous, p = polar, Rdn = reduction, SC = soft curd, a = aerobic, fa = facultative aerobe.

TABLE 2.

Activity of Selected Psychrophiles and Mesophiles on Saturated Fatty Acids

Organism	Microliters of Oxygen Uptake in 120 minutes			
	C_2	C_4	C_6	C_8
Pseudomonas sp. 1M	129	-16	126	89
Pseudomonas sp. 4M	155	34	170	197
Flavobacterium sp. 2M	121	-8	-2	9
Pseudomonas sp. 1B	60	16	59	86
Pseudomonas sp. 7B	36	60	74	123
Pseudomonas sp. 9B	34	45	64	85
Pseudomonas sp. 17C	183	22	94	129
Ps. fluorescens	217	29	122	150
Achromobacter sp.	145	5	111	123
Alcaligenes sp.	184	67	180	283
Ps. geniculata	241	47	132	166
Flavo. rhenanum	60	0	0	0
Serr. marcescens	33	0	54	58
Sarcina flava	45	23	74	46
Aero. aerogenes	83	5	17	18

Substrate concentration = 10 micromoles of sodium salt, M/20 phosphate buffer at pH 7.4, incubation at 30 C, air atmosphere, values corrected for endogenous rates.

TABLE 3.

Microkjeldahl Nitrogen Determinations of Whole Cell Suspensions

Organism	Optical Density of Cell Suspension at 540m	Mg. Nitrogen per ml of Suspension (av. of duplicates)
Serr. marcescens	0.345	0.945
Ps. geniculata	0.345	0.938
Pseudomonas sp. 4M	0.345	0.968
Sarcina flava	0.470	1.128

TABLE 4.

Temperature Characteristic (μ) Values for the Oxidation of Octanoate

Organism	(μ) (cal/mole)	Temperature Range °C
Pseudomonas sp. 4M	7630	15 - 37.5
	16,030	7.5 - 15
Ps. geniculata	6870	15 - 35
	14,660	7.5 - 15
Sarcina flava	9160	25 - 37.5
	22,900	15 - 25
Serr. marcescens	9160	20 - 35
	18,320	7.5 - 20

TABLE 5.

Activity of *Pseudomonas* sp. 4M Acetone Powder on Sodium Octanoate

Substrate Concentration	Cofactors Added	Oxygen Uptake in Microliters Time (minutes)					
		30	60	90	120	150	180
2 micromoles	None	0	0	0	-	-	-
2 micromoles	ATP	1.0	0	0	-	-	-
2 micromoles	CoA	2.3	6.1	5.7	-	-	-
2 micromoles	CoA ATP	3.7	6.5	9.8	13.5	19.8	21.5
5 micromoles	CoA ATP	16.7	20.0	39.8	37.5	46.9	52.2

The reaction flasks contained 1 ml of the acetone powder (25 mg/ml), 2 moles of $MgCl_2$, 0.12 mg CoA, 0.1μmole GSH, 1μmole ATP, 5μmoles phosphate, in .05M tris buffer at pH 7.4, and substrate as indicated. Incubation at 30 C, air atmosphere with 0.2 ml 20% KOH in the center well. The total volume of the reaction mixture was 3.2 ml. All data are corrected for endogenous respiration in absence of substrate.

TABLE 6.

Activity of *Pseudomonas geniculata* Acetone Powder on Sodium Octanoate

Cofactors Added	Oxygen Uptake in Microliters Time (minutes				
	30	60	90	120	150
None	1.9	6.4	4.9	5.8	5.1
CoA	0.4	2.7	1.8	5.2	4.2
ATP	4.5	18.0	21.9	29.3	30.3
CoA ATP	1.5	9.9	14.9	23.5	24.5

The reaction mixtures are as described in Table 5, except the substrate concentration was 2 μ moles.

TABLE 7.

Activity of *Pseudomonas geniculata* Cell-Free Extracts on Sodium Octanoate

Cofactors Added	Oxygen Uptake in Microliters Time (minutes)			
	30	60	90	120
None	11.9	12.7	16.4	18.7
ATP	9.2	12.4	12.6	14.4
CoA	19.0	23.6	26.9	26.3
α-ketoglutarate 1 μmole	12.4	16.5	21.7	23.7

The reaction mixtures contained 1 ml of the extract (11 mg protein/ml), 2 μmoles of $MgCl_2$, 0.12 mg CoA, 0.1 mole GSH, 1μmole ATP, 5μmoles phosphate, in 0.05 M tris buffer at pH 7.4, and substrate concentration of moles. Incubation at 30 C, air atmosphere with 0.2 ml 20% KOH in the center well. The total volume of the reaction mixture was 3.2 ml. All data are corrected for endogenous respiration in absence of substrate.

TABLE 9.

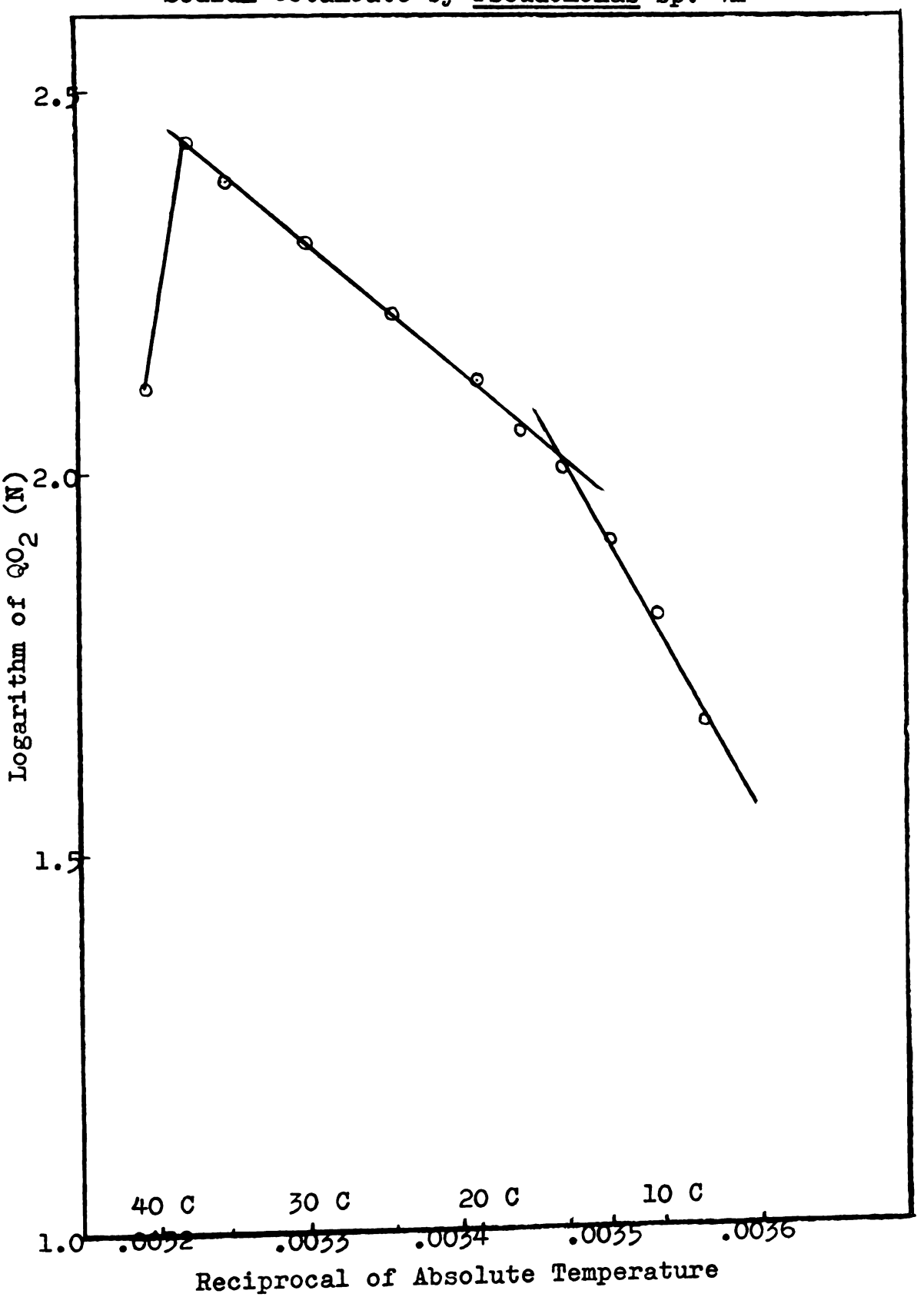
Activity of *Serratia marcescens* Cell-Free Extracts on Sodium Octanoate

Cofactors Added	Oxygen Uptake in Microliters Time (minutes)		
	30	60	90
None	0	0	0
ATP	1.0	0	0
CoA	2.7	6.3	5.4
CoA ATP	17.3	20.4	21.5

Reaction mixture same as in Table 7, except the extract consisted of 7 mg protein per ml.

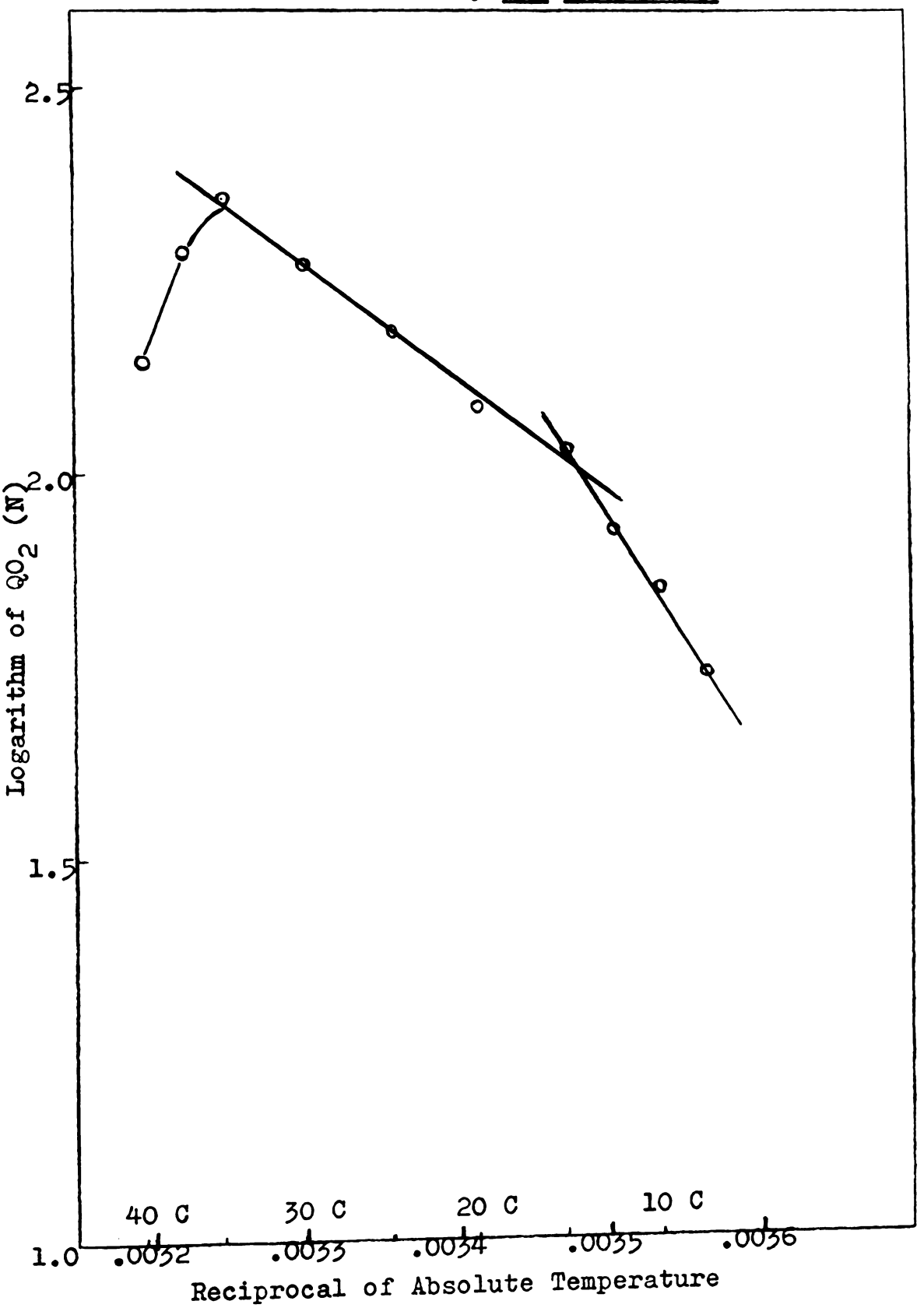
GRAPH 1.

Temperature Characteristic Plot of the Oxidation of Sodium Octanoate by Pseudomonas sp. 4M



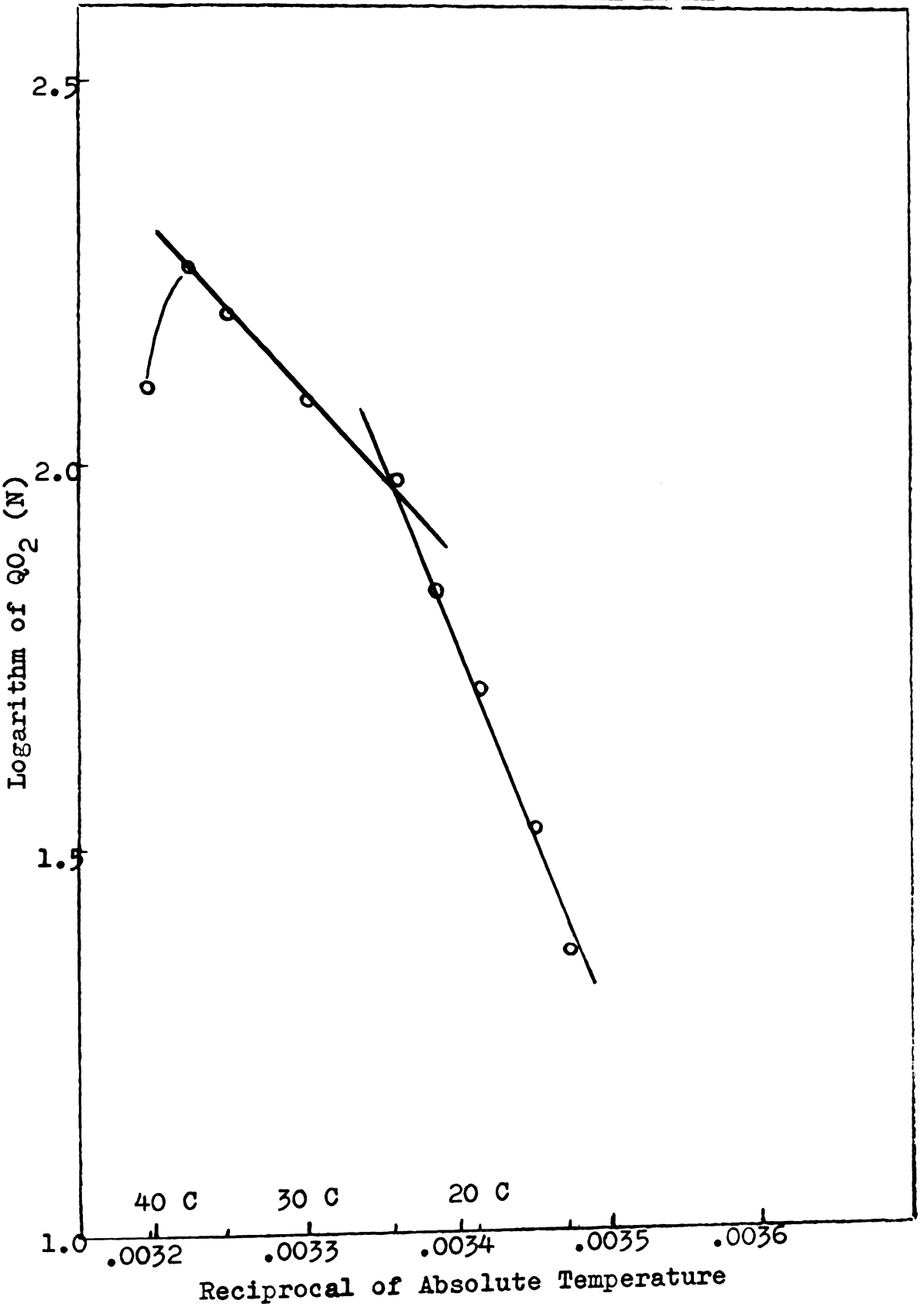
GRAPH 2.

Temperature Characteristic Plot of the Oxidation of Sodium Octanoate by Ps. geniculata



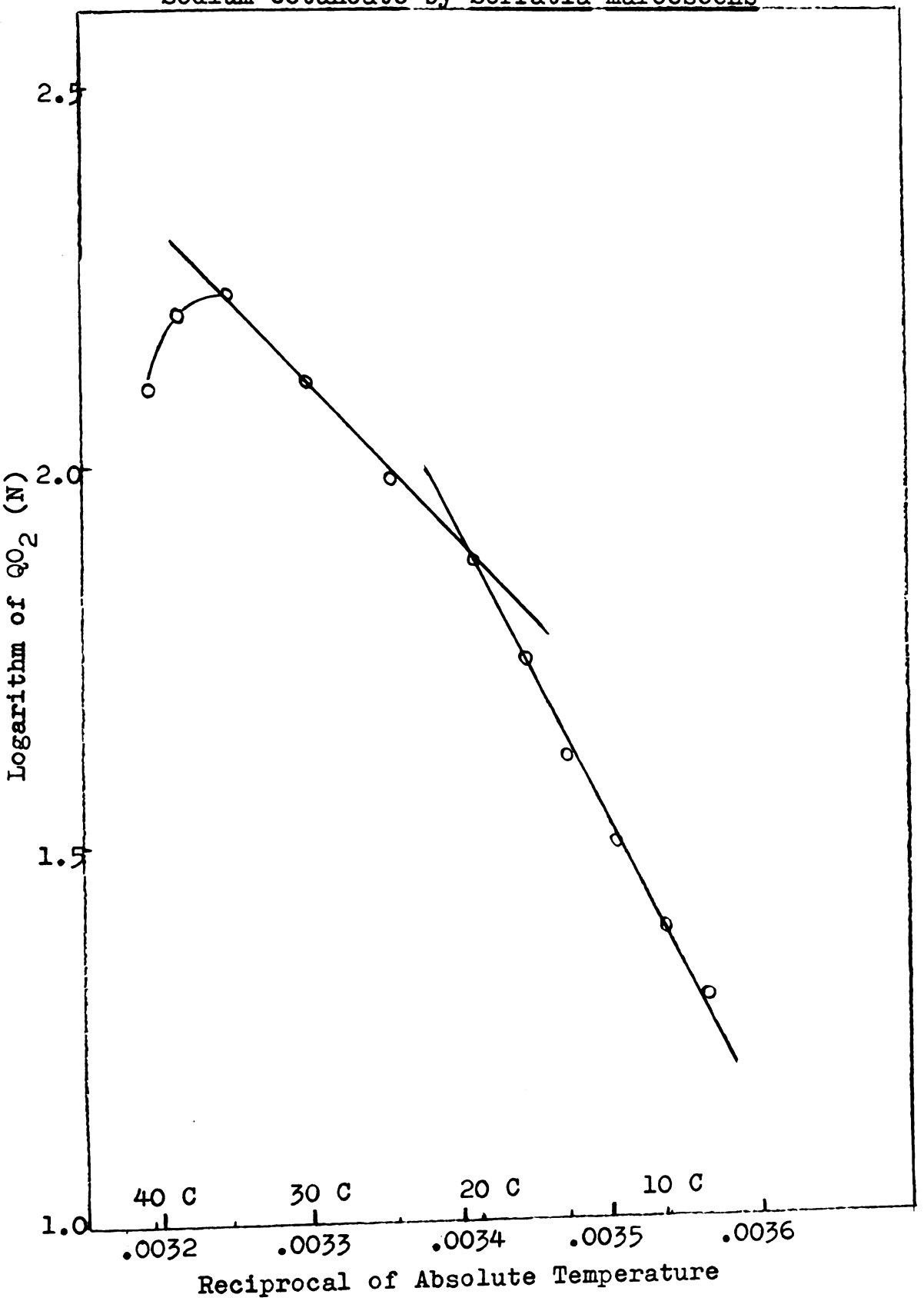
GRAPH 3.

Temperature Characteristic Plot of the Oxidation of Sodium Octanoate by *Sarcina flava*



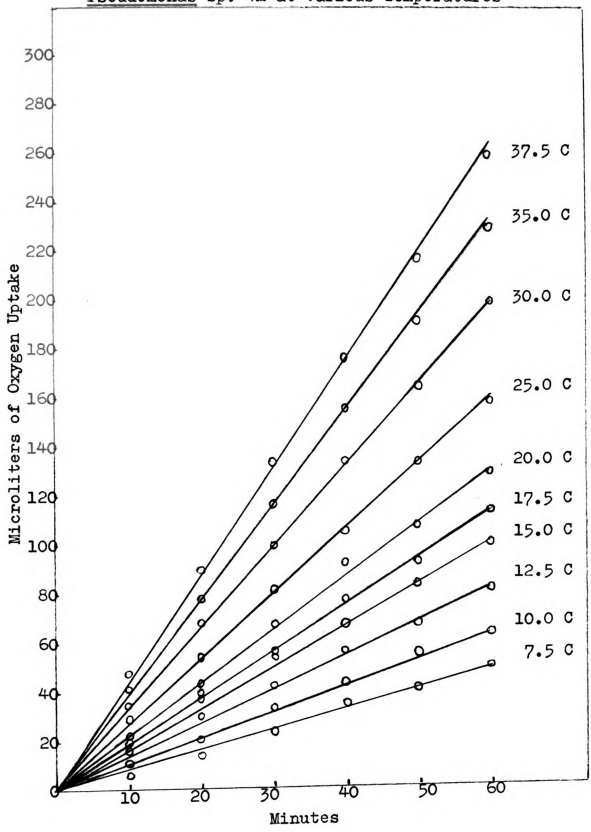
GRAPH 4.

Temperature Characteristic Plot of the Oxidation of Sodium Octanoate by Serratia marcescens



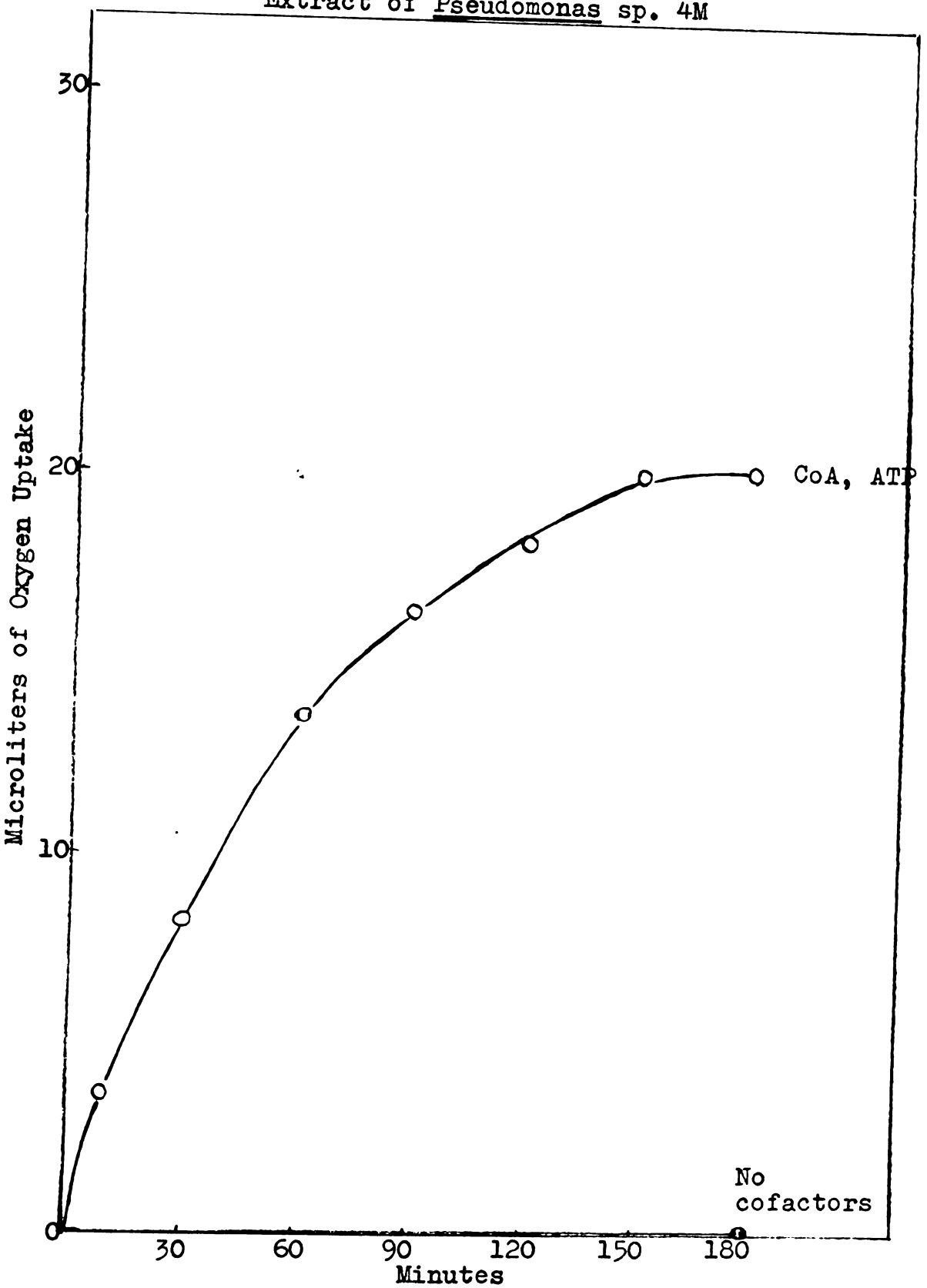
GRAPH 5.

Oxidation of Sodium Octanoate by
Pseudomonas sp. 4M at Various Temperatures



GRAPH 6.

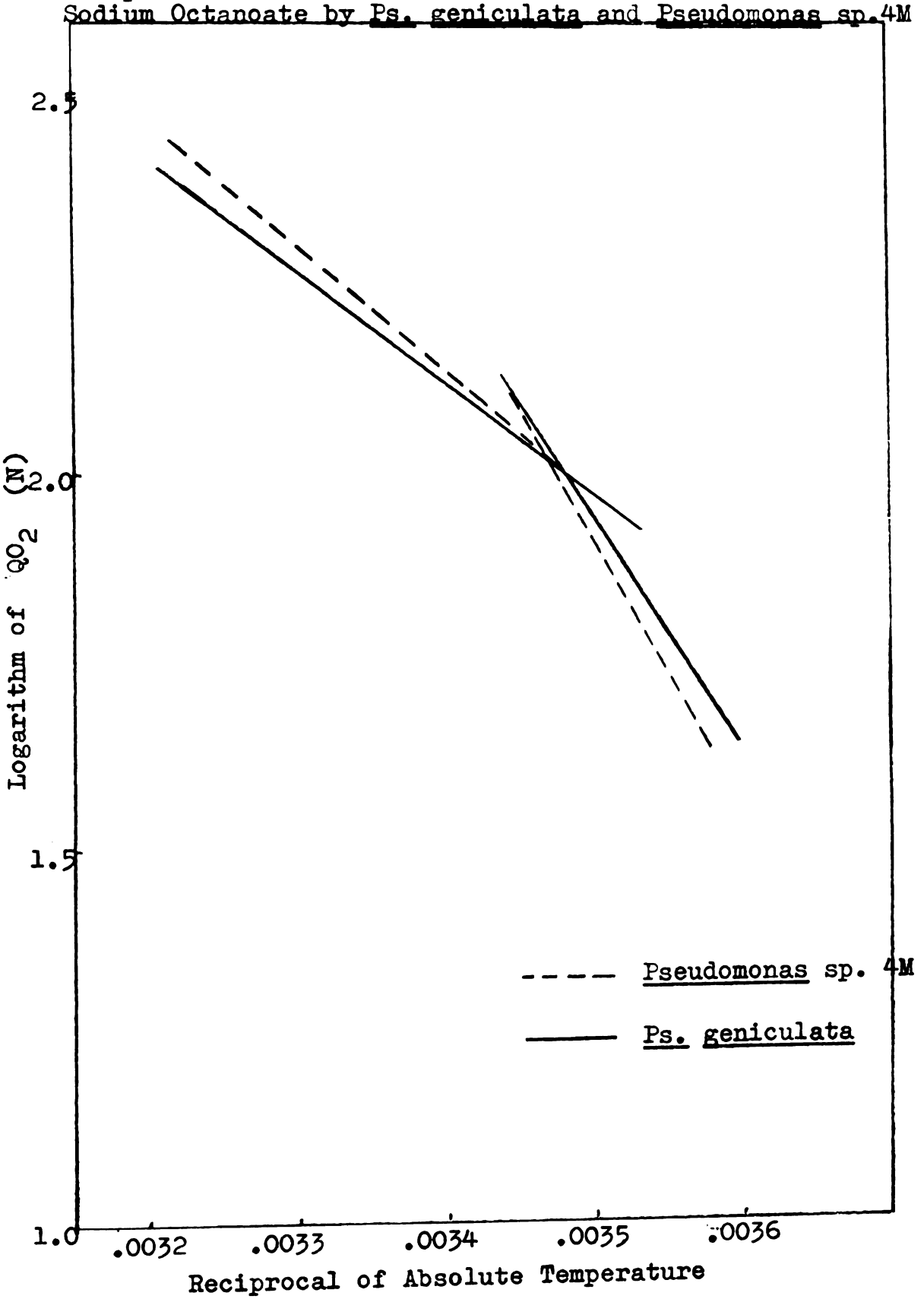
Oxidation of Sodium Octanoate by Cell-Free Extract of Pseudomonas sp. 4M



Reaction mixtures as described in Table 5, except the extract consisted of 15 mg. protein per ml.

GRAPH 7.

Temperature Characteristic Plot of the Oxidation of Sodium Octanoate by Ps. geniculata and Pseudomonas sp. 4M



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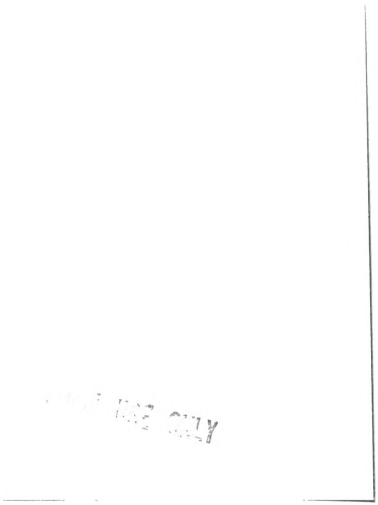


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