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FATTY ACIDS OF *BACILLUS POLYMYXA* AND *BACILLUS STEAROTHERMOPHILUS* AS INFLUENCED BY TEMPERATURE

by

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INTRODUCTION

Microorganisms have a wide range of tolerance to heat. Some psychrophiles hardly survive at normal room temperature (25°C), while a few thermophiles can grow well at 70 and 80°C. Vegetative cells of bacteria are destroyed almost instantly at 100°C but spores of certain species can withstand prolonged exposure to such a high temperature without seemingly affecting their viability.

Thermobiosis is of considerable interest because the phenomenon dictates the inability of some bacteria to metabolize and reproduce at temperatures which are suitable for most other forms of life. Two explanations have been offered for thermophily: first, the theory of the "dynamic nature of thermophily" has been suggested by Allen (1953). She holds that proteins of thermophiles are readily denatured at temperatures at which the cells grow well. The cells, however, maintain their viability through a rapid synthetic process or repair of damaged protein. A second theory is that the thermophiles contain proteins with greater heat stability or have a factor(s) which confers thermal stability to the corresponding proteins. This is exemplified by the findings of heat resistant enzyme systems in thermophiles (Marsh and Militzer, 1952; Campbell, 1955; Brown et al., 1957) or in the heat stabilizing effect of fatty acids on proteins (Boyer et al., 1946).

Many hypotheses have been advanced on the thermal resistance of spores, from the simple explanation of reduced water content to the more sophisticated biochemical binding of spore constituents. Essentially, thermal destruction of organisms is thought to be caused by the coagulation or denaturation of proteins. Peptide chains of closely folded native protein cannot unfold unless water flows into the space between the chain; therefore, dry proteins are apt to be more resistant to denaturation than moist proteins. An additional hypothesis has been proposed by Lewis et al. (1960) to the effect that spores contain a composite protein complex which limits the water uptake; the water content within the spore is controlled through pressure exerted by slow contraction of the cortex during maturation. The cortex, is a distinct layer between the spore coat and the spore wall.

Dipicolinic acid (DPA) is almost exclusively found in bacterial spores, and the fact that the ratio of DPA and calcium in the spore is always close to one has been interpreted to mean a possible formation of a complex of DPA, calcium, and other compounds such as proteins which could enhance heat stability. DPA is an extremely good chelating agent for bivalent metal ions; calcium is capable of balancing the dispersing effect of monovalent cations on colloidal aggregates. When negatively charged groups in aggregates are linked through calcium ions, a stronger bond may develop be-

tween colloidal groups which confers greater resistance to thermal unfolding of peptide chains (Powell and Strange, 1953; Sugiyama, 1951).

Evidence accumulated from the past has shown that certain constituents can vary between vegetative cells and spores. Thermally stable antigens, for instance, have been found in the spore (Baille and Norris, 1964). Halvorson (1962) claimed the presence of heat resistant proteins in spores. Nucleic acids, believed to be responsible for the control of metabolism, production, and growth of cell, have also been found to have a different composition (Fitz-James, 1955).

The study of bacterial lipids is relatively new; the lack of information in this area may be attributable to the difficulty in isolation and characterization of these compounds. It is generally agreed, however, that lipids and fatty acids are chemically complex and metabolically more active than suspected in the past. Considerable changes in composition of fatty acids, for example, are found in bacteria under different growing conditions. Spores produced at high temperatures usually have increased resistance to heat while demonstrating a tendency of decreased lipid content. Because of the complexity of any biological system and the spotty knowledge on such topics, no definite conclusion has been derived yet on the role of lipid in the "inertness" of spores. However, it is agreed that thorough understanding of the

occurrence of fatty acids is essential in further understanding of such factors as microbial lipid synthesis and degradation, function and immunological properties of lipids found in cells. In addition, knowledge of the lipid composition of a cell may even be used as a tool for taxonomic studies.

The present work has been undertaken 1) to determine if any qualitative differences could be detected in the lipid fraction of vegetative cells and spores of mesophile and a thermophile; 2) to correlate with the lipid fraction, if possible, any differences in the stability of the spore to heat; and 3) to gain a better appreciation of the role of lipids in thermophily.

REVIEW OF LITERATURE

Lipids are found as structural components and energy reserves in all organisms except a few single viruses. White et al. (1964) have defined lipid as that fraction of any biological material which is extractable by non-polar solvents. Lately, through improvements in analytical techniques, especially vapor phase chromatography, interest in the role of lipids in the cell especially those of lipids in bacteria has received increased emphasis. Several reviews ranging in subject from synthesis and degradation, intracellular distribution and function, to the immunological properties and the nature of lipid complexes attest to interest in the field (Asselineau and Lederer, 1960; O'leary, 1962a; Kates, 1964; Asselineau, 1966).

The intracellular distribution of lipids varies. Lipid inclusions, for instance, have been found in Azotobacter, Rhizobium, Spirillum, Bacillus, and Corynebacterium, but very few gram negative bacteria show such deposits (Burdon, 1958). Marked differences in lipid content between the cell wall of gram positive and gram negative bacteria have been observed by Salton (1960). Gram negative bacteria have cell walls that are multilayered, with the lipid amounting to about 10-20% of the wall. Roughly, the lipid in the wall consists of lipoprotein in the outermost layer and lipo-

polysaccharides in the middle layer. Weidel and Primosigh (1958) even went so far as to claim that the cell wall of Escherichia coli contains 80% lipoproteins.

Alimova (1958) studied the distribution of lipid in the cell wall of Corynebacterium and found that it contained a variety of fatty acids ranging from C_{14}-C_{24} with the acids occuring either free or as esters of trehalose. Azotobacter vinlandii also contains C_{14}-C_{18} acids with palmitoleic being the major components (Marr and Kaneshiro, 1960).

Cytoplasmic membranes, which are believed to be selective, semipermeable barriers of bacterial cells, are rich in lipid materials. Gilby and co-workers (1958), for instance, found the membrane of Micrococcus lysodeikticus to be made up of 28% lipid. The fatty acid composition in membranes differs from one species to the other. Branched C_{15} acid was noted as the major acid in Bacillus megaterium (Thorne and Kodicek, 1962b) while palmitic, cis-vaccenic and lactobacillic acid are the main components in Lactobacillus casei.

Although such studies on fatty acid components are being pursued at present, there is yet no conclusive information concerning the distribution of lipid in bacterial cells.

Fatty Acids

Fatty acids are integral constituents of most simple and complex lipids. Large numbers of fatty acids have been isolated from bacterial lipids. A comprehensive description of these various fatty acids was published by Asselineau (1966). Fatty acids seldom occur in nature in the free state; instead, fatty acids are bound or combined with other materials through ester linkages.

Saturated straight chain acids

Saturated, straight chain acids have been found in bacteria and are identical to saturated acids found in other forms of life. The shorter chain acids (those with less than 12 carbon atoms) have been detected in small amounts (O'leary, 1962a). The major portion of the acids, however, seem to be those with a higher number of carbon atoms (C_{12}-C_{20}). Fatty acids have been detected in many kinds of bacteria with C_{16} being a predominant fraction. Kaneshiro and Marr (1961) noted that as much as 40% of the fatty acids in *Escherichia coli* was palmitic acid. Law *et al.* (1963) found an even higher concentration of this acid in *Serratia marcescens*, and *Azotobacter agilis*. Gram positive bacteria have a more diverse form of acid components as indicated by the results of Macleod and Brown (1963) in which they reported a significant amount of myristic acid to

be present as well. Macfarlane (1962b) detected a large quantity of myristic and arachidic acid in Clostridium perfringens. Acids greater than C_{20} are not prominent although such acids as behenic (C_{22}), lignoceric (C_{24}), and octacosanoic (C_{28}) acid have been found in Corynebacterium diphtheriae (Asano and Takahashi, 1945). The odd number straight chain fatty acids such as C_{15} and C_{17} acids have occasionally been recovered in Pseudomonas aeruginosa (James and Martin, 1956); C_{15} in Clostridium butyricum (Goldfine and Bloch, 1961); C_{13}, C_{15}, C_{17} in Clostridium bifermentans (Moss and Lewis, 1967); and C_{15}, C_{17} in Mycobacterium phlei (Lennarz et al., 1962).

Branched chain acids

Branched chain acids are more or less characteristic of the Eubacteriales; the more common ones found are the iso acids and the anteiso acids. Iso acids are the branched chain fatty acids having one methyl group at the penultimate position of the longer carbon chain while the anteiso acids refer to the branched fatty acids having one methyl group at the antepenultimate position. In fact, the aerobic bacilli have as their major constituents branched chain acids with more than 13 carbons. Saito (1960), and Kates et al. (1962), working with Bacillus subtilis and Bacillus cereus respectively, have found that as much as 70% of the fatty acids may be identified as branched chain acids. Other

bacteria known to possess such acids are Staphylococcus aureus (Macfarlane, 1962a) and Micrococcus lysodeikticus (Macfarlane, 1961). When Akashi and Saito (1960) hydrolyzed their crude phosphatidic acid extracts, they isolated a liquid, saturated fatty acid they first thought to be C_{15} but later identified as iso C_{15}. Since they also extracted similar material from a Sarcina culture, it is probable that Sarcina species contain branched chain acids as well. Gubarev and his co-workers (1951) fractionated the lipids of Corynebacterium and positively identified several acids present including corinnic and diphtheric acids. These acids are thought to have a melting point of 64 C and 44-45 C respectively and have as their empirical formula $C_{35}H_{68}O_2$,

Cyclopropane acids

The first acid of this type to be discovered in bacteria has been named lactobacillic acid. This compound was first reported by Hofmann and Lucas (1950) when they isolated the ester of a $C_{19}H_{26}O_2$ acid by fractional distillation of the ester mixture from the lipids of Lactobacillus arabinosus. Further examination led to the conclusion that the cyclopropane ring is located on carbon atoms 11 and 12. Similar acids had also been isolated from Agrobacterium tumefaciens (Hofmann and Tausig, 1955), Escherichia coli (Law, 1961), and Clostridium butyricum (Goldfine and Bloch, 1961).

Although C_{19} cyclopropane acids seem to be the most prevalent cyclic acids in bacteria, more recent studies have shown that other cyclopropane acids do occur. A C_{17} cyclopropane acid was detected in the lipids of Escherichia coli (Chalk and Kodicek, 1961). By examining the oxidation product of the esters and with the aid of infrared and mass spectra comparison, the acid has now been identified as 9,10 methylenehexadecanoic acid. The acid has since been detected in Clostridium butyricum (Goldfine and Bloch, 1961) and Pasteurella pestis (Asselineau, 1961). Employing the gas chromatography and radioisotope tracer technique on Aerobacter aerogenes, O'leary (1962b) claimed the presence of a large quantity of C_{17} cyclopropane acid. Furthermore, he claimed that the S-adenosylmethionine functions as a donor of a one carbon unit to be used in the formation of ring structure in the synthesis of cyclopropane acids. Recent refinements in separation techniques have shown the possible presence of C_{13} and C_{15} cyclopropane acids in Clostridium butyricum (Goldfine and Bloch, 1961).

Unsaturated acids

It is widely accepted that bacteria are incapable of synthesizing polyenoic fatty acids (Lennarz, 1966), although linoeic acid has been detected in the cells of Rhodopseudomonas particilis (Hands and Bartley, 1962) and a $C_{20:4}$ fatty acid was listed by Huston and Albro (1964) in

their studies on Sarcina lutea. Bacteria, however, have been shown to synthesize monoenoic acids. Cis-vaccenic acid (cis-11-octadecenoic acid) was found in Lactobacillus arabinosus (Hofmann et al., 1952), oleic acid has been definitely identified in streptococci (Hofmann and Tausig, 1955). Monoenoic C_{16} as well as other higher fatty acids were also found quite regularly in various species of bacteria (Kaneshiro and Marr, 1961; Bishop and Still, 1963; Thorne and Kodicek, 1962a; Kates, 1964).

Hydroxy acids

Hydroxy acids from bacteria usually belong to the D-series when comparing the configuration of their asymmetric carbon. The more commonly found hydroxy acids are 3-hydroxybutyric acid (Smithies et al., 1955; Forsyth et al., 1958), and 3-hydroxy-decanoic acid (Cartwright, 1957; Law, 1961).

3-D hydroxybutyric acid (or β-hydroxybutyric acid) will be discussed in more detail because of its relative abundance in Bacillus species. Lemoigne et al. (1944) first identified its presence in B. subtilis in polymer form. Weibull (1953) also noted the acid as a major lipid constituent when he characterized the nature of the sudanophilic granules isolated from "ghosts" of B. megaterium. However, it was not until 1958 that Williamson and Wilkinson demonstrated unequivocally that the intracellular granules in Bacillus cereus consisted

of poly-hydroxy-butyric acid (PHBA).

Lemoigne et al. (1949) claimed that this acid constituted up to 50% of the total lipids in B. megaterium. The polymers were also found, though in lesser quantity, in Bacillus cereus, Bacillus mycoides, Bacillus anthracis (Lemoigne et al., 1944). Among other bacteria thought to contain PHBA were Chromobacterium, Azotobacter, (Forsyth et al., 1958), Rhizobium (Forsyth et al., 1958; Vincent et al., 1962), Hydrogenomonas (Schlegel et al., 1961), Micrococcus (Sierra and Gibbons, 1962), Nitrobacter (Tobback and Landelout, 1965), Sphaerotilus (Rouf and Stokes, 1962), Spirillum and Vibrio (Hayward et al., 1959).

Macrae and Wilkinson (1958) studied the effects of various cultural conditions on the synthesis of PHBA in Bacillus megaterium. They found an increased deposition of the acid in bacteria after exhaustion of the nitrogen source and in the presence of energy source or after increasing the glucose concentration in the medium. Furthermore, aerobic conditions were needed for such production although an oxygen concentration (v/v) of greater than 5% could inhibit assimilation. Doudoroff and Stanier (1959) observed that the major portion of the carbon from the substrate was accumulated as PHBA. When the substrate was eventually removed, rapid intracellular degradation of the polymer occurred suggesting that PHBA is a reserve material

in the cell. A similar conclusion was arrived at by Stanier et al. (1959) when they showed a tremendous drop in PHBA content in the presence of carbon dioxide and nitrogen with a concomitant increase in both carbohydrate and nitrogen content in the cell.

Slepecky and Law (1961) showed that if conditions were not favorable for utilization of PHBA, Bacillus megaterium did not sporulate readily. However, if conditions were favorable, the organism used the nutrient and sporulation proceeded at a much faster rate. They also noted that sporulation occurred only in cells containing large amounts of PHBA, which suggested that a system for metabolizing PHBA developed before sporulation, and its function was to supply energy and carbon for the sporulation process.

Fatty acids of spore forming bacteria

Fatty acids in spores have not been studied too extensively, mainly because of difficulty in extraction and isolation of these acids from the tough spore coat. Abel et al. (1963) tried to classify microorganisms by analyzing the fatty acid content of different genera. They found spores of Bacillus subtilis to be made up of $C_{15}^{=}$, as the major component, followed by C_{17} and some other acids ranging from C_{10}-C_{22}. Spores of Bacillus anthracis were found to have many more fractions; however, $C_{15}^{=}$ was still the major component among the acids ranging from C_5-C_{19} in

length. Kaneda (1966a; 1967), working mainly with cells of the Bacillus group, noticed a remarkable concentration of branched chain acids (from C_{15}-C_{17}); these acids constituted over 60% of the total lipids. The same was shown by Saito (1960) in his work with Bacillus subtilis. Moss and Lewis (1967) ran tests on 40 strains of Clostridium and found that they could divide the anaerobes into 4 different groups based on differences in fatty acid composition. They further found a change in the level of acid at different stages of growth with more unsaturated acid present in the younger cells. Pheil and Ordal (1967) extracted lipids from the spores of Clostridium thermosaccharolyticum and found the most important fractions to be C_{14}, C_{16} and C_{18}. Evidently, different species contained different fatty acids which vary in concentration depending upon the cultural conditions.

Influence of Cultural Conditions on Bacterial Lipids

Studies on bacterial lipids must take into consideration cultural conditions because lipids in the cell are known to vary with the composition of the culture medium, the age of the culture, and the temperature. Lemoigne et al. (1949) found a consistent decrease in percentage of PHBA and a relative increase of other lipids even though the total lipid

decreased as the culture aged. Lactobacillus arabinosus collected in the stationary phase consistently showed a higher percentage of saturated acid as compared to the organism collected during the logarithmic phase (Croom and McNeill, 1961). A similar effect was found in Escherichia coli by Marr and Ingraham (1962) and Law et al. (1963); they noticed an increase in C_{17}, C_{19} cyclopropane acid and a decrease in C_{16} and C_{18} monoenoic acids as the culture aged. Phosphatidyl glycerol content of E. coli was much higher during the log phase than during the stationary phase. (Lennarz et al., 1962; Kanfer and Kennedy, 1963; Kates et al., 1964). Law et al. (1963) noticed an increasing change in lipid composition of Serratia marcescens; the lipid content would rise to a maximum value then decrease to a minimum during the first half of the logarithmic phase but would rise again to the initial value during the latter part of the logarithmic phase and the early stationary phase.

Mesophilic and psychrophilic bacteria grown at temperatures less than the optimum usually showed an increased content of unsaturated fatty acids. Marr and Ingraham (1962) found a progressive increase of hexadecenoic acid in E. coli as the incubation temperature was progressively decreased. Little change in monoenoic and cyclopropane acids in Serratia marcescens was observed when the temperature of incubation was reduced from 37 to 30 C (Bishop and Still,

1963). However, there was an increase in the amount of β-hydroxy acids. If the temperature was dropped to 10 C, there was a definite decrease in the unsaturated and cyclic acids. Thermophiles demonstrated an opposite effect; Long and Williams (1960) found an increase in unsaturation when the bacteria were grown at higher than optimal temperatures.

Burdon et al. (1942) contended that the type of medium was responsible for accumulation of lipid granules in B. subtilis; Grelet (1952) concluded that Mn^{++} was required for lipid synthesis in Bacillus megaterium. Compounds such as iron, glycerol, and acetate influence the lipid content of Mycobacterium species (Asselineau, 1966). Acetate and mevalonic acid were shown to be precursors of fatty acids and unsaponifiable lipids in lactobacilli (Thorne and Kodicek, 1962a), while Tween 40 has been shown to stimulate the production of fatty acids in lactobacilli (Hofmann et al. 1957). Cho et al. (1964) studied the effect of diphenylamine on fatty acid composition of mycobacteria, Sarcina, and Chromobacterium; they concluded that this compound did not produce any significant change in total lipid content. However, the concentration of individual acids was shown to have changed. A definite increase in palmitic, methylene hexadecanoic acid with a concurrent decrease of unsaturated acids in E. coli occurred when the bacteria were grown in a nitrogen-limiting medium rather than in a carbon-limiting

medium (Marr and Ingraham, 1962). Furthermore, they claimed that the proportions of palmitic and unsaturated acid depends more on the material present in the medium than on the growth rate of the bacteria; glucose minimal medium, for instance, provided the highest content of palmitic and least unsaturated acids.

Cultural variation not only changes the composition of the bacteria, it also may affect the heat resistance of the bacteria. Murrel and Warth (1965) tested the resistance of Bacillus cereus spores and concluded that the farther away from the optimum temperature, the more heat sensitive the spores became. A similar observation was observed with spores of the anaerobes (Sugiyama, 1951; Wynne, 1957). No evidence, however, has been presented to indicate whether changes in fatty acid composition is the cause or result of the shift in thermal resistance of spore.

As mentioned before, fatty acids seldom exist as free fatty acids in bacterial cells, but rather they are combined with other compounds to form complex lipids. Knowledge of fatty acid content does not necessarily provide any clue as to the kinds of lipid bacteria contain; however, since fatty acids do not function as an individual substance but rather as a complex substance, a short discussion of the more prevalent complex lipids will be made.

Phospholipids

Phospholipids are widespread in bacteria and frequently comprise the major portion of the total lipid content of the microorganism. The content, however, can vary quite drastically between different genera and even between species (Cmelik, 1954; Kaneshiro and Marr, 1962; Rosenberg, 1963). Escherichia coli, for instance, was found to contain more than 5% of its dry cell weight as phospholipid (Law, 1961) while negligible amounts were found in Bacillus species (Lemoigne et al., 1949; Kates et al., 1962).

Phospholipids are extremely important because they tend to aggregate at interfaces and thus appear as constituents of the cell membrane system. As a group, they are thought to be capable of mediating the active transport of molecules and ions; facilitating the diffusion of compounds like CO_2; or even imparting charges for the propagation of electrical impulses along the membrane (Trumbore, 1966).

Phosphatidic acids

Phosphatidic acids are the basic structure upon which phospholipids are built. Bloch (1936) isolated a nitrogen-free compound from the phosphatide fraction of human tubercle bacilli which he identified as diglyceride phosphoric acid. Later, inositol glycerol diphosphoric acid was detected in

this same organism by DeSütö-Nagy and Anderson (1947). More recently, Vilkas and Lederer (1956) isolated from several streptomycin-resistant strains of mycobacteria a magnesium salt of phosphatidic acid. Similar results have been reported for other bacteria such as the work of Matches et al. (1964) on cells and spores of Bacillus polymyxa. Huston et al. (1965) noted that approximately 5% of the total lipid of Sarcina lutea is phosphatidic acid. Although phosphatidic acid has been detected quite regularly, a doubt still exists as to whether it is merely the hydrolytic product of other complex lipids (Asselineau, 1966).

Lecithin and cephalin

Lecithin and cephalin are both derivatives of phosphatidic acids. Lecithin is the choline ester of phosphatidic acid while cephalin is the ethanolamine ester of phosphatidic acid. As early as 1939, Geiger and Anderson tried to characterize the phosphatides they isolated from Agrobacterium tumefaciens by identifying the saponification products of the phosphatides; they found these products to be choline and ethanolamine. They concluded that phosphatides were a mixture of lecithin and cephalin. Similar observations were made by Kaneshiro amd Marr (1962) in the same bacteria as well as by Matches et al. (1964) in Bacillus polymyza.

Bacteria frequently have only one nitrogen base as a component of the phospholipids. Choline, for instance, has

been found in Neisseria gonorrhea (Stokinger et al., 1944), Sarcina lutea (Huston et al., 1965), and Streptococcus lactis (Macleod and Brown, 1964) while ethanolamine was found in Clostridium butyricum (Goldfine, 1962) Micrococcus halodentrificans (Kates et al., 1961), Staphylococcus aureus (Few, 1955), and Serratia marcescens (Kates et al., 1964). Both phosphatidyl serine and phosphatidyl ethanolamine were found in Escherichia coli (Kanfer and Kennedy, 1963).

Phosphatidyl glycerol

Phosphatidyl glycerol is a complex phosphatidic acid in which there may be several glycerol or phosphatide residues. Several possible structural formulae have been proposed by workers in these field (Pangborn, 1947; Hanahan, 1957; Lecocq and Ballou, 1964). Phosphatidyl glycerol is said to be involved in active transport, more specifically the β-galactoside permease activity (Tarlov and Kennedy, 1965). This lipid is also involved as a co-factor in the malate oxidizing system in mycobacteria (Tobari, 1964).

Evidence of such a compound in bacteria has been considerable. Gilby et al. (1958) and Macfarlane (1961) found a lipid component from the protoplast of Micrococcus lysodeikticus similar to the structure proposed by Pangborn (1947). Kates et al. (1961) were not able to identify several fractions in their extracts obtained from Micrococcus halodentrificans. They suggested the possibility of poly-

phosphatidic acid by virtue of similarity in R_f values. In Halobacterium cutirubrum (Kates et al., 1963), 2% of the total cell was said to be extractable lipids with most of the lipids identified as phosphatides. Further examination of the phosphatide enabled the characterization of the isolated pure lipid as phosphatidyl glycerophosphate. Some other bacteria known to contain such lipids are Thiobacillus thioxidans (Jones and Benson, 1965), Bacillus cereus (Kates et al., 1962), Bacillus megaterium (Haverkate et al., 1962), and Bacillus polymyxa (Matches et al., 1964).

Plasmalogens

Plasmalogens are a group of phosphatides in which the fatty acid at the α position is replaced by an α, β unsaturated ether. Upon hydrolysis, the ether gives rise to an aldehyde. Such a compound was found by Wegner and Foster (1963) when Bacteroides succinogens was grown in a medium containing iso-butyric or valeric acid. Goldfine (1964) and Bauman et al. (1965) also isolated this compound from Clostridium butyricum.

Plasmalogen is thought to be an essential part in the make up of the functional units of organized mitochondrial enzyme systems (Joel et al., 1958), and perhaps to a lesser extent, as an energy source in the absence of sufficient carbon source (Hartree and Mann, 1959).

Amino acid-containing phospholipids

Phospholipids containing amino acids have been observed in many instances. Their function appears to be acting as intermediates in the metabolism of amino acids and possibly other compounds (Pilgeram and Greenburg, 1955).

A series of O-amino acid esters of phosphatidyl glycerol in Clostridium perfringens and Staphylococcus aureus was isolated by Macfarlane (1961; 1962b) Houtsmuller and van Deenen (1963) working with Bacillus cereus, Bacillus megaterium, Pseudomonas stutzeri, and Serratia marcescens found the presence of ornithine bound in ester linkage to glycerophosphate. Lysine was found to be bound to phospholipids in Lactobacillus casei, Lactobacillus plantarum, and Streptococcus faecalis while D-alanine was found in Leuconostoc mesenteroides (Ikawa, 1963).

Glycerides

Glycerides are esters of fatty acids and glycerol and are used most likely as an energy source (Lennarz, 1966). Before the 1950's, evidence for the presence of glycerides in bacteria was mostly indirect. Their presence had been assumed because of the detection of glycerol, which is the principal water-soluble constituent of the neutral fat after saponification.

Bloch and co-workers (1957) used a magnesium silicate

column to separate lipids and found some mono-, di-, and tri-glycerides. Noll and Jackim (1958) fractionated acetone soluble fat extracts from Mycobacterium tuberculosis and found that one of the fractions behaved as a monoglyceride with an infra-red spectrum similar to the spectrum of 1-monostearin in β-crystalline form while another fraction behaved like α-monoglyceride of mycolic acid. Glycerides have also been reported for Staphylococcus aureus (Macfarlane, 1962a), Bacillus cereus (Kates et al., 1962), and Corynebacterium diphtheriae (Asselineau, 1961).

Apparently, the understanding of the role of lipid in bacteria is not clear. Lipids in recent years have been shown to be capable of producing an immunological response, being toxic to other cells, inhibiting enzyme activities, and serving as structural support in cell formation, Furthermore, some fatty acids might possibly impart a protective effect on protein and stabilize the protein to adverse conditions such as heat. Whether such factor contributes significantly to the thermal tolerance of a thermophile or of a bacterial spore has not been established; however, further research should shed more light on this question.

MATERIALS AND METHODS

The Organisms

Bacillus polymyxa strain 1A39 from the culture collection of the Department of Bacteriology at Iowa State University was grown in "G" medium. (Stewart and Halvorson, 1953). The medium consisted of 1 g K_2HPO_4; 2 g $(NH_4)_2SO_4$; 2 g yeast extract; 4 g glucose; 0.1 g $MnSO_4 \cdot H_2O$; 0.8 g $MgSO_4$; 0.01 g $ZnSO_4$; 0.01 g $CuSO_4 \cdot 5\ H_2O$; 0.001 g $FeSO_4$; 0.01 g $CaCl_2$. The ingredients were dissolved in one liter of distilled water and adjusted to pH 6.8 before sterilization. For production of spores, the concentration of $CaCl_2$ was increased tenfold (0.1 g/l of water).

The inoculum for the production of vegetative cells and spores was prepared by washing the growth from the surface of 10 stock culture agar slants which had been incubated overnight at 30 or 37°C (depending on the experiment). The organisms, washed free from the slants, were then transferred to 1.5 l of "G" medium. Overnight incubation at the prescribed temperature on a gyrotory water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.) produced sufficient growth to inoculate 12 l of "G" medium in a 15 l fermenter. Sterile air was forced through the medium at a rate of 1.9 liters/min as measured by a Manostat flowmeter (The Emil Greiner Co., New York, N.Y.). The air was sterilized by filtering through glass wool packed in a steel tube

(6-1/2" x 1-1/2"). Vegetative cells were collected after approximately 12 hrs. and spores after approximately 24 hrs. of growth.

The cells or spores were collected by centrifuging the suspension at 10,000 rpm (12,100 x g) with an automatic refrigerated centrifuge RC-2 (Ivan Sorvall, Inc., Norwalk, Conn.). The collected cells and spores were washed with acid water (pH 2.5) and distilled water. Vegetative debris and proteins were removed from spores by washing in 35% dioxane.

Bacillus stearothermophilus (ATCC 7953) was grown at 55 and 45°C in a medium consisting of nutrient broth (Difco), 8 g; $CaCO_3$, 0.5 g; $MnCl_2$, 2 mg; $NaNO_3$, 2 mg. The ingredients were dissolved in one liter of distilled water and the pH adjusted to 7.0 $\pm$ 0.2. The organism was grown in essentially the same manner as B. polymyxa. Compressed air was forced through the medium at a rate of 1.9 liters/min although on occasion, increasing the flow rate seemed to be helpful in the sporulation process. The cells were collected after approximately 16 hrs. incubation at 55 $\pm$ 2 C while spores were collected after 24 hr incubation. When the temperature of incubation was lowered to 45°C, an additional 12-24 hrs. was sometimes needed before the organisms were ready for collection. Again, the collection and washing procedure were done in the same manner as with B. polymyxa. Cultures were checked under the phase microscope to determine if they

were ready for harvesting. Spores were considered ready for collection when 95-100% sporulation was observed under the microscope.

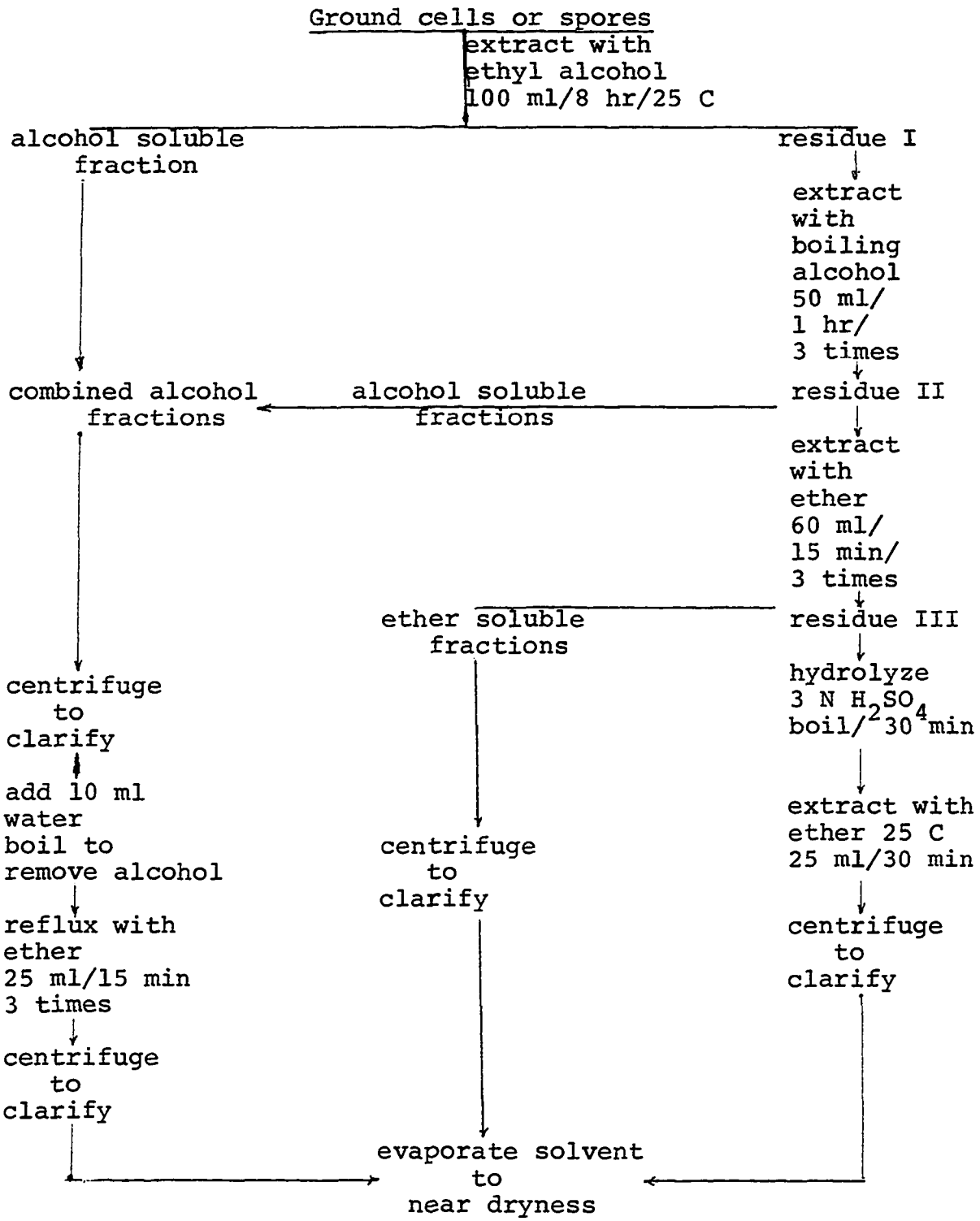
Isolation of lipids

Lipids were collected using a modification of the method of Long and Williams (1960) in which the cells were suspended in ethanol and mixed with glass beads. The mixture was ground in a mortar grinder (Fischer Scientific, Chicago, Ill.) until the cells were broken. The glass beads were of the size 29 μ (Type 500-5005) and made by Minnesota Mining and Manufacturing Company (St. Paul, Minn.). Again the cells were examined under the microscope to verify the destruction of cellular structure. The process usually required approximately half an hour. The ground mixture was then extracted with boiling alcohol as well as ether to insure the recovery of most of the lipids. A more detail description of the extraction procedure is shown in Figure 1.

Spores were extracted by the same procedure except that a much longer grinding period was needed to disrupt the spores-- between 2-4 hours.

Another method of extraction as suggested by White and Cox (1967) was tried for the extraction of cellular lipids. The procedure consisted of refluxing the cells with 3 N KOH containing 50% ethanol for 12 hours followed by five extractions with petroleum ether. The method produced essentially

Figure 1. Extraction of lipid


Ground cells or spores
extract with ethyl alcohol 100 ml/8 hr/25 C
alcohol soluble fraction
residue I
extract with boiling alcohol 50 ml/ 1 hr/ 3 times
combined alcohol fractions
alcohol soluble fractions
residue II
extract with ether 60 ml/ 15 min/ 3 times
ether soluble fractions
residue III
hydrolyze 3 N H_2SO_4 boil/ 30^4 min
centrifuge to clarify
add 10 ml water boil to remove alcohol
centrifuge to clarify
extract with ether 25 C 25 ml/30 min
reflux with ether 25 ml/15 min 3 times
centrifuge to clarify
centrifuge to clarify
evaporate solvent to near dryness

the same results. Since the experiments were initiated using the method of Long and Williams, it was decided to continue without changing to other extraction methods.

Isolation and esterification of fatty acids

Preparation of ion exchange resin Strong base anion exchange amberlite IRA 400 (Mallinckrodt Chemical Works, St. Louis, Mo.) was pretreated as suggested by Bills (1964) to eliminate a possible source of contamination and to avoid leaching of low molecular weight polymers. The procedure consisted of mixing with a magnetic stirrer ten grams of Amberlite IRA-400 with 20 ml of 1 N NaOH for 5 minutes. The resin was washed twice with distilled water until free of NaOH; after this treatment, it was washed twice with 20 ml portions of 95% ethanol, once with absolute alcohol, and three times with petroleum ether (B.P. 60-70 C). Secondly, sixty milligrams of stearic acid were dissolved in 10 ml of petroleum ether and stirred with the resin for 15 minutes. The solvent was decanted and the resin washed three times with petroleum ether, once with absolute alcohol, and twice with 95% ethanol. In the third step, the resin was stirred for 20 min in 10 ml of 5-10% HCl-methanol. The HCl-methanol was prepared by dropping concentrated sulfuric acid on hydrochloric acid, and bubbling the HCl gas through methanol until the desired acidity (5-10%) was obtained as determined by titration. After decanting the solvent, the resin was

washed with HCl-methanol for 5 minutes, followed by two washings with distilled water. The resin was then subjected to the above treatment two more times except the petroleum ether washing was omitted in the first step and the second step was skipped entirely. After which the resin was treated with 20 ml of 1 N NaOH for five minutes, washed free of alkali, rinsed in methanol and stored under a layer of methanol in the refrigerator. The Amberlite IRA-400 was ready for use at this point. Resin prepared in this manner keeps for several weeks without losing its effectiveness.

Esterification of fatty acids The prepared resin was washed with 10 ml of absolute alcohol and twice with petroleum ether. The lipids, collected from previous extraction of cells or spores, were dissolved in 10 ml of petroleum ether and added to the resin. The solution was stirred with the resin for 20 minutes and decanted. Four washings with 10 ml of petroleum ether, two with ethanol and two with methanol removed most of the lipids. The free fatty acids, which were still adsorbed to the resin, were esterified by stirring in 10 ml of five to ten percent HCl-methanol for 20 minutes; the supernatant was then carefully decanted into a separatory funnel containing 5 ml of cold distilled water. The esterification was repeated using 5 ml of the reagent and stirring for 5 min with the resin. The combined reagent and water

were mixed with 10 ml of petroleum ether (B.P. 30-40 C) and shaken vigorously for one minute. The aqueous phase was removed and extracted with petroleum ether two more times. The petroleum ether extracts were combined, washed twice with cold distilled water, and dried overnight with anhydrous sodium sulfate at -20 C.

In order to increase the keeping quality of the resin, it was appropriate to revert the resin to its more stabilized form, namely, the basic form. The manner in which the regeneration of the resin was accomplished was by washing the resin twice with methanol and twice with distilled water, followed by 5 minute treatment of 1 N NaOH. The excess NaOH was rinsed away with distilled water and methanol. The resin was then stored under a layer of methanol in the refrigerator for later use.

A slight modified procedure suggested by Metcalfe et al. (1966) was used to collect the unadsorbed lipids from the washing. The solvent was heated over a steam bath. Four milliliters of 0.5 N methanolic NaOH was added to the lipids until everything was dissolved. Five ml of boron trifluoride-methanol (prepared by bubbling 125 grams of BF_3 into one liter of methanol) was added to the mixture and boiled for 2 minutes. The mixture was then transferred to a separatory funnel where about 20 ml of petroleum ether (B.P. 30-40 C) was added. The funnel was shaken vigorously for 1 minute and the layer

allowed to separate. The lower layer was drained off and discarded and the top layer was collected for the fatty acid analysis.

Gas chromatographic separation of the methyl esters

Fatty acid methyl esters were analyzed by gas chromatography. Helium was used as the carrier gas with an outlet flow rate of 65 ml/min and a column inlet pressure of 40 psi. Two 1/4 inch O.D. copper columns, six feet in length, were packed with 15% LP-71 (ethylene glycol succinate) (F & M Scientific Corp., Avondale, Pa.) on 60-80 mesh Diatoport ST-111. The effluent was monitored by H_2 flame ionization detector in an analytical gas chromatograph (Model 810-29 F & M Scientific Corp. Avondale, Pa.). A programmed temperature operation was employed at the start. The program was set for a temperature range of 80-175^{0}C with a 10^{0}C rise per minute. The results, however, did not give any significant improvements over isothermal runs as far as separation of fractions was concerned. Finally, the experiments were run isothermally at 169 and 180^{0}C with attenuation set at 10. Samples were injected in such amount that a maximal number of peaks were detected without having the major peaks overlap the smaller ones. Because of the difficulty in determining the precise amount of extraction from the bacteria, especially those of the spores, no quantitative analyses were attempted on these sets of experiments. Standards used for identification purposes were

obtained from Applied Science Laboratories. (State College, Pa.) The retention time of those standard saturated straight chain C_4- C_{18} (even number acid), as well as those of $C_{12}^{=}$, $C_{14}^{=}$, $C_{16}^{=}$, $C_{18}^{=}$, iso C_{18}, anteiso C_{19}, iso C_{20}, and anteiso C_{21} were taken to compare with the retention time of the sample peaks. To further substantiate the identification, standards were mixed into the sample and injected into the chromatograph. Identical acid esters should reinforce each other and give much larger peaks than when no standards were added. Retention time of each peak was measured as the distance from the injection point to the tip of the curve. The concentration of each acid was roughly calculated as the area under the peak and above the baseline, and was used to estimate the relative amounts of these acids in the bacteria, (Heftmann, 1967).

Identification of the fatty acids was based essentially on the well known linear relationship between the logarithm of the retention volume and the number of carbon atoms among homologous fatty acids (James and Martin, 1952; Merritt et al., 1964), The identification also made use of the criteria described by Landowne and Lipsky (1961) whereby they claimed that: 1) An increase in the operating temperature of the column decreases the separation factor for saturated straight chain component. (2) An increase in the operating temperature of the column decreases the separation factor for

branched-chain saturated isomers relative to the preceding saturated straight chain component. (3) An increase in the operating temperature of the column increases the separation factor for unsaturated straight chain components relative to the preceding saturated straight chain component.

Colorimetric determination of hydroxy acids Hydroxy acids were analyzed qualitatively by heating the sample with concentrated hydrochloric acid to form an aldehyde and an acid. The breakdown products were tested for by the use of 2,4 dinitrophenylhydrazine and saturated alcoholic KOH. A yellow, or red color indicated the presence of hydroxy acid. (Veibel, 1966; Shriner et al., 1965).

RESULTS AND DISCUSSION

Identification of Fatty Acids in B. Polymyxa and B. Stearothermophilus

The experimental results will be presented and discussed in two parts. The first part will deal with fatty acids composed of less than 12 carbon atoms isolated from vegetative cells and spores of Bacillus polymyxa and Bacillus stearothermophilus; and the second, with fatty acids composed of 12 or more carbon atoms isolated from the same sources. The principal reason for this is that the short chain fatty acids were present in relatively small quantities. Also the short chain fatty acids were eluted from the ethylene glycol succinate column relatively fast following the introduction of the sample into the gas chromatograph because of their low boiling point. Separating the short chain fatty acids into a different section would tend to minimize the effect of a possible misidentification of the short chain acids because of poor separation of these particular fractions on the columns used in this work. The grouping of the more abundant acids ($>C_{12}$) separately would provide a more meaningful interpretation of the results.

Sporeformers have always been considered a nuisance in any food processing. Thermophiles, in particular, are noted for their ability to cause flat souring in canned foods (Desrosier, 1963). The strain of Bacillus stearothermophilus

used in these experiments is frequently used in the canning industry to determine processing times for various products; for this reason, the organism was considered a logical choice for additional studies of factors influencing heat resistance. Since previous work on the phospholipids in vegetative cells and spores in this laboratory was done with B. polymyxa, the same organism was selected as a representative of the mesophiles for the sake of comparison.

Fatty acids with less than 12 carbons

Fatty acids extracted from spores and vegetative cells of the bacteria with less than 12 carbons are listed in Table 1. These acids constituted only 1-2% by weight of the total fatty acid, an insignificant amount in-so-far as total acid content is concerned. Short chain acids have not been discussed in many studies, not even by those people concerned in using lipid composition as one criterion for taxonomic classification of bacteria (Abel et al., 1963; Lewis et al., 1967). Church et al. (1956) extracted a certain lipid fraction from Bacillus cereus with a resulting reduction in resistance to ethylene oxide. Their analysis of the lipid fraction indicated that the acids were mainly of the C_2-C_8 chain length. In this work, however, either because of the limitation of the method used, or because of the relatively low yield of such acids, the short chain acids were not successfully recovered. The shortest chain

acid recovered had 7 carbons.

Table 1 and Figures 2 and 3 show that acids consisting of less than 12 carbon atoms in both cells and spores were mostly iso acids, a few normal acids, and almost no anteiso acids. B. polymyxa seemed to have more of the short chain acids by volume while B. stearothermophilus had more variety of acids. Iso C_9 and iso C_{10} were the most frequently identified acids. Since normal acids usually have a higher melting point than their respective isomers, the presence of more normal acids for the spore as compared with the cells may have some significance in reference to heat tolerance. The fatty acids, having a higher boiling point, can combine with the protein and enhance the stabilization of the protein (Boyer et al., 1946). By the same token, the thermophiles were noted to contain considerably more branched chain acids than the mesophiles; possibly the thermophiles have derived some of their heat resistance capability from such modification in the lipid content. The results are not as comprehensive as was desired for the short chain acids; and furthermore, the separation and identification of the acids was not detailed enough to make any conclusive statements. Therefore, more studies on the short chain acids as well as on the chemical and biological effect of such acids on the cellular constituents should be made before a final conclusion can be made.

Table 1. Fatty acids containing less than 12 carbon atoms found in vegetative cells and spores of Bacillus polymyxa and Bacillus stearothermophilus grown at different temperatures (Values are expressed as % relative weight)

Chain length of fatty acids	Growth temp.	Bacillus stearothermophilus 55 C		45 C		Bacillus polymyxa 37 C		30 C	
		Vegeta-tive cells	Spores	Vegeta-tive cells	Spores	Vegeta-tive cells	Spores	Vegeta-tive cells	Spores
i C_8	[a]	0.32[b]		0.04	0.06		0.24		1.51
i C_9		0.46	0.27	0.05	0.06	0.49	0.49	0.32	
n C_9			0.57	0.12	0.06				0.66
i C_{10}		0.29	0.50			0.96	0.54	0.42	
n C_{10}			0.75		0.15		0.31		
i C_{11}		0.19		0.21		0.65			
a C_{11}									0.34

[a] i=iso acid, n=normal acid, a=anteiso acid.

[b] Blank spaces indicate that no fatty acid of this type was detected.

Figure 2a. Characteristic gas chromatographic profile of methyl esters of fatty acids consisting of less than 12 carbon atoms extracted from vegetative cells and spores of B. stearothermophilus grown at different temperatures

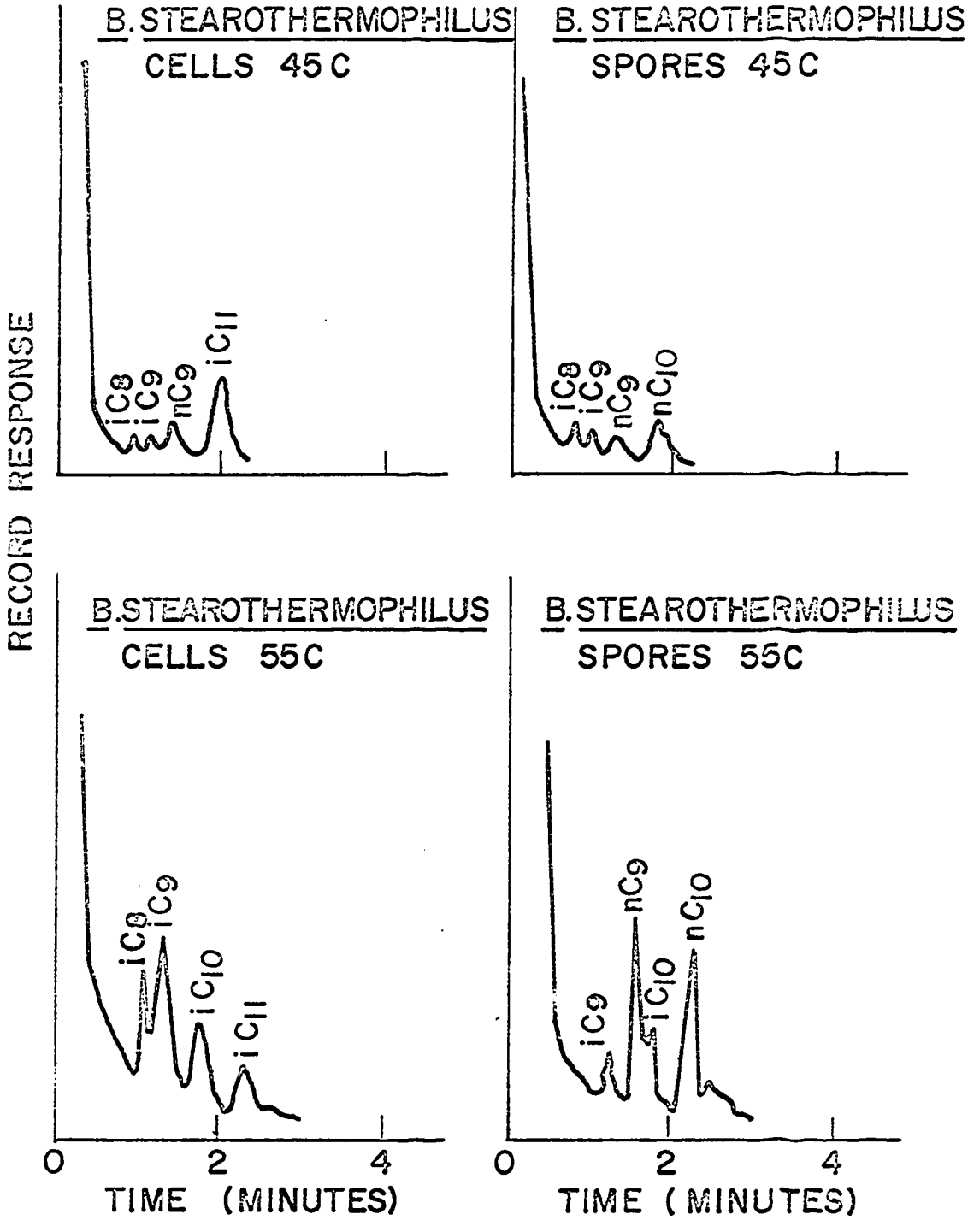

B. STEAROTHERMOPHILUS
CELLS 45C
iC8
iC9
nC9
iC11
B. STEAROTHERMOPHILUS
SPORES 45C
iC8
iC9
nC9
nC10
RECORD RESPONSE
B. STEAROTHERMOPHILUS
CELLS 55C
iC8
iC9
iC10
iC11
B. STEAROTHERMOPHILUS
SPORES 55C
iC9
nC9
iC10
nC10
0
2
4
TIME (MINUTES)
0
2
4
TIME (MINUTES)

Figure 2b. Characteristic gas chromatographic profile of methyl esters of fatty acids consisting of less than 12 carbon atoms extracted from vegetative cells and spores of B. polymyxa grown at different temperatures

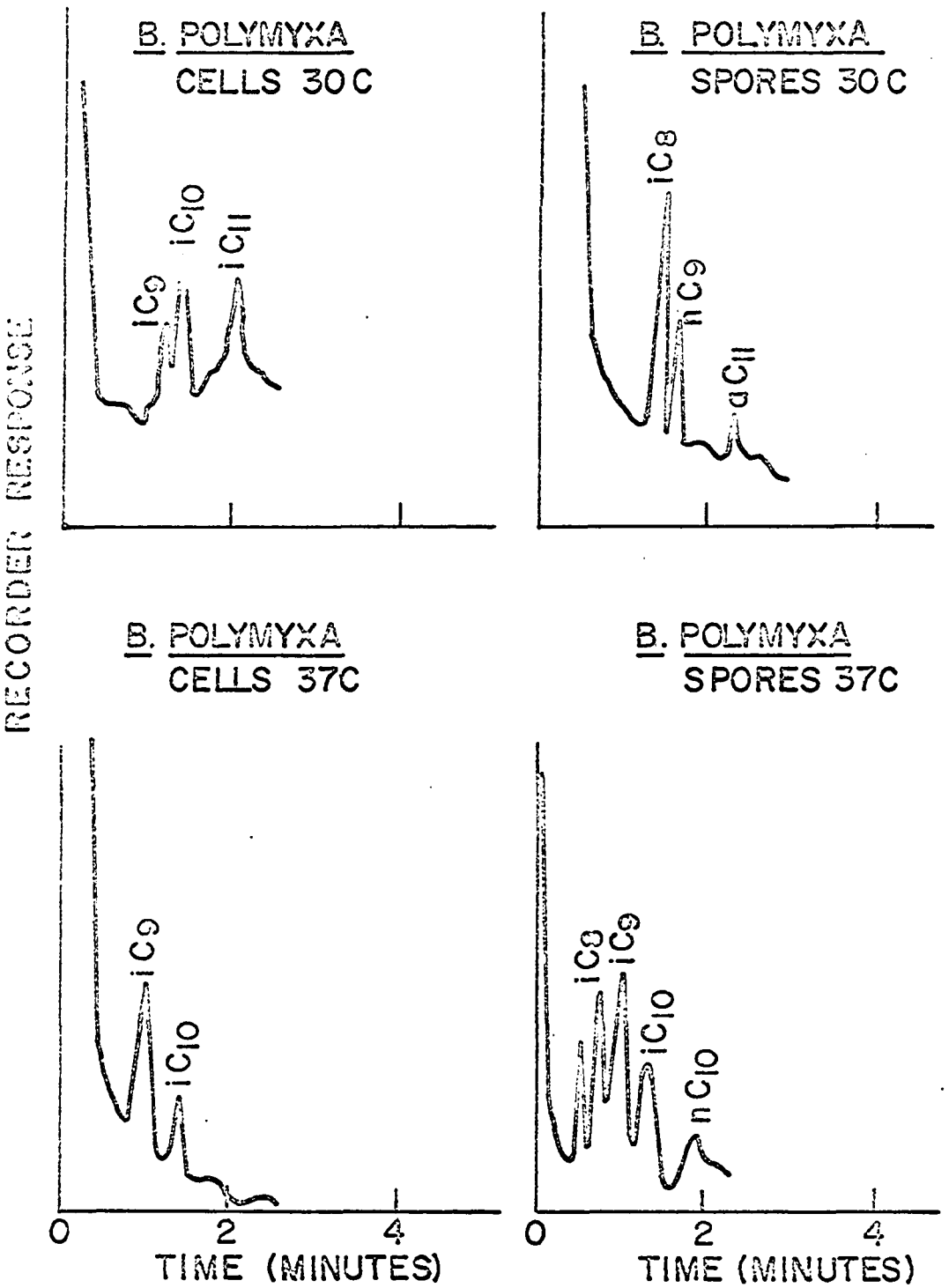
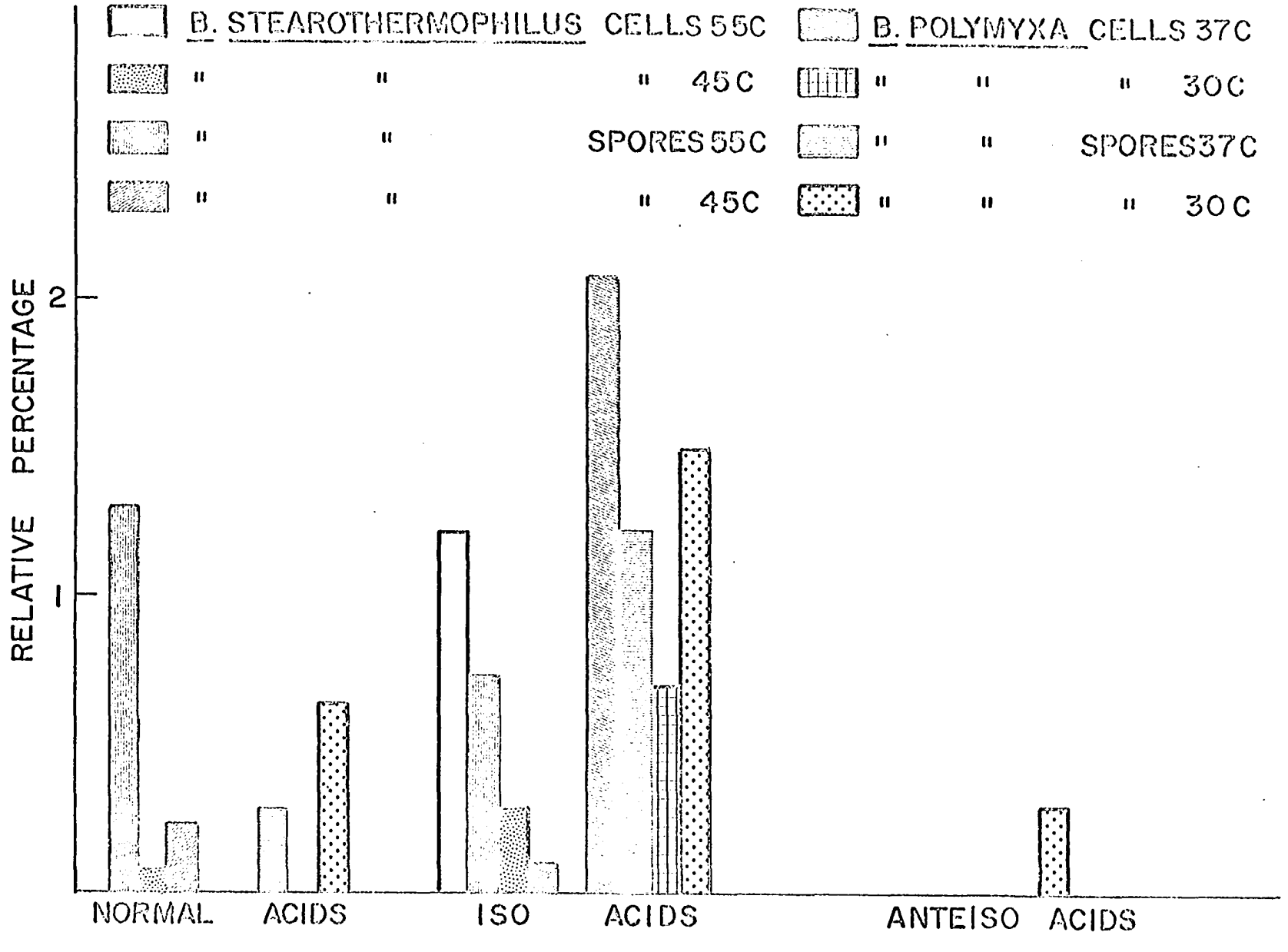

B. POLYMYXA
CELLS 30C
iC9
iC10
iC11
B. POLYMYXA
SPORES 30C
iC8
nC9
aC11
B. POLYMYXA
CELLS 37C
iC9
iC10
B. POLYMYXA
SPORES 37C
iC8
iC9
iC10
nC10
RECORDER RESPONSE
0
2
4
TIME (MINUTES)
0
2
4
TIME (MINUTES)

Figure 3. Relative amount of different types of fatty acids ($<C_{12}$) extracted from vegetative cells and spores of B. polymyxa and B. stearophilus grown at different temperatures


B. STEAROTHERMOPHILUS CELLS 55C
" " " 45C
" " SPORES 55C
" " " 45C
B. POLYMYXA CELLS 37C
" " " 30C
" " SPORES 37C
" " " 30C
RELATIVE PERCENTAGE
2
1
NORMAL ACIDS
ISO ACIDS
ANTEISO ACIDS

Fatty acids with more than 12 carbons

The fatty acids present in great quantities were those with chain lengths between C_{12}-C_{18}. The composition of the fatty acids are listed in Table 2. The most obvious item is the apparent lack of acids (C_{12} and C_{13}) in the thermophile. The C_{17} acid is absent in the cells of the mesophile except for a trace of normal C_{17} in the spore fraction. Because of the increased number of acids in the mesophiles, no one acid is as dominant in concentration as in the thermophile. The incubation temperature changes the composition of the bacteria for both the mesophile and thermophile as evidenced in a shift of the major peak from C_{16} to C_{15} acid. The overall change in fatty acid composition between the cell and spore is not as pronounced. Some differences do appear upon closer scrutiny and will be presented in more detail later. The results of the experiment will be subdivided into four sub-headings where each topic will be discussed in its relation to thermophily and heat resistance.

Mesophiles vs. thermophiles

Fatty acids of B. polymyxa differed quite extensively from those found in B. stearothermophilus (Figure 4). Fatty acids containing 12 and 13 carbon atoms were detected solely in B. polymyxa while acids containing 17 carbon atoms were almost non-existent. This variation could be attributed to

Table 2. Fatty acid (>C_{12}) composition of vegetative cells and spores of *Bacillus polymyxa* and *Bacillus stearothermophilus* grown at different temperatures (in % relative weight)

Chain length of fatty acids*	Growth temp.	*Bacillus stearothermophilus* 55°C		*Bacillus stearothermophilus* 45°C		*Bacillus polymyxa* 37°C		*Bacillus polymyxa* 30°C	
		Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores	Vegetative cells	Spores
i C_{12}						0.70	1.22	1.35	1.81
n C_{12}						0.32	0.28	1.14	0.27
i C_{13}				0.09		13.22	10.34	25.64	4.42
a C_{13}									2.47
i C_{14}		0.29	1.16	0.47	0.39	2.31	6.53	3.77	10.92
n C_{14}		2.28	3.68	0.25	0.80	2.50	2.44	2.21	2.48
i C_{15}		19.60	11.70	43.20	36.15	24.65	40.38	23.58	8.48
a C_{15}		12.99	5.22	22.67	24.67	13.03			5.71
i C_{16}		2.06	3.48	2.41	2.66	3.91	6.37	2.31	4.15
n C_{16}		42.11	47.98	1.97	14.32	18.98	14.0	27.03	44.73
$C_{16}^{=}$		10.90	5.52			18.92	15.19	11.58	11.09
i C_{17}				16.91	11.23				
a C_{17}		7.60	17.55	11.61	9.45				
n C_{17}		0.91	1.62				1.67		0.96

*i=iso acid, n=normal saturated acid, a=anteiso acid, C=monoenoic acid.

Figure 4a. Characteristic gas chromatographic profile of methyl esters of fatty acids consisting of more than 12 carbon atoms extracted from vegetative cells and spores of B. polymyxa grown at 30 C

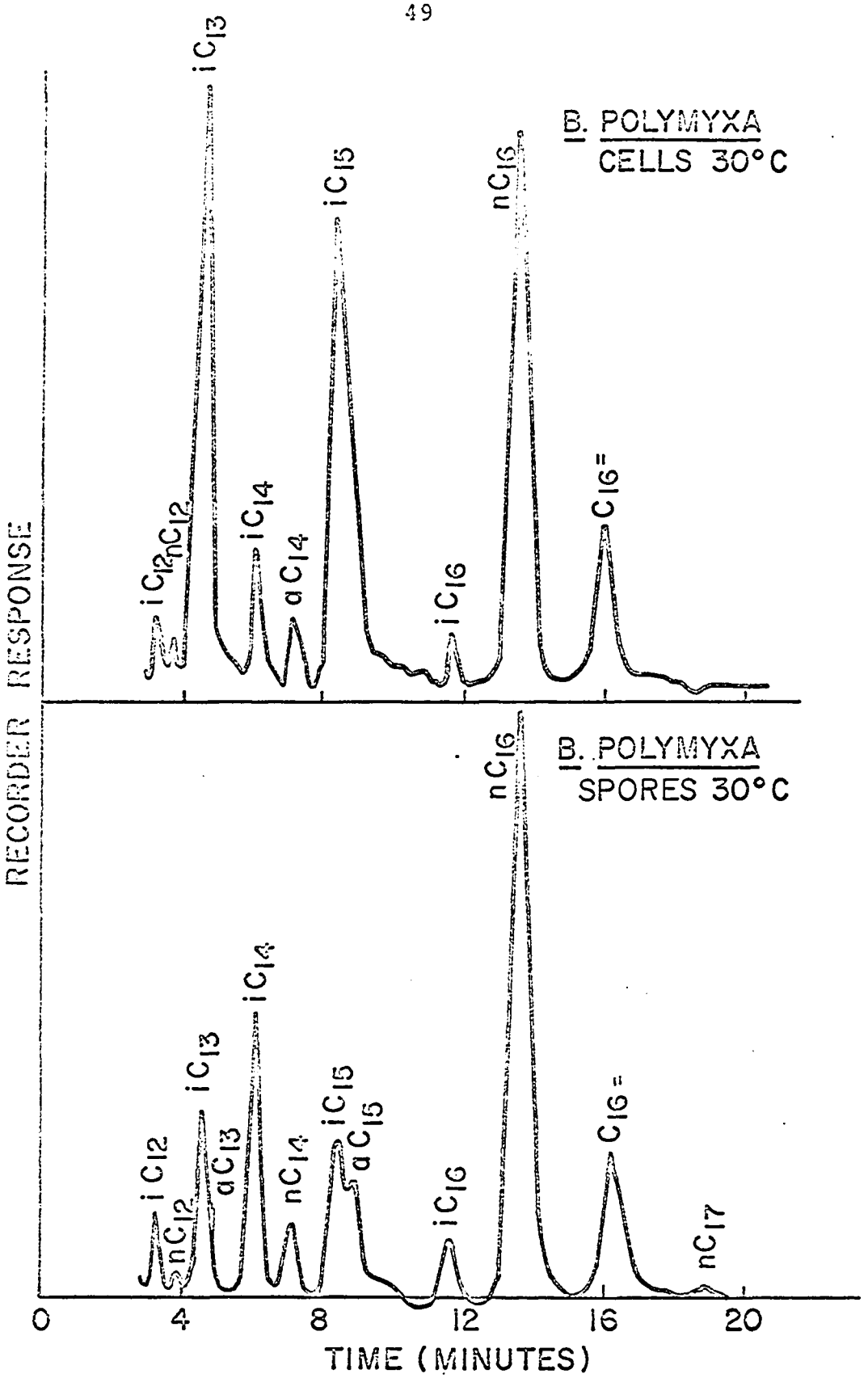

B. POLYMYXA
CELLS 30°C
iC13
iC12
nC12
iC14
aC14
iC15
iC16
nC16
C16=
B. POLYMYXA
SPORES 30°C
nC16
iC12
nC12
iC13
aC13
iC14
nC14
iC15
aC15
iC16
C16=
nC17
RECORDER RESPONSE
0
4
8
12
16
20
TIME (MINUTES)

Figure 4b. Characteristic gas chromatographic profile of methyl esters of fatty acids consisting of more than 12 carbon atoms extracted from vegetative cells and spores of B. polymyxa grown at 37 C

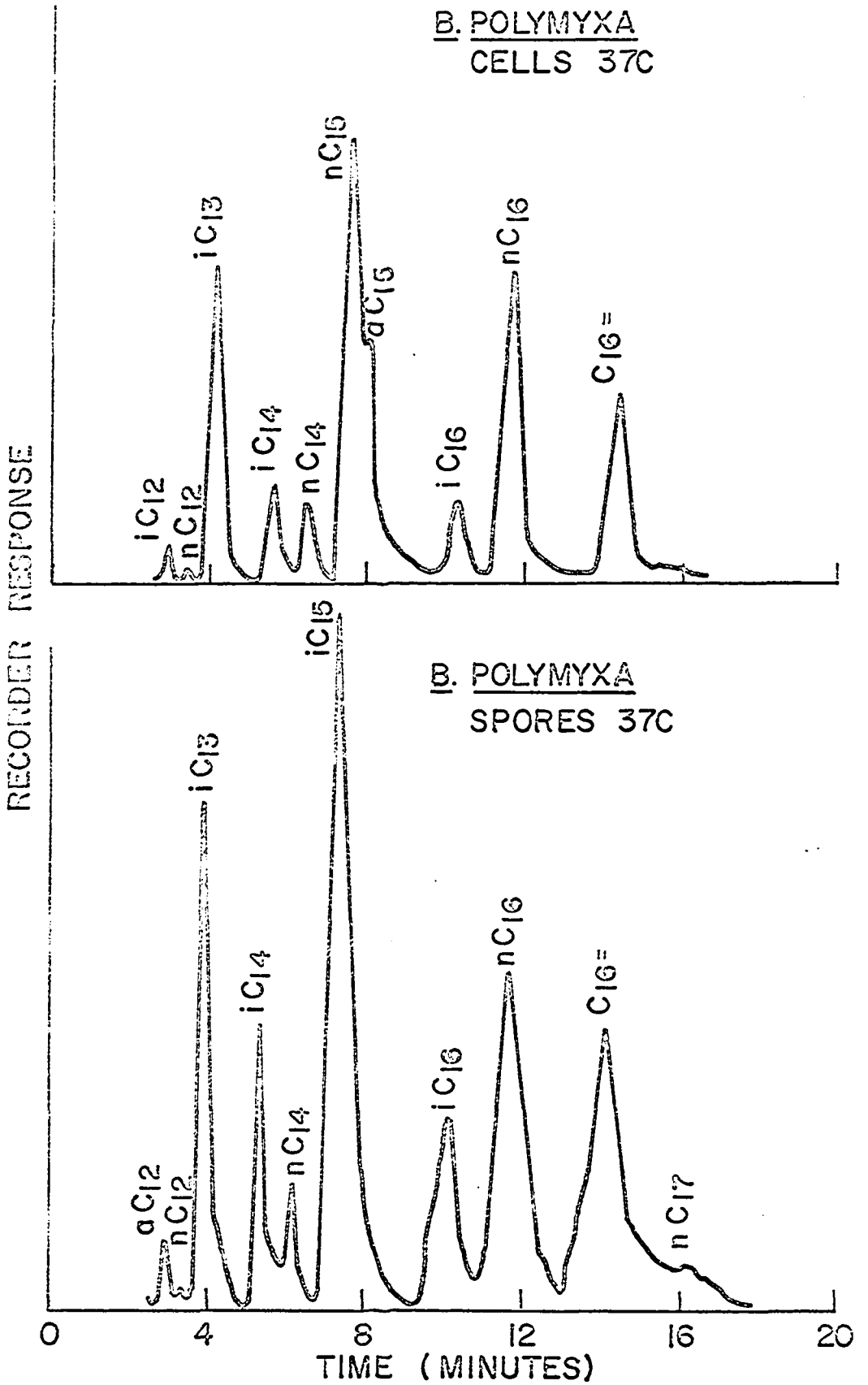

B. POLYMYXA
CELLS 37C
iC12
nC12
iC13
iC14
nC14
nC15
aC15
iC16
nC16
C16=
B. POLYMYXA
SPORES 37C
aC12
nC12
iC13
iC14
nC14
iC15
iC16
nC16
C16=
nC17
RECORDER RESPONSE
0
4
8
12
16
20
TIME (MINUTES)

Figure 4c. Characteristic gas chromatographic profile of methyl esters of fatty acids consisting of more than 12 carbon atoms extracted from vegetative cells and spores of B. stearothermophilus grown at 45 C

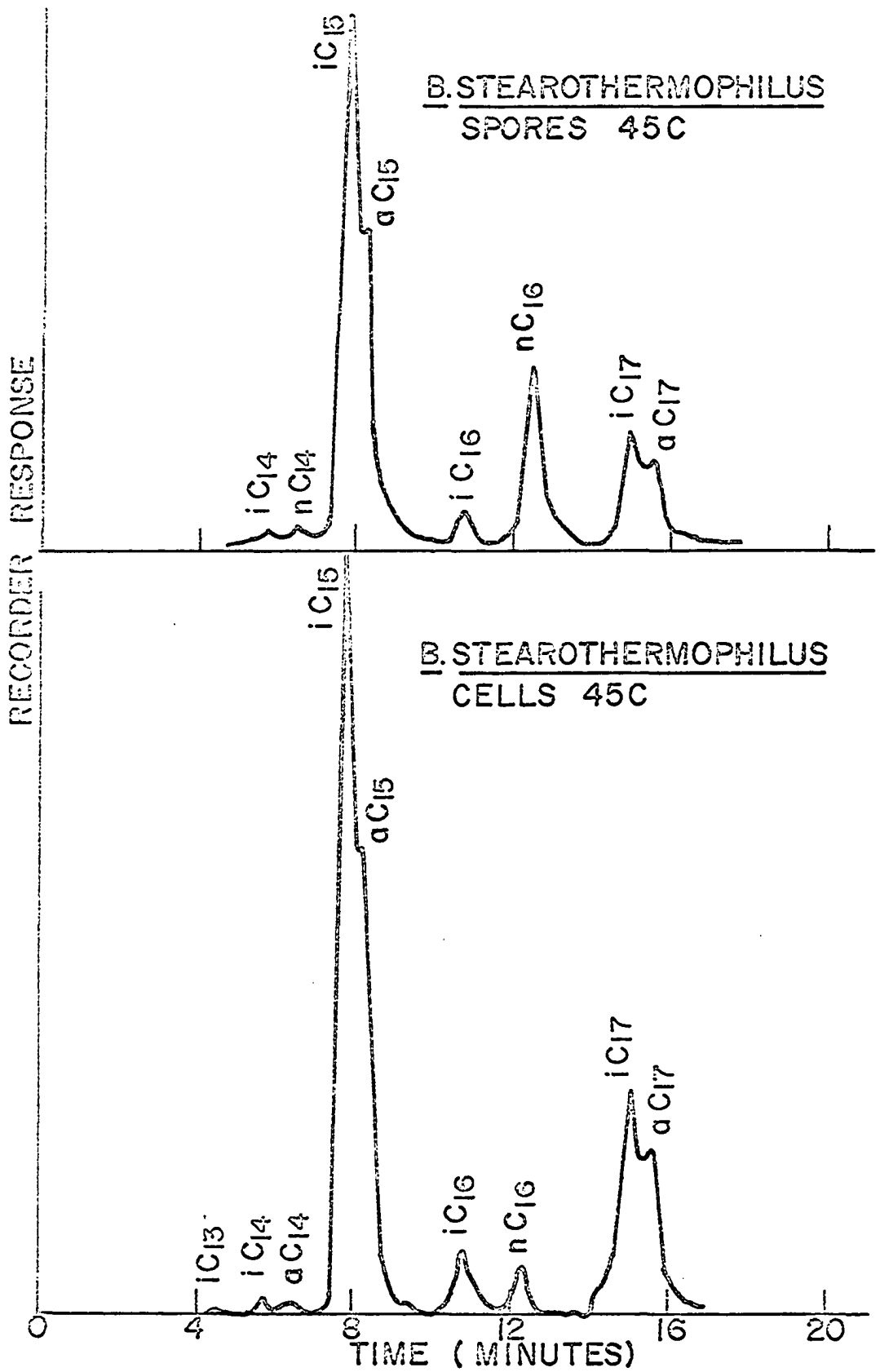
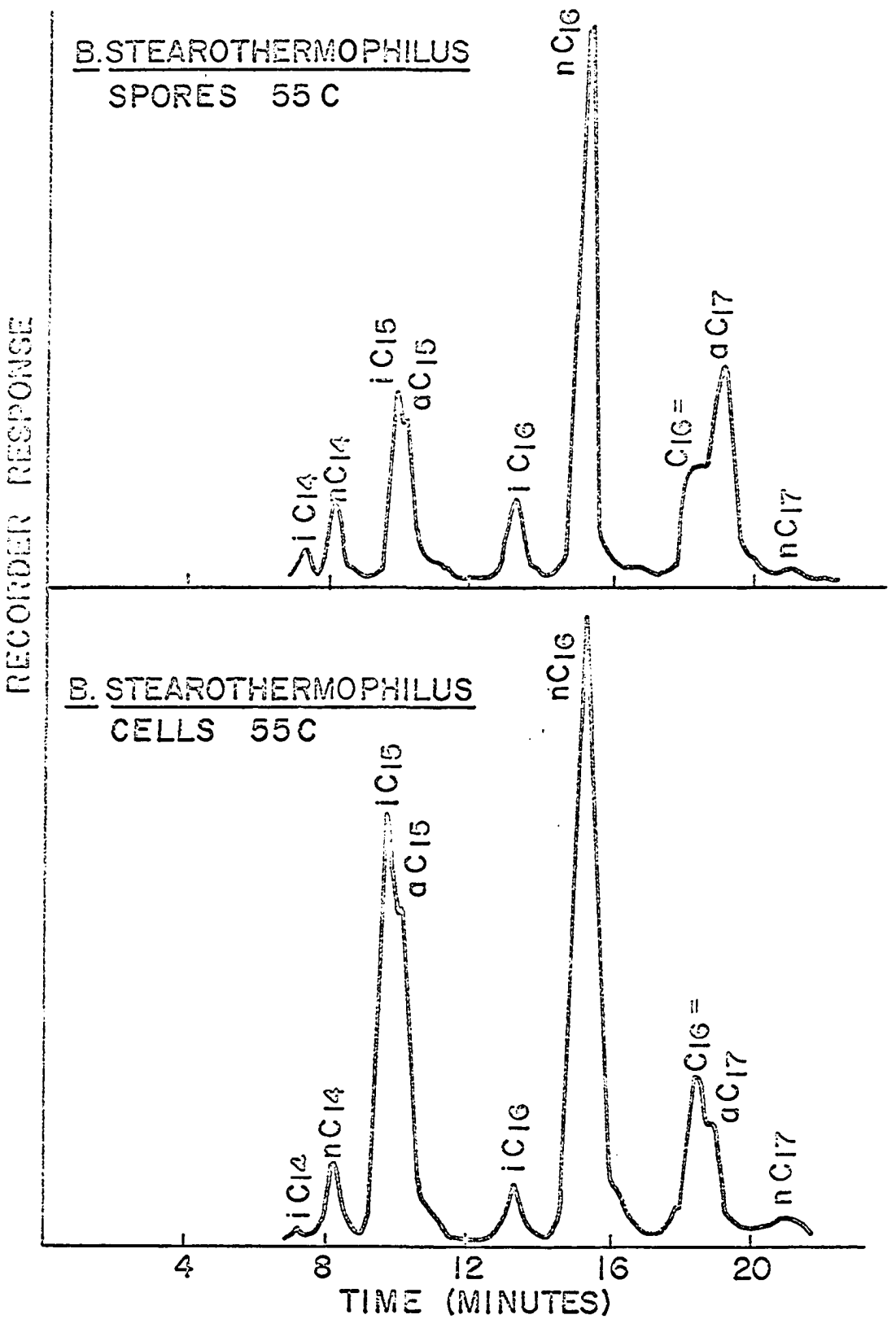

B. STEAROTHERMOPHILUS
SPORES 45C
B. STEAROTHERMOPHILUS
CELLS 45C
RECORDER RESPONSE
iC13
iC14
nC14
aC14
iC15
aC15
iC16
nC16
iC17
aC17
0
4
8
12
16
20
TIME (MINUTES)

Figure 4d. Characteristic gas chromatographic profile of methyl esters of fatty acids consisting of more than 12 carbon atoms extracted from vegetative cells and spores of B. stearothermophilus grown at 55 C


B. STEAROTHERMOPHILUS
SPORES 55 C
iC14
nC14
iC15
aC15
iC16
nC16
C16=
aC17
nC17
B. STEAROTHERMOPHILUS
CELLS 55C
iC14
nC14
iC15
aC15
iC16
nC16
C16=
aC17
nC17
RECORDER RESPONSE
4
8
12
16
20
TIME (MINUTES)

differences in the culture media (Marr and Ingraham, 1962). Also it could be a case of the thermophiles maintaining the acids in forms which possess the highest boiling point; that is, the cellular constituents of the bacteria are of the more heat tolerant variety and, therfore, able to stand up to higher temperatures of incubation (Ingraham, 1962).

Thermophiles seemed to have a greater variety of long chain fatty acids than do mesophiles. Moreover, the thermophile always seemed to have one extremely large fraction which comprised approximately half the total acids. This fraction was apparently influenced by the incubation temperature because palmitic acid was noted as the major fraction for the thermophile when the bacteria were grown at 55 C; however, when the growth temperature was changed to 45 C, the major peak shifted to iso C_{15}. Sugiyama (1951) tested the heat resistance of C. botulinum grown at the different temperatures and came to the conclusion that spores of this anaerobe showed the highest heat tolerance when they were grown at the optimal growth temperature. The increase in concentration of fatty acids with higher melting points in the organisms grown at 55 C could be one reason for the increased heat resistance of such bacteria over the ones grown at 45 C since this indicates a possible stability of cellular structure such as proteins or even membrane structures (Brock, 1967).

Horning et al. (1961) devised a scheme for the synthesis of branched chain fatty acids in bacteria. They demonstrated the synthesis of branched chain fatty acids with a partially purified enzyme system primarily responsible for the synthesis of myristic and palmitic acids in the presence of sufficient substrates. Kaneda (1963; 1966) obtained a similar mechanism for the synthesis of branched chain fatty acids in B. subtilis with the exception that the initiator of the synthesis had to be an α-keto acid. In other words, branched acid syntheses are built up from three acyl CoA compounds (isovaleryl, isobutyryl, 2-methylbutyryl CoA). Since the different CoA compounds and their respective enzyme systems are needed in the de novo synthesis of branched chain acids, the change in composition derived from changing incubation temperatures may be simply a matter of depletion of nutrients whereby one system predominates.

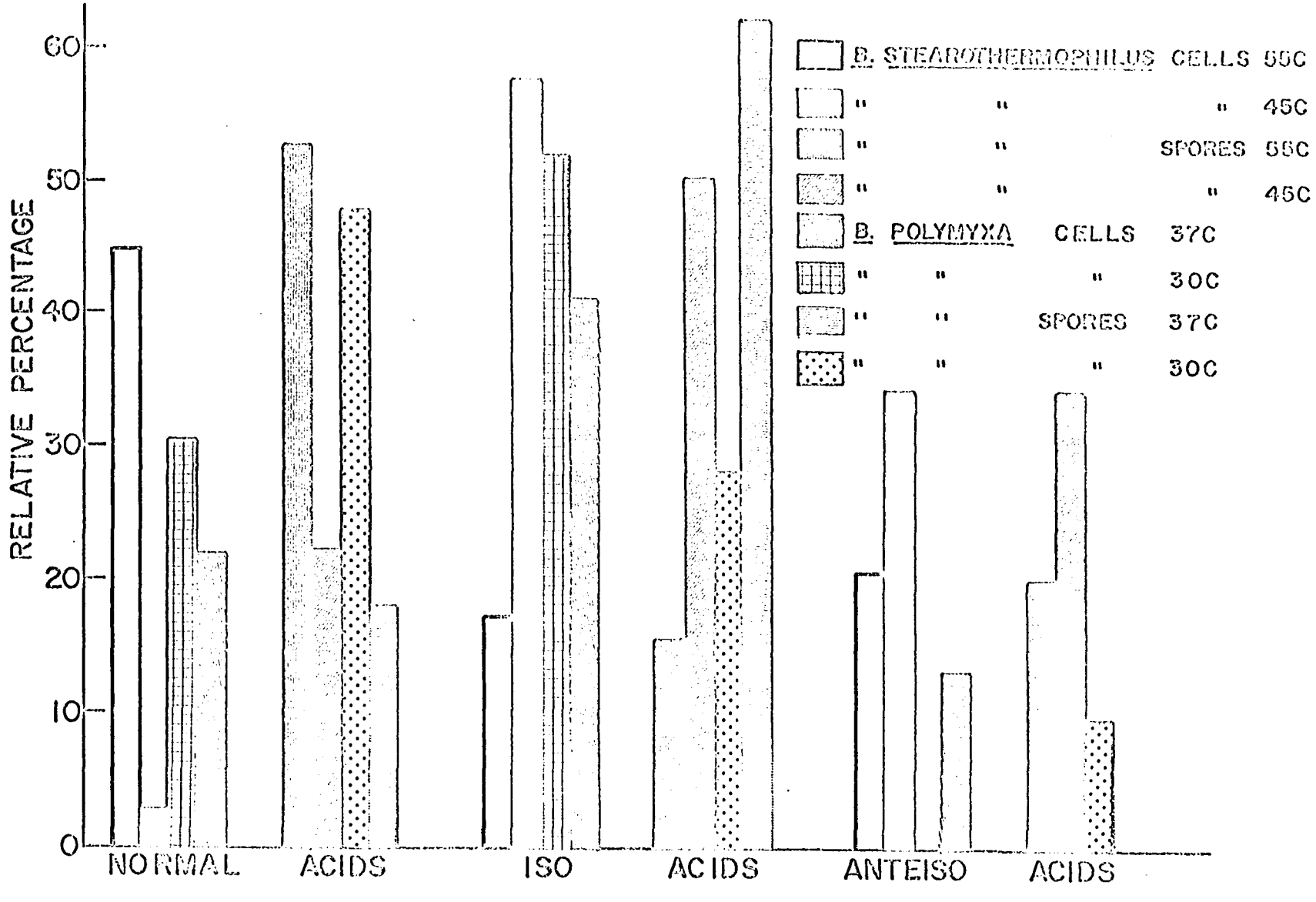
Mesophiles were shown to react similarly with regard to change in incubation temperature. Similar to the thermophile, the major fraction for the bacteria grown at their optimal temperature was n C_{16}; but when the temperature was switched to 37 C, iso C_{15} became the maximum peak for both vegetative cells and spores (although in the case of vegetative cells, the difference was more pronounced). There were some changes in the distribution of fatty acids in the vegetative cells of the mesophile when the temperature was

changed; the most notable of couse is the more even distribution amongst several fractions thus reducing the predominance of one particular fraction. Another point was the higher concentration of the longer chain fatty acids when the temperature of incubation was increased.

Grouping the normal acids in one group, iso acids in another, and anteiso in still another, thermophiles were shown to have a very significant drop in amount of normal acids when grown at other than the optimal growth temperature (Figure 5). Furthermore, the number of anteiso acids increased considerably. Apparently, there were changes in the biological system such as a change in activities of certain enzymes, activation and/or inactivation of metabolic control systems, or even the formation of some new biological pathways. Also the fact that B. stearothermophilus grew more slowly at 45 C may indicate a possible effect attributable to age differentials. The presence of more anteiso acids when grown at a sub-optimal temperature can mean a more active α-keto acid dehydrogenase enzyme system or the mere presence of a more favorable condition for the accumulation of isoleucine which may be the precursor of such acids (Kaneda, 1963b;1967).

Spores and vegetative cells of B. polymyxa exhibited a similar decrease in number of normal acids when grown at other than optimal temperature. Furthermore, the mesophile

Figure 5. Relative amounts of different types of fatty acids extracted from vegetative cells and spores of B. polymyxa and B. stearothermophilus grown at different temperatures


RELATIVE PERCENTAGE
60
50
40
30
20
10
0
NORMAL
ACIDS
ISO
ACIDS
ANTEISO
ACIDS
B. STEAROTHERMOPHILUS CELLS 55C
" " " 45C
" " SPORES 55C
" " " 45C
B. POLYMYXA CELLS 37C
" " " 30C
" " SPORES 37C
" " " 30C

showed a tendency to have less pronounced changes between the two temperatures. Perhaps the temperature differential was not as great as that used with the thermophiles, or it could be attributed to the shorter cultivation time (the age of the culture was about the same as that of the thermophiles).

Two principal explanations have been offered for the ability of thermophiles to grow at elevated temperatures: first, thermophiles contain proteins with greater than normal heat stability; there have been a number of occasions where thermostable proteins and enzyme systems were found in the thermophiles. Malic dehydrogenase from B. stearothermophilus was much more stable than that found in Lactobacillus arabinosus (Marsh and Militzer, 1952). α-amylase, purified to remove any protective factors from influencing stability, has also been found to be more heat resistant (Campbell, 1955). Many other enzyme systems have reacted similarly; namely, pyrophosphatase (Brown et al., 1957), ATP-sulfurylase (Akagi and Campbell, 1962). In fact, some other cellular constituents such as flagella (Dimmick, 1965) and ribosomes (Saunders and Campbell, 1966) have been found to be more heat stable when isolated from a thermophile. Secondly, the theory of "dynamic nature of thermophily" has been expounded by Allen (1953) in which she suggests that a rapid turnover of biological materials existed within the bacterial cell. Although this hypothesis has been challenged by Brock

(1967), it is nevertheless worthwhile to mention the possibility of accumulating more end products by the thermophiles through their rapid reproduction. Such an accumulation may in fact be used to explain the presence of higher concentrations of long chain fatty acids in vegetative cells and spores of thermophiles than in those of mesophiles.

Cells vs. spores

Table 2 shows that bacterial cells of both mesophiles and thermophiles contained about the same fraction of fatty acids as their spores. However, a considerable shift in the lipid synthesis apparently occurred when the bacteria were grown at different temperatures. N C_{16}, for instance, was the major fatty acid component when either of these organisms was grown at their respective optimal temperatures. However, when the incubation temperature was changed, the fatty acid distribution also changed; iso C_{15} became the predominant fatty acid of all the fractions. It is possible that such a change may be the result of deviation from the normal pattern of biochemical reactions involved in the fatty acid metabolism of aerobic microorganisms. One such possibility is the modification or inactivation of the permease systems such as those studied in E. coli (Kepes and Cohen, 1962). The bacteria were grown in media containing valine at 37 C. Accumulation of the amino acid was significantly higher when compared with the accumulation of valine in the

same bacteria but grown in a valineless medium. Furthermore, the valine accumulation may be replaced competitively by another valine molecule or by some structurally related amino acids, such as leucine or isoleucine. Since the biosynthesis of fatty acids of aerobic microorganisms may have several pathways depending on the products, branched chain fatty acids require the presence of three amino acids (valine, leucine, and isoleucine) as precursors. A change in the temperature of incubation could possibly inhibit the premease system of the Bacillus; perhaps, not destroying the system completely but, rather, altering the system to such an extent that accumulation of the amino acid is sufficiently reduced. A study of such a system, for instance, has been reported by Bernlohr (1965) in which he tested the oxidation rate of different amino acids and noticed a drastic decrease in the concentration of valine, leucine, and isoleucine during sporulation. Indeed, a slight decrease in the relative amount of branched chain fatty acid in the spore was found when compared to the vegetative cell (Figure 5).

Gas chromatograms of lipid extracts from B. polymyxa cells grown at 30 C showed the presence of at least 12 different acids; the majority of these acids were branched chain acids. Iso C_{13}, iso C_{15}, n C_{16} were the predominant constituents followed by monoenoic C_{16}, iso C_{16}, n C_{14}, and

iso C_{14}. When the incubation temperature was raised to 37 C, the gross composition changed very little except perhaps the relative amounts of some of the acids. As shown in Table 2, the fatty acids were more evenly distributed throughout the fractions, the predominant fraction, now, being iso C_{13}, iso C_{15}, anteiso C_{15}, n C_{16}, and $C_{16}^{=}$. The monoenoic C_{16} concentration did not show much variation all through the experiment.

Essentially the same conclusion can be made for the spore extracts of B. polymyxa. The spores, however, did have some distinguishing characteristics of their own. These spores have one principal fraction with the rest distributed fairly equally among a number of acids. The predominant fraction for spores grown at 30 C was n C_{16}. Other prominent fractions were iso C_{13}, iso C_{14}, iso C_{15}, anteiso C_{15}, iso C_{16}, and $C_{16}^{=}$. Changing the incubation temperature from 30 C to 37 C shifted the main component from n C_{16} to iso C_{15}. The less prominent fractions were iso C_{13}, iso C_{14}, n C_{16}, and $C_{16}^{=}$. The spore extracts also showed the presence of n C_{17} which was not detected in the extracts from the vegetative cell (Figure 4). The concentration of iso C_{14} and iso C_{16} acids in the spore is about twice as much as the concentration in the vegetative cells. The same thing is true for the prominent fraction in the spore when compared to the cell. This may very well be due to a shift in the metabolic

systems in the bacteria as a result of differences in ages of the cell. The relative decrease of valine, leucine, and isoleucine (Bernlohr, 1965) during sporulation may also be due to such an accumulation of fatty acids.

Extracts of B. stearothermophilus resembled those of B. polymyxa, in that the fatty acids were made up mostly of branched chain acids. As was observed in B. polymyxa, the fatty acids were more evenly distributed in vegetative cells than in spores. Unlike B. polymyxa, however, the bacterium had fewer fatty acid fractions. Furthermore, the cell extracts had one fatty acid fraction which was predominant just as in the spore extracts. When the bacteria were grown at 45 C, the major fractions were iso C_{15}, anteiso C_{15}, iso C_{17}, anteiso C_{17} with iso C_{15} comprising almost half the total acids. In the spore extracts, the major fraction was also iso C_{15}. However, the n C_{16} increased by more than 7 times in relative volume compared with the ones in vegetative cell extracts. When the incubation temperature was raised to 55 C, some significant changes also emerged as n C_{16} became the major fraction, with additional major fractions such as iso C_{15}, anteiso C_{15}, monoenoic C_{16}, and anteiso C_{17}. The same thing held true for the fatty acid composition of spore extracts. Again, the quantitative changes resulted in the possible absence of significant peaks for n C_{17} in the 45 C culture. The variation in fatty acid composition

can probably be best explained in terms of changing cultural conditions through age.

Since no quantitative examinations were performed on the bacteria, there is no way to compare the fatty acid output per individual cell. The seemingly high relative volume of normal acid in spores as compared to vegetative cells (Figure 5) may be due to the extreme stability of its carrier protein (Alberts et al., 1963); that is, the cell continues to produce the same amount of normal fatty acid while the enzyme systems producing other fatty acids vary with temperature.

Cyclic acids are not commonly found in the family Bacillaceae. Kaneda (1963a) and Pheil and Ordal (1967) were not able to detect such acids in either aerobic or anaerobic spore formers. Apparently, the acids, if they are present, are in such minute quantities that they can be considered negligible. Colorimetric tests for hydroxy acids did not give any indication of the presence of such acids, no attempts were made to detect possible presence of hydroxy acids by chromatographic methods.

Saturated straight chain acids vs. saturated branched chain acids

Saturated, straight chain fatty acids have long been known to occur in bacteria (O'leary, 1962a; Kates, 1964). Fatty acids of longer than 12 carbon chain length constitute

a much larger proportion of the total fatty acid content than the shorter chain ones. In these experiments most of the straight chain acids contained an even number of carbon atoms of which the predominant one was the C_{16} acid. Branched chain acids seldom occur in higher forms of life, but appear in great quantity in bacteria, especially in spore formers. Branched chain C_{13}, C_{15}, C_{17} have been found by a number of researchers (Kates et al. 1962; Saito, 1960; Kaneda, 1963a). From Figure 4 and 5, the iso acids were the predominant branched chain acids. Iso C_{13}, and iso C_{15} were the major branched chain acids in B. polymyxa whereas in B. stearothermophilus, the branched chain acids were more evenly distributed among iso C_{15}, anteiso C_{15}, iso and anteiso C_{17}. When the bacteria were grown at their optimum temperatures, the total of branched chain acids was roughly equivalent to that of saturated, straight chain acids. When the bacteria were grown at their optimum temperatures, the total of branched chain acids was roughly equivalent to that of saturated, straight chain acids. When the bacteria were grown at sub-or super-optimal temperatures, however, the total branched chain acids were much higher than the straight chain acids.

The presence of significant amounts of both straight and branched chain fatty acids in microorganisms has been well established. Lennarz (1966) took note of the fact that acetyl

CoA acted as precursor of the methyl terminal portion of straight chain fatty acids and of malonyl CoA, the mechanism controlling the formation of relatively large amounts of branched-chain fatty acids; he suggested three possible ways in which branched chain fatty acid synthesis could be favored: (1) that there is a large intracellular pool of branched chain CoA esters, relative to acetyl CoA; (2) that there are two separate enzymes present in the organism, one specific for branched chain CoA esters and the other for acetyl CoA; (3) that there is only one enzyme that has a marked but not absolute specificity for branched chain CoA esters. Whichever pathway functions, the changes in the composition of spore formers had to be initiated by some environmental factor such as a change in temperature of growth or a change in physiological age of the culture. This means that precise knowledge of growth conditions and the effect of these conditions on the fatty acid composition will be needed; this is particularly applicable if bacterial lipids are to be used as a tool in classification of organism as suggested by Abel et al. (1963).

Kaneda (1967) showed the presence of C_{17} acids in his studies on Bacillus species, in which one of the samples was the vegetative cell of B. polymyxa. In our work, C_{17} was hardly ever found in the mesophile. The differences may be attributable to individual strain variation, differences in

culture media or even extraction procedure. However, since the acid appeared in the spore extract, the tendency would be to explain the difference on the basis of the low concentration of such acid.

Kodicek (1962) pointed out that the structure of a cellular membrane must have enough branched chain acids, highly unsaturated acids, or cyclic acids to resist being packed closely into a film and to also remain elastic. Whether there is a change in the composition of the cellular membrane in bacteria grown at a temperature other than the optimum remains to be proven. If the membrane does become more premeable, it would be possible to assume that reduced heat resistance of these organisms is caused by the leakage of cellular constituents through the membrance of increased porosity.

Unsaturated acid vs. saturated acids

Long and Williams (1960) contended that in B. stearothermophilus the unsaturation of the lipid material decreased as the incubation temperature was lowered. This agrees well with the findings in these experiments in which unsaturated acids were found in the bacteria grown at 55 C but not in 45 C. One can only guess, however, whether this change is due to the absence of the reaction mechanism, or some other reason. Kates (1964), working with Serratia marcescens, claimed that young cells synthesized monoenoic

acid but did not convert them to cyclopropane acids. If this fact holds true for the bacteria used in the experiment, then it would partially explain the disappearance of $C_{16}^{=}$ in cultures grown at 45 C as being used to form the cyclopropane C_{17} acid as the C_{17} branched chain acid concentration had increased considerably. The retention time of the cyclic acid on the chromatographic column may be close to that of the branched C_{17} acid and so was assimilated into one larger peak. Some reservations must be made in relation to such a proposition since cyclic acids are not commonly found in Bacillus species (Kaneda, 1963a).

B. polymyxa was found to contain monoenoic C_{16} acids in both cells and spores and at both temperatures. There was a slight decrease in concentration of the unsaturated acid when growth was at 30 C. This difference was not great enough to establish any meaningful support to the contention that more unsaturated lipids can be expected when the organisms were grown at lower temperatures. The spores did show the presence of a new fraction, mainly n C_{17}, and some decrease in relative volume of the unsaturated fatty acid. Again, the new fraction could be merely a result of increased acid concentration or it could be the result of cyclic acid production through changes in the age of the culture.

Apparently, bacteria change their lipid composition considerably whether through temperature or age variation.

The thermophile exhibited a higher concentration of long-chain fatty acids when compared with B. polymyxa. However, the thermophily of bacteria can hardly be attributed to the lipid since the fatty acid composition of these organisms had much in common when they were grown at their optimum temperature. It would be interesting to test whether the accumulation of long chain fatty acids were the cause or result of heat tolerance of any bacteria. The tolerance of spores to extreme heat does not seem to be the result of fatty acid variation due to the inconsistency of results between mesophile and thermophile. Nevertheless, such studies might prove useful in further understanding of the biosynthesis, metabolism of lipids and their relation and function with other compounds. The utilization of fatty acid content as a taxonomic tool may have some application if one is aware of the effect of cultural conditions on the accumulation of these materials in bacteria.

SUMMARY AND CONCLUSIONS

Vegetative cells and spores of Bacillus polymyxa and Bacillus stearothermophilus were produced in suitable media. Vegetative cells and spores of Bacillus polymyxa were produced at 30 and 37 C while those of Bacillus stearothermophilus were grown at 45 and 55 C. Lipids were extracted from vegetative cells or spores with alcohol and ether. The esters of the fatty acids were then run through a vapor phase chromatograph equipped with ethylene glycol succinate column for further separation.

The different fatty acid components were shown as peaks coming out of the chromatogram and identified by their retention time within the column. Isomers and unsaturated acids were distinguished from straight chain saturated acid by comparing the relative changes in their respective retention time when the columns were run at two temperatures. Further identification of the fatty acids included the use of standard fatty acids in conjunction with the sample. The relative volumes of the peaks were measured by the area each peak covered.

Fatty acids ranging from C_9-C_{17} were detected in the process, with C_{13}-C_{17} constituting the core of the acid fraction. Spores and vegetative cells had about the same number of fractions. However, the relative quantities of the

acids varied between cells and spores. Growing the bacteria at temperatures other than the optimum resulted in the decrease of normal acids, an increase in branched chain acids, and a decrease or absence of unsaturated acids. Also, the thermophile was found to contain a greater number of long chain acids than the mesophile qualitatively as well as quantitatively.

From this work, the following conclusions may be made:

(1) Fatty acids are present in bacterial cells and spores.

(2) Extraction of fatty acids from the spore requires a much harsher treatment than does extraction from vegetative cells.

(3) Cultural conditions such as temperature of incubation produced extensive changes in fatty acid composition of bacteria.

(4) Branched chain fatty acids comprised a major part of the total fatty acids in the two Bacillus species studied.

(5) The thermophile (B. stearothermophilus) contained more types of fatty acids than did the mesophile (B. polymyxa). The most obvious difference is the absence of C_{12} and C_{13} acid in the thermophile and the absence of C_{17} in the mesophile.

(6) The fatty acid differences between vegetative cells and spores gave little indication of being a factor in heat resistance of spores.

(7) Bacteria grown at other than the normal optimum temperature showed no particular relationship between the heat resistance of bacteria and its fatty acid changes.

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