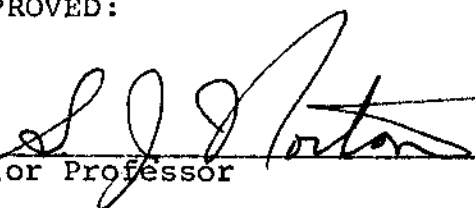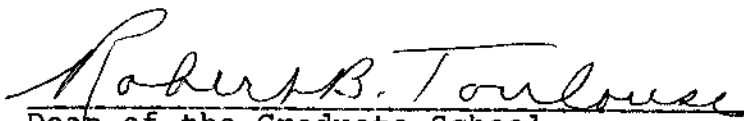STUDIES CONCERNING THE PRODUCTION OF LACTOBACILLIC ACID IN

LACTOBACILLUS PLANTARUM

APPROVED:

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Halper, Laura A., Studies Concerning the Production of Lactobacillic Acid in Lactobacillus Plantarum. Master of Science (Chemistry), May, 1973, 49 pp., 7 tables, bibliography, 79 titles.

This study is concerned with certain factors affecting the content of lactobacillic acid (cis-11,12-methyleneoctadecanoic acid) in Lactobacillus plantarum (ATCC 8014). Lactobacillic acid is one of several cyclopropane fatty acids which have been found in a variety of bacterial species. Although these fatty acids may occur in the membrane in rather high concentrations, no satisfactory explanation of their function has been proposed to date.

Three main areas of investigation are reported herein. The effects of both the oxygen tension and the pH of the culture medium on the accumulation of lactobacillic acid were determined. In addition, monolayer studies were conducted to determine the influence of cyclopropane fatty acid content on the molecular packing of membrane lipids.

The content of lactobacillic acid in bacterial samples was determined by extraction of the cell lipids, followed by saponification and esterification of the fatty acids. Gas chromatography was used to analyze for the presence of specific fatty acids and their relative concentrations. Preliminary experiments showed no major differences in the fatty acid spectra of the membrane and of the whole bacterial cell. Therefore, subsequent analyses were performed on whole cells.

The oxygen level of the growth medium was adjusted by sparging with nitrogen or by adding reducing agents to the solution. It was found that the content of lactobacillic acid increases with increasing bacterial growth regardless of the oxygen level. Thus, oxygen tension affects the production of cyclopropane fatty acid only insofar as oxygen acts as a growth-promoter.

Studies concerning the effect of pH on cyclopropane fatty acid production showed that bacteria in the exponential phase of growth produce more lactobacillic acid as the pH of the growth medium is decreased, and less lactobacillic acid as the pH is increased. It was also found that the content of cyclopropane fatty acid is increased under low pH conditions during all phases of growth. Previous workers had examined pH effects only on bacteria harvested in the stationary phase and cultured at constant pH values.

Bacteria which have been grown into the stationary phase at neutral pH respond in a different manner when the pH is suddenly decreased. Under these conditions, the lactobacillic acid content actually decreases at the lower pH. A sudden increase in salt concentration causes a similar reduction in lactobacillic acid content.

The dramatic effects of low pH conditions may indicate that cyclopropane fatty acids function to control membrane permeability to hydrogen ions. This possibility was investigated by the use of a Langmuir film balance. Lipids extracted

from bacteria grown at low and neutral pH were used in mono-layer studies on water at low and neutral pH. The film pressure-area curves obtained were essentially identical when determined on substrate maintained at 21° C.

The study concludes that the production of cyclopropane fatty acid does not function mainly to protect unsaturated membrane lipids from oxidation. This conclusion is based on the fact that lactobacillic acid content depends upon the amount of bacterial growth regardless of the oxygen tension of the medium. It is still unknown whether or not lacto-bacillic acid affects membrane permeability to hydrogen ions. Additional film balance studies at the temperature of bac-terial growth are needed to answer this question.

An important finding is that lactobacillic acid content decreases after sudden imposition of high acidity or salt concentration in cells grown into the stationary phase at neutral pH. The data indicate possible preferential cata-bolism of lipids containing cyclopropane fatty acid, or the catabolism of the lactobacillic acid itself. This paper represents the first report of such metabolism of cyclopro-pane fatty acid.

STUDIES CONCERNING THE PRODUCTION OF LACTOBACILLIC ACID IN

LACTOBACILLUS PLANTARUM

THESIS

Presented to the Graduate Council of the

North Texas State University in Partial

Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

by

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Denton, Texas

May, 1973

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CHAPTER I

INTRODUCTION

Occurrence

The natural occurrence of a fatty acid containing a
cyclopropane ring was first reported by Hofmann and Lucas (1).
On the basis of various chemical and physical properties of
the compound, Hofmann and co-workers (2) identified relatively
large amounts of 11,12-methyleneoctadecanoic acid (lacto-
bacillic acid) in lipid extracts of Lactobacillus arabinosus
(L. plantarum). Also identified was cis-11,12-octadecenoic
acid (cis-vaccenic acid). No oleic acid was found. Lacto-
bacillic acid was later discovered in lipid extracts of
L. casei, L. delbrueckii, and Agrobacterium tumefaciens (3-5).

Other cyclopropane fatty acids have been discovered
since that time. The lipids of Escherichia coli contain
cis-9,10-methylenehexadecanoic acid (6,7). Salmonella
typhimurium contains cis-9,10-methyleneoctadecanoic acid
(dihydrosterculic acid) (8). Other bacterial lipids have
been reported to contain C_{13}-, C_{15}-, and C_{21}-cyclopropane
fatty acids of unidentified structure (9-11). Plasmalogens
containing C_{17}- and C_{19}-cyclopropane aldehydes have been
identified in Clostridium butyricum (12). Table I (13,p.6)
shows the abundance of C_{19}-cyclopropane fatty acid (not

1

necessarily lactobacillic acid) and C_{17}-cyclopropane fatty acid in a number of bacteria.

All of the polar lipid classes contain similar proportions of cyclopropane fatty acids in L. casei (14), A. tumefaciens (15), and E. coli (16). The cyclopropane fatty acids usually occupy the 2-position of phospholipids, a site otherwise normally occupied by unsaturated fatty acids. The 1-position is usually occupied by saturated fatty acids whether or not cyclopropane fatty acids are present. Phospholipids with fatty acids in the usual positions include the phosphatidylethanolamines (cephalins) of E. coli and Serratia marcescens, and the cephalins and phosphatidylcholines (lecithins) of A. tumefaciens (15,17). However, C. butyricum contains cephalins in which the unsaturated and cyclopropane fatty acids occupy mainly the 1-position (15).

Biosynthesis

A precursor relationship of cis-vaccenic acid to lactobacillic acid was postulated by Hofmann and co-workers (18,19). They found that when L. delbrueckii and L. plantarum were grown in the presence of cis-vaccenic acid, large amounts of lactobacillic acid were formed. The presence of lactobacillic acid in the growth medium did not result in production of unsaturated fatty acids. Hofmann et al. (19) demonstrated that stearic acid and cis-vaccenic acid exhibit different biotin-sparing activities, indicating that they are not

TABLE I

OCCURRENCE OF CYCLOPROPANE FATTY ACIDS*

Bacteria	Percent Cyclopropane Acid	
	C_{17}	C_{19}
Escherichia coli	24	24
	6	1
	9	3
Salmonella typhimurium	16	4
Serratia marcescens	44	9
	32	3
	28	12
	20	1
Aerobacter aerogenes	25	6
Agrobacterium tumefaciens	..	13
Lactobacillus plantarum	..	30
	..	47
	..	15
Lactobacillus casei	..	16
	..	45
	..	35
Lactobacillus acidophilus	..	30
Lactobacillus delbrueckii	..	9
Streptococcus lactis	..	20
Streptococcus cremoris	..	18
Streptococcus agalactiae	..	2
Streptococcus uberis	..	8
Clostridium butyricum	9	5
Micrococcus cryophilus	..	1
Rhodomicrobium vanielii	..	4
Pseudomonas fluorescens	3	8

* From Reference 13, p. 6.

interconverted. These workers also failed to find any "fatty acid dehydrogenase system" which could convert stearic acid to cis-vaccenic acid.

Proof that cis-vaccenic acid is converted to lacto-bacillic acid was provided by carbon-14 and tritium labelling in L. plantarum and E. coli (6, 20-23). The immediate donor of the methylene group was found to be S-adenosylmethionine (24,25). Two of the methyl hydrogen atoms are incorporated into lactobacillic acid (26), while the vinyl protons of the olefinic precursor are retained (27). Evidence that oleic acid is not isomerized to cis-vaccenic acid was provided by demonstrating that Lactobacillus converts oleic acid to cis-9,10-methyleneoctadecanoic acid (28).

Enzymatic Studies

Crude enzyme extracts of C. butyricum and S. marcescens were found to catalyze the transmethylation reaction shown in Figure 1 (25). Chung and Law (29) were able to purify the enzyme (cyclopropane synthetase) from C. butyricum 50-fold by using DEAE-cellulose and Hypatite C columns. These workers demonstrated (30) product inhibition of the reaction by S-adenosylhomocysteine in cell-free extracts. C. butyricum was found to contain an enzyme which hydrolyzes S-adenosyl-homocysteine to adenine and other unidentified products. These workers proposed that this hydrolysis reaction may

Figure 1

Biosynthesis of Lactobacillic Acid

$$H_2C-O-\overset{\overset{O}{\parallel}}{C}(CH_2)_{14}CH_3$$

$$CH_3(CH_2)_5CH=CH(CH_2)_9\overset{}{C}-O-\overset{}{CH}$$
$$\underset{O}{\parallel}$$

$$H_2C-O-\overset{\overset{O}{\parallel}}{P}OCH_2CH_2NH_2$$
$$\underset{OH}{}$$

1-palmitoyl,2-cis-vaccenoyl-L-phosphatidylethanolamine

+

S-adenosylmethionine

$$HOC\overset{O}{\diagup}$$
$$HC(CH_2)_2-\overset{\oplus}{S}-H_2C$$
$$\underset{NH_2}{}\quad\underset{CH_3}{}$$

↓ cyclopropane synthetase

$$H_2C-O-\overset{\overset{O}{\parallel}}{C}(CH_2)_{14}CH_3$$

$$CH_3(CH_2)_5HC\overset{\overset{H\ \ H}{\diagup\diagdown}}{\underset{}{C}}C(CH_2)_9\overset{}{C}-O-\overset{}{CH}$$

$$H_2C-O-\overset{}{P}-O-CH_2CH_2NH_2$$
$$\underset{OH}{}$$

1-palmitoyl,2-lactobacilloyl-L-phosphatidylethanolamine

+

S-adenosylhomocysteine

$$HOC\overset{O}{\diagup}$$
$$HC(CH_2)_2-S-H_2C$$
$$\underset{NH_2}{}$$
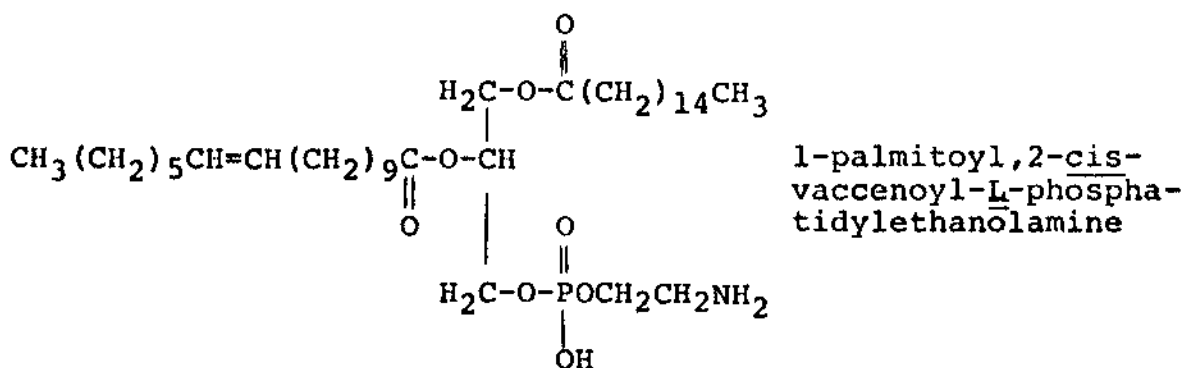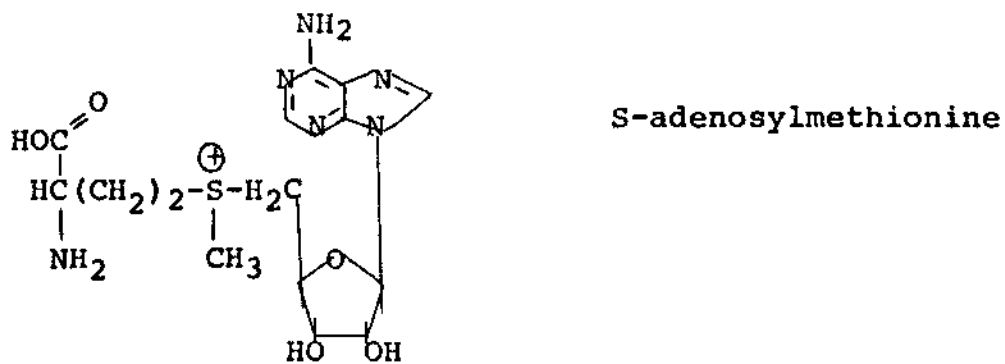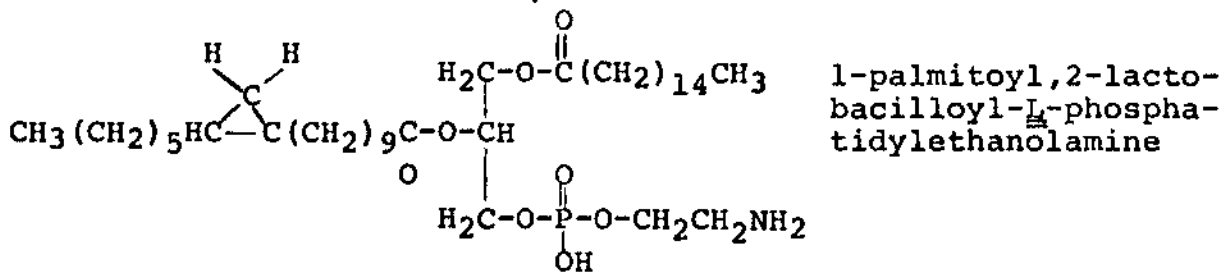
represent a method of regulating cyclopropane fatty acid synthesis in vivo.

Cyclopropane synthetase from C. butyricum is highly specific for the L-glycerol phosphatides and has a definite, though not absolute, preference for the 1-acyl group (15,31). Using gel filtration, Law (32) calculated a molecular weight of about 80,000 for a crude enzyme preparation from this bacterium.

The synthetase reaction is stimulated by anionic detergents and inhibited by cationic and neutral detergents. Free fatty acids and fatty acyl CoA derivatives are not substrates (29). Phosphatidic acid and the phosphatides of ethanolamine, serine, and glycerol are all substrates, but phosphatidyl-choline is not. Phosphatidyl choline inhibits the reaction with substrate phosphatidylethanolamine, probably due to the positive charge on lecithin (31). The reaction rate is also dependent upon the state of the lipid dispersion (31). The surface charge on the dispersed lipid probably is affected by detergents (33, 34). The resulting surface charge evidently influences the approach and binding of the lipid to the enzyme, and so affects reaction rates (31).

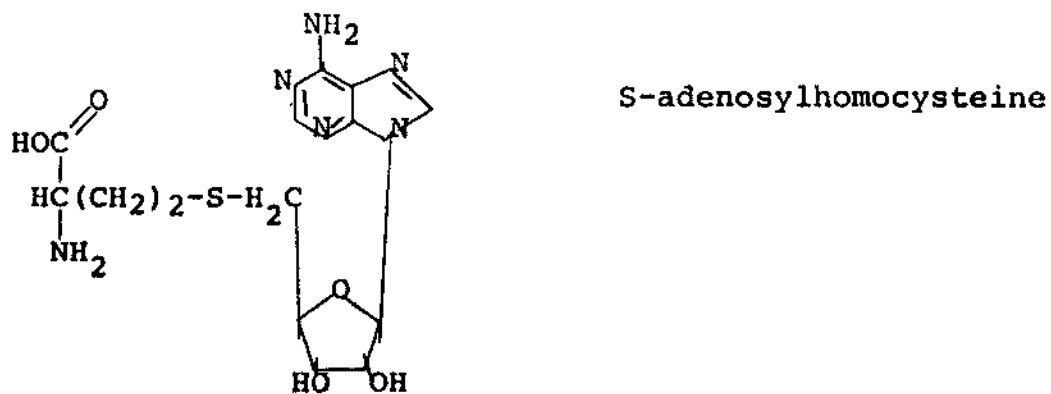Conflicting data have been obtained regarding cofactor requirements. Chung and Law (29) failed to find any cofactors involved in the C. butyricum enzyme system. However, Henderson et al. (35) found that folic acid coenzymes appear to take part in the reaction in L. plantarum. These workers

calculated that less energy would be needed to regenerate S-adenosylmethionine from S-adenosylhomocysteine and methionine via folic coenzyme intermediates than to regenerate the active species from methionine and ATP.

Phospholipids in which the alkyl chains are linked by ether bonds are also substrates (31, 36). The reaction mechanism, therefore, does not involve removal of the acyl chain from the phosphatide backbone with subsequent esterification at the active site of the synthetase. Christie (13, p.35) presents a brief summary of theories concerning possible intermediates in the transmethylation reaction mechanism.

Environmental Factors

Cyclopropane fatty acids accumulate during the late exponential and stationary phases of growth in E. coli (17, 37, 38), A. tumefaciens (17), S. marcescens (17, 39), Pseudomonas fluorescens (40), and L. plantarum (41). Most of the early investigations of environmental effects of cyclopropane fatty acid accumulation have been done with E. coli. Knivett and Cullen (42) found that the accumulation of cyclopropane fatty acid (mainly C_{17}) is increased by acidic medium, poor oxygen supply, or high growth temperatures. The presence of citrate or lack of Mg^{2+} decreases cyclopropane fatty acid accumulation. These workers later showed (43) that a marked accumulation of C_{17}-cyclopropane fatty acid occurs when either NH_4^+ or PO_4^{3-} is growth limiting with

adequate amounts of glycerol; under these conditions, some C_{19}-cyclopropane fatty acid is also formed. A mixture of glycerol, Mg^{2+}, and SO_4^{2-} stimulates cyclopropane fatty acid formation in resting cells.

Crowfoot and co-workers (44) found that increasing the oxygen level of the growth medium decreases the accumulation of C_{17}-cyclopropane fatty acid in P. fluorescens during all phases of growth. It was later shown that the level of cyclopropane synthetase increases in P. fluorescens grown at low oxygen tensions, and that the enzyme can be induced by decreasing the oxygen tension of the growth medium (45). Production of cyclopropane fatty acids decreases when the organism is grown at 5°C rather than at 30°C, although the proportion of unsaturated fatty acids (plus cyclopropane fatty acids) increases at the lower temperature (40).

Limiting amounts of methionine or very low levels of thymidine with adequate methionine have been shown to decrease the accumulation of cyclopropane fatty acid in Streptococcus faecalis (46). Studies with Salmonella typhimurium have shown that after thermal shock, fewer cyclopropane fatty acids are synthesized, and concomittantly more of the parent monoenoic fatty acids are synthesized (47). These results were interpreted as indicating partial inactivation of the cyclopropane synthetase system during thermal injury.

Functions of Cyclopropane Fatty Acids

It is difficult to rationalize a vital function for the cyclopropane fatty acids because they are not found in all bacteria, and yet they are found in bacteria of several different orders (see Figure 1). There is as yet no explanation which accounts for this limited distribution. There appears to be no metabolic turnover of the cyclopropane fatty acids (17), which means that the acids are not intermediates for further metabolic reactions. The suggestion has been made (17) that the cyclopropane fatty acids remove an undesirable or excess metabolic product (S-adenosylmethionine or unsaturated fatty acids), but such a control mechanism seems wasteful of cellular energy.

An antioxidant function also has been proposed (17). The unsaturated fatty acids in cell membranes may be subject to oxygen or free radical peroxidation. Addition of a methylene bridge would protect the integrity of the cell membrane by preventing lipid peroxidation. On the basis of phospholipid changes in E. coli, Cronan (48) suggested that the accumulation of cyclopropane fatty acids in the later stages of growth may protect the phospholipids from degradation when metabolism is slow. However, experiments with E. coli (42) and P. fluorescens (44) have demonstrated that high oxygen levels in the growth media actually decrease the amount of cyclopropane fatty acids synthesized. These data indicate that an

antioxidant function is not the primary role of cyclopropane fatty acids in these bacterial membranes.

It is not known what structural effects occur when an unsaturated fatty acid in the membrane is converted to a cyclopropane fatty acid. The biotin-sparing activity of unsaturated fatty acids, indicating an involvement of biotin in the biosynthesis of unsaturated fatty acids, has been known for some time (49-51). Lactobacillic acid also has biotin-sparing activity in L. plantarum (52). L. delbrueckii, L. plantarum, and L. casei do not synthesize cis-vaccenic acid when they are grown in the presence of lactobacillic acid (18, 19); evidently lactobacillic acid can substitute for cis-vaccenic acid both metabolically and structurally.

The effects of cyclopropane fatty acid accumulation on the physical properties of the membrane of P. fluorescens have been investigated recently by means of a Langmuir-type film balance (40). The molecular packing of cyclopropane fatty acids was found to be intermediate between that of the corresponding cis- and trans-monoenoic fatty acids. Phosphatidylethanolamine with seventy-six percent unsaturated (plus cyclopropane) fatty acids exhibited a slightly more expanded force-area curve as compared with cephalin containing sixty-six percent unsaturated (plus cyclopropane) fatty acids. Temperature effects on packing were found only near the liquid crystalline transition temperature. The conclusion

was drawn that accumulation of cyclopropane fatty acid does
not greatly affect the physical properties of bacterial
membranes.

Film Balance Studies of Membrane Lipids

The Langmuir film balance (53) provides a simple method
of studying membrane lipids under various conditions of molec-
ular packing in the air-water interface. Certain precautions
should be taken when drawing analogies between film balance
monolayers and biological membranes (54). Current information
indicates (55) that the most likely membrane structure is
some kind of bimolecular leaflet (56). Not only does the air-
water interface present a different environment from that
existing in most biological membranes, but also the behavior
of lipids in a monolayer may be different from their behavior
in a biomolecular leaflet membrane.

In addition, the question arises as to which part of the
film pressure-area curve is most relevant to biological
membranes. Using a bimolecular leaflet model, Haydon and
Taylor (57) calculated the effective pressure in the lipid
layer to be more than thirty dynes per cm. More important,
however, was their conclusion that the molecular situation at
the air-water interface entails quantitative, rather than
qualitative differences from the situation in a bimolecular
leaflet structure. Thus, film balance studies can provide
useful information about lipid behavior in membranes.

Among the many factors which affect monolayer behavior are those of the class of lipids studied, the chain length and degree of unsaturation of the fatty acids, and the temperature, pH, and salt content of the subphase. A brief review of the effects of these variables on monolayers of known lipid composition follows.

Chapman and co-workers (54) found that phosphatidyl-choline monolayers are more expanded than are phosphatidyl-ethanolamine monolayers of corresponding fatty acid composition. Such expansion is believed due to the effect of the methyl groups of the choline moiety on the packing of the fatty acid hydrocarbon chains (58). It has been found also that cis-unsaturated phospholipid monolayers are more expanded than those of the trans-unsaturated isomers (54).

Studies of mixed monolayer systems have shown that saturated lecithins of similar chain length exhibit ideal mixing in the monolayer (59). If the chain length or degree of unsaturation is very different between two lecithins, then the molecular area of the higher melting compound becomes larger in a mixed monolayer of the two lipids. This effect has been attributed to the increased configurational freedom of the hydrocarbon chains in the mixed monolayers.

In a similar manner, the permeability of liposomes (60) to glycerol has been found to increase with increasing unsaturation or decreasing chain length of the constituent lecithins (61). The increase in unsaturation of bacterial fatty acids

as the growth temperature is decreased has been suggested
to result in a greater membrane permeability (37).

Various workers have found that the temperature of mono-
layer phase transitions depends upon the specific lipid studied
and the temperature of compression (59, 62, 63). Monolayers
in either the limiting condensed or liquid-expanded phases
are relatively invariant with temperature (63). However, the
phase transition region of the curves is sensitive to temper-
ature.

The pH of the subphase would be expected to affect the
ionization state of lipid material, and therefore affect the
compression curves. Both lecithin and cephalin are isoelectric
at pH 5.5, and so exist as essentially neutral monolayers at
this pH (63-65).

Conflicting data are reported in the literature for the
interaction of phosphatides with monovalent cations. Rojas
and Tobias (66) found that lecithin, cephalin, and phospha-
tidylserine isotherms on pH 5.7 water are essentially invari-
ant regardless of whether the substrate contains NaCl or KCl
in concentrations of either 10 or 100 mM. Shah and Schulman
(67) found the compression curves of dipalmitoyl lecithin to
be identical in the presence of 0.02M Na^+, K^+, $Li+$, SO_4^{2-},
NO_3^-, or Cl^-. However, Vilallonga and co-workers (68) have
found that the presence of 0.15M NaCl, KCl, or LiCl displaces
the curves of dipalmitoyl lecithin towards larger molecular
areas in the order Na^+ >$K+$> Li^+> H_2O.

Studies with known, single lipid species are necessary for an understanding of the effects of various parameters on molecular packing. However, a natural extract should produce a monolayer whose composition is much closer to that of a biological membrane. Even though the lipid species present may not be characterized, use of these extracts should give information about membrane behavior as reflected in monolayer properties.

Objectives of This Investigation

Three main areas have been studied with $\underline{L}$. plantarum in this investigation. The effect of oxygen tension on the accumulation of cyclopropane fatty acid was studied to test further the hypothesis that these acids protect membrane lipids from oxidation. Although the effect of oxygen levels has been investigated in $\underline{E}$. coli (42) and in $\underline{P}$. fluorescens (44), these organisms undergo changes in metabolism and in the level of membrane respiratory components as the oxygen level is varied. Such complications are absent in $\underline{L}$. plantarum; therefore, possible antioxidant functions of cyclopropane fatty acids should be more obvious in this organism.

The second area of study was that of the effect of pH on cyclopropane fatty acid formation. Knivett and Cullen (42) have found that stationary cells of $\underline{E}$. coli grown below pH 6.2 accumulate more C_{17}-cyclopropane fatty acid as the pH of the growth medium is lowered. The present study was undertaken in part to determine if the accumulation of these acids is greater at all phases of growth when bacteria are grown at a low pH, or if such accumulation only occurs during the stationary phase of growth. If the latter case were true, then cyclopropane synthetase may be inducible by low pH only during the stationary phase. It was also of interest to determine whether or not cyclopropane fatty acid production

can be induced by a sudden decrease in pH after growth at a neutral pH has reached the stationary phase.

The third main area of investigation concerned the effect of cyclopropane fatty acids on membrane packing. The large accumulation of these acids at low pH may imply that these compounds function in the control of hydrogen ion permeability through the bacterial membrane. To investigate this question, the molecular packing of lipid extracts from L. plantarum containing high and low proportions of cyclopropane fatty acid were examined with a Langmuir film balance on substrates of both high and low pH.

L. plantarum was chosen as the experimental organism for two main reasons. First, most of the previous studies concerning cyclopropane fatty acid production have been done with E. coli, a Gram-negative facultative anaerobe. Little work has been done concerning environmental effects on the accumulation of this acid in Gram-positive aerobic organisms such as L. plantarum.

Second, this bacterium does not contain cytochromes, nor does it have a respiratory electron transport chain (69, p.662). Therefore, energy is obtained by fermentation regardless of the oxygen level of the growth medium. In this respect, L. plantarum may be considered to be a simpler system to study than would be a facultative anaerobe which undergoes major changes in energy-yielding metabolism as the oxygen level is varied.

CHAPTER II

EXPERIMENTAL METHODS

Bacterial Culture

Cultures of L. plantarum (ATCC 8014) were maintained at
5°C by monthly transfers into agar stab tubes containing
Micro Inoculum Broth (Baltimore Biological Laboratory). Cul-
tures were grown routinely at 37°C in an acid-hydrolyzed
casein medium whose composition is given in Table II. The
pH was adjusted with sodium hydroxide or hydrochloric acid
as required.

For the studies with controlled pH, a four-liter flask
was used which had been equipped with a reservoir of alkali
solution with stopcock flow control. After sterilization with
ninety-five percent ethanol, a pH electrode was immersed in
the medium. Periodic pH readings were taken and the pH
adjusted by the addition of either sterile alkali or eighty-
five percent lactic acid solution as required.

Cell growth was monitored by reading absorption at 540nm.
Samples were withdrawn from a small spout at the bottom of
the growth flask; the medium was mixed on a magnetic stirrer
only just prior to sampling. An initial plot was made of
absorption versus cell mass, and samples were diluted such
that the turbidity readings corresponded to the linear por-
tion of the graph.

TABLE II

GROWTH MEDIUM

Component	Amount Added for One Liter	
Acid-hydrolyzed casein	14	g
Sodium acetate, anhydrous	12	g
1-Asparagine	200	mg
1-Cysteine	200	mg
1-Methionine	200	mg
1-Tryptophan	200	mg
Salts A[a]	40	ml
Salts B[b]	10	ml
Vitamin supplement[c]	2	ml
Purine and pyrimidine supplement[d]	4	ml
Glucose	10	g

a Salts A:

K_2HPO_4	25	g
KH_2PO_4	25	g
Water	250	ml

b Salts B:

$MgSO_4 \cdot 7H_2O$	10	g
NaCl	500	mg
$FeSO_4 \cdot 7H_2O$	500	mg
$MnSO_4 \cdot 4H_2O$	500	mg
Water	250	ml

c Vitamin Supplement:

Thiamin	3	mg
Nicotinic acid	3	mg
Pantothenic acid	3	mg
Riboflavin	3	mg
Pyridoxine	50	mg
Inositol	15	mg
Biotin	0.5	mg
p-Aminobenzoic acid	0.15	mg
Folic acid	0.15	mg
Boiled distilled water	15	ml
95% Ethanol	15	ml

TABLE II--<u>Continued</u>

d Purine and Pyrimidine Supplement:
Adenine.. 100 mg
Guanine.. 100 mg
Uracil... 100 mg
Water.. 100 ml

Fatty Acid Analysis

Bacteria which had been grown under the various conditions were collected by centrifugation, washed with 0.85 percent saline solution, and lyophilized. Lipids were extracted into chloroform:methanol (2:1, v,v) and dried under nitrogen (70). The lipids were saponified with methanolic sodium hydroxide, and methyl esters were prepared with boron trichloride:methanol solution (71). Water was added, and the esters were extracted into either diethyl ether or petroleum ether. The esters were analyzed on a Varian Aerograph Model 204-1C gas chromatograph with flame ionization detectors, using a column of 15 percent diethylene glycol succinate polyester on Chromosorb W (60/80 mesh) at 180°C. Identification of the peaks was made by comparison with methyl ester standards and with published retention times (70). Peak areas were calculated using the method of Carroll (72).

Each fatty acid was reported as percent of the total fatty acids present. Examination of the fatty acid spectra obtained reveals that the lactobacillic acid content is inversely related to the cis-vaccenic acid content. The proportions of the other fatty acids remained rather constant under the various growth conditions, with no evident trends in percentages. Thus, environmental effects could be monitored by noting changes either in percent lactobacillic acid or in the ratio of lactobacillic acid to cis-vaccenic acid.

Adjustment of Oxygen Levels

The gross level of oxygen in the growth medium was adjusted by three different methods, although the oxygen tension was not measured quantitatively. In all cases, rubber stoppers were used in flasks containing reduced media, and cotton plugs were used in flasks containing non-reduced media.

In the first method, deoxygenated ("reduced") medium was obtained by sparging a hot autoclaved medium with nitrogen until cool. Less severely reduced medium was obtained by passing nitrogen over the surface of the hot solution until cool. Normal levels of oxygen were obtained by allowing the medium to cool with a cotton plug in the flask to permit transport of air to the liquid surface. The effect of continuous shaking as a method to increase aeration of growing cultures was also investigated.

In the second method, reduced medium was obtained by adding sodium thioglycollate (or thioglycollic acid) before autoclaving, followed by flushing nitrogen over the surface of the medium until cool. A minimum concentration of 0.5 ml of thioglycollate per liter of medium was chosen in consideration of the standard thioglycollate medium, which has a concentration of 0.3 ml per liter (73). Sodium thioglycollate (or thioglycollic acid) was added to control flasks with cotton plugs to determine whether or not the thioglycollate itself affected cyclopropane fatty acid accumulation.

The third method of adjusting the oxygen level involved the use of flasks which were tightly sealed with rubber stoppers and septa. The medium was sparged with nitrogen after autoclaving, and then known volumes of sterile air were injected through the septa above the surface of the medium.

Film Balance Studies

The Wilhelmy slide method was used to determine the film pressure-area curves (74). Changes in film pressure were transmitted through a platinum plate, suspended in the substrate surface, which had been sandblasted to improve wetting. A zero-degree contact angle was observed for every run. Substrate was contained in a rectangular pyrex tray, the top edges of which had been coated with hard paraffin. Pressure changes were detected by means of a Statham Universal Transducing Cell (Model UC2) fitted with a Model UL5 microscale accessory. A Statham Universal Readout (Model SC1000) connected to a strip chart recorder provided data readout. Lipid films were compressed by manually sliding paraffin-coated glass barriers through measured distances. During some runs, temperature control was provided by pumping water from a constant temperature bath through a glass serpent immersed in the substrate. All glassware used for the extraction, esterification, and film balance studies was cleaned in a chromate:sulfuric acid bath.

Substrate water was deionized and distilled in an all-glass apparatus. The ionic strength of the substrate was adjusted to 0.1 with sodium chloride which had been roasted to remove organic impurities. Spreading solutions were prepared from lipid extracts which had been lyophilized and weighed on an analytical microbalance. The lipid was redissolved in chloroform:methanol (1:1, v,v) which had been redistilled and percolated through activated silica gel before use. Spreading solutions were stored in a solvent-saturated atmosphere at 5°C. The solution was applied from a Hamilton microliter syringe in the form of small droplets gently touched to the substrate surface. No noticeable deterioration of the solutions had occurred after a period of three weeks as indicated by reproducibility of the film pressure-area curves.

CHAPTER III

RESULTS AND DISCUSSION

Previous studies have indicated that nearly all lipids
of Gram-positive bacteria are present in the cell membrane
(75). However, Thorne and Kodicek (76) reported that the
membrane of L. casei contains four odd-numbered acids not
found significantly in the whole cells, and that lactobacillic
acid is a less important constituent of the membrane than of
the whole cell. It was of interest, therefore, to compare
the fatty acids from whole cell and membrane preparations of
L. plantarum.

Bacteria were sampled in the early logarithmic phase of
growth. The membranes were prepared by the method of Vorbek
and Marinetti (66). No major differences were found in the
fatty acid spectra of the whole cell and of the membrane
preparation upon gas chromatography (Table III). Therefore,
fatty acids in all subsequent experiments were analyzed
from whole cell lipid extracts.

The Effects of Oxygen Level

The microaerophilic nature of L. plantarum (77) was
evidenced by the fact that growth was best at reduced oxygen
tensions. Growth was extremely poor in the most reduced
medium, and was best in cotton-plugged flasks which were not

TABLE III

FATTY ACID SPECTRA OF MEMBRANE AND WHOLE CELLS

Fatty Acid	Percent of Total Fatty Acids in Membrane	Percent of Total Fatty Acids in Whole Cells
Myristic	0.3	0.3
Palmitic	25.8	28.2
Palmitoleic	4.4	3.7
Stearic	3.2	3.8
cis-Vaccenic	35.0	33.6
Lactobacillic	31.3	29.8
Others	--	0.7

shaken. When the media had sufficient oxygen to support growth, the amount of cyclopropane fatty acid produced correlated with the amount of growth. It was found also that addition of thioglycollate to cotton-plugged flasks did not significantly affect either the amount of growth or the accumulation of lactobacillic acid.

Typical results are summarized in Table IV. Production of lactobacillic acid increased with the amount of growth regardless of the oxygen level. Two factors may account for such increases. First, the accumulation of cyclopropane fatty acids is known to increase as growth continues through the exponential phase and into the stationary phase in L. plantarum (41) as well as in other bacteria (17, 37-40). Therefore, the cultures exhibiting the highest relative growths would be expected to contain the highest percentages of lactobacillic acid.

Second, the pH of the medium falls as growth continues due to the excretion of lactic acid from the bacteria. The pH was not controlled during these experiments because of the difficulties inherent in manually adjusting the pH of the medium without affecting its oxygen level. Production of cyclopropane fatty acid, measured in the stationary phase, has been shown to increase as the pH is decreased in E. coli (42), and a similar effect might be expected in L. plantarum.

These two factors are sufficient to explain the results shown in Table IV. The only evident effect of oxygen on

TABLE IV

EFFECT OF OXYGEN LEVEL[a] ON LACTOBACILLIC ACID CONTENT

Method of Adjusting Oxygen Level	Relative Growth[b] (Percent of Control Growth)	Lactobacillic Acid Content (Percent of Control Content
N_2 Sparged Into Medium	12[c]	52
Cotton-Plugged Flasks Continually Shaken	76[d]	83
5% O_2 Injected	105	110
N_2 Flushed Over Surface	122[e]	138

[a] See Experimental Methods for details of adjusting oxygen levels.

[b] Growth relative to growth during the same experiment in a cotton-plugged flask with no shaking ("control").

[c] Growth poor due to anaerobic conditions.

[d] Growth intermediate due to aerobic conditions.

[e] Growth best due to microaerophilic conditions.

lactobacillic acid production is due to growth promotion.
Any direct effect of the oxygen level per se on lactobacillic
acid production must be so slight as to be hidden by the
effects of the stage of growth and possibly the pH of the
culture. It must be assumed that if cyclopropane fatty acids
have an antioxidant function in the cell membrane, then that
function must not be the sole or even major function. This
conclusion is supported by the observations of other workers
that cyclopropane fatty acids actually increase at low oxygen
levels in E. coli (42) and in P. fluorescens (44).

The Effects of pH

Very little previous work has been done to study the
separate effects of pH and the phase of growth on cyclopropane
fatty acid production. Knivett and Cullen (42) have shown
that E. coli sampled in the stationary phase and grown at
constant pH contains a higher percentage of cyclopropane fatty
acids when grown at a low pH. Their results leave many ques-
tions unanswered, however, since bacteria in the stationary
phase undergo major changes in their chemical composition,
and therefore in their metabolic behavior (69, p. 307).

The present study was concerned with answering several
questions. First, does the accumulation of cyclopropane fatty
acid in the stationary cells at low pH indicate synthesis of
these compounds during stationary phase? Low pH may increase
the synthesis of these compounds during an earlier growth

phase, whereas the stationary cells themselves may not actually be producing cyclopropane fatty acids at all. A second, related question concerns whether low pH effects greater accumulations of these lipids during all phases of growth, or only during the stationary phase.

Third, what is the effect of a sudden decrease or increase in pH in bacteria which have been grown into the stationary stage at a neutral pH? Do cells in the stationary phase maintain the ability to produce cyclopropane fatty acids in response to low pH conditions?

Fourth, is the increased production of cyclopropane fatty acids a general response to adverse growth conditions, or is it specific to hydrogen ion concentration? For example, does a sudden increase in salt concentration affect cyclopropane fatty acid production?

Initial experiments were conducted to determine the effect of pH on exponentially growing cells. Preliminary experiments proved growth to be quite poor below pH 4.5 or above pH 8.5. It was found that increased acidity had little effect on lactobacillic acid accumulation until a minimum pH threshold was reached. Thus, the accumulation of lactobacillic acid was only slightly increased when bacteria were grown at pH 5.5, and slightly decreased at pH 9.0, as compared with cells grown at pH 7.0

When an inoculum of late exponential cells at pH 5, containing a high proportion of lactobacillic acid, was introduced

into fresh medium controlled at pH 7.5, the percent of lactobacillic acid decreased as growth continued. When the inoculum was introduced into fresh medium controlled at pH 4.5, the percent of lactobacillic acid increased. Typical results are shown in Figure 2. Thus, during exponential growth, lactobacillic acid production is increased as the pH is lowered, and decreased as the pH is raised.

To compare the effects of pH on the production of lactobacillic acid during various stages of growth, cultures were grown with the pH maintained at 4.7, 7.0, and 8.0. Figure 3 shows the growth curve for each culture and the times at which samples were taken for fatty acid analyses. The results of the analyses are shown in Table V.

The bacteria grown at pH 4.7 and pH 7.0 showed an increase in lactobacillic acid as growth continued into the stationary phase. The percentage of lactobacillic acid in cells grown at pH 8.0 went through a minimum during late logarithmic growth, and showed a much less dramatic increase in cyclopropane fatty acid with growth than did the other two cultures. At all phases of growth, however, the acidic pH enhanced lactobacillic acid production, and the basic pH repressed lactobacillic acid production as compared with the bacteria grown at neutral pH.

After the cells grown at pH 7.0 had passed into the stationary phase, they were not used in a subsequent experiment to determine whether   a sudden decrease in pH could

Figure 2

Effect of pH Change on the Lactobacillic Acid Content

of Late Exponential Phase Lactobacillus plantarum

An inoculum containing a high percentage of lactobacillic acid was introduced into fresh medium and allowed to grow for three hours. The pH was then adjusted and maintained at either pH 4.5 or pH 7.5. Samples were taken for fatty acid analyses at the times indicated in the figure.

▲ , sample of cells grown at pH 4.5

● , sample of cells grown at pH 7.5

PERCENT LACTOBACILLIC ACID / PERCENT cis-VACCENIC ACID
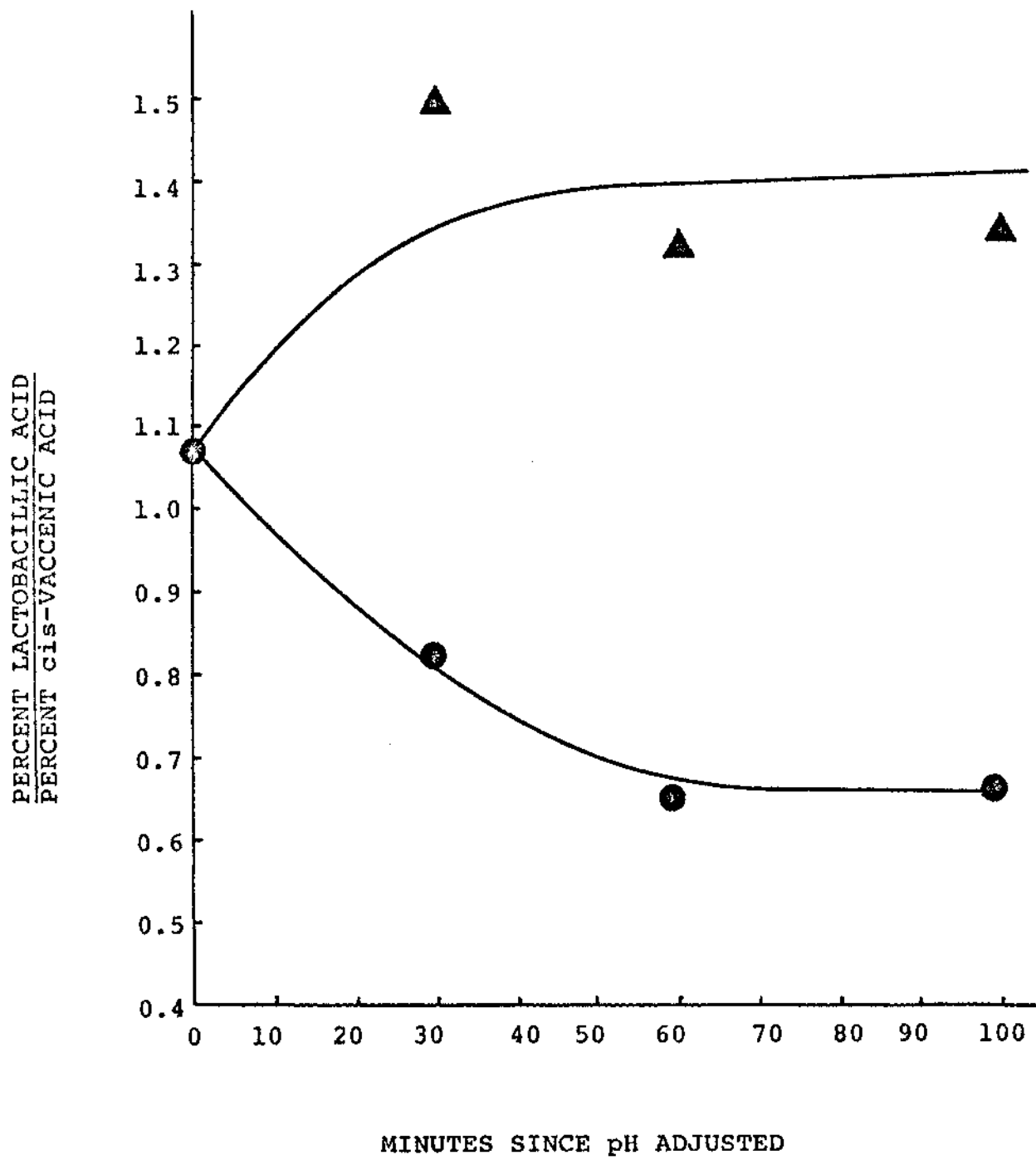
MINUTES SINCE pH ADJUSTED

Figure 3

Growth Curves of <u>Lactobacillus plantarum</u> grown at

Constant pH

Media were inoculated and growth was continued for two hours
at neutral pH. The pH of the cultures was then adjusted and
maintained at pH 4.7, 7.0, or 8.0. Samples were taken for
fatty acid analyses at the times indicated on each curve.

▲ , sample of cells grown at pH 4.7

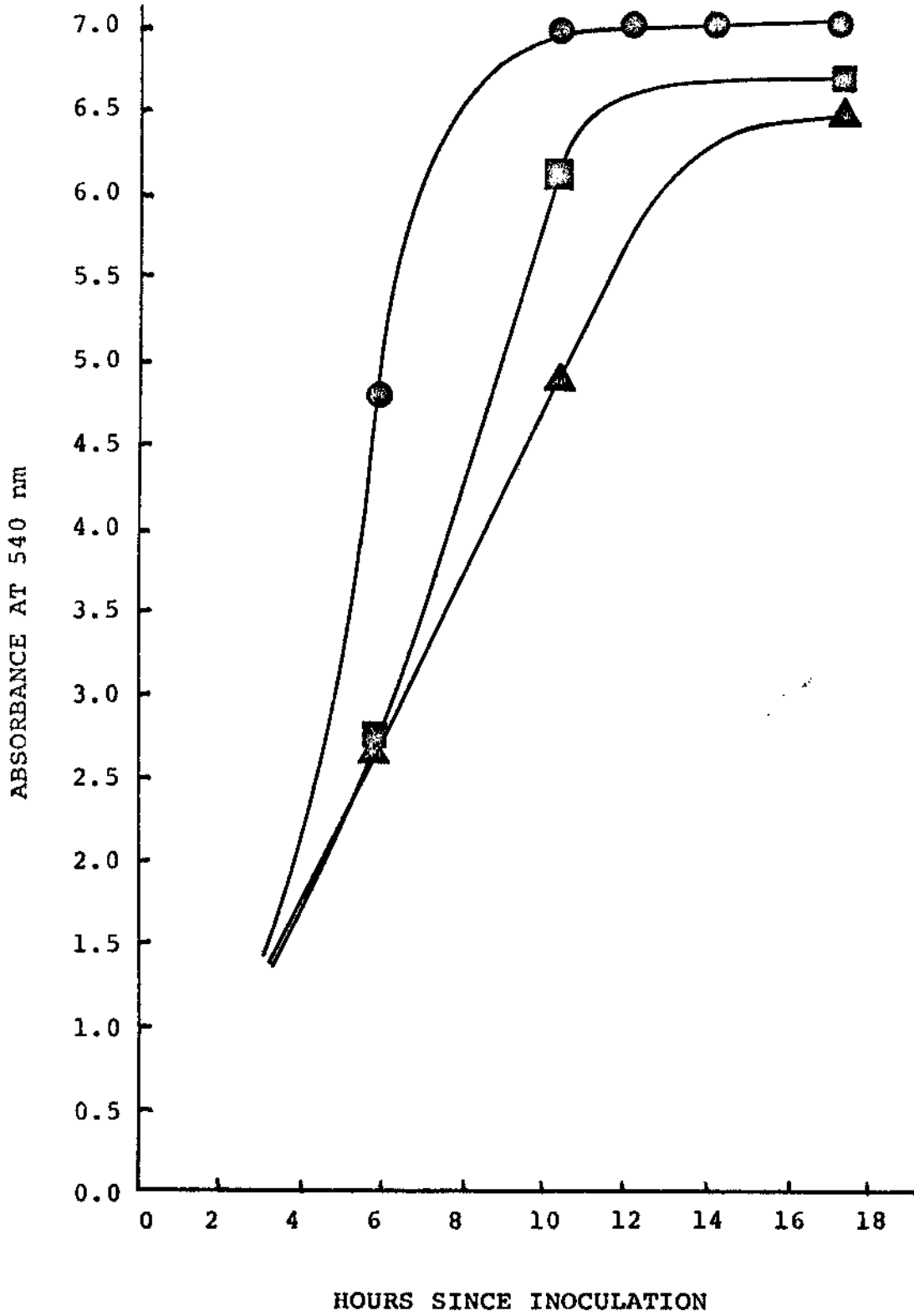● , sample of cells grown at pH 7.0

■ , sample of cells grown at pH 8.0

HOURS SINCE INOCULATION

TABLE V

EFFECT OF pH AND GROWTH PHASE*
ON LACTOBACILLIC ACID ACCUMULATION

Phase of Growth	Lactobacillic Acid Content (Percent of Total Fatty Acids)
Early Logarithmic	
pH 4.7	38.4
pH 7.0	29.7
pH 8.0	29.6
Late Logarithmic	
pH 4.7	49.2
pH 8.0	25.0
Early Stationary	
pH 7.0	33.2
Late Stationary	
pH 4.7	56.6
pH 7.0	39.7
pH 8.0	31.9

* The sampling times are indicated on the growth curves in Figure 3.

induce cyclopropane fatty acid synthesis in stationary cells. The effect of salt was also investigated to determine the generality of cyclopropane fatty acid production as a response to growth-limiting conditions.

The stationary phase cells at pH 7.0 were divided into four aliquots. The pH of one aliquot was maintained at 7.0, and two other aliquots were adjusted to pH 4.7 and 8.0, respectively. Sufficient sodium chloride was added to the fourth aliquot to reach a concentration of four percent. Preliminary experiments had shown that this salt concentration depresses, but does not totally inhibit, the growth of active cultures of L. plantarum. The pH of this aliquot was maintained at 7.0. Bacteria were collected after two hours and again after five hours of growth under the new conditions. The measured cell turbidities indicated that no appreciable lysis had occurred. The results of the fatty acid analyses are shown in Figure 4.
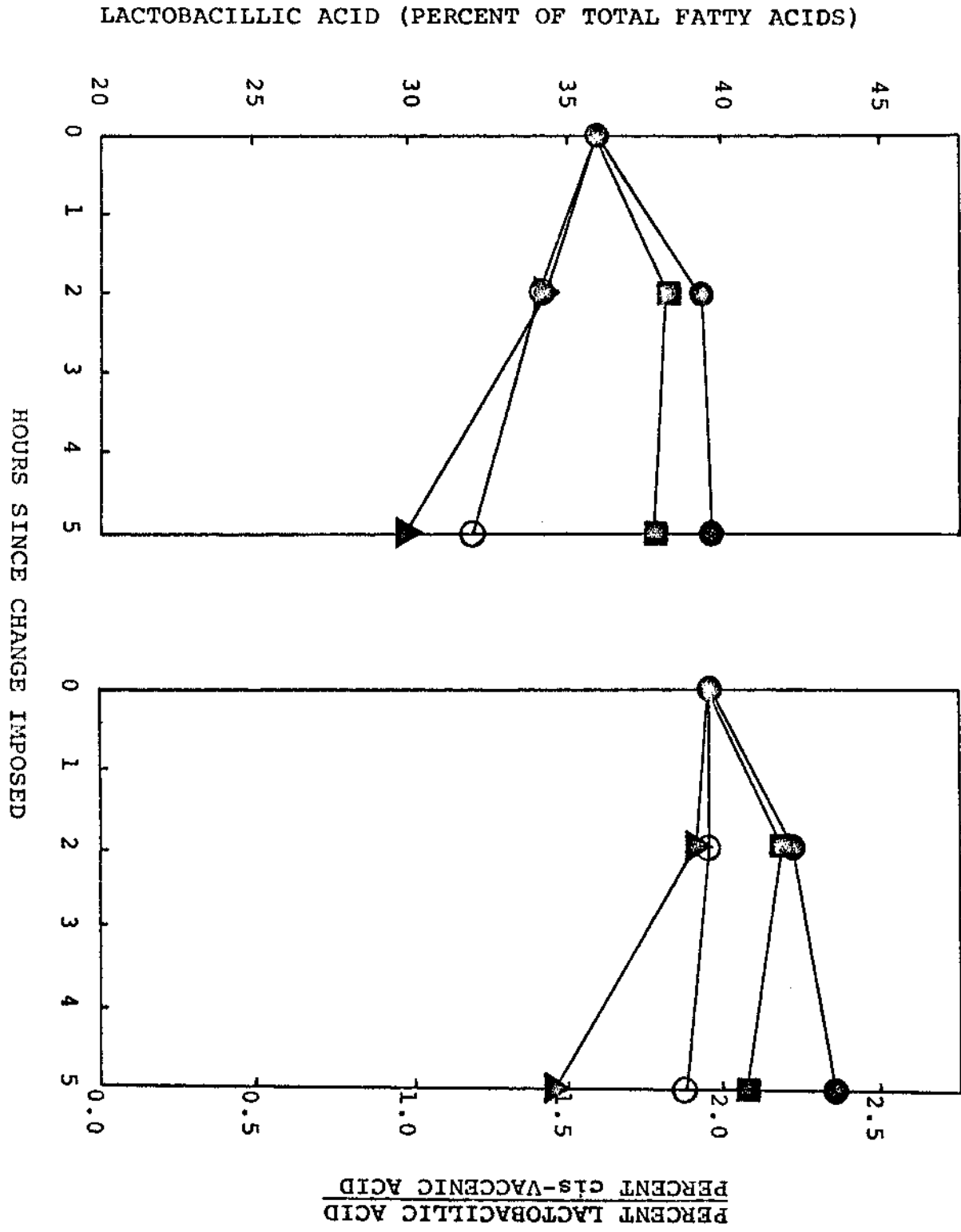
The production of cyclopropane fatty acid continued well beyond the early stationary phase, but essentially ceased during the later stationary phase. Thus the percent total lactobacillic acid increased during the first two hours of the experiment, but remained nearly constant for the next three hours in the bacteria maintained at pH 7.0. As was expected on the basis of the earlier experiments, the percent total cyclopropane fatty acid was somewhat smaller in the pH 8.0 cells as compared with the pH 7.0 cells. Sudden

Figure 4

Effect of Sudden Changes in pH or Salt Concentration

During Stationary Phase

Bacteria were grown into the stationary phase at pH 7.0.
The cells then were divided into four aliquots which were
adjusted for pH and salt concentration as indicated.
Samples were taken for fatty acid analyses at the times in-
dicated in the figure.

● , medium maintained at pH 7.0

■ , medium maintained at pH 8.0

▲ , medium maintained at pH 4.7

○ , medium maintained at pH 7.0; NaCl added to a total
concentration of 4%

LACTOBACILLIC ACID (PERCENT OF TOTAL FATTY ACIDS)

HOURS SINCE CHANGE IMPOSED

PERCENT cis-VACCENIC ACID
PERCENT LACTOBACILLIC ACID

imposition of either low pH or high salt concentration during
early stationary growth considerably reduced the percent
total cyclopropane fatty acid. These results are extremely
interesting, as there are no reports in the literature in
which lowering the pH results in a decreased cyclopropane
fatty acid content of bacteria.

At least two explanations can be given for the percent-
age decrease following sudden changes in pH or salt concentra-
tion. If the stationary phase bacteria responded to the im-
posed adverse conditions by synthesizing lipid containing
fatty acids other than lactobacillic acid, then the percent
total of the latter compound would decrease. Such sudden
metabolic activity would be unexpected, however, because the
ribosomal level of bacteria declines in the stationary phase
(69, p. 307) so that protein synthesis must be slow during
this stage of growth. In addition, the excretion of lactic
acid decreases abruptly when the bacteria enter the stationary
phase, indicating a decrease in glycolysis. Therefore,
energy-requiring metabolism must greatly decrease at this time.

The second possibility is that lactobacillic acid is
metabolized in such a way that it is lost to the method of
fatty acid analysis used in these experiments. Although
further experimentation is necessary to determine which of
several possibilities may occur, the data in Table VI indicate
that lactobacillic acid may be converted to cis-vaccenic
acid when pH 7 stationary cells undergo rapid changes in pH

TABLE VI

EFFECT OF pH OR SALT DURING STATIONARY GROWTH

Time Since Change in Salt Concentration or pH Imposed*	Lactobacillic Acid Content (Percent of Total Fatty Acids)	% Lactobacillic Acid/% cis-Vaccenic Acid	% Lactobacillic Acid + % cis-Vaccenic Acid
pH 7.0:zero hours	36.0	1.96	54.4
pH 7.0:two hours	39.3	2.23	56.9
pH 8.0:two hours	38.3	2.19	55.8
pH 4.7:two hours	34.4	1.93	52.2
pH 7.0+NaCl: two hours	34.2	1.90	52.2
pH 7.0:five hours	39.7	2.34	56.7
pH 8.0:five hours	37.9	2.13	55.7
pH 4.7:five hours	29.7	1.47	49.9
pH 7.0+NaCl: five hours	32.0	1.88	49.0
Bacteria Grown Into Late Stationary At Constant pH:			
pH 7.0	39.7	2.34	----
pH 8.0	31.9	1.84	----
pH 4.7	56.6	10.5	----

*Bacteria were grown into the stationary phase at pH 7.0 before changes in pH or salt concentration were imposed.

or salt concentration. The total percentage of lactobacillic acid plus cis-vaccenic acid decreased in bacteria undergoing any of the three treatments. These decreases may indicate preferential catabolism of these two fatty acids. In addition, both the percent total lactobacillic acid and the ratio of lactobacillic acid to cis-vaccenic acid decreased as compared with the cells maintained at pH 7.0.

The unanticipated lowering of lactobacillic acid content is not solely a result of late stationary metabolism. Data for bacteria grown into the late stationary phase with the pH maintained at 7.0 throughout growth indicate no decrease in lactobacillic acid content. Rather, the percentage lactobacillic acid continues to increase to a maximum at the late stationary phase in those cells. Therefore, the observed decreases in cyclopropane fatty acid must result from the sudden changes in pH or salt concentration imposed during the stationary phase. The fate of lactobacillic acid under these experimental conditions is of great interest as these results seem to be the first indication of metabolic turnover of the cyclopropane fatty acids in bacterial membranes.

Film Balance Studies

Because dramatic increases in the lactobacillic acid content are found when L. plantarum is cultured at low pH, the suggestion may be made that the presence of lactobacillic acid affects membrane permeability to hydrogen ions. The

formation of cyclopropane fatty acid may be a mechanism to maintain cell viability under high acidity conditions. Increased permeability to hydrogen ions may be reflected in expanded force-area curves on low pH substrates. Bacterial lipid produced at low pH then could be expected to exhibit monolayer characteristics different from those of bacterial lipid produced at neutral pH.

L. plantarum was cultured into the stationary phase at pH 7.0 and 4.7. The fatty acid spectrum for each culture is shown in Table VII. Film pressure-area curves of each lipid extract were made on water which was 0.1M in NaCl at both pH 7.0 and 4.5. There were no detectable differences in the curves obtained over the temperature range of 20-22°C. The data are shown in Figure 5.

The striking similarity among all four curves was unanticipated. The ionization states of the various polar groups of the membrane lipid most probably vary with subphase pH. Therefore, different molecular packing at the two experimental pH values would be expected. There are at least three factors to be considered in examining these data. It is possible that these factors interact such that similar force-area curves are produced under the experimental conditions.

First, different proportions of the various lipids may be produced depending upon the pH of the growth medium. Changes in lipid composition in response to changes in pH have been

TABLE VII

FATTY ACID SPECTRA OF MONOLAYER LIPIDS

Fatty Acid	Percent of Total Fatty Acids in Lipid from Bacteria Grown at pH 7.0	Percent of Total Fatty Acids in Lipid from Bacteria Grown at pH 4.7
Palmitic	30.6	32.3
Palmitoleic	6.0	5.1
Stearic	4.4	2.5
cis-Vaccenic	18.3	6.9
Lactobacillic	40.6	53.2

Figure 5

Film Pressure-Area Curves of Monolayers of Lipid Extracted

From Bacteria Grown at pH 4.7 or 7.0

The subphase was water adjusted to 0.1M in NaCl at 21°C.
Each curve represents an average of three runs.

⬤ , lipid grown pH 7.0:subphase pH 7.0

▲ , lipid grown pH 4.7:subphase pH 4.5

◯ , lipid grown pH 7.0:subphase pH 4.5

△ , lipid grown pH 4.7:subphase pH 7.0
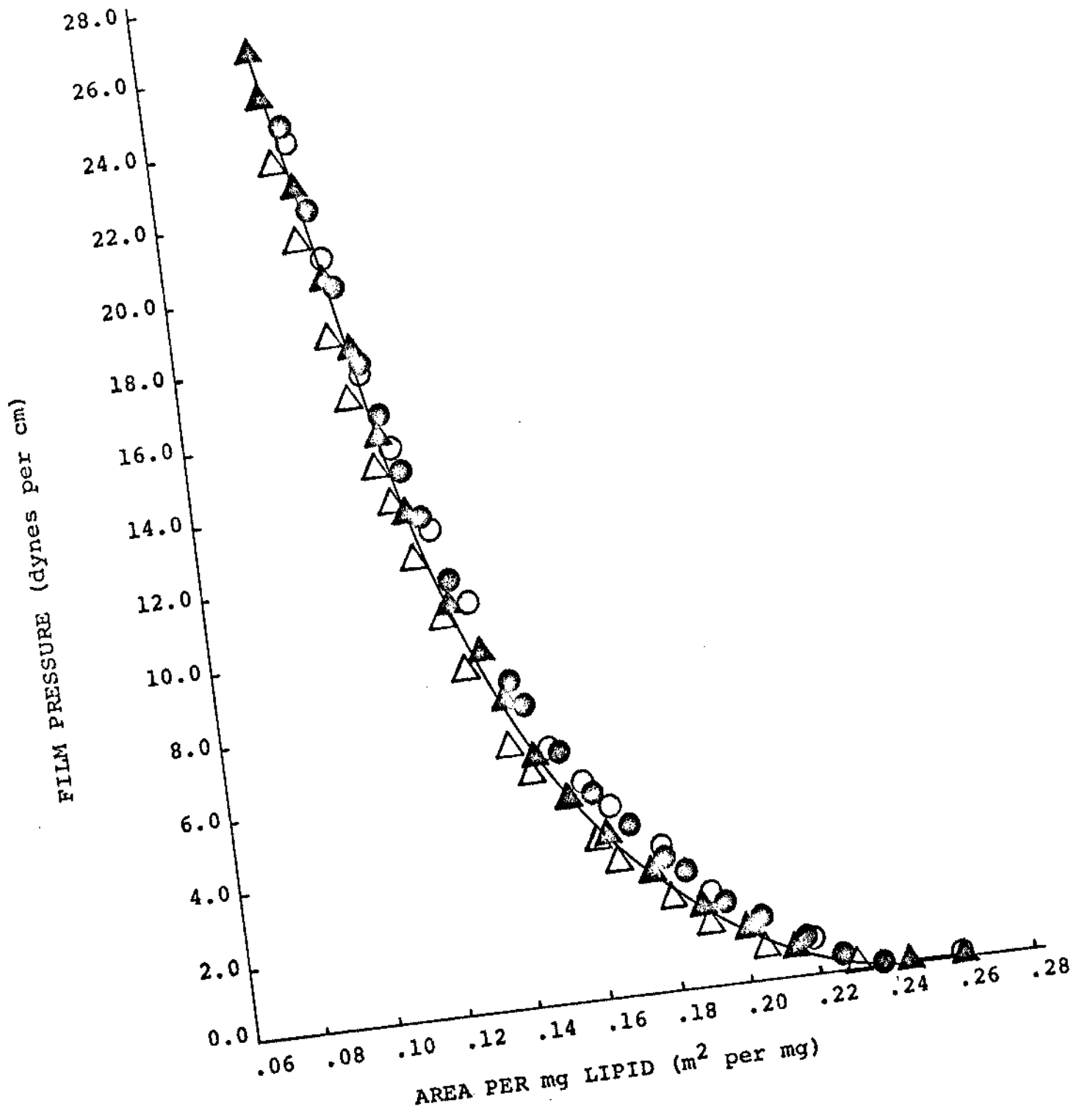
reported in several bacteria (78, 79). Second, the effect of NaCl on the force-area curves of the bacterial lipids is not known. The extract contains a mixture of lipids, and these compounds may exhibit different degrees of cation interaction at the different values of pH investigated.

Third, there may be different monolayer characteristics at the compression temperature (21°C) and the bacterial growth temperature (35°C). It has been reported that the liquid crystalline transition temperatures of stearoyl and palmitoyl 1,2-diacyl phosphatidylcholines dispersed in water are 58°C and 41°C, respectively (59). These transition temperatures are lowered when unsaturated lecithins are added. Thus, the temperature of phase transition at least partly depends upon the fatty acids present. Because the fatty acid species present within each class of extracted lipid are not known, the behavior of the monolayers at 35°C (or other temperatures) cannot be predicted from the data obtained at 21°C. Further experiments are necessary to determine the packing characteristics of the lipid extracts at the growth temperature.

CHAPTER IV

CONCLUSION

This investigation answered several questions concerning the production of lactobacillic acid in L. plantarum. These experiments represent the first study of the effect of oxygen on the lactobacillic acid content of bacteria in which glycolysis is the main energy-producing pathway. It was found that the major function of cyclopropane fatty acid most probably is not that of a lipid antioxidant. Although oxygen does not affect lactobacillic acid production, the stage of growth and the pH of the growth medium have major effects.

The separate effects of the stage of growth and pH were studied further. It was found that cyclopropane fatty acid content in L. plantarum increases in the later stages of bacterial growth, in agreement with reports by previous workers with this and other bacteria. Lactobacillic acid content decreases with basic pH and increases with acidic pH at all stages of growth into the early stationary phase. Exponentially growing cells respond to increases or decreases in acidity by accumulating more or less lactobacillic acid, respectively. However, cells in the late stationary phase respond very differently. In these bacteria, the percent total lactobacillic acid actually decreases with a decrease

in pH. This study is the first report of such a phenomenon, and clearly indicates the need for further work in this area.

Monolayer studies were conducted to determine whether or not the presence of lactobacillic acid affects membrane permeability to hydrogen ions. Film balance studies at temperatures below the growth temperature indicated no significant differences in monolayer behavior between lipid extracts of L. plantarum grown at low and neutral pH. However, other workers have shown major temperature effects on the packing behavior of certain lipids. It is still not known whether or not lactobacillic acid functions in membrane permeability at the temperature of bacterial growth.

The present study indicates that membrane cyclopropane fatty acids quite probably have an important role in the life cycle of L. plantarum. This conclusion is based upon the rather sudden enhancement of synthesis or apparent degradation of this lipid species, elicited by changes in chemical or physical environment.

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