

CANADIAN THESES ON MICROFICHE

I.S.B.N.

THESES CANADIENNES SUR MICROFICHE



National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

MULTINUCLEAR NMR STUDIES OF THE YEAST-LIKE
FUNGUS, AUREOBASIDIUM PULLULANS

by

Barbara A. Blackwell
MSc., Toronto

Thesis submitted to the School of Graduate
Studies, University of Ottawa in partial
fulfillment of the requirements for the
Degree of Doctor of Philosophy in Chemistry

Ottawa, Ontario
February, 1982



Barbara A. Blackwell, Ottawa, Canada, 1982.



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

A multinuclear approach has been taken to the Nuclear Magnetic Resonance Study of a complex Polymorph, Aureobasidium pullulans, in order to determine the advantages and limitations of using NMR to elucidate problems of metabolism and membrane structure in vivo. The organism has been characterized by using proton, carbon-13 and deuterium NMR to look at the lipid components of the cell, high power phosphorus NMR to look at the phospholipid component, and high resolution phosphorus NMR plus ^{13}C NMR to look at metabolism in vivo under various perturbing conditions.

The lipid component of the organism has been labelled with carbon-13 and deuterium biosynthetically, using labelled acetate as the precursor, thus allowing the determination of the quadrupole splitting pattern of the perdeuterated lipid chain and the ^{13}C relaxation times of the individual carbon atoms in the lipid chain. The results for the organism in vivo are compared with two types of crude membrane preparations from the labelled organism. This study shows that the average membrane component of the organism is highly fluid in nature in spite of its protein component, and that the motion of lipid molecules in the intact membrane compares well with a model system of lipid vesicles in water. The average lipid of the organism is much more highly unsaturated than those

previously studied by NMR (i.e., Acholeplasma laidlawii or E. coli), since approximately 80% of the fatty acid chains are C-18:1 and C-18:2, as befits an organism that is more highly evolved and complex.

Since the yeast has a highly rigid polysaccharide wall surrounding it, the function of the plasma membrane, as well as the other internal membranes, is to provide a physical (in the case of plasma and nuclear membranes) or osmotic (as in case of plasma membrane) barrier, rather than maintaining the integrity and shape of the cell. Phosphorus NMR of the phospholipid component shows the natural phospholipid mixture to be predominantly in the bilayer form when isolated and to be remarkably stable with respect to temperature. In a total lipid mixture, the phospholipid adopts a bilayer form plus a substantial isotropic component, as has been observed for other natural lipid systems.

When the organism was subjected to a condition of lack of carbon in the medium, the ^{13}C label which was stored as triglyceride in the cell can be followed by ^{13}C NMR to result in labelled amino acids, but not glucose. It appears that the acetate label is taken up and used exclusively to synthesize lipids and amino acids, as precursors to protein, through the TCA cycle. The organism utilizes its storage triglyceride to maintain lipid and to build up the amino acid pool, but the

polysaccharides and sugars appear to be formed purely from the glucose storage pool that is also maintained. Glucose and trehalose appear to be preferred storage forms over glycogen.

Phosphorus NMR studies were undertaken to look at the phosphorus metabolism of A. pullulans under this "starvation" condition, as well as under other conditions leading to cell death, in order to determine the role of the third major storage form in the cell, the polyphosphate. Intracellular pH and metabolite levels were followed by NMR as a function of time and correlated to viability curves. Polyphosphate does not appear to be required for cell viability, but is maintained under conditions of abundant nutrients. It forms a storage pool for ions and phosphate to be used under conditions of stress. The appearance of pyrophosphate, as an evolutionarily primitive form of phosphate bond energy, appears to be correlated with the disappearance of ATP as the culture begins to lose viability. The effect of aeration under the above conditions was also observed.

The picture that emerges is one of a rather primitive eukaryote, whose viability under all sorts of deleterious conditions results from flexibility in biochemistry and in morphology. The viability of the organism under conditions that result in cell death for other species of yeast result from

an ability to utilize older forms of energy storage, such as pyrophosphate and polyphosphate, to store large amounts of polyphosphate, acylglycerol and glucose during periods of abundant nutrition and to regulate the use of these storage forms, as well as to change morphology and biochemistry to suit the external environment. The membrane structure of the organism appears highly fluid in comparison to those previously studied by NMR, but reflects the lipid structure. The close correlation between in vivo cell and model systems show that the results of study of the lipid components of a membrane may indeed reflect the behaviour of the "operating" membrane.

TABLE OF CONTENTS

Chapter		Page
	ABSTRACT	ii
	TABLE OF CONTENTS	vi
	ACKNOWLEDGEMENTS	ix
	LIST OF ABBREVIATIONS	xi
	LIST OF PLATES	xiv
	LIST OF TABLES	xvi
	LIST OF FIGURES	xviii
I	INTRODUCTION	1
II	MORPHOLOGY	15
III	BASIC EXPERIMENTAL TECHNIQUES	34
	Growth Conditions	34
	'Starvation' Conditions	36
	Lipid Staining	36
	Viability Curves	37
	Membrane Preparations	39
	(a) Enzymatic Preparation	39
	(b) Lysis by Nystatin Method	44
	Lipid Extractions	46
	Preparation of Purified Phospholipid Fraction	49
	Precipitation of Pullulan	49
	Analyses	50
	(a) For Fatty Acids	50
	(b) For Amino Acids	50
	Perchloric Acid Extracts	51
	NMR Spectra	52

Chapter		Page
IV	CHARACTERIZATION OF THE ORGANISM	57
	Growth Characteristics	57
	Incorporation of the ^{13}C and ^2H Labels	58
	Optimization of Label Incorporation	67
	Classification of Lipid Content of <u>A. pullulans</u>	69
	(a) Acyl Chain Content	70
	(b) Lipid Classes	73
	(c) Phospholipid Headgroup Analysis	78
	Effect of Carbon Sources	82
V	^{13}C STUDIES OF MEMBRANE STRUCTURE	88
	Introduction	88
	Theory of ^{13}C Relaxation Times	99
	Results	111
	T_1 Profile of Fatty Acid Chains	138
	Membrane Preparations - Comparison to Intact Cell Profile	145
	(a) Starved Cells	145
	(b) Enzyme-Prepared Membrane Preparation	146
	(c) Nystatin Membrane Preparation	151
	^1H NMR of <u>A. pullulans</u>	165
	Discussion	168
	Appendix to Chapter V	176
VI	^2H AND ^{31}P OF <u>A. PULLULANS LIPIDS</u>	182
	(a) ^2H of <u>A. pullulans Lipids</u>	184
	(i) ^2H Experimental Parameters	185
	(ii) ^2H Results	187
	(b) ^{31}P NMR of <u>A. pullulans Lipids</u>	193
	(i) ^{31}P Experimental Parameters	196
	(ii) ^{31}P Results	197
	(iii) Discussion	211

Chapter		Page
VII	METABOLIC RESULTS FROM ^{13}C NMR	217
	Introduction	217
	Experimental Conditions	224
	(a) Acetate Utilization During Growth	225
	(b) Path of Label During Starvation	243
	(c) Metabolic Pathways from Labelled Acetate	255
	(d) Amino Acid Analysis	266
	(e) Pullulan	268
	Discussion	283
VIII	METABOLIC STUDIES BY ^{31}P NMR	299
	Introduction	299
	Experimental Conditions	309
	Results	311
	(a) Effect of Aerobicity	311
	(b) Effect of Starvation	335
	(c) Effect of Nystatin	348
	(d) Effect of Methyl Mercury	367
	(e) Effect of Magnesium (Mg^{2+})	380
	Discussion	385
IX	ADDITIONAL EXPERIMENTS AND CONCLUSIONS	402
	(a) ^{199}Hg NMR of Methyl Mercury Chloride (MeHgCl)	402
	(b) The Fate of ^{13}C -l Galactose in Cell Suspensions of <u>A. pullulans</u>	406
	(c) Growth of <u>A. pullulans</u> on Inositol	409
	SUMMARY	416
	REFERENCES	421

ACKNOWLEDGEMENTS

For the following pages, I am indebted to Dr. I.C.P. Smith, who not only gave me advice and scientific stimulation, but also provided a multidisciplinary laboratory with a distinctively international flavour. I enjoyed the opportunity to interact with scientists from different parts of the world, who presented new points of view to the problems of science and of life in general. The variation in fields of expertise within the laboratory made it easy to find someone to help with any aspect of a scientific problem. I am grateful to Dr. K. Butler, Dr. A. Byrd, Dr. H. Jarrell and Dr. M. Rance for enlightenment of subjects biochemical, magnetic, chemical and theoretical respectively. For sharing the problems (and their solutions) of being a graduate student, I thank fellow students Dr. M. Taylor, Dr. H. Casal, Dr. Y. Boulanger, Eric Kelusky and Erick Dufourc. Special credit is also due to Jan Johns for her friendship and encouragement.

It is also a pleasure to acknowledge the aid of many of the scientists and support staff of the Division of Biology in solving my scientific problems. Specifically, appreciation is extended to Dr. M. Yaguchi for the amino acid analyses, Dr. S. Martin for culturing the fermentor growth of A. pullulans, Dr. J. Phipps for the methyl mercury viability

curves, and Mr. F. Cooper for the GCMS analyses. I also thank Harry Turner for the photographic plates and for helpful suggestions for the visual presentation of scientific data.

I would like to specifically thank Anne Joyce and Dr. Roxanne Deslauriers, of this laboratory, for their friendship and advice. Roxanne provided for me a model of patience, perseverance and self-discipline. Anne has taught me the technique of microbiology, and specifically has performed many of the fatty acid analyses and the isotopic optimization study of A. pullulans. Her skills at calligraphy are evident in several of the diagrams of this thesis.

Finally, I would like to thank Mrs. Yvonne Rowe for her patient and skillful typing of this thesis, and the National Research Council and Government of Ontario for financial support during the course of this work.

LIST OF ABBREVIATIONS

A. pullulans	Aureobasidium pullulans (Pullularia pullulans)
ACP	Acyl carrier protein
ADP	Adenosine diphosphate
ALA	Alanine
ARG	Arginine
ASN	Asparagine
ASP	Aspartic acid
ATP	Adenosine triphosphate
C-16:0	Ester of hexadecanoic (palmitic) acid
C-18:0	Ester of octadecanoic (stearic) acid
C-18:1	Ester of cis-9-octadecenoic (oleic) acid
C-18:2	Ester of cis-9,12-octadecadienoate (linoleic) acid
Card	Cardiolipin
CIT	Citrulline
CoA	Coenzyme A
CSA	Chemical shift anisotropy
CYS	Cysteine
DMPC	1,2 dimyristoyl-3-sn-phosphatidylcholine
DPPC	1,2 dipalmitoyl-3-sn-phosphatidylcholine
E _a	Activation energy
EDTA	Disodium ethylene diamine tetraacetate
EM	Electron microscopy
F6P	Fructose-6-phosphate
FDP	Fructose diphosphate
G3P	Glycero-3-phosphate
G6P	Glucose-6-phosphate
GCMS	Gas chromatography - mass spectroscopy
GLC	Glucose
GLN	Glutamine
GLU	Glutamate
GLY	Glycine

GPC	Glycero-3-phosphatidylcholine
GPE	Glycero-3-phosphatidylethanolamine
GPI	Glycero-3-phosphatidylinositol
GPS	Glycero-3-phosphatidylserine
HIS	Histidine
ILE	Isoleucine
LEU	Leucine
LYS	Lysine
lyso PC	Glycero-3-phosphatidylcholine, monoalkyl ester
MDG	Mono and diglycerides
MET	Methionine
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NP	Nucleotide phosphate
ORN	Ornithine
P	Inorganic phosphate, orthophosphate
P _n	Polyphosphate
PP	Pyrophosphate
P _{n-1} ^P	Terminal phosphate of polyphosphate
P _{n-x} ^{P_x}	Penultimate phosphates of polyphosphate
PA	Glycero-3-phosphatidic acid, dialkyl ester
PC	Glycero-3-phosphatidylcholine, dialkyl ester
PE	Glycero-3-phosphatidylethanolamine, dialkyl ester
PHE	Phenylalanine
PI	Glycero-3-phosphatidylinositol, dialkyl ester
PL	Phospholipid
PRO	Proline
PRPP	Phosphoribosyl pyrophosphate
PPM	Parts per million
PS	Glycero-3-phosphatidylserine, dialkyl ester
r _{CH}	C-H bond length
SE	Sterol ester
SER	Serine

Sph	Sphingomyelin
τ_c	Correlation time
T	Tesla
T_1	Spin lattice relaxation time
T_2	Spin-spin relaxation time
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle, Krebs's acid cycle
TG	Triglyceride
THR	Threonine
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TRE	Trehalose
trilinolein	tri (cis-9,12-octadecadienoate) glycerol
triolein	tri (is-9-octadecenoate) glycerol
TRP	Tryptophan
TYR	Tyrosine
UDPG	Uridine diphosphate glucose

LIST OF PLATES

- Plate I Fresh culture, three days old, magnified x 914, showing granular nature of the active yeast phase
- Plate II Magnification x 2 of Plate I
- Plate III Septate, 'swollen' cell. The size is approximately twice that of the yeast cell
- Plate IV An example of the mycelial phase of A. pullulans. The germ tube originated from the large cell at the top of the plate. This germ tube will later differentiate into a septate mycelium which will become more branched
- Plate V A. pullulans yeast phase grown on deuterated acetate. The cells are smaller and more uniform than in regular cultures but the cytoplasm is still granular
- Plate VI A. pullulans yeast phase grown on inositol as the sole carbon source. Cells are smaller and uniform in size. Many appear dominated by a single vacuole, and there is usually only one nucleus per cell
- Plate VII Cells of A. pullulans after three days in a medium lacking in carbon (starved cells). Cells continue to bud, but cytoplasm is less granular and cells are dominated by one or two vacuoles
- Plate VIII A. pullulans, after treatment with nystatin. The dark cells are lysed and show ragged edges at the pole. The halo produced by the phase contrast is not as pronounced as in healthy cells, indicating they may be somewhat flattened. Vacuoles are clear
- Plate IX Cells after nystatin treatment showing lysing effect of nystatin. The swollen cell at the left shows that it has lost the ability to maintain its shape

Plate X

Membrane preparation of A. pullulans made from snail gut enzyme, magnified x 914. Although there is a lot of cellular debris, the membranes are about half the size of the original cells, and are free of cytoplasmic contents

LIST OF TABLES

Table III.1	90° pulse lengths as measured on standard samples
Table IV.1	Extent of deuteration in fatty acid from <u>A. pullulans</u> supplemented with D ₂ O/H ₂ O (1:1,v/v) plus 0.2% methyl d ₃ -acetate
Table IV.2	Optimization of growth and incorporation of labelled substrate
Table IV.3	Fatty acid distribution of total cell lipids
Table IV.4	Lipid class distribution of <u>A. pullulans</u>
Table IV.5	Phospholipid composition of <u>A. pullulans</u>
Table V.1	Chemical shifts and assignments for labelled lipid peaks in cells
Table V.2	Summary of T ₁ values at 20 MHz
Table V.3	Summary of T ₁ values at 75 MHz
Table V.4	Summary of linewidths in lipid spectra (Hz)
Table V.5	Summary of ¹³ C NOE values
Table V.6	Data for temperature dependence of T ₁
Table VII.1	¹³ C chemical shifts of glucose and related compounds

Table VII.2	^{13}C chemical shifts of some common amino acids
Table VII.3	Peak assignment of labelled whole cells (75 MHz)
Table VII.4	^{13}C assignments of spectra of supernatant from labelled nystatin membrane preparations
Table VII.5	Assignment of some peaks of aqueous portion of Bligh-Dyer extract, pH=6.6
Table VII.6	Assignment of ^{13}C resonances of starved cells
Table VII.7	Predicted appearance of ^{13}C label in the amino acid pool from acetate incorporation via various metabolic pathways
Table VII.8	Amino acid analysis of starved cells
Table VII.9	pH behaviour of peaks observed in pullulan spectra
Table VII.10	Analysis of acid hydrolysate of isolated pullulan

LIST OF FIGURES

- Figure II.1 The relationship between different morphological forms in the life cycle of A. pullulans (adapted from Ramos and Garcia-Acha, 1975(a))
- Figure IV.1 Growth curve for A. pullulans in shaken culture at 26°C
- Figure IV.2 Diagram of the biosynthetic pathway of acetate-derived deuterium into fatty acid chains of lipid, through the ACP mechanism
- Figure V.1 Biosynthetic pathway for acetate incorporation into lipid via fatty acid chain elongation. The dot and asterisk mark the metabolic fate of ^{13}C -2 acetate and ^{13}C -1 acetate respectively
- Figure V.2 ^{13}C NMR spectrum (20 MHz) of packed whole cells of A. pullulans in D_2O at 30°C. The cells were grown on media enriched in ^{13}C -1 sodium acetate
- Figure V.3 Same as Figure V.2 for cells supplemented with ^{13}C -2 sodium acetate
- Figure V.4 20 MHz natural abundance ^{13}C NMR spectrum of whole packed cells at 30°C. The central resonances due to storage glucose represent the same concentrations as observed in Figures V.2 and V.3, and the lipid resonances represent the sum of resonances observed in these two previous figures. The spectrum represents 9000 accumulations

Figure V.5

75 MHz ^{13}C spectrum of packed whole cells from a 50 ml culture labelled with ^{13}C -1 acetate. This represents a typical infinity spectrum in a T_1 measurement. The spectrum is a result of 200 accumulations of 8K data points, using a 90° pulse and 4 sec recycle time

Figure V.6

Same as Figure V.5 for cells labelled with ^{13}C -2 acetate. Comparison of the intensities of labelled to unlabelled peaks between this and the previous spectrum allow an estimation of the level of acetate incorporation

Figure V.7

A typical inversion recovery sequence for whole cells labelled with ^{13}C -2 acetate. The 90° pulse was 17 μsec ; the recycle time 5 sec. Each spectrum is a result of 400 transients at 75 MHz

Figure V.8

Plot of NT_1 values vs carbon number assuming a model of a C-18:2 acyl chain for whole cells ($\square$ — $\square$), extracted total lipids sonicated in D_2O ($\square$ ---- $\square$), and extracted total lipid in chloroform ($\blacksquare$ — $\blacksquare$). The T_1 values were determined at 20 MHz and represent an average of 8 experiments. The average deviation between the different cultures used for these measurements is generally within the size of the symbols used in the plots

Figure V.9

Variation of (a) NOE and (b) relaxation time with correlation time assuming overall

isotropic motion. The calculations show the effect of field strength for ^{13}C nuclei resonating at 75 MHz and 20 MHz. Only for slow motions beyond the T_1 minimum is T_1 (solid line) unequal to T_2 (broken line)

Figure V.10

Effect of correlation time on linewidth (calculated from T_2) at two magnetic fields assuming isotropic reorientation

Figure V.11

NT_1 profile for whole cells measured at 20 MHz ($\square$) and 75 MHz (Δ) using models representing the dominant acyl chain species: C-18:1 (broken lines) and C-18:2 (solid line). The two models can be resolved in some regions of the acyl chain due to appearance of resonances that are due to only one chain species

Figure V.12

NOE profile as a function of carbon number for whole cells at 75 MHz for both acyl chain models, C-18:1 (broken line) and C-18:2 (solid line)

Figure V.13

20 MHz ^{13}C NMR spectra of pure triglycerides, (a) triolein and (b) trilinolein in CDCl_3 . Assignments of these spectra aided in the assignment of lipid resonances in whole cells

Figure V.14

^{13}C NT_1 values at 20 MHz for pure triglycerides in CDCl_3 . Results are listed as a function of carbon number for triolein

and trilinolein as pure solutions. The figures in brackets were measured for a 2:1 mixture of the two triglycerides. Only positions C-9, C-12-15, and C-16 of the triolein component can be resolved in the mixture

Figure V.15

Plot of data in Figure V.14 compared to total extracted lipid in chloroform (■) in order to determine the profile across the double bond region. The plots are identified as follows: trilinolein (□-----□), triolein (□- · -□), total lipid as per C-18:1 chain (■- · -■) and C-18:2 chain (■————■)

Figure V.16

75 MHz ^{13}C NMR spectrum of enzyme membrane preparation of cells from a 500 ml culture supplemented with ^{13}C -1 acetate. The spectrum is a result of 800 accumulations of 8K data points using a 90° pulse. Note the absence of resonances due to internal small molecules

Figure V.17

75 MHz ^{13}C NMR spectrum of "floating lipid" isolated during preparation of the membranes shown in the previous figure. The chemical shifts of the carbonyl and glycerol resonances confirm that this is triglycerides from cellular storage pool. The predominant peaks are due to unlabelled sorbitol used as an osmotic stabilizer in the membrane preparation. The spectrum is a result of 2000 transients of 16K data points using a 45° pulse

Figure V.18

75 MHz ^{13}C NMR spectra of cells from 100 ml cultures supplemented with both specific labels, after lysis with nystatin. The resonances are assigned according to Table V.1, showing those resonances which can be assigned to individual chain species. Note absence of peaks due to glucose indicating loss of cytoplasmic contents

Figure V.19 (a)-(b) Temperature behaviour of T_1 's of nystatin membrane preparation, observed at three temperatures. Linear plots result from those resonances assignable to specific positions

Figure V.20 (a)-(d) NT_1 vs carbon number profiles plotted for models C-18:1 and C-18:2 at both 75 MHz ($\blacktriangle$) and 20 MHz ($\blacksquare$) for whole cells (—), enzyme membrane preparation (---) and nystatin membrane preparation (.....). The data points are averages of several experiments

Figure V.21

100 MHz ^1H NMR spectrum of a) whole cells, and b) extracted lipid vesicles in D_2O at 30°C

Figure V.22

^{13}C NT_1 values of the 18:1 chain of the lipids of A. pullulans cells at 30°C and in the 16:0 chains of dipalmitoyl lecithin vesicles at 52°C (Lee et al., 1976). Where no points are given for a particular carbon, this is due to lack of resolution of individual

resonances; in this case an average value is given, as for C-4 to C-13 of dipalmitoyl lecithin and C-4 to C-7 of the 18:1 chains

Figure VI.1

46 MHz ^2H NMR spectra of 45 mg/ml of extracted lipid from cells grown in 50% D_2O and d_3 -acetate and the debris after extraction, in deuterium depleted water. Spectra (C) show the large isotropic component with some quadrupole pattern in the base of the peak. The spectra represent 56000 and 36000 accumulations respectively, using a quadrupolar echo pulse sequence where the pulse spacing was 60 μs , quadrature detection on 2K data points, 125 KHz spectral window and 0.5 s recycle time. Spectra (A) are a result of subtracting an isotropic component of 150 Hz linewidth (the linewidth of the water line), (spectra (B)) from the FID before the Fourier transform. The arrows correspond to quadrupole splittings of 38, 22, 16, 8, and 1 KHz for the lipid spectrum and 40, 17, 9 and 1.3 KHz for the debris spectrum

Figure VI.2

121 MHz high resolution ^{31}P spectrum of purified phospholipid mixture from A. pullulans in CHCl_3 . The major phospholipid head groups in their correct proportions can be identified from the spectrum

Figure VI.3

121 MHz ^{13}P spectra of 100 mg/ml purified phospholipid liposomes as a function of

temperature. These spectra, representing 2500 accumulations, employed a 90° pulse, 1 s recycle time, 50 KHz spectral window and high power gated decoupling during the acquisition. Each spectrum is a composite of a powder pattern plus an isotropic component. Above 27°C , the system is stable to any further increase in temperature

Figure VI.4

Plot of the chemical shift anisotropy as measured from the spectra of Figure VI.3 as a function of temperature. The powder pattern decreases in width from 5°C to 25°C ; at higher temperatures there is no change

Figure VI.5

High power ^{31}P and ^2H spectra of the sample of deuterated lipid (45 mg/ml) presented in Figure VI.1 at 30°C . Of the total lipid sample, ~ 10 mg is phospholipid. The differences between the relative amounts of the isotropic and anisotropic components is due to parameters involved in determining the spectra. The relatively narrow CSA pattern (37 ppm) and the small quadrupolar splittings are consistent with the highly fluid nature of the lipid extract. The ^{31}P spectrum was accumulated using gated decoupling to avoid sample heating, 90° pulses, 0.8 s recycle time, and 6000 transients

Figure VI.6

A DANTE experiment performed on the ^{31}P spectrum of total lipid extracted from deuterated A. pullulans, in order to determine

the shape of the powder pattern without the isotropic component. Spectrum (C) is the same as in the previous figure. The powder pattern was irradiated at the low field side with a series of 4000 soft pulses (0.5 μ s duration, 50 μ s spacing, followed by a delay time of 1 ms to allow for ringdown of the saturation pulse train, and then by a normal 90° pulse and acquisition. The resultant spectrum (B) is essentially only the isotropic component. The difference spectrum (A) is the anisotropic component only, and has the same shape as the powder pattern for purified phospholipid at 30°C

• Figure VI.7

Sample spectral simulation assuming that the powder lineshape is a sum of overlapping powder patterns. This can be compared with a single powder pattern lineshape assuming component linewidths of 500 Hz

Figure VII.1

20 MHz ^{13}C spectrum of whole cells grown on ^{13}C -1 glucose. The peaks corresponding to intracellular constituents are labelled as follows: L, lipid; P, protein; C, carbohydrate; G, glucose

Figure VII.2

75 MHz ^{13}C spectrum of supernatant from nystatin membrane preparation from 100 ml culture of cells supplemented with ^{13}C -1 acetate. The total supernatant was lyophilized and resuspended in 1 ml D_2O . The spectrum is a result of 18,500 scans, using a 45° pulse and 0.5 sec recycle time

- Figure VII.3
- a) 20 MHz ^{13}C spectrum of water soluble components of the supernatant described in Figure VII.2. This sample was not derived from the same culture as the previous figure, accounting for the differences between the relative amounts of mannitol and glucose. The spectrum represents 100,000 accumulations
- b) 20 MHz ^{13}C spectrum of aqueous fraction from lipid extraction of unlabelled cells. This spectrum is a result of 100,000 accumulations on approximately 25 mg of material per ml of D_2O at pH=6.6

Figure VII.4

20 MHz ^{13}C spectrum of 100 ml culture of ^{13}C -1 acetate-derived cells after three days in starvation medium. The spectrum represents the accumulation of 27,000 transients, using a 45° pulse and a 1 sec recycle time

Figure VII.5

Same as Figure VII.4 for ^{13}C -2 acetate-derived cells

Figure VII.6

75 MHz ^{13}C spectrum of starved unlabelled cells, showing large pool of trehalose. The interplay between trehalose and free glucose as storage pools appears to be a sensitive function of the growth time. Spectrum is a result of 18,000 transients, 60° pulses, and 1 sec recycle time

Figure VII.7^{1/2}

Representation of the possible points of entry of labelled acetate, either from the medium or as a product of fatty acid oxidation, into the Krebs' acid cycle, and the resultant incorporation into those amino acids which are immediate products of the cycle

Figure VII.8

Metabolic pathways for biosynthesis of spectrally observed amino acids from α -ketoglutarate

Figure VII.9

Metabolic pathways for those amino acids which are derived from pyruvate and oxaloacetate

Figure VII.10

Structure of the two glucose oligomers commonly found in this study - pullulan and trehalose. Both contain α linkages of glucose units. Pullulan has a repeating unit of maltotriose, joined by 1,6 linkages. The symmetry of the trehalose molecule is responsible for its simple spectrum

Figure VII.11

20 MHz ^{13}C spectra of pullulan, isolated from cultures that were both unlabelled and specifically labelled. Each spectrum represents a concentration of 50 mg of isolated material per ml at pD=13. All spectra were accumulated under the same conditions of 115,000 transients, 45° pulses and 0.5 sec recycle time

Figure VIII.1

Summary of the cellular location of the constituents of phosphate metabolism, as found in filamentous fungi (N. crassa) and yeast (S. cerevisiae)

Figure VIII.2

121 MHz ^{31}P spectrum of cells of A. pullulans at 2×10^9 cells per ml a) 0.5 hours and b) 10.0 hours after harvesting. The spectra are a result of 2000 accumulations with a 45° pulse and 0.5 sec recycle time. The large peak at high field is attributable to polyphosphate. Smaller peaks that appear in the enlargement (x 10) of the spectrum are due to internal and external inorganic phosphate (P_{int} , P_{ext}), penultimate and terminal phosphate groups of polyphosphate ($\text{P}_{\text{n-1P}}$, $\text{P}_{\text{n-x}}$, P_{x}) and ATP. The broad peak downfield of inorganic phosphate is due to sugar phosphate. Spectrum (b) shows the appearance of a new peak due to pyrophosphate (PP)

Figure VIII.3

121 MHz ^{31}P NMR spectrum of a perchloric acid extract of fresh cells. The peaks are assigned on the basis of the titration curves (Figure VIII.4). The enhanced resolution allows the observation of additional peaks hidden in the spectra of Figure VIII.2, as well as the unambiguous assignment of the existing peaks. The sugar phosphate region can be resolved to show the presence of both G6P and fructose phosphates (FP). In addition to the species observed in the previous figure, the presence of ADP, UDPG, nucleotide phosphated (NP) and glycerol-3-phosphatidylcholine (GPC) are confirmed

Figure VIII.4

Titration curves for the spectrum of the previous figure, spectral peaks are identified by comparison of titration curves to those of

standards. Curves are identified as follows:
A, G6P; B, FDP; C, F6P; D, inorganic phosphate;
E, GPC; F, nucleotide phosphate; G, terminal
polyphosphate; H, γ -ATP; I, pyrophosphate;
J, α -ADP; K, α -ATP; L, UDPG; M, β -ATP; N,
penultimate or smaller polyphosphate; P,
polyphosphate

Figure VIII.5

Key to symbols used in time course plots of
phosphate metabolite levels (Figures VIII.6
to VIII.30)

Figure VIII.6

Relative intensity of spectral peaks of
Figure VIII.2 as a function of time. Meta-
bolite intensities are correlated with the
viability curve of the culture under the
conditions of the NMR experiment

Figure VIII.7

^{31}P 121 MHz spectrum of whole cells suspended
at 2×10^9 cells per ml and oxygenated. The
spectra change very little in the course of
10 hours

Figure VIII.8

Relative peak intensities for the aerated cell
suspension as correlated with the viability
curve. ATP levels are very low but remain
through the course of the experiment.

Figure VIII.9

Time course of internal and external pH as mea-
sured from the chemical shift of inorganic phos-
phate for the anaerobic and aerobic cultures of
Figures VIII.2 to VIII.8. The solid lines
refer to internal phosphate pools and the
dashed lines represent external pH. Triangles

(Δ) and squares (□) refer to the anaerobic and aerobic experiments respectively

Figure VIII.10

Time course of ^{31}P spectra of rehydrated cells in 10 mM phosphate buffer. No differentiation between external and internal phosphate pools was observed. Polyphosphate and sugar phosphate pools decreased with time

Figure VIII.11

Time course of metabolite intensities for the spectra of the previous figure. Cells were no greater than 5% viable under these conditions. Changes observed are therefore due to simple enzymatic degradation in the absence of active respiration

Figure VIII.12

Time course of metabolite intensities of an aerated suspension of rehydrated cells. The resolution of two pools of inorganic phosphate and polyphosphate ~7 hours may be correlated with the increased viability of the culture

Figure VIII.13

121 MHz ^{31}P spectrum of anaerobic cell suspension of starved cells, 0.25 hours after harvesting. ATP levels are very low and no polyphosphate peak is observed

Figure VIII.14

Titration curves of spectral peaks for a perchloric acid extract of starved cells. Identities of the curves are the same as for Figure VIII.4. Additional peaks were observed and are identified as GPS or GPI (X) and

GPE (Y). ATP is present in low levels (curves H, K and M), too low to be observed in the spectrum of the intact cells (previous figure)

- Figure VIII.15 Time course of metabolite levels in the anaerobic starved cell spectrum, as correlated with the viability curve. The resolution of three inorganic phosphate pools disappeared after 2 hours, concomitant with the disappearance of GPC and appearance of a larger sugar phosphate pool
- Figure VIII.16 Time course of 121 MHz phosphorous spectra of an aerated suspension of starved cells. Small levels of ATP can be seen in the initial spectrum. The unphased peak at ~25 ppm is due to the transmitter
- Figure VIII.17 Metabolite levels as a function of time for inorganic phosphate and GPC from the spectra of the previous figure. Under oxygenation, the viability of the culture is enhanced and then falls off
- Figure VIII.18 pH dependence as a function of time for anaerobic and aerobic starved cell suspensions. The codes are the same as for Figure VIII.9
- Figure VIII.19 Time course of ^{31}P NMR spectra for an anaerobic cell suspension treated with 5 mg nystatin
- Figure VIII.20 Metabolite levels as a function of time for the experiment of the previous figure, as correlated with the viability curve

Figure VIII.21

Time course of metabolite levels for an anaerobic cell suspension treated with only 1 mg nystatin (spectra not shown). The viability curve is not as precipitous as with the higher dosage, and pyrophosphate appears in the cell spectrum.

Figure VIII.22

pH behaviour of the cell suspensions treated with 1 mg (∇) and 5 mg (Δ) nystatin, and rehydrated cells (⊠). A small pH difference between inner and outer pools was observed for the first spectrum of the 5 mg treated suspension, but was gone by the second. The lack of appearance of two pools for the lesser dosage may be due to a delay in starting the experiment. The rehydrated cells show that the measured pH is approximately that as expected for the phosphate buffer.

Figure VIII.23

Time course of ^{31}P NMR spectra, for an aerobic suspension of cells treated with 5 mg nystatin, showing the appearance of two pools of polyphosphate.

Figure VIII.24

Viability, inorganic phosphate and polyphosphate curves for the experiment of the previous figure. The appearance of a second polyphosphate pool may be due to undergrated polyphosphate released into the medium by cell lysis.

Figure VIII.25

Initial and final spectra from an aerobic cell suspension treated with 1 mg nystatin. Pyrophosphate appears as for the anaerobic culture, and pH gradients appear to be preserved.

Figure VIII.26

Time course of relative intensities of phosphorus-containing metabolites for aerobic culture treated with 1 mg nystatin. The viability curve shows that aeration helps the organism resist the effect of the nystatin. Decrease in polyphosphate and appearance of pyrophosphate can be correlated with the loss of viability

Figure VIII.27

The time dependence of pH for the experiments of Figures VIII.24 and VIII.26. No pH gradient was observed for the higher dosage of nystatin ($\square$), whereas inner and outer pools were preserved throughout for treatment with 1 mg nystatin ($\square$)

Figure VIII.28

Time course of ^{31}P NMR spectra for a cell suspension treated with $6 \times 10^{-4} \text{ M MeHgCl}$. The cells do not appear to lyse, as indicated by the preservation of inner and outer pools of inorganic phosphate. The polyphosphate peak is reduced in size with time but also broadens. Pyrophosphate is produced after 5-6 hours, and also appears to be in two pools

Figure VIII.29

Time course of metabolites for methyl mercury-treated cells. The viability curve in this case represents a slightly aerated culture, as opposed to strictly anaerobic. The three inorganic phosphate pools are preserved for longer than for other treatments perhaps indicating very rapid respiration. The appearance of pyrophosphate is concomitant

with the disappearance of one of the inorganic phosphate pools. The increase of the peak due to terminal polyphosphate, shows that the polyphosphate chain length is being reduced

Figure VIII.30

Time course for an experiment similar to that of the previous figure, using an oxygenated cell suspension. The change in polyphosphate levels during the first hour may not be significant

Figure VIII.31

Time dependence of pH for aerobic ($\square$) and anaerobic (Δ) cell suspensions treated with MeHgCl . As before, dashed lines refer to the outer pool, and solid lines to the inner pool of phosphate. The discrimination between inner and outer pools is not as clear for the anaerobic suspension, and it is assumed that the pool of initial $\text{pH}=5.5$ is the external pool. This particular pool becomes quickly very acidic, and the internal pH is lower than that for other experiments

CHAPTER I

INTRODUCTION

In recent years, Nuclear Magnetic Resonance has been used as a non-invasive probe in the study of biological membranes and of metabolic processes (Seelig and Seelig, 1980; Wennerström and Lindblom, 1977; Smith and Deslauriers, 1981). The biological system offers a remarkable assortment of nuclei that are NMR-active - including ^{13}C , proton, deuterium, ^{31}P , ^{17}O , ^{14}N and ^{15}N as the most abundant, along with ^{113}Cd , ^7Li , ^{23}Na , ^{39}K , ^{199}Hg , ^{35}Cl and ^{19}F as the more exotic. The major advantage of the NMR technique is that it allows one to look at the specific molecular environment without disturbing the system (other than by placing it in a high magnetic field (Tenforde, 1979)); not only on a static basis, but on a dynamic basis. Since NMR is a time dependent phenomenon, it not only allows the observation of the chemical environment of the nucleus, but also of the detailed motion of the nucleus, as long as this motion is of the order of the NMR time scale. The chief disadvantage of the technique is its inherent insensitivity since detection involves looking for minute changes in field, which are expressed in frequency as Hertz, within a probing signal in Megahertz. This is compounded by the inherent sensitivity of the nucleus, which is a result of its gyromagnetic ratio and its natural abundance.

In a natural biological system, there is further dilution of the species under examination. Thus the researcher can follow one of three paths:

- (a) observe nuclei whose natural abundance is virtually 100%, such as proton, phosphorus and nitrogen-14;
- (b) look at model systems, i.e., lipid/water systems for membranes;
- (c) biosynthetically or synthetically enrich the species under investigation with a rare spin and introduce it into the biological system.

The first and the third route have been chosen in this study of a single organism, the yeast-like fungus Aureobasidium pullulans. It has been attempted here to characterize a living system by NMR by applying to this whole organism many of the NMR techniques used for more specific studies, in order to determine what information can be extracted and what are the limitations to the technique.

The complexity of the whole cell renders the interpretation of spectral results not unambiguous, which thus limits the absolute information that can be obtained. However, spectral results can be compared with those of model systems, as well as with the results of standard biochemical tests in order to substantiate the trends observed and establish correlations which then can be applied to other organisms.

The applications of NMR to biological problems can be roughly divided into two categories - studies of the membrane properties and studies of the cytoplasm. Properties of membranes can be investigated by using model systems which are chemically pure (i.e., liposomes), fractionated membranes prepared from organisms such as E. coli or A. laidlawii, or reconstituted membranes (i.e., removing the molecular protein and replacing it). Elucidation of the molecular properties of the membrane is essential to the understanding of the living organism, since membranes form selective barriers to nutrients, act as sites of active synthesis of biomolecules and of active transport, as well as forming physical barriers. All membranes are composed basically of lipid and protein, in the fashion of the accepted fluid mosaic model of Singer and Nicholson (Singer and Nicolson, 1972), in which the lipid molecules form a liquid crystalline bilayer sufficiently fluid to allow the protein molecules to function. NMR studies on the protein component are awaiting advances in technology, since the rigidity, conformation and molecular weight of the average protein present problems on the NMR time scale. Thus, to date, most of the NMR studies have concentrated on the role of the lipid. The effect of the protein upon the dynamics of the lipid phase are as yet in controversy, i.e., whether this effect is on a micro scale (in the immediate environment of the individual protein molecule), or is felt "on the average"

throughout the lipid phase. Since NMR is essentially a probe of the average nuclear environment in the lipid phase, resolution of this question must await observation of the protein component. In any case, the lipid construction of a membrane must allow for the conformational changes required for protein function, and at this point we must be content with comparing the "average" lipid properties of a natural membrane with those of a pure lipid in order to determine the "average" effect of the protein component.

Nature has provided us with a wide variety of lipid classes, and structures in order to accommodate the requirements of each individual type of membrane. Lipids come alone, as phospholipids or triacylglycerols; esterified to sterols, or linked to proteins and carbohydrates as proteolipid or glycosyl-lipid. The acyl chain moieties of natural lipids range widely in length and degree of unsaturation and many biochemical studies, as well as NMR studies, have attempted to make structure/function correlations. In phospholipids alone, there are many different head groups which yield decidedly different properties (Büldt and Wohlgemuth, 1981; Seelig and Seelig, 1980).

The properties of membrane lipids have been described in terms generally used for anisotropic fluids. Thus, properties measured by NMR, such as order parameters and relaxation times are said to be due to the "fluidity" of the lipid phase.

Fluidity involves two concepts. One relates to molecular ordering (intramolecular) and may be probed by the order parameter (Mantsch et al., 1977), which in deuterium NMR is a measure of the distribution of trans and gauche conformers of the fatty acyl chain or backbone of the lipid molecule. The other relates to the overall molecular mobility and is a measure of the relative amplitude and rate of motion of the molecule as a whole. The latter behaviour may be probed with the dynamic properties of NMR, such as relaxation times. In any one technique it must be remembered that these two properties are not necessarily separable. The dipolar or quadrupolar relaxation of a nucleus is dependent on both order and mobility. The relaxation time represents a sum of all correlation times of the individual motions of the nucleus, and thus provides a probe of not only intramolecular motion, but also of the intermolecular forces contributing to motion of the molecule as a whole.

Carbon-13 has several advantages for the study of membrane functions, including a large chemical shift range (about 200 ppm) to resolve components more effectively, and a simple relaxation mechanism through proton-carbon dipole interaction, which permits the interpretation of relaxation times in terms of individual motions (Lee et al., 1974). In this study, the "fluidity" of the lipid moiety of an organism has been studied via the biosynthetic incorporation of carbon-13 (from ^{13}C

labelled acetate) into alternate positions on the fatty acid chain portion. Thus, almost every position in the average lipid carbon chain can be viewed by NMR without the problems of overlapping peaks or ^{13}C - ^{13}C couplings. In this way, one may obtain the relaxation profile of the entire chain. The relative "fluidity" of the average lipid component in the living cell can be compared with the model system (vesicles) formed from that same lipid in order to test the adequacy of the model system. Since the lipid signal in the complete organism is a composite of plasma, vacuolar, nuclear, and mitochondrial membranes, as well as lipidic particles, the results obtained from the organism can be compared with lysed cells or a preparation in which the non-membrane lipid has been removed. Since the organism can also be grown on deuterated acetate, the CH_2 segments of the lipid chain can be deuterated and the order of the average perdeuterated chain can be probed. In addition, ^{13}C relaxation times have been measured as two different magnetic fields in order to ascertain unambiguously the correlation times corresponding to these relaxation times (Levy et al., 1977).

Having looked at the acyl moiety of the lipid in situ, one may then go on to look at the headgroup of the phospholipid component by phosphorus NMR. In the present case, this proves to be very difficult since the organism chosen has a very small percentage of phospholipid and the NMR signal from phospholipid

in vivo is dominated by signals from metabolites. However, one can look at the phosphorus NMR signal from the extracted phospholipid or in a model system constructed from the extracted lipid of the organism. The average order of this mixture can then be compared with that of individual phospholipids if the components of the natural lipid mixture are known. Thus, the lipid environment of the cell has been studied in order to relate the concepts that have been developed in pure lipid liposomes by NMR, namely the order parameter profile, the gel to liquid crystal transition, the headgroup CSA parameter, etc. to a system that contains a much greater percentage of unsaturated acyl chains than those previously studied. Other studies of integral membrane systems have been largely based on A. laidlawii (by deuterium, (Rance et al., 1980; Davis et al., 1980; Smith et al., 1979)) and E. coli (by deuterium NMR, (Yang et al., 1979; Nichol et al., 1980), by ^{15}N NMR (Irving and Lapidot, 1977) and by phosphorous NMR (Burnell et al., 1980a, b)). Individual studies that have incorporated ^{13}C labelled acyl chains have dealt with the yeast Candida albicans (London et al., 1975), E. coli (Birdsall et al., 1975) and virions (Stoffel et al., 1976).

The second experimental approach employing NMR is to study cytoplasmic processes, which have traditionally been dealt with by biochemical techniques. The chief advantage is that the process may be studied in situ, without extracting from the

cell the component or reaction system under study. The disadvantage of NMR is sensitivity, so that the processes studied must be carefully chosen and the presence or absence of small quantities of enzymes or intermediates must be inferred from the presence (or absence) of NMR signals assignable to reaction products specific to those enzymes. The ideal situation is, of course, a system accessible by both NMR and biochemistry, in order to verify experimental results. Physiological studies as well, such as uptake of nutrients, response to stress, growth cycles, etc. may also be complemented by information from NMR. High resolution NMR is responsive mainly to the highly mobile (smaller molecular weight) components of the cytoplasm, so that the presence of rigid components such as cell walls or organelles do not interfere. Depending on the amount of signal averaging required, the response of an organism can be followed over time courses ranging from milliseconds to days.

Cytoplasmic studies have employed ^{31}P NMR, ^{13}C NMR and proton NMR (e.g., Ogino et al., 1980), with specific application to the imaging technique for proton NMR (Andrew, 1980; Damadian, 1980) and ^{15}N NMR (Jacob et al., 1980; Irving and Lapidot, 1977). The carbon metabolism may be easily followed in viable systems by introducing ^{13}C enriched substrates and observing where the label ends up after a period of time. The ^{13}C nucleus thus provides a tracer and enhances sensitivity.

Chemical shifts of mono and disaccharides occur in a specific region of the ^{13}C spectrum, and are very sensitive to substitution on carbon-1 and carbon-6, as well as to conformation of linkage. The α -carbons of amino acids and small peptides, as well, occur in a specific region and are sensitive to linkage and to pH. Thus, unusual metabolic products can be easily detected and relative contributions from different biochemical pathways assessed. The process most extensively studied has been gluconeogenesis in rat liver cells (Cohen et al., 1979), E. coli (Brown et al., 1978) and as a model eukaryote, the yeast Saccharomyces cerevisiae (den Hollander et al., 1979). In the present study, an alternate approach was taken. The organism was allowed to utilize its carbon-13 enriched storage lipid and the randomization of the label was observed. This information, along with that from phosphorus NMR, provides some insight as to how various storage pools are mobilized in the cell.

Phosphorus NMR, on the other hand, has been much more widely used for metabolic studies due to the large chemical shift range (~30 ppm for biological phosphates) and 100% natural abundance, combined with relatively little contribution from signals other than metabolites (for reviews, see Gadian et al., 1977; Hollis et al., 1980; Shulman et al., 1979). Most of the cellular phosphorus is in the form of phosphate, alone or as mono or di esters, and the chemical shifts of the

phosphate group are very sensitive to pH, ester ligand, ionic strength, and the presence of paramagnetics such as Mn^{+2} or diamagnetic ions such as Mg^{+2} , which form complexes with the phosphate. Thus the technique has been widely used for determining intracellular and subcellular pH, specifically for yeast (Salhany et al., 1975; Gillies et al., 1981), yeast spores (Barton et al., 1980), bacteria (Setlow and Setlow, 1980; Urgurbil et al., 1978) and amoeba (Deslauriers et al., 1981). Intracellular pH changes under anaerobic conditions, under environmental stress, dormancy and under the influence of drugs. These changes can be correlated with phosphorus-containing metabolite levels in the same experiment and therefore, elucidate the survival tactics of microorganisms.

Since many yeasts can survive under both aerobic and anaerobic conditions, they are a suitable system for such studies. The phosphorus spectrum of A. pullulans was therefore studied as a function of perturbants and of time in order to monitor pH changes within the cell plus the metabolism of the large polyphosphate store carried by this particular organism.

In previous studies the approach has been to follow the metabolism of cells under conditions that are as close to growth conditions as possible, after a particular nutrient has been added to the sample. In the present study, the approach was to follow the metabolite levels by NMR as a function of time after the cells were removed from their source of nutrition,

and to correlate these levels and intracellular pH with the relative viability of the cell suspension. Other ^{31}P studies have been performed on baker's yeast (Salhany et al., 1975) and S. cerevisiae (Gillies et al., 1981; Navon et al., 1979), monitoring glucose and phosphate metabolism. These form an interesting comparison to the polymorphic yeast, A. pullulans.

As has been alluded to above, A. pullulans is not a commonly studied organism, largely because it is a polymorph and because it is not typical fungus. S. cerevisiae is commonly chosen by biochemists who wish to study yeast metabolism, since strains have been well characterized, and often Neurospora crassa is chosen as a typical mycelial fungus. A. pullulans belongs to the class known as "fungi imperfecti" - a class created for those fungi which do not possess a sexual phase to their life cycle. Other fungi, such as yeast, reproduce both by budding (asexual phase) or by producing spores (haploid phase). Many of the class fungi imperfecti may be reduced forms which have lost the power to reproduce sexually, or may never have evolved the ability, and therefore are of interest from an evolutionary point of view. There are many species of this class that cause important plant diseases, including potato and celery blights and apple blotch, as well as superficial and systematic human diseases. Ringworm, athlete's foot, thrush, moniliasis, sporotrichosis, torulosis and "valley fever" are all caused by members of this class. The persistence

of these infections against treatment is largely due to ability of the organism to survive in either hyphal, single cell or spore-like forms.

Aureobasidium pullulans, or Pullularia pullulans (two of a variety of names it has been given (Cooke, 1959)), is widely distributed in nature and is commonly referred to as the "black yeast", due to its production of black melanin-like pigment granules. It is important industrially as a causative agent in the deterioration of paint and of the discolouration of lumber (Zabel and Terracine, 1980). It has been isolated from the lymph nodes of patients with Hodgkin's granuloma (Wynne and Gott, 1956) and from the inflamed joints of patients with rheumatoid arthritis (Merdinger, 1969), and it has been associated with superficial skin infections in tropical countries (Cooke, 1959). In addition, A. pullulans is a general plant pathogen. There is much confusion in the early literature regarding the proper classification and naming of this species, which has arisen largely from the fact that it has adapted to such a wide variety of environments. As well as being found on a wide variety of fruits, vegetables and leaves (Akochova-Kratochvilova et al., 1980), the organism has been isolated from nectar, butter and cheese, leaf litters, chickens, shrimp (some strains are salt water tolerant (Phaff et al., 1952), all stages of the lumber and pulp and paper industry, optical instruments, leather (both old and during tanning),

old books, canvas, cotton, rubber and from a variety of insects (Cooke, 1959). Reports of the species have come from all over the world; it seems especially common in Panama, central Asia and India and South America, but it has been found in Ontario and many states of the U.S.A. Wherever materials subject to mould action are to be found, this species will also be found. The distribution of the organism is due to its ability to survive for long periods of time (up to two years) without nutrients. The organism can survive dessication, grow easily in the temperature range from 20°C to 65°C and although basically an aerobe, has a low oxygen requirement as compared to other fungi, which probably accounts for its ability to survive within paint layers.

A. pullulans is resistant to chlortetracycline, UV radiation, CS₂, organotin compounds (O'Neill and Drisko, 1980) and other common soil treatments, but can be successfully controlled with general anti-fungal treatments, including sorbic acid, formalin, iodine and mercuric acetate, although greater doses are often required. The present study shows that nystatin and methyl mercury chloride are also lethal, but their effects are strongly dependent on dose and aeration.

Industrially, the organism is quite important both for the formation of the black pigment (Merdinger, 1964) and for the production of pullulan. Pullulan is a simple α 1 $\rightarrow$ 4, 1 $\rightarrow$ 6 glucan (for structure, see Chapter VII) which is produced by

the organism and released into the medium (exocellular polysaccharide). This glucan has the ability to form strong, resilient films and fibres with possible applications to food packaging and preserving. To date, the commercial aspects have been investigated only in Japan (Rose, 1978).

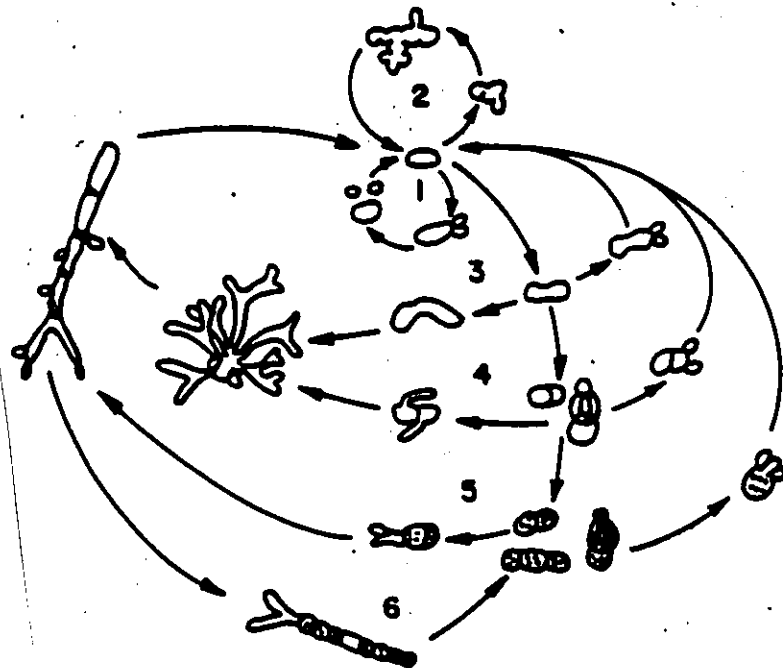
A. pullulans can owe its survival capabilities to the presence of multiple forms and an ability to utilize a broad variety of nutrient sources. These two attributes cause special difficulties in performing an NMR study, since it is difficult to isolate one form and also it is not easy to manipulate the lipid composition of any one form. However, the results of the study may shed some light on the relationship between evolutionary, morphological and biochemical aspects of this ubiquitous organism.

CHAPTER II

MORPHOLOGY

Aureobasidium pullulans assumes several morphological forms depending on the carbon source, nitrogen source, aeration rate, substrate, pH, growth temperature and inoculum size. It has a yeast phase which is single celled, multinucleate and elliptical, that reproduces by budding, primarily at the two poles. The size of the yeast cell is $3 \times 4-6 \text{ nm.}$, which serves to differentiate it from another phase, called large cells or 'swollen cells' which are $7 \times 10-14 \text{ nm.}$ (Ramos et al., 1975). As the culture ages, these large cells become thicker walled, pigmented chlamydospores, which may either bud to recycle into the yeast phase, or sprout a germ tube and differentiate into yet another phase, that of mycelia. The mycelial phase of this organism is septate. There are also a variety of intermediate forms.

The complex life cycle of this organism is shown in Figure II.1 (from Ramos & Garcia-Acha, 1975(a)). When grown on agar, the mycelial form dominates, whereas in liquid media, the morphology is highly dependent on nutrients. Subcycle 1 dominates under growth conditions used in these experiments. The yeast cells or blastospores reproduce by budding at the ends, leaving a weakened part of the cell wall which seems more subject to lysis under stress (see Plate VIII) than the rest of

LIFE CYCLE OF A. PULLULANS

1. YEAST PHASE
2. PSEUDOMYCELIAL PHASE
3. LARGE CELL PHASE
4. SEPTATE CELL PHASE
5. CHLAMYDOSPORE PHASE
6. SEPTATE CHLAMYDOSPORE PHASE

(ADAPTED FROM RAMOS AND GARCIA-ACHA, 1975(a))

Figure II.1 - The relationship between different morphological forms in the life cycle of A. pullulans (adapted from Ramos and Garcia-Acha, 1975(a))

the cell wall. In the strain used here, multiple budding, as indicated in Figure II.1, was never observed - only single daughter cells were formed. Subcycle 2 involves the formation of pseudomycelia and again these were not observed in present cultures.

Some of the yeast phases differentiate into large cells (subcycle 3) which form either mycelia or swollen septate cells (subcycle 4) which appear to be large cells which have begun to divide mitotically, but have not separated. This septate form was occasionally observed in these experiments but in small quantities. If left in the medium for longer periods of time (up to 14 days), these septate cells are converted to chlamydospores (subcycle 5). The walls thicken and the cells become dark. The heavy cell wall is melanin-rich and granular (Brown et al., 1973). Chlamydospore formation is favoured when ammonia is used as the nitrogen source (Ramos & Garcia-Acha, 1975 (a)). These large spores were observed during early experiments when cultures were grown for five days; however, using amino acids as a nitrogen source, and harvesting after approximately 72 hours, there were very few chlamydospores present. The chlamydospores generally produce sperm tubes which develop into mycelia (subcycle 5), although the yeast phase can be derived from chlamydospores directly. Mycelia can also be produced from swollen cells and mycelia will also give rise to the yeast phase.

The major forces in determining the dominant morphology in liquid culture appear to be the sources of carbon and nitrogen. This aspect has been investigated by Sevilla et al. (1977) who have shown that the organism can be grown on a wide variety of carbon and nitrogen sources. The use of glucose and acetate as carbon sources and asparagine and cysteine as nitrogen sources should result in a mixture of yeast phase, large cells and some mycelia (due to the effect of cysteine), and this composition was observed consistently in this study.

In the course of attempting to manipulate the lipid composition and increase the labelling capacity of the organism, it was observed that cultures grown with 1% glucose plus 0.2% acetate contained considerably fewer mycelia than those grown on glucose alone, and that almost 100% yeast phase was produced under conditions of inositol, octadecane and glycerol as the sole carbon source, as well as under conditions of "carbon starving". When glucose was used as the major carbon source, the mycelial phase was enhanced when aeration was high (i.e., when grown in a fermentor) and when the cells were packed at high density. It was often observed that after a few days in an NMR tube at concentrations greater than 10^9 cells per ml, the mycelial content was much greater than when the culture was first harvested. Cultures grown in a medium using D_2O instead of H_2O resulted in almost 100% mycelial material.

Under normal preparation however, the cultures were about 90% yeast phase and 10% 'swollen' cell phase, with a negligible amount of mycelia.

The physical appearance of the organism also reflects the conditions under which it was grown, and the metabolic state. It is important to have a visual concept to relate to the results of metabolism by NMR and thus the picture of the organism as shown in the following few plates can be correlated qualitatively to the different metabolic levels observed by phosphorous NMR (Chapter VIII).

Plate I shows the average three day old culture grown on acetate plus glucose. The cells are a creamy pink in colour. Some are actively budding and the cytoplasm appears very granular and dark due to the abundance of mitochondria, endoplasmic reticulum and many small vacuoles. This is an indication of young cells at high metabolic activity (Ramos et al., 1975). There is often more than one nucleus, especially prior to budding. Plate II shows a magnification of Plate I. There is a thin cell wall surrounding the cell and a bud scar at the polar end. The cell wall is a single layer and smooth (Ramos et al., 1975). The many small vacuoles hold the variety of storage forms of the cell - polyphosphates, glucose and triglycerides. The dark cell in the centre has lysed. With the lipophilic stain, Sudan Black, the plasma membrane inside the wall was stained, although somewhat intermittently, on the long

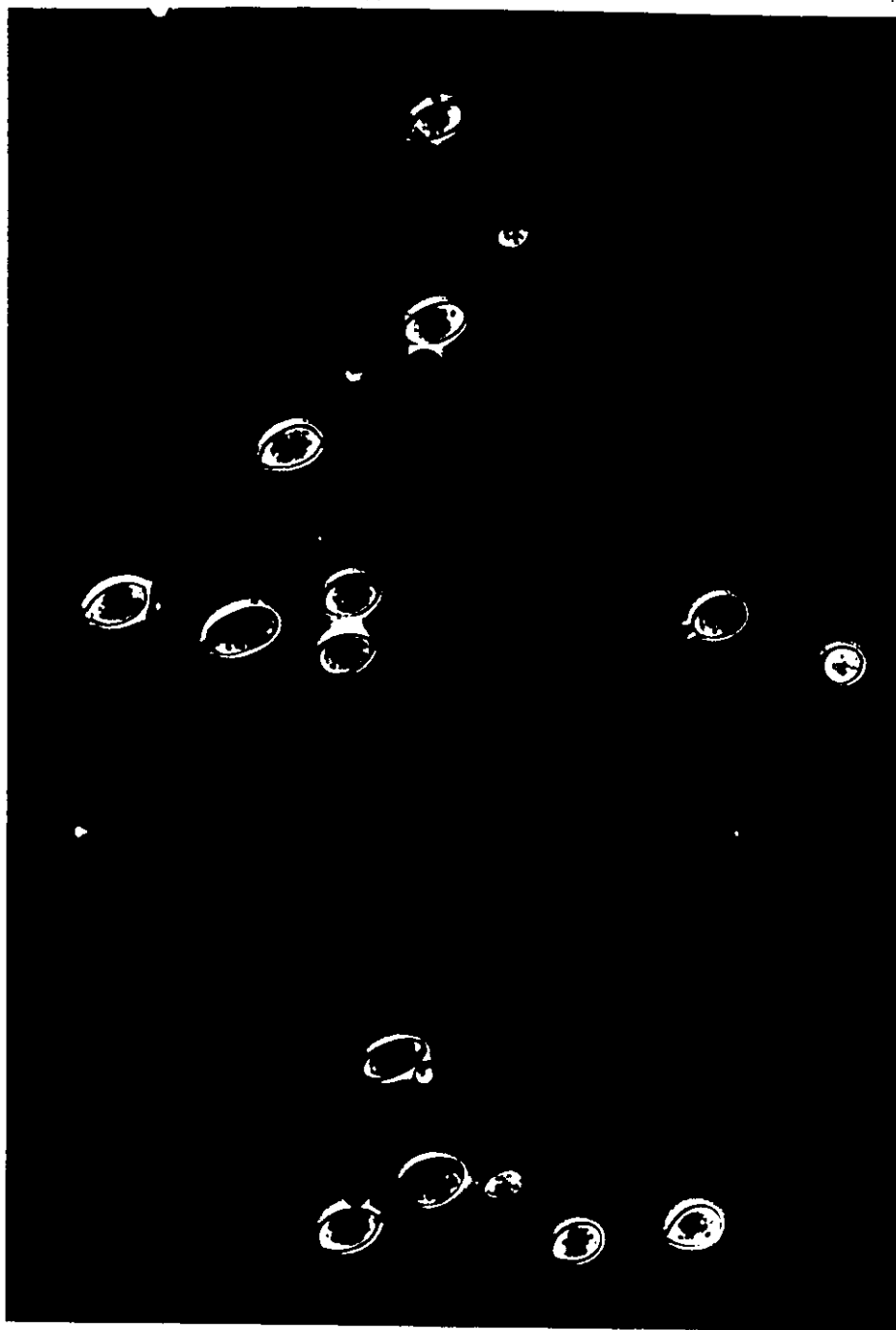


Plate I - Fresh culture, three days old, magnified x 914,
showing granular nature of the active yeast phase



Plate II - Magnification x 2 of Plate I

sides of the cell. The polar ends were heavily stained. The vacuolar membranes stained as well as specks in the cytoplasm, which are likely triglyceride droplets.

Plate III shows an example of a swollen septate cell. The wall appears smooth indicating that this cell has not differentiated into a chlamydospore. The cytoplasm is very granular and more than one nucleus is usually observed. Plate IV shows the mycelial form of A. pullulans. The germ tube originates from the large cell at the top of the plate and has differentiated into a septate hypha. The cell wall appears ruptured in the centre of the hypha. When stained with Sudan Black, only the inner wall and septum stained heavily, along with vacuolar membranes. The cytoplasm did not take the stain.

Plates V and VI show the organism when grown for three days on glucose plus deuterated acetate and on inositol, respectively. There appears to be a small isotope effect. Normally, deuterated cultures showed more lysis bodies than regular cultures. The yeast cells are more uniform in size and generally smaller. However, they appear active as indicated by the granular nature. Cells grown on inositol also appear uniform and small in size. Although some have granular cytoplasm, there are many cells dominated by a single large vacuole, indicating older or less active cells (Sevilla et al., 1977).

It is well known that under conditions of abundant sugar and oxygen supply, yeasts accumulate lipids as potential

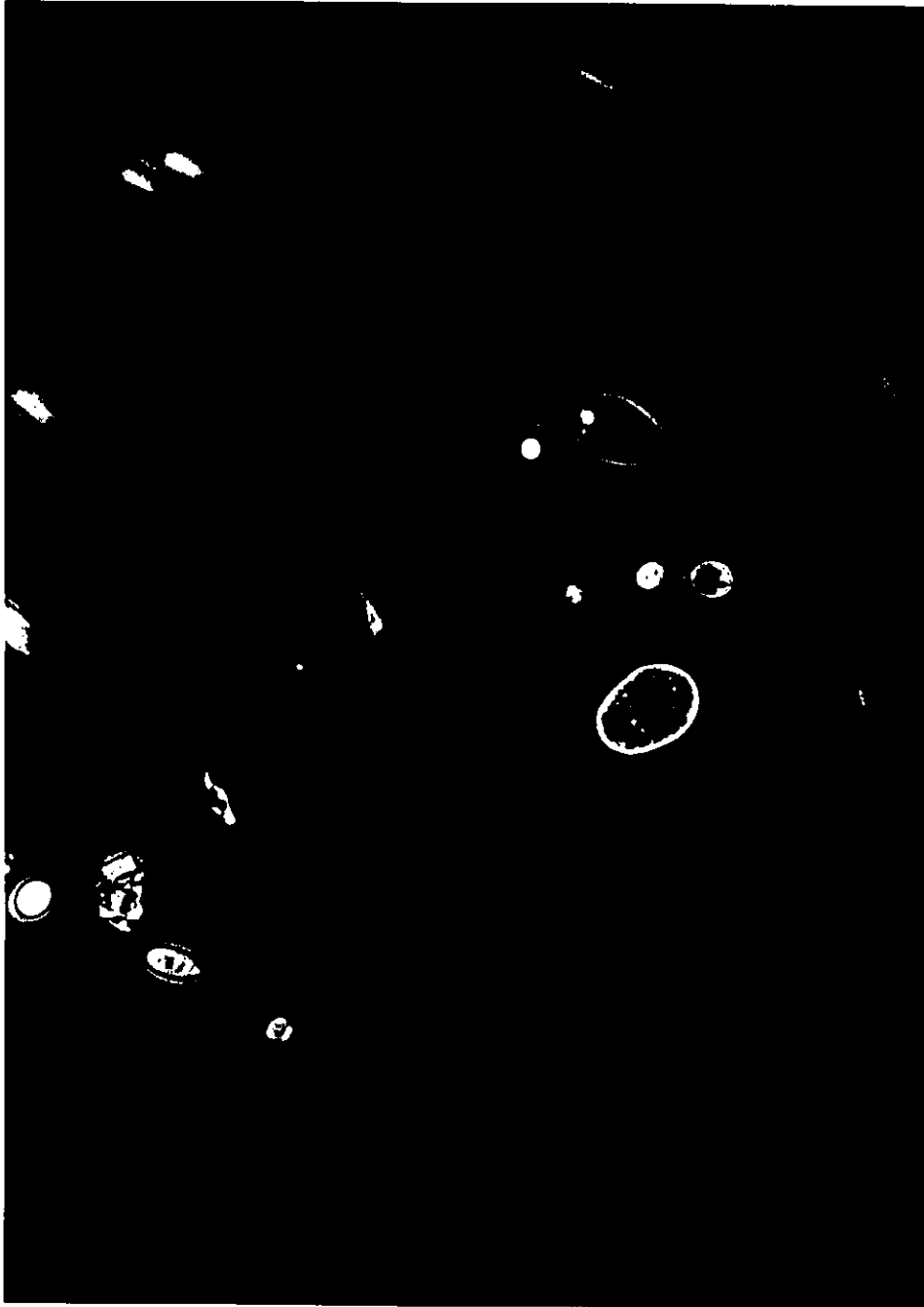


Plate III - Septate "swollen" cell. The size is approximately twice that of the yeast cell



Plate IV - An example of the mycelial phase of A. pullulans. The germ tube originated from the large cell at the top of the plate. This germ tube will later differentiate into a septate mycelium which will become more branched

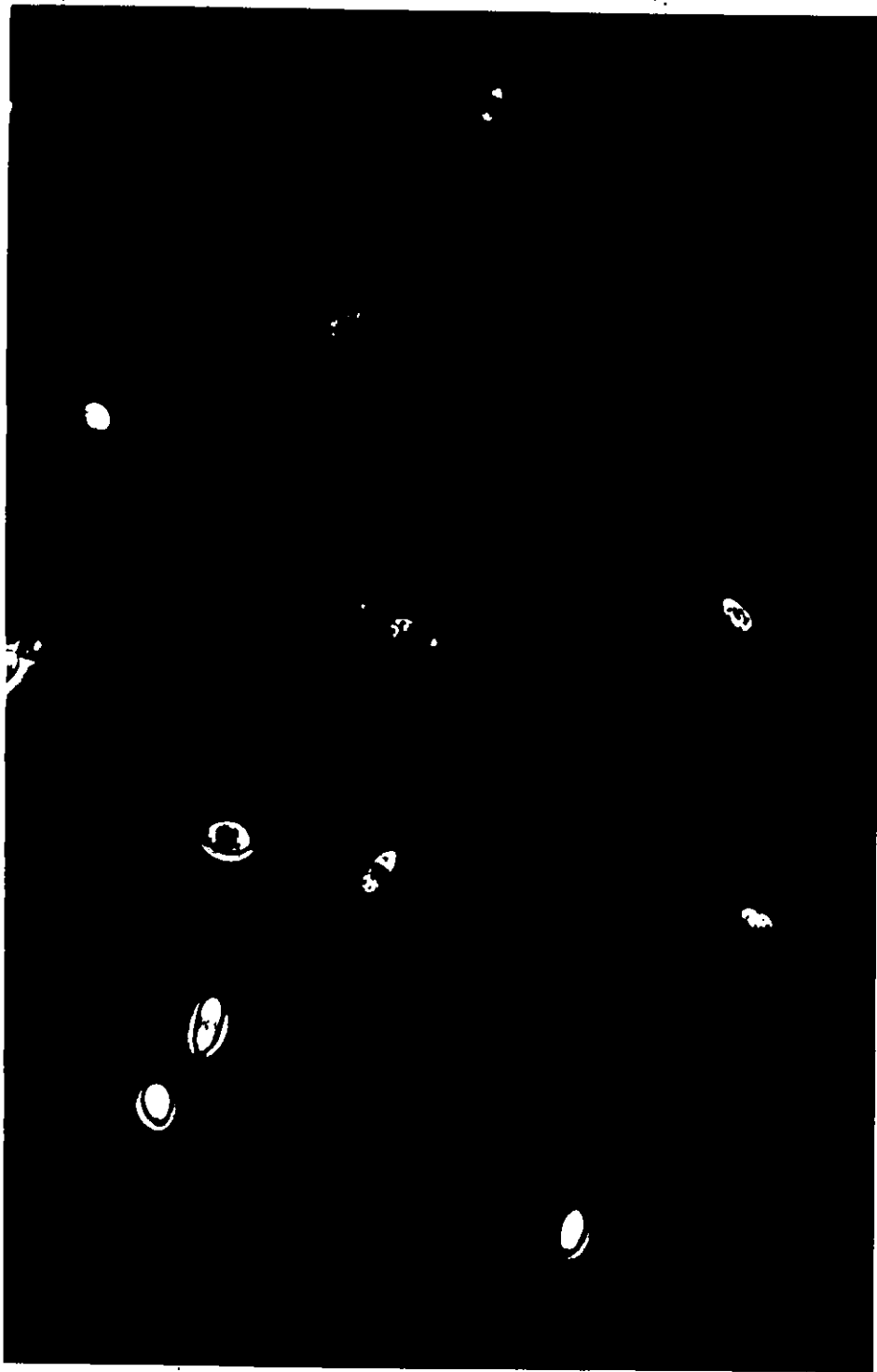


Plate V - A. pullulans yeast phase grown on deuterated acetate.
The cells are smaller and more uniform than in regular
culture but the cytoplasm is still granular

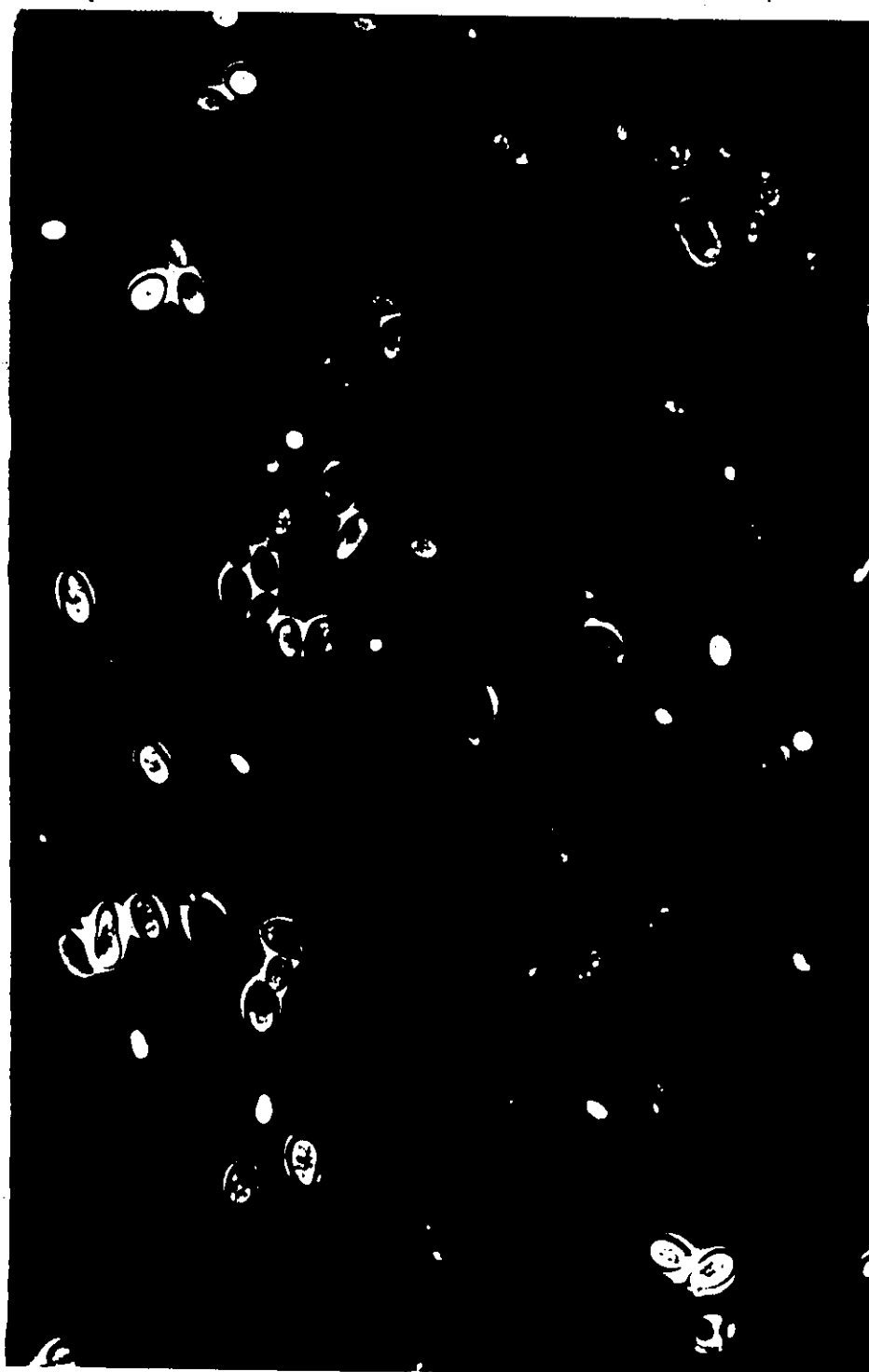


Plate VI - A. pullulans yeast phase grown on inositol are the sole carbon source. Cells are smaller and uniform in size. Many appear dominated by a single vacuole, and there is usually only one nucleus per cell.

reserves of energy and carbon. These accumulated triglycerides have posed a problem to studying the membrane lipids by NMR and it was desirable to induce the organism to use up some of this lipid supply without reducing its viability. This was accomplished by suspending the culture in a medium lacking in carbon for a minimum of three days. This procedure was called 'starving' the cells. Plate VII shows an example of starved cells. The cells are still quite viable as shown by abundant budding and there is still a range in cell size. Since nutrient starving has been previously used as a method of synchronizing cultures, this may well represent a relatively synchronous system (Neff et al., 1964). The cytoplasm is much less dense, not as granular and the cells are dominated by one or two large, more dense vacuoles. The lack of abundant mitochondria and endoplasmic reticulum indicate that the cells are much less active. When stained with Sudan Black, the cytoplasm did not take the stain as much as it did in fresh cells and only the plasma vacuolar and nuclear envelopes were strongly stained. This indicates that cytoplasmic triglyceride has been reduced. The appearance of this form is very similar to the diploid strains of A. pullulans developed by Imshenetskii and Kondrat'eva (1973).

One of the methods of making a crude membrane preparation in this study was based on the observation that treatment with nystatin consistently lysed cells under conditions of high cell

[20 μ m]

Plate VII - Cells of *A. pullulans* after three days in a medium lacking in carbon (starved cells). Cells continue to bud, but cytoplasm is less granular and cells are dominated by one or two vacuoles

density. The effect of nystatin is shown in Plates VIII and IX. For the purposes of photography, nystatin was simply added to the cell culture, so that lysis is not complete. However, the cells at the centre of the plate are typical of the effect of nystatin. The vacuoles become clear, while the cytoplasm turns dark and fibrillar. Some of the sub-cellular organelles remain, but become shrunken and flattened. The halo surrounding the cell due to the phase contrast becomes much smaller, indicating that the cell has lost its avoid shape. The cells consistently lyse at the bud scar, which then appears ragged and broken. This may be due to weakening of the cell wall at this point due to budding or due to the proximity of hydrolytic enzymes which act on the polysaccharide wall during budding (Ramos et al., 1975). Plate IX shows a swollen cell which has lost its ability to maintain its shape and is in the process of lysing. The cells above it and below it in the plate are also beginning to lyse. The cell at upper left shows empty vacuoles and a broken bud end.

Plate X shows an example of the product of a membrane preparation made from lysed spheroplasts of the organism. The cell wall is first enzymatically degraded with snail gut enzyme and the resultant spheroplast is lysed, rinsed, lyophilized and rehydrated. There is a lot of cellular debris which is normally centrifuged away, but the membranes left are mostly plasma, vacuolar and nuclear. The halo surrounding each shows that it

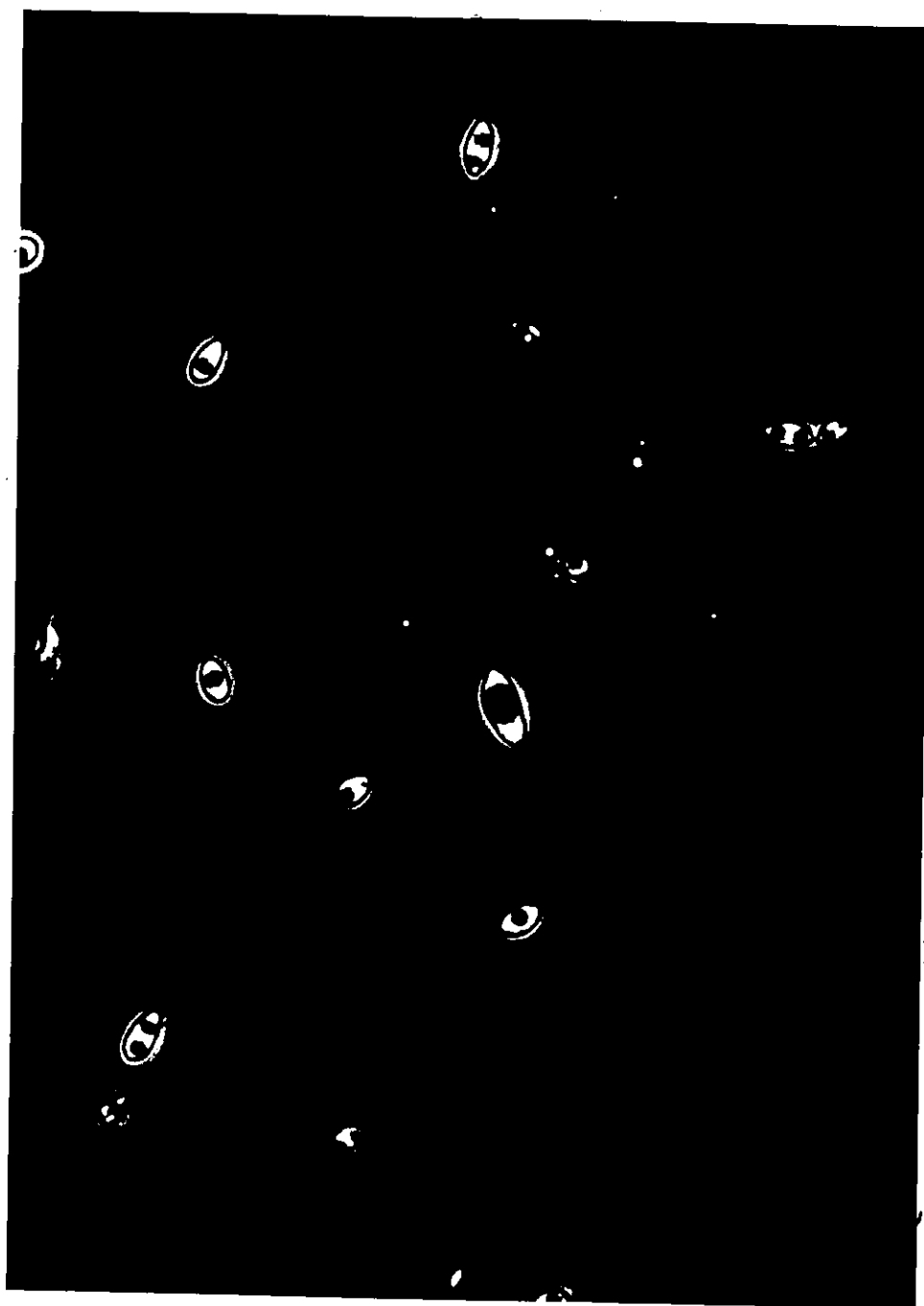


Plate VIII - A. pullulans, after treatment with nystatin.
The dark cells are lysed and show ragged edges at the pole. The halo produced by the phase contrast is not as pronounced as in healthy cells, indicating they may be somewhat flattened. Vacuoles are clear

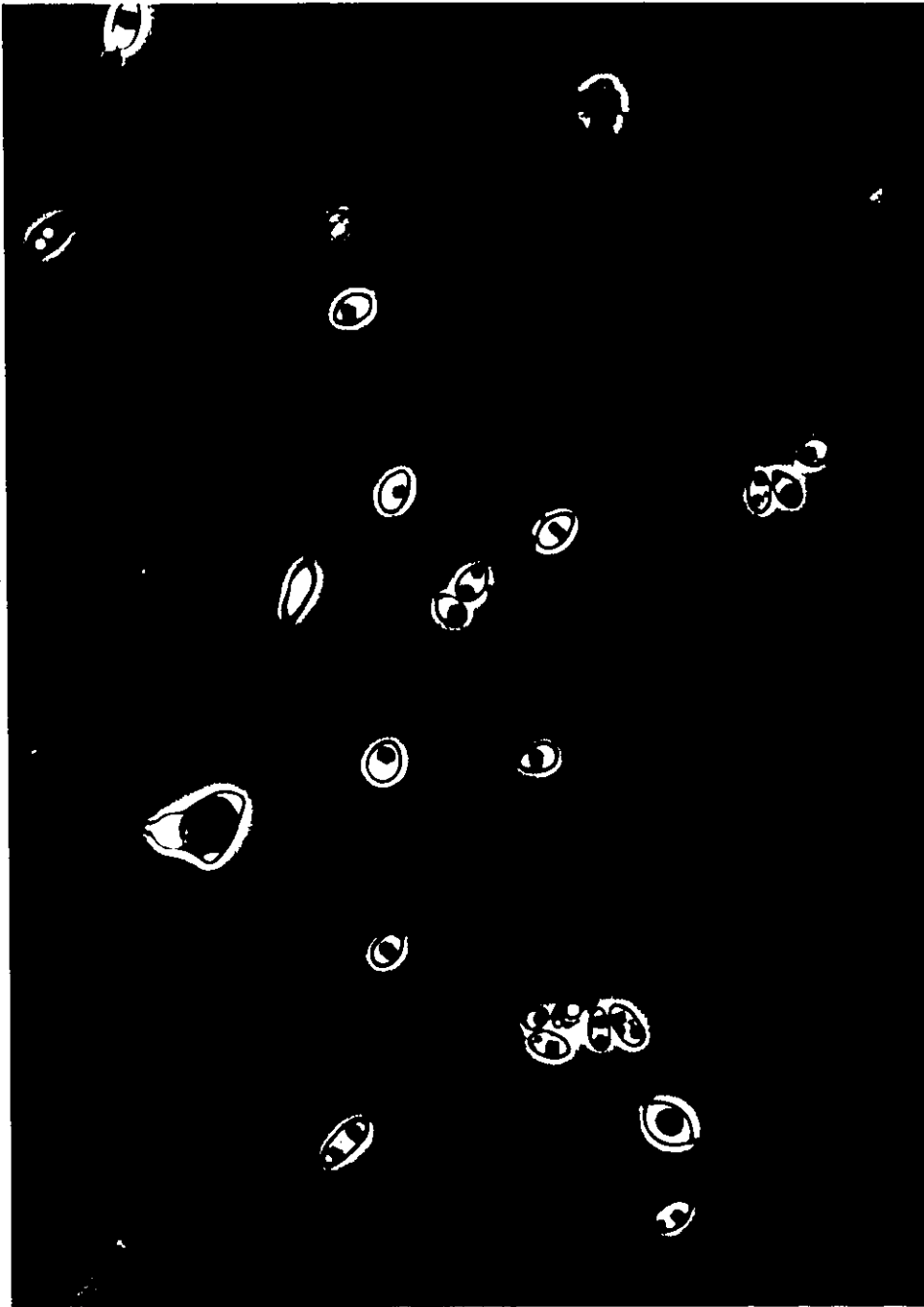


Plate IX - Cells after nystatin treatment showing lysing effect of nystatin. The swollen cell at the left shows that it has lost the ability to maintain its shape

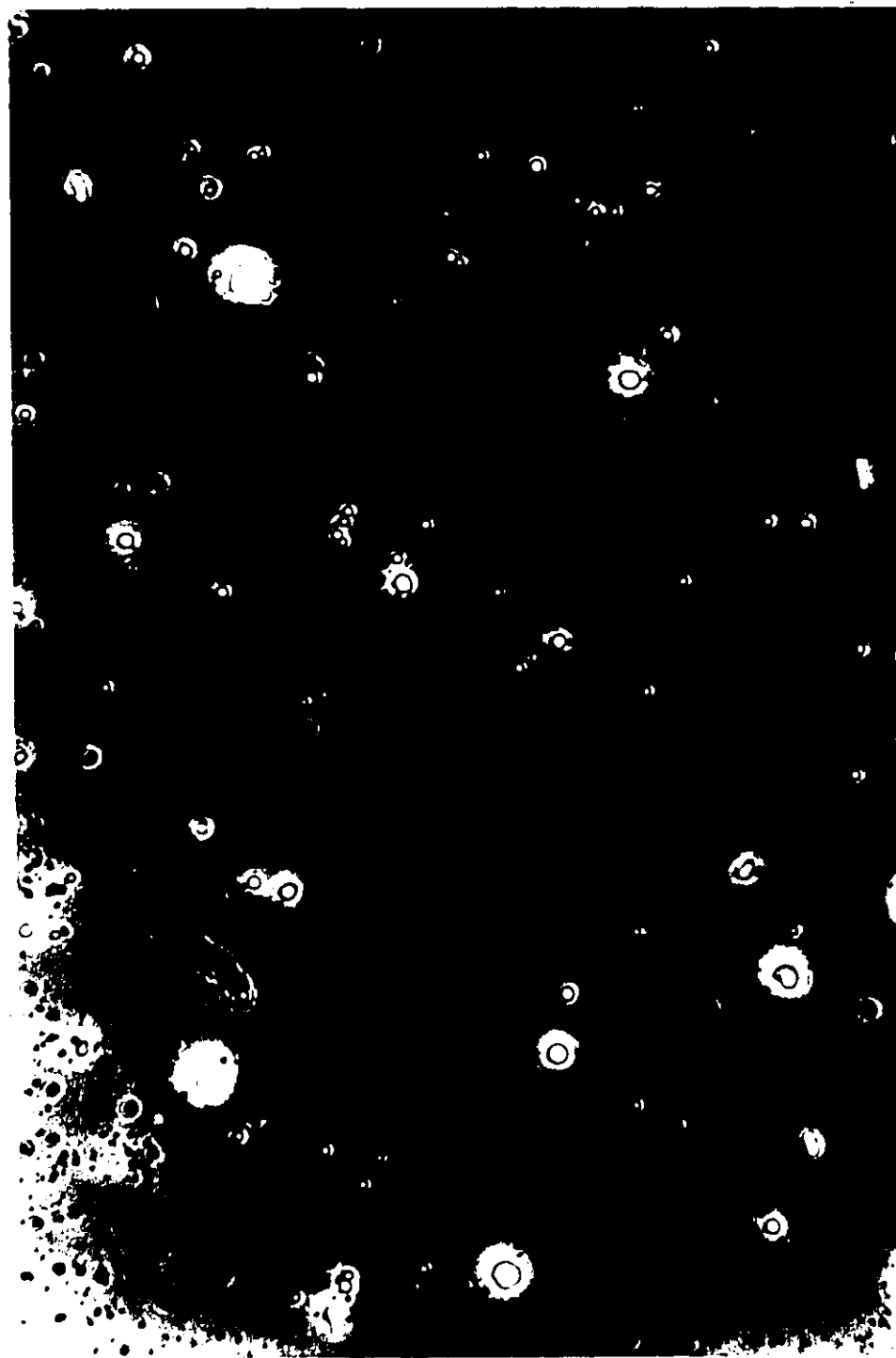


Plate X - Membrane preparation of A. pullulans made from snail gut enzyme, magnified x 914. Although there is a lot of cellular debris, the membranes are about half the size of the original cells, and are free of cytoplasmic contents

is likely spherical and most are about one half the size of the original cell. Most appear empty of cytoplasmic structure.

The relative physiology of the organism after these different treatments should be kept in mind when interpreting the results seen by NMR. For example, the differences seen in the phosphorous spectra between the 'starved cell' and the normal cell culture can be related to the absence of small vacuoles and lipid staining droplets in the former, thus indicating the possible locations for triglyceride, sugars and polyphosphate in the cell (see Chapter.VIII).

CHAPTER III

BASIC EXPERIMENTAL TECHNIQUES

Growth Conditions

Aureobasidium pullulans (ATCC 9348) was grown on a minimal medium (Sowa et al., 1963) on a rotary shaker at about 140 rpm and at 26°C. The medium consisted of:

- 10.0 g/l glucose
- 2.0 g/l sodium acetate
- 1.0 g/l K_2HPO_4 (dibasic)
- 1.0 g/l l-asparagine
- 0.5 g/l cysteine - HCl
- 0.5 g/l $MgSO_4 \cdot 7H_2O$
- 10.0 mg/l (trace) thiamine - HCl
- 0.1 g/l yeast extract

For whole cell ^{13}C studies 100 ml growths were used. For most of the metabolism studies 50 ml growths were employed, while for membrane preparations normally 500 ml or 1 l cultures were prepared. The pH of the medium was about 6.7. Due to the low phospholipid content of the organism, one 200 l culture was grown in a fermentor with the above medium and the resulting cell yields were simply frozen wet and stored at -20°C for subsequent bulk lipid extractions.

Cultures were inoculated with 5% by volume of an inoculum culture grown on the above medium and started from 1 ml agar slants which were stored at 4°C or from sterile, freeze-

dried yeast cells kept at -20°C . It was found that the viability of the agar slants dropped markedly after 3 or 4 months while the freeze-dried cells remained viable for at least a year and only a few milligrams of freeze-dried cells were required to start a healthy inoculum.

Carbon and deuterium labelled cultures were grown on the above medium substituting an equal quantity of C-1 or C-2 labelled sodium acetate ($\text{CH}_3^{13}\text{COONa}$ and $^{13}\text{CH}_3\text{COONa}$, respectively, 90 atom % ^{13}C , Merck, Sharp and Dohme, Montreal, Canada), or d_3 -sodium acetate (CD_3COONa , 99 atom % D, Merck, Sharp and Dohme), for the unlabelled acetate. The ^{13}C glucose study was carried out using C-1 labelled glucose provided by Dr. P. Moyna, University of the Republic, Montevideo, Uruguay. In addition, some of the deuterated cultures were grown on D_2O alone or 50% $\text{D}_2\text{O}/\text{H}_2\text{O}$ (v:v) plus deuterated acetate.

For investigations of various carbon sources, cultures were grown without acetate, with only 1% of the particular carbon source and using glucose as a control. For labelling experiments involving inositol, 0.2% acetate and 1% inositol were used.

Due to the production of black pigment granules by the yeast phase (Merdinger, 1964), the cultures tended to turn greyish black. During harvesting, granules sedimented at the top of the cell pellet and were difficult to remove. It was discovered that by wrapping the culture flasks in aluminum foil and/or growing the cultures in the dark, the cultures

remained creamy white and the harvested cells were light pink and free of pigment granules. The addition of acetate to the cultures also tended to keep the cultures white. Glucose alone in the medium tended to produce grey cultures much more quickly.

Cells were harvested in the early stationary phase, after three days growth, by centrifugation at 3000 x g for 10 minutes at 4°C. Cultures were rinsed twice with distilled water, and either freeze-dried, used directly for NMR analysis, or used as indicated in the various preparations.

'Starvation' Conditions

Cells were 'starved' of carbon for varying lengths of time (normally three days) in order to deplete their fatty acid storage pool biosynthetically. Cells were harvested under sterile conditions by centrifugation and resuspended in twice the original volume of minimal medium prepared without glucose and acetate. All other ingredients were the same as above, so that essentially the medium provided only amino acids and salts. The pH was about 6.5. The flasks were returned to the rotary shaker at 26°C for the 'starvation' period.

Lipid Staining (Hartman, 1940)

In order to visualize more clearly the location of lipid under the microscope, cells were stained with Sudan Black B

(2,3 dihydro-2,2 dimethyl-6-(4-phenylazo-1-naphthylazo)-perimidine), a relatively non-specific lipid stain for tissue fat. The Sudan Black powder was dissolved in a ratio of 0.7 gm per 100 ml in propylene glycol at 100°C, and filtered twice - once while hot with filter paper, and once cool through a scintered glass filter. Slides were prepared by smearing culture suspensions on a slide and fixed by passing through a flame. The slides were stained for 5-7 minutes, differentiated for 5 minutes in 85% propylene glycol and washed with distilled water. The lipid stained essentially blue-black under the microscope.

Viability Curves

The viability of the organism, under the conditions imposed upon it in the NMR tube, was determined as a function of time after harvesting, by a plating and cell counting method. Cells were counted in the medium before harvesting with a hemocytometer in order to determine the total cell count. The cells were harvested at 3000 x g for 10 minutes in sterile centrifuge tubes, rinsed once with sterile distilled water, and suspended to a cell density of $1.5 - 2 \times 10^9$ cells per ml in 10 mM phosphate buffer, pH 5.5. The buffer was not necessary but was used to reproduce conditions of phosphorous NMR metabolic studies (Chapter VIII). Cells suspended in D₂O or H₂O to the same cell density gave essentially the same viability curve as

with phosphate buffer. Water does not lyse these cells. They were at one point found to be growing in D₂O with no added nutrients.

Plates were prepared from sterile solutions of Bacto-Yeast Malt Extract Agar (Difco Laboratories) at 38 gm/l supplemented with 5 g/l (0.5%) glucose. Contamination was easily seen as the organism grew as large round white fluffy colonies whereas bacteria grew as smaller, 'wet-looking' colonies in a variety of colours.

Cells at the density of 2×10^9 per ml were sampled as a function of time by taking a 50 ul sample and diluting by a factor of 100, three times, vortexing between each dilution to ensure adequate mixing. Sterile water was used for dilutions (i.e., 50 ul in 5 ml, 50 ul of that solution in 5 ml, etc.). From the final solution of about 2×10^3 cells per ml, three 100 ul samples were taken and plated onto three plates. Plates were incubated at 36°C for two days or until colonies of about 1/8 inch diameter were formed. Longer incubation caused too large or fused colonies that were difficult to count. The counts from the three plates were averaged and normalized to the count taken at time zero, immediately after packing. In most cases this initial count represented 100% viability, i.e., the count was 150-200 colonies.

Cells often tended to clump together, or a bit of mycelia would get sampled and thus plates would be occasionally

overloaded due to uneven sampling or dilution. These plates would show an even sheet of grey mycelia with only tiny demarkations for colonies, instead of the usual round creamy, fluffy colonies. These plates would not be included in the count. Viability curves usually represent the average of two separate trials.

Membrane Preparations

(a) Enzymatic Preparation

The choice of reagent in making a membrane preparation is very important, since the enzyme used must have the capability of digesting the major component of the cell wall. The standard method of making membranes from yeast cells is by first forming spheroplasts by enzymatic degradation of the polysaccharide cell wall, followed by controlled lysis by osmotic shock (Wiley, 1974). Further purification by sucrose density gradient centrifugation allows one to select pure plasma, vacuolar, nuclear or mitochondrial membrane fractions (Schibaci et al., 1973).

The cell wall of A. pullulans is composed of 3 types of β -glucan; a linear 1,3 linked glucan, a branched 1,3 glucan with single glucose residues in the 6 position and an amorphous glucan containing 1,3 and 1,6 linkages plus a heteropoly-saccharide containing a 1,6 mannopyranose backbone (Brown and

Lindberg, 1967). The only previous spheroplasting preparation of the organism was made by employing the $\beta(1,3)$ glucanase lytic system of Micromonospora chalicea (Ramos and Garcia-Acha, 1975). Since growth of this additional organism in the lab was not desirable, it was resolved to use 'glusalase' as the enzyme reagent since it was easily available and well characterized as possessing along with various other enzymes, β -glucanase (Villaneuva and Garcia-Acha, 1971). Glusalase is unable to degrade α -glucans (Schwencke and Nagg, 1978; Brown et al, 1973).

After many trials with different osmotic stabilizer preparations with varying degrees of success, the following workup was chosen as yielding the best and most consistent yield. Purity must be sacrificed to yield in the case of these NMR experiments, since the yield of membrane fraction was about 30% at best, and about 20 mg of dry weight preparation was required to perform T_1 measurements in a reasonable length of time. For the same reason, the membrane preparation was not further purified by the sucrose density gradient method, since the costs in terms of NMR time, and ^{13}C precursor would not be justified by the relatively small gain in information. Pretreatment of the cells with a solution of 25 mM EDTA and 0.2 M 2-mercaptoethanol did not seem to increase the yield significantly and thus was discarded as part of the preparation (Wiley, 1974, p. 611). Stabilization of the spheroplasts by

0.7 M MgSO_4 during enzymatic degradation did not produce as uniform a mixture as did the use of sorbitol as the stabilizer. There was greater lysis of the spheroplasts resulting in additional losses of yield. The use of sorbitol produces greater risk of bacterial contamination than the use of salt, but this can be adequately controlled with an antibiotic, ampicillin, added to the reaction mixture. Stabilization of the spheroplasts with a small amount of Concanavalin A proved useful as the particles were then easier to spin down (Scarborough, 1975), but it was difficult to rid the sample of the 'clumped' particles for NMR analysis.

Subsequent lysis of the spheroplasts was also attempted by various methods including osmotic shock, gentle and severe (with homogenizer), addition of alcohol, sonicating and treatment with phospholipase C. Treatment with phospholipase C produced good but inhomogeneous yields and perturbed the lipid structure (as seen by TLC). Homogenization produced tiny fragments of membrane and simply suspending the spheroplasts in distilled water with stirring did not always produce sufficient lysis.

The following method is a variation of that of Bussey et al., (1979) and employs recommendations of Hutchinson and Hartwell (1967). The concentration of cells in the mixture is important to attain efficient conversion of cells to spheroplasts. During the course of finding the best preparation, it

was discovered that centrifuging the sorbitol-glucalase mixture at low speed (about 2500 x g for 15 minutes) resulted in inadequate sedimentation, due to the high density of the sorbitol solution, of particles such as those shown in Plate X - i.e., those vacular membranes and spheroplasts that had been lysed during the glucalase treatment. Often, fat particles centrifuged to the top during the first spin, appearing as a yellow scum on the surface of the cloudy supernatant and was identified as predominantly triglyceride by TLC. This fraction was always discarded.

The preparation method involved centrifugation of whole spheroplasts plus untreated cells after glucalase treatment, followed by lysis of the spheroplasts, sedimentation at low speed of unlysed fragments and cell walls, with subsequent sedimentation at high speed of the total membrane fraction. Since a Bligh-Dyer extraction of the supernatant from an initial low speed centrifugation in 1.2 M sorbitol - Tris gave a significant lipid yield that showed the presence of all four major lipid classes observed for lipid extracts from whole cells and membrane preparations, it was concluded that the particles giving this lipid were smaller membrane fragments that were too light to spin down in the dense medium. Thus, after glucalase treatment, the suspension was spun until the supernatant was clear and showed no particles such as are illustrated in Plate X. The mixture was then rinsed with

1.2 M sorbitol, 10 mM Tris-HCl to rid the system of the enzyme and resuspended in 10 mM Tris-HCl, pH 7.6 for lysis of the whole cell fragments and spheroplasts.

The following is the preparation used in NMR experiments. Glusalase (Glusalase, Diagnostic Reagent) was purchased from Endo Laboratories Ltd. and had a glucoronidase activity of 127,232 IU/ml. Cells (freeze-dried or fresh) ~~were~~ suspended to a density of about 1.0 to 1.2×10^8 cells per ml in 1.2 M sorbitol, 10 mM Tris-HCl, pH 7.6. Ampicillin (Sigma Chemical Co.) was added to a concentration of 50 ug/ml to prevent bacterial contamination. A lytic mixture of glusalase, 0.5 M NaCl and 1% cysteine HCl in the ratio 1:1:0.5 (v/v) was added to a total glusalase concentration of 1%, and the mixture was incubated on a rotary shaker at 37°C for 2 to 4 hours, checking the mixture periodically under the microscope. The cysteine solution was required to remove the effect of organic mercury added as a preservative to glusalase (Villaneuva and Garcia-Acha, 1971). The length of time required to complete the enzymatic degradation varied with the age of the culture and growth conditions, e.g., deuterated cells appeared more fragile. If left too long, many of the spheroplasts would lyse, forming particles that were more difficult to spin down (see above). The reaction was terminated when the majority of cells had 'rounded up', losing their ability to remain oval, and most showed thin spots on the outer perimeter - signs of

cell wall damage as determined by phase contrast microscopy. The mixture was spun at 5800 x g for 15 minutes, and the pellet rinsed twice with the stock solution (1.2 M sorbitol, 10 mM Tris, pH 7.6), spinning at a higher speed (10,000 x g for 20 minutes) for the rinses. The pellet was suspended in 10 mM Tris-HCl, pH 7.6, with 50 ug/ml ampicillin (normally to the same cell density) and left on a rotary shaker for a few hours. If such treatment was not sufficient to lyse the remaining spheroplasts, the mixture was sonicated gently for 10 minutes. The suspension was centrifuged at 1000 x g for 10 minutes to remove broken cell fragments, cell walls, etc. The supernatant was then centrifuged at 40,000 x g for 30 minutes to sediment the membrane fragments. The pellet was rinsed twice with 10 mM Tris-HCl, pH 7.6 and then freeze-dried and stored at -20°C until ready for use. The yield of membrane produced by this method was about 30% on a dry weight basis of original starting material. Approximately 30 mg of dry membranes were produced from a 250 ml culture of yeast. To prepare this sample for NMR analysis, the 30 mg sample was hydrated in 1 ml D_2O and vortexed until homogeneous before transferring to a 10 mm NMR tube. The rehydrated form was the same as shown in Plate X.

(b) Lysis by Nystatin Method

The following description produces what is not technically a membrane preparation, but more properly a lysed cell, which

is freeze-dried and rehydrated when ready for use. Lysis by nystatin produces essentially a complete cell - cell wall intact - which has a hole at the polar (budding) end, and which retains some of the fibrillar components of the cytoplasm plus vacuolar membrane (collapsed) without the majority of the cytoplasm.

It was discovered during the course of the high resolution phosphorus work on the organism, that certain doses of nystatin administered at the concentrated cell density required for NMR produce complete loss of viability and, when examined under the microscope, complete lysis within a few hours. Lysis was confirmed by ^{31}P NMR (see Chapter VIII). The method was generalized as follows. The nystatin used in all experiments was from two sources, ICN (with an activity of 5700 IU per mg) and Sigma Chemical Co. (with an activity of 5600 IU per mg). They were used equivalently.

Cells were harvested by centrifugation at $3000 \times g$ for 5 to 10 minutes, rinsed with sterile distilled water and suspended in 10 mM phosphate buffer, pH 5.5 at a cell density of about 2×10^9 . In the case of freeze-dried cells, cells were hydrated at about 2×10^8 cells per ml in sterile medium for two hours before centrifugation and suspension in the phosphate buffer. Nystatin was added to a concentration of 2.5 mg/ml, vortexed well and the mixture incubated at room temperature for 5 hours, or until more than 90% of cells

appeared dark under phase contrast microscopy, and the polar ends of cells were ragged (see Plate VIII). The mixture was diluted x 15 and centrifuged at 3000 x g for 10 minutes. The pellet was rinsed twice with distilled water and then freeze-dried and stored at -20°C until ready for use. Not all the nystatin was removed as indicated by the pale yellow colour of the sample. The supernatant from the first NMR spin was also lyophilized for subsequent hydration and NMR analysis to check for metabolites, sugars and labelled triglycerides. The yield of membranes by this method was about 40% on a dry weight basis and the lyophilized supernatant accounted for about 60% of the original starting material. To prepare for NMR analysis the sample was hydrated in 1 ml D_2O or distilled water, vortexed and transferred to a 10 mm NMR tube.

Lipid Extractions

Lipid extractions were performed by the method of Bligh and Dyer (1959). The freeze-dried cells were hydrated for an hour with stirring before adding organic solvent. The following proportions were used to extract a 50 ml growth of cells: 4 ml water, 10 ml MeOH and 5 ml chloroform. The preparation was then scaled up to extract larger growths. For a wet cell pellet, only 3 ml water was used in order to get better separation between the aqueous and organic phases.

The mixture was usually stirred overnight. After centrifugation at 3000 x g for 10 minutes, the pellet was discarded and 5 ml water and an equal amount of chloroform was added to the supernatant. It was found to be unnecessary to re-extract the pellet, since a negligible amount of lipid was obtained, except in the case of very large quantities of cells (see below). In some cases, the debris was washed twice with water and freeze-dried for NMR analysis. The two phases were separated by centrifugation at 3000 x g for 10 minutes and the aqueous phase was discarded or dialyzed against distilled water and freeze-dried for NMR analysis. The organic phase was dried over sodium salicylate and evaporated. The lipid was dried under vacuum and if stored, taken up in distilled chloroform, flushed with nitrogen and stored at -20°C .

In the extractions involving very large cell masses (0.8 kg wet weight) preliminary to preparing the pure phospholipid fraction, a tractable amount of solvent was used (total about 5 l) and the cells re-extracted after filtering, rather than centrifugation.

The Bligh-Dyer extraction removed about 90 to 95% of the lipid. NMR analysis on the debris from ^{13}C labelled cells showed that a very small amount (less than 10%) of 'strongly bound' lipid remained in the debris. In one experiment, extraction by the method of Folch et al. (1957) was compared with the above procedure. The total lipid extracted by the

Folch procedure (chloroform:methanol, 2:1) was less, although it contained less water soluble material, than the Bligh-Dyer procedure. The 'strongly bound' lipid which remained behind could be extracted by treatment with boiling 10% HCl for three hours before extraction and was found to possess the same lipid classes (by TLC) and a similar fatty acid composition as the more easily extractable lipid. Fatty acid analysis of a Folch extraction with an internal standard showed that much greater portion of the lipid was saponifiable lipid, whereas Bligh-Dyer extractions had a considerable portion of unsaponifiable material (carbohydrates, hydrocarbons, etc. see Chapter III). A Bligh-Dyer extraction of the cellular debris from a Folch extraction yielded a small amount of additional material, leaving about 5 to 10% of total lipid behind as 'bound' lipid. The amount of lipid left behind in the debris was too small to measure ^{13}C T_1 values on the labelled debris in a reasonable period of time, but linewidths were similar to those in the cells, indicating similar lipid structure. Sudan Black staining showed some staining of fragments in a thin layer inside the cell wall. The triglyceride of the 'bound' lipid fraction is generally hydrolysed to fatty acid by the strong acid, so that this fraction is of little use in characterizing the lipid structure of the cell. A complete study of 'free' and 'bound' lipid in a different strain of A. pullulans has been previously reported (Elinov et al., 1975).

Preparation of Purified Phospholipid Fraction

The following is an adaptation of that used to purify egg PC (Singleton et al., 1965). Frozen cells from a 200 l fermentor growth of A. pullulans were thawed and subjected to Bligh-Dyer extraction. The 14.2 g of total lipid (from 0.75 kg wet weight cells) was then suspended just until dissolved in redistilled acetone. The acetone solution was cooled quickly and left at -20°C for about 12 hours, whereupon the phospholipid fraction appeared as a white precipitate. The precipitate was collected by suction filtration, washed with cold acetone, and recrystallized from fresh aliquots of cold acetone. The precipitate was dried under vacuum yielding 3.2 gm, judged to be greater than 95% pure by TLC. It was then dissolved to known concentration in chloroform/methanol (2:1) and stored under nitrogen at -20°C .

Precipitation of Pullulan

The pullulan fraction was recovered from the medium by the method of Sowa et al. (1963). Four volumes of acetone were slowly added with stirring to the medium which was decanted after centrifugation of the cell pellet. The precipitate, a white, spongy polymer mass was collected, washed several times with ethanol and lyophilized after redissolving in water. The yields from 500 ml medium were about 1.4 ± 0.1 gm. To prepare

for NMR analysis, about 50 mg were dissolved in 1.5 ml D_2O with sonication and the addition of NaOH to $pD=13.0$ to aid in dissolution (Colson et al., 1979).

Analyses

(a) For Fatty Acids

Methanolic HCl reagent was prepared by dissolving 14 ml acetyl chloride slowly into 250 ml cold redistilled methanol. Lipid samples (1 to 10 mg) were dried down from chloroform or chloroform/methanol (2:1) solutions in a side-arm flask along with a known amount of $C_{17:0}$ fatty acid as a standard. 4.5 ml of methanolic HCl reagent were added and the mixture refluxed for 2 hours. After cooling, water was added to make the mixture 9:1 (methanol:water) and the methyl esters extracted with petroleum ether or hexane. Fatty acid analyses were performed on a Hewlett-Packard 5710A Gas Chromatograph. Mass spectrometry analyses were performed on a Hewlett-Packard GC-Mass Spectrometer (No.5985) on total lipid extracts after hydrolysis and methylation.

(b) For Amino Acids

Amino acids were analyzed on a Durrum single column amino acid analyzer (model D-500), after a boiling water extraction of harvested starved cells, by a method described by Basabe.

et al. (1979). Small peptides were re-analyzed after acid hydrolysis.

Perchloric Acid Extracts

Perchloric acid extracts of cells were prepared by a method similar to that of Navon et al., (1979). Due to difficulty in extracting the cells (i.e., simple incubation, freeze-thawing or mild sonication with perchloric acid were not adequate to extract a sufficiently large sample for NMR analysis), a larger growth of cells was used for this purpose than for the whole cell NMR.

A 500 ml growth (or 1 l of starved cells) were harvested by centrifugation, rinsed with distilled water and re-centrifuged in glass centrifuge tubes. To the pellet was added 0.5 volume of ice cold 35% perchloric acid and this was mixed well. The mixture was kept at 0°C in an ice bath and sonicated in a Branson 350 cell disruptor with microtip for 10 minutes with a 40% duty cycle and 0.4 attenuation. The mixture was centrifuged for 12 minutes at 12,000 x g at 4 C and the pellet discarded. The supernatant was neutralized with a saturated solution of potassium carbonate in an ice bath. The extract was adjusted to pH=6.0-6.5 and passed through a small column of Chelex-100 resin in the acidic form which essentially removed most of the bound ions (as indicated by phosphorous NMR, see Chapter VIII). The extract was collected,

EDTA was added and the sample freeze-dried after re-adjusting the pH to 6.8. The pH of the rehydrated sample was adjusted with 1 M HCl and 1 M NaOH during NMR analysis.

NMR Spectra

^{13}C NMR spectra were obtained on a Varian CFT 20 and Bruker CXP-300 spectrometer operating at 20 and 75 MHz, respectively. Samples were contained in sample tubes of 10 mm OD at 30°C with continuous broad band proton decoupling. All chemical shifts are reported with respect to external tetramethylsilane. D_2O was used as a field lock for experiments run on the Varian spectrometer and any pH measurements made with respect to NMR samples in D_2O are reported as pD, or corrected for deuterium activity in order to compare with aqueous samples ($\text{pD} = \text{pH} + 0.4$ (Glasoe, 1960)), i.e., for amino acid solutions. T_1 measurements were carried out using an inversion - recovery sequence ($180 - \tau - 90$) with a waiting time of 3 to $5 \times T_1$ (for the longest estimated T_1) between cycles. Proton spectra were obtained on a Varian CFT 20 spectrometer operating at 80 MHz at 30°C with sample tubes of 5 mm OD. High resolution phosphorus spectra were obtained at 121.47 MHz in aqueous solution in sample tubes of 10 mm OD, normally at 30°C with continuous proton decoupling. All chemical shifts are reported with respect to 85% H_3PO_4 in a sealed concentric melting point tube. Spectra were obtained with quadrature detection and a phase alternating pulse

sequence to increase S/N ratio and to reduce spectral aberration due to spectrometer ringing. Phosphorus spectra for an experimental series were obtained by collecting 2000 scans with a 45° pulse and 0.5 s recycle time every half hour for 9-11 hours. Spectra were transformed and plotted, normalized to the tallest peak of the initial spectrum, such that intensities of constituent peaks could be compared. High power phosphorus spectra were obtained in short sample tubes of 10 mm OD in a cross-coil configuration. Gated proton decoupling was employed to reduce sample heating. Temperatures were regulated by a Bruker BVT 1000 temperature unit and monitored with a thermocouple in the probe. After a temperature change, the sample was allowed 15 minutes to come to equilibrium. Chemical shifts are reported relative to 85% H_3PO_4 .

Deuterium spectra were also obtained in tubes of 10 mm OD in a home built probe of cross coil configuration at 46.063 MHz. Spectra were obtained using the quadrupolar echo technique (Davis et al., 1976), with phase alternation applied along the x direction. Spectra were accumulated in quadrature with the carrier frequency at the centre of the isotropic peak; using a spectral width of 125 KHz, 90° pulses, 60 or 40 μsec pulse spacing, and a recycle time of 0.5 sec.

Preliminary attempts at ^{199}Hg NMR were made at 53.6 Mhz in a broad band probe with tubes of 15 mm OD, at ambient temperature. In all experiments both the probe impedance and

resonance frequency were adjusted using a reflection bridge. Specifics of sample preparations and spectral accumulation are given with results. Approximate 90° pulse lengths for the various nuclei employed are given in Table III.1.

Table III.1

90° PULSE LENGTHS AS MEASURED ON STANDARD SAMPLES

<u>Spectrometer</u>	<u>Nucleus</u>	<u>Frequency</u> (MHz)	<u>90° Pulse</u> (μsec)	<u>Sample</u>
Varian CFT20	^{13}C	20.0	24	ethylbenzene
Varian CFT20	^1H	60.0	19	ethylbenzene
Bruker CXP-300	^{13}C	75.47	16-17	Dioxane/D ₂ O 2:1, v/v
Bruker CXP-300	^{31}P (HR)	121.47	16	H_3PO_4
Bruker CXP-300	^{31}P (HP)	121.47	12	
Bruker CXP-300	^2H	46.063	5.5	D ₂ O
Bruker CXP-300	^{199}Hg	53.6	52	Hg(Ac) ₂

CHAPTER IV

CHARACTERIZATION OF THE ORGANISM

Because the morphology of A. pullulans varies so extensively as a function of its environment, it is quite likely that its chemical composition is equally as adaptive. Many of the previous studies on the organism have concentrated on the various polysaccharides to be found in the cell, since these are of industrial interest; few have explored the lipid composition. Since not all previous studies used the same strain, it is difficult to tell whether differences are due to strain or growth conditions. Some interesting correlations have developed. The organism has been shown biochemically to manufacture a variety of simple α -glucans; pullulan, trehalose (Merdinger, 1969; Catley and Kelly, 1979) and glycogen (Kikuchi et al., 1974). Pullulan production has been associated with the yeast phase, while trehalose is found exclusively in mycelia. The cell walls, however, are almost exclusively β -glucan (Brown and Lindberg, 1976). The lipid composition has been investigated by Merdinger and coworkers (Merdinger, 1966; Merdinger et al., 1968; Merdinger, 1969) and more recently by Ponton et al., (1980). None of these studies used the particular strain of this study, and the fatty acid composition and phospholipid distribution are different from those presented here. However, features in common with most

yeasts, such as high triglyceride to phospholipid ratio, and a relative abundance of linoleic acid, are observed.

Growth Characteristics

The growth curve for a normal culture, grown under conditions outlined on page 34, is shown in Figure IV.1. The optical density was measured at the absorbance maximum, using distilled water to adjust for 100% transmission. Most cultures were harvested after approximately 70 hours (as shown by the arrow) or in early stationary phase. In preliminary experiments, cultures were allowed to grow for 5 days (late stationary phase), but since this resulted in the formation of black pigment granules, the growth time was shortened. Viability after preparation for NMR was virtually 100%, as measured by plating count compared to visual count. Cell density at harvesting was $\sim 7-8 \times 10^7$ cells per ml. Viability after NMR is discussed in Chapter V.

No growth curve was monitored for the starved cultures. However, after three days in the carbon-free medium, cells had retained their creamy pink colour (i.e., pigment was not produced), their morphology ($\sim 90\%$ yeast phase) and were budding actively. The cell density at harvest was $\sim 5 \times 10^7$ cells per ml, but since the volume of the starvation medium was twice that of the original growth medium, this represents an increase in cell mass of $\sim 30-40\%$. Viability was 100% of the cell count.

Incorporation of the ^{13}C and ^2H Labels

The level of label incorporation was checked by GC-mass spectroscopy of the total extracted lipid. The relative incorporation of label was the same for all fatty acid classes. For cultures grown on glucose plus ^{13}C acetate, there was a distribution of the number of ^{13}C atoms incorporated per chain, which peaked at 3-4 atoms. The intensity distribution of the parent ion peaks, corrected for natural abundance ^{13}C content show that 93% of the total lipid is labelled, with the following distribution: 9% having one ^{13}C per chain, 19% having 2 carbons, 23% having 3 carbons, 21% with 4, 14% with 5, 5% with 6 and 2% with 7 carbons. There is a small variation between incorporations of different fatty acids, with palmitoyl incorporation peaking at 3 carbon atoms and stearoyl incorporation peaking at 4 or 5 carbons. Since the incorporation of the labelled acetate appears to be completely random in terms of position, the ensemble of lipid chains can be represented by a single chain with a ^{13}C atom in every alternate position down the chain (see Figure V.1 for incorporation pathway). The unlabelled carbons are largely a result of glucose-derived carbon from the TCA cycle, since the original acetate is 90% enriched. This even distribution results in no intensity aberrations when comparing ^{13}C labelled lipid spectra to unlabelled lipid spectra, and also results in no ^{13}C - ^{13}C couplings to confuse spectral

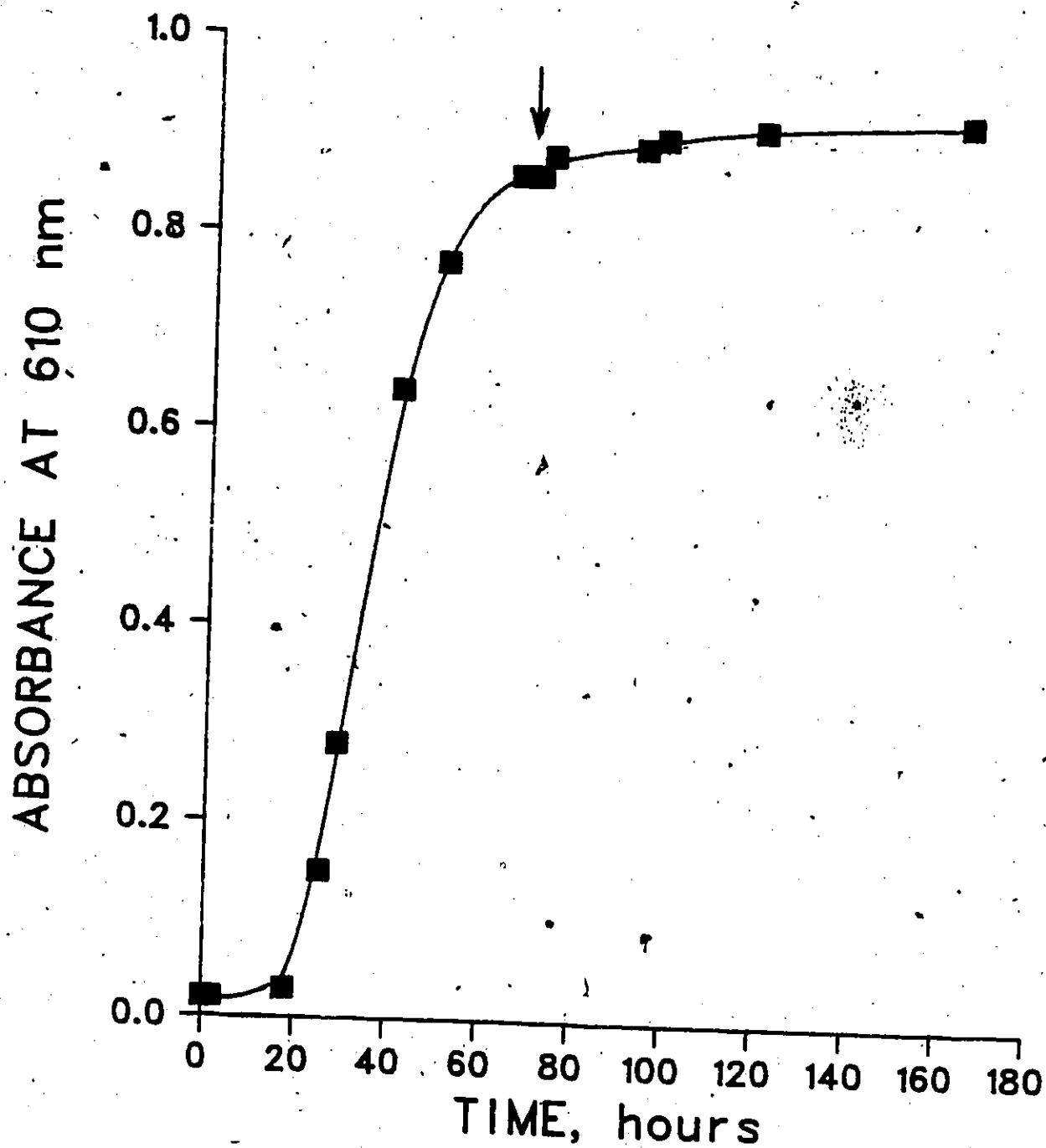


Figure IV.1 - Growth curve for A. pullulans in shaken culture at 26°C

interpretation. The intensity of such coupling would be proportional to the probability of a natural abundance ^{13}C atom occurring next to an acetate-derived atom in any one chain, and thus is only marginally greater than in natural abundance spectra.

A similar pattern was found for deuterated lipid derived from methyl d_3 -acetate. Since the deuterium label to be incorporated is only on the methyl group of the acetate, only the even carbons of the lipid ought to be deuterated. More of the deuterons appear to be lost through scrambling in biosynthesis than for ^{13}C label. The mass spectral results show that 85% of the total fatty acid chain population is deuterated, with 2% having 1 deuteron per chain, 22% with 2 deuterons, 20% with 3, 12% with 4, 7% with 5 and 2% with 6 deuterons per chain. This low incorporation makes it very difficult to observe what is essentially a perdeuterated ^2H NMR signal in a reasonable length of time, against the background signal from natural abundance deuterium of the more mobile components of the cell, plus the randomized deuterons from the acetate (see Chapter VI).

A previous study (White, 1980) indicated that the stoichiometry of deuterated fatty acids from E. coli grown on methyl d_3 -acetate is due to three deuterated populations - one with only the terminal methyl group deuterated, a second with one labelled hydrogen for each even carbon, derived via

NADPH. The relative incorporation was much higher than for A. pullulans, but since there is no evidence of the NADPH pathway in the ^{13}C acetate-derived lipid, it is not very surprising that the deuterium incorporation pattern is very similar to the C-13 pattern. It is also not unusual that odd numbers of deuterons are incorporated.

The fact that, for both A. pullulans and E. coli, only half of the expected amount of deuterium is incorporated from each acetate unit was explained by White (1980) as a kinetic isotope effect, but the effect can be easily explained by the mechanism of fatty acid synthesis via malonyl CoA (Metzler, 1977). Figure IV.2 shows the mechanism of the condensation reaction which forms the link between malonyl CoA and the lengthened chain. In the formation of malonyl CoA, the methyl-derived deuterons become activated. The condensation reaction occurs after CoA has been replaced by acyl carrier protein (ACP), and the unstable intermediate (in brackets) has lost one of the original deuterons. On decarboxylation, a proton is added from water. Thus, the resultant fatty acid consists of CDH groups on even numbered carbons. The subsequent loss of the keto group proceeds through an unsaturation step (step 5). The final CH_2 group contains a proton derived from NADPH (step 6). Thus, due to biosynthesis, there is only a 33% change of any CH_2 segment containing a deuteron derived from the original deuterated acetate.

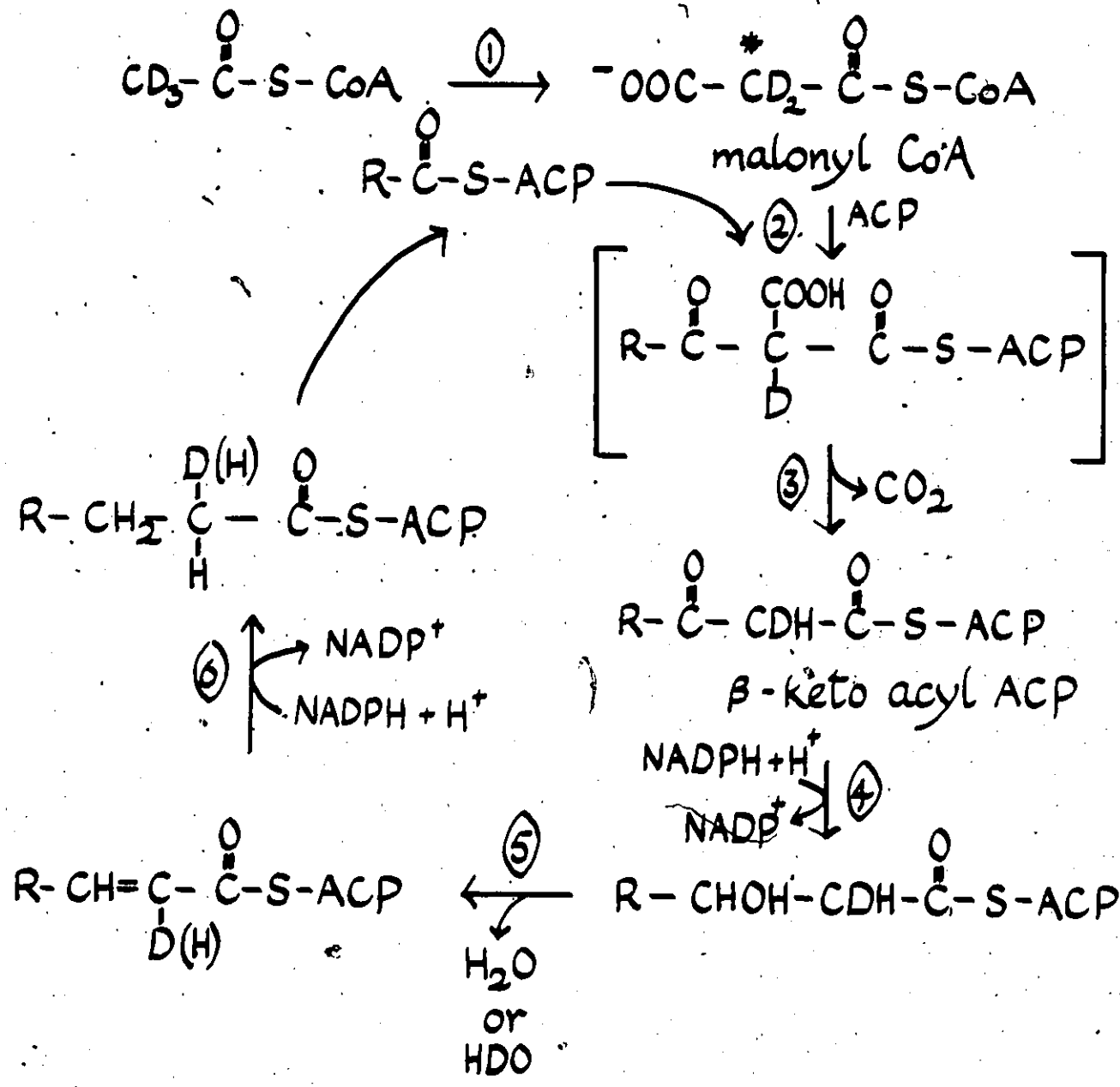


Figure IV.2 - Diagram of the biosynthetic pathway of acetate-derived deuterium into fatty acid chains of lipid, through the ACP mechanism

It follows therefore, that replacing the water in the medium with D_2O would enhance the deuteration rate. The incorporation of deuterium into fatty acid chains of rat liver through perfusion of tissue cells by a medium controllable in D_2O content has been measured by Patton and Lowenstein (1979). When 100% D_2O is used as a perfusate, 24 atoms are incorporated per chain for C-16:0 fatty acid and 26 atoms are incorporated for C-18:0, which is slightly more than one-half the available hydrogen. The incorporation is approximately a linear function of D_2O/H_2O ratio. Since A. pullulans produces only mycelia in 100% D_2O medium, the organism was grown on a 50% D_2O medium, supplemented with deuterated acetate and unlabelled glucose. From Patton and Lowenstein (1979), this should lead to an increase in incorporation of 8 to 10 atoms per chain. This increase was observed by GC-MS, with an interesting dependence of incorporation on fatty acid identity.

All the fatty acids were deuterated to some extent, but the ranges of the number of deuterons incorporated was a sensitive function of fatty acid length and degree of unsaturation. The relative distributions over the ranges of incorporation were, however, independent of fatty acid identity, indicating that these differences are mechanistic in origin. Table IV.1 gives the results of GC-MS of fatty acid esters derived from a total lipid grown under the above

Table IV.1

EXTENT OF DEUTERATION IN FATTY ACIDS FROM A. PULLULANS
 SUPPLEMENTED WITH
 D_2O/H_2O (1:1, v/v) PLUS 0.2% METHYL d_3 -ACETATE

<u>Fatty Acid</u>	<u>Relative Abundance (%)</u>	<u>Range of Number of Deuterons Per Chain</u>	<u>Average Number of Deuterons Expressed as:</u>	
			<u>(a) #</u>	<u>(b) % of Available Protons</u>
C-16:0	10.6	6-15	10	32.6
C-18:0	11.3	8-17	12	35.6
C-18:1	39.5	8-16	12	36.4
C-18:2	34.4	6-14	10	32.6

conditions. The average deuteration in the acyl chains is one-third of the total available hydrogen. The maximum incorporation for the saturated fatty acids is one-half the available hydrogen, corresponding to one deuteron per carbon and 2-3 per methyl group. Upon a single desaturation from C-18:0 to C-18:1, the range is reduced by one deuteron and the average number stays the same, indicating that if there is an equal probability of a deuteron occurring on C-9 as on all the other carbons; deuterium is retained with an average of 12 deuterons per chain. If one considers the maximum degree of deuteration as giving only CDH groups, this represents the reaction $CDH - CDH \rightarrow CD = CH$, normalized by the probability of deuterium occurring on C-9 and C-10. If a further unsaturation occurs, (between C-12 and C-13), there is definitely an isotope effect, in which deuterons are preferentially lost and protons retained. White (1980) has also observed this slight change in enrichment during desaturation from palmitic to palmitoleic acid, and has attributed this to two opposing factors: first, a previously observed, specific isotope effect against removal of deuterium from C-9 (Schraepfer and Bloch, 1965) and second, a mass effect - i.e., a greater chance of desaturation of less deuterated molecules. The results shown here indicate that there does indeed appear to be an isotope effect, which is directed towards the greater ease of loss of deuterium in desaturation. The greater affinity of C-9 for deuterium, for whatever reason, appears

to hold here as well. The exact mechanism of sequential desaturation of fatty acids is not well understood (Wakil, 1970; Metzler, 1977); however, the mechanism may proceed through a hydroxyl intermediate as in Figure 3, step 4, from which deuterium is more easily lost (step 5). There is a small amount of C-18:3 fatty acid produced by A. pullulans, but this is insufficient to check by CG-MS for any isotope effect on the third desaturation reaction (from C-18:2 to C-18:3). The fact that the unsaturated fatty acids are composed of only cis-double bonds may have some bearing on the mechanism leading to this isotope effect. There may be conformational specificity of the CDH groups as they are incorporated which leads to discrimination against deuterium on all carbons which lose a hydrogen except for the first in the chain (C-9).

The incorporation of deuterium into lipids is sufficient by this method to do NMR, but the background isotropic deuterium signal from D₂O incorporated into amino, hydroxyl and other exchangeable sites on proteins, sterols, etc., as well as deuterium from small metabolites and cytoplasmic water has increased, such that care must be taken to remove exchangeable deuterium before attempting spectra of whole cells or membrane preparations.

Optimization of Label Incorporation

In order to maximize the utilization of labelled acetate, the relative amounts of glucose and acetate were adjusted in order to give maximum ^{13}C incorporation plus maximum cell yield. The organism can be grown on acetate as the sole carbon source, but cell yield is low, and the cost of labelled acetate is prohibitively high. This supporting work was performed by Anne Joyce of this laboratory, and is as follows.

Test cultures of 50 ml were grown with 1% and 2% glucose plus 0.1% and 0.2% sodium acetate. Fatty acid content was quantitated and relative ^{13}C incorporation was measured by GC-MS. The results are shown in Table IV.2. Of the four cultures, the greatest relative enrichment is produced on lower glucose content, and specifically culture 2, with higher acetate content. Since the relative enrichment factor takes into account total fatty acid produced and is normalized by the amount of acetate, it is apparent that acetate is much more efficiently incorporated in the presence of less glucose, even though cell yield is greater with more glucose. The conditions of culture 2 were therefore used in all subsequent experiments.

Table IV.2 also shows that the fatty acid composition is also a strong function of glucose content in the medium. Total cell yield is a function of total available carbon,

Table IV.2

OPTIMIZATION OF GROWTH AND INCORPORATION OF LABELLED SUBSTRATE

Culture	Glucose %	Acetate %	Cell Yield (dry wt., mg)	Fatty Acid Yield (% of dry cell wt.)	Relative Enrichment ¹	Fatty Acid Composition		
						16:0	18:0	18:1 18:2
1	2%	0.2%	267	10.9	3.06	22.5	9.7	32.0 35.9
2	1%	0.2%	152	8.0	5.56	18.7	6.1	29.3 45.8
3	2%	0.1%	164	12.4	1.70	19.0	9.0	33.0 39.0
4	1%	0.1%	131	8.3	4.04	21.3	4.6	29.2 44.9

¹ Relative enrichment is proportional to the total ¹³C incorporated, normalized by the fatty acid yield and the amount of acetate used.

regardless of source. However, cultures 2 and 4 have substantially less total lipid content. It appears that the availability of glucose determines lipid accumulation. Thus culture 2 is also a good choice for whole cell NMR experiments, since these growth conditions likely minimize the production of non-membrane triacylglycerol. In addition, the amount of available glucose influences the acyl chain composition. Reduced glucose results in a slightly greater population of unsaturated lipid. In particular, C-18:2 content is increased at the expense of C-18:1 and C-18:0. Initial experiments used the conditions of culture 1, and the fatty acid analyses reflected this difference in glucose content.

Classification of Lipid Content of A. Pullulans

In an attempt to quantify the amount of non-membrane triglyceride in the various preparations used for ^{13}C NMR spectra, total extractable lipid was characterized in terms of the relative lipid classes. The following results should be kept in mind when interpreting the results of ^{13}C T_1 measurements of Chapter V, since the ^{13}C results represent the weighted average of all lipid classes and lipid organizations. The results may be compared with those of Ponton et al. (1980), which is the only detailed study on the lipid composition of this organism.

Under normal culture conditions, the total extractable lipid (by the Bligh and Dyer method) is 2% (w/w) wet weight cells or 10% of lyophilized cells. This places the organism as a medium content species (yeast lipid content ranges from 2 to 30% by weight). Of this total lipid, approximately 25% is phospholipid.

(a) Acyl Chain Content

The general fatty acid composition is highly unsaturated, and consists of only three major and two minor components, so that its simplicity is suited for NMR studies. The major components are C-16:0, C-18:1 and C-18:2 and the minor ones are C-18:0 and C-18:3. Often the C-18:3 content was less than 1% and was therefore ignored.

Table IV.3 shows sample fatty acid compositions of lipid extracts for the various systems used in these experiments. The fatty acids are largely determined by the growth conditions. The lipids from membrane preparations produce essentially the same distributions as whole cell lipids. Analyses of growths under conditions of 1% glucose and 0.2% acetate were consistent over the duration of this study, to the relative error shown for the first entry of Table IV.3. There is a high total unsaturated chain content (~75%). In all cases, there is no 16:1 chain content, and 18 carbon chains are preferred (>80%). There appears to be some isotope effect for the deuterated

Table IV.3

FATTY ACID DISTRIBUTION OF TOTAL CELL LIPIDS

Growth	Fatty Acid % Composition					% Unsaturated Chains
	16:0	18:0	18:1	18:2	18:3	
glucose plus acetate	19.0 ±0.5	8.0 ±0.1	25.0 ±2.0	47.0 ±2.0		72%
3-day starved	15.4 ±0.5	5.8 ±0.5	36.0 ±1.0	43±2		79%
6-day starved	13.3 ±0.4	2.8 ±0.5	23±1	61±1		84%
deuterated acetate plus glucose	19.0	8.5	33.6	38.2		72%
glucose plus d ₃ -acetate plus H ₂ O/D ₂ O	11.0	11.9	41.1	36.0		77%
purified phospholipid	16.3	2.4	19.4	58.5	2.6	80.5%
MeOH supplemented	21.0 ±0.5	7.0 ±0.5	30±1	41±1		71%
MeOH plus starvation	16.8 ±0.5	4.2 ±0.2	24.0 ±1.0	55±1		79%
glucose	18.3	6.2	34.8	40.7		75.5%
inositol	17.5	4.0	15.6	62.9		78.5%
acetate plus inositol	16.6	4.4	18.1	60.8		79%
octadecane	14.8	24.3	14.1	46.8		61%

cultures, which is even more marked when D_2O is added to the medium. This effect militates against unsaturation in the C-18 chains, lending support to the kinetic explanation for deuterium being preferentially lost during acylation. This could be due to a kinetic isotope effect involved in the desaturation or simply the organism's physiological response to a general toxin.

Small amounts (<2%) of C-18:3 chains were occasionally observed, but were lost through degradation, depending on how carefully and over what period of time the lipid was treated. The purified phospholipid fraction from the fermentor growth has a somewhat greater unsaturated chain content. This is not just due to careful treatment and storage under nitrogen, but reflects to some extent the greater mycelial content of this culture, plus the slightly greater amount of unsaturation to be found in the phospholipid fraction over the neutral lipid fraction (see Section (b)). Over a period of storage for one year, this phospholipid analysis changed by less than 0.2%. Thus degradation is a small part of the discrepancy between results.

Cells from cultures starved for three days appear to have the same acyl composition as for the normal three day culture. The total amount of unsaturation is somewhat greater. Starvation for longer periods of time results in a greater extent of unsaturation, reflected in an increase in the 18:2

content to the detriment of 16:0 and 18:0. This may be a result of a tendency for shorter chain and saturated storage lipids to be used preferentially for β -oxidation, or simply a requirement for greater membrane fluidity under this stressed condition. Starved cells were somewhat easier to lyse or to extract by perchloric acid than normal cells.

Since C-18:2 forms almost 50% of the total acyl chain content, it serves as a model for the analysis of spectra, with C-18:1 as the second most abundant. This composition seems fairly typical for yeasts, but is much more unsaturated than for bacteria such as E. coli and A. laidlawii (Kates, 1964). Candida utilis yeasts have been shown to have a similar content of unsaturated fatty acids, but they are more evenly distributed between oleic, linoleic and linolenic acids (London et al., 1975). Previous reports of lipid composition of A. pullulans (Merdinger et al., 1968; Ponton et al., 1980), which were grown on glucose, show only 50-60% unsaturation, with oleic acid (C-18:1) as the major unsaturated species.

(b) Lipid Classes

The chloroform-soluble portion of the cell consists of phospholipid, sterol, sterol ester, mono-, di- and triacylglycerols, plus other non-saponifiable material. A small portion of lipid is lost as proteolipid which appears at the

interface between the water and chloroform phases in the process of extraction. A small amount also remains behind in the cellular debris, as was seen by Sudan Black staining, by re-extraction after boiling HCl treatment, and by deuterium NMR (Chapter VI), which is due to fragments bound strongly to the cell wall, perhaps as lipopolysaccharide.

Lipid classes were quantified as follows. A known weight of total lipid was layered onto a precleaned silica gel plate and the plate was developed in 5% ether/benzene. Four groups of lipids were identified with iodine vapour - phospholipids, mono- and di-glycerides plus sterols, triglycerides and sterol esters. The areas corresponding to these classes were scraped off the plate and methyl esters made directly, according to the procedure described previously, adding a known amount of a standard fatty acid (C-17:0). The four classes were analyzed by GC and the total weight of fatty acids were converted to lipid weight by the appropriate factors (Christie, 1973).

Table IV.4 shows the results of such an analysis for the systems used in ^{13}C NMR experiments. Whole cells show a substantial amount of triglycerides, although the major class is phospholipids. The portion of total lipid that is acyl chain containing is only 60%, the rest being unsaponifiables, in agreement with Ponton *et al.*, (1980). Only a small portion of the remaining 40% is due to sterols, and small

Table IV.4
LIPID CLASS DISTRIBUTION OF A. PULLULANS

System	Lipid Content (% of dry wt.)	Lipid Classes (mg)				% Recovery	TG/PL Ratio
		PL	MDG	TG	SE		
Whole Cells	8-10%	2.25	0.04	1.25	0.02	60%	.57
Starved Cells	5- 7%	1.77	0.04	0.37	0.05	41%	.21
Inositol Cells	4- 6%	1.37	0.04	0.57	0.02	28%	.39
Nystatin Membrane preparation	6%	2.87	0.04	0.70	0.02	40%	.24
Enzyme Membrane preparation	7%	1.26	0.06	0.07	0.01	35%	.10

PL - Phospholipid
MDG - Mono- and Diglycerides
TG - Triglycerides
SE - Sterol Esters

amounts of sterol were found in the mono- and diglyceride portion, (in this solvent system, ergosterol runs with diglyceride), and in the sterol ester portion. Although not quantitated, sterols were consistent with ergosterol, squalene, zymosterol, stigmasterol and possibly lanosterol, using GC standards. Both Merdinger et al. (1968) and Ponton et al. (1980) agree that the sterol content is only 5% of the total lipid extract, but the former identifies ergosterol and stigmasterol as the major components, and the latter finds ergosterol, zymosterol and desmosterol.

7
The relative fatty acid compositions of each of the lipid classes was similar, reflecting the overall distributions of Table IV.3. The only consistent difference was that the phospholipid fraction contained slightly greater amounts of unsaturated fatty acids, in all cases, by about 10%. This is not consistent with Ponton et al., who found that phospholipids contained less unsaturated fatty acid than nonpolar lipids. The average distribution of the phospholipid class is well represented by that of the purified phospholipid fraction, as given in Table IV.3.

When the cells were starved, the triglyceride content was much reduced, and the total lipid content as % dry cell weight was also reduced. This represents efficient β -oxidation of the cytoplasmic triglyceride as a carbon source. This is also reflected in a reduced TG/PL ratio. The enzyme

preparation also shows a small triglyceride content, although it can easily be visualized by TLC. Thus, the results of NMR for this sample represent essentially those of the phospholipid moiety. Reasons for such a low lipid recovery (35%) are uncertain. There may be a greater proportion of sterols in this total lipid extract. In addition, the recovery of sterol esters by this method may be anomalously low. The sterol ester fractions in all systems of Table IV.4 should be therefore regarded as the minimum quantities.

The nystatin preparation shows triglyceride content which is reduced, but not as efficiently as that of the enzyme preparation. Some of the fatty globules may stick to the cell walls and not be completely removed. However, extraction of the supernatant after the cell wall and membrane material was removed, shows that triglyceride was present in that fraction. This evidence was supported by NMR (Chapter V). The inositol grown cells also show a sharp reduction of triglycerides, as has been observed previously (see next section).

Thus, the results of Table IV.3 show that in all cases, the majority of the relaxation results of Chapter V are attributable to the phospholipids of the system. The presences of triglyceride with all the preparations should not necessarily be regarded as an impurity due to unpurified membranes, since triglyceride has been reported to be associated with purified plasma and mitochondrial membrane fraction of yeast (Hunter and Rose, 1971).

(c) Phospholipid Head Group Analysis

The total phospholipid content of the organism is quite low, only 2% of cell dry weight, as compared with other yeasts. Prior to the paper of Ponton et al. (1980), the identities of the head groups composing the phospholipid portion were neither well-defined nor quantitated. Since this distribution is essential for interpreting the phosphorus NMR spectra, the head groups were quantitated in the following way.

The phospholipid purified from lipids of the fermentor-grown culture were used for this purpose, since quantities were too small from cultures of up to 1 l. However, lipids from normal and labelled cultures, starved cell cultures, inositol and deuterated cultures were checked by TLC, and were visually the same as the fraction quantitated. The head groups were first identified by TLC. The phospholipid mixture in chloroform/methanol was run on silica gel plates in several solvent systems including; (a) chloroform, methanol, water (65:25:4, v/v); (b) chloroform, methanol, acetic acid, water (25:15:4:2, v/v) and (c) chloroform, acetone, methanol, acetic acid, water (6:8:2:2:1, v/v). In order to confirm that all spots were indeed phospholipids, staining was done with phospholipid-specific reagent (Dittmer reagent (Dittmer and Lester, 1964)), which was prepared according to Kates (Techniques in Lipidology, p. 437, 1972). Eight major components were present,

identified as PC, PE, PS, PI, Cardiolipin, PA, Sphingomyelin, lyso PC, and a glycerophospholipid. Since the last component was only 1% of the mixture, it was not characterized further.

The head group species were quantitated in the following way (see Kates, Techniques of Lipidology). Prewashed silica gel plates were layered with a known weight of phospholipid and were developed in solvent system (a) above. The fractions were identified with standards and stained with iodine vapour. Since a blank run showed that the relative amount of silica gel from the plate influenced the recovery of the lipid, a minimum width of the plate was used - just enough to allow for adequate separation of the bands. Each fraction was scraped from the plate and placed in a small side-arm flask. Methyl esters were made in the usual way (see experimental section). Extraction of the methylation mixture was performed with both petroleum ether and hexane. The more polar solvent increased the total yield, but phospholipids distributions were unaffected by the extraction procedure. Fatty acids for each fraction were analyzed by GC and total mg of fatty acids calculated. Each fraction was then converted to mg of lipid by multiplying by the appropriate factor (see Table IV.5a), which take into account the head group weight and the fatty acid distribution.

In most solvent systems it is difficult to resolve PS and PI at the same time as the zwitterionic components.

Therefore, one sample of phospholipid was developed in (d) chloroform, methanol, acetic acid, water (65:25:5:5, v/v), in which the PI component was sufficiently separated from PS, but the other components (PC, PE, Card) were not resolved. The difference between the % of PI from this experiment, and the % of PI plus PS was therefore attributed to PS. The fact that PI concentration dominates that of PS was confirmed by observing the colouration of the PS/PI spot with Dittmer reagent. The sample spot turns pinkish-blue, whereas samples of pure PI turn pinkish-blue, and that of pure PS turn blue.

Table IV.5b shows the resultant phospholipid distribution as compared with the results of Ponton et al. (1980). The latter results are from a one day culture of a different strain. Noticeable differences are the increased amounts of PE and PA and decreased PS and cardiolipin in the three day culture. Small differences in the fatty acid distributions of each component were observed, but were not as large as those observed by Ponton et al. The predominant species are PC and PE as zwitterionic lipids and PS plus PI as charged lipids. There was virtually no PG present, but some cardiolipin. Thus the natural phospholipid mixture could be modelled as a PC/PE/charged lipid mixture in the weight ratio 1:1:0.7, where PS acts as a model charged lipid (see Chapter VI).

Table IV.5

PHOSPHOLIPID COMPOSITION OF A. PULLULANSa) Conversion Factors from Total mg Methyl Esters to mg Phospholipid

<u>Lipid</u>	<u>X Factor</u>
PC	1.38
PE	1.31
PI	1.52
PS	1.39
Card	1.28
lyso PC	1.78
PA	1.23
Sph	2.60

b) Distribution of Phospholipid Headgroups in Total Phospholipid

<u>Lipid</u>	<u>% Total PL</u>	<u>Results of Ponton, et al.</u>
PC	32.6	34.2
PE	29.2	18.1
PS	6	17.4
PI	15	12.6
Card	5.7	12.9
Sph	3.2	4.8
PA	6.8	
glycero-PL	1.1	1.0
lyso PC	0.4	

Effect of Carbon Sources

Table IV.3 also shows the effect on the acyl chain composition of growth on several carbon sources that had not been previously studied with this organism (Sevilla et al., 1977). This was attempted in order to manipulate the lipid content to the extent that differences could be seen in the ^{13}C relaxation times. Unfortunately, the versatility of the organism proved to be a disadvantage here, since it was able to utilize the added factor as a sole carbon source, thus masking the desired effect.

The organism was grown on glycerol plus glucose, with the intention that ^{13}C labelled glycerol could be incorporated preferentially into the backbone moiety of the lipids to look at the relaxation times of this part of the lipid. Yeasts can utilize glycerol as a carbon source provided they possess glycerol kinase, the enzyme which catalyzes the phosphorylation of glycerol to G3P (which then condenses with fatty acyl CoA to give PA - the precursor to all lipids) (Gancedo et al., 1968). A. pullulans had been previously observed to utilize glycerol (Clarke and Wallace, 1958). In this way, growth of an organism supplemented with ^{14}C glycerol is often used to quantitate the lipid content of cells. However, some organisms can utilize glycerol as the sole carbon source and grow well in that medium. Some strains have been shown to incorporate glycerol efficiently and exclusively into the backbone of

lipid, whereas acetate goes into acyl chains, when the organism is given a choice of carbon substrates (Kleinig and Kopp, 1978). It was discovered, however, that A. pullulans can utilize glycerol as a sole carbon source, with cell yields greater than on acetate as the sole carbon source. The cells from such a culture are devoid of mycelia, but many are swollen, some of which were observed to be dividing mitotically. The cells are much less active, as indicated by the dominance of most by one large vacuole, which does not take the Sudan Black stain, indicating that it is not a lipid storage vacuole. Cell yields were somewhat enhanced on glucose plus glycerol medium, but labelling experiments were not attempted due to the high cost of ^{13}C labelled glycerol, plus the likelihood of greater scrambling of the label than for acetate as indicated by the ability to use the substrate as sole carbon source. Such experiments to achieve lipids totally labelled biosynthetically could possibly be tried in the future.

Growth of A. pullulans supplemented by alkanes was also attempted in the light of evidence that the fatty acid composition of yeast could be altered by supplementing the medium with alkanes of various chain lengths. In particular, Candida cells have shown a factor of two increase in content of the lipid acyl chain of the length of the alkane substrate (Fukui et al., 1972). Growth on octadecane gave a lipid content that was 91% in 18-carbon long acyl chains and C-18:1

formed 67% of the total lipid composition. A. pullulans was grown on minimal medium with 1% octadecane miscible with the medium. Growth was sluggish and cell yield low. All the cells were in the yeast phase but of low metabolic activity, as indicated by the presence of few cytoplasmic bodies and only one large vacuole per cell. Table IV.3 shows the effect of this carbon source on the fatty acid composition. The C-18:0 content increased by a factor of three over that of normal growth conditions, to the detriment of the C-18:1 content. However, the overall change in the eighteen carbon composition is minimal, and the relative increase in stearoyl chains is not large enough to see a significant change in the ^{13}C relaxation times of the "average" lipid molecule.

For similar reasons, growth was also attempted on medium supplemented with 1% methanol. Many yeasts can utilize long chain alcohols as well as ethanol as carbon sources (Sols et al., 1971). However, many small alcohols also act as general anesthetics (Richards, 1978), which appear to have an effect of fluidizing natural and model membranes (Patterson et al., 1972; Miller and Pang, 1976), perhaps because they are highly soluble in lipid. Organisms grown in the presence of small doses of anesthetic show the tendency to alter their fatty acid composition to oppose this fluidizing effect (Nandini-Kishore et al., 1977), by producing more saturated

fatty acid. A. pullulans growth was not inhibited by 1% methanol added to the medium, but neither was the acyl chain content altered (see Table IV.3). When methanol grown cells were starved, the unsaturated chain content increased in the same manner as for normal cultures. It is possible that the methanol was simply used as a carbon source or that the amount was not high enough to have any effect. Since the organism survives on paint films, it is quite likely that small amounts of organic solvent can be easily tolerated. Further characterization would require toxicity and ^{14}C tracer studies. ^{13}C spectra of three day grown and starved methanol-supplemented cultures were identical to those of Chapter V.

A fourth perturbation was attempted in order to reduce the triglyceride content of the yeast, without the concomitant reduction in ^{13}C incorporation which occurs with starvation. There has been much attention paid recently to the role of myo-inositol in metabolism and many studies have correlated inositol-deficiency with triacylglycerol accumulation in yeasts (Challinor and Daniels, 1955; Lewin, 1965; Daum et al., 1979; Paltauf and Johnston, 1970). The only source of inositol in the medium used in these experiments is the small amount of yeast extract added, so that these conditions might be described as inositol-deficient. Inositol appears to be an essential vitamin for mammalian tissue (Hawthorne and White, 1975), but its mechanism of action is unknown. It

appears to be essential for mitochondrial function (Daum et al., 1977) and phospholipid production. Inositol is directly incorporated into phosphatidylinositol and from there into other phospholipids (Domínguez et al., 1978), and PI levels are enhanced when inositol is added to the growth medium (Daum et al., 1977).

Only a few species of yeast are known to utilize inositol as a sole carbon source (Hunter and Rose, 1971). A. pullulans was shown to be able to utilize inositol very efficiently as a sole carbon source and gave the morphology shown by Plate VI. Cell yields from 1% inositol medium were equal to those from normal medium. Yields from glucose or acetate supplementation of inositol were even greater. The fatty acid composition of these cells, as shown in Table IV.4, tends to be more unsaturated, with linoleic chains the predominant species (~62%). The triglyceride content is reduced as indicated by Table IV.5. Labelling studies of cultures grown on inositol plus labelled acetate are discussed in Chapter IX.

CHAPTER V
¹³C STUDIES OF MEMBRANE STRUCTURE

Introduction

While the ensemble of membranes in a unicellular organism perform a variety of specific tasks, the basic functions are threefold:

- (a) to form an expandable cover and protective barrier for the entire cell (plasma membrane) or for its sub-components (nuclear or vacuolar membranes);
- (b) to act as an organelle for the control of entry and exit of solutes for cellular metabolism;
- (c) to act as a synthetic organelle - a site at which cellular building materials are synthesized. The plasma membrane provides the site of synthesis of the cell wall polysaccharides and the mitochondrial membrane the site of protein synthesis.

The inherent properties of membranes, such as solute transport, flexibility and tensile strength, must be related to both the packing and individual mobility of the lipid molecules that compose the majority of the membrane. Research has tended to focus on the lipid moiety, since the properties of pure lipids are better defined than those of other membrane components. The effects of the addition to lipid of sterols, proteins and glycolipids are not yet well understood and are

the topic of much present research. The approach to date has been from the point of view of effects on the lipid matrix - few studies have attempted to understand these interactions from the protein or sterol point of view.

All functioning biological membranes are modelled on a lipid matrix behaving as a liquid-crystal. In general, an organism will adjust its lipid composition in order to ensure that the transition temperature (T_c) for the gel to liquid-crystal phase transition for the predominant phospholipid species is below the growth temperature. In the current model of the membrane (Singer-Nicholson model), the phospholipid molecules are oriented parallel to the normal of the bilayer, with the hydrophobic chains together toward the centre of the bilayer and the hydrophilic headgroups in the aqueous medium. The glycerol backbone is oriented almost parallel to the bilayer surface and the sn1 chain is kinked such that it appears shorter than the sn2 chain (Seelig and Seelig, 1980). The packing properties of the acyl chains define the cooperativity of motion between individual lipid molecules and can be probed by the order parameter obtained from ^2H NMR. The overall mobility of the acyl chains is the sum of all motions available to the molecule and can be probed by the NMR relaxation times (T_1 's). In ^{13}C NMR, the motion of the C-H dipole causes effective dipolar relaxation between the spin system and the lattice. The measured ^{13}C T_1 is therefore sensitive to the amplitude and rate of motion allowed by the immediate

environment about the ^{13}C nucleus. Since the spectrum probes the ensemble average of lipid molecules in the bilayer, the relaxation time represents the sum of all the individual motions of the acyl chains, which are a result of:

- (a) overall motion of the macroform, either vesicle, cell or liposome;
- (b) diffusion of the lipid molecule in the plane of the bilayer;
- (c) rapid rotation of the molecule about its long axis, and
- (d) the rapid gauche-trans isomerization which is characteristic of the liquid crystal phase.

The rate of gauche-trans isomerization is $\sim 10^{10}$ Hz, which is on the order of the correlation time of the ^{13}C nucleus, thus making ^{13}C relaxation times sensitive to this type of motion.

The object of the present research was to observe the average mobility of a functioning membrane as compared to model systems, such as liposomes or vesicles, which are composed of pure lipid. A higher unicellular organism was chosen not only for convenience, but to look at the mobility of a bilayer containing a greater extent of unsaturation in the acyl chain distribution. Bacterial membranes, on the whole, are much more saturated and are more subject to lysis. The plasma membrane, particularly, of yeast must be flexible.

in order to survive desiccation, changes in osmotic pressure and the process of sphaeroplasting. In general, a greater average mobility has been associated with more unsaturation, since the cis conformation of the carbon-carbon double bonds requires that the acyl chains occupy a greater average volume in the liquid-crystal phase (Seelig and Seelig, 1977; Seelig and Waespe-Sarcevic, 1978). The increasing packing disorder in cis unsaturated chains is reflected in a decreasing T_C with increasing unsaturation (Legaly et al., 1977). Since the whole cell is extremely complex, we cannot hope to elucidate differences between lipid sites or even different membranes within the cell. Such a study would require careful biochemical characterization as well as purification procedures that would result in very small samples, which would make the measurement of relaxation times prohibitively long. An attempt has been made therefore to compromise the requirements of the NMR experiment and the complexity of the system in order to determine the applicability of model system studies to the real situation.

The comparison of whole cell relaxation times to model lipid bilayers allows the estimation of the motional contributions of the protein and sterol fractions in their natural proportions, at least to those motions sensitive to the ^{13}C nucleus. It has generally been established that sterols have an ordering effect in the membrane, as determined from model systems studied by various techniques (Demel and de

Kruijff, 1980; Stockton et al., 1976; Paphadjopoulos and Kimelberg, 1973; Seelig and Seelig, 1980 and references therein). While the effect of sterol (specifically cholesterol) on the acyl chains is one of ordering (Gally et al., 1976; Stockton et al., 1976; Jacobs and Oldfield, 1978), the effect on the lipid headgroup appears to be one of disordering (Brown and Seelig, 1978; Oldfield et al., 1978). Cholesterol is normally chosen for experimental studies since this is the main sterol in mammalian tissue and has therefore direct medical application. However, lanosterol has been shown to have a much smaller immobilization effect on lipid bilayers than cholesterol (Yeagle et al., 1977), so it is possible that such effects are a function of the individual sterol composition. The sterols of A. pullulans, like all yeasts, are more simple than cholesterol (such as ergosterol, zymosterol, and lanosterol (Ponton et al., 1980), and may have a relatively small effect on the overall mobility of the lipid bilayer, by analogy.

The role of protein is not as well understood and likely is not as simple, since the conformation of proteins is an important factor in their activities as enzymes. Some researchers have claimed that the protein induces a local rigidity (or lipid annulus) in the membrane (Davoust et al., 1980; Kung et al., 1980; Owicki and McConnell, 1979), while others have proposed that the flexibility of the protein matches that of the surrounding lipid so that there is no

net effect (Bloom, 1979; Seelig and Seelig, 1980). This latter proposal is based on evidence that there is no large effect of protein on the overall order (as determined from quadrupole splittings in ^2H NMR) or overall dynamics (as determined from NMR relaxation times). One factor supporting the presence of a lipid annulus is that the surrounding lipid environment is required for protein function, suggesting that there is a strong lipid-protein interaction (Warren et al., 1975). In contrast to the effect of sterols, there is some evidence that the effect of protein is perhaps greater at the headgroup than in the acyl chains (Rajan et al., 1981). The variation in experimental results is likely a function of the relative lipid to protein ratio (Chapman et al., 1979), but the reconciliation of these differing interpretations lies in the contribution of protein to the different parameters probed by the various experimental techniques. There may be a profound effect of protein on the lipid matrix, but not at the frequencies of motions sensed by conventional NMR experiments. The greatest effect of protein may be at the low frequency motions of lipid (Deese et al., 1981), such as coordinated chain wagging or motions sympathetic with those of protein. Perhaps the most satisfactory explanation is one which requires that the lipid molecules of the lipid annulus are in fast exchange on the NMR timescale (Watts et al., 1981). Since the protein to lipid ratio in many yeasts is approximately 1:1 (Hunter and Rose, 1971), any effect of the protein component

on the cis-trans mobility of the lipid moiety should be easily observed by the ^{13}C relaxation times.

Since the inherent sensitivity of the ^{13}C nucleus is weak, it is difficult to perform NMR relaxation experiments in a reasonable length of time. The sensitivity is further decreased by the inherent dilution of the lipid species within the intact cell. Few intact membrane systems have therefore been studied in natural abundance. It is somewhat easier to look at the linewidths of spectral lines (see, for example, of mitochondrial and chloroplast membranes (Keough *et al.*, 1973) to evaluate T_2 , although there are inherent difficulties in accurately evaluating T_2 by this method. Thus, most natural abundance studies have been performed on model membrane systems (single bilayer vesicles) composed of pure lipids (Lee *et al.*, 1976; Levine *et al.*, 1972; Sears, 1975) or composed of natural lipid mixtures extracted from cells (Degani *et al.*, 1980). From studies of this type, authors seem to agree that the average lipid environment as sensed by the ^{13}C nucleus is motionally restricted in a bilayer as compared with a solvated or aggregated form. The effect of cholesterol on the ^{13}C relaxation times appears generally to reduce the degree of acyl chain mobility as determined both by T_1 (Brainard and Cordes, 1981) and T_2 (Lancee-Heimkens and de Kruijff, 1977). However, the effect of protein on the acyl chain relaxation times is not clear. Isolated lipoproteins show T_1 values

which are similar to those of pure lipid (Williams et al., 1974) and the addition of cytochrome C to cardiolipin-PC vesicles does not significantly change the lipid T_1 values (Brown and Wüthrich, 1977), while other authors have observed profound changes in acyl chain T_1 's upon association with protein (Stoffel et al., 1977).

In addition to the time factor, the spectral resolution of the lipid component in a natural abundance spectrum is not sufficient to resolve individual positions within the acyl chain. Most natural abundance studies show that only the terminal methyl, penultimate carbon and any unsaturated carbons can be resolved from the bulk methylene resonance. The time problem may be overcome by isotopically enriching the lipid moiety, while both time and resolution problems can be overcome simultaneously by using specific enrichment of certain positions of the acyl chain. While this procedure has been used extensively in model membrane studies by ^2H NMR, it proves to be an expensive and tedious procedure in ^{13}C NMR, since significant enrichment is required to reduce the time factor in measuring T_1 's. Specifically (and chemically) labelled lipids have been employed in only a few studies, both in systems in vitro (Schmidt et al., 1977; Stoffel et al., 1977) and in viral membranes by biosynthetic incorporation (Stoffel et al., 1976; Stoffel and Bister, 1975). The uptake of labelled fatty acids by most organisms is small unless the

de novo fatty acid biosynthesis is blocked. Although this method has been used with much success in producing deuterated lipid in A. laidlawii (see, for example, Rance et al., 1980) and E. coli (see, for example, Nichol et al., 1980), it has not been used for ^{13}C incorporation, except for one preliminary study (Metcalf et al., 1972).

The highest incorporation of ^{13}C into intact membranes has been obtained by biosynthetic incorporation into lipids from labelled precursors, the most successful precursor being acetate. Since the process of fatty acid chain elongation employs the incorporation of acetate units from acetyl CoA, acetate will be preferentially incorporated into the lipid moiety when the organism is presented with a variety of carbon sources. Few microorganisms will grow on acetate as a sole carbon source, but many yeasts and fungi are exceptions to this rule. Growth of an organism on labelled acetate as a sole carbon source is useful for obtaining highly labelled lipid, but does nothing towards simplifying the spectrum of the whole cell. The membrane structure of C. utilis has been studied by London and coworkers (Eakin et al., 1972; London et al., 1975) in which the signal to noise problem has been reduced by culturing the cells on 20% randomly enriched ^{13}C acetate as a sole carbon source. The relaxation times of the lipid moiety are easily measured, but spectral resolution of the lipid is not aided by random enrichment. Due

to the highly unsaturated nature of yeast lipids, however, the spectral lines are relatively narrow as compared to bacteria (Birdsall et al., 1975; Joyce and Smith, 1979), and several regions of the acyl chain may be resolved. The situation is further complicated by the heterogeneous distribution of acyl chain species - the lipid composition of C. utilis is almost equally divided between four different chain species.

If the labelled acetate is supplemented with an alternate unlabelled carbon source, the acetate is used preferentially for lipid synthesis by some organisms. This has been illustrated for Tetrahymena (Nwanze et al., 1977), and fibroblast cells (Howard, 1977), as well as in the present study. It is also possible to mutate E. coli to achieve the same result (Cronan and Batchelor, 1973). This method achieves an efficient use of the ^{13}C label, with little being lost as a metabolic byproduct. In addition, the ^{13}C spectrum will be simplified by the elimination of resonances due to peptide and carbohydrate.

The spectrum can be further simplified by the use of specifically labelled acetate, where the label is either exclusively on the methyl or carbonyl positions. Since the entire acetate unit is incorporated into the fatty acid chain (as opposed to the process of keto-chain elongation, see Chapter VII), this results in the labelling of alternate

carbon positions down the chain. This biochemical pathway has been shown by ^{13}C NMR in E. coli (Birdsall et al., 1975), M. freudenreichii (Joyce and Smith, 1979) and Tetrahymena (Nwanze et al., 1977), although the method has not been used to study membrane structure in these organisms.

In addition to providing a method for studying membrane structure in situ, the preferential use of acetate also provides an easy route to specifically and efficiently labelled lipid, both by carbon isotopes ^{13}C and ^{14}C , and by deuterium (White, 1980).

The biosynthetic scheme for acetate incorporation into the acyl chains of lipids is illustrated in Figure V.1. In the absence of externally supplied acetate, acetate units are derived from glucose. Malonyl CoA, produced from acetyl CoA, provides the condensing reagent. Additions of the acetate unit are accomplished on the substrate of acyl carrier protein (ACP). The acyl chains are elongated to give the correct distribution of chain lengths required by the cell. Desaturation by the various desaturases occurs after the removal of the ACP substrate. The fatty acids are then condensed onto the glycerol phosphate backbone to form phosphatidic acid, the precursor to all phospholipids. Excess fatty acids are stored in the cytoplasm, either in free form or as triglycerides. The condensation pattern mediated by ACP results in the even-numbered carbons of the acyl chain deriving from

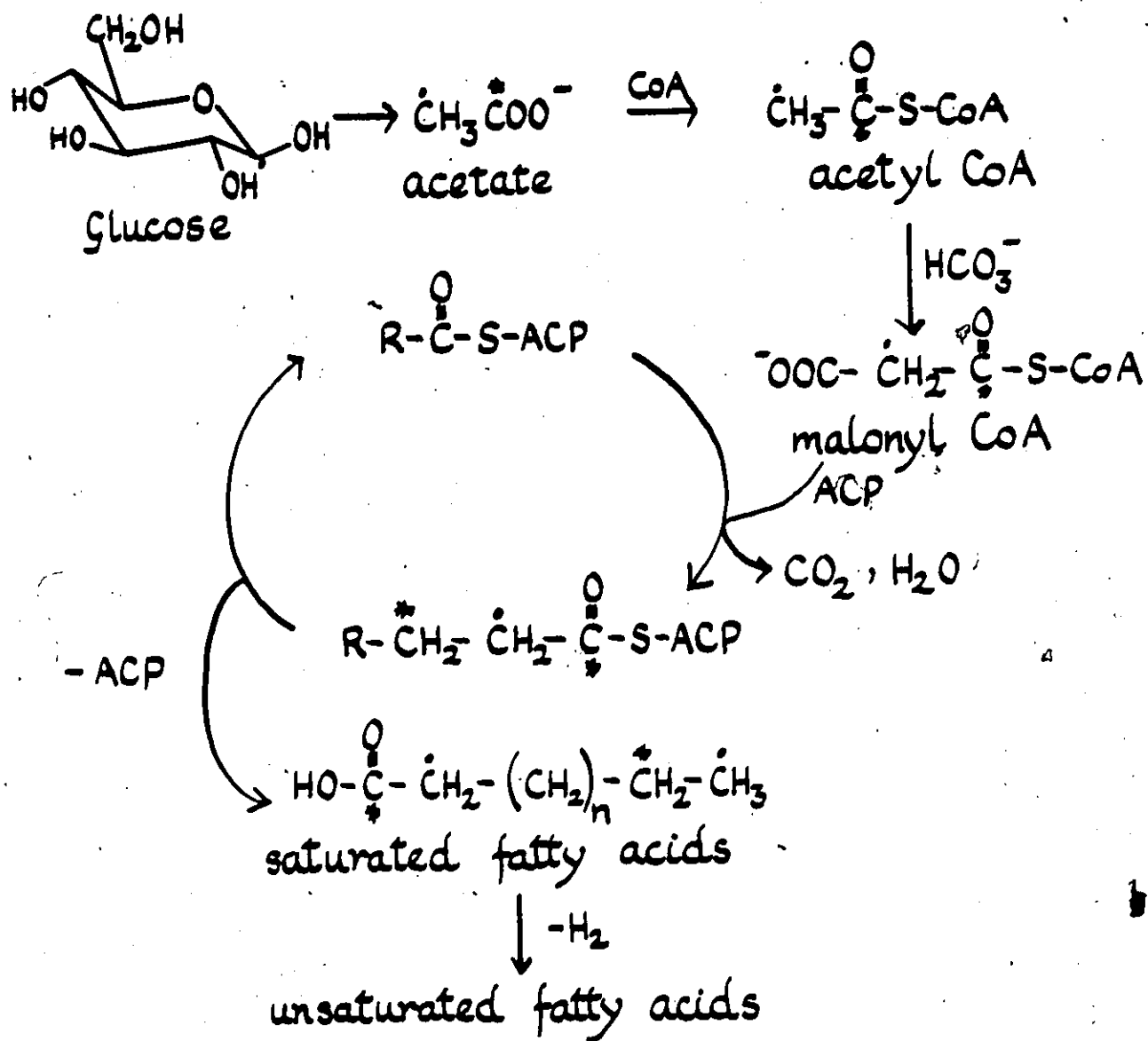


Figure V.1 - Biosynthetic pathway for acetate incorporation into lipid via fatty acid chain elongation. The dot and asterisk mark the metabolic fate of ^{13}C -2 acetate and ^{13}C -1 acetate respectively

the methyl group of the acetate unit, while the odd-numbered carbons originate from the carbonyl carbon. This biosynthetic pathway also eliminates spectral complication due to ^{13}C - ^{13}C couplings, since each labelled carbon has a natural abundance carbon as its immediate neighbour.

Theory of ^{13}C Relaxation Times

The loss of magnetization of the ^{13}C nucleus as observed by NMR spectra is exponential and may be characterized by the following equation (see Weiss *et al.*, 1980):

$$S(\tau) = S(\infty) [1 - \{1 + A(1 - e^{-W/T_1})\} e^{-\tau/T_1}] \quad (\text{V.1})$$

where $S(\tau)$ and $S(\infty)$ refer to the spectral intensities at times τ and ∞ , respectively, in the pulse sequence, "A" refers to the flip angle, which under conditions of perfect 90° and 180° pulses is equal to 1. If T_1 is measured by inversion recovery pulse sequence, $W=0$. Thus equation V.1 reduces to the more familiar form:

$$S(\tau) = S(\infty) [1 - 2e^{-\tau/T_1}] \quad (\text{V.2})$$

There are many pulse techniques by which to measure T_1 , but the one best characterized and most suitable for the purposes of these experiments is the inversion recovery pulse sequence ($[180^\circ - \tau - 90^\circ - (T)]_n$ for varying τ values). To follow the recovery curve, the magnetization must be sampled at several values of τ (i.e., $S(\tau)$),

including one in which τ is very long ($S(\infty)$). At short settings of τ , the spectra show inverted lines, which later pass through the null condition and become normal positive-going signals. A rough estimate of T_1 may be obtained by sampling the null condition. Thus $T_1 = 1.45\tau_0$, where τ_0 is the value at which $S(\tau) = 0$. Each pulse sequence must be separated from the previous one by a time T , which is long compared with the relaxation time, in order for thermal equilibrium to be reestablished. For accurate measurement, $T = 5 \times T_1$ for the longest T_1 . In the present experiments, T was chosen to be 5 sec for the ^{13}C -2 acetate-derived spectra and 4 sec for the ^{13}C -1 acetate-derived spectra, except for the case of lipid in chloroform, where T_1 's are much longer. This choice adequately satisfies the $5 \times T_1$ condition for all resonances except that of the terminal methyl group. The waiting time in the latter case is not less than $3 \times T_1$ (CH_3). However, the error introduced by using a T of $3 \times T_1$ (FIRFT experiment) is relatively small compared to the inherent error in measuring T_1 (Canet et al., 1975).

The accuracy in determining relaxation times as well as the optimization of methods in terms of spectrometer time, has been the subject of much theoretical research in the last decade. All the aspects of T_1 measurement - from the choice of pulse method used to determine T_1 , the choice of parameters such as τ values and the method of data analysis, to the model

used to explain the observed relaxation times - must be carefully chosen for the particular system under study in order to maximize the accuracy of the T_1 measurement. It is generally agreed that in systems where relaxation times are relatively short (i.e., < 5 sec) that the most accurate method of measuring T_1 is by the inversion-recovery pulse sequence (IRFT) where $T \geq 5T_1$ (Becker et al., 1980; Levy and Peat, 1975). When T_1 's are long, the required spectrometer time becomes prohibitive and other methods of determination are preferred, such as progressive saturation or saturation recovery). Since the systems used here have short accumulation times due to isotopic enrichment and T_1 's less than 1.5 seconds, the relaxation times can be measured by the IRFT method in a relatively short time, including accurate determination of the infinity value ($S(\infty)$).

The τ values must be properly chosen in order to characterize accurately the exponential decay curve. This is especially difficult when a simultaneous measurement of several relaxation times is attempted. For very short τ values, the accuracy of the spectrometer hardware becomes important (more so at lower field), while for values greater than $2xT_1$, inaccuracies in the determination of $S(\infty)$ introduce changes in linearity of the relaxation plot (Gerhards and Dietrich, 1976). For the present experiments, T_1 's were calculated

using only those points where $\tau \leq 1.5 \times T_1$ of the particular resonance. Fortunately, it has been shown that evenly spaced τ values are not necessary for accuracy (Harris and Newman, 1976), since this condition would be difficult to achieve in an experiment which simultaneously measures relaxation times spanning an order of magnitude. A choice of values spanning the range $0 < \tau < 2T_1$ is adequate. A range of τ values ensures that there is no additional error introduced due to poor signal to noise for τ values near the null point.

Authors employ the term "optimization" in these theoretical investigations, since the object is to ensure maximum accuracy for the minimum number of τ values (i.e., minimum spectrometer time; see for example, Weiss et al., 1980; Bernassau and Hyafil, 1980). This appears to be a sensitive function of the system under study and theoretical formulae for parameter optimization must be used with caution. For example, the procedure suggested by Bernassau and Hyafil (1980) was employed on a few occasions in measuring the relaxation times for whole cell and membrane preparations. Optimal τ values are calculated for simultaneous measurement of T_1 's for several resonances for which the maximum and minimum T_1 can be accurately estimated. The τ values are designed to minimize the number of spectra required for complete determination of the set of T_1 's to the same degree of accuracy. It appears that this procedure works best if the set of T_1 's are evenly distributed between the minimum and

maximum values; a case which is not likely to occur in a real biological system. In the present experiments, the majority of T_1 values fall within a factor of two, with one or two values significantly longer than the rest.

Application of the optimization procedure resulted in the majority of T_1 values not characterized by enough points to ensure accuracy, especially if τ values greater than $2xT_1$ were ignored. The procedure of evenly spacing τ values to cover the range of T_1 values was therefore preferred, and did not result in any significant increase in spectrometer time.

Since spectrometer conditions varied substantially over the period that these experiments were conducted, it was useful to have a standard sample, whose T_1 was accurately known and could be measured in a few minutes. The sample used for this purpose was a 2:1 (v/v) solution of dioxane in D_2O , in the manner of Armitage et al., (1974). This ensured that T_1 's from different spectrometers could be confidently compared and reduced errors from experiment to experiment due to misset pulses or pulse programming.

The results of an inversion recovery experiment are commonly analyzed by a linear regression plot of Equation V.2 in the familiar log form:

$$\ln(S(\infty) - S(\tau)) = \ln 2S(\infty) - \frac{\tau}{T_1} \quad (V.3)$$

Other possible methods of analysis include a two parameter exponential fit of Equation V.2, in which the T_1 value and the infinity value are fitted simultaneously, and a three parameter fit, which corrects in addition for miset pulses. The relative accuracy of these methods has been discussed by Pitner and Whitby (1979). The linear regression method is fairly insensitive to instrumental inaccuracy and allows one to assess the relative accuracy of experimental points, but an accurate determination of the infinity value is essential. The relative accuracy can be roughly assessed by comparison of the intercept of the plot with the theoretical value of $2S(\infty)$. There is no significant difference in accuracy between the two and three parameter exponential fits, but statistical errors are only as good as the algorithm used to converge to the best fit values. These fitting procedures are preferred when accuracy is of utmost importance and signal to noise is good (Pitner and Whitby, 1979; Gerhards and Dietrich, 1976; Weiss et al., 1980). The two parameter fit is more sensitive to miset pulses but does not require an accurate determination of the infinity value. For these reasons and for consistency, the linear regression technique was used for T_1 measurements at 20 MHz (CFT-20), and a two parameter fit was used for the data obtained at 75 MHz (CXP-300). Cross checks were performed at both frequencies and the results of the three analysis procedures (linear regression, two and three parameter fits) were the same

(within 5%) for experiments with good signal to noise. This choice was made since the pulse length for a 90° pulse was much longer at the lower field and could not be as accurately controlled over the spectral frequency range as at 75 MHz. The increased sensitivity at higher field made it possible to ensure that the pulse length was correct for the sample. The exponential fitting procedure was performed with the algorithm of the Nicolet NMR software package (NTCFT-1280). Reproducibility error was determined by running 3-5 sets of T_1 's from different cultures and separate membrane preparations. Statistical error was much more difficult to estimate. For linear regression plots, the scatter of experimental points provides a good estimation. The Nicolet exponential fit, however, gave an anomalously small value of error, determined from the degree of convergence to the two fitted values. Several sets of data were calculated using an algorithm of MacDonald (1980), in order to estimate more accurately the statistical error in performing the exponential fit. This error was 4-10% on most experiments, but up to 20% for experiments with poor signal to noise.

The infinity value was normally measured twice; at the beginning and at the end of the series of τ values. At 75 MHz, these two values were consistently within measured error. However, at 20 MHz, the infinity value was often reduced during the experiment, perhaps due to settling of the

sample. This problem was reduced by allowing the sample to settle for an hour before starting the experiment, but this reduced the viability in the case of live cells. If the infinity values differed by more than 10%, they were corrected for each τ value by assuming a linear change between the initial and final values. On a linear regression plot, which is sensitive to errors in $S(\infty)$, this correction improved the linearity of the plot (i.e., reduced the curvature introduced by using an average $S(\infty)$ value).

The correlation time, which is extracted from the relaxation time of the NMR experiment, is model-dependent. The model must account for the field dependence of relaxation times as well as other empirically observed results. Pure dipolar relaxation may be expressed in terms of the spectral density function $J(\omega)$ which is frequency dependent:

$$\frac{1}{NT_1} = \frac{\hbar^2 \gamma_C^2 \gamma_H^2}{20r^6} [J_0(\omega_H - \omega_C) + 3J_1(\omega_C) + 6J_2(\omega_H + \omega_C)] \quad (V.4)$$

where N is the number of directly bonded protons, r is the C-H bond distance, γ_C and γ_H are the gyromagnetic ratios of the ^{13}C and ^1H nuclei respectively, and ω_C and ω_H are the Larmor frequencies in radians per sec of the two nuclei. This expression is derived from consideration only of the motion of the local field set up by the C-H dipole within the static magnetic field (see Abragam, Chapter 8, (1961);

Carrington and McLaughlin, 1967). The expression for $J(\omega)$ assumes a form of the correlation function - the function which expresses the correlation between the ^{13}C and directly attached proton - in terms of τ_C , the correlation time (the average time the two nuclei remain in the same relative position on the NMR timescale). Thus the correlation time represents the period of motion of the C-H dipole. If it is assumed that the total motion of each dipole is essentially isotropic, the spectral density function takes the form of Equation V.5.

$$J(\omega) = \frac{2\tau_C}{1 + \omega^2 \tau_C^2} \quad (\text{V.5})$$

Substitution of Equation V.5 into Equation V.4 gives the following:

$$\frac{1}{NT_1} = \frac{\hbar^2 \gamma_C^2 \gamma_H^2}{10r^6} \left[\frac{\tau_C}{1 + (\omega_H - \omega_C)^2 \tau_C^2} + \frac{3\tau_C}{1 + \omega_C^2 \tau_C^2} + \frac{6\tau_C}{1 + (\omega_H + \omega_C)^2 \tau_C^2} \right] \quad (\text{V.6})$$

(Doddrell et al., 1972). It is this expression which is plotted in Figure V.9, for $N=1$. While the assumption of isotropic overall motion may appear crude for an unsymmetric molecule or an oriented system, the model is satisfactory in predicting the observed T_1 minima, field dependence and

temperature behaviour in many systems. Other models have been developed in order to interpret more accurately relaxation results from membrane systems. These include Woessner's model of isotropic overall motion with anisotropic internal motion (Woessner, 1961), Howard's model based on librational motion with overall rotation (Howard, 1979), and other more statistical models assuming small amplitude jump motions (Wittebort and Szabo, 1977; London and Avitabile, 1977; and Brainard and Szabo, 1981). All models behave quite similarly in the region of fast motion, and reduce, in the limit of very fast motion to the expression,

$$\frac{1}{NT_1} = \frac{h^2 \gamma_C^2 \gamma_H^2}{r^6} \tau_C \quad (V.7)$$

The model of effective isotropic reorientation is very sensitive to the value chosen for r . This value is normally chosen from solid state studies (x-ray crystallography) and may not accurately apply to more liquid-like phases. It has been suggested that an accurate choice of r may allow this model to more satisfactorily account for experimental results on a wide variety of systems, than more sophisticated and more restricted models (Dill and Allerhand, 1979). Since the motions sensed by the ^{13}C relaxation times are relatively fast segmental motions, they are quite adequately described

in terms of a single correlation time (Brown, 1979), as a first approximation. Further details of ^{13}C relaxation theory may be found in a review by Wright *et al.* (1979).

If the isotropic model is assumed, a T_1 minimum, which occurs at the Larmor frequency, is predicted. Thus, a measure of the field dependence of T_1 will aid in correctly determining an effective correlation time. Since any value of T_1 can give rise to two values, it is sufficient to measure T_1 at two sufficiently different fields, in order to determine the presence or absence of a field dependence. This can also be used to determine the co-operativity of various types of motion contributing to the observed relaxation (Levy *et al.*, 1977). If the relaxation mechanism is exclusively dipolar, the measurement of the Nuclear Overhauser Enhancement (NOE) will also aid in determining whether the motions contributing to relaxation are slower or faster than the Larmor frequency. The NOE effect arises from enhancement of ^{13}C resonance intensity during proton irradiation (decoupling) due to strong dipolar coupling between ^{13}C nuclei and their attached protons. The expression for the NOE factor is given by:

$$\text{NOE} = 1 + \frac{\gamma_H}{\gamma_C} \left[\frac{\frac{6\tau_C}{1+(w_H+w_C)^2\tau_C^2} - \frac{\tau_C}{1+(w_H-w_C)^2\tau_C^2}}{\frac{\tau_C}{1+(w_H-w_C)^2\tau_C^2} + \frac{3\tau_C}{1+w_C^2\tau_C^2} + \frac{6\tau_C}{1+(w_H+w_C)^2\tau_C^2}} \right] \quad (\text{V.8})$$

and is plotted in Figure V.9. In the limit of fast motions, a full NOE of 2.99 will be developed, while in the slow motion limit, a minimum value of 1.15 is predicted. As with T_1 measurement, it is very difficult to determine NOE values accurately, and several precautions must be observed. Anomalous low values of NOE are obtained unless 90° pulses and recycle times $>5T_1$ are employed in obtaining spectra (Canet, 1976). For these reasons, NOE measurements were made using 10 sec recycle times in all cases. Even with care, NOE reproducibility was no better than 10% (a significant factor if one is looking for small differences near the T_1 minimum) and measurements were made only for spectra with good signal to noise values.

At higher magnetic fields, the chemical shift anisotropy (CSA) mechanism can become more important in the relaxation of the ^{13}C nucleus, particularly for carbons of high substitution or low symmetry. Since the overall observed T_1 is a sum of all mechanisms contributing to the relaxation in the manner:

$$\frac{1}{T_{1\text{OBS}}} = \frac{1}{T_{1\text{D}}} + \frac{1}{T_{1\text{CSA}}} + \dots \quad (\text{V.9})$$

the presence of CSA will cause the measured T_1 's to be longer (i.e., relaxation is faster). A dampening of effects caused by different motional environments will also be predicted. In addition, spin rotation may contribute to the relaxation

process, but its effects will only be observed in the terminal methyl group of the acyl chain, which has full rotational freedom. The observed contribution of these mechanisms to the system under study will be discussed in the next section.

Results

The spectra of whole cells grown on media supplemented with the two specifically labelled acetates are shown in Figures V.2 and V.3. The dominant resonances can be assigned to lipid (using a C-18:1 acyl chain model as one of the predominant lipid species), showing that that acetate has been preferentially incorporated into the lipid fraction. The additional peaks in the carbohydrate region of the spectrum may be assigned to natural abundance glucose, as well as free amino acids (see Chapter VII). The complementarity of these two spectra can be seen by comparison to the natural abundance spectrum of whole cells (Figure V.4). Superposition of the lipid portion of each of the specifically labelled spectra gives the total lipid spectrum of the unlabelled species. The enhanced sensitivity of the labelled lipid can be seen by comparison to the latter spectrum where the unlabelled lipid is less intense than the natural abundance glucose. Thus, specific enrichment aids greatly in the spectral resolution of the various acyl chain positions. Only certain resonances can be assigned to more than one chain position, and even these are due to a maximum of two positions. The spectral

P. pullulans cells
Acetate - $^{13}\text{C}_1$

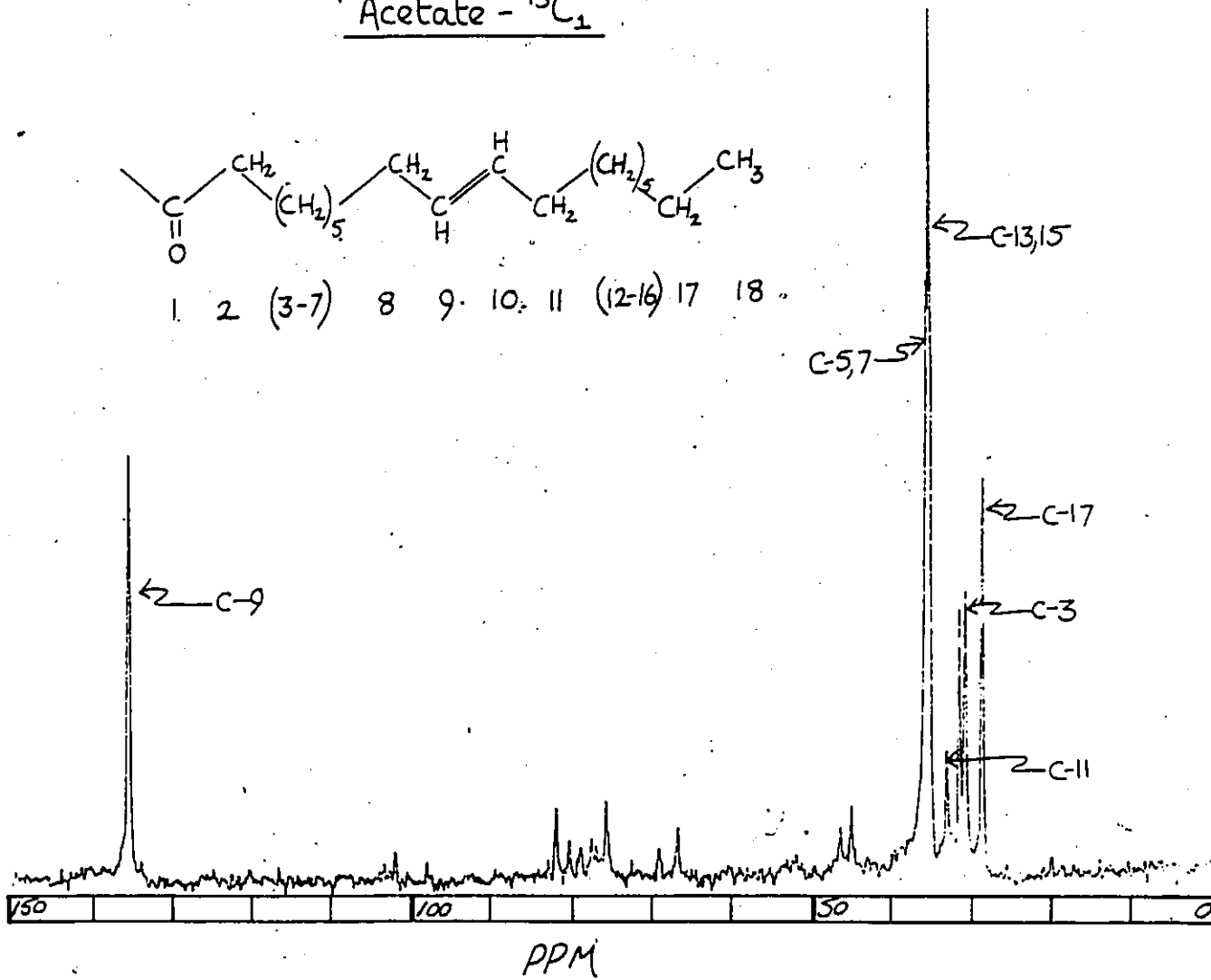
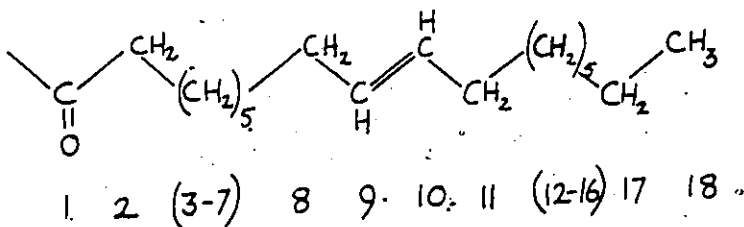


Figure V.2 - ^{13}C NMR spectrum (20 MHz) of packed whole cells of A. pullulans in D_2O at 30°C . The cells were grown on media enriched in ^{13}C -1 sodium acetate

P. pullulans cells
Acetate- $^{13}\text{C}_2$

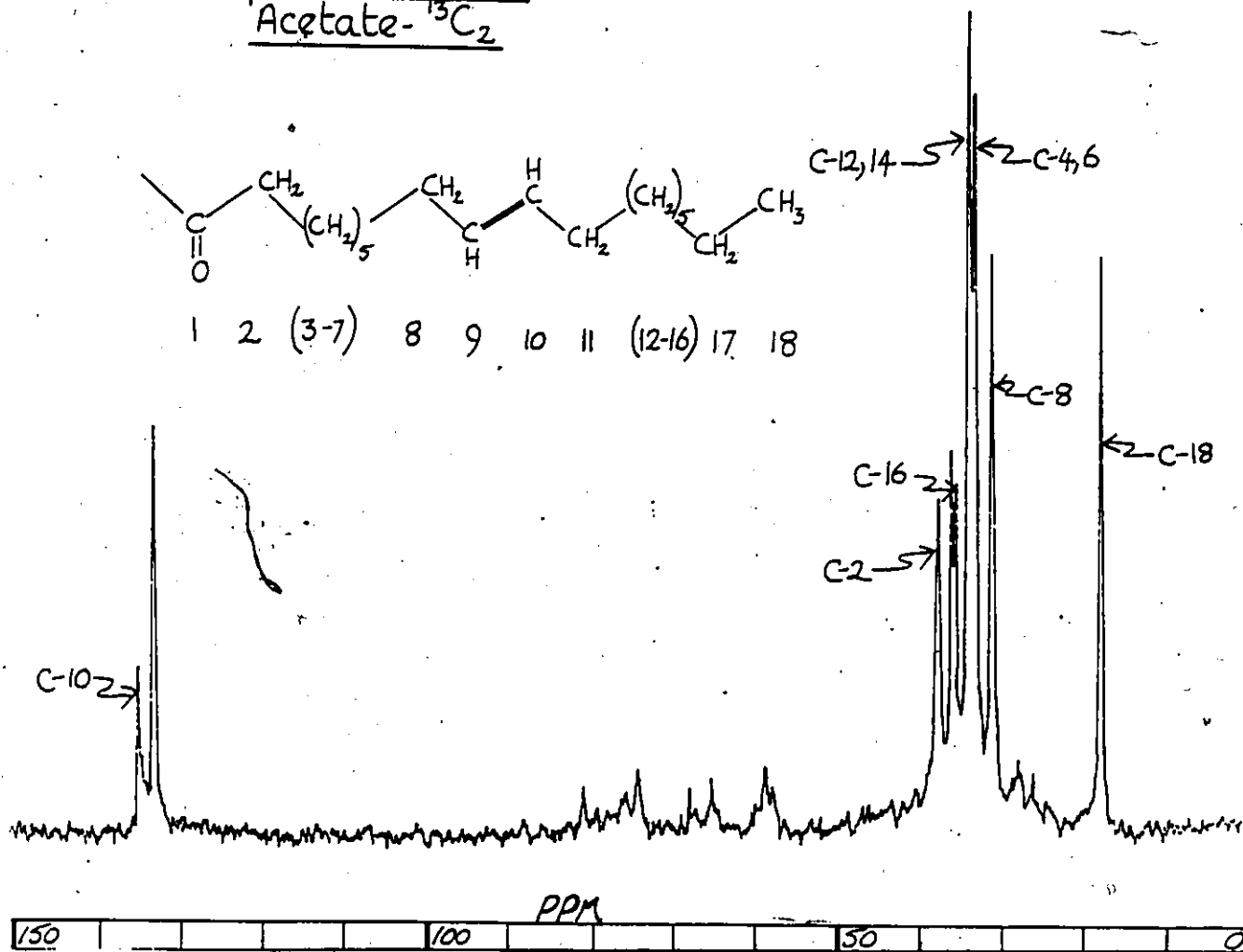


Figure V.3 - Same as Figure V.2 for cells supplemented with ^{13}C -2 sodium acetate

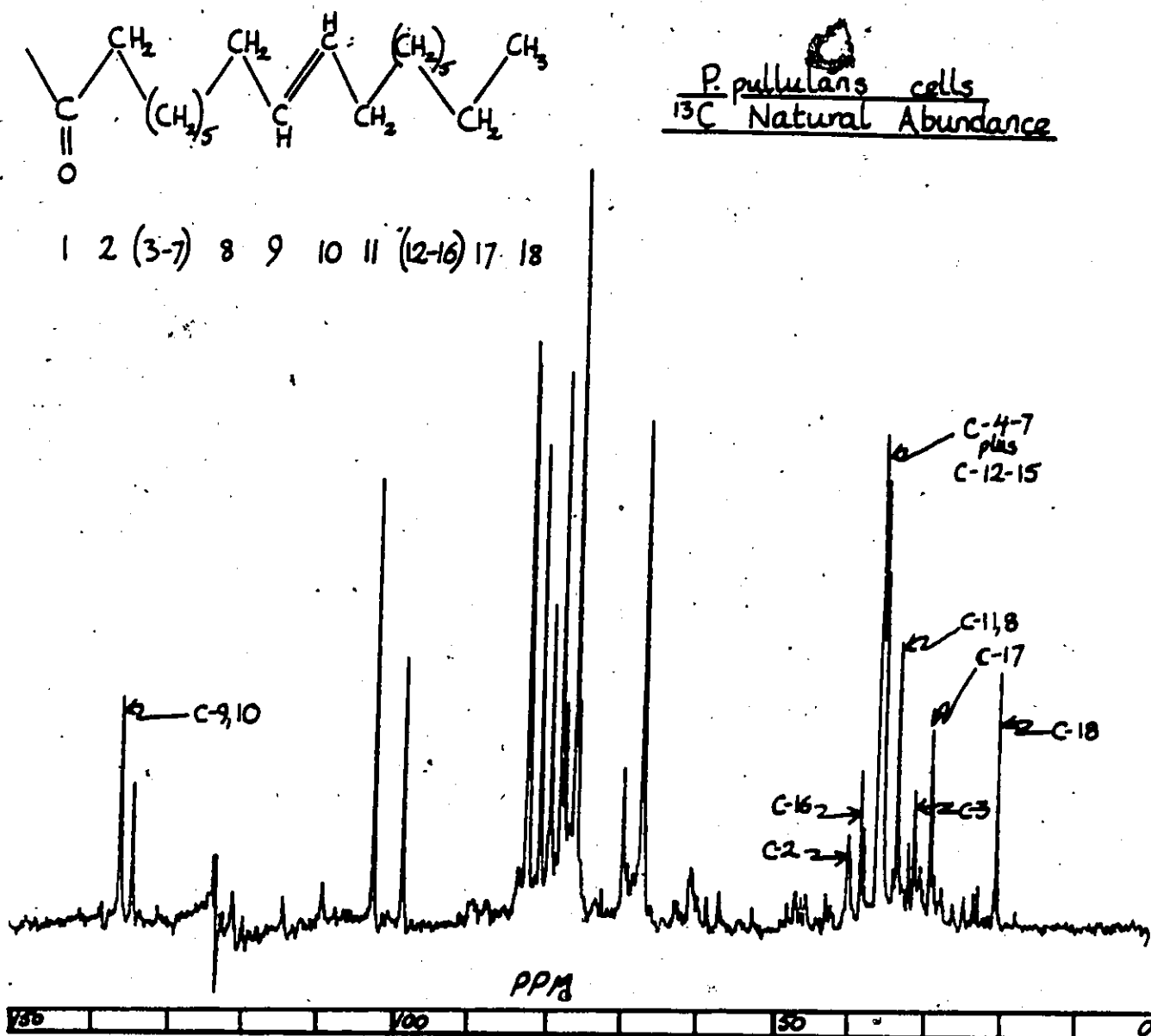


Figure V.4 - 20 MHz natural abundance ^{13}C NMR spectrum of whole packed cells at 30°C. The central resonances due to storage glucose represent the same concentrations as observed in Figure V.2 and V.3, and the lipid resonances represent the sum of resonances observed in these two previous figures. The spectrum represents 9000 accumulations

lines are well resolved for such an ordered system, due to the high degree of unsaturation of the fatty acids, which leads to a high fluidity (high degree of motion). Much poorer resolution was observed on bacterial systems where fatty acids were more saturated (Joyce and Smith, 1979). The specifically labelled systems are also shown at higher field in Figures V.5 and V.6 (75 MHz). These latter spectra are typical infinity spectra in a T_1 sequence. The enhanced resolution and sensitivity can be noted from the fact that these spectra are a result of ~ the same number of accumulations (200-300) at half the cell concentration used for the lower field measurement. Comparison of the intensities of labelled to unlabelled species in the two spectra (see, for example, the methyl and olefinic unlabelled peaks in the ^{13}C -1 acetate-derived spectrum) show that the peak intensities are enhanced by a factor of 15-20 times over natural abundance. The GCMS results show the presence of an average of 3-4 ^{13}C atoms per chain. The natural abundance content (1.1%) is 0.2 ^{13}C per chain, assuming a predominance of C-18 chains. This also gives an enhancement figure of x 15-20. The lipid analysis shows that up to 75% of the total fatty acid content is due to C-18:1 and C-18:2 acyl chains, with the latter composing almost 50% of the total. Therefore the spectrum can be assigned on the basis that it arises largely from these two chain species. The chemical shifts and the assignments of the seven lipid peaks

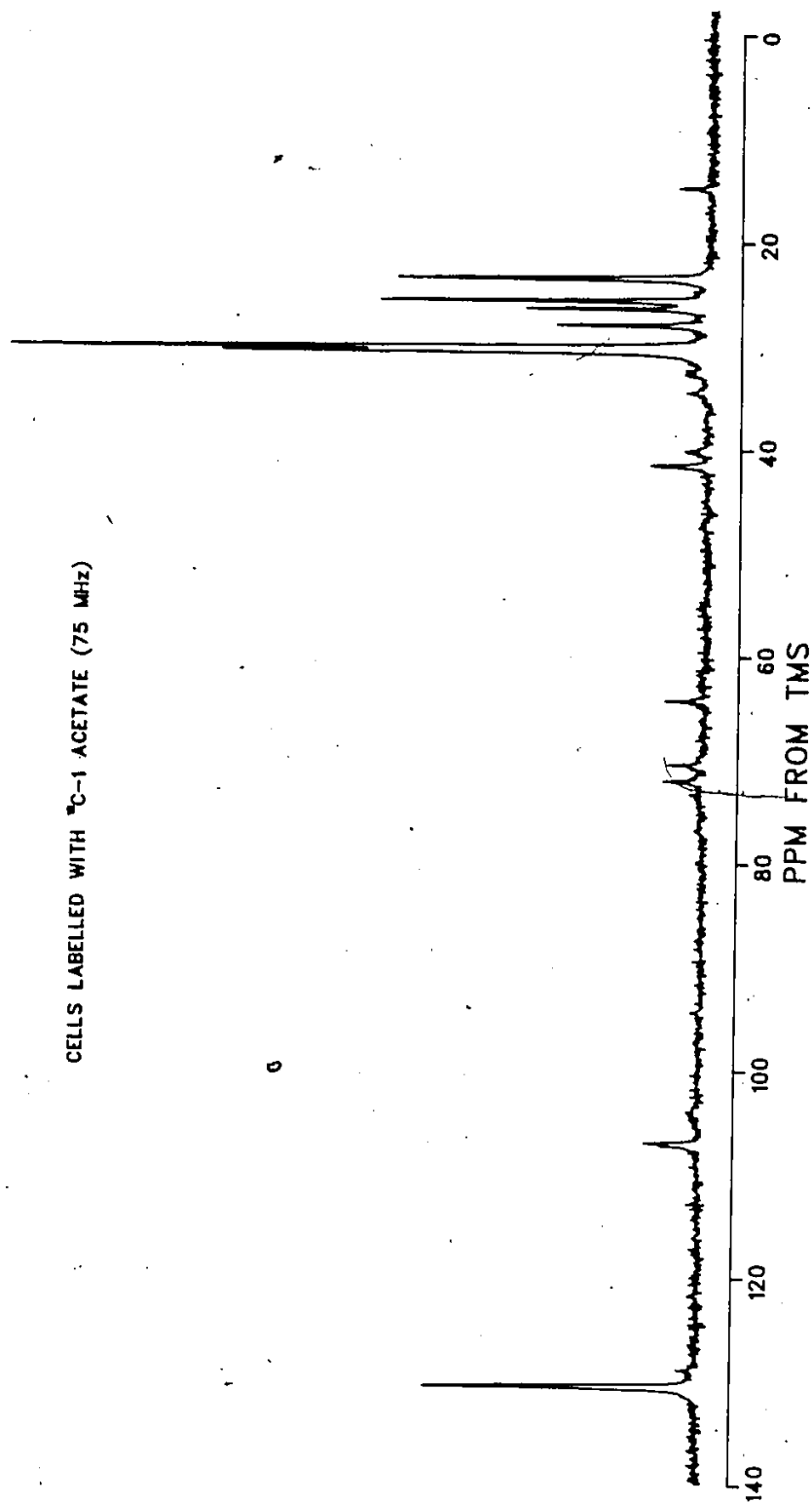


Figure V.5 - 75 MHz ^{13}C spectrum of packed whole cells from a 50 ml culture labelled with ^{13}C -1 acetate, showing the enhanced resolution and sensitivity at higher field. This represents a typical infinity spectrum in a T_1 measurement. The spectrum is a result of 200 accumulations of 8K data points, using a 90° pulse and 4 sec recycle time

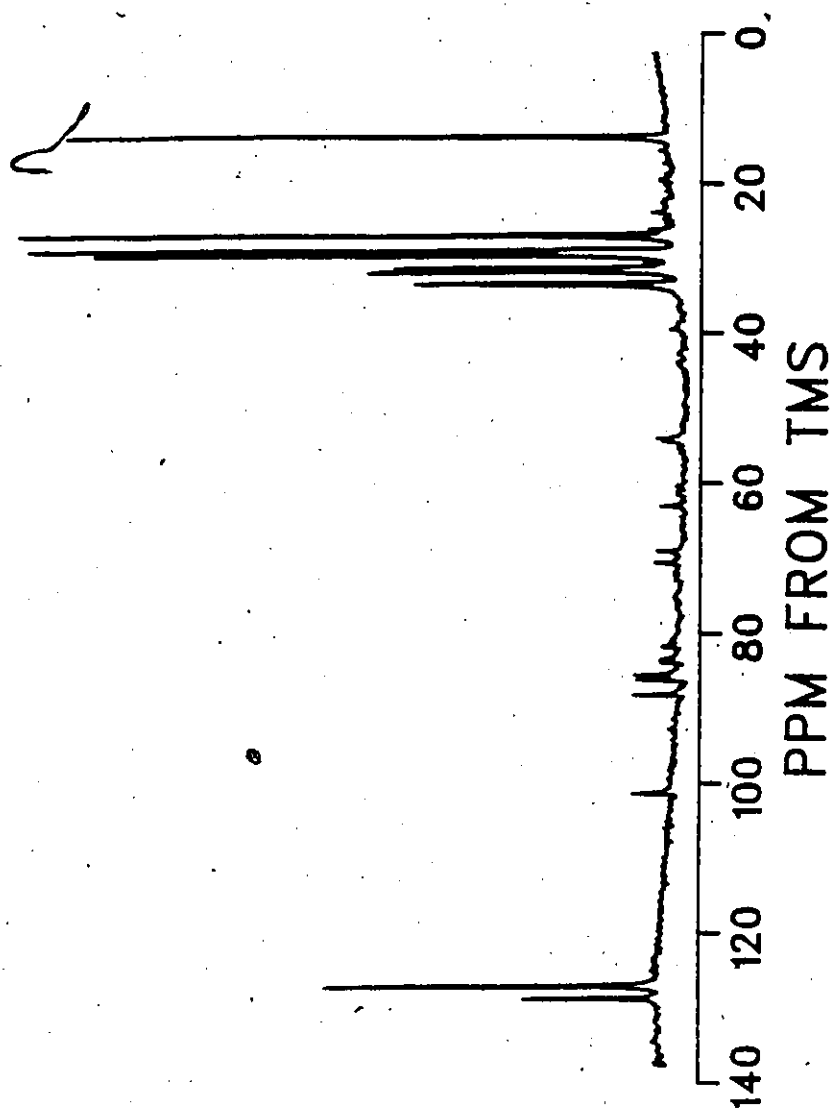
CELLS LABELLED WITH ^{13}C -2 ACETATE (75 MHz)

Figure V.6 - Same as Figure V.5 for cells labelled with ^{13}C -2 acetate. Comparison of the intensities of labelled to nonlabelled peaks between this and the previous spectrum allow an estimation of the level of acetate incorporation

observed in the ^{13}C -1 acetate-derived spectra and the 9 peaks of the ^{13}C -2 acetate-derived spectra based on these two models are shown in Table V.1. Assignments were based on comparison with data for pure fatty acids and phospholipids (Gunstone et al., 1977; Barton, 1975) as well as triglyceride standards.

Chemical shifts of the acyl chain segment are not sensitive to the solvent or head group identity. There were no differences in chemical shifts at the two observed fields. The chemical shifts of the olefinic carbons are sensitive to conformation (cis-trans) (Bus et al., 1977) and the spectra indicate that the predominant conformation is cis, as expected for most natural lipid systems. The carbonyl region was neglected for purposes of relaxation measurements since the relaxation mechanism for these resonances is not totally dipolar. In addition, the carbonyl region is complicated by resonance due to amino acids (see Chapter VII).

A typical inversion recovery sequence is shown in Figure V.7. The τ times refer to the delay between the 90° and 180° pulses in the pulse sequence. These particular spectra are of whole cells, and are typical of the quality of spectra used for most of these relaxation measurements. The results for all relaxation analyses at the two fields are tabulated in the Appendix to this chapter, along with the number of replicate performed for each system, for which the reported T_1 is an average. All systems were done in duplicate, once with each

Table V.1

CHEMICAL SHIFTS AND ASSIGNMENTS FOR
LABELLED FOR RESONANCES

<u>Spectrum</u>	<u>Peak</u>	<u>δ (ppm from TMS)</u>	<u>Assignment</u>
¹³ C-1 Acetate grown, cells	1	23.7	$\underline{\text{CH}}_2 - \text{CH}_3$
	2	25.9	$\underline{\text{CH}}_2 - \text{CH}_2 - \text{COO} -$
	3	26.6	$\text{CH} = \text{CH} - \underline{\text{CH}}_2 - \text{CH} = \text{CH}$
	4	28.2	$\text{CH} = \text{CH} - \underline{\text{CH}}_2$
	5	30.4	$(\text{CH}_2)_n$ (C-13,15)
	6	30.8	$(\text{CH}_2)_n$ (C-6,7)
	7	130.7	$\underline{\text{CH}} = \text{CH}$
¹³ C-2 Acetate grown cells	1	15.0	CH_3
	2	28.2	$\underline{\text{CH}}_2 - \text{CH} = \text{CH}$
	3	30.2	$(\text{CH}_2)_n$ (C-4,6)
	4	30.9	$(\text{CH}_2)_n$ (C-12,14)
	5	32.6	$\underline{\text{CH}}_2 - \text{CH}_2 - \text{CH}_3$ (C-18:1)
	6	33.1	$\underline{\text{CH}}_2 - \text{CH}_2 - \text{CH}_3$ (C-18:2)
	7	34.8	$\underline{\text{CH}}_2 - \text{COO} -$
	8	129.0	$\text{CH} = \underline{\text{CH}} - \text{CH}_2 - \underline{\text{CH}} = \text{CH}$
	9	130.7	$\text{CH} = \underline{\text{CH}}$

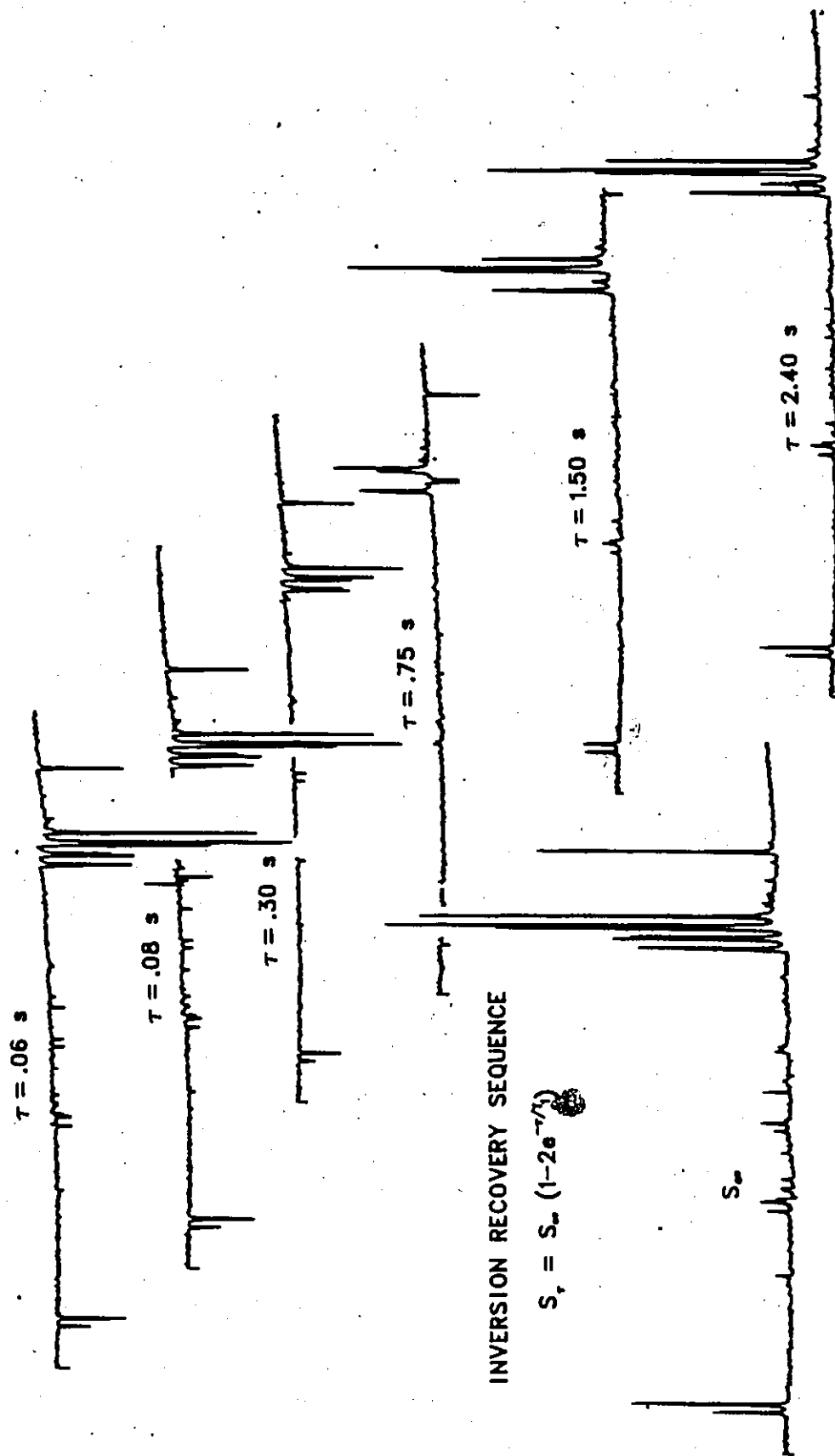


Figure V.7 - A typical inversion recovery sequence for whole cells labelled with ^{13}C -2 acetate. The 90° pulse was $17 \mu\text{sec}$; the recycle time 5 sec . Each spectrum is a result of 400 transients at 75 MHz

acetate label, although initial experiments employed less expensive ^{13}C -1 acetate. The reported deviation represents the reproducibility from culture to culture and is on the same order as the statistical error in determining T_1 . The reproducibility is also checked through the agreement between T_1 's for the two specific labels in the methylene region of the spectrum. For example, the relaxation time measured for positions C-4,6 in the ^{13}C -2 acetate spectrum agrees with that measured for the peak assigned to C-5,7 in the ^{13}C -1 acetate spectrum.

Using the somewhat simplified argument that NT_1 is a measure of average mobility, a qualitative picture of the dynamics of the fatty acid chains in the various systems studied may be obtained. The T_1 profile at 20 MHz of whole, viable cells is shown in Figure V.8, along with that for vesicles formed from total extracted lipids, and total lipid dissolved in chloroform. The profile assumes the model of a C-18:2 acyl chain. The reproducibility is within the size of the symbols used in the plot (see Table V.2, Appendix). In the bilayer on proceeding from C-2 to C-8, there is a slight but significant increase in the mobility of the carbon segments. This is similar to the behaviour found in dipalmitoyl (Levine et al., 1972) and egg (Sears, 1975) lecithin, and can be attributed to restriction of the polar regions of the lipid due to electrostatic interactions, with increasing mobility

CELLS AND EXTRACTED LIPID (20 MHz)

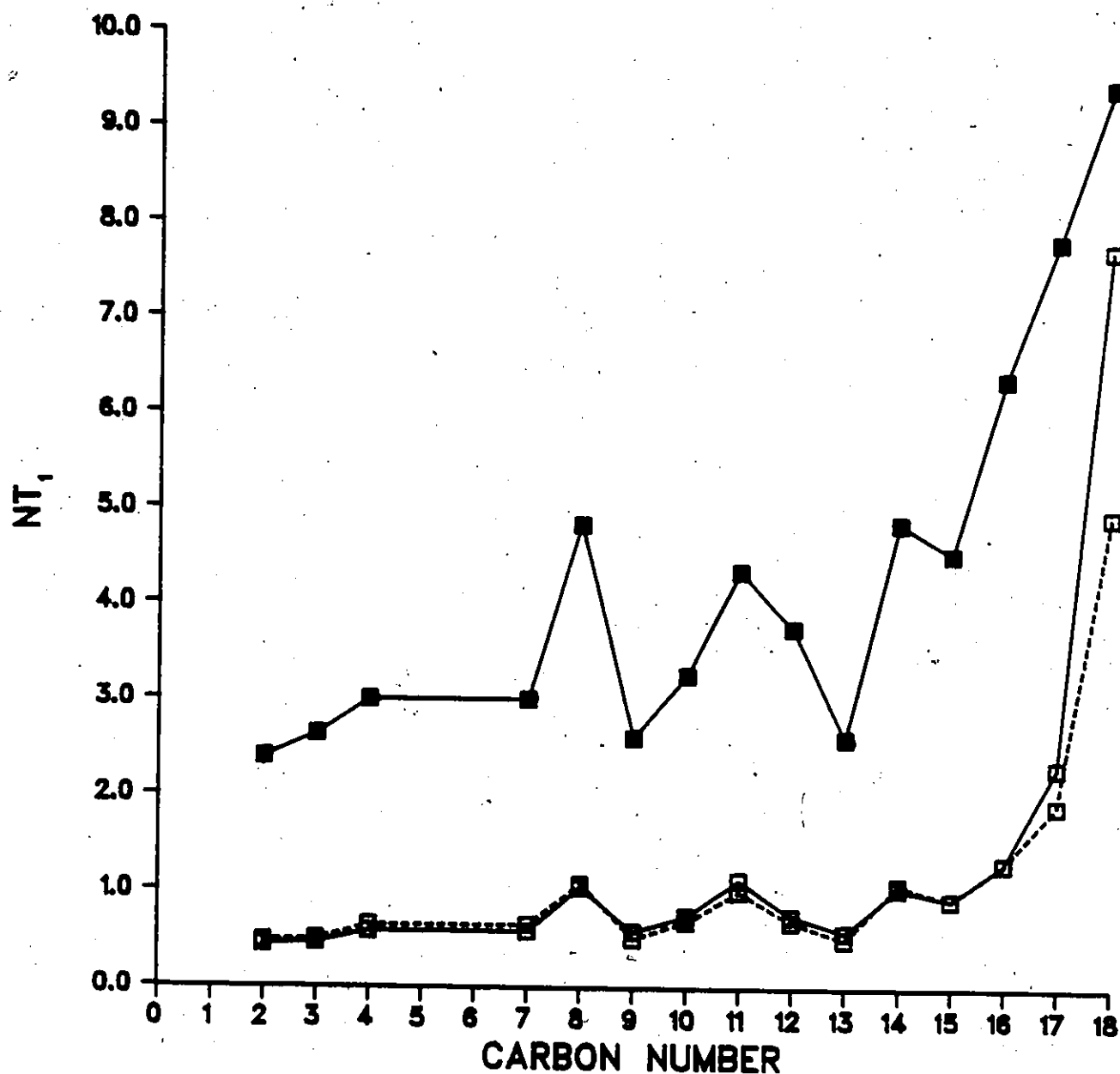


Figure V.8 - Plot of NT_1 values vs carbon number assuming a model of a C-18:2 acyl chain for whole cells (□—□), extracted total lipids sonicated in D_2O (□----□), and extracted total lipid in chloroform (■—■). The T_1 values were determined at 20 MHz and represent an average of 8 experiments. The average deviation between the different cultures used for these measurements is generally within the size of the symbols used in the plots

mobility away from this region due to rapid gauche-trans interconversions. The mobility is also restricted by the packing of the acyl chains in the bilayer as compared to free in solution. There is, however, a drop in NT_1 about the two double bonds, situated in positions C-9,10 and C-12,13 which are the carbons of the cis double bonds. The central methylene, C-11, shows an increase mobility, due to its freedom to rotate between the two cis double bonds. This decreased mobility over the double bond is due to the lack of rotational freedom about the C-9,10 and C-12,13 bonds. Moving further away from the double bond toward the terminal methyl group an increase in mobility is observed which becomes dramatic for C-17 and C-18. The relaxation time for the terminal methyl group is likely anomalously high due to a contribution from spin rotation to the relaxation mechanism. In theory, the NT_1 values for the olefinic carbons should be corrected for the difference in C-H bond length between the olefin and methylene groups. This has the qualitative effect of increasing the value of these particular T_1 's. Since the dependence of T_1 on r has a power of six, a small difference in r can make significant difference in T_1 (see Equation V.6). However, the olefinic bond lengths are not accurately known. The correction, based on the uncertainty in r (olefin) ranges from 0-12% of the value of NT_1 . Including this correction does not render the observation of mobility decrease in the

region of the double bond insignificant. The correction was not made throughout the data, since it was not significantly greater than the error in determining T_1 values.

The NT_1 values observed for the isolated lipid vesicles are very similar to those of the intact cells. Lipid vesicles were prepared in the standard way; by sonicating to a pale milky colour a suspension of dry lipid in D_2O . This similarity might be taken to indicate the absence of protein-lipid or carbohydrate-lipid interaction on the level of the motions sensed by the ^{13}C relaxation times. The profile displays qualitatively the same features as that of whole cells, with the possible exception of a less dramatic increase towards the terminal methyl group of NT_1 . This may be due to the differences in packing constraints imposed in the central zone of the bilayer of the vesicle, due to its much smaller size. The agreement between the two systems is remarkably good despite the difference in size and therefore in overall tumbling rate of the two systems. This shows that the ^{13}C T_1 's are more sensitive to the gauche-trans motion of the individual lipid molecules than to the diffusive motion of the molecule. This interpretation is somewhat complicated by the fact that there is a substantial quantity of storage triglyceride contributing to the whole cell spectra. Thus the measured relaxation times are a combination of contributions from the membrane fractions and the triglyceride fraction in the form of small vesicles.

(The relative proportions of these components is estimated on the basis of the relative amounts of the extracted phospholipid and triglyceride tabulated in Table IV.5). This aspect has been resolved by preparing membranes (vide infra).

An attempt was also made to make much larger single bilayer vesicles than are formed by sonication, in order to compare the relaxation times more realistically with the situation of the plasma membrane in the whole cell. Unfortunately it was found that these larger vesicles were not sufficiently stable to survive the time required to perform the relaxation experiment. This was attributed to the highly unsaturated nature of the lipid mixture. The large unilamellar vesicles (LUV) were prepared from total lipid extract by the ether injection method (Deamer and Bangham, 1976). The lipid was dissolved in ether and slowly bubbled through a crimped 22 gauge needle into D_2O . The production of a uniform population of vesicles was a sensitive function of the lipid concentration in ether, the needle bore, and the bubbling rate. The resultant mixture was pale and milky. Electron microscopy showed that the lipid formed uniform vesicles of $\sim 400 \text{ \AA}$ in diameter. These were stable only for a few hours, after which only fractions of bilayer, visible as striations in the EM, were present.

The NT_1 values of isolated lipids in chloroform are significantly longer than those of lipids in the intact cells or dispersed in water. This is consistent with the formation of small inverted micelles, which should have a much looser packing of the fatty acyl chains, as well as an increased rate of micelle rotation contributing to the T_1 values. This change in structure is not only apparent in an increase on all the NT_1 values, but also a larger relative effect between carbon positions down the acyl chain. This reflects the increased freedom of rotation of carbon bonds in the central portion (C-8 to C-15) of the chain. The same profile is observed about the olefinic carbons, indicating that the profile is due, in fact to the presence of the unsaturations. The relative mobility of C-11 may be increased because this carbon describes a larger arc of motion during rotation of the molecule about its long axis, due to the constraints imposed by the two cis double bonds. In addition, there may be a greater rate of gauche-trans motion about the bonds on either side of C-11, to adjust the relative positions of the two double bonds in order to comply with packing constraints. The verification of these trends will be discussed below.

In order to verify the approximation that the NT_1 profile represents a mobility profile and to extract a crude estimation of the correlation time, the studies were carried out at two

fields; 2.3 T (20 MHz for ^{13}C) and 7.05 T (75 MHz for ^{13}C). The expected behaviour of T_1 , T_2 and NOE at these two fields, assuming effective isotropic rotation, is shown in Figure V.9. The calculations were performed using a program written by I. Peat (1973) employing the equations presented in the previous section for effective isotropic rotation. The value chosen for r was 1.096 Å (Tables of Interatomic Distances and Configuration in Molecules and Ions, 1965), after surveying more recent literature employing both x-ray and neutron diffraction results. The calculation predicts that beyond $\tau = 2 \times 10^{-10}$, a difference will be observed for both the NOE values and T_1 values at the two field strengths. Linewidths, as calculated from T_2 (dipolar) (Figure V.10), will only be different at the two field strengths at correlation times where the NOE values are between their maximum and minimum values. However, the linewidth that is measured from the spectrum has other contributing factors including spectrometer conditions and the inherent viscosity of the sample, so that the comparison of linewidths to determine such small differences is intrinsically inaccurate.

The combined results for whole cells at the two frequencies are plotted in Figure V.11. Since results from all observed resonances are included, there is a profile for both C-18:1 (broken line) and C-18:2 (solid line) chains, as there are resonances solely assignable to each chain species. At both

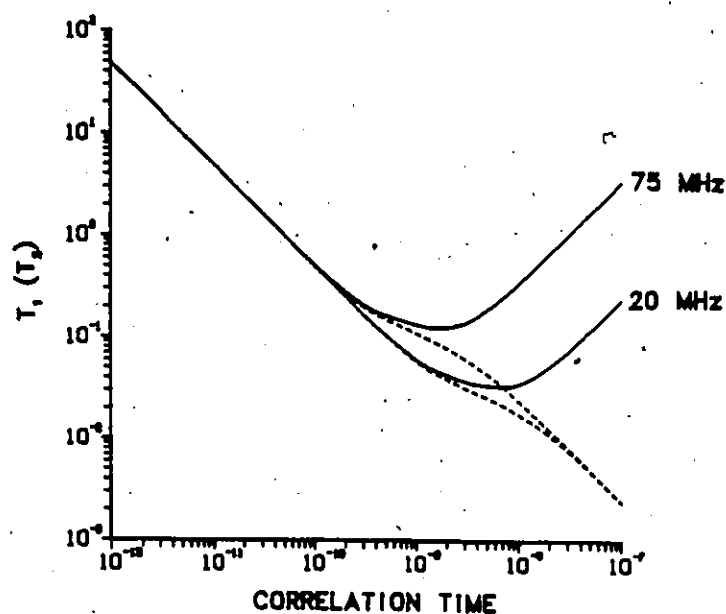
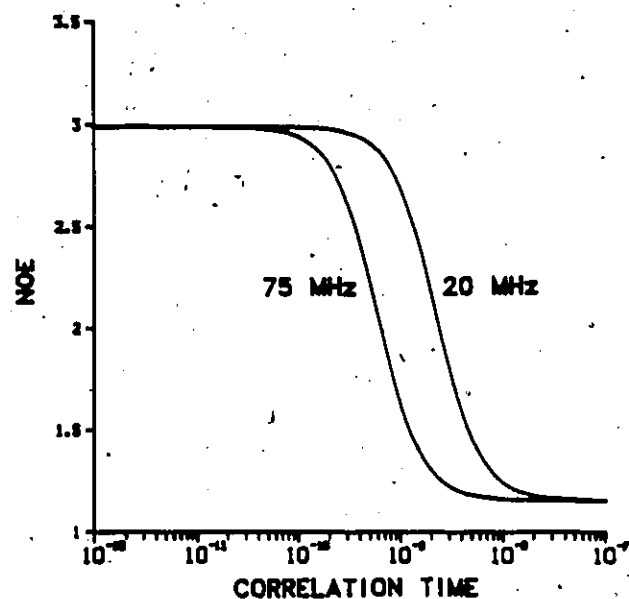


Figure V.9 - Variation of (a) NOE and (b) relaxation time with correlation time assuming overall isotropic motion. The calculations show the effect of field strength for ^{13}C nuclei resonating at 75 MHz and 20 MHz. Only for slow motions beyond the T_1 is T_1 (solid line) unequal to T_2 (broken line)

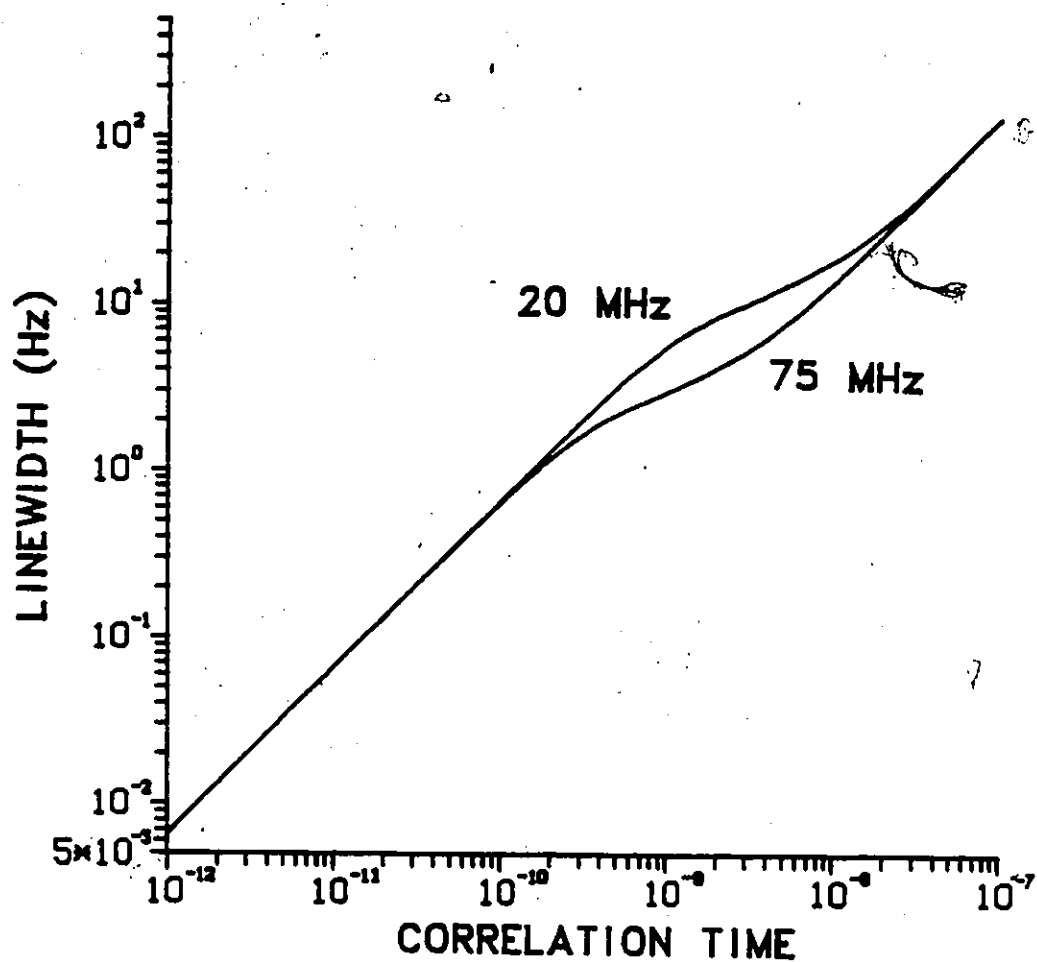


Figure V.10 - Effect of correlation time on linewidth as calculated from T_2 at two magnetic fields for isotropic reorientation

WHOLE CELLS (75 MHz AND 20 MHz)

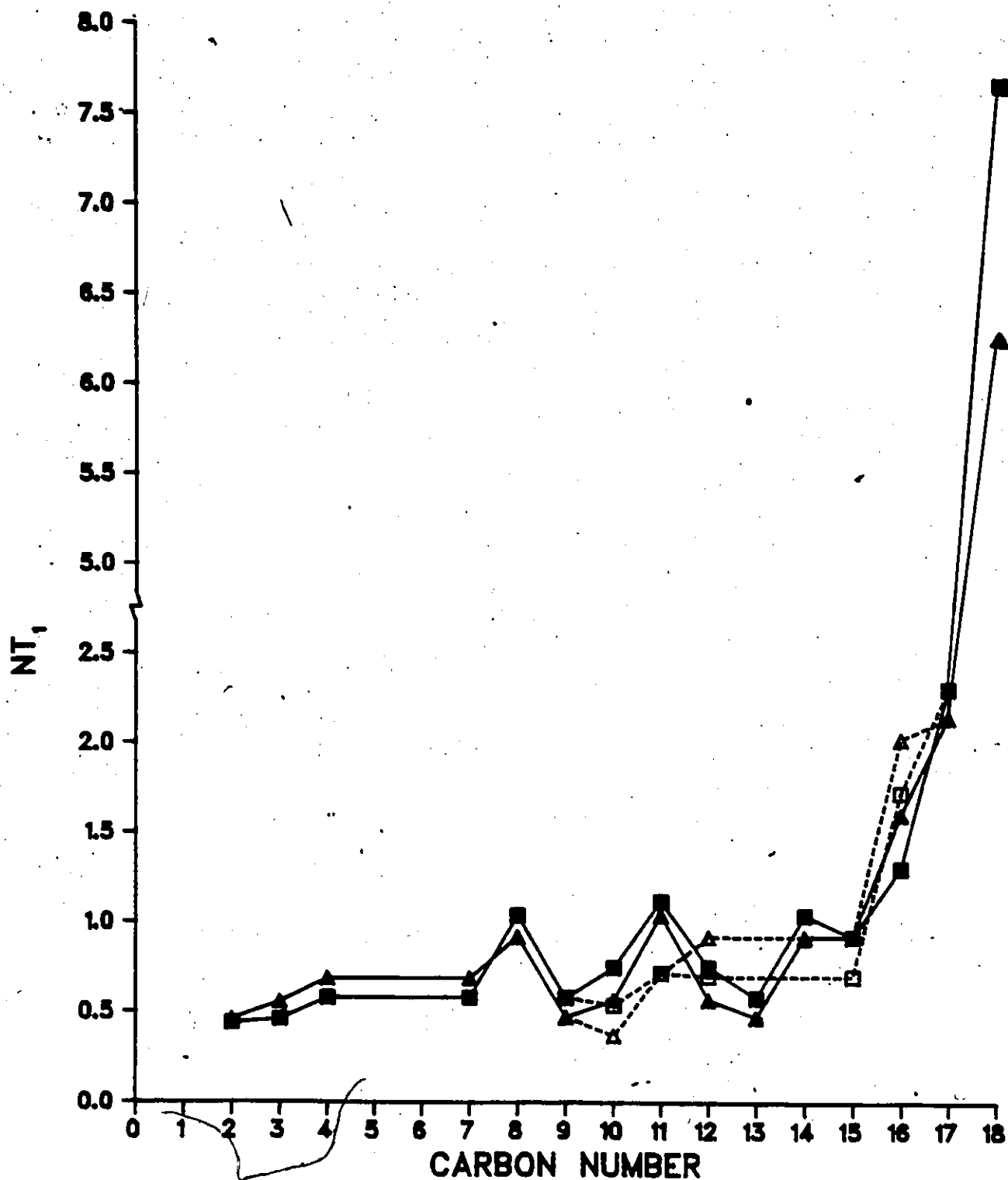


Figure V.11 - NT_1 profile for whole cells measured at 20 MHz ($\square$) and 75 MHz (Δ) using models representing the dominant acyl chain species: C-18:1 (broken lines) and C-18:2 (solid line). The two models can be resolved in some regions of the acyl chain due

frequencies, the qualitative features for both chain models are the same. Whereas there is a symmetrical pattern in the 18:2 chain from C-8 to C-14, there is a drop in T_1 across the double bond of the 18:1 chain which is then followed by an increase towards the end of the chain. The relaxation time at C-11 is significantly less for C-18:1 than C-18:2. This is due to the differences in packing requirements required by the two chains. The volume required for a chain with only one cis double bond is much less than that required for a chain with two, and thus the relative freedom of C-11 is greater in the latter case. If the rate of gauche-trans motion is approximately the same at this position in both chains, this difference then reflects the fact that C-11 can describe a greater arc of motion in C-18:2 during rotation of the molecule as a whole. The relaxation time at C-16 is also significantly greater when only one double bond is present. This reflects the difference in the cumulative degrees of freedom at this position. The restriction in gauche-trans freedom imposed by the conjugated double bonds is felt further down the chain for C-18:2. Unfortunately, the two chain species cannot be resolved at C-17 and C-18 to determine if this effect continues to the end of the chain.

It is difficult to ascertain whether the differences observed at the two field strengths are significant. Since the differences are small, it can be concluded that the

correlation times are on the motional-narrowing side of the T_1 minima and are perhaps approaching the minimum at 75 MHz. The absolute values of the NT_1 results, ranging from 0.4-2.0 sec, predict, from the model of Figure V.9, that there should be no field dependence. Note that Figure V.9 has been plotted for $N=1$, so that the NT_1 values should be used to determine correlation times (see Equation V.6). Since the signal-to-noise ratio was quite high for whole cell samples, the statistical error in determining T_1 was quite small. The reproducibility between cultures was approximately 4%, which is smaller than the symbol size in Figure V.11. Since all relaxation experiments were performed in the same manner, the errors common to the relaxation experiment can be eliminated, and it is therefore likely that the observed differences are significant. The temperature was controlled at the higher field to match the probe temperature at lower field so that temperature is not a cause of these differences. However, it does not necessarily follow that the differences are due to the value of the correlation time. There is a greater contribution of the CSA mechanism to results at higher field and especially to those carbons exhibiting a more anisotropic chemical shift tensor. The general effect of CSA to relaxation would be a dampening of the sensitivity of T_1 to mobility differences, and this does not appear to be significant from Figure V.11. The difference in NT_1 value of the terminal

methyl is likely due to the relative contributing mechanisms. The error in determining this T_1 value is also higher due to experimental conditions.

The question arises whether the relaxation times apply to the viable as well as intact system and whether this has any significance for membrane mobility. The viability curves were checked under the conditions with which these samples were prepared. In general the viability curve of whole cells suspended in D_2O was similar to that presented for the anaerobic culture in Figure VIII.4. There was virtually no effect on viability of a factor of two in cell concentration (experiments at higher field were performed on 50 ml cultures reduced to two ml sample volume, while lower field used 100 ml cultures), or of using D_2O or H_2O instead of 10 mM phosphate as the sample medium. The relaxation measurements were normally run over a period of 8-12 hours, and therefore the cell suspensions were losing viability over that time. However, there was no effect of viability on the relaxation measurements. On several occasions, experiments were performed on the same sample, both fresh and one week old, with no change in T_1 . Since the cells do not lyse when they lose viability, this procedure simply determined the effect of viability. Thus, at least this aspect of the mobility of the membrane is simply a function of the integrity of the membrane and not of its activity.

The NOE values, obtained under conditions of 90° pulses and $10 \times T_1$ recycle times, were measured and linewidths were noted. Linewidths were consistent from sample to sample and were always wider than a standard sample used for shimming purposes. The linewidths were therefore not determined principally by field inhomogeneity. Linewidths at 75 MHz were approximately a factor of 3 higher than at 20 MHz (see Table V.4, Appendix), showing that more than the dipolar mechanism was contributing to the spectral lineshape. This could be due to lack of resolution of components due to different chain species exhibiting slightly different chemical shifts. This would lead to the observed linewidth increase since the field is increased also by a factor of three. These lines are not further resolved at higher field due to the inherent linewidth determined by the viscosity of the system. The NOE values for whole cells (see Table V.5, Appendix) were only crudely measured at 20 MHz, but indicated that NOE's at this field strength were almost full (3.0). Due to increased sensitivity, the NOE values could be more easily performed at 75 MHz, and were quite reproducible.

The NOE values presented in Table V.5 for whole cells are plotted in Figure V.12, as a function of carbon position. The NOE values exhibit the same qualitative pattern for both 18:1 and 18:2 chains as the relaxation times. Reproducibility in NOE values was estimated to be ± 0.1 so that this profile

NOE VALUES FOR WHOLE CELLS (75 MHz)

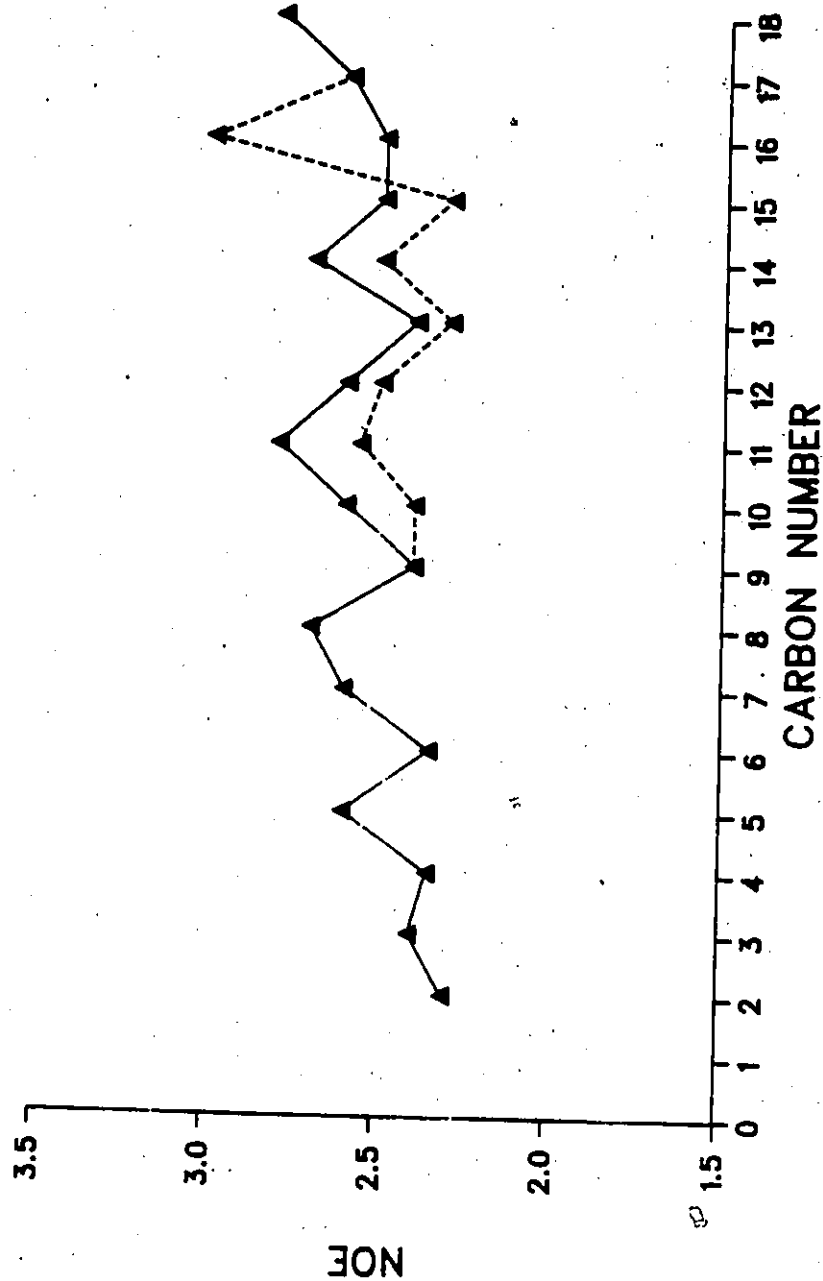


Figure V.12 - NOE profile as a function of carbon number for whole cells at 75 MHz for both acyl chain models, C-18:1 (solid line) and C-18:2 (broken line)

is significant. The fact that almost full NOE's can be developed also indicates that the CSA mechanism has only a small contribution to relaxation. If the relaxation profile is due to differences in correlation time, the same premise must be applied to NOE values, and therefore this dependence of NOE on position indicates that the correlation times are in the region where NOE is time-dependent. The correlation times predicted for this range of NOE values is $2-6 \times 10^{-10}$ sec (see Figure V.9(a)). This range of correlation times also predicts that NOE values at 20 MHz are full.

If the correlation times calculated from the NOE values are applied to the relaxation curves (Figure V.9(b)), they predict a small but consistent field dependence of T_1 , which was not observed. Conversely, the values of τ_c that can be calculated from Figure V.9(b) for the T_1 values present in Figure V.11 range from 1×10^{-11} at the terminal position to 1×10^{-10} at the C-2 position. This discrepancy between the correlation times as determined from the two measurements can stem from a number of causes. Firstly, there may be a slight contribution from the CSA mechanism which has the effect of decreasing both NOE and T_1 . Qualitatively, correction for this effect would increase the estimate of τ_c (dipolar). However, the lack of change of sensitivity to position with field as well as the temperature dependence (vide infra) at 75 MHz, indicate that this contribution is not significant.

Secondly, the discrepancy may be due to the inadequacy of the model of isotropic rotation when applied to a system which is not exactly isotropic. Introduction of a two correlation time model; one time for overall molecular reorientation plus one for gauche-trans motion, has the effect of shifting the relaxation curve up and down with respect to the T_1 (T_2) axis (for certain values of overall reorientation), without appreciably changing the shape of the curve (Doddrell et al., 1972). This would also qualitatively converge the values of τ_c obtained from the two sources. However, as was mentioned in the introduction to this chapter, Dill and Allerhand (1979) have observed that discrepancies between correlation times observed at different field strengths may be the result of an inaccurately chosen value of r_{CH} . While the NOE values are independent of the choice of r_{CH} , the relaxation times are strongly dependent. The authors point out that the use of a value of r_{CH} derived from the solid state may be inaccurate for a more liquid like state. The use of a value for r_{CH} , which has been corrected for vibrational motion of the C-H bond (a correction which increases the value of r_{CH} (Vold et al., 1977)), shifts the relaxation curve up on the T_1 (T_2) scale. Although this calculation was not performed in this study, the qualitative improvement made by a small increase in r_{CH} is one of converging

the correlation time values as determined from both NOE and T_1 . Increasing the value of r_{CH} to 1.12 has the effect of increasing T_1 values by 14%, and values up to 1.15 have been reported in some peptides (Llinas *et al.*, 1977). Since the error in determining NOE values and T_1 values must also be taken into account, the data are not sufficiently accurate to determine a value for r_{CH} in this system, by fitting the data for an optimum value of r_{CH} . For the degree of accuracy reported here, this explanation is sufficient to resolve any discrepancy in the determination of correlation times, and thus a convergence to correlation times over a range 5×10^{-11} to 5×10^{-10} for intact membrane systems is concluded from these studies. In order to determine accurately the value of r_{CH} to be used in the calculations for lipid systems, an accurate study of T_1 and NOE as a function of field would have to be carried out on a pure, well-characterized lipid (such as DPPC), in order to minimize errors intrinsic in measurements of this type.

T_1 Profile of Fatty Acid Chains

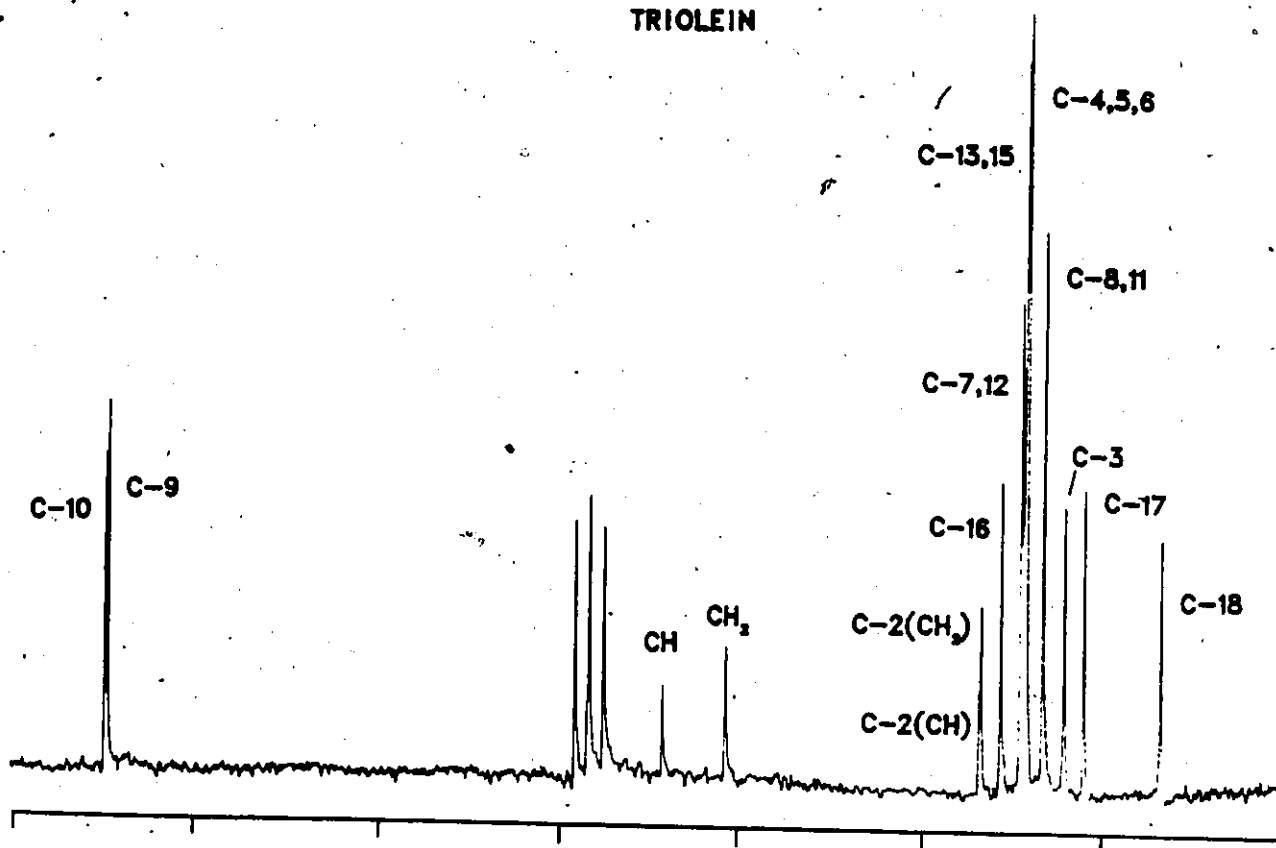
The relaxation time profile which appears to be so characteristic of the composition of the fatty acid may be somewhat anomalous due to the pattern of overlapping resonances from which T_1 must be determined. The pattern for 18:2 chains is symmetrical in the region C-8 to C-14 due to the fact the

resonances for C-8 and C-14 overlap as well as those of C-9 and C-13 plus C-10 and C-12. In addition, there may be an effect on the profiles of the individual chain types (C-18:1 and C-18:2) as a result of their interaction in a 2:1 mixture. Since the profile in CDCl_3 qualitatively reflects the profile in the intact cell, the validity of the profile was ascertained by comparison with that for triglyceride samples, which could be easily obtained with chain purity.

The use of these triglyceride spectra in CDCl_3 also aided in the unambiguous assignment of the lipid chain spectrum. The spectra of triolein (C-18:1) and trilinolein (C-18:2) are shown in Figure V.13. The use of an organic solvent allows the resolution of many of the carbon positions, even though the sample is not isotopically labelled. The individual double bond positions can be resolved so that T_1 's can be measured uniquely on all olefinic carbons for the pure chain species. In addition, substitution of the acyl chain at the 2-position on the glycerol backbone can be resolved from the 1,3-positions, for C-2 of the acyl chains. The glycerol backbone positions can also be seen. These latter carbons were not observed in intact cells partly because the glycerol backbone does not derive from acetate and therefore does not receive any label, but also because these resonances are broadened in aqueous solution. The triglyceride to phospholipid

A.

TRIOLEIN



B.

TRILINOLEIN

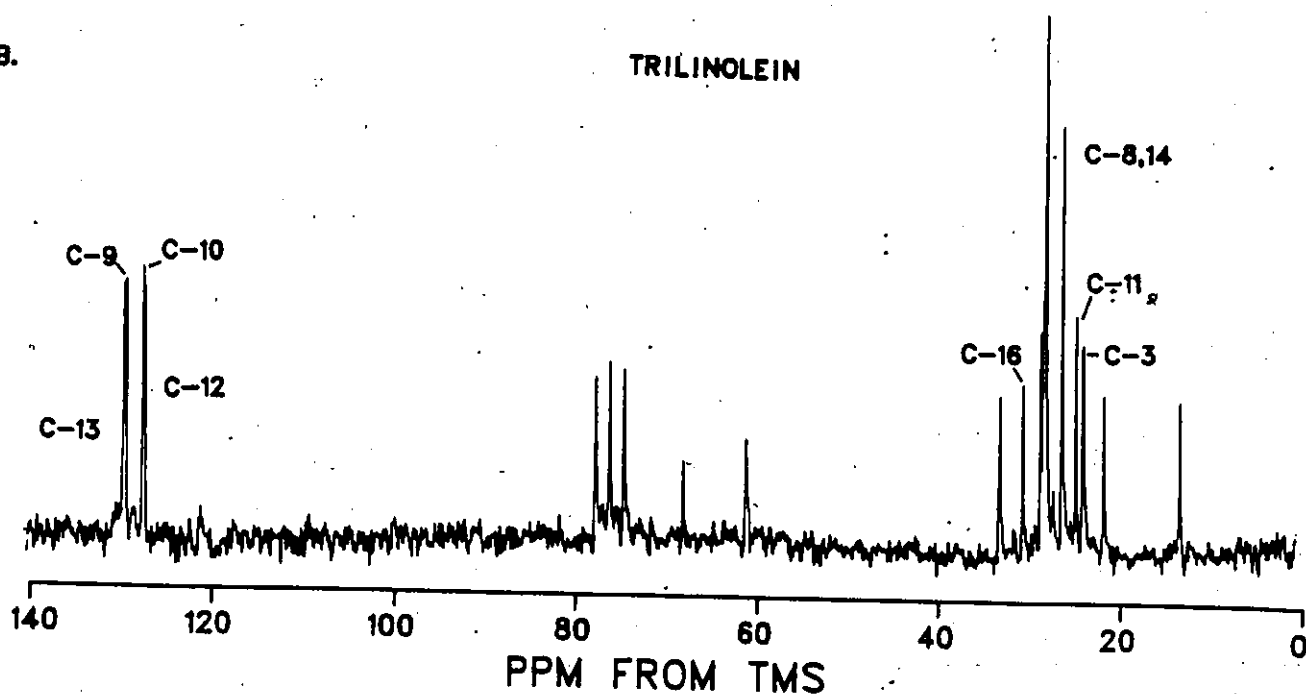


Figure V.13 - 20 MHz ¹³C NMR spectra of pure triglyceride, (a) triolein and (b) trilinolein in CDCl₃. Assignments of these spectra aided in the assignment of lipid resonances in whole cells

ratio is such that there is insufficient concentration of triglyceride to be observed even in natural abundance. A mixture of trilinolein/triolein (2:1), which mimics the fatty acid composition of the cell lipid, effectively provided a fingerprint spectrum of the total lipid extract, which could be easily assigned from the spectra of the pure triglycerides. The NT_1 values for the pure triglycerides along with those for the mixture are shown in Figure V.14, and these values are compared with the labelled lipid profile of Figure V.8 in the following figure (Figure V.15). Note that in Figure V.14, the double bonds are represented as being in the trans conformation, solely for the convenience of presentation. The NT_1 values increase down the chain in the same manner as we have already observed. It is interesting to note that the T_1 value at the sn-2 position of the glycerol backbone is shorter than that at the 1,3 positions, but the C-2 position of the acyl chain attached sn-2 experiences a longer T_1 . This may be due to the conformation adopted by the triglyceride in the solvent. The effect of mixing the two chain types (although these are not mixed on the same molecule - which may be the case in the cellular lipids), appears to be small although the T_1 values are slightly greater from C-2 to C-14 of the 18:2 chain. Figure V.15 shows that the profile of the pure chain species is the same in the region C-8 to C-14, where each position can be separately determined, as

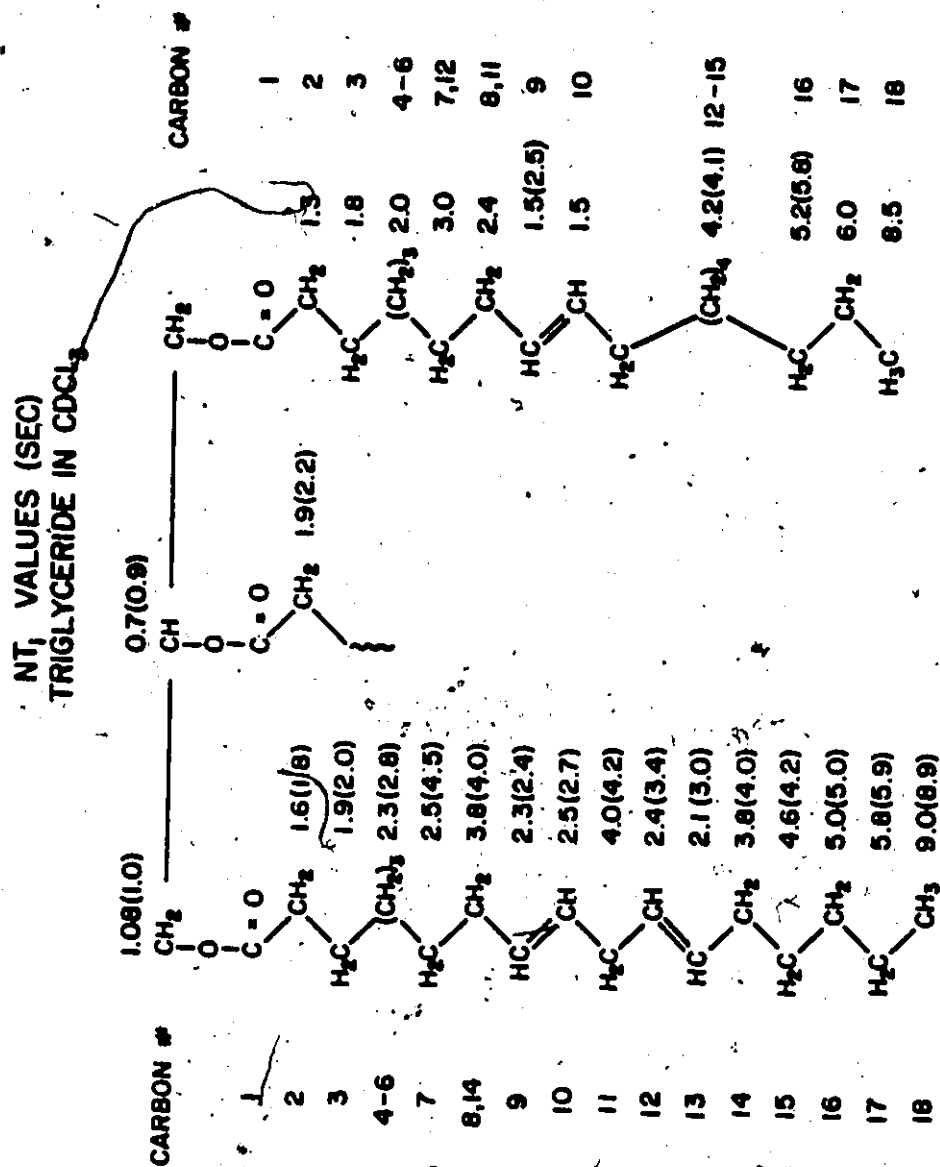


Figure V.14 - ^{13}C NT1 values at 20 MHz for pure triglycerides in $CDCl_3$. results are listed as a function of carbon number for triolein and trilinolein as pure solutions. The figures in brackets were measured for a 2:1 mixture of the two triglycerides. Only positions C-9, C-12-15, and C-16 of the triolein component can be resolved in the mixture

TRIGLYCERIDE IN CHCl_3
(18:1, 18:2 AND TOTAL LIPID)

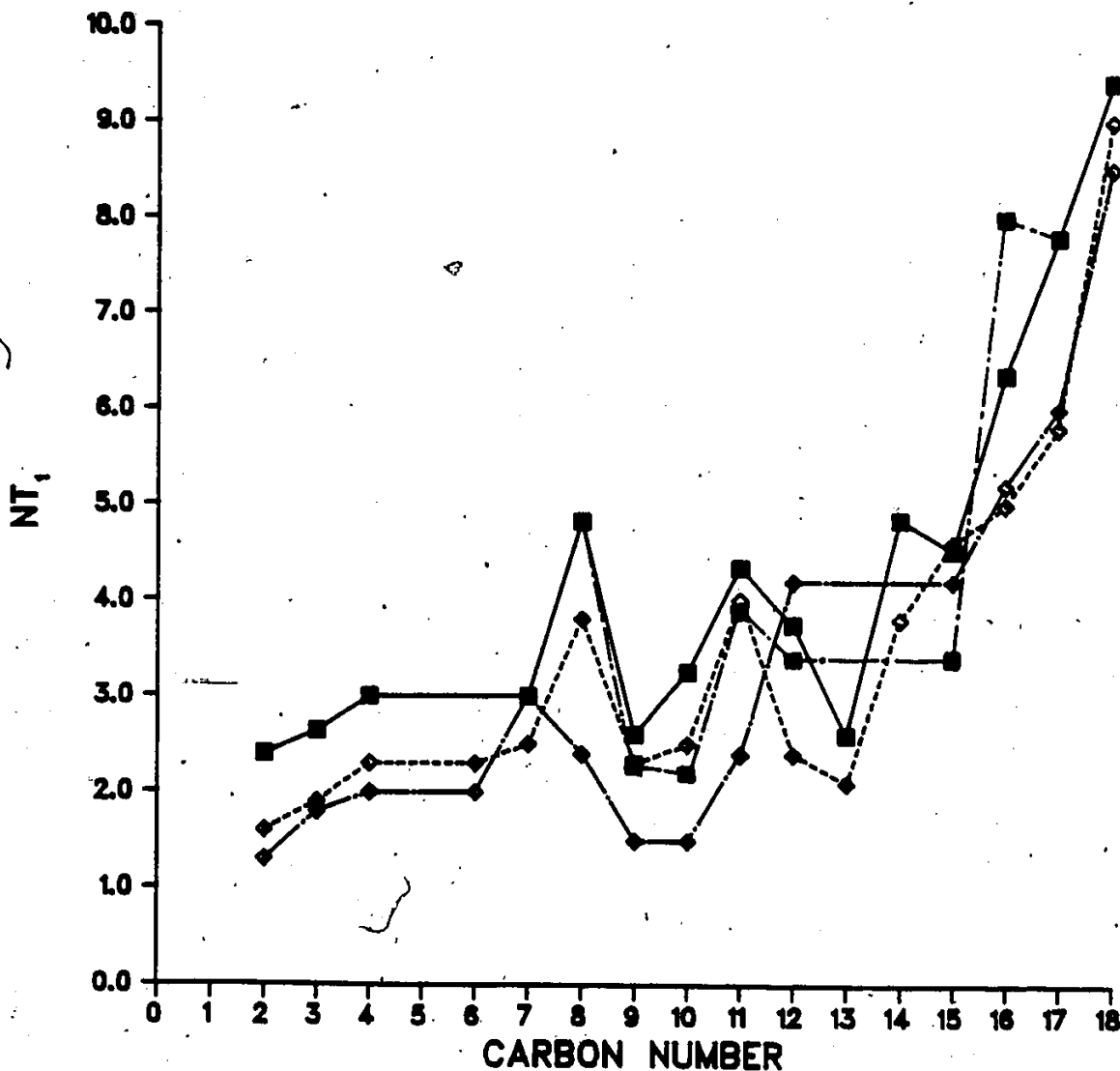


Figure V.15 - Plot of data in Figure V.14 compared to total extracted lipid in chloroform (■), in order to determine the profile across the double bond region. The plots are identified as follows: trilinolein (◇-----◇), triolein (◇-.-◇), total lipid as per C-18:1 chain (■-.-■) and C-18:2 chain (■—■).

in the labelled species, where the resonances overlap. There is an increase in T_1 from C-9 to C-10, which is followed by a decrease from C-12 to C-13. The T_1 's of positions C-9 and C-10 are smaller in C-18:1 than C-18:2, and are essentially equal. In $CDCl_3$, the C-9 can be resolved for each chain species in the labelled lipid extract, and this shows a similar trend. This position is not resolvable in an aqueous environment, and therefore the drop in T_1 from C-9 to C-10 in the 18:1 chain is a result of obtaining only one T_1 value for position 9. There is little difference in NT_1 for the two chains at positions C-17 and C-18, but the value at C-16 is higher in the 18:1 chain, and even more so in the stoichiometric mixture. The T_1 measured for position C-8 is much lower in 18:1 chains than in 18:2. Since this position cannot be resolved in labelled lipid, the observed value is anomalously high for the 18:1 chain but accurately represents the 18:2 chain. The result for C-11 in the triolein sample may be low due to overlap with C-8, and therefore not represent the true mobility at this position. For this position, the labelled spectrum (where this overlap has been removed) is more indicative of the correct value. These results indicate that the profiles observed in the whole cells and various membrane preparations can be treated with confidence as representing the true relaxation profile, and are not a result of spectral overlap in which the T_1 's do not differ by enough to observe non-exponential behaviour in the T_1 curve.

V

Membrane Preparations - Comparison to Intact Cell Profile

It has been noted previously that since yeast cells store triglyceride, there is a significant contribution to intact cell spectra from this source, which is present in the cell in small vesicles or micelles or generally in a form that is not bilayer in structure. The magnitude of this component has been quantitated in Table IV.5, from the composition of the extractable lipid. In order to reduce this component and determine only the profile of the bilayer component several procedures were attempted, the experimental details of which were discussed in Chapter III.

(a) Starved Cells

The cell cultures were starved for various periods of time in order to reduce the triglyceride stores through active metabolism. This reduced the available label, making the T_1 measurement longer and confusing the spectrum with the labelled products of β -oxidation (see Chapter VII). The starved cell suspension remained viable for longer periods of time under the conditions of the NMR measurement, so that these relaxation measurements are truly of a viable culture. The optimum starvation period was three days, although the relaxation times did not change significantly with this variable. For longer periods, the turnover in lipid content

had reduced the level of ^{13}C label sufficiently that T_1 measurements were prohibitively long. At this point the triglyceride to phospholipid ratio had been reduced by a factor of 2.5, although triglyceride was still present. The T_1 values were measured only at 20 MHz and are summarized in Table V.2. Within the error in measuring T_1 , there were no differences observed between starved cells and the freshly cultured cells.

The debris after a Folch's extraction of the lipid component showed a spectrum quite similar to that of the whole cell. Since this procedure did not extract as much lipid as the Bligh and Dyer procedure, there was sufficient labelled lipid left to perform a T_1 measurement. This fraction then represents "bound" lipid, which may be associated with the cell wall through lipopolysaccharides. This concept was confirmed by lipid staining. The debris was only checked at 20 MHz, and there were not sufficient replicates to determine the same degree of accuracy as for the other systems, but, as Table V.2 shows, there is no significant differences between the T_1 values of the debris and those of the whole cells. This provides further evidence that the T_1 's measured in the whole cells are representative of the membrane component.

(b) Enzyme-Prepared Membrane Preparation

This preparation, while the most efficient at ridding the system of most of the triglyceride (see Table IV.4), was

the least efficient in the use of the ^{13}C label. The spectrum of Figure V.16 is a result of the preparation obtained from a culture five times larger than that used for whole cell measurements, and the accumulation time is approximately 2-3 times longer. The resonances are, however, slightly broader at both fields, indicating that there may be some reduction in mobility with respect to whole cells. There are no resonances due to cytoplasmic contents, and the membranes appear to be hollow vesicles of various sizes (there is likely a mixture of plasma, nuclear, and vesicular membranes), though smaller than the original cell (see Plate X, Chapter II). The efficient removal of triglyceride by this preparation was confirmed in several ways. The TG/PL ratio measured from the extractable lipid was reduced by a factor of 5 from whole cells. In addition, during the preparation, a yellow scum appeared at the top of the supernatant after centrifuging to isolate the membranes from the lytic mixture. A spectrum of this portion isolated during the preparation of cells labelled with ^{13}C -1 acetate is shown in Figure V.17. The predominant peaks are due to the sorbitol used as an osmotic stabilizer during the preparation, but there is a significant amount of labelled lipid present, which shows the now familiar labelling pattern. The carbonyl carbons are also labelled from ^{13}C -1 acetate. Attribution of these resonances to triglyceride can be confirmed from the glycerol backbone peaks, which

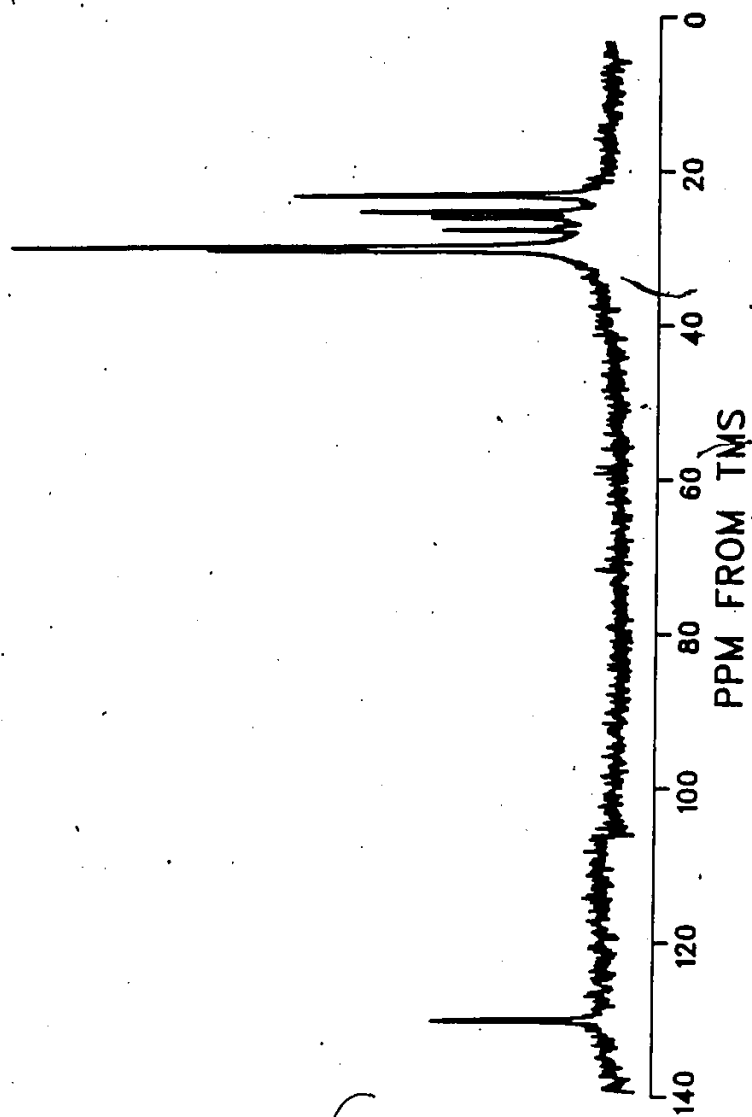
MEMBRANE PREPARATION FROM ^{13}C -1 ACETATE DERIVED CELLS (75 MHz)

Figure V.16 - 75 MHz ^{13}C NMR spectrum of enzyme membrane preparation of cells from a 500 ml culture supplemented with ^{13}C -1 acetate. The spectrum is a result of 800 accumulations of 8K data points using a 900 pulse. Note the absence of resonances due to internal small molecules

LIPID FROM ¹³C-1 ACETATE MEMBRANE PREPARATION

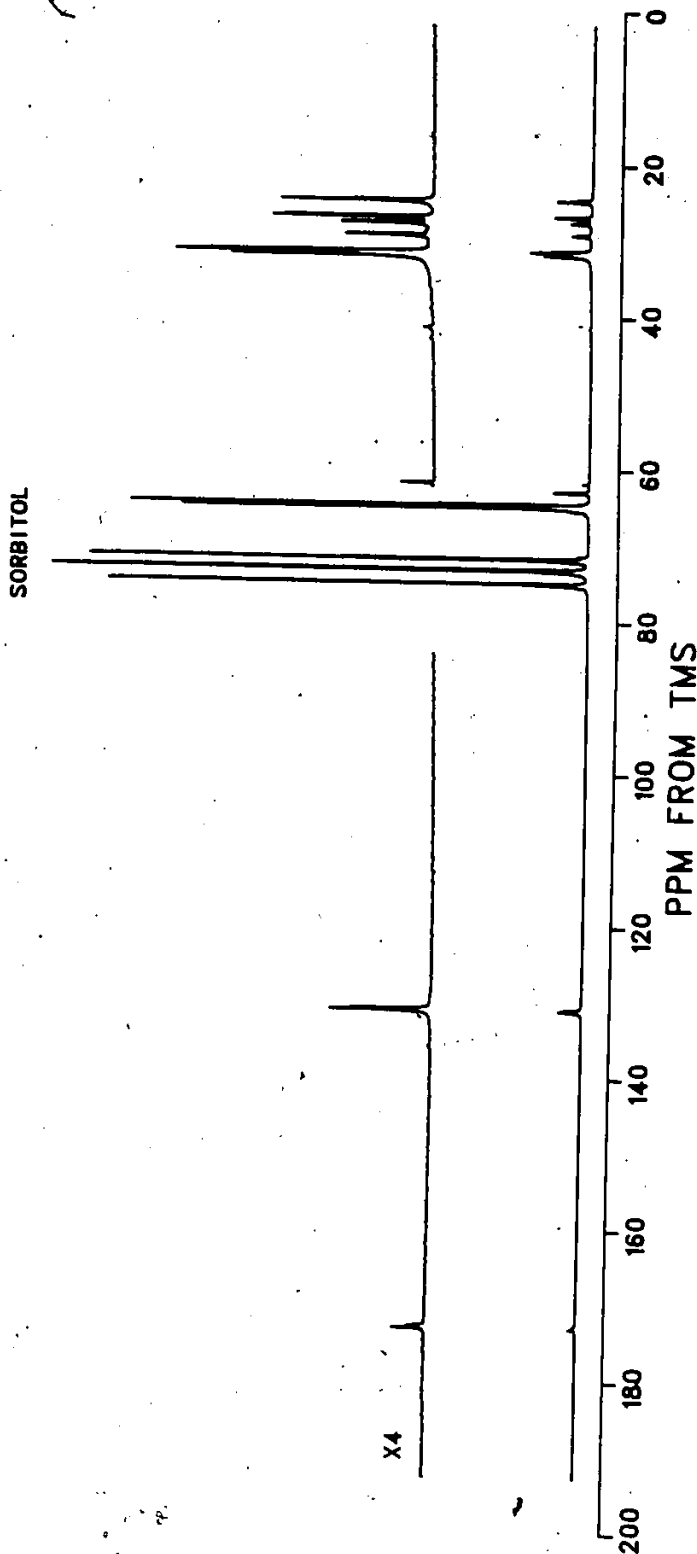


Figure V.17 - 75 MHz ¹³C NMR spectrum of "floating lipid" isolated during preparation of the membranes shown in the previous figure. Inspection of the chemical shifts of the carbonyl and glycerol resonances confirm that this is triglyceride from cellular storage pool. The predominant peaks are due to unlabelled sorbitol used as an osmotic stabilizer in the membrane preparation. The spectrum is a result of 2000 transients of 16K data points using a 450 pulse

are observed upfield from 40 ppm, and from the chemical shift and multiplicity of the carbonyl peaks. In general, the carbonyl carbons of triglyceride are found about 3 ppm removed from those of phospholipid (Avila *et al.*, 1978). This could theoretically be used as a method for determining the TG/PL ratio in the intact system, but in practice, the PL peak is overlapped by carbonyls from storage amino acids. The carbonyl peak also shows the 2:1 intensity ratio of the two positions of the glycerol backbone. The presence of triglyceride was also confirmed by TLC.

The relaxation times measured for this preparation are presented for both field strengths in Tables V.2 and V.3 and the NT_1 values are compared with both whole cells and the preparation from lysis by nystatin in Figure V.20(a) through (d). The mobility profile is identical for both chain models to that of whole cells or extracted lipid, but the T_1 values are generally lower than those of whole cells at 20 MHz. Although the magnitude of the difference is still within the 10% error in determining T_1 , the experiments are sufficiently reproducible to make this a significant observation. The effect of removing the triglyceride fraction is to reduce the average mobility of the entire ensemble of lipid molecules to that representing the bilayer contribution. The difference in T_1 values, however, does not significantly alter the estimation of τ_c , since this is mainly an order of magnitude calculation.

(c) Nystatin Membrane Preparation

The best compromise between efficient use of label for relaxation measurements, and the optimum reduction of triglyceride content was found to be the method of cell lysis by nystatin, which removed cytoplasmic contents. This gave a preparation which retained the structural features of the cell (see Plate VIII) but removed the triglyceride only to the same extent as the starvation procedure (see Table IV.4). The spectra of the rehydrated preparation for both specific labels is shown in Figure V.18. Spectral resonances are assigned to show those due to individual chain species. The absence of the glucose resonances confirms the absence of cytoplasmic contents. These spectra are a result of 200-300 transients using a 90° pulse and represent the product from a culture of twice the volume of that used for intact cell measurements. Thus T_1 's can be easily performed in the same time period as those of whole cells. This property, along with the stability of this preparation to degradation, made this a better system to use for temperature dependence studies, since relaxation times could be recorded with some confidence as to their reproducibility.

The values of T_1 at three temperatures for each specific label are recorded in Table V.6, for nystatin membranes. The relaxation times all increase with temperature as would be

NYSTATIN MEMBRANE PREPARATION

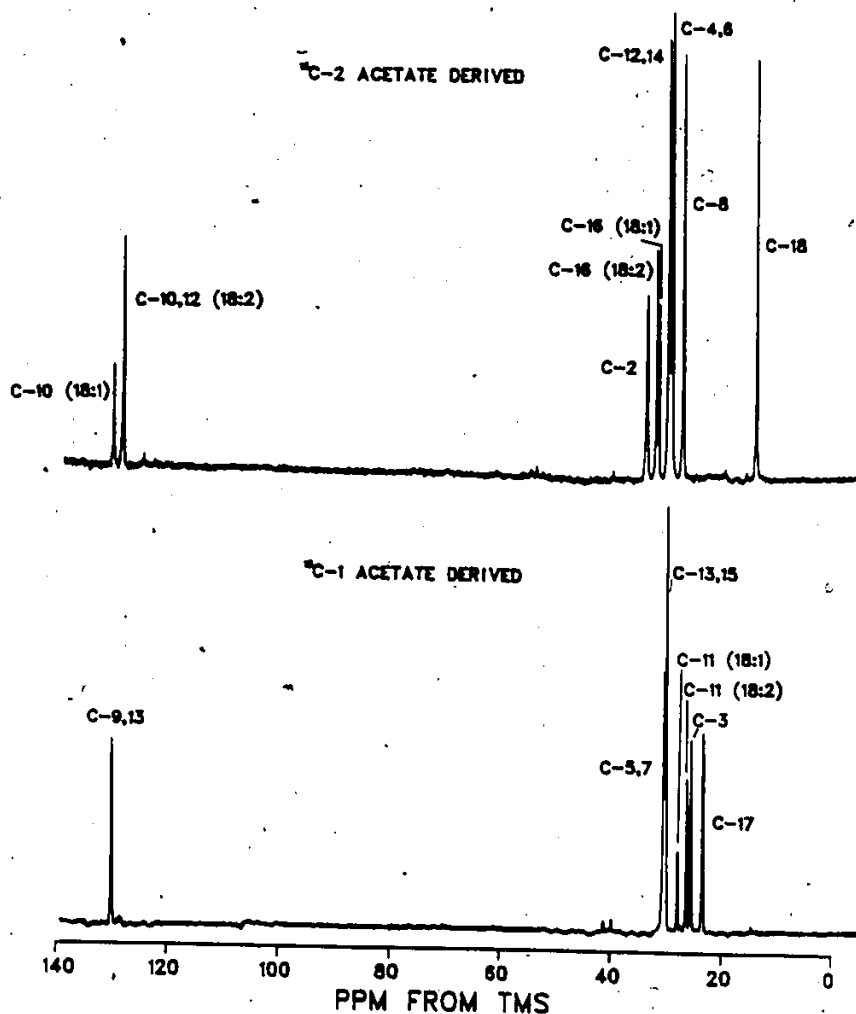


Figure V.18 - 75 MHz ^{13}C NMR spectra of cells from 100 ml cultures supplemented with both specific labels, after lysis with nystatin. The resonances are assigned according to Table V.1, showing those resonances which can be assigned to individual chain species. Note absence of peaks due to glucose indicating loss of cytoplasmic contents

expected for correlation times on the motional narrowing side of the relaxation curve. Increasing the temperature increases the degree of motion available at each position and therefore decreases the correlation time. This general observation held true for whole cells and enzyme membrane preparation even though complete sets of measurement were not performed. The Arrhenius plots of these relaxation times are shown in Figure V.19(a) and (b). Not all plots are linear, indicating a variation in the relaxation times of the various ^{13}C nuclei contributing to the resonance. Those resonances which exhibit linear plots can be assigned to a single position in the acyl chain or to two positions having similar T_1 values. The decrease in T_1 at high temperature for positions C-17 and C-18 show the influence of the spin rotation contribution to the magnetic relaxation of these positions. Spin rotation exhibits an inverse temperature dependence and the relative contribution of this mechanism in overall relaxation will increase at higher temperature (Farrar and Becker, 1971).

For those positions exhibiting linear Arrhenius plots, a crude estimate of the activation energy (E_a) for the motion about the C-C bonds in the alkyl chains of the bilayer can be made from the slope of the curves. This calculation yields the values of 2.3 kcal/mole for the C-4,6 positions, 2.7 kcal/mole for the C-8,14 positions, 2.7 kcal/mole for the C-9, 2.6 kcal/mole for the C-16 positions of both chain types, 2.9

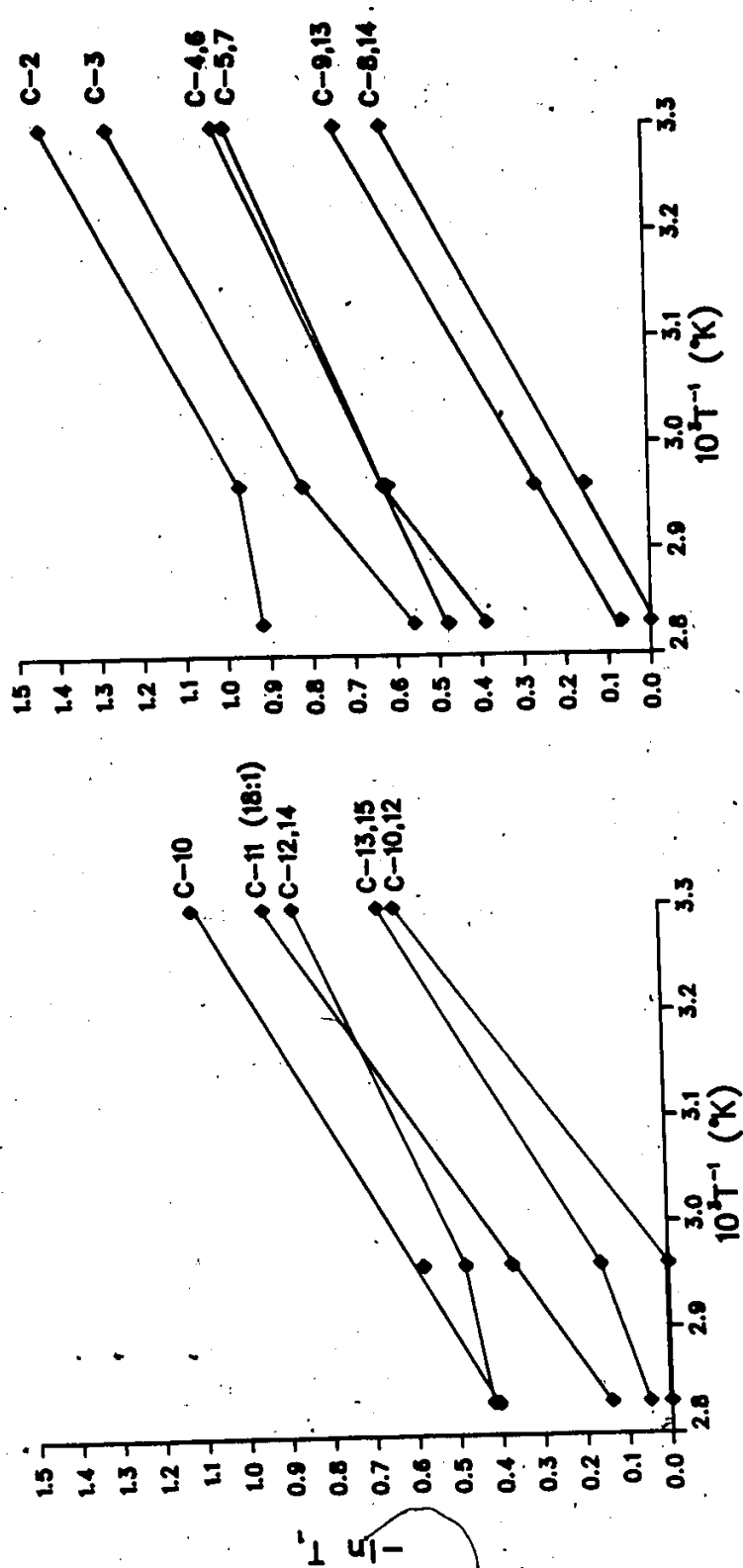
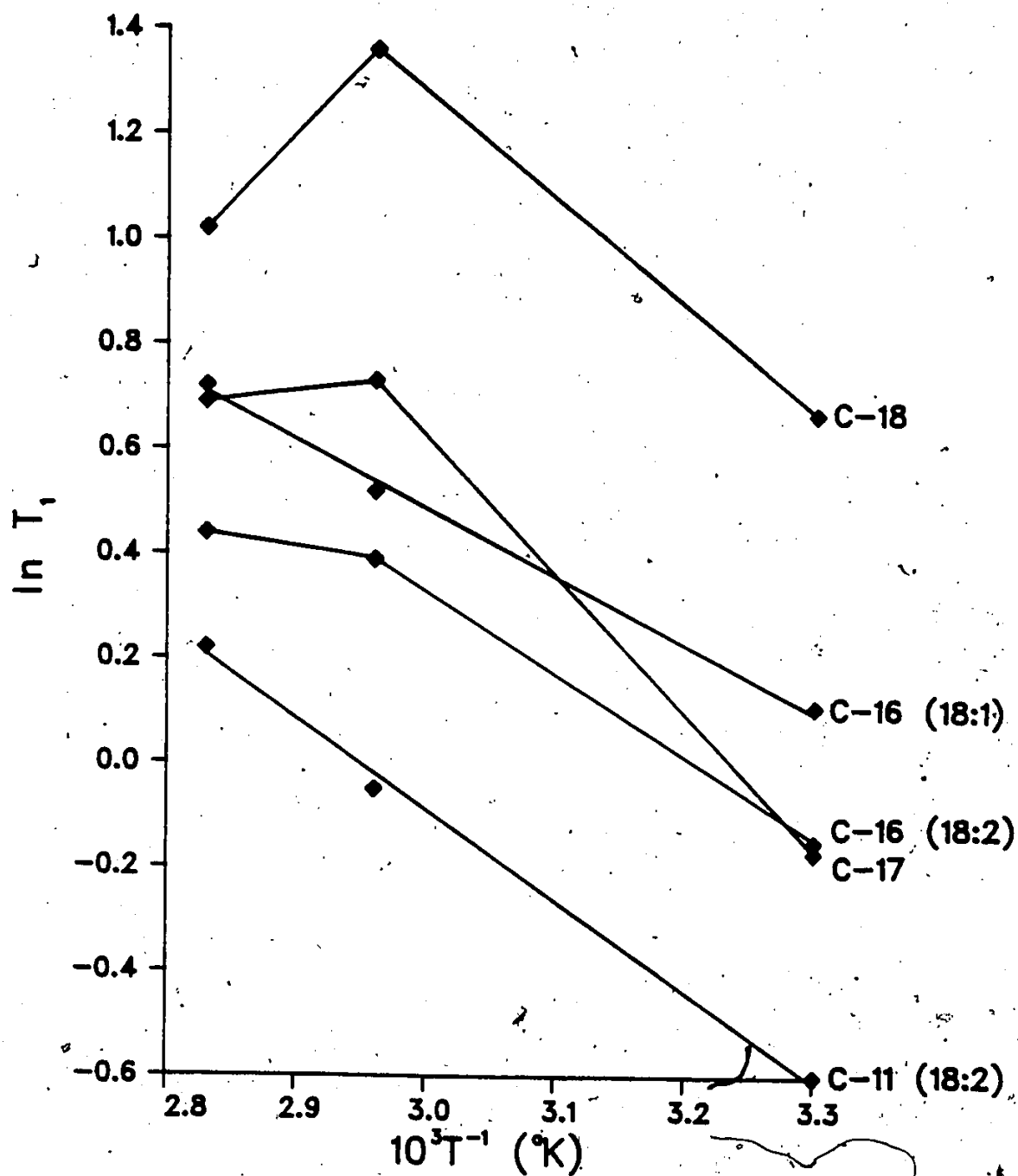
TEMPERATURE BEHAVIOR OF MEMBRANE T₁'S

Figure V.19(a)-(b) - Temperature behaviour of T₁'s of nystatin membrane preparation, observed at three temperatures. Linear plots result from those resonances assignable to specific positions

TEMPERATURE BEHAVIOR OF MEMBRANE T_1 'S

kcal/mole for the C-10 position in the C-18:1 chain and 3.4 kcal/mole for both C-11 resonances. A crude estimate for the C-2 position, for which the Arrhenius plot is not as linear as 2.4 ± 0.4 kcal/mole. These activation energies appear to be relatively independent of position, except that position C-11 has a significantly higher value. The values may be compared with a figure of 4.5 kcal/mole obtained for DMPC bilayers (Lee et al., 1974) and 4.0-4.8 kcal/mole in egg PC/PE vesicles (Gent and Rrestegard, 1974), also obtained from ^{13}C relaxation times. These values are in the range expected for the trans-gauche conformational changes in alkanes, London et al. (1975) have reported somewhat greater activation energies of 4-6 kcal/mole for fractionated Candida utilis membranes. Their results also show a higher value of E_a for the C-11 position than for other alkyl positions. The estimates for A. pullulans lipids appear somewhat lower than previous results. This may in part be due to the highly unsaturated nature of the lipid. Greater motional freedom due to reduced packing constraints would reduce the overall barrier to independent trans-gauche freedom between all C-C bonds, since the requirements for coordinated motion between sequential bonds are reduced. Candida utilis membranes, however, exhibit a considerable extent of unsaturation, although the overall composition is not dominated by any one chain species. The temperature range used for the Candida study was somewhat lower

than for A. pullulans, and the authors note that activation energies are invariably higher at lower temperatures (London et al., 1975). This could possibly contribute to the observed difference. Another factor which must be considered is that the results for A. pullulans were recorded at 75 MHz, while previous studies were carried out at 20-25 MHz. The T_1 values at 75 MHz are approaching the T_1 minimum, a region in which the temperature dependence is not as strong. For correlation times longer than 2×10^{-9} sec, an inverse temperature dependence would be expected. Preliminary temperature dependence studies on the enzyme membrane preparation (for results, see Table V.6), indicated a reduced temperature dependence and several resonances gave a nonlinear temperature dependence. In spite of the greater error in the determination of T_1 's for this type of membrane preparation, the behaviour is consistent with the slightly lower T_1 values obtained for this system as compared with whole cells. If the overall relaxation times are reduced as the triglyceride content is reduced, the temperature will be dictated by the relative values of the correlation time, which is in the region of the T_1 minimum. This is also consistent with the observation of a somewhat stronger temperature dependence for the intact cell system.

The activation energy for the free lipid will be affected by the degree of coordination of motions at successive C-C bonds. Restriction by intermolecular forces in the bilayer

will increase E_a , due to this requirement for coordination about bonds at different positions down the chain. This can be shown by analogy to ΔE (the energy difference between gauche and trans conformations, where $\Delta E < E_a$). The value of ΔE for a coordinated trans-gauche-trans conformation has been calculated to be 3.6 kcal/mole (Seelig and Niederberger, 1974). This includes a 0.5 kcal energy requirement per trans conformation (as suggested by Batchelor et al., 1972) plus an intermolecular contribution due to the extra volume required by the chain in this "kinked" conformation. The smaller values of E_a for A. pullulans may therefore reflect less coordination of conformation about successive C-C bonds. The volume in the bilayer occupied by the C-18:2 chain may be defined by the two cis double bonds through rotation of the lipid molecule about its long axis, in which case, less coordination of motion is required for trans-gauche freedom above C-9 and below C-13 in the acyl chain. The activation energies for the olefinic and methylene carbons are similar, even though there is no trans-gauche freedom in the former case. This was also observed in C. utilis, and has been explained as being due to the concerted motion of the bonds about the double bond (London et al., 1975). The value for the C-11 position in the C-18:2 chain is ~0.5 kcal/mole higher than E_a for the olefinic carbons; this may reflect the small additional energy requirement for the gauche-trans motion of the C-C bonds between the two

inflexible cis double bonds. However, due to the error involved in the calculation of E_a , more detailed studies on a purified lipid model membrane system would be required in order to more closely examine these trends.

The NT_1 profiles of the nystatin membrane preparation are compared with the other systems in Figure V.20(a) to (d). The data are plotted for both chain models and at both field strengths. In all systems, the mobility profiles are identical to the pattern observed for whole cells and lipid vesicles; the rationale behind which has been discussed in the previous section. At 20 MHz, the profiles appear to be approximately in the order of their respective triglyceride content. As triglyceride decreases the entire T_1 profile moves toward shorter T_1 's. Thus, whole cells have the highest average value of T_1 , the nystatin preparation is somewhat reduced and enzyme preparations have the shortest T_1 values. Since differences between these curves are at the limit of the statistical accuracy in determining T_1 , it is not possible to make a quantitative correlation between the value of T_1 and the relative proportion of lipid in membrane and non-membrane structures. At higher field, the same relationship does not hold true, and the profiles for the two preparations appear to bracket the profile for the intact cells. This may be due to the greater proximity of the NT_1 values for all systems to the minimum in the T_1 curve at 75 MHz as well as greater sensitivity on T_1 at higher field to differences in the size of the

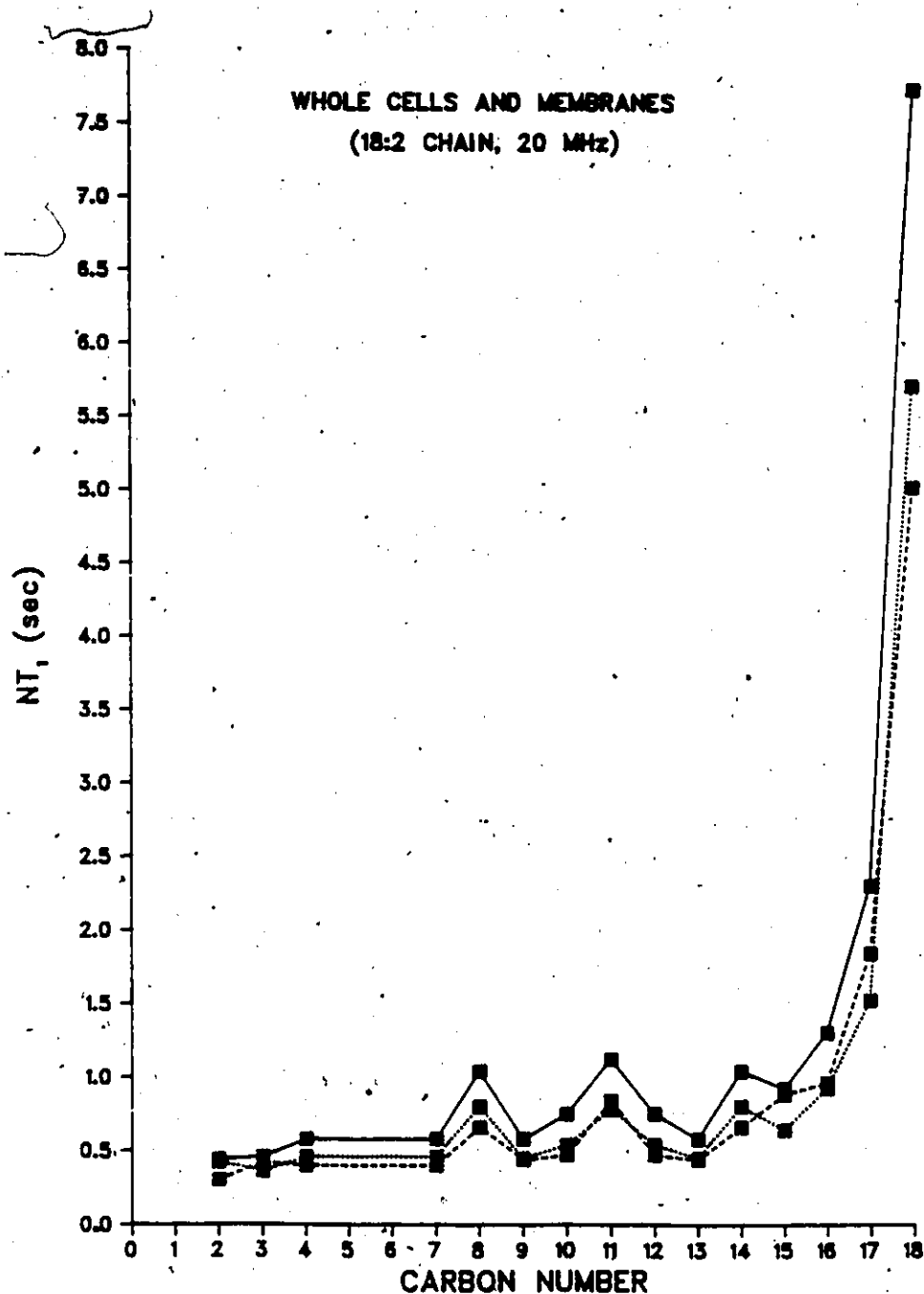
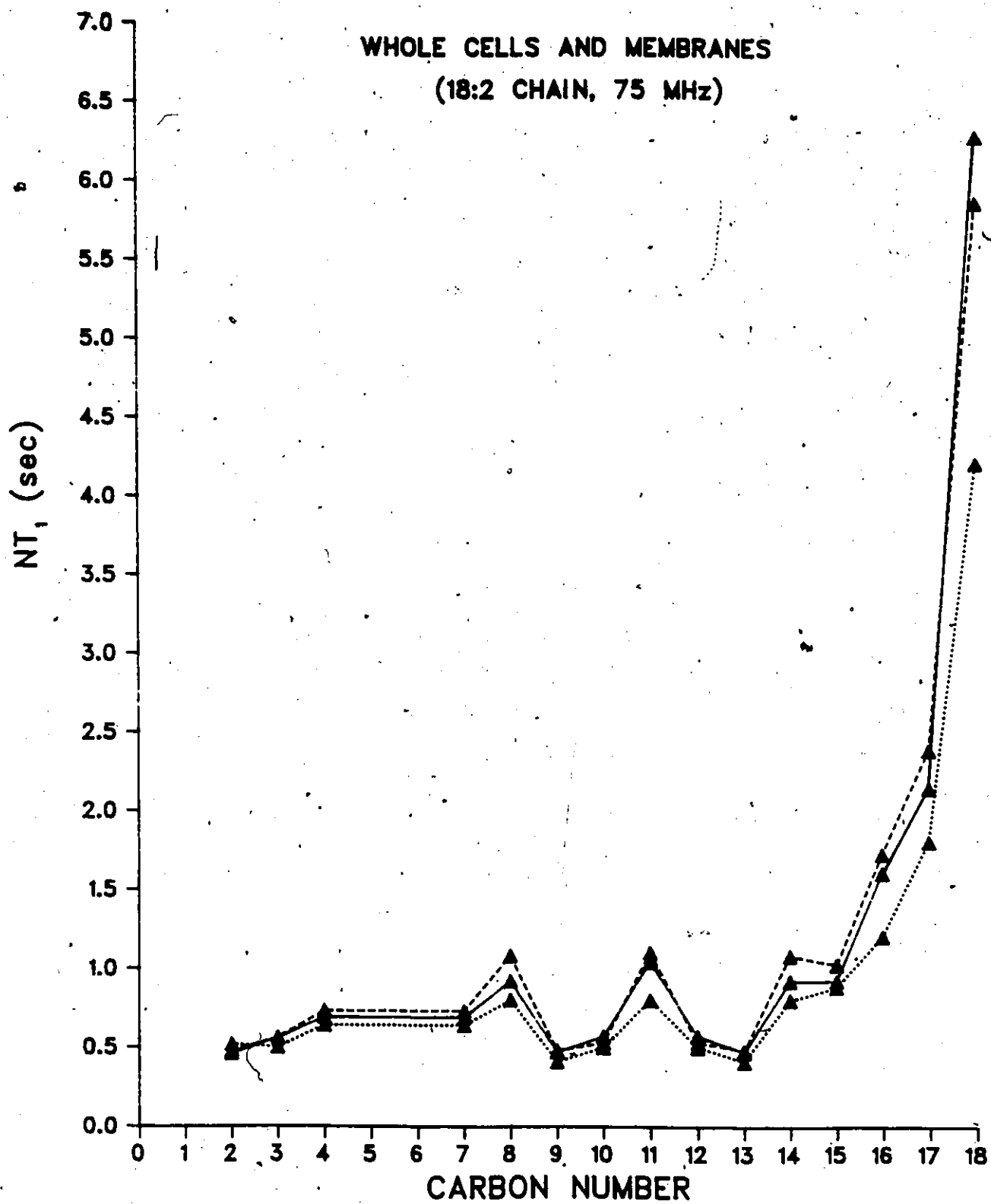
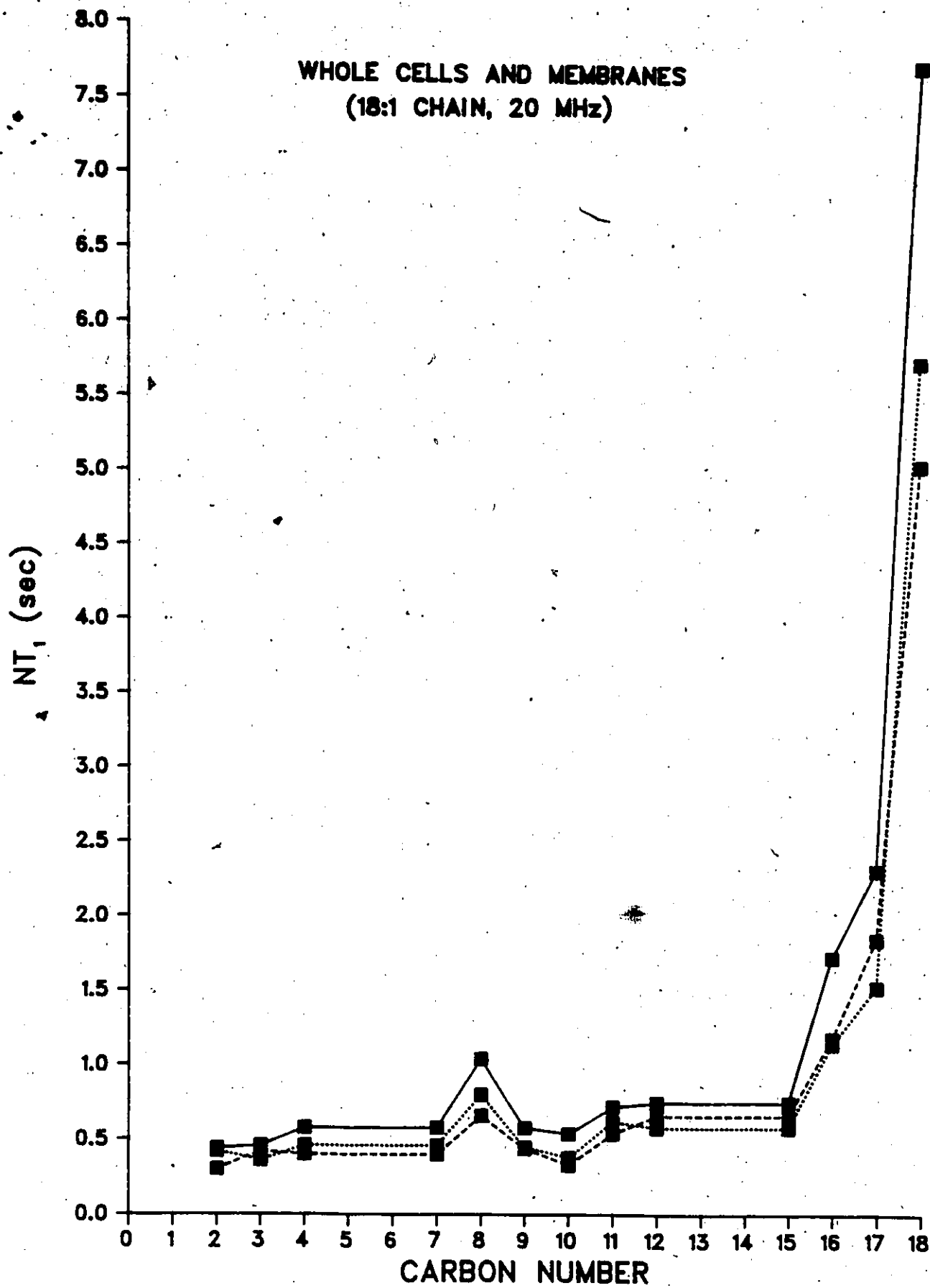
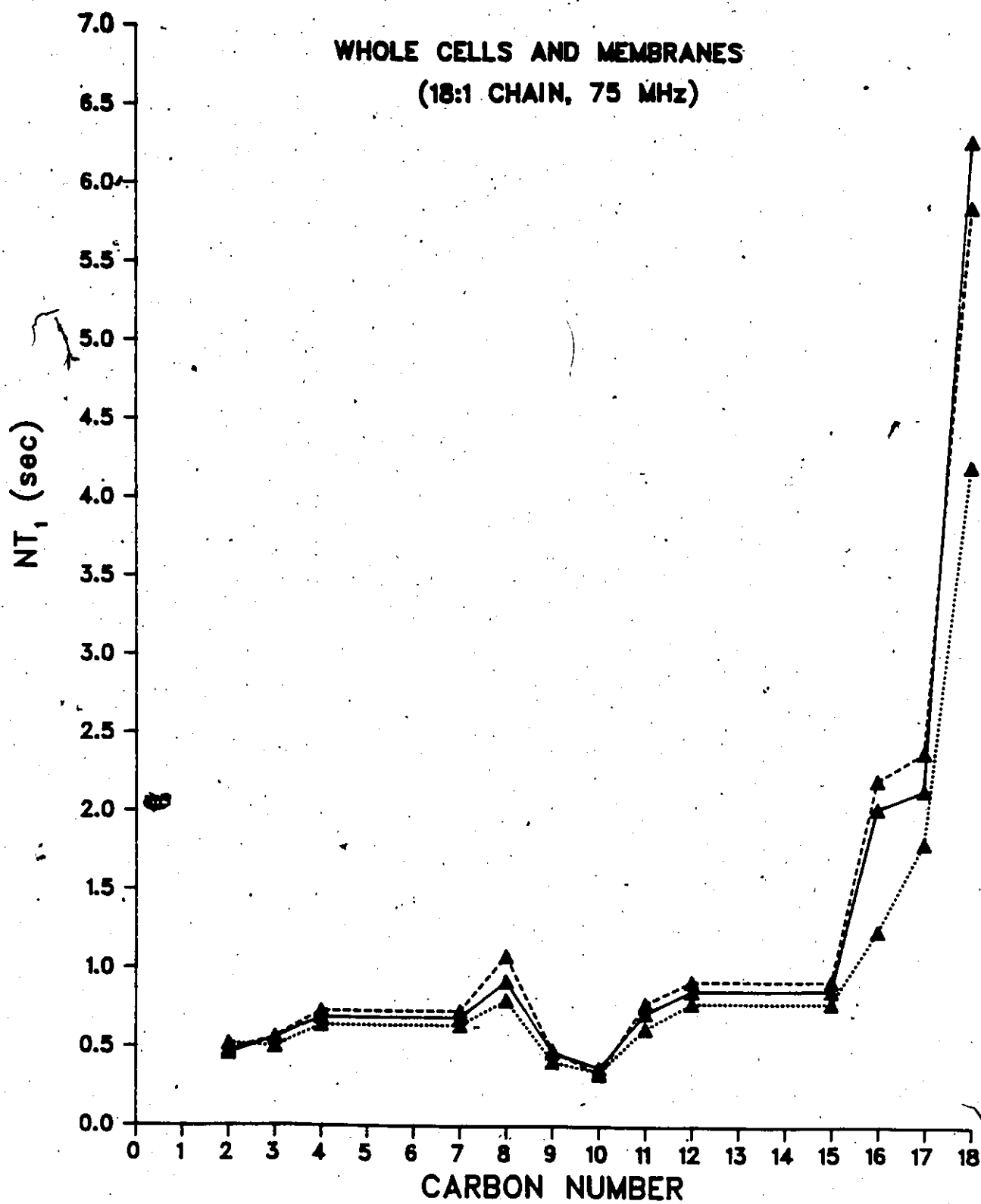


Figure V.20(a)-(d) - NT_1 vs carbon number profiles plotted for models C-18:1 and C-18:2 at both 75 MHz (Δ) and 20 MHz ($\blacksquare$) for whole cells (—), enzyme membrane preparation (----) and nystatin membrane preparation (.....). The data points are averages of several experiments.







structures. The enzyme preparation formed structures much smaller than the intact or lysed cells.

In none of the various preparations attempted - starvation, lysing, or extracting the membrane component - was it possible to remove completely the triglyceride component, as was shown in Table IV.4, although it was considerably reduced. While this may be due to lack of purity in the membrane preparation, it is possible that a small amount of triglyceride forms an integral part of the membrane. Neutral lipids have been reported to be associated with both plasma and mitochondrial membranes of yeast (Hunter and Rose, 1971), and the presence of triglyceride has been noted with membrane preparations from other species (R. Deslauriers, private communication). While the NT_1 profile of whole cells appears to be influenced by the presence of nonmembrane lipid, it is not a dominant feature of the ^{13}C relaxation of the lipid fraction. Thus, for a cellular system such as A. pullulans, the phospholipid fraction is the major determinant of the relaxation profile. The relaxation of ^{13}C nuclei of the lipid chain appears to be largely determined by the conformation of the lipid chain which is a function of the average fatty acid composition. The presence of nonbilayer lipid serves to decrease slightly the average correlation time determined by the ensemble of environments in which each particular carbon position can be found within the cell.

^1H NMR of *A. pullulans*

Figure V.21 shows a 100 MHz ^1H spectrum of whole cells as compared to vesicles formed from extracted lipid. The former spectrum is dominated by resonances due to the glucose storage pool, consistent with the natural abundance ^{13}C spectrum. The large water peak, which occurs in spite of rinsing the cell suspension with D_2O , has been reduced by a saturating rf irradiation at the frequency of the H_2O resonance. Resonances that can be assigned to lipid acyl chains are resolved, but comparison to the extracted lipid spectrum shows that lipid resonances are heavily overlapped with peaks due to protons of amino acids and small peptides. Assignment of the lipid spectrum was aided by comparison to published spectra of egg PC (Hauser *et al.*, 1975; Jendrasiak and Estep, 1977). Several of the additional resonances of the whole cell spectrum are consistent with chemical shifts of protons of the major intracellular free amino acids, arginine and lysine (Wüthrich, 1976; see Chapter VII).

In spite of the broad and nonspecific nature of the whole cell proton spectrum, relaxation measurements yielded linear, reproducible T_1 plots. Proton relaxation times were somewhat shorter than expected from the dipolar relationship.

$$\frac{NT_1(^1\text{H})}{NT_1(^{13}\text{C})} = \frac{(r_{\text{HH}})^6}{(r_{\text{CH}})^6} \frac{\gamma_{\text{C}}^2}{\gamma_{\text{H}}^2} \quad (\text{V.9})$$

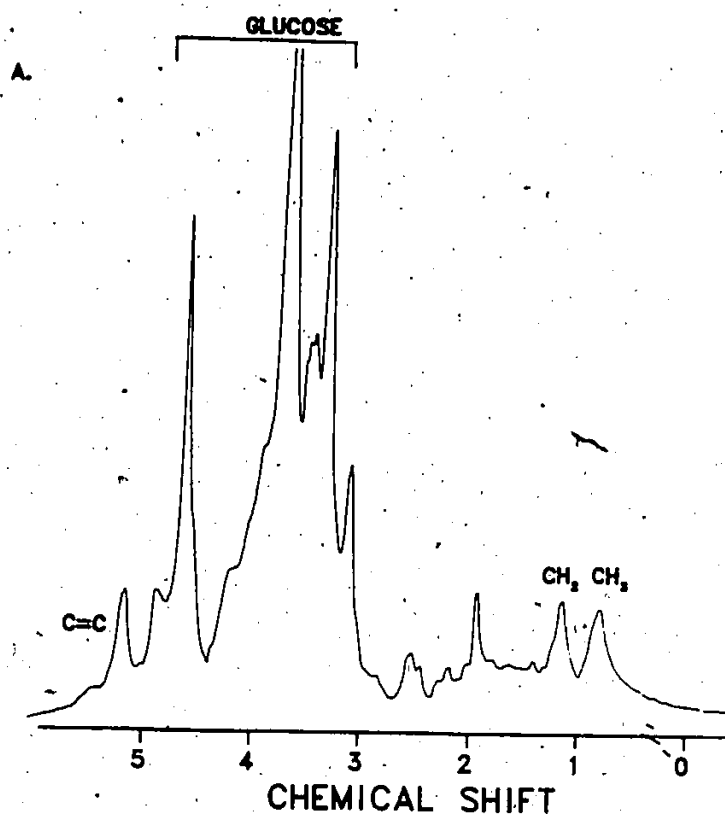
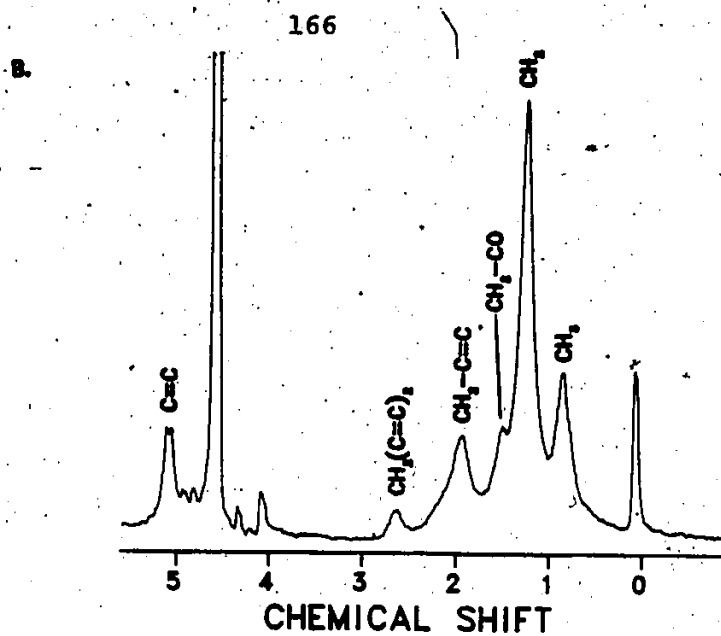


Figure V.21 - 100 MHz ^1H NMR spectrum of a) whole cells, and b) extracted lipid vesicles in D_2O at 30°C

which reduces to ~ unity for CH_2 groups, assuming a H-C-H bond angle of 109° . T_1 values ranged from 0.3 sec for the methylene peak to 0.6 sec for the methyl peak. The T_1 of the olefinic protons was 0.7 sec, while the glucose protons has longer values of 0.9-1.0 sec. The trend of increasing T_1 toward the end of the acyl chain is also present in the proton T_1 results, although the trend is not as dramatic. This is likely due to the lack of a contribution of spin rotation to the proton relaxation mechanism of the methyl groups or contribution from spin diffusion to the ^1H relaxation time.

The proton T_1 values compare well to those results obtained from distearoyl lecithin vesicles (Kainosho et al., 1978) and oriented multilayers of dimyristoyl lecithin (van der Leeuw and Stulen, 1981). Since effects of chain length and overall vesicle size appear to be very small (van der Leeuw and Stulen, 1981; Feigenson and Chan, 1974), the T_1 values for lipid vesicles and whole cells can be confidently compared. Thus, the T_1 values measured on the whole cell system appear to represent adequately the bilayer fraction. No significant increase in T_1 due to the highly unsaturated nature of the lipid appears to be present (see Figure V.22, for equivalent comparison of ^{13}C T_1 values).

Due to the inherent lack of resolution of the proton spectrum of the bilayer structure, the proton relaxation times are not sufficiently specific to elucidate mobility

properties. The rather broad linewidth of both the vesicle and whole cell spectra is due to the dipolar component of the more ordered protons of the bilayer (Bloom et al., 1978). The rather crude T_1 measurements do, however, serve to confirm that the ^{13}C relaxation times are dominated by the bilayer structure. A better approach to the study of bilayer dynamics using proton NMR would employ the polarization transfer technique of the INEPT pulse sequence (Morris, 1980). This technique allows the determination of the proton relaxation times via the resolution of the ^{13}C spectrum, and would be an ideal tool for determining the exact relationships between the ^{13}C and ^1H relaxation times experienced by the acyl chains of the bilayer.

Discussion

The results of this study indicate that the lipids of a naturally-occurring, intact, functioning membrane have motional properties giving rise to effective correlation times on the order of 10^{-10} sec, and down to 10^{-11} sec at the centre of the bilayer. This is not an unusual result by comparison to model bilayer systems. A similar estimate has been obtained from the ^2H T_1 's of selectively deuterated DPPC bilayers (both single bilayer vesicles and multilamellar dispersions) (Brown et al., 1979). The ^2H relaxation profiles also showed a plateau in T_1 for positions C-2,9 of the acyl chain, corresponding

to a $\tau \approx 10^{-10}$ sec, and an increase in T_1 toward the end of the chain corresponding to a $\tau \approx 10^{-11}$ sec. A significant increase in $^2\text{H } T_1$'s was observed upon introducing a single unsaturation into the acyl chain. For the latter case, the $^2\text{H } T_1$ also decreased across the 9,10 cis double bond. The behaviour of the ^{13}C relaxation times is also qualitatively similar to that of selectively deuterated potassium palmitate in the liquid crystalline phase (Davis et al., 1978), where a similar order of magnitude was obtained for the correlation time. This comparison serves to point out that the dynamics of the individual chains appear to be the most important aspect in determining spin lattice relaxation times, whether quadrupolar or dipolar, while the macroscopic order of the system appears to be of less importance. The plateau behaviour was observed in the potassium palmitate system only for temperatures just above the gel to liquid-crystal phase transition; at higher temperatures the relaxation profile resembles that for the free species - a steadily increasing T_1 upon moving away from the carboxyl end of the acyl chain. In the lipid bilayer, this plateau region is preserved at temperatures well above the phase transition. This shows the effect of additional intermolecular forces between the acyl chains of lipid molecules in phospholipids as opposed to soaps.

The measurement of deuterium T_1 's also allows a comparison of the dynamics of the bilayer as determined from the T_1 , to

the degree of order within the bilayer as determined from quadrupolar splittings, for each position of the acyl chain. By analogy with systems for which order parameters have been measured, it might be expected that the NT_1 values decrease more at positions closer to the carboxyl group. While there is no necessary correlation between order and mobility, it appears intuitively that mobility would increase as packing constraints decrease. Brown et al., (1979) have observed that the behaviour of the order parameter as a function of position can be roughly correlated with the T_1 profile for DPPC bilayers, in that a plateau region is maintained at higher order from C-2 to C-9 followed by a decrease to lower order towards the end of the chain. While the order parameter profile has not yet been determined for the C-18:2 system, it has been determined for 15 positions of specifically deuterated oleic acid (C-18:1) incorporated into the membranes of A. laidlawii (Rance et al., 1980), which provides a crude comparison with the ^{13}C T_1 profile of the A. pullulans unsaturated system. These deuterated membranes show a steady decrease in order parameter from C-2 to C-18, with a sharp decrease at positions 10 and 11. While there is no strict plateau observed in the order parameter profile, the overall rate of decrease in order with position is much less from C-2 to C-15, than for the last two positions. The rigid double bond system leads to an overall decrease in order (S_{CD}) as well as a slight decrease in mobility.

The decrease in order then allows a greater freedom of motion at the next flexible position (C-11), which was observed in the T_1 profile. By analogy, it would be expected that these effects would be amplified in the order profile for a doubly unsaturated system. Thus, the qualitative features of the T_1 profile reflect intermolecular effects which are more sensitively displayed by the order parameter profile.

The ^{13}C T_1 profile may also be compared to that of other systems, both model and intact membranes, although the present work provides greater resolution of chain position. Figure V.22 compares the ^{13}C NT_1 values in the C-18:1 chain model of the lipids of A. pullulans at 30°C and in the 16:0 chains of dipalmitoyl lecithin at 52°C (Lee et al., 1976). The difference in temperature provides a comparison that roughly accounts for the temperature difference of the gel to liquid-crystal phase transition of the lipids of the two different systems. This has proved an important factor in comparing order profiles of different lipid systems (Rance et al., 1980). Due to the lack of unsaturation of the palmitoyl chains, it is difficult to resolve, in the natural abundance spectrum, any of the individual resonances in the region C-4 to C-13, so that average values are given. However, some similarities can be noted. In a now familiar pattern, the apparent mobilities of the carbon atoms are essentially constant for all but the last two carbon atoms, which are considerably more mobile. This

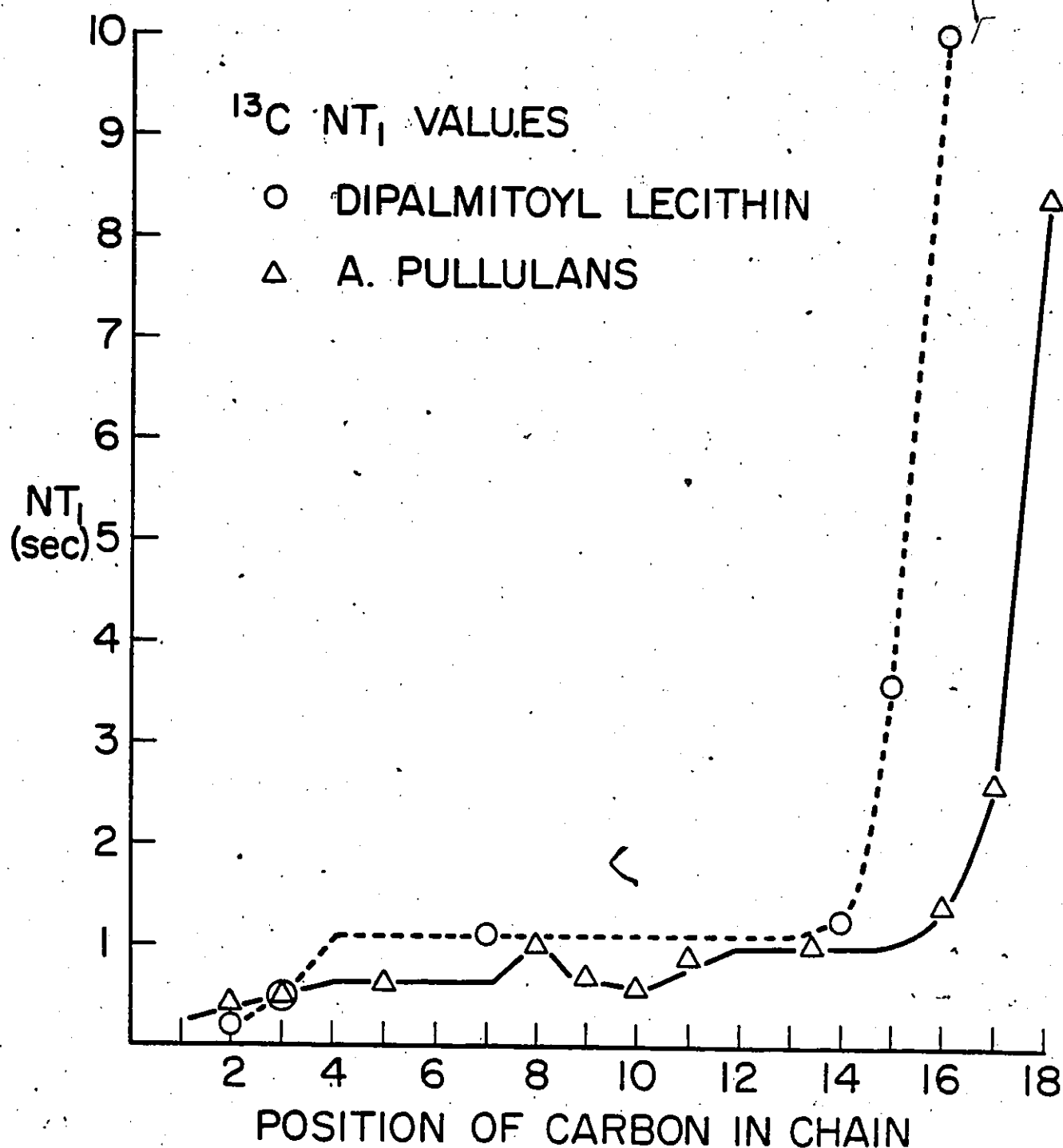


Figure V.22 - ^{13}C NT₁ values in the 18:1 chains of the lipids of *A. pullulans* cells at 30°C and in the 16:0 chains of dipalmitoyl lecithin vesicles at 52°C (Lee et al., 1976). Where no points are given for a particular carbon, this is due to lack of resolution of individual resonances; in this case an average value is given, as for C-4 to C-13 of dipalmitoyl lecithin and C-4 to C-7 of the 18:1 chains.

correlation between NT_1 and position has been demonstrated to hold, regardless of chain length (Lee et al., 1976).

Furthermore, the terminal T_1 values are relatively independent of chain length, indicating that the intermolecular interaction extends the full length of the chain in the bilayer (Lee et al., 1976). Comparison of this aspect to non-bilayer motion of n-alkanes (Levine et al., 1974), shows that the profiles of the latter increase much more sharply for shorter chain lengths. The mobility profiles of alkanes do not show a plateau region, but rather a steady increase of NT_1 from the centre of mass of the molecule to the ends. The plateau region of the bilayer profile has been extensively discussed in terms of the trans-gauche mobility model by Wright et al., (1979). The macroscopic order determines the overall T_1 and the overall rate of axial motion of the lipid molecule. The plateau region is determined by rapid axial motion plus equal trans-gauche motion about individual C-C bonds. The lower T_1 of this region is due to the lower rate of trans-gauche motion. This, in turn, is due to molecular interactions which result in coordinated rotations that favour a roughly straight chain configuration to minimize these interactions. Toward the end of the chain, the axial motion remains the same while the rate of trans-gauche motion increases, thus increasing T_1 . This model predicts that the overall macroscopic order is compatible with the overall T_1 , and adequately accounts for the relative

positions of the T_1 profiles in various solvents, for example as a bilayer in water, and as an inverted micelle in chloroform.

The comparison of Figure V.22 further points out that while the mobility profile is highly sensitive to the properties of individual chains, and that the relative positions of the T_1 profile is largely determined by the overall order, that ^{13}C T_1 's are relatively insensitive to local variations within the bilayer structure. The good agreement between the profiles of the natural membrane and the total lipid vesicles shows that these motions are not affected by the significant protein component. The further good agreement with a pure lipid system also shows that the sterol fraction, isolated with the total lipid, also seems to have little effect. This shows the limitation of this method to determine specific interactions of this type. These mechanism of these interactions are perhaps better probed by other parameters, such as ^2H order parameters. However, the sensitivity to chain composition illustrated by this study shows that this may be a valuable tool in studying the mobility properties of longer and more unsaturated lipid systems such as C-22:4 and C-20:4 which are common components of membranes of higher organisms, or of unusual lipid systems such as the tetra ether lipids which span the membranes of some thermophilic methanogenic bacteria (Kushwaha et al., 1981).

The results presented for A. pullulans also agree well with a similar study performed on the non-specifically labelled

membranes of Candida utilis (London et al., 1975). Although the system is not as well defined in fatty acid composition, the qualitative features of mobility increase at position C-8 and the decrease at the double bond region are present. A greater variation in the profile is observed from C-2 to C-4 in the latter system, which may be due to the greater heterogeneity of the lipids.

A valuable aspect of this cellular system is that it provides at the very least a simple and inexpensive route to the synthesis of ^{13}C (or ^2H) enriched fatty acids. It is possible to incorporate these labelled fatty acids specifically into the membranes of other organisms (e.g., A. laidlawii). This would provide the correlation of ^{13}C T_1 values and order parameters for membranes of a variety of organisms, as a basis for the complete picture of the spatial and temporal aspects of lipid organization in the intact membrane.

APPENDIX TO CHAPTER V

The following tables summarize the data obtained in relaxation time measurements for all systems studied. The deviations are average deviations from the mean of the given number of experiments.

Table V.2

 T_1 VALUES AT 20 MHz (sec)A) ^{13}C -1 Acetate Label

Peak #	Assignment	Whole Cells (8 expts)	Starved Cells (4 expts)	Debris (1 expt)	Memb. Preparations Nystatin (2 expts)	Enzyme (3 expts)	Extracted Lipid D_2O (vesicles) (6 expts)	CDCl_3 (5 expts)
1	C-17	1.15 $\pm$.1	1.17 $\pm$.1	1.73	.92 $\pm$.06	.76 $\pm$.06	.95 $\pm$.1	4.2 $\pm$.4
2	C-3	.23 $\pm$.01	.28 $\pm$.01	.22	.21 $\pm$.01	.18 $\pm$.02	.25 $\pm$.02	1.3 $\pm$.2
3	C-11(18:2)	.56 $\pm$.05	.47 $\pm$.08	.50	.42 $\pm$.04	.39 $\pm$.03	.50 $\pm$.05	2.2 $\pm$.3
4	C-11(18:1)	.36 $\pm$.05	.40 $\pm$.10	.39	.27 $\pm$.02	.31 $\pm$.03	.33 $\pm$.01	1.9 $\pm$.2
5	C-13,15(18:1), C-15(18:2)	.46 $\pm$.08	.52 $\pm$.05	.45	.44 $\pm$.08	.32 $\pm$.02	.46 $\pm$.06	(18:1) 1.8 $\pm$.06
6	C-5,7	.28 $\pm$.02	.25 $\pm$.02	.30	.22 $\pm$.02	.21 $\pm$.03	.30 $\pm$.03	(18:2) 2.3 $\pm$.1
7	C-9,13(18:2), C-9(18:1)	.58 $\pm$.03	.44 $\pm$.10	.54	.44 $\pm$.01	.45 $\pm$.02	.51 $\pm$.05	1.5 $\pm$.1
								2.6 $\pm$.3

B) ^{13}C -2 Acetate Label

Peak #	Assignment	Whole Cells (8 expts)	Starved Cells (4 expts)	Debris (1 expt)	Memb. Preparations Nystatin (2 expts)	Enzyme (3 expts)	Extracted Lipid D_2O (vesicles) (6 expts)	CDCl_3 (5 expts)
1	C-18	2.56 $\pm$.2	2.45 $\pm$.30	2.65 $\pm$.1	1.67 $\pm$.03	1.90 $\pm$.1	1.64 $\pm$.08	4.7 $\pm$.4
2	C-8,14(18:2), C-8(18:1)	.52 $\pm$.04	.47 $\pm$.07	.46 $\pm$.05	.33 $\pm$.01	.40 $\pm$.05	.54 $\pm$.05	2.4 $\pm$.2
3	C-4,6	.30 $\pm$.04	.34 $\pm$.08	.25 $\pm$.03	.18 $\pm$.00	.25 $\pm$.03	.37 $\pm$.06	1.5 $\pm$.2
4	C-12,14(18:1)	.35 $\pm$.04	.33 $\pm$.08	.34 $\pm$.03	.22 $\pm$.01	.26 $\pm$.03	.42 $\pm$.08	1.6 $\pm$.1
5	C-16(18:1)	.86 $\pm$.07	.81 $\pm$.10	.75 $\pm$.10	.59 $\pm$.06	.57 $\pm$.02	.87 $\pm$.05	4.0 $\pm$.3
6	C-16(18:2)	.65 $\pm$.04	.73 $\pm$.05	.68 $\pm$.03	.48 $\pm$.05	.46 $\pm$.03	.65 $\pm$.07	3.2 $\pm$.3
7	C-2	.22 $\pm$.05	.23 $\pm$.03	.16 $\pm$.01	.13 $\pm$.00	.21 $\pm$.02	.24 $\pm$.02	1.2 $\pm$.2
8	C-10,12(18:2)	.75 $\pm$.05	.77 $\pm$.10	.71 $\pm$.1	.47 $\pm$.02	.54 $\pm$.04	.69 $\pm$.04	3.2 $\pm$.3
9	C-10(18:1)	.54 $\pm$.04	.40 $\pm$.10	.53 $\pm$.05	.33 $\pm$.00	.38 $\pm$.04	.41 $\pm$.08	2.2 $\pm$.03

Table V.3

 T_1 VALUES AT 75 MHz (sec)

A) $^{13}\text{C-1 Acetate Label}$				
Peak #*	Whole Cells (3 expts)	Membrane Preparations Nystatin (1 expt)	Enzyme (4 expts)	Lipid Vesicles in D_2O (1 expt)
1	1.07 $\pm$.09	1.19	.9 $\pm$.1	.88
2	.28 $\pm$.01	.28	.25 $\pm$.03	.32
3	.52 $\pm$.02	.55	.40 $\pm$.05	.33
4	.36 $\pm$.02	.39	.31 $\pm$.03	.33
5	.46 $\pm$.02	.51	.44 $\pm$.05	.42
6	.36 $\pm$.02	.37	.33 $\pm$.03	.24
7	.47 $\pm$.02	.48	.41 $\pm$.09	.42
B) $^{13}\text{C-2 Acetate Label}$				
1	2.09 $\pm$.2	1.95	1.4 $\pm$.2	-
2	.46 $\pm$.05	.54	.39 $\pm$.05	.31
3	.33 $\pm$.02	.36	.31 $\pm$.03	.24
4	.39 $\pm$.02	.42	.34 $\pm$.05	.29
5	1.01 $\pm$.1	1.12	.62 $\pm$.09	.55
6	.80 $\pm$.1	.86	.60 $\pm$.08	.48
7	.23 $\pm$.02	.24	.26 $\pm$.03	.19
8	.57 $\pm$.06	.53	.50 $\pm$.05	.44
9	.37 $\pm$.05	.33	.34 $\pm$.03	.41

* Assignments are as presented in Table V.2

Table V.4.

LINEWIDTHS* (Hz)

<u>Field</u>	<u>Whole Cells</u>	<u>Membrane Preparations</u> <u>Nystatin</u>	<u>Enzyme</u>	<u>Lipid Vesicles</u>
20 MHz	4-5	4-5	8	4-5
75 MHz	12	12	15-17	24-30

* Based on minimum linewidths from 2 or 3 peaks which are assignable to a single carbon position

Table V.5

NOE VALUES

Peak	Membrane Preparations				Whole Cells (75 MHz)
	Nystatin		Enzyme		
	(20 MHz)	(75 MHz)	(20 MHz)	(75 MHz)	
A)	<u>¹³C-1 Acetate</u>				
1	2.2	2.4	2.8		2.6
2	2.3	2.2	2.7		2.5
3	2.6	1.8	3.1		2.8
4	2.3	2.4	3.1		2.6
5	2.6	2.5	2.5		2.5
6	2.4	2.2	2.1		2.4
7	2.7	2.5	2.2		2.4
B)	<u>¹³C-2 Acetate</u>				
1	2.6	3.1	2.5	2.5	2.8±.1
2	2.6	3.1	2.5	2.8	2.7±.01
3	2.4	2.8	2.3	2.5	2.6±.02
4	2.6	2.8	2.4	2.2	2.3±.01
5	2.9	3.1	2.8	2.4	3.0±.1
6	2.9	3.1	2.6	2.7	2.5±.06
7	2.7	2.4	2.3	2.2	2.3±.06
8	2.7	2.8	2.7	2.9	2.6±.1
9	2.7	2.7	2.7	2.6	2.4±.06

Table V.6

TEMPERATURE VARIATION OF T_1 (75 MHz)A) ^{13}C -1 Acetate Label

Peak	Membrane Preparations					Whole Cells
	Nystatin			Enzyme		
	303 ^o K	338 ^o K	353 ^o K	303 ^o K	310 ^o K	
1	1.19	2.08	2.1	.76	.89	
2	.28	.44	.57	.23	.24	
3	.55	1.05	1.25	.35	.42	
4	.39	.69	.87	.29	.34	
5	.51	.85	.95	.38	.46	
6	.37	.53	.68	.29	.32	
7	.48	.76	.93	.32	.38	

B) ^{13}C -2 Acetate Label

	303°K	338°K	353°K	303°K	323°K	348°K	303°K	358°K
1	1.95	3.9	2.8	1.50	1.26	1.17	1.74	2.75
2	.54	.86	1.0	.42	.43	.47	.42	1.06
3	.36	.53	.62	.32			.30	.67
4	.42	.62	.66	.35	.35	.37	.34	.72
5	1.12	1.68	2.06	.70	.67	.73	.87	1.96
6	.86	1.47	1.55	.63	.70	.79	.67	1.52
7	.24	.38	.40	.23	.23		.21	.44
8	.53	1.0	1.0	.52	.53	.60	.45	1.04
9	.33	.56	.66	.34	.37	.45	.25	.80

CHAPTER VI

^2H AND ^{31}P of A. PULLULANS LIPIDS

In order to characterize further the membrane properties of this organism, those NMR experiments which reflect the degree of order in the biological membrane were attempted. The degree of order in a natural lipid chain in a bilayer can be accurately determined from the analysis of the quadrupole splittings patterns of specifically deuterated chain, and the properties of the phospholipid head groups can be determined from the phosphorus CSA patterns. Such information is of a complementary nature to that of the ^{13}C T_1 's since the information reflects to a greater extent the intermolecular forces involved in membrane dynamics. These experiments proved not to be as easily performed as the high resolution ^{13}C studies, for a variety of reasons. Both ^2H and ^{31}P spectra are dominated by their respective isotropic components in both the intact cell system and the membrane preparation. In addition, the species under observation is much more diluted in the intact system than the model membrane system. The ^{31}P spectra are complicated by the dilution of the phospholipid species and by the relatively much larger concentration of isotropic inorganic phosphate. The deuterons on the lipid chains experience both the dilution due to the dispersion of the lipid molecules, and the dilution of the label over the even-numbered chain

positions. As was pointed out in Chapter IV, the culture of the organism on perdeuterated acetate leads to the incorporation of only half the number of deuterons expected from the incorporation of ^{13}C . In addition, structures such as the micellar triglycerides contribute to the isotropic component.

These factors made it extremely difficult to observe the anisotropic components, both in intact cells and in membrane preparations. The use of the enzyme-prepared membranes should have been feasible, at least for the deuterium spectra, by analogy to the success achieved with A. laidlawii. However, the deuterated cells proved to be somewhat more fragile than those grown with ^{13}C labelled or unlabelled acetate, and the preparation resulted in a high loss of labelled material as well as a greater fragmentation of the membrane fraction. The use of isolated lipids not only permitted a much higher concentration of the nucleus under observation, but served as well to eliminate interfering isotropic signals (internal phosphate and exchangeable deuterium). The ^{13}C results permit to some extent, extrapolation of the results obtained from the total lipid system to the intact membrane. More powerful pulse techniques, such as cross-polarization in the case of ^{31}P powder spectra, could be employed in order to excite specifically the nuclei of the dilute species in the intact system without the interference of the isotropic component (A. Byrd, unpublished).

(a) ^2H NMR of A. pullulans Lipids

The NMR of selectively deuterated lipid models has proven to be a reliable method for the determination of the order parameter, S_{CD} , associated with a particular C-D bond direction with respect to the magnetic field. This feature is due to the quadrupole moment of the ^2H nucleus. The quadrupole interaction with the magnetic field causes a splitting of the Zeeman resonances, whose frequencies are dependent upon the angle between the C-D bond and the magnetic field. In a slowly tumbling membrane, all orientations of the C-D vector with respect to the field will be populated and the resulting spectrum will be a superposition of individual spectra due to the many orientations. This "powder pattern" has well defined peaks and shoulders, whose splittings correspond to the 90° and 0° orientations of the C-D bond angle, respectively. The quadrupole splitting of the shoulders is twice that of the powder peaks. The additional rapid motion of the lipid system reduces the values of the splittings in a manner proportional to the rate of motion, such that if the motion is assumed to be axially symmetric, the order parameter can be described as:

$$\Delta\nu_Q = \left(\frac{3}{4}\right) \left(\frac{e^2 q Q}{h}\right) S_{\text{CD}} \quad (\text{VI.1})$$

where $e^2 q Q/h$ is the static deuterium coupling constant of approximately 170 KHz for aliphatic C-D bonds.

A more detailed account of the theory behind ^2H NMR as applied to the lipid bilayer, which is not necessary for the present discussion, can be found in Mantsch et al., (1977) and Seelig (1977). Due to the nature of the incorporation of deuterium, the spectrum of A. pullulans lipids represents the spectral sum of all powder patterns for all deuterated positions of the lipid chain, and therefore can not give the detailed information as a function of position that is possible in specifically deuterated systems or from the high resolution ^{13}C experiment. In theory, it should be possible to extract these individual splittings, and assign them by comparison to the specifically deuterated model, provided that the positions are deuterated to a sufficient extent to give the required spectral quality (Davis et al., 1980; Bloom et al., 1981). This quality is intrinsically difficult to achieve using a method of biosynthetic incorporation from a non-specific precursor such as acetate. The purpose of these few experiments was to determine the envelope of quadrupole splittings, and perhaps the magnitudes of these splittings, in order to compare with the oleate profile in A. laidlawii (Rance et al., 1980).

(i) ^2H Experimental Parameters

The use of a model membrane system permitted an increased ^2H incorporation, by supplementing the growth medium with

D₂O. In an intact system this would only serve to increase the isotropic component. Lipid extractions were performed on cells grown on the defined medium, deuterated acetate and 50% D₂O. Dry lipids were hydrated with deuterium-depleted water, sonicated and relyophilized repeatedly until the lipid appeared to disperse easily in water. This had the effect of both removing the exchangeable deuterium from the crude lipid and removing the traces of organic solvent which make an even dispersion difficult to obtain. The affinity of the lipid for organic solvent seems to be a property of the more unsaturated lipid mixtures extracted from higher microorganisms (H. Jarrell and R. Deslauriers, private communication). The resulting 45 mg of lipid was taken up in 1 ml deuterium depleted water using a vortex mixer to suspend the sample. The sample was then cycled through several freeze-thaw-vortex cycles, until a homogeneous mixture was obtained. This procedure was an attempt to maximize the uniformity of particle size in the liposome suspension. This was important since the total lipid mixture had been through no purification process to remove the triglyceride component. From the analysis of crude lipid extracts by GC, approximately 10 mg of this 45 mg was phospholipid; the remainder is due to TG, sterol and hydrocarbon (see Chapter IV).

The spectra were observed using conditions outlined in Chapter III. The solid echo technique of spectral accumulation was used in order to maximize the detection of the short T_2 component (Davis et al., 1976). The basic echo pulse sequence consists of an initial 90° pulse applied along the x direction in the rotating frame followed by a 90° pulse in the y direction at a time τ later. The echo peak occurs at a time τ after the second pulse. Combined with a fast digitization rate, and a rapid recycling time compared to the T_1 of the isotropic component, this technique enhances the observation of the powder component in the presence of the isotropic component.

(ii) ^2H Results

In spite of these precautions, there was a large isotropic component in the liposome spectrum. Figure VI.1 shows the spectra of the extracted lipid liposomes and the cellular debris (after extraction and rinsing with deuterium-depleted water). The lipid spectrum is a result of 38000 accumulations and the debris spectrum, of 56000 accumulations. All other spectral parameters were the same for the two systems. The spectra are represented as difference spectra in order to enhance the portion of the spectra that shows the quadrupole splittings. The initial points of the FID

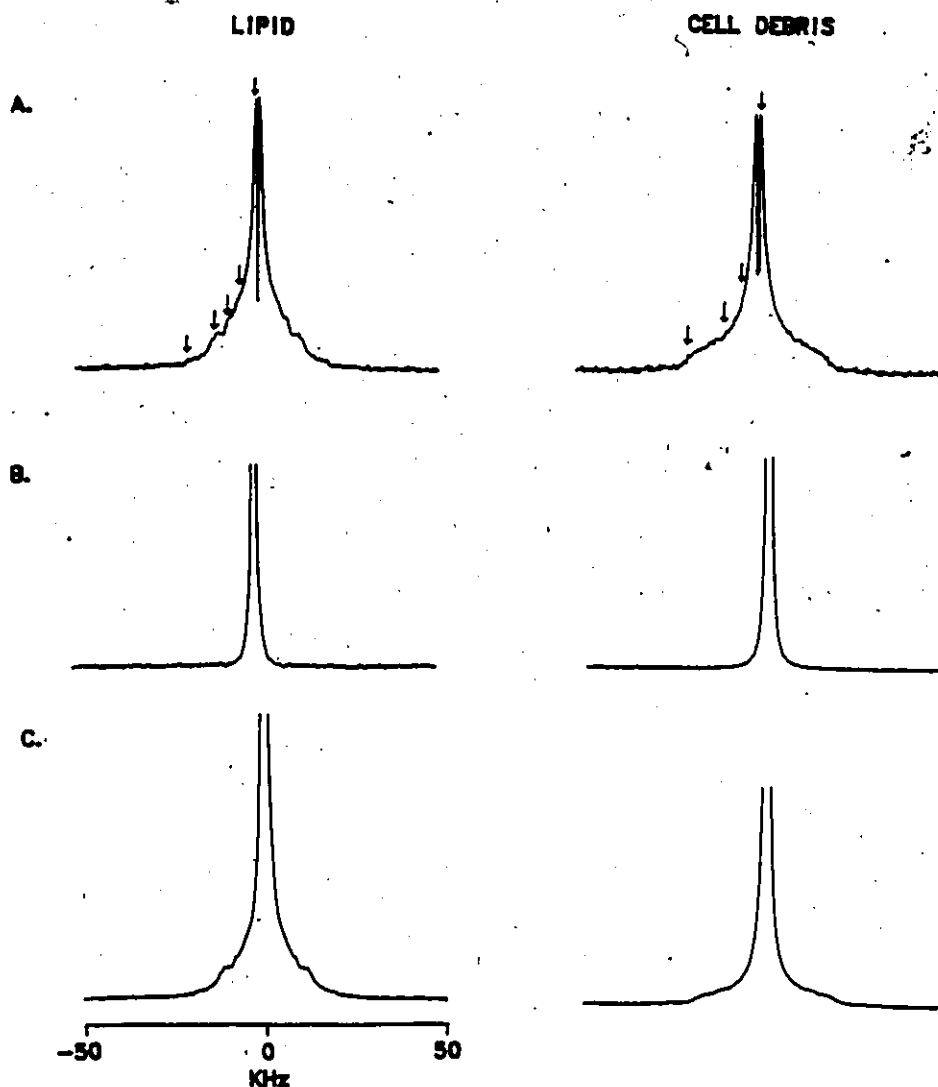
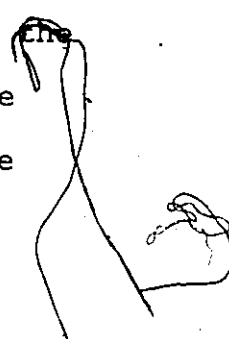


Figure VI.1 - 46 MHz ^2H NMR spectra of 45 mg/ml of extracted lipid from cells grown in 50% D_2O and d_3 -acetate and the debris after extraction, in deuterium depleted water. Spectra (C) show the large isotropic component with some quadrupole pattern in the base of the peak. The spectra represent 56000 and 36000 accumulations respectively, using a quadrupole echo pulse sequence where the pulse spacing was 60 μs , quadrature detection on 2K data points, 125 KHz spectral window and 0.5 s recycle time. Spectra (A) are a result of subtracting an isotropic component of 150 Hz linewidth (the linewidth of the water resonance), (spectra (B)) from the FID before the Fourier transform. The arrows correspond to quadrupole splittings of 38, 22, 16, 8 and 1 KHz for the lipid spectrum and 40, 17, 9 and 1.3 KHz for the debris spectrum.

were deleted, until the resultant line had a linewidth of 150 Hz, which was the linewidth of the water line under these spectral conditions. This portion of the FID was then subtracted from the original FID, effectively eliminating that fraction with a long T_2 time. The natural linewidth of the entire spectrum was 250 Hz. The subtracted FID was then subjected to 6K of zero filling, left shifted to the top of the FID (note that the second delay time in the echo sequence is shorter than the first so that accumulation begins before the peak of the quadrupolar echo), and Fourier transformed.

The spectra of Figure VI.1 show that there are definite "shoulders" of quadrupole splittings on the perdeuterated profile. These defined splittings are shown by the arrows on the figure. The maximum observed splitting was 38-40 KHz, and the profiles in the two systems are approximately the same, given the reduced signal of the debris spectrum. The shoulders appear at 38, 22, 16 and 8 KHz in the liposome spectrum and 40, 17 and 9 KHz in the debris spectrum. Smaller quadrupole splittings are present since significant signal down to 1 KHz splitting is maintained after spectral subtraction of the isotropic line. The lack of resolution of these smaller splittings is likely due to the choice of delay time in the echo sequence. Previous experiments have shown that the spectral shape can be strongly influenced by this choice



(Rance et al., 1980), due to the angular dependence of T_2 across the powder spectrum.

The similar quadrupole splitting pattern of the two systems, liposomes and cellular debris, could be expected. A close agreement was observed between the order parameter profiles of specifically labelled dioleoylphosphatidylcholine (Seelig and Waespe-Sarcevic, 1978) and oleate-labelled A. laidlawii membranes (Rance et al., 1980) except in the first two positions of the lipid chain. There appears to be very little bulk effect of the additional components of the natural membrane on the overall order. It is likely that the magnitudes of quadrupole splittings in the liposome spectrum reflect that in the natural membrane of A. pullulans.

The oleate-supplemented membranes of A. laidlawii show quadrupole splittings of 20-25 KHz for the initial positions of the chain, 4-5 KHz for positions C-10 and C-11 due to the single double bond, an increase to ~15 KHz for the latter part of the chain and a decrease to 3 KHz in the terminal two positions of the chain. The authors show that the results for C-9 and C-10 (C=C) at 16 and 5 KHz respectively, indicate that the cis double bond is ~7-8° removed from the bilayer normal. The reduction in splitting at position C-10 has been explained as being due to the change in the angle between the C-D bond and the bilayer normal that is introduced by the inflexible double bond (Seelig and Waespe-Sarcevic, 1978).

This does not reflect a decrease in order (S_{MOL}), since the double bond is in fact highly ordered, as would be expected from the ^{13}C T_1 results.

Simple steric considerations of the conformation of a cis-9,10, cis-12,13 diene system, which is attempting to maintain an average conformation which minimizes the total chain volume, predict that the plane of the second double bond will be 90° with respect to that of the first. This in turn predicts a small angle between the C-D bond and the bilayer normal for position 13. This angle at position C-12 would be expected to be similar to that at C-9. A detailed calculation of the angles between the C-D bond and the bilayer normal for positions C-11 to C-14 would be required to confirm this estimation. Thus, it would be expected that the diene system would show similar quadrupole splittings as the monoene system. The initial portion of the C-18:2 chain (up to C-11) should exhibit roughly the same pattern as oleate. The additional ordering of the diene system might be reflected in slightly higher quadrupole splittings for the region C-15 to C-17 than the C-18:1 chain. This overall behaviour is also consistent with the ^{13}C T_1 profile, where similar T_1 's were observed for pairs of carbons C-8 and 14, C-9 and 13, and C-10 and 12. The observed quadrupole splitting pattern of A. pullulans lipids is not inconsistent with this interpretation. The maximum quadrupole splitting of 38 KHz

is higher than the maximum for A. laidlawii, for reasons unknown. The 22 KHz plateau is likely due to the C-4 to 7 positions of the chain, the 16 KHz splitting to positions C-8,14 to 16, and the smaller splittings to the remaining unsaturated portion and terminal positions. These smaller splittings blend into a broader isotropic component which is due to the smaller structures formed by TG and hydrocarbon components, so that the minimum splitting cannot be estimated. In general, the spectra are consistent with the conclusion that the order profile for C-18:2 would not be significantly different from that of C-18:1 except in the region C-10 to 15.

In order to confirm these trends for the C-18:2 lipid chain, it would be necessary to obtain either specifically deuterated phospholipid or significant quantities of the purified perdeuterated phospholipids. Specific deuteration could not be accomplished with this organism, although it would be possible to use A. laidlawii labelled with the perdeuterated fatty acids isolated from A. pullulans. Purification of perdeuterated phospholipid from A. pullulans would improve the signal quality sufficiently to analyze the spectrum using the "de-Paking" technique (Bloom et al., 1981), but would require gram quantities of deuterated acetate as well as several litres of D₂O. This would not be a cost-efficient method of producing deuterated phospholipid.

While significant effort was required to produce this preliminary result, it points the way to future possibilities in measuring molecular order in more unsaturated systems than have been previously studied. At the same time, these preliminary results show that the order profile for the complex mixture of unsaturated phospholipids of the A. pullulans membranes is not inconsistent with those of other more pure unsaturated systems.

(b) ^{31}P NMR of A. pullulans Lipids

A great deal of recent NMR literature has been focussed on the measurement of the degree of organization of the lipid headgroup by ^{31}P NMR. The basis of the method is the anisotropy of the chemical shift of the phosphodiester resonance. The motion about the long axis of the lipid chain is not sufficient to time average the elements of the ^{31}P chemical shift tensor to give the isotropic chemical shift. The orientation of the phosphodiester function, such that the lipid headgroup is approximately parallel to the membrane surface, defines the magnitude of these reduced elements of the chemical shift tensor. The CSA powder pattern corresponds to the right-handed half of the quadrupolar powder pattern for deuterium (since $\text{spin}=1/2$, not 1). Like deuterium, the static powder pattern of a phosphodiester has a defined width (corresponding to the difference between the 90° and 0°

orientation), which has a value of 125 ppm. This is referred to as the chemical shift anisotropy. The rapid motion of the phosphate headgroup causes further axially symmetric averaging of the tensor components, thereby reducing the width of the powder pattern, as measured by the CSA parameter. The phospholipid headgroup motion appears to be one of hindered bond rotations - completely free rotation or completely hindered models are not consistent with published data (Seelig and Seelig, 1980), so that the motion is one of "wagging" of the entire lipid molecule about its long axis. Lipid phases with other structures, such as micellar, cubic and hexagonal (H_{II}) give rise to different spectral shapes and therefore offer the opportunity to distinguish between different forms of lipid aggregation. The micellar and cubic forms, as well as rapidly rotating vesicles, give rise to an isotropic spectrum, while the hexagonal phase gives rise to a tensor of reversed sign and reduced magnitude. The powder pattern of the hexagonal phase is "left-handed" and the width is reduced by a factor of two. Further details of theory behind ^{31}P powder spectra can be found in reviews by Buldt and Wohlgemuth (1981), Seelig (1978) and Cullis and de Kruijff (1979). The roles of the various lipid structures in membranes are discussed by Cullis et al., (1980(a) and (b)) and Verkleij et al., (1980).

In general, it has been observed that each phospholipid headgroup exhibits a particular behaviour which is not significantly influenced by the rest of the molecule. PC, PE and PG have similar headgroup structure and basically similar motional properties as measured by ^{31}P NMR. The charged headgroup of PS is definitely less mobile (Browning and Seelig, 1980). Other charged lipids (i.e., PI) have not been investigated. The effect of sterol, which increases the order in the acyl chain, appears to cause either little effect (Brown and Seelig, 1978) or greater disorder (Cullis *et al.*, 1978(a)) in the headgroup region of the bilayer, depending on the system. The effect of protein is not yet clear. The ^{31}P spectra of natural phospholipid mixtures are not well understood. The behaviour of mixtures cannot be predicted from that of the composite species and appears to be highly dependent on the relative proportions of different headgroup species, pH and the presence of ions. In general, much larger isotropic components are associated with natural lipid systems and intact membranes (Deslauriers *et al.*, 1981; de Kruijff *et al.*, 1980(b); Burnell *et al.*, 1980(a)). The isotropic component has been correlated with macroscopic structures (Burnell *et al.*, 1980(b)), indicating that its function is perhaps associated with the membrane fusion process (Verkleij *et al.*, 1980).

(i) ³¹P Experimental Parameters

The total phospholipids of A. pullulans were extracted as described in Chapter III. The mixture was essentially free of mono, di and triglycerides and sterols as determined by TLC. The fatty acid analysis showed that ~80% of the lipid was unsaturated (see Table IV.3).

100 mg of dry lipids were hydrated with vortexing and cycled through several freeze-thaw-vortex cycles to obtain a homogeneous dispersion. Neglecting this cycling procedure gave a dispersion which looked homogeneous, but which produced a hysteresis effect in temperature-dependent spectra. As the temperature was increased above 30°C, the isotropic component increased much more than if the cycling procedure was followed. Returning the sample to 30°C produced a spectrum with a much larger isotropic component than initially, indicating that the morphology of the sample had been changed. The cycling procedure produced identical room temperature spectra before and after heating.

Lipid dispersion spectra were accumulated on 1K data points using quadrature detection with the transmitter on the high field side of the powder pattern. The high power decoupling was gated to reduce sample heating. A 50 KHz spectral window, 1 sec recycle time, 10 msec acquisition and 90 pulse were used. FID's were transformed from 4K data

points. CSA parameters were measured from the point of half height of the low field shoulder to the high field side of the peak of the powder spectrum, which corresponds to the difference between the 90° and 0° orientations.

(ii) ^{31}P Results

The isotropic ^{31}P spectrum of phospholipid in CHCl_3 is shown in Figure VI.2. The major phospholipid species can be identified from the spectrum in approximately the same proportions as determined by GC analysis. The spectrum was assigned by comparison to pure standards and previously published spectra (Henderson *et al.*, 1974). Since the phospholipid chemical shifts are highly solvent and pH dependent, this spectrum is not indicative of the isotropic chemical shifts in aqueous medium. The latter may be determined through the addition of detergents to the aqueous lipid suspension (Plückthen and Dennis, 1981; London and Feigensen, 1979). The lowfield shoulder of the spectrum can be assigned to Cardiolipin and PA. The entire phospholipid mixture is composed of PC, PE, PS plus PI, Cardiolipin and PA in the ratio 1.0:1.0:0.7:0.2:0.2 respectively, which is consistent with the relative peak intensities. With careful attention to the factors influencing the chemical shifts of phospholipids (even acyl chain composition has some effect), the NMR method can be a quick way to roughly determine the head-group composition of a lipid mixture.

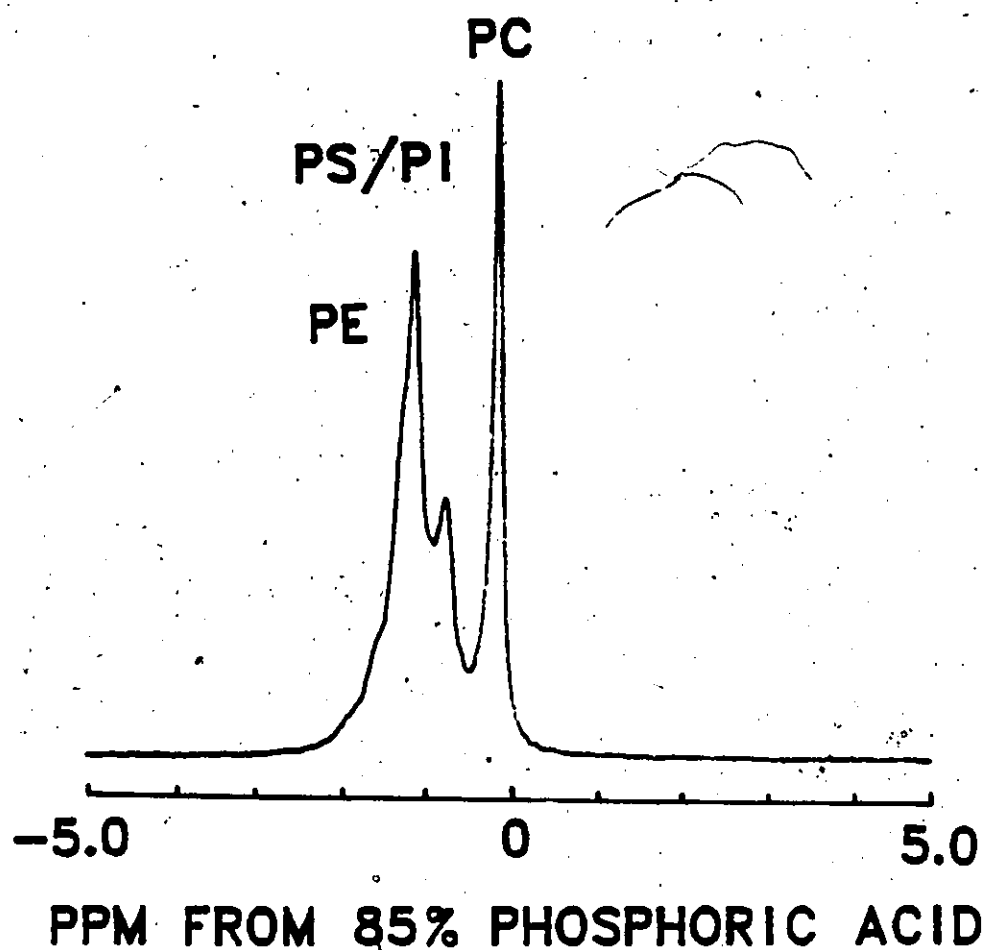
TOTAL PHOSPHOLIPID (CHCl_3)

Figure VI.2 - 121 MHz high resolution ^{31}P spectrum of purified phospholipid mixture from A. pullulans in CHCl_3 . The major phospholipid headgroups in their correct proportions can be identified from the spectrum.

Figure VI.3 shows the temperature dependence of the ^{31}P NMR spectrum of the aqueous dispersion of A. pullulans phospholipids. At 5°C , the lipid appears to be entirely in the bilayer phase. As the temperature is increased through the growth temperature (26°C), the powder pattern decreases in width and grows more sharp, with a concomitant increase in the isotropic component. Above 30°C , the spectrum is essentially unchanged, indicating that the bilayer is stable to temperatures up to 90°C . No hexagonal phase was observed, in contrast to the lipids of Tetrahymena (Deslauriers et al., 1981), E. coli (Burnell et al., 1980(a)) and rat liver (Cullis et al., 1980(b)). This stability in structure is somewhat unusual for a lipid mixture containing a substantial proportion of highly unsaturated PE. Aqueous dispersions of natural PE are particularly prone to undergoing a bilayer to hexagonal phase transition (Cullis and de Kruijff, 1978(a)). However, the uniformly unsaturated lipids of E. coli (PE plus PG, 90% C-18:1) also show no hexagonal phase (Gally et al., 1980). The appearance of the hexagonal phase may be a sensitive function of the ratio of PE to other components, the identity of those other components, and may also be a function of the heterogeneity of fatty acyl composition. The appearance of hexagonal phase in lipid dispersions may also be a result of phase separation during sample preparation, since generally only bilayer and isotropic phases are associated

TOTAL PHOSPHOLIPID (LIPOSOMES)

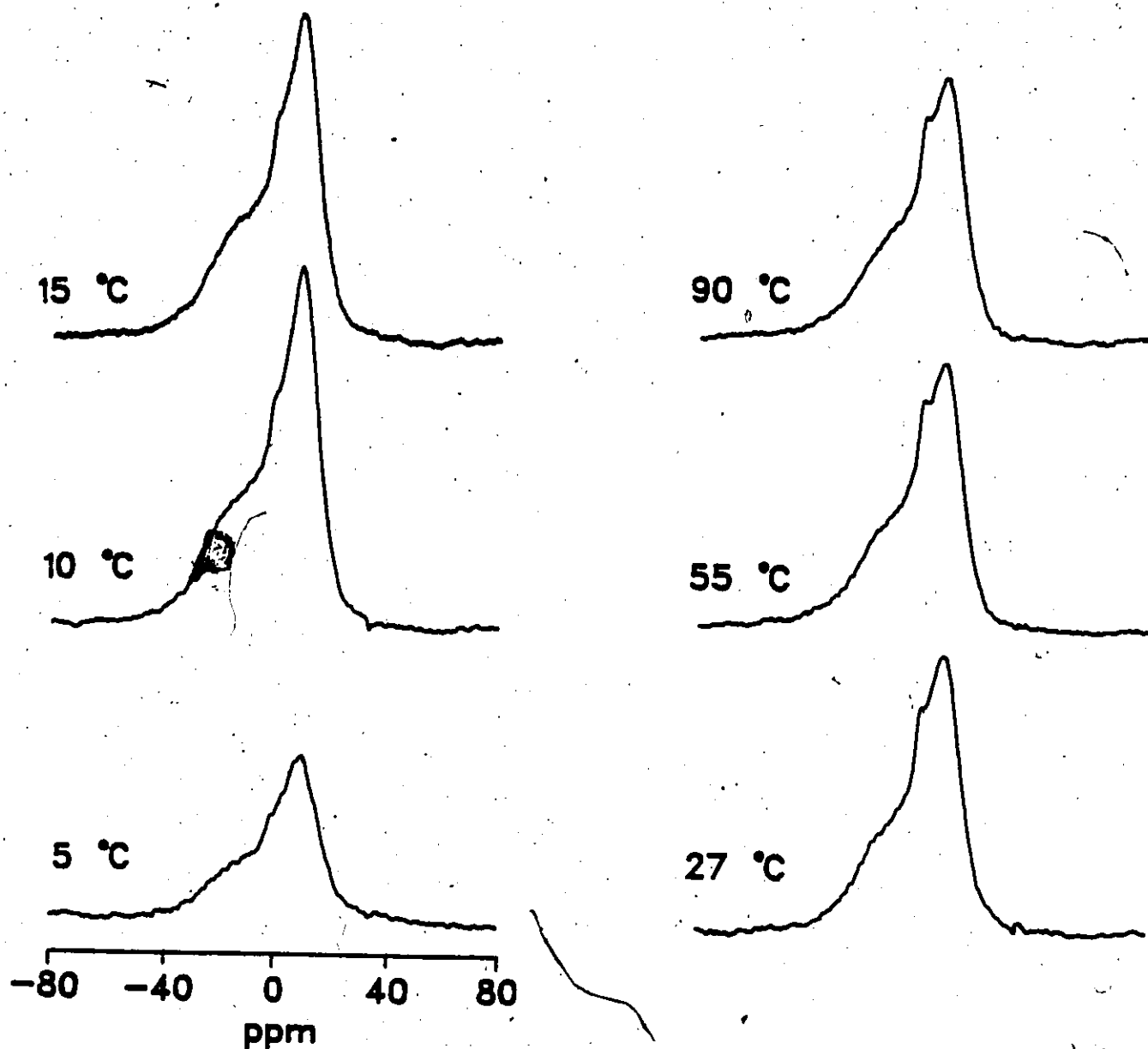


Figure VI.3 - 121 MHz ^{31}P spectra of 100 mg/ml purified phospholipid liposomes as a function of temperature. These spectra, representing 2500 accumulations, employed a 90° pulse, 1 s recycle time, 50 KHz spectral window and high power gated decoupling during the acquisition. Each spectrum is a composite of a powder pattern plus an isotropic component. Above 27°C , the system is stable to any further increase in temperature.

with intact membranes.

The variation of the CSA parameter (as measured from the powder spectra) with temperature is shown in Figure VI.4. At 5°C, the CSA is 44 ppm and decreases to 37 ppm by 27°C. Above this temperature there is no further change. This decrease in CSA may be indicative of the end of the gel-liquid crystal phase transition which occurs over a very wide temperature range because of the heterogeneity of the lipid composition. The growth temperature lies just at the end of this transition so that the phospholipid behaves essentially as a liquid crystal in the intact membrane (assuming that there is no further effect by other membrane components). This transition appears to be at somewhat higher temperature than expected for dilinoleyl PC. This may be due to the presence of a significant proportion of charged lipid (PS plus PI), which possesses a higher transition temperature and appears generally to stabilize the bilayer. The absolute values of the CSA parameters are somewhat smaller than obtained for pure lipid components (see Seelig, 1978), which may be due to the heterogeneity of the lipids and the higher unsaturated content. The faster overall motion experienced by the lipids containing a significant amount of C-18:2, may narrow the powder pattern to some extent, although this has not been observed in pure lipids. The particle size may also influence the shape of the powder pattern (Burnell et al., 1980(c)).

TOTAL PHOSPHOLIPID

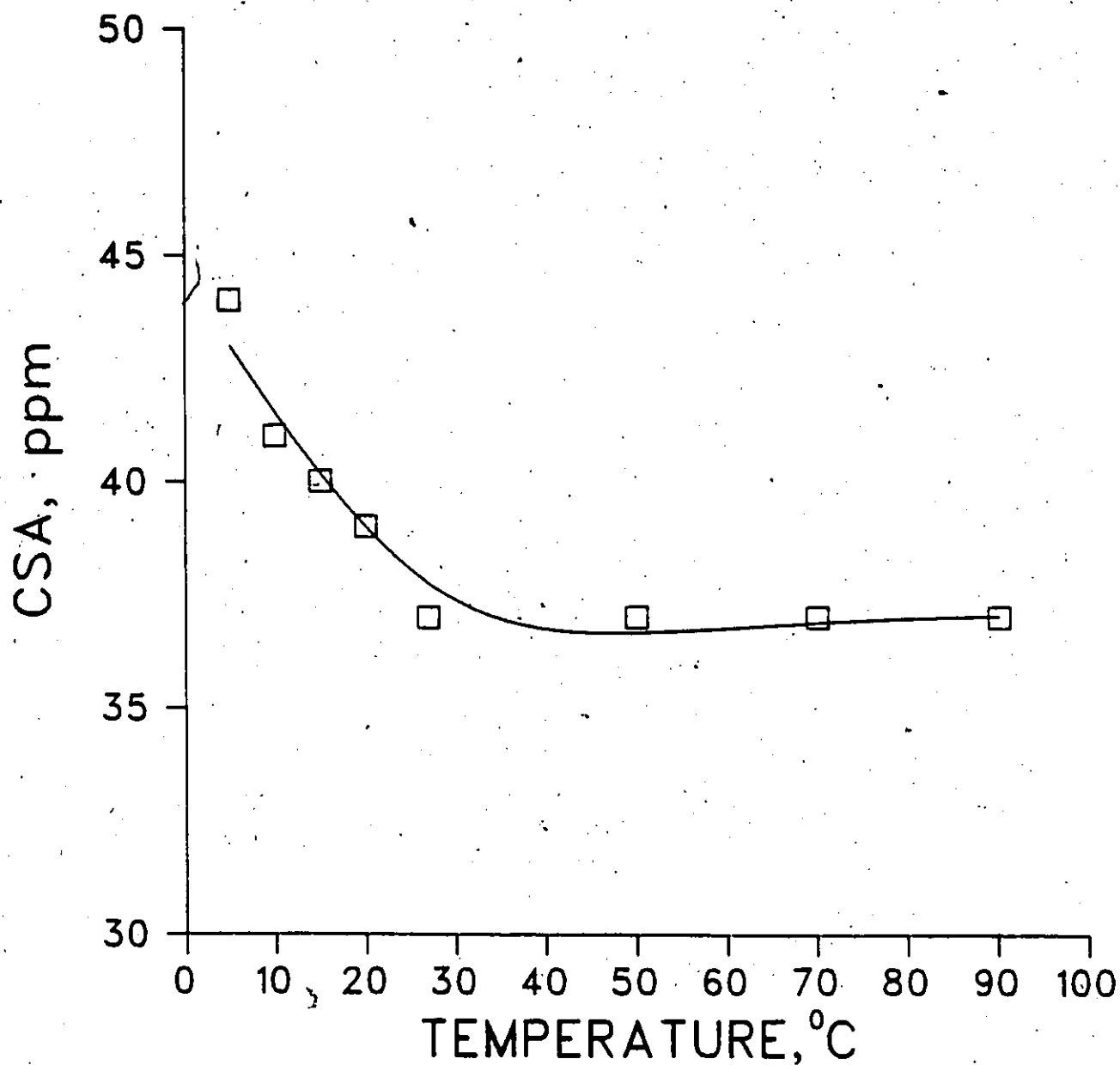


Figure VI.4 - Plot of the chemical shift anisotropy as measured from the spectra of Figure VI.3 as a function of temperature. The powder pattern decreases in width from 5°C to 25°C; at higher temperatures there is no change.

Smaller particle sizes cause partial averaging due to particle tumbling and the powder spectrum becomes narrower with edges less well-defined.

The behaviour of the phospholipid mixture can be compared with that of a 1:1 PC/PE mixture, composed totally of C-18:1 fatty acid (Cullis et al., 1978(a)). This system showed substantial bilayer phase up to 50°C, but becomes completely isotropic by 90°C. No hexagonal phase was observed, unless cholesterol was added. The additional stability of the A. pullulans mixture may be due to the presence of a significant portion of charged lipid, PS and PI. As was mentioned above, the influence of charged lipids appears to stabilize the bilayer, especially in the presence of ions such as calcium (Browning and Seelig, 1980; Hope and Cullis, 1980). It has been suggested that the role of PE, which tends to destabilize the bilayer structure, is to form phases that may be related to important membrane functions, while bilayer stability is controlled predominantly by the fatty acid composition (Cullis and de Kruijff, 1978(a)). This role does not appear to be suggested by the behaviour of A. pullulans lipids, in spite of the substantial amount of PE present.

The stability of the bilayer structure of these lipids may be responsible for the organism's ability to grow at a wide variety of temperatures. During relaxation studies, it was observed that the cells were not lysed even after

enduring a period of time at 85°C. The organism is also well represented in tropical habitats. This stability for temperatures above the growth temperature indicate an ability to withstand a range of growth conditions without altering the lipid composition. The organism can also grow at temperatures lower than 26°C, but what effect this has on the relative phospholipid composition is unknown. It is likely that the composition would be adjusted to allow the growth temperature to occur within the region of pure liquid-crystal phase. It is interesting to note that the motion of the headgroups in the bilayer phase (as measured by the stability of the CSA parameter) do not change over a range of temperature that causes a large increase in the acyl chain mobility (as measured by the ^{13}C T_1 's). This indicates that the increase in temperature affects only the gauche-trans populations and interconversion rates of the lipid chains, without disturbing the overall integrity of the bilayer or the axially symmetric motion of the phospholipid headgroup.

The effect of the triglyceride and sterol components on the phospholipid dispersion spectrum can be estimated by comparing the powder pattern of Figure VI.3. with that of Figure VI.5. The phosphorus spectrum of the latter represents the structure of the phospholipid portion of the deuterated total lipid dispersion used for ^2H NMR. The ^2H spectrum

LIPID FROM DEUTERATED A. PULLULANS

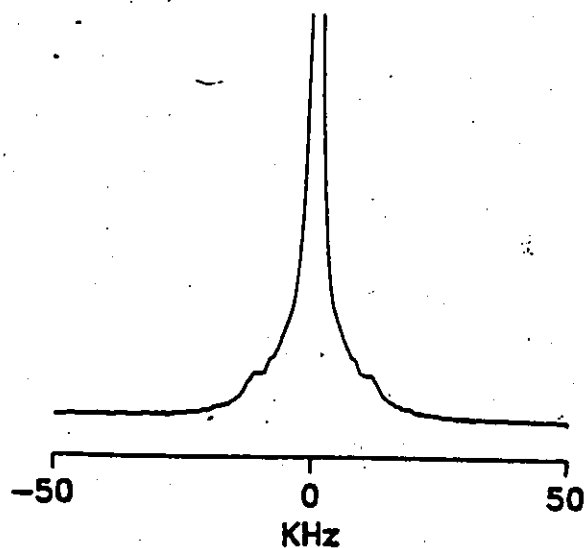
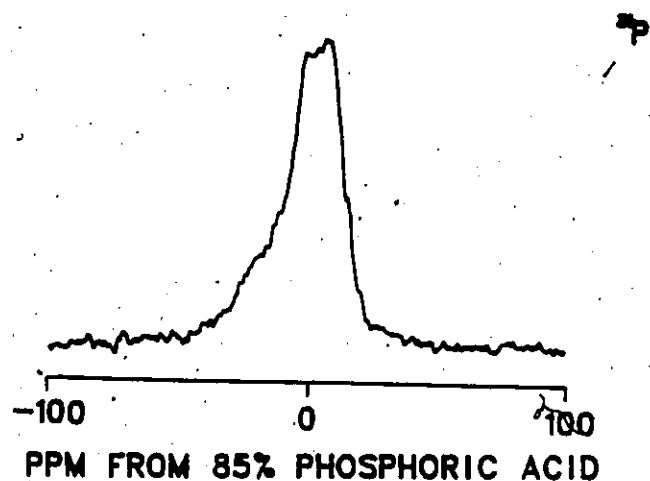


Figure VI.5 - High Power ^{31}P and ^2H spectra of the sample of deuterated lipid (45 mg/ml) presented in Figure VI.1 at 30°C. Of the total lipid sample 10 mg is phospholipid. The differences between the relative amounts of the isotropic and anisotropic due to parameters involved in determining the spectra. The relatively narrow CSA pattern (37 ppm) and the small quadrupolar splittings are consistent with the highly fluid nature of the lipid extract. The ^{31}P spectrum was accumulated using gated decoupling to avoid sample heating, 90° pulses, 0.8 s recycle time, and 600 transients.

(without subtracting the isotropic component) is shown for comparison. In the total lipid dispersion, much of the phospholipid is still in the bilayer phase, but the contribution of the isotropic component has increased. Since only the phospholipid fraction is detected by ^{31}P NMR, this suggests that the presence of neutral lipid has a slight disordering effect on the PL structure. The presence of isotropic phase has been associated with natural membranes. In rat liver microsomes, a significant portion of the phospholipids undergo isotropic motion (de Kriujff et al., 1980 (b)) at 37°C . The bilayer phase increases with reduction in temperature. A. laidlawii membranes also show a substantial isotropic component (de Kriujff et al., 1976), and E. coli cytoplasmic membranes show an isotropic phase at and above the growth temperature (Burnell et al., 1980(a)). Below the growth temperature, the bilayer phase again increases. Gally et al., (1980) observed that E. coli membranes show a much larger isotropic component than does the dispersion of the membrane lipids. It is also important to note that the presence of sterol (Cullis and de Kriujff, 1978(a)) and calcium (Cullis et al., 1980(a)) also increase the isotropic component in lipid mixtures of biological origin. This isotropic phase could arise from the tumbling of small vesicles, from rapid exchange between bilayer and hexagonal phases or from non-bilayer structures. The behaviour suggests

a role for the triglyceride fraction in the natural membrane of A. pullulans. The inability to remove the triglyceride fraction completely from the membrane preparations suggests an association of triglyceride with intact membranes. This component may serve to disrupt the phospholipid structure, which alone is quite stable, to form the isotropic plus bilayer phases normally seen in the lipids of non-triglyceride containing organisms.

In this context, it may be mentioned that a large isotropic component was observed in the ^{31}P NMR spectrum of nystatin-prepared membranes (spectrum not shown). The bilayer component was very similar to that observed for purified phospholipid. It was difficult to obtain sufficient spectral quality to estimate the relative isotropic and bilayer contributions due to the dilution of the phospholipid species in the sample.

In an attempt to deconvolute the ^{31}P NMR spectrum of Figure VI.5 and compare the bilayer portion to the spectrum of the purified phospholipid, the saturation technique as used by de Kriujff et al., (1980(b)) was applied to the spectrum. A specific saturation sequence (the DANTE pulse sequence, (Morris and Freeman, 1978)) was applied to the component on the right side of the spectrum. A series of "soft" pulses were applied at a frequency corresponding to the 90° orientation of the powder pattern. If the saturating pulse train is maintained long enough for all species responsible for

the powder spectrum to rotate through the 90° angle, the entire spectrum will be saturated, leaving only the isotropic component. Such an experiment is shown in Figure VI.6. The total spectrum (Figure VI.6(c)) was subjected to a series of 4000 pulses of 0.5 μ sec duration and 50 μ sec separation, followed by a delay of 10 μ sec to allow for the ringdown of the saturation pulse and a normal 90° -acquire sequence. The resultant DANTE spectrum is shown in Figure VI.6(b).

It is important to adjust the DANTE parameters to achieve maximum saturation of the powder pattern (de Kriujff et al., 1980(a)). The delay between pulses must be short enough to permit total saturation of the resonance at the transmitter frequency. The number of pulses must be such that the saturation power is on for a sufficient period of time to allow for diffusion to occur to all parts of the spectrum. This parameter was adjusted until the low field shoulder of the pattern disappeared. The delay between completion of the pulse train and the 90° sensing pulse must be long enough to allow for pulse ringdown, but not to allow relaxation of the powder component. This was adjusted to minimize signal on the high field shoulder. Spectral subtraction of the two FID's results in the bilayer component (Figure VI.6(a)). The shape of the powder component in the total lipid extract is virtually the same as in the purified phospholipid,

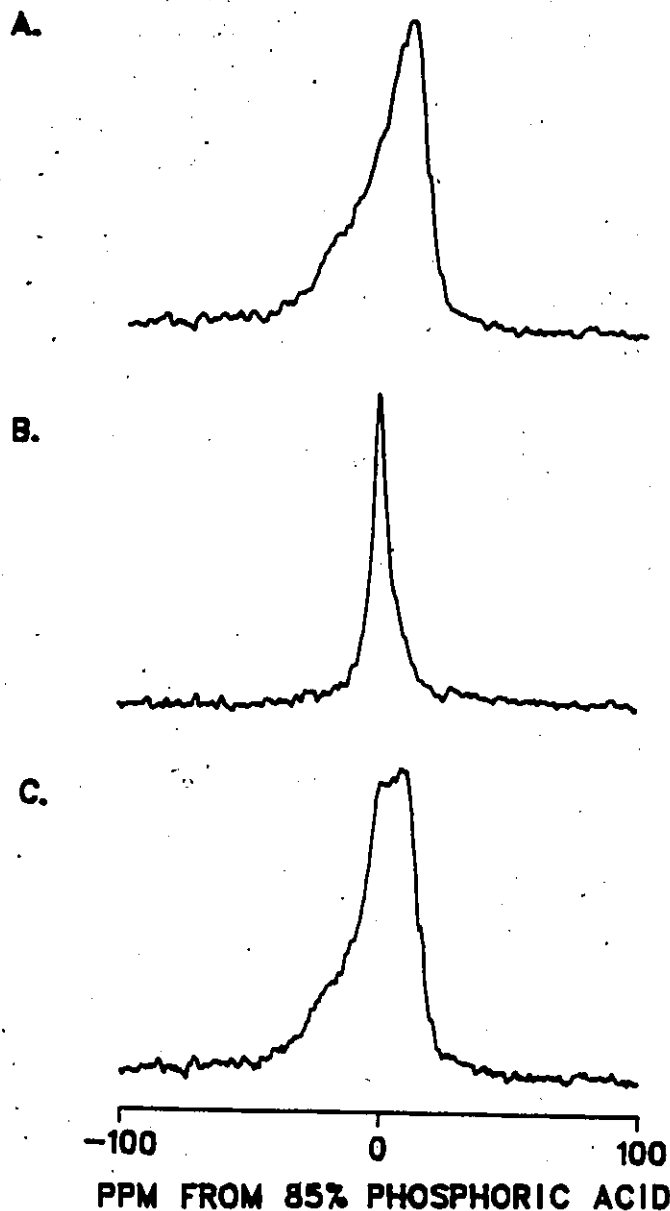
LIPID FROM DEUTERATED *A. PULLULANS*

Figure VI.6 - A DANTE experiment performed on the ^{31}P spectrum of total lipid extracted from deuterated *A. pullulans*, in order to determine the shape of the powder pattern without the isotropic component. Spectrum (C) is the same as in Figure VI.5. The powder pattern was irradiated at the low field side with a series of 4000 soft pulses (0.5 μs delay), followed by a delay time of 1 ms to allow for ringdown of the saturation pulse train, and then by a normal 90° pulse and acquisition. The resultant spectrum (B) is essentially only the isotropic component. The difference spectrum (A) is the anisotropic component only, and has the same shape as the powder pattern for purified phospholipid at 30°C .

thereby indicating that the degree of bilayer structure as indicated by the shape of the powder pattern is a property of the specific phospholipid composition.

The DANTE technique was also applied to the deuterium spectrum, but without success due to the inability to adjust the rf pulse train power of the ^2H transmitter. Further work would require hardware modification. The feasibility of applying this technique to the intact system is limited by the relative proportions of the isotropic and bilayer phases. In systems where the bilayer spectrum is weak, it is difficult to adjust parameters in order to extract the correct bilayer lineshape. Other techniques which involve distinguishing between phases on the basis of differences in relaxation time has more potential in these conditions (A. Byrd, private communication).

It was also observed in the course of this work that the phospholipid powder pattern could be perturbed with nystatin. While it proved impossible to observe the effect of nystatin on the lipid acyl chains in the membranes at the concentrations required to cause cell lysis, it was possible to look at the effect that nystatin had on the bulk phospholipid fraction. The experiment of Figure VI.3 was repeated using 100 mg of purified A. pullulans phospholipid in a 3:1 mole ratio with nystatin. The spectra are not shown as the experiment was performed only once. The 20°C spectrum was very similar to

that of the total lipid extract. The basic powder pattern was the same but the isotropic component was increased. Above 37°C, the isotropic phase increased at the expense of the bilayer phase until by 65°C, the spectrum consisted of one broad isotropic line. This suggests that nystatin has a disordering effect on the yeast membrane, which may account for its lytic behaviour. The nystatin appears to attack the plasma membrane at its weakest point, the bud scar, where the polysaccharide wall is the thinnest and where there is a concentration of enzymes responsible for weakening the cell wall during the budding process. Attack by disordering the membrane at this point causes complete lysis of the cell by relatively small quantities, since only a small region of the plasma membrane is affected.

(iii) Discussion

It is difficult to interpret unambiguously the spectral shape of the powder pattern for this phospholipid mixture, i.e., whether the phospholipids behave as a single bilayer, the spectral shape being determined by the average phosphate environment, or whether the spectrum is a result of a combination of overlapping powder patterns corresponding to the individual species. The overall spectral shape is somewhat more diffuse than expected for a powder pattern of CSA of 37 ppm. A simulated spectrum of a single powder pattern

with a CSA of 37 ppm is shown in Figure VI.7, for comparison with Figure VI.3. The simulated pattern is composed of overlapping resonances with individual linewidths of 500 Hz. The shoulders of the natural phospholipid spectrum are more sloping and the intensity peaks do not occur at the same frequencies with respect to the isotropic chemical shift (approximately 0 ppm). Since the powder spectrum is arranged symmetrically about the isotropic chemical shifts which vary over ~3 ppm for the species present in this lipid mixture (London and Feigensen, 1979), it is possible that such a lineshape results from the distribution in isotropic chemical shifts in the bilayer structure. Arnold *et al.*, (1981) have shown that it is possible to analyze the powder pattern for an equimolar mixture of DPPC and DPPE in terms of two overlapping powder patterns of different CSA values.

The factors which influence the behaviour of mixtures of lipid headgroups is not well understood, since the properties displayed by a single lipid species are not necessarily carried over into lipid mixtures. Pure cardiolipin gives rise to a bilayer of greater CSA than that of PC (Cullis *et al.*, 1978(b)). Together (PC plus cardiolipin), the PC bilayer is somewhat destabilized (de Kriujff *et al.*, 1979). Unsaturated PE tends to prefer the hexagonal phase (Cullis and de Kriujff, 1978(a)), but in equimolar combination with PC, shows no hexagonal phase (Cullis *et al.*, 1978(a)).

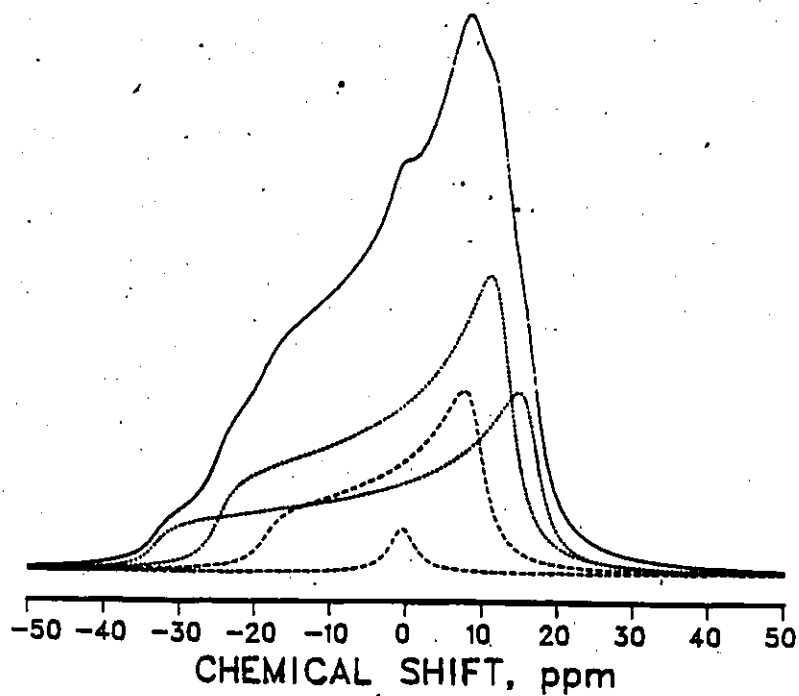
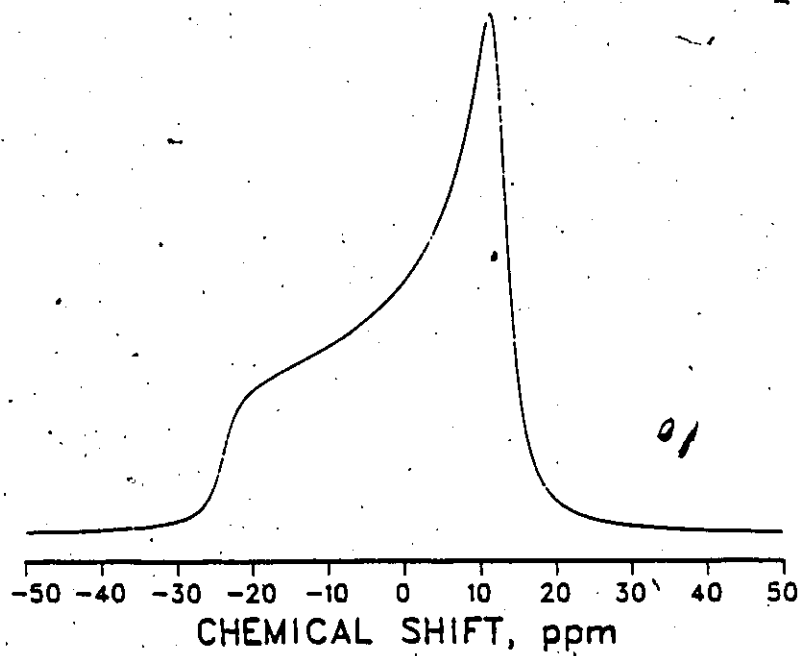


Figure VI.7 - Sample spectral simulation assuming that the powder lineshape is a sum of overlapping powder patterns. This can be compared with a single powder pattern lineshape assuming component linewidths of 500 Hz.

Model membranes of PG give a broad stable bilayer up to 80°C (Wohlegemuth et al., 1980), and also serve to stabilize the bilayers of unsaturated PE (Gally et al., 1980). Although there have been many studies of various combinations of all the common phospholipid headgroups, no common mode of behaviour has emerged.

Since the major components of the phospholipid mixture are PC, PE and charged lipid, the dispersion was modelled on three representative components with PS representing the charged lipid. Spectral simulations were performed using the aqueous isotropic chemical shifts of PC, PE and PS (as determined by Pluckthun and Dennis, 1981; and London and Feigenstein, 1979). The CSA parameters and the relative contributions of the components were varied in an attempt to mimic the spectral lineshape. The simulation which most correctly fitted the experimental powder pattern is also shown in Figure VI.7. The simulated spectrum is a combination of three powder patterns of width 38, 28 and 50 ppm representing the fractions PC, PE and PS plus PI in the ratio 1.0:0.5:0.7. Addition of a 10% isotropic component completes the spectral shape to that observed at 27°C for the purified phospholipids.

The fitting of the lineshape to a combination of powder patterns whose relative composition reflects that of the component species does not necessarily infer that this is the correct interpretation of the phospholipid behaviour. Such

diffuse spectral shapes can be due to smaller particle sizes (vide supra), although this does not explain why such lineshapes have been observed in intact membranes which are of much larger size than a liposome. They could also arise from chemical exchange between different lipid phases. The exchange phenomenon can be slowed by cooling the sample and would result in a sharper bilayer pattern at lower temperature. This behaviour was not observed in the lipids of A. pullulans. A lineshape interpretation in terms of overlapping powder patterns should be viewed with caution, in the light of evidence that the spectral shape is profoundly influenced by spectral accumulation conditions (M. Rance, unpublished). These ^{31}P spectra were not observed with an echo sequence, and it can be shown that this has an effect on the intensities of the component orientations, even to the point of altering the CSA parameter. Thus, spectral simulation can be misleading. It would be possible to determine whether the phospholipid pattern was a result of overlapping powder patterns by performing the echo experiment using long delays between the 90° pulses. This effectively reduces the pattern to that of the component chemical shifts of the 90° orientation. The appearance of several resonances under these conditions would tend to confirm the concept of phospholipid phase separation.

The combination of phospholipids in A. pullulans appears to be designed for maximum bilayer stability over the range of growth temperatures. The relative headgroup as well as fatty acid composition may be adjusted in response to environmental factors. It has been previously noted that A. laidlawii cells adjust the polar headgroup composition in response to environmental factors. It is possible to explain this cellular response by the phase behaviour of the component species (Weislander et al., 1981). These authors conclude that in general, cells avoid lipid compositions resulting in non-lamellar phases of bulk lipids. This interpretation also applies to the lipid structure of A. pullulans.

CHAPTER VII

METABOLIC RESULTS FROM ^{13}C NMR

Introduction

Natural abundance ^{13}C NMR studies of the more mobile components of intact systems* (i.e., soluble components of the cytoplasm) can only be carried out if these components are present in high quantities or if the systems are stable over the long accumulation times that are required to produce a spectrum. Thus, there have been relatively few such natural abundance studies. The information that can be obtained from such studies is limited to the identification of the mobile components that give rise to the spectra, such as fluid lipid in membranes, amino acid pools or simple sugars. Such studies might be described as "static", since the cytoplasmic components identified from the spectra are the steady state products of metabolism. Thus, information concerning the various metabolic pathways of the organism, may be inferred from the type or levels of these cytoplasmic components. Examples of systems that have been studied by natural abundance ^{13}C NMR in this way include intact plant seeds (Schaefer and Stejskal, 1975; Kainosho, 1976), whole tissue samples from marine organisms (Norton, 1979), chromaffin granules (Sharp and Richards, 1977), excised mammalian

nerve (Williams et al., 1973), and cysts of amoeba (Deslauriers et al., 1980). All of these systems represent dormant or stable systems which do not change during the spectral accumulation time. The marine organisms contain large cytoplasmic amino acid pools, which are proposed to have an osmoregulatory function. The process of fermentation in Baker's yeast has been investigated by natural abundance ^{13}C NMR, showing that this yeast prefers to store sugars in the form of trehalose (α, α -glucose) (Kainosho et al., 1977), and intact fungal mycelia show pools of mannitol (Matsunaga et al., 1980) in their carbon spectrum; these are the only two studies of simple eukaryotes by this "static" method reported to date.

The majority of metabolic ^{13}C studies have employed ^{13}C enrichment and can be described as "dynamic" studies, since the progress of the labelled precursor is followed through the metabolic pathway in the NMR tube, from the point at which the label is added to the external medium. The enriched components only require short accumulation times and the spectra are simplified since there is no background interference from unlabelled species. This is the basis for the now classic in vivo studies of glycolysis and gluconeogenesis from ^{13}C labelled glucose by Shulman and coworkers. Such tracer studies have been carried out in suspensions of rat liver cells, using ^{13}C labelled glycerol (Cohen et al.,

1979; Cohen and Shulman, 1980) or ^{13}C -labelled alanine (Cohen et al., 1978; Cohen et al., 1981), and in E. coli using ^{13}C -labelled glucose (Brown et al., 1978), and S. cerevisiae (den Hollander et al., 1979). Labelled acetate has been used as a metabolic precursor in a recent study on perfused rat heart (Bailey et al., 1981). In most of these studies conclusions can be reached about the presence of glycolytic enzymes or the relative importance of various metabolic pathways by observing the disappearance of peaks due to the labelled glucose, glycerol or alanine with the concomitant appearance of peaks due to metabolic products such as lactate, or intermediates in the Krebs' acid cycle, such as succinate or glutamate. Spectra are normally taken in a matter of minutes and metabolic levels of labelled components are monitored for a few hours, depending on the integrity of the system. The study on E. coli cells in anaerobic suspension shows that the glucose label appears in lactate, succinate, acetate, ethanol and alanine, whereas under more aerobic conditions more of the label went into glutamate (Brown et al., 1978). The labelling pattern of the glutamate showed that the label entered the citric acid cycle as acetyl-CoA, as might be expected. It was also shown that intermediates in glycolysis, such as fructose di-phosphate could be seen in the spectrum. In a more recent study, the fermentation of totally labelled glucose in the

spirochete, T. phagedenis, has been followed by NMR (Ohsaka et al., 1981) showing that this bacterium produces acetic acid, ethanol, formic acid and alanine as major end products from glucose in the growth medium.

When labelled glucose is fed to anaerobic suspensions of yeast cells, the principal products of glycolysis, namely glycerol and ethanol are observed (den Hollander et al., 1979). In addition, comparison of the labelling patterns appearing in fructose di-phosphate from C-1 labelled glucose and C-6 labelled glucose show that the formation of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate from FDP are in equilibrium in the cell suspension. An earlier study also showed the same end products from ^{13}C glucose metabolism in another strain of yeast, Candida utilis (Eakin et al., 1972). In this case, however, glucose-6-phosphate was observed.

The metabolism of plants may be studied by labelling with $^{13}\text{CO}_2$, which is not accessible to aerobic organisms. The label appears as an overall labelling of all carbons of the simple sugars first, followed by labelling of the lipid fraction, showing that some of the sugar is oxidized via the pentose phosphate cycle to provide acetyl-CoA for lipid biosynthesis (Schaefer et al., 1975).

When acetate is used as a source of ^{13}C label in "dynamic" NMR experiments, the majority of the label appears in the

immediate products of the Krebs's acid cycle, since acetate can quickly cross the mitochondrial membrane. As was discussed in Chapter V, acetate is often a preferred source for fatty acid synthesis and thus labelled acetate appears to label the acyl chains of phospholipids efficiently.

When randomly enriched acetic acid was used as a sole carbon source for Candida utilis, the label appears in all carbons of the mobile components of the cell; amino acids, peptides, and simple sugars as well as lipid chains (London et al., 1975; Eakin et al., 1972). The dynamic metabolism of specifically labelled acetate has been studied only in rat heart (Bailey et al., 1981) and S. cerevisiae (den Hollander et al., 1981). In the former study, ^{13}C -2 labelled acetate in the perfusate of the heart is immediately metabolized into the γ -carbon of glutamate, followed by the β -carbon and eventually the α -carbon on the second turn of the Krebs's acid cycle. The presence of ^{13}C spin coupling between the β and γ carbons of glutamate indicates that acetate is the preferred substrate rather than glycogen or endogenous triglycerides. Alanine, which was present in large quantities, did not receive any of the acetate label since it is pyruvate-derived and the pyruvate appears to result from glycogen catabolism. At longer times, the label appears in aspartate, which is derived directly from the Krebs's cycle. In S. cerevisiae, very similar results are seen, in spite of the

large difference in the cell system. The ^{13}C -2 acetate label appears first in the γC of glutamate, followed by the α and β -carbons. Upon exhaustion of the acetate, the label appears in the α and β -carbons of aspartate. The coupling patterns of the glutamate indicate that the αC and βC are equally labelled, but to a lesser extent than the γC . The label also appears in C-1,2,5 and 6 of the major sugar storage form, trehalose, indicating that the acetate is being used for glucose biosynthesis via pyruvate. The results with carboxy-labelled acetate support the above; the label appearing in C-3 and C-4 of trehalose and the δC (carboxyl) of glutamate. The results are interpreted in terms of the interplay between the Kreb's acid cycle and the glyoxylate cycle. This study also shows that aspartate and glutamate are the largest constituents of the soluble amino acid pool in this species of yeast.

Whereas this last study looked at the detailed time course of utilization of acetate in the NMR tube in a suspension of viable cells, the present chapter is concerned with looking at the steady state and products of utilization of labelled acetate during growth of the yeast phase of A. pullulans. During the course of the membrane study of this organism it was observed that other constituents of the cell had been labelled in a very specific manner during growth on an acetate-supplemented medium, and that during a period of

carbon-deprivation it was possible to observe the ^{13}C label from stored triglyceride appear in the products of β -oxidation. These observations are therefore of the "static" variety, since the levels observed represent the steady state concentrations of labelled components in the cell culture at the point of harvesting. The conditions in the NMR tube are such that any metabolism in the cells is much slowed and the cells are slowly dying. The information thus obtained relates to the ability of this particular organism to compartmentalize its food resources and the results can be compared to those outlined above.

This brief summary of ^{13}C labelling studies shows the wide variety of intracellular molecules that become labelled as a result of the peculiarities of the metabolism of the particular organism. In general, however, labelling studies employing glucose are used to follow glycolytic activity whereas the smaller acetate units are more active in the immediate products of the citric acid cycle. This largely results from the fact that acetate simply diffuses into the cell and across most of the intracellular membranes, whereas glucose requires facilitated diffusion to cross the cytoplasmic membrane and its motion within the cell is much more restricted. More in-depth reviews of ^{13}C NMR in cellular systems may be found in Shulman et al., (1979); Scott and Baxter, (1981); Norton, (1980); and Smith and Deslauriers, (1981).

Experimental Conditions

The spectra of this chapter were obtained under the conditions outlined for the T_1 experiments. They represent 50 or 100 ml cultures of cells at $2-5 \times 10^9$ cells per ml in water or D_2O . The labelled extracts are the total supernatant from the nystatin membrane preparation of a 100 ml culture of cells, or the aqueous fraction after a Bligh and Dyer type extraction of this supernatant to remove any lipid component. The spectrum in Figure VII.3b represents a portion of the aqueous phase of a bulk lipid extraction (outlined in Chapter III, to obtain a large amount of the purified phospholipid), which has been concentrated by lyophilizing and resuspending in a small amount of D_2O .

Most spectra were accumulated using 45° pulses, at $30^\circ C$. The spectra of Figures VII.4 and VII.5 were acquired under the same conditions in order to compare the relative intensities of the metabolite signals. Spectral peaks were assigned by comparison with published spectra or by running spectral standards when published spectral chemical shifts were in doubt. Amino acids were identified by running standard solutions of known mixtures of amino acids, adjusted to the approximate pH of the internal cellular environment as determined by ^{31}P NMR.

(a) Acetate Utilization During Growth

The culture of Aureobasidium pullulans was originally begun to characterize the ^{13}C spectrum of the exopolysaccharide, pullulan. This study was done using natural abundance material (Colson et al., 1974; Jennings and Smith, 1973). Since a ^{14}C -glucose study had shown that labelled glucose in the medium resulted in labelled lipid, sterol, and storage glucose in the form of trehalose (Merdinger, 1969), the introduction of the ^{13}C labelled analogue was expected to label the same moieties. In addition, since trehalose is not a major constituent of these cells under conditions of high nutrition, the label would be expected to be transferred to other polysaccharides, in particular, pullulan. For this reason, the organism was grown on ^{13}C -1 glucose, and the isolated pullulan spectra showed that the label was preserved in the C-1 positions of the glycosyl residues of this glucan, thus confirming spectral assignments.

The spectrum of the harvested whole cells grown on ^{13}C -1 glucose as a sole carbon source is shown in Figure VII.1. Since the cells are washed during harvesting, the labelled components are intracellular and not due to residual labelled glucose of the medium. The major areas of the spectrum are assigned to lipid (L), carbohydrate (C), and glucose (G). Protein (P) is largely hidden in the baseline due to its

^{13}C 20 MHz

WHOLE CELLS P. PULLULANS

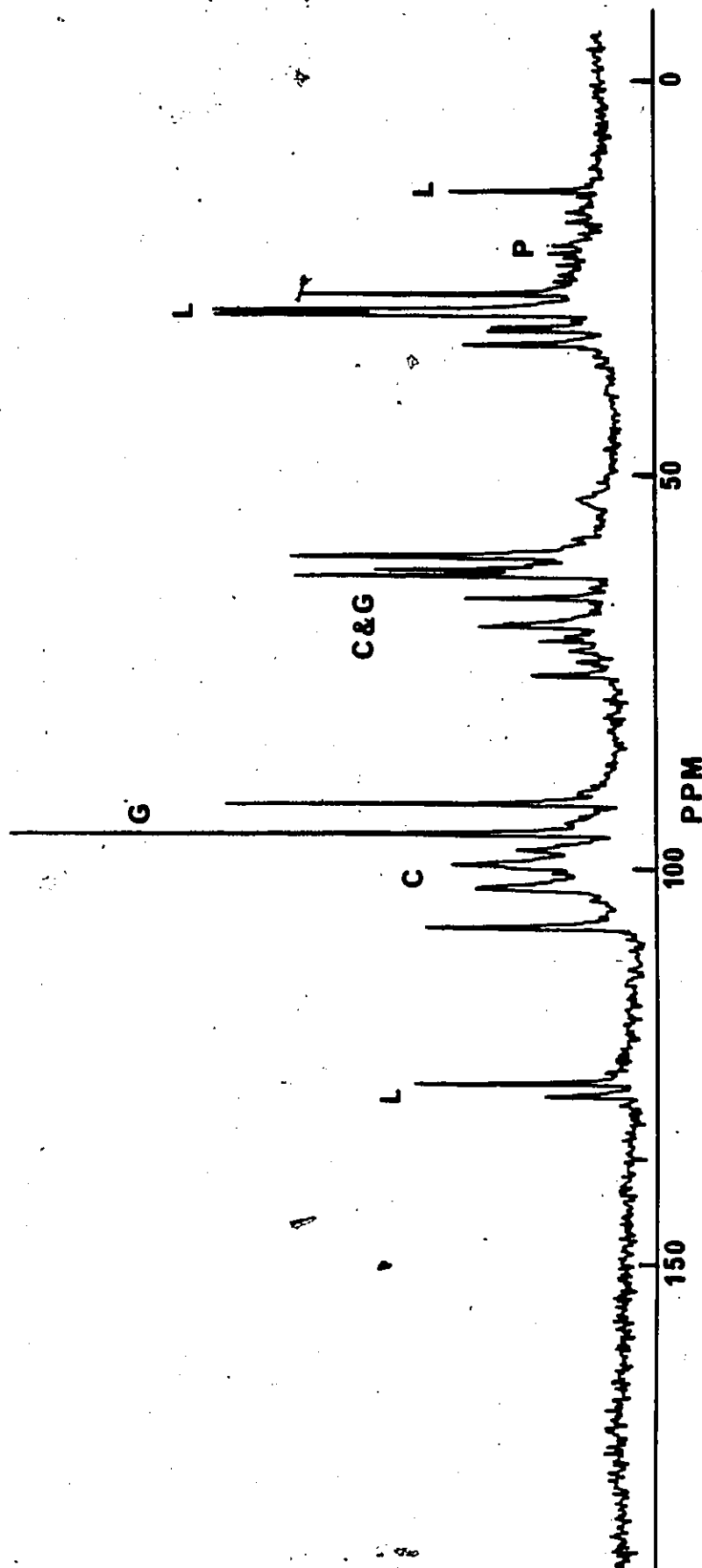


Figure VII.1 - 20 MHz ^{13}C spectrum of whole cells grown on ^{13}C -1 glucose. The peaks corresponding to intracellular constituents are labelled as follows: L, lipid; P, protein; C, carbohydrate; G, glucose

short T_2 . The labelling pattern in the lipid is very similar to that seen in Figure V.3 for cells grown on ^{13}C -2 acetate. This would be expected since the label in the C-1 position of glucose results in C-2 labelled acetyl-CoA in the catabolism of glucose. However, the labelling of the lipids is not nearly as efficient as for acetate, as can be seen by the relative signal heights of the labelled lipid and unlabelled glucose in comparison with Figure V.3. The spectrum also shows that the glucose is predominantly stored as free glucose and not as glycogen. The large peaks at 93 and 97 ppm are due to the α and β conformers of the C-1 position of free glucose. The additional peaks between 98 and 110 ppm are due to labelled C-1 carbons of glucose derivatives such as maltotriose and cellobiose which are metabolic precursors of glycogen and cellulose. The assignment of the peak at ~108 ppm is uncertain, but it does appear consistently in whole cell spectra. The chemical shift is consistent with the C-1 position of an alkylated glycoside - perhaps of glycolipid, or a precursor thereof. The intensity of the peaks at 61 to 64 ppm (assignable to the C-6 carbons of glucose and derivatives) show that some of the ^{13}C label has scrambled to these positions. This can occur if the specifically labelled glucose is catabolised via the Aldolase triangle (Shulman et al., 1979) and returned to the storage glucose pool. The intervening peaks between 70 and 75 ppm are largely

due to unlabelled glucose. The labelled peak at 64 ppm may be due to the C-1 and C-6 carbons of mannitol which derives from glucose via fructose or fructose 6-phosphate (Boonsaeng et al., 1976). This component has been previously observed in mycelial fungi (Matsunaga et al., 1980), but not in yeast. The ease with which the organism can assume different morphological forms may account for the presence of mannitol in what is otherwise a normal yeast. The labelling pattern of this spectrum shows the complementarity of glucose and acetate when compared to the spectra of Chapter V. The acetate is obviously preferred for fatty acid synthesis, while the intracellular glucose pool is maintained from externally supplied glucose and used for polysaccharide and carbohydrate synthesis. The glucose pool appears to be in anomeric equilibrium (approximately 2:1, to anomers), just as it would in solution. No peaks due to ethanol are seen since this yeast has no fermentation capability. Tables VII.1 and VII.2 summarize the chemical shifts of compounds that have been identified in the cell spectra of this chapter, along with those compounds that would be expected to appear on the basis of common metabolic pathways. Reference to these tables will show why certain peaks have been identified as they have in the following spectra. Table VII.1 gives the chemical shifts of glucose and related compounds, including those that one would expect to find in the functioning cell

Table VII.1

¹³C CHEMICAL SHIFTS OF GLUCOSE
AND RELATED COMPOUNDS

<u>Sugar</u>	<u>Anomer</u>	<u>C-1</u>	<u>C-2</u>	<u>C-3</u>	<u>C-4</u>	<u>C-5</u>	<u>C-6</u>	<u>Ref.</u>
Glucose	α	93.1	72.5	73.8	70.7	72.5	61.7	(a)
	β	97.0	75.2	77.0	70.7	76.8	61.8	
Trehalose	αα	94.4	73.3	73.7	70.9	72.2	61.7	(b)
Pullulan	α1,4	101.1	74.6	75.2	80.5	72.7	61.7	(b)+(c) pD = 11
	α1,6	99.2	73.7	75.2	71.5	71.0	68.0	
Glucose-6-phosphate	α	93.3	72.6	73.7	70.3	71.6	65.0	(b)
	β	97.2	75.2	76.6	70.3	76.0	65.2	
Fructopyranose	α	65.9	99.1	70.9	71.3	70.0	61.9	(d)
	β	64.7	99.1	68.4	70.5	70.0	64.1	
Fructofuranose	α	63.8	105.5	82.9	77.0	82.2	61.9	(d)
	β	63.6	102.6	76.4	75.4	81.6	63.2	
Fructose-6-phosphate	α	63.8	105.3	82.6	76.9	81.4	67.5	(e)
	β	63.8	102.4	76.2	75.4	80.8	65.4	
Fructose-di-phosphate	α	66.9	105.7	82.4	77.5	82.7	65.4	(e)
	β	66.9	102.0	76.9	75.1	80.4	65.4	
Maltotriose (glycogen)	m ⁺	101.1	74.3	75.1	71.2	73.4	62.4	(f)
Cellobiose (cellulose)	n ⁺	103.9	74.7	77.2	71.1	77.4	62.4	(f)
Mannitol		64.6	72.2	70.6	70.6	72.2	64.6	(g)

+ m middle unit

n non-reducing unit

(a) Colson et al. (1975)

(b) Determined by standard measurement

(c) Colson et al. (1974)

(d) Koerner et al. (1978)

(e) Koerner et al. (1973)

(f) Usui et al. (1973)

(g) Matsunaga et al. (1980)

Table VII.2

¹³C CHEMICAL SHIFTS OF SOME COMMON AMINO ACIDS

Amino Acid	C=O	α C	β C	γ C	δ C	ϵ C	LC	pH	Ref.
GLY	173.5	42.6						6.9	a
GLY-GLY	177.6	44.4						6.5	a
	168.7	41.9							
ALA	177.0	51.9	17.5					4.5	b
	176.3	51.1	16.8						c
ILE	174.7	60.4	36.6	25.2	11.8				c
				15.4					
LEU	176.7	54.6	41.0	25.3	23.15			6.8	a
					22.06				
	176.1	54.2	40.5	24.9	22.7				c
					21.6				
THR	173.5	61.0	66.6	20.0					c
PRO	175.7	62.4	30.1	24.9	47.2			6.9	a
	174.1	61.1	29.2	23.9	46.0				c
ASP	175.4	53.46	37.73	178.7				6.3	a
	175.5	53.46	37.78	178.7				6.7	b
GLU	173.3	53.9	26.7	31.7	178.3			2.0	a
	175.8	55.9	28.2	34.7	182.4			7.0	b
GLN	173.3	53.7	26.1	30.6	177.5			2.0	a
	175.5	55.2	28.3	33.0	179.5				c
MET	174.8	54.7	30.5	29.6		14.7			c
LYS	175.6	55.7	31.0	22.5	27.5	40.3		6.6	a
	175.8	55.9	31.2	22.6	27.7	40.54		6.0	b
ARG	175.6	55.5	28.7	25.0	41.66		158.0	6.9	a
(ARG) ^N	174.4	54.3	29.5	25.6	41.76		157.8	6.9	a
ORN	175.3	55.3	28.6	23.9	40.05			7.4	a
	175.3	55.5	28.7	24.03	40.32			5.0	b
CIT	175.7	55.7	28.9	26.1	40.4		162.7	6.5	d
Amino Acid	C=O	α C	β C	Ring	Carbons				Ref.
HIS	174.4	55.3	28.5	C2	136.7				c
				C4	117.7				
				C5	131.0				
TRP	175.2	56.1	27.4	C2	126.0				c
				C5	122.9				
				C3	108.4				
				C4	120.3				
				C6	119.3				
				C7	112.8				
				C8	137.3				
				C9	127.5				

(a) Determined by Standard Measurement

(b) Rattle, (1981)

(c) Wüthrich, (1976), (determined in neutral D₂O)(d) Bruker ¹³C Data Bank, Vol. I

as intermediates in glycolysis, dissolved in aqueous solution. The chemical shifts of the C-1 position are generally the key to identification since they are a sensitive function of type and conformation of linkage. The C-1 positions of the middle units of trisaccharides are characteristic of the longer polysaccharide. Thus a peak at 101 ppm is indicative of the presence of glycogen and a peak at 103.9 ppm indicates the presence of cellulose. Table VII.2 gives the chemical shifts of some common amino acids in aqueous solution. Since the shifts of the α and carboxyl carbons are highly dependent on pH, this was taken into account in assigning spectra. Standard solutions of amino acids adjusted to pH=6.3-6.9, which is approximately the pH of the internal environment of the cell, have been run at 20 MHz and these chemical shifts are included in the table and compared with the published data. Shifts due to incorporation into small peptides can be calculated (Christl and Roberts, 1972), and thus the form of the amino acid in the spectrum can be determined. Since the chemical shifts of glycine do not seem to follow the peptide rules, a diglycine sample was run in aqueous solution at the approximate pH of the cell.

The acetate-derived spectra of whole cells presented in Chapter V show, in addition to labelled lipid, small amounts of unlabelled glucose (or related compounds), and peaks that appear to be labelled and are characteristic of amino acids.

The spectrum derived from ^{13}C -1 acetate is of greater interest since the carboxyl group of the acetate is normally lost to CO_2 during metabolism, and therefore the occurrence of labelled peaks other than lipid are a result of a specific biosynthetic path. Under the conditions of spectral accumulation, only compounds which have received the label or are present in high quantities will appear. The spectra of Figure V.2 and 5 show two peaks at approximately 40 ppm that can be assigned to the ϵC of lysine and δC of arginine. It can be shown that these particular carbons derive from the carboxyl group of acetate through the Krebs' acid cycle (vide infra). The additional peaks in the 20 MHz spectrum can be assigned to the two conformers of glucose in their correct proportions. However, the 75 MHz spectrum (Figure V.5) shows only three peaks in the carbohydrate region. These could be due to two sources. The peak at 64.5 ppm is indicative of either glucose-6-phosphate, fructose phosphates or mannitol. Since fructose phosphates show characteristic peaks at 80 and 102 ppm, this can be eliminated. Glucose-6-phosphate has a spectrum which resembles glucose, except for the chemical shift of the C-6 position. In addition, the peak at 70.6 could be assigned to C-4 of glucose, which could derive from the carboxyl of acetate, and therefore be labelled. However, the most consistent assignment of the three observed peaks is to mannitol, for the following reasons. Under the conditions imposed on the cell in the NMR

tube, one would not expect high quantities of sugar phosphates. This is confirmed by phosphorus NMR results (see Chapter VIII). Paper chromatography of extracts of fresh cells showed the presence of large quantities of free glucose, as well as fructose di-phosphate as the major sugar phosphate. In all spectra, sugars were never observed to be labelled. Thus, in any one spectrum all peaks of a particular sugar or sugar derivative should be seen in the correct ratio. Comparison to extract spectra (see below) also shows that the three peaks assignable to mannitol occur in approximately equal intensities. For these reasons, the peak at 64.5, which occurred consistently in all spectra was assigned to mannitol. Whenever peaks in spectra of fresh cells appeared that could be assigned to phosphorylated sugars, these were assigned to fructose phosphates. This assignment procedure gave satisfactory consistency in all the observed spectra. The relative amounts of glucose, mannitol and other glucose oligomers in the cells varied from culture to culture and appeared to be a very sensitive function of the growth time of the culture.

The assignments of the peaks observed in Figures V.5 and 6, that are of other than lipid origin, are presented in Table VII.3. The spectra of these two figures can be compared since they represent the same accumulation time for the same sample size. Whereas the ^{13}C -1 acetate derived spectrum shows amino acid peaks at 40 ppm, the ^{13}C -2 acetate derived spectrum

Table VII.3

LABELLED WHOLE CELLS (75 MHz)
 (Assignment of peaks other than lipid)
 (See Figures V.5 and V.6)

<u>Spectrum</u>	<u>Chemical Shift (TMS)</u>	<u>Assignment</u>
¹³ C-1 Acetate	38.8	Unlabelled Lipid
Grown Cells	40.4	εC LYS
	41.8	δC ARG
	64.5	C-1 Mannitol
	70.6	C-3 Mannitol
	72.2	C-2 Mannitol
	107.2	C-1 Alkylated Glycoside
¹³ C-2 Acetate	25.0	γC ARG
Grown Cells	55.7	αC AA'S
	61.7	C-6 TRE
	70.9	C-4 TRE
	72.2	C-5 Trehalose
	73.3	C-2 TRE
	73.7	C-3 TRE
	94.3	C-1 TRE
	64.5	C-1 Mannitol
	~83+85	FDP, F6P
	103.1	C-6, FDP

shows a peak at 55 ppm that can be assigned to the α -carbons of amino acids, likely arginine and lysine, in order to be consistent with Figure V.6. There is also a peak at 25 ppm which may be assigned to γ C arginine rather than lipid, since it appears in a water soluble extract (see below). The three large peaks between 60 and 72 ppm can again be assigned to mannitol, although the small peaks in the carbohydrate region are more consistent with trehalose than glucose, in this case. The additional peaks are indicative of fructose phosphates and the peak at 103.1 is assigned to FDP rather than cellobiose (see Table VII.1) simply on the basis of relative intensity to the peaks in the 85 ppm region.

The above analysis suggests that as well as labelled lipid, there exist substantial pools of unlabelled sugar and labelled arginine and lysine in the cell. The relative proportions of these species can be seen in the natural abundance ^{13}C spectrum (Figure V.4). It can easily be seen that the free glucose and mannitol pools are relatively large and the amino acid pools are small. The region between 40 and 60 ppm indicates presence of more amino acids than those that appeared in the labelled spectrum, but the peaks at 40 and 55 ppm are both present.

The appearance of the ^{13}C label in the three day grown cells can be further studied by extracting the water soluble components of the cell and looking at the non-lipid labelled

material. In the process of making the nystatin membrane preparation, the cells were lysed and the cytoplasmic components remained in the supernatant after removal of the membrane components. Figure VII.2 is a spectrum of this fraction. The predominant peaks are the same as those observed in the whole cell spectrum, and appear to be labelled to a greater extent than the other components. Some of the peaks are due to labelled triglyceride that is lost on lysis. Peaks in the 170-180 ppm region, due to the carboxyl groups of the amino acids and lipids, also derive from the carboxyl of acetate. In an attempt to clarify the assignments, a Bligh-Dyer extraction of the supernatant was performed, in order to separate the lipid from the water soluble components. The aqueous phase spectrum is shown in Figure VII.3a. TLC on the chloroform-soluble portion confirmed the presence of triglyceride. The carboxyl region is better resolved in this spectrum and the predominant peaks in this region can be assigned to the non-amino carboxyls of glutamate and aspartate, as well as to the amino carboxyls of all observed amino acids. Comparison of the labelling pattern can be made with Figure VII.3b, which is a spectrum of the aqueous phase of a Bligh-Dyer extract of unlabelled cells. Although the spectra are of different sample quantities and accumulation times, the constituents of each spectrum are present in the same relative proportions. The unlabelled spectrum

SUPERNATANT FROM ^{13}C -1 ACETATE DERIVED
NYSTATIN MEMBRANES

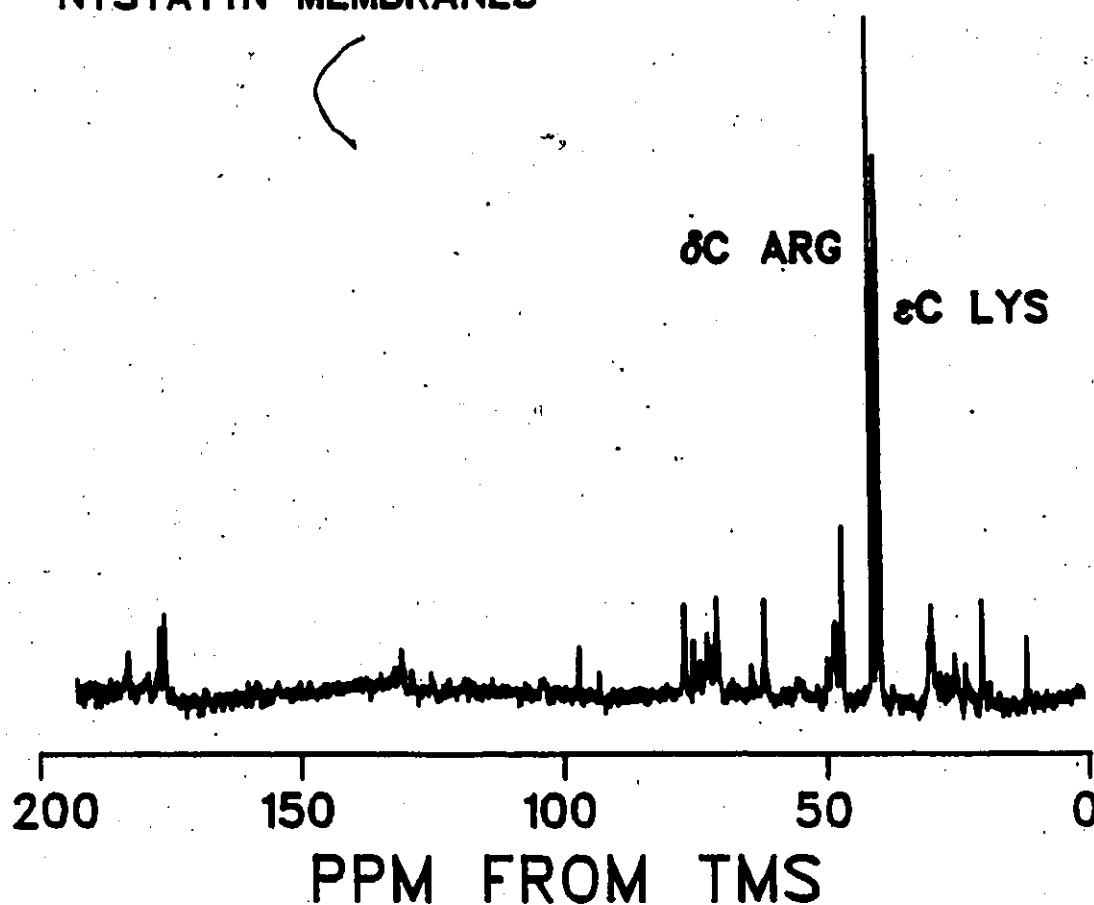


Figure VII.2 - 75 MHz ^{13}C spectrum of supernatant from nystatin membrane preparation from 100 ml culture of cells supplemented with ^{13}C -1 acetate. The total supernatant was lyophilized and resuspended in 1 ml D_2O . The spectrum is a result of 18,500 scans, using a 45° pulse and 0.5 sec recycle time

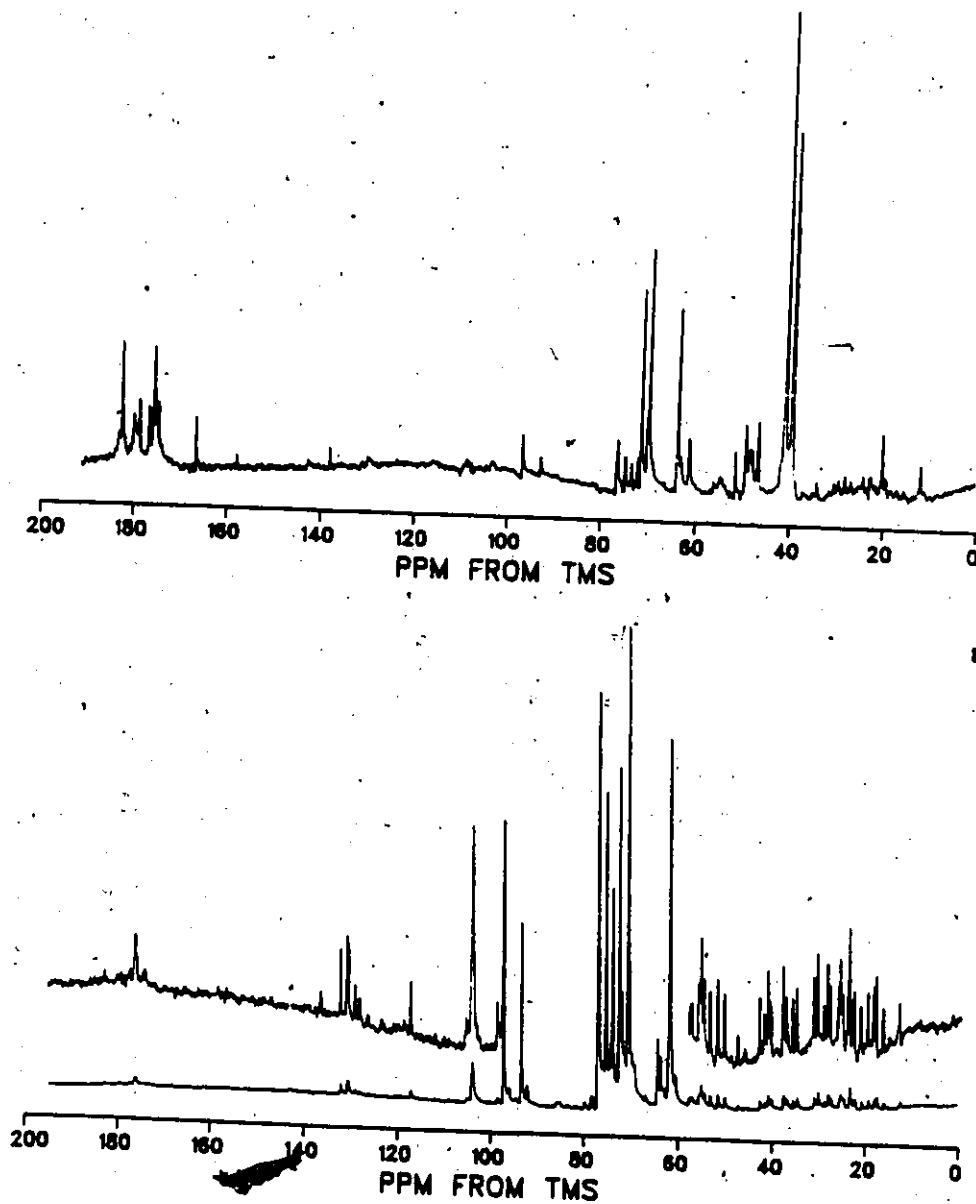


Figure VII.3 -

- a) 20 MHz ^{13}C spectrum of water soluble components of the supernatant described in Figure VII.2. This sample was not derived from the same culture as the previous figure, accounting for the differences between the relative amounts of mannitol and glucose. The spectrum represents 100,000 accumulations
- b) 20 MHz ^{13}C spectrum of aqueous fraction from lipid extraction of unlabelled cells. This spectrum is a result of 100,000 accumulations on approximately 25 mg of material per ml of D_2O at pH=6.6

shows only the carboxyl peak of the accumulated amino acid pool, indicating that in the labelled spectrum the δC glutamate and γC aspartate receive more of the label than the amino carboxyls. The forest of lines in the 40-60 ppm range in the unlabelled spectrum show that there are a great deal more amino acids and small peptides present than can be inferred from the labelled spectrum. The region from 115-140 ppm in the unlabelled spectrum shows peaks that can be assigned to all the ring carbons of histidine, whereas the labelled spectrum shows only the C-2 position on the ring (which appears as the smallest peak in the unlabelled spectrum). The carbohydrate region in both Figures VII.2 and 3 contain varying proportions of mannitol and free glucose but all peaks are consistent with no labelling of these pools. Comparison of the peaks at 40 ppm with the glucose peaks in the two spectra of Figure VII.3 show how efficiently the label has gone into the two specific amino acid peaks. Other than these two carbons and the carboxyl groups, very few amino acid carbons have received much of the ^{13}C label. In the region below 40 ppm, only two carbons have been significantly labelled. The peak at 20.5 ppm can be assigned to the γC of threonine and the peak at 11.8 ppm to the δC of isoleucine. Both these carbons are derived from the carboxyl carbon of acetate by the accepted metabolic pathway.

The same procedure was repeated for the supernatant of the nystatin preparation of the ^{13}C -2 acetate derived cells, but the spectra are not shown. Labelling patterns are consistent with the preferred labelling of the glutamate, arginine, and lysine pools. The assignments of the spectra in Figures VII.2 and 3a, as well as the ^{13}C -2 analogue are given in Table VII.4. Peaks marked (L) are those which appear to be labelled. Common peaks occurring in spectra derived from the two labels likely do not bear any of the acetate ^{13}C , whereas the labelled peaks show a complementarity of origin. The peak at 166 ppm, for example, occurs in the spectra from both labels. This peak can be assigned to carbonate or to urea. At the pH of growth, most of the CO_2 released as a result of metabolism would be expelled into the air rather than dissolved as carbonate. Urea, on the other hand, would be expected to be present if there were relatively large amounts of arginine present, due to the urea cycle. Since the urea carbon derives from CO_2 in metabolism, it might be expected that it would be labelled in the ^{13}C -1 acetate-derived spectrum if the CO_2 were taken directly from the products of metabolism rather than from the atmosphere. The occurrence of this peak in the spectra of both labelled extracts probably means that it is not significantly labelled and that the urea is derived from atmospheric CO_2 .

Table VII.4

¹³C ASSIGNMENTS OF SPECTRA OF SUPERNATANT
FROM NYSTATIN MEMBRANE PREPARATIONS

<u>Spectrum</u>	<u>Chemical Shift (TMS)</u>	<u>Assignment</u>
¹³ C-2 Acetate	165.6	Urea or Carbonate
Grown Cells	107.3	C-1 Alkylated Glycoside
(Water-Soluble	94.3	C-4 TRE
Fraction)	97.0, 93.1	C-6 GLC
	77.0	C-3 βGLC
	76.8	C-5 βGLC
	75.3	C-2 βGLC
	73.8	C-3 αGLC
	72.6	C-2,5 αGLC
	70.6, 70.7	C-4 GLC
	61.8, 61.9	C-1 GLC
	55.6 (L)	αC GLU, ARG
	54.5 (L)	αC LYS
	34.3 (L)	γC GLU
	28.0	βC GLU
	27.5 (L)	δC LYS
	25.0 (L)	γC ARG
¹³ C-1 Acetate		
Grown Cells	182.4 (L) a,t	δC GLU
(See Figures VII.2	179.9 (L) a	δC GLN
and VII.3(a))	178.7 (L) a	δC ASP
	175.8, 176.6 (L) a,t	CO LYS, ARG, ASP, GLN

Table VII.4 Con't

<u>Spectrum</u>	<u>δ (TMS)</u>	<u>Assignment</u>
^{13}C -1 Acetate	166.5 a	Urea
Grown Cells	136.5 a	C-2 HIS
	130.6	{Lipid C=C
	128.7 }t	
	97.0 a,t	C-1 β GLC
	93.2 a,t	C-1 α GLC
	94.0 a	C-1 TRE
	77.0 a,t	C-3 β GLC
	75.2 a,t	C-2 β GLC
	73.9 t	C-3 α GLC
	72.5 a,t	C-2 α GLC
	72.1 a,t	C-3 Mannitol
	70.6 a	C-2 Mannitol
	70.4 a,t	C-4 α, β GLC
	64.3 a,t	C-1 Mannitol
	61.9 a,t	C-6 GLC
	47.2 (L) a,t	δ C PRO
	41.6 (L) a,t	δ C ARG
	40.2 (L) a,t	ϵ C LYS
	30.0 - 32.0 (L) t	Lipid
	20.5 (L) a,t	γ C THR
	11.8 (L) a,t	γ C ILE

(L) Indicated that the peak is likely labelled

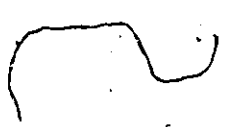
a Appears in aqueous solution portion of supernate from nystatin membrane preparation (Figure VII.3a)

t Appears in total supernate from nystatin membrane preparation (Figure VII.2)

Some of the peaks in the natural abundance spectrum of Figure VII.3b are assigned in Table VII.5. Complete assignment of the spectrum was not attempted, although peaks due to all amino acids found by amino acid analysis can be accounted for. The pH of this unlabelled aqueous extract was adjusted to make chemical shifts approximately the same as in the whole cell.

(b) Path of Label During Starvation

The reduction of non-membrane triglyceride during the period of starvation of the culture has been shown by lipid analysis (see Table IV.5), but it can also be inferred from the spectra of the starved cells. Figures VII.4 and 5 show the spectra from 100 ml cultures of starved cells which were originally grown in specifically labelled acetate. These spectra were taken under identical accumulation conditions and for approximately the same cell density for comparison. They represent spectra that were used for T_1 analysis (Chapter V). Comparing these spectra to those of Figures V.2 and 3, it is easily seen that the label in the lipid is reduced, and the glucose pool appears to have increased, along with the appearance of peaks that were not seen in the unstarved spectrum. It is obvious that much of the labelled lipid has been metabolized during starvation. The extent of labelling within the lipid pool, however, has not been



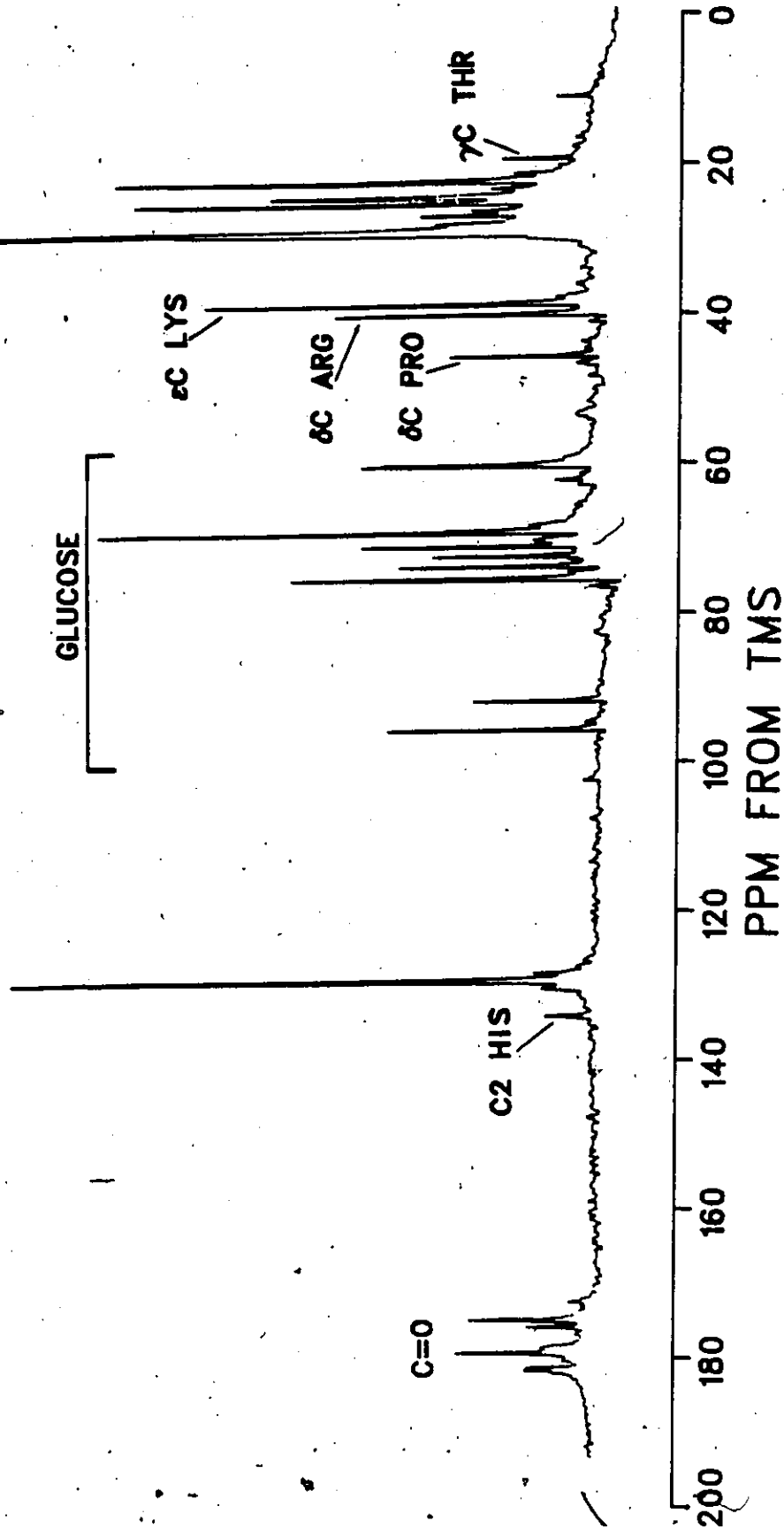
^{13}C -1 ACETATE GROWN STARVED CELLS

Figure VII.4 - 20 MHz ^{13}C spectrum of 100 ml culture of ^{13}C -1 acetate-derived cells after three days in starvation medium. The spectrum represents the accumulation of 27,000 transients, using a 45° pulse and a 1 sec recycle time.

¹³C-2 ACETATE GROWN STARVED CELLS

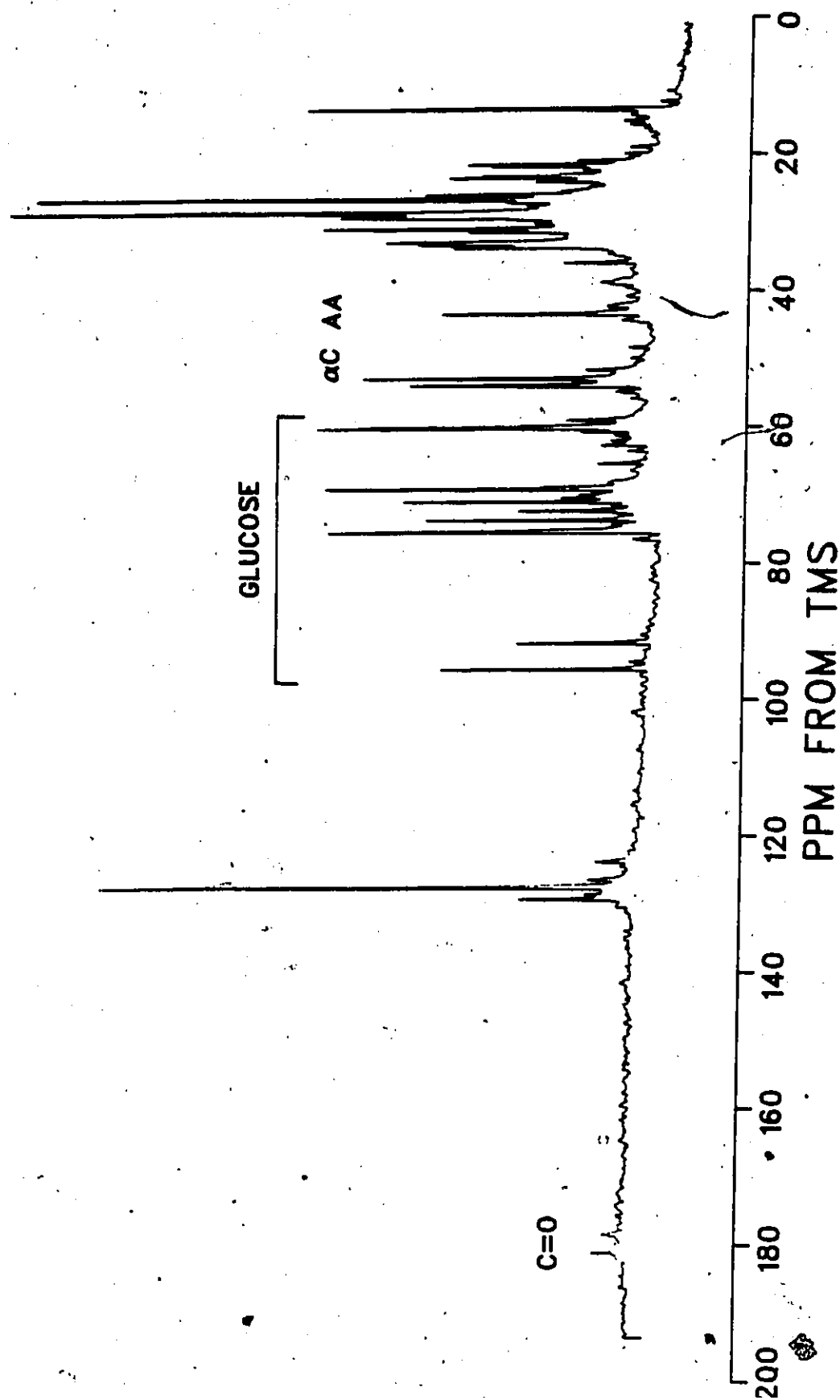


Figure VII.5 - Same as Figure VII.4 for ¹³C-2 acetate-derived cells

Table VII.5

ASSIGNMENT OF SOME PEAKS OF AQUEOUS PORTION
OF BLIGH-DYER EXTRACT, pH=6.6

The following table assigns some of the peaks seen in
Figure VII.3b

<u>Chemical Shift (TMS)</u>	<u>Assignment</u>
20.6	γ C THR
23.1	γ C LYS
27.9	δ C LYS, β C ARG
30.1	β C PRO
30.9	β C LYS
37.6	β C ASP
40.8	ϵ C LYS
41.6	δ C ARG
47.3	δ C PRO
54.6	α C LYS
55.1	α C ARG (HIS)
61.8	C-6 GLC
63.8	C-1 F6P
64.3	C-6 F6P, mannitol
70.7	C-4 GLC, mannitol
72.0	C-2 mannitol
72.5	C-5,2 α GLC
73.8	C-3 α GLC
75.2	C-2 β GLC
76.9	C-3 β GLC
93.1	C-1 α GLC
96.9	C-1 β GLC
103.7	C-2 FDP
117.0	C-5 HIS
131.8	C-4 HIS
136.5	C-2 HIS

substantially reduced, as can be seen by comparison of unlabelled to labelled lipid peak heights in the two spectra. A spectrum of the corresponding unlabelled cells looks not unlike that of Figure V.4, except that the sugar and amino acid pools have increased slightly relative to the lipid pool. (This spectrum is not shown, but the peaks are listed and assigned in Table VII.6). Thus, the first response of the organism to a reduced carbon source appears to be to maintain the levels of pooled sugar, while utilizing fatty acid for maintenance of metabolism.

The specific labelling of the amino acid region in the spectra of Figure VII.4 and 5 is the key to identifying the specific components, and the identities are supported by the appearance of the appropriate peak from the alternate label. As before, the ^{13}C -1 acetate spectrum provides a more simple labelling pattern. In addition to the peaks due to lysine and arginine, the δC of proline, the C-2 of the histidine ring, the γC of threonine, the γC of methionine (at 29.6 ppm), and the γC of leucine (at 24.8 ppm), can be seen in the cell spectrum. The identities are supported by observing the neighbouring carbons at about the same intensity in the spectrum of growths with the alternate label (i.e., γC proline, the βC threonine, βC methionine, etc.), as would be expected from the incorporation of a single acetyl-CoA unit (deriving from oxidation of fatty acid chains)

Table VII.6
 ASSIGNMENT OF ^{13}C RESONANCES OF STARVED CELLS

<u>Spectrum</u>	<u>Chemical Shift (TMS)</u>	<u>Assignment</u>
Unlabelled Cells (75 MHz) (see Figure VII.6)	40.4	$\epsilon\text{C LYS}$
	45.8	$\alpha\text{C GLY}$
	55.1, 56.7	$\alpha\text{C AA}$
	61.8	C-6 TRE
	65.5	C-1 Mannitol
	70.95	C-4 TRE, Mannitol
	72.3	C-5 TRE, Mannitol
	73.3	C-2 TRE
	73.8	C-3 TRE
	75.9	C-5 βG6P
	76.7	C-5 βGLC
	94.4	C-1 TRE
	104	C-2 FDP
	109	C-1 Alkylated Glycoside
	129, 130	Lipid C=C
	180, 182	C=O Lipid and AA
Unlabelled Cells (20 MHz) pH=6.5	15.1	C-18 Lipid
	23.9	C-17 Lipid
	25.2	$\gamma\text{C ARG}$
	25.9	C-3 Lipid
	26.7	$\delta\text{C LYS}$, $\beta\text{C GLU}$
	28.3	C-3,8 Lipid
	29.0	$\beta\text{C ARG}$, HIS
	30.6, 31.0	C-4-7, 12-15 Lipid
	32.7	$\gamma\text{C GLU}$
	33.1	C-16 Lipid
	35.1	C-2 Lipid
	37.8	$\beta\text{C ASP}$
	40.8	$\epsilon\text{C LYS}$, $\delta\text{C ORN}$
	41.9	$\delta\text{C ARG}$
	55.8	$\alpha\text{C ARG}$, GLU, LYS, HIS
	62.1	C-6 GLC
	63.9	C-1 F6P
	64.6	C-1 Mannitol
	70.7-77.2	C-2-5 GLC, Mannitol
	93.5	C-1 αGLC
	97.3	C-1 βGLC
	104.0	C-2 F6P or FDP
	117.2	C-4 HIS

<u>Spectrum</u>	<u>Chemical Shift (TMS)</u>	<u>Assignment</u>
Unlabelled Cells (20 MHz) pH=6.5	129.1, 130.8 132.1 158.3 173.3, 175.5, 179.8	Lipid C=C C-5 HIS C ARG CO AA, Lipid
¹³ C-1 Acetate Grown Starved Cells (20 MHz) (See Figure VII.4)	182.8 182.5 180.2 179.6 176.7 175.7 173.5 135.3 131.8, 130.7, 129.7 97.05 93.2 77.04 76.9 75.3 73.9 72.6 70.6 64.6 61.8, 61.9 47.25 41.6 40.2 30.7, 30.4 29.6 28.2 27.6 26.6 25.7 24.8 23.6 20.6 14.9	¹³ C GLU CO Lipid C ASP, C GLN CO LEU, ALA CO LYS, GLU, PRO, ARG, ASP, HIS, MET CO THR, GLY C-2 HIS C=C Lipid (41 Hz ¹³ C Coupling) C-1 BGLC C-1 aGLC C-3 BGLC C-5 BGLC C-2 BGLC C-3 aGLC C-2,5 aGLC, Mannitol C-4 GLC, Mannitol C-1 Mannitol C-6 GLC C PRO C ARG C LYS, C ORN Lipid C MET Lipid Lipid (unlabelled) Lipid C LEU Lipid C THR Lipid C-18
¹³ C-2 Acetate Grown Starved Cells (20 MHz) (See Figure VII.5)	182.2, 179.5 130.8, 129.0 125.3 97.0 93.2 77.04	Unlabelled CO, Lipid, AA Lipid C=C ? C-1 aGLC C-1 BGLC C-3 BGLC

<u>Spectrum</u>	<u>Chemical Shift (TMS)</u>	<u>Assignment</u>
¹³ C-2 Acetate Grown	76.9	C-5 β GLC
Starved Cells	75.3	C-2 β GLC
(20 MHz) (See Figure VII.5)	73.9	C-3 α GLC
	72.6	C-2,5 α GLC, Mannitol
	70.8	C-4 GLC, Mannitol
	67.0	β C THR
	64.6	C-1 Mannitol
	62.4	α C PRO
	61.9	C-6 GLC
	60.5	α C THR
	55.65	α C GLU, ARG
	54.6	α C LYS, LEU, MET
	53.3	α C ASP
	51.0	α C ALA
	45.1	α C GLY (peptide)
	40.2	β C LEU
	37.6	β C ASP
	35.4	γ C GLU
	34.9, 34.5	Lipid
	33.3, 32.6	Lipid C-16
	30.8, 30.2	Lipid, β C LYS, ¹³ C GLU
	28.6	β C ARG
	28.2	Lipid, β C GLU
	27.5	δ C LYS
	25.9	Lipid (unlabelled)
	25.0	δ C ARG, γ C PRO, γ C LEU
	23.5	Lipid (unlabelled)
	23.1	γ C LYS, δ C LEU
	22.6	δ C LEU
	17.0	γ C ALA
	14.9	Lipid C-18

into these amino acids from the Kreb's acid cycle. In addition, the carboxyl groups of the amino acids have been labelled, showing the presence of the δC of glutamate, the γC of aspartate, as well as the amino carboxyls of those amino acids that are found to have labelled carbons in the other portions of the spectrum. In addition, there appears to be two peaks attributable to δC of glutamate, that are consistent with free and bound (as in a small peptide) species in approximately the same amount; a fact which was born out by an amino acid analysis (see below).

The additional peaks of Figure VII.4 can be assigned to labelled lipid, free glucose in anomeric equilibrium, and a small amount of mannitol. The peaks at the foot of the peak due to C-9 and 13 of the lipid chain are not consistent with aromatic amino acids, and must therefore be due to ^{13}C -coupling between neighbouring ^{13}C nuclei on the lipid chain. The distance between these peaks is 41 Hz, which is a reasonable figure for such coupling. The appearance of this coupling in the methylene region of the spectrum is obscured.

The predominant peaks from the cells deriving from the alternate label (Figure VII.5) arise from the α -carbons of amino acids. In the freshly grown cells only a single broad peak due to the α -carbons appeared at 55 ppm, but here this peak is resolved into two peaks which can be assigned to the αC of glutamate and arginine (55.6 ppm) and the αC of lysine.

and leucine (54.6 ppm). A little further upfield are the small α C due to aspartate (53.3 ppm) and alanine (51.0 ppm). The large peak at 45.1 ppm cannot be due to other than the α C of glycine, which did not appear to be labelled in the original growth. The chemical shift of this carbon is consistent with glycine existing almost totally as an amino terminal unit in a small peptide and not free in solution (see Table VII.2 and Jung et al., 1970). There appear to be more small peaks between 60 and 70 ppm in this spectrum, and these can be assigned to the β C of threonine, and the α C of proline. The α C of threonine also appears at 60.5 ppm. The additional resonances in the methylene region are largely due to lipid, however, small peaks which do not overlap with any lipid peaks show the presence of the intervening carbons in the pools of arginine, lysine, aspartate, leucine, glutamate etc. which derive from the ^{13}C -2 carbon of acetate.

All the resonances of Figures VII.4 and 5 are listed with their assignments in Table VII.6. All assignments are consistent with the relative contents of each amino acid as measured by an amino acid analysis, as well as being consistent within the metabolic pathway. For example, the presence of a labelled acetate unit within any amino acid must appear in both spectra with the same approximate intensity. Included are the assignments of the unlabelled starved spectra, at 20 MHz (not shown) and 75 (MHz) (Figure VII.6), which confirm the presence of the species assigned in the labelled spectra,

and give some idea of their relative abundance in the cell.

Figure VII.6 is included to show that, on some occasions, large amounts of trehalose, instead of free glucose, were observed in the starved cell. This spectrum is of a culture that was a day or so older than the normal three day growth, when suspended in the starvation medium. As a result, the storage form has been retained as trehalose. Trehalose is a non-reducing disaccharide, whose structure is shown in Figure VII.10. Due to the molecular symmetry, there is only one conformer, which simplifies the NMR spectrum. Trehalose has a characteristic chemical shift at 94.4 ppm for C-1; otherwise the shifts resemble those of glucose. Throughout the study, varying amounts of trehalose were observed in the cells, although the dominant form in cells harvested after three days was free glucose. Trehalose has been found in a variety of microorganisms, including other strains of yeast (den Hollander *et al.*, 1981) and amoeba (Deslauriers *et al.*, 1980), and is thought to function as a storage form of carbon for organisms which can survive dessication, since reducing sugars interact irreversibly with protein (Lea *et al.*, 1950). Trehalose has been observed previously in this organism, but only after three days growth in liquid culture (Merdinger, 1969). Spectra taken of cells from older cultures, of 6 or 12 days growth in minimal medium, show exclusively trehalose in the sugar pool. Thus, when suspended in the

STARVED UNLABELLED CELLS (75 MHz)

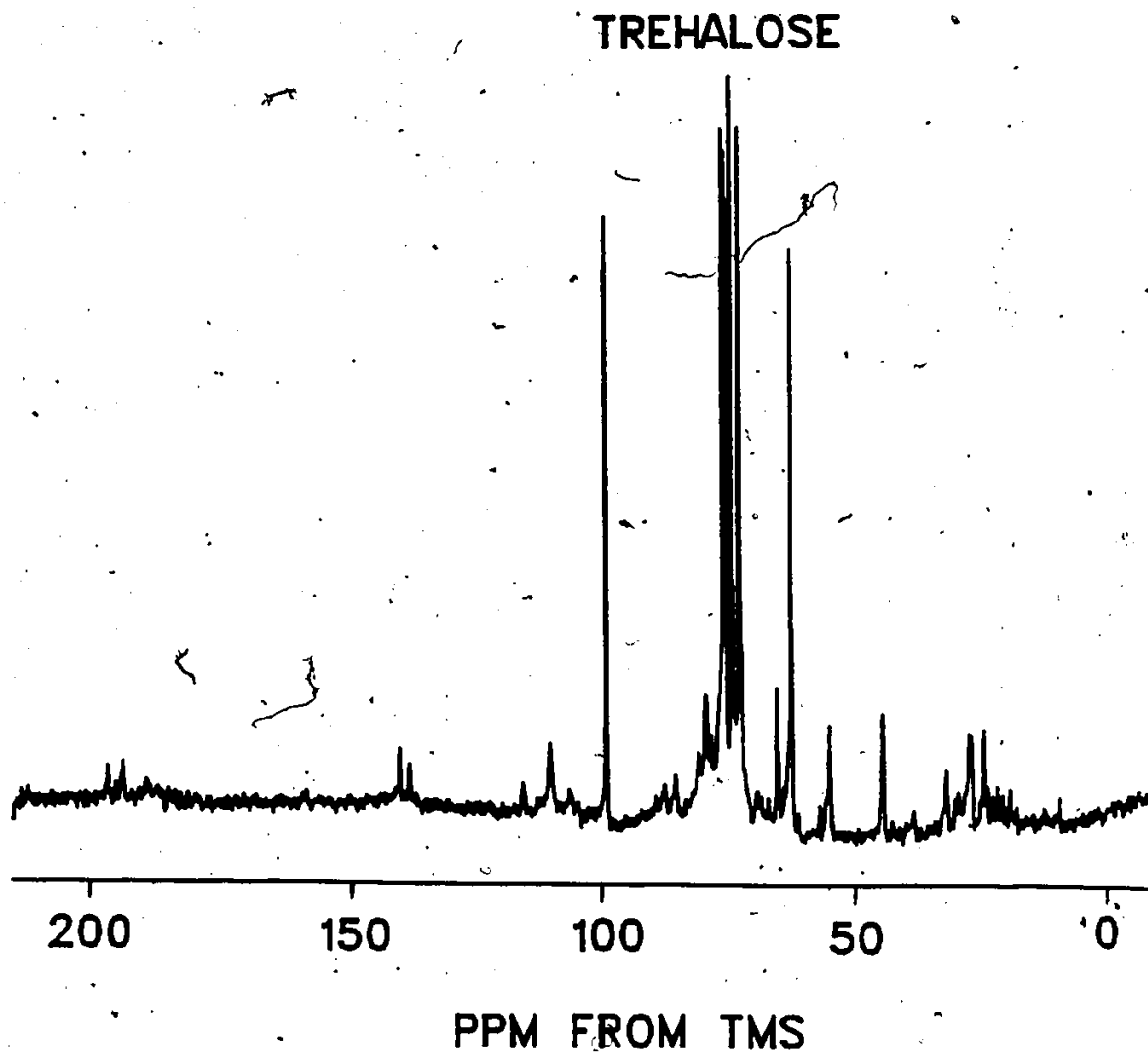


Figure VII.6 - 75 MHz ^{13}C spectrum of starved unlabelled cells, showing large pool of trehalose. The interplay between trehalose and free glucose as storage pools appears to be a sensitive function of the growth time. Spectrum is a result of 18,000 transients, 60° pulses, and 1 sec recycle time

starvation medium, the organism retains, and perhaps increases, the sugar pool in the form it preferred at the time of removal from the growth medium. In all other respects, the spectrum of Figure VII.6 resembles that of starved cells that were suspended in the starvation medium after three days. There is a small amount of mannitol, and the amino acid region shows that perhaps the most abundant amino acids present are glycine (45.8 ppm) and glutamate (55.1 ppm and 182 ppm).

(c) Metabolic Pathways from Labelled Acetate

The labelling pattern observed in the previous sections can be rationalized on the basis of known biochemical pathways. Figure VII.7 is a schematic diagram of the Krebs' acid cycle and the pathways from which the particular amino acids arise. Acetyl-CoA, derived from either exogenous acetate or from fatty acid oxidation, enters the cycle in a condensation with oxaloacetate to form citrate. On one complete turn of the cycle, oxaloacetate itself will become labelled and equally so at both ends of this four-carbon molecule, due to the symmetry of the succinate molecule. If the glyoxylate shunt is operative, one unit of acetyl CoA can also condense with glyoxylate to form malate. Thus the label can enter oxaloacetate directly, but in this case only on one end of the molecule. In the formation of glyoxylate from isocitrate, any label obtained from the oxaloacetate plus acetyl-CoA

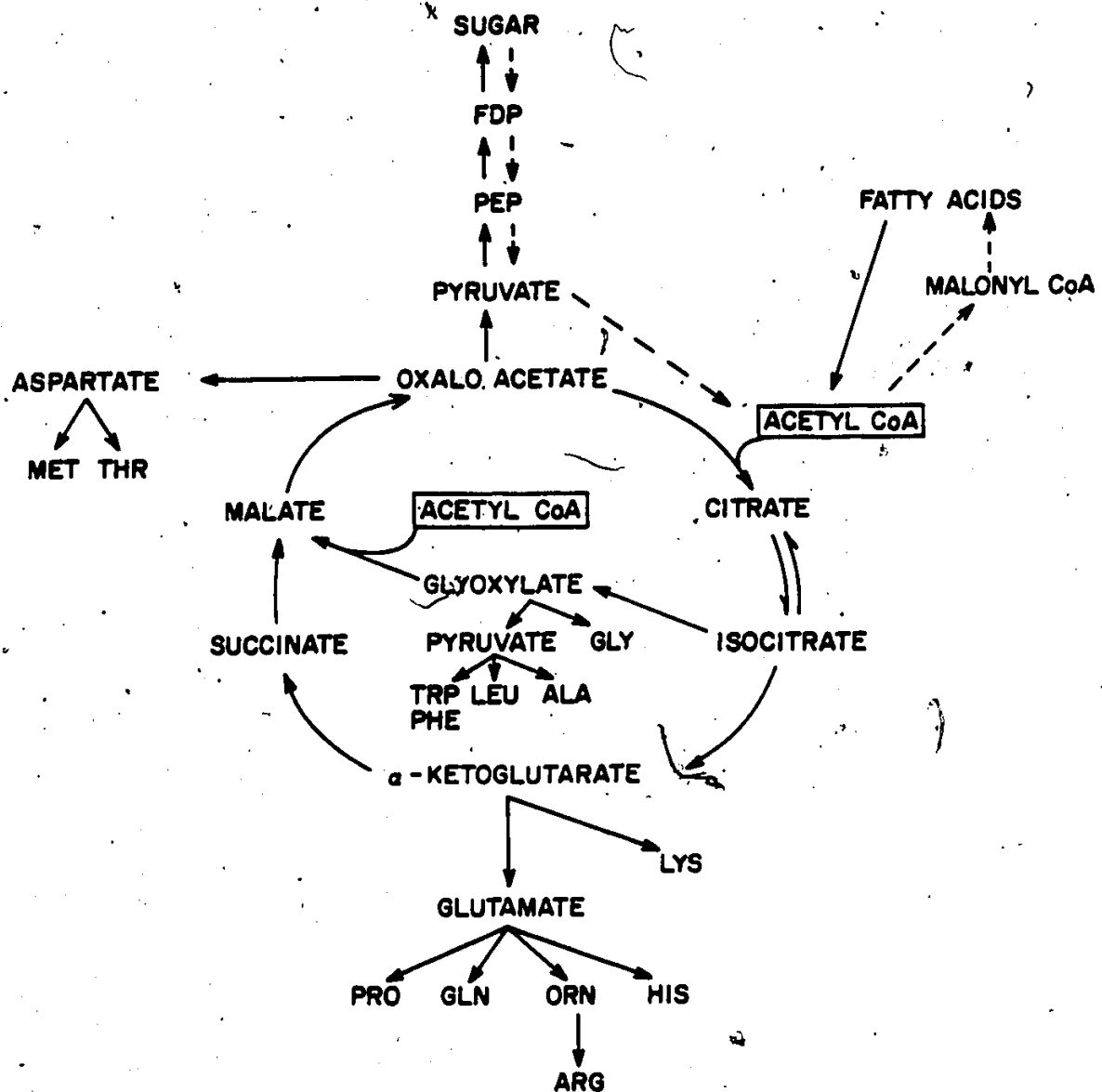


Figure VII.7 - Representation of the possible points of entry of labelled acetate, either from the medium or as a product of fatty acid oxidation, into the Krebs's acid cycle, and the resultant incorporation into those amino acids which are immediate products of the cycle

condensation is retained in succinate (i.e., isocitrate - oxaloacetate plus succinate), so that on the first turn of the cycle, glyoxylate is not labelled.

Although oxaloacetate gives rise to synthesis of sugars, this has been shown in the spectra not to occur under conditions of adequate glucose nutrition. This would be the pathway by which labelled sugars would be observed. The reverse pathway, however, by which sugar is catabolized via pyruvate to give acetyl-CoA, is operative, since we observe that fatty acids are labelled to the extent of only 5 labelled acetate units per chain (see Chapter IV).

The derivation of the amino acids is based on common metabolic pathways, but only those which are known to operate in yeast (Robichon-Szulmajster and Surdin-Kerjan, 1971). There are differences in the biosynthesis of certain amino acids between fungi, bacteria and mammalian tissue. Lysine, for example, arises from α -ketoglutarate in fungi and from aspartate in mammalian systems. Other common amino acids which are not shown, such as cysteine and serine, are derived from sugar and are therefore not observed. In addition, since cysteine and asparagine are supplied in the medium, one would not expect to see them labelled. The only other observed amino acid not included in the figure is isoleucine, which is derived directly from threonine.

The majority of the labelled amino acids appearing in spectra of fresh cells are derived from α -ketoglutarate. Figure VII.8 shows the detailed derivation of these species. A labelled acetate unit entering α -ketoglutarate from condensation to produce citrate, ends up as the γ and δ C's of glutamate. These are then carried through the synthesis of arginine to result in the same carbons of this molecule. Thus $^{13}\text{C-1}$ acetate labels the δ C and $^{13}\text{C-2}$ acetate labels the γ C. The same is true for proline, which results from a cyclization of glutamate: the $^{13}\text{C-1}$ label is carried through to the δ C and the $^{13}\text{C-2}$ label to its neighbour the γ C. Histidine is an exception, since it is the only aromatic amino acid which derives from glutamate. It is formed from a condensation of glutamate with PRPP (phosphoribosyl pyrophosphate), which comes from FDP. The only glutamate carbon which remains in the histidine molecule is the C-2 position in the ring (between the two ring nitrogens). All other carbons derive from the opened ring of PRPP. If the biosynthesis is followed carefully, it turns out that this C-2 was the original δ C of glutamate, which is labelled from $^{13}\text{C-1}$ acetate. Thus, this is the only histidine carbon seen in the labelled spectra, and it appears in the $^{13}\text{C-1}$ acetate-derived spectrum. Recall that all histidine carbons can be found in the unlabelled aqueous extract of the cells.

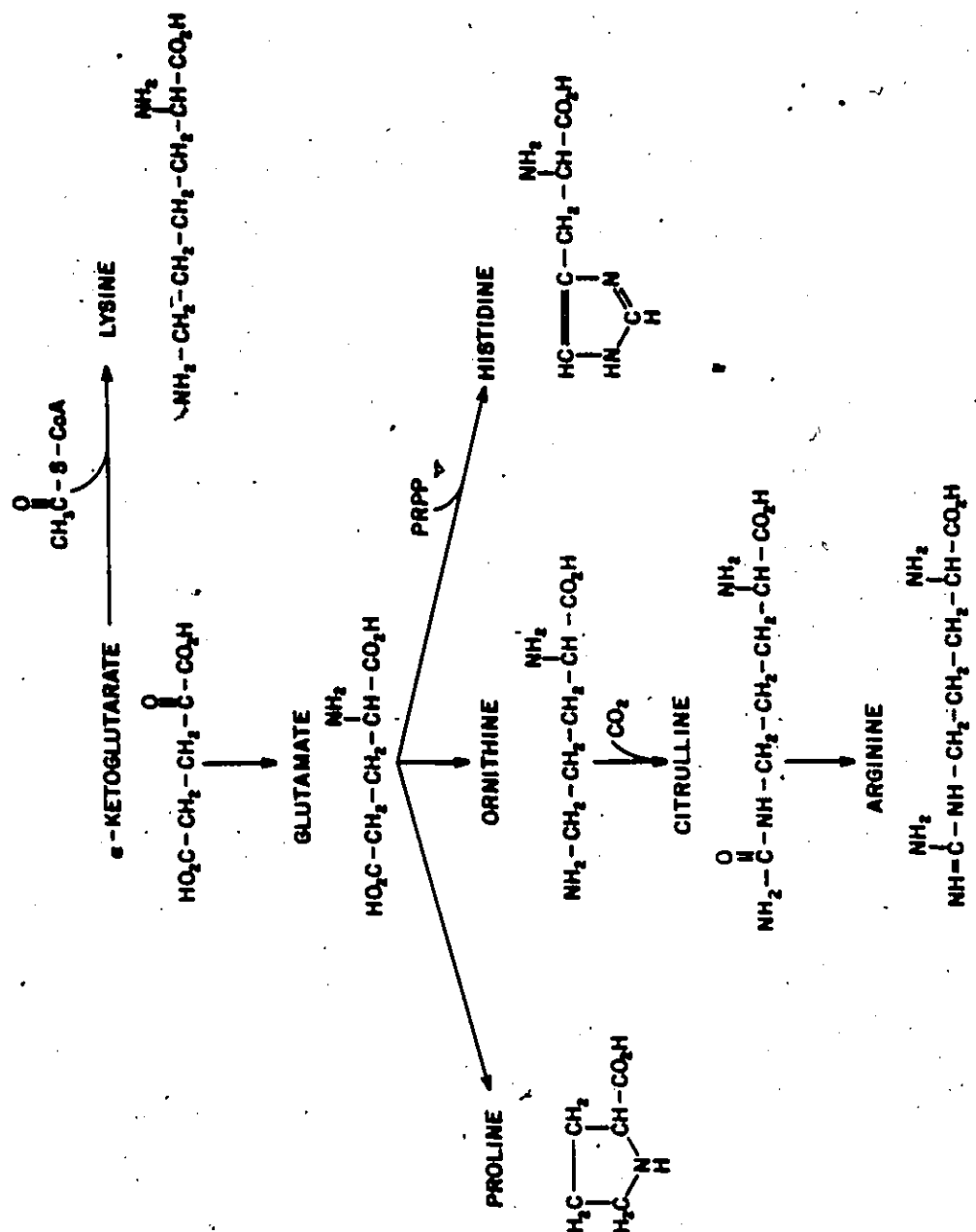


Figure VII.8 - Metabolic pathways for biosynthesis of spectrally observed amino acids from α -ketoglutarate

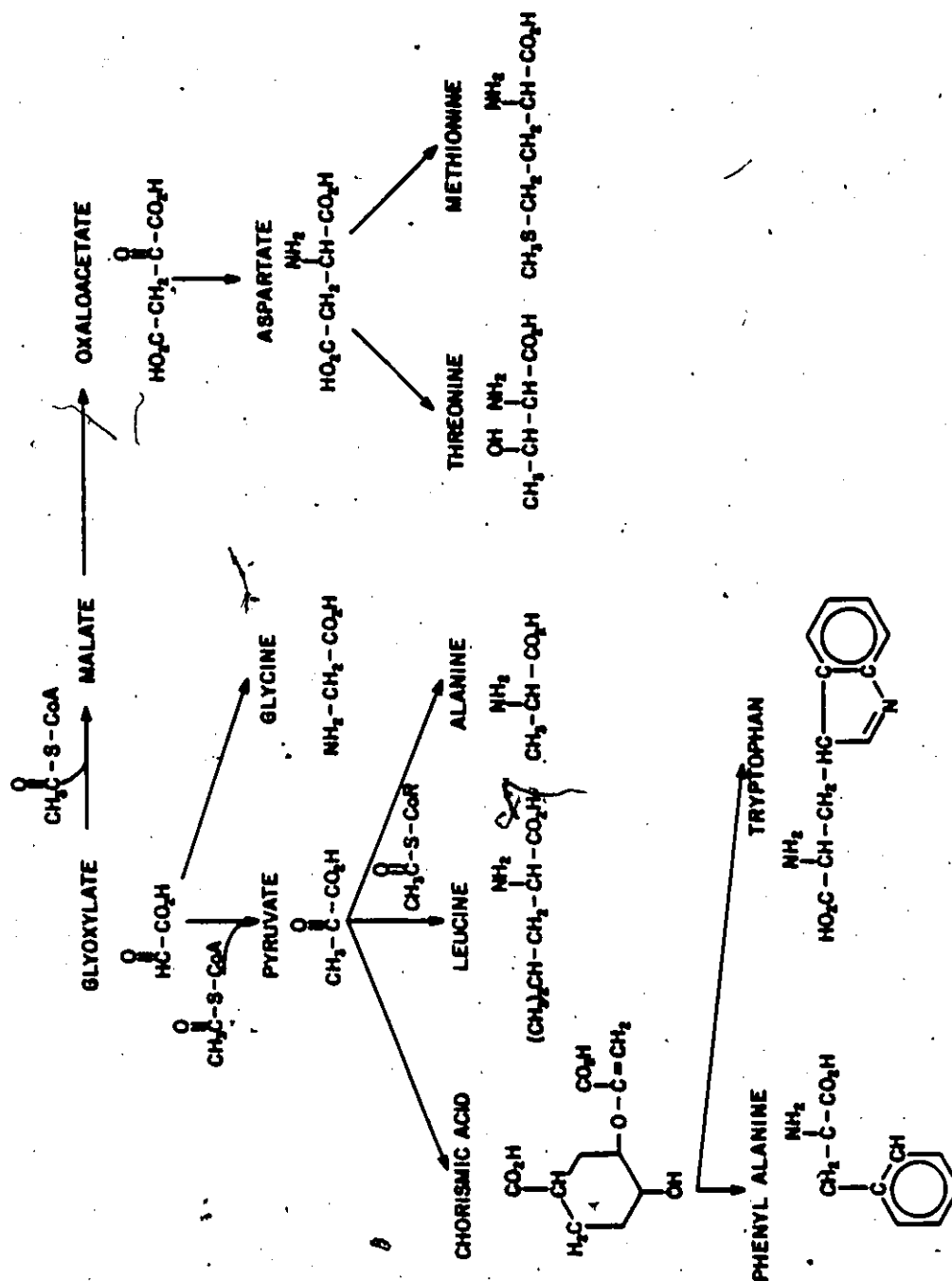


Figure VII.9 - Metabolic pathways for those amino acids which are derived from pyruvate and oxaloacetate

If acetyl-CoA enters by condensation with glyoxylate, this will appear in the α C and carboxyl carbon of glutamate, and the same carbons of arginine and proline. These carbons can also be labelled through the symmetry of succinate, without invoking the glyoxylate cycle, but this predicts an even labelling of both the α C and the β C. These carbons can be seen only in the ^{13}C -2 acetate-derived spectra, since the C-1 label from the acetyl unit which gives rise to the β C, is lost as CO_2 in the step, isocitrate $\rightarrow$ α -ketoglutarate. Since the spectra show only labelling of the α C, this indicates that much of the acetate resulting in the ^{13}C label comes from the glyoxylate shunt.

The biosynthesis of lysine shows that there is a third path by which acetate can enter the amino acid pool. The label resulting from citrate ends up in the δ and ϵ C's of lysine, accounting for the large ϵ C peak in the ^{13}C -1 acetate-derived spectra. The second pathway (via glyoxylate) results in labelling of the β C only (which is unfortunately buried in labelled lipid peaks in the spectrum). The six carbons of lysine result from keto-chain elongation of the glutarate molecule, a process in which the carbon backbone is lengthened by one carbon for every unit of acetyl-CoA condensed. In the process, one carbon originating from the carboxyl of acetate is lost. This third acetate unit ends up on the α and carboxyl carbon of lysine.

In the course of the synthesis of arginine, the amide carbon enters through a condensation of CO_2 with citrulline. This CO_2 could possibly be labelled from the carboxyl of acetate units discarded in reactions of the Krebs's cycle. However, this carbon was not observed in the ^{13}C -1 acetate-derived spectra. Urea was observed in spectra from both labels, and the urea carbon is derived from this αC of arginine in the urea cycle, providing further evidence that this carbon is not labelled. This observation is consistent with the compartmentalization of the label, since the urea cycle is cytosolic in origin (see Discussion).

Most of the remaining common amino acids derive either from pyruvate or from aspartate. The aromatic amino acids are a result of two units of pyruvate and one of erythrose-phosphate, which comes from the sugar pool. Pyruvate can result from keto-chain elongation of glyoxylate or from oxaloacetate, but both sources give rise to the same labelling pattern. Glycine arises simply from transamination of glyoxylate. It is notable that none of these amino acids appear in the fresh cell spectrum (or those of the extracts), whereas some, especially glycine, appear strongly labelled in the spectra of starved cells. This observation is consistent with the lack of labelling of the βC of glutamate-derived acids (see above), since the αC of glycine and βC of glutamate both come from the aldehyde carbon of glyoxylate.

The labelling pattern in aspartate and its derivatives confirms the above observation that much of the label enters via the glyoxylate cycle, rather than from a complete pass around the citric acid cycle. The latter would predict even labelling of the two halves of the aspartate molecule, with the resultant observation of the α C's of aspartate, threonine and isoleucine in the ^{13}C -2 acetate-derived spectrum. These peaks are not observed in the fresh growth extracts. However, the peaks due to the β and γ C's are easily seen. The γ C of aspartate and the γ C of threonine have characteristic shifts, which were observed in the ^{13}C -1 acetate cells. This labelling pattern can be passed on to isoleucine, resulting in the labelling of the γ C (which derived from the γ C of threonine) in the ^{13}C -1 acetate-derived spectrum and the γ C (which derives from the β C of threonine, in the ^{13}C -2 labelled spectrum. Unfortunately, this last peak overlaps with the γ C of arginine, which is also labelled. The remaining peaks of isoleucine derive from pyruvate, and therefore are not observed. The glyoxylate pathway is further supported by evidence from the spectra of pullulan (see next section) that aspartate is strongly labelled in the β and γ positions, but no peak due to the α position was observed.

The predicted appearance of peaks in spectra of each label is summarized in Table VII.7, for all methods of incorporation discussed above. The peaks that appear to be strongly

Table VII.7

PREDICTED APPEARANCE OF LABELLED CARBONS
IN AMINO ACID POOL FROM ACETATE
INCORPORATION VIA VARIOUS METABOLIC PATHWAYS

<u>Mechanism</u>	<u>Spectrum</u>	<u>Labelled Carbons</u>
A) <u>Acetyl CoA</u> <u>Plus Oxaloacetate</u>	¹³ C-1 Acetate Grown	δC GLU, ARG, PRO εC LYS C-2 HIS
	¹³ C-2 Acetate Grown	γC GLU, ARG, PRO δC LYS
B) <u>Acetyl CoA</u> <u>Plus Glyoxylate</u>	¹³ C-1 Acetate Grown	γC ASP, THR, MET CO GLU, ARG, PRO
	¹³ C-2 Acetate Grown	βC ASP, THR, MET βC LYS αC GLU, ARG, PRO
C) <u>Glyoxylate Labelled</u>		
i) Directly from Glyoxylate (via Pyruvate)	¹³ C-1 Acetate Grown	CO GLY, ALA, PHE γC LEU C-3 TRP C-3,4 GLC
	¹³ C-2 Acetate Grown	αC GLY, ALA, PHE βC LEU C-1 PHE C-9 TRP C-2,5 GLC
ii) From Incorporation into α-ketoglutarate	¹³ C-1 Acetate Grown	CO ASP, THR, MET
	¹³ C-2 Acetate Grown	αC ASP, THR, MET γC LYS βC GLU, ARG, PRO
D) <u>Keto Chain Elongation</u>		
i) Directly	¹³ C-1 Acetate Grown	CO LYS, LEU αC LYS, LEU
ii) Via Pyruvate	¹³ C-2 Acetate Grown	βC ALA δC LEU C-8 TRP βC, C-2,6 PHE C-1,6 GLC
E) <u>From ¹³CO₂</u>	¹³ C-1 Acetate Grown	ζC ARG, CIT

labelled in the spectra of the fresh cells are those resulting from mechanism A and B, which imply direct incorporation into the citric acid cycle. All the peaks listed under these two mechanisms were observed or are hidden under strongly labelled lipid peaks. Following the period of starvation, many more peaks were observed due both to enhanced labelling and higher amino acid levels. In addition to mechanisms A and B, these peaks are consistent with labelling of the glyoxylate molecule, either through one complete cycle around the entire cycle, or one complete cycle around the shunt. Thus the α C's of glycine, alanine, aspartate, threonine, along with the β C's of glutamate, arginine and proline, appear in the ^{13}C -2 acetate-derived spectrum. The effect is reflected in greater labelling of the carboxyl region, and the appearance of the γ C of leucine in the ^{13}C -1 spectrum. Peaks that result from direct keto-chain elongation (i.e., α C of LYS and LEU) seem to appear in both fresh and starved spectra. There is also some indication that some pyruvate is produced by keto-chain elongation of glyoxylate in the appearance of the β C of alanine and δ C's of leucine. Thus, under conditions of high nutrition and active protein synthesis, the labelled acetate is incorporated once in the Krebs' cycle and released into the amino acid pool, while during starvation, there is more incorporation into the amino acid pool and the intermediates of the cycle become more fully labelled before they are released into the cytoplasmic pools.

(d) Amino Acid Analysis

The assignments of the peaks of the starved cell spectra were confirmed by an amino acid analysis of the cytoplasmic components. Table VII.8 shows the results of such an analysis. Before the total extract was run, protein was precipitated so that only the amino acids and small peptides which would appear in the spectra would be detected. The results show significant quantities of arginine, urea, lysine, histidine, glutamate, serine and threonine. There is an anomalously large amount of methionine, but this may be due to the overlap of small peptides with the methionine peak, since the peak reduces in size upon hydrolysis. Since some amino acid elution times overlap; i.e., glutamine and asparagine overlap with serine and threonine, the mixture was hydrolysed for a short period of time in order to convert all amino acids to the form that they would appear on protein hydrolysis. Many of the amino acids appear to increase in amount after this procedure, but this is likely due to the fact that different carrier solvents were used in the two analyses. The initial analysis was run in TCA after protein precipitation, whereas the hydrolysis products were run in the appropriate buffer at pH=2. The urea disappears on initial hydrolysis, since it is easily converted to ammonia. This second analysis shows that most of the glutamate is

Table VII.8

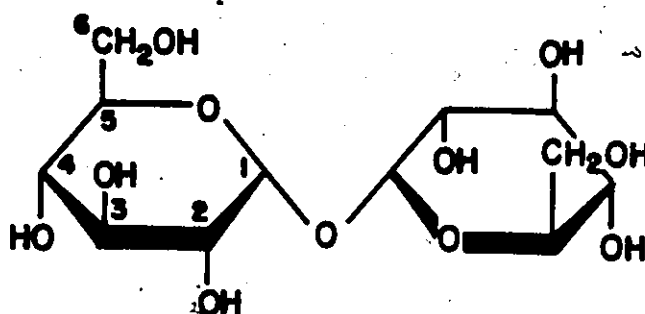
AMINO ACID ANALYSIS OF STARVED CELLS

<u>Amino Acid</u>	<u>Quantity (nmoles per μl extract)</u>		
	<u>Extract Before Hydrolysis</u>	<u>After 4.5 Hours Hydrolysis</u>	<u>After 20 Hours Hydrolysis</u>
ARG	1.4	1.7	1.8
Urea	6.0	1.9	3.1
ORN	-	1.1	1.9
LYS	2.1	2.5	3.7
HIS	0.7	1.1	1.5
PHE	0.1	0.6	0.8
TYS	0.1	0.2	-
LEU	0.2	1.2	1.7
ILE	0.1	0.9	1.4
MET	1.4	1.0	0.7
ALA	0.5	2.6	4.0
GLY	0.4	3.6	5.7
GLU	1.9	4.0	6.2
SER	1.1	2.5	2.1
THR	0.7	2.3	2.7
ASP	0.6	2.7	4.0
CYS	0.5	-	-

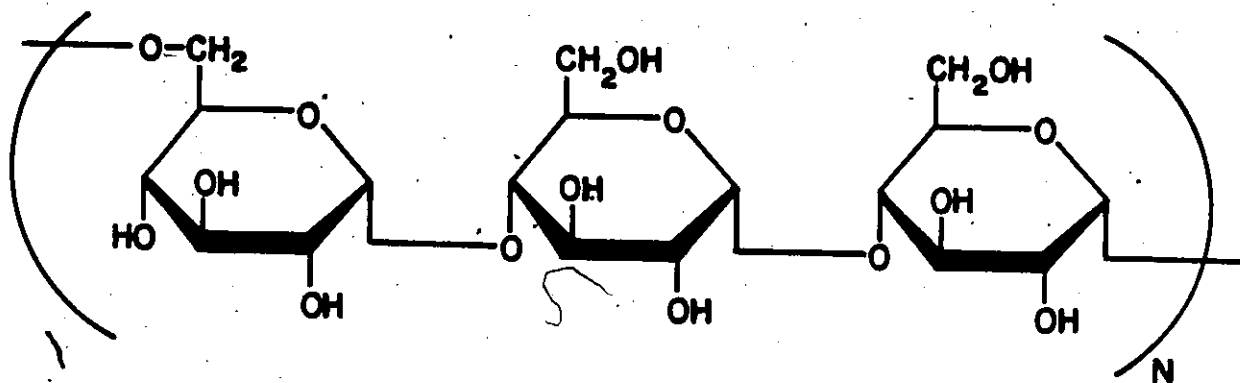
present as glutamate and not glutamine, as was indicated by the carboxyl region of the labelled spectrum. A further hydrolysis for 20 hours reduces the small peptides to their composite residues. A comparison of the third column to the second shows that significant amounts of alanine, glycine, aspartate and glutamate exist in small peptides. In the case of glycine, the majority exists in peptide form (as was indicated by NMR). Since glycine is easily hydrolyzed, it may be partially degraded on the first hydrolysis. Arginine, lysine, histidine, leucine and threonine, on the other hand, exist as free amino acids. Note that there are approximately equal amounts of serine and threonine, yet only threonine is spectrally observed, as serine derives from the sugar pool.

(e) Pullulan

The exopolysaccharide, pullulan, which is produced by this organism, is of great commercial interest. Many microorganisms produce exocellular products, which provide a alternative source of food, fuel, and polymers to those furnished from the oil industry. Dextran, for example, which is secreted by dextran bacteria, is another α -glucan, which is industrially well-established. The form of pullulan is depicted in Figure VII.10. The repeating unit is maltotriose (α 1,4 glucose), joined by α 1,6 linkages. This is the predominant



α,α TREHALOSE



PULLULAN

Figure VII.10 - Structure of the two glucose oligomers commonly found in this study - pullulan and trehalose. Both contain α linkages of glucose units. Pullulan has a repeating unit of maltotriose, joined by 1,6 linkages. The symmetry of the trehalose molecule produces its simple ^{13}C spectrum.

structure of pullulan, as there is no unique structure. As isolated, pullulan normally contains small amounts (~10%) of other glycosyl linkages (Jeanes, 1977). The molecular weight, linkage purity, and production are maximized on growth on a glucose medium. The properties of the polymer relate to its structure; the $\alpha 1,6$ bond introduces flexibility, and the interruption of regularity makes pullulan relatively water soluble. The spacing of the linkages makes this polymer relatively inert to degradation and increases its ability to form tough resilient films. The pullulan films have a high tensile strength and are impervious to oxygen (Yuen, 1974), thus making it a desirable coating for preserving food and pharmaceuticals. Pullulan can also be added directly to food, to reduce caloric value, aid in achieving proper consistency, and act as a preservative. Pullulan inhibits fungal growth, which may point to the reason for its existence. A. pullulans cannot metabolize pullulan (Catley, 1970), but, like other fungi, may produce it in order to inhibit competition with other species (O'Neill and Drisko, 1980; Zabel and Terrazino, 1980).

The biosynthetic mechanism by which pullulan is produced is unknown. Maximum production is associated with the yeast phase and in log phase cultures (Catley, 1973). As the culture ages, pullulan production slows down. It is perhaps significant that, at this stage, trehalose is preferred over

glucose as a storage form of sugar. ^{14}C studies have shown that glucose is the preferred precursor for pullulan and that acetate is not conducive to its production. Other conditions under which pullulan production has been studied are reviewed by Catley, 1979.

Industrially, pullulan is isolated by solvent precipitation similar to that described in Chapter III. The purified powder is a tasteless, fine, white powder. Figure VII.11 shows the spectra of pullulan, dissolved in D_2O , which was isolated from media of three 500 ml cultures that were both specifically labelled and unlabelled. These spectra represent equal pullulan concentrations and identical spectral conditions. The central portion of each spectrum is assigned to the 1,4 and 1,6 linkages of glucose carbons by analogy to maltotriose (Jennings and Smith, 1973). There are, in addition to the glycosyl peaks, peaks at 43.8 ppm and 136.5 ppm in the unlabelled spectrum, which appear to be labelled in the ^{13}C -2 acetate-derived spectrum. There are peaks due to ^{13}C couplings at the base of the 43.8 ppm peak that are of approximately the same size as the unlabelled peak, indicating coupling with the natural abundance ^{13}C neighbouring carbon. The ^{13}C -1 acetate-derived spectrum shows labels appearing in the carboxyl region. The pullulan itself appears to be unlabelled, as expected, since it is derived from the unlabelled glucose storage pool. These labelled peaks are consistent with amino.

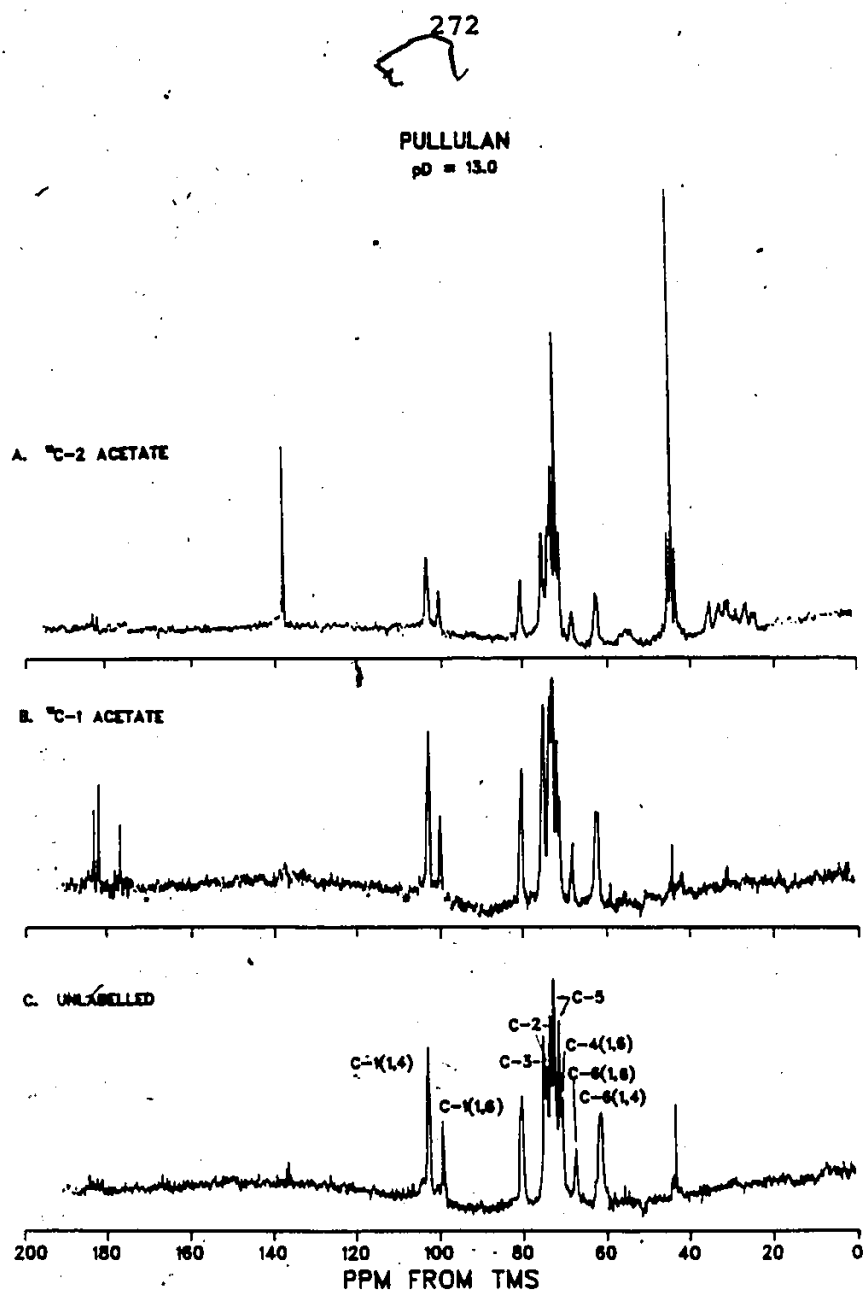


Figure VII.11 - 20 MHz ^{13}C spectra of pullulan, isolated from cultures that were both unlabelled and specifically labelled. Each spectrum represents a concentration of 50 mg of isolated material per ml at pD=13. All spectra were accumulated under the same conditions of 115,000 transients, 45° pulses and 0.5 sec recycle time.

acid residues, and are therefore assigned to terminal residues of protein, for the following reasons. All water soluble components should remain in the medium, since the extraction solvent is acetone and the extract has been well washed with ethanol. If this is indeed a protein (or proteins), it must be strongly associated with pullulan, since acetone does not normally precipitate proteins. This observation is not an anomaly of the isolation procedure, as this observation has been made previously (A. Martin, unpublished results). No extra labelling was observed for pullulan isolated from growths on labelled glucose. Note that no other peaks due to amino acids appear (for example, there is no characteristic α -carbon at 55 ppm), even in the unlabelled spectrum. Since these peaks have been assigned to terminal residues, it may be that the conformation of the protein is such that these particular carbons are the only ones that can be resolved on the NMR time scale. There is a hint of a fairly broad peak at 55 ppm in the unlabelled spectrum. In addition, these peaks appear to be efficiently labelled.

Further evidence that these peaks are amino acid residues is provided from the pH behaviour of the chemical shifts. While the resolution of the pullulan peaks is poor at pD=7, due to the incomplete dissolution of the sample, the molecular species responsible for the labelled resonances appear to be unaggregated and their chemical shifts were easily observed.

The pH behaviour is summarized in Table VII.9. The chemical shifts were observed at pH=7 and pH=13, corresponding to a titration of free amino groups of peptide residues. The 136.5 ppm peak is not pH-dependent, as would be expected for a benzylic carbon. At pH=7, there are two peaks in the 37-41 ppm region, the larger at 37.1 ppm, which merge on titration to pH=13. The titration shifts of these carbons are consistent with that expected for free ($\Delta\text{pH}=6.7$) and bound ($\Delta\text{pH}=3.0$) β -carbons of amino acid residues (Wüthrich, 1976). The chemical shifts are consistent with the β -carbons of free and residue aspartate or asparagine. Since asparagine is provided in the medium, it is not expected to be highly labelled. The pH behaviour is consistent with aspartate, both free (Rattle, 1976), and as a residue (through measurement of pH behaviour of the peptide Asp-Phe (Deslauriers et al., 1974)). Since free amino acid would not be isolated with pullulan, it is possible that this resonance is due to γ C-linked terminal aspartate. Although there is a common analogue for this type of linkage for glutamate in glutathione (Jung et al., 1970), there is no precedent for a γ C-linked aspartate in naturally occurring peptides. Regardless of the linkage type, however, these observed peaks are consistent with the β -carbons of aspartate or a derivative.

The aromatic peak at 136.5 is not pH dependent as would be expected for a benzyl carbon. If this peak is assumed to

Table VII.9

pH BEHAVIOUR OF LABELLED PEAKS OBSERVED
IN PULLULAN SPECTRUM (FIGURE VII.11)

<u>Spectrum</u>	<u>Chemical Shifts (TMS)</u>		<u>ΔShift (ppm)</u>
	<u>pD=7</u>	<u>pD=13</u>	
^{13}C -1 Acetate	172.8	175.8	3.0
Derived		181.0	4.3 (8.2)
	176.7	182.1	5.4
^{13}C -2 Acetate	37.1	43.8	6.7
Derived	40.6	43.8	3.0
	136.5	136.5	0

be due to an amino acid, in order to be consistent with the labelling patterns observed in the whole cell and in the other peaks of the pullulan spectrum, then it can only be assigned to the C-8 ring carbon of tryptophan. The C-8 is the ring fusion carbon next to the pyrrole nitrogen in the ring system. The chemical shift is also consistent with the C-2 of histidine, but the latter was observed only in the ^{13}C -1 acetate-derived spectrum for the whole cells, and here the label origin is ^{13}C -2 acetate. In addition, this carbon of histidine has a weak pH dependence (Freedman *et al.*, 1973). The linkage of tryptophan within a protein, requires that the ring system extend from the protein backbone, thereby having somewhat greater freedom of movement, and perhaps accounting for the appearance of this carbon as a well-resolved peak. Other possible aromatic carbons from phenylalanine and tyrosine can be ruled out as chemical shifts are not consistent with the expected labelling pattern (see Table VII.2 and 7). The C-8 of tryptophan is derived from the methyl group of pyruvate, the remaining pyruvate carbons occupying the other ring fusion position and the γC position on the pyrrole ring. All other ring carbons are derived from chorismic acid and PRPP (see Figure VII.9). The methyl group of pyruvate can become labelled from the addition of acetyl-CoA to glyoxylate, either directly or via oxaloacetate. Both the βC of aspartate and the methyl of pyruvate can be derived from the same carbon

of oxaloacetate. While the singular appearance of this peak is consistent with the lack of labelling of the glyoxylate moiety as observed in the whole cells, there was no observation of peaks due to amino acids derived from the methyl groups of pyruvate in the cell spectra (i.e., the δC of leucine and the βC of alanine). This inconsistency could possibly be explained on the basis of relatively different amounts produced, or differences in cellular location of synthesis. It is possible that large amounts of highly labelled oxaloacetate are shunted into pyruvate and aspartate that are used exclusively for protein synthesis, since there is no requirement for sugar synthesis from pyruvate.

Turning to the carboxyl region, it is therefore expected that the peaks can be assigned to the carbons deriving from the other half of the acetate unit that is incorporated into oxaloacetate. This carbon is lost in the synthesis of pyruvate, but is retained in the γC of aspartate. Only two peaks were observed at $pD=7$, at 172.8 and 176.7 ppm, which split into three at $pD=13$. There is an uncertainty as to the origin of each peak in the titration behaviour, but they are correlated in order to make the chemical shift changes reasonable in terms of the expected behaviour (Wüthrich, 1976). The titration shift that gives rise to the 181.0 peak is uncertain. If it derives from the peak at 172.8, the observed $\Delta\delta$ of 8.3 is that expected for the amino carboxyl of a free amino terminal.

Since it is not expected that the amino carboxyl is labelled, the alternative origin (the 176.7 ppm) is favoured. The peak at 176.7 ppm is consistent with the free γ -carboxyl of aspartate. The titration effect is consistent with that expected for free aspartate (Rattle, 1981), and amino terminal aspartate (Deslauriers *et al.*, 1974). Why the peak splits into two upon titration is uncertain. There could be differences in conformation leading to slight differences in the effect of protonation of the amino group or it is possible that this represents two carboxyl groups in the same position, as for β -carboxy aspartic acid, which has been recently found in *E. coli* (Christy *et al.*, 1981). The steric effect of an additional carboxyl in the γ position would be expected to cause slightly different pH shifts. Since this is a recently discovered amino acid, a pure sample was not available to check this hypothesis by NMR.

The peak at 172.8 ppm which experiences a relatively smaller pH effect, is consistent with the γ C of a γ -linked terminal aspartate, by analogy with glutamate in glutathione. The shift in resonance of the δ C of glutamate upon incorporation into glutathione is approximately -6 ppm (Jung *et al.*, 1970), and the effect of titration of the terminal amino group of glutamate is +2 ppm (Feeney *et al.*, 1974). The predicted chemical shift of γ C of aspartate would therefore be 172.7 ppm (178.7 - 6), and the amino group titration would

be expected to produce a larger effect than 2 ppm, yet smaller than that for the free γ C (4 ppm).

This assignment of the labelled peaks associated with pullulan is largely speculative and is based on the use of established patterns in assigning peptide spectra. While the isolation of the protein (or proteins) in question and the linkage characterization is a time-consuming and technically difficult procedure, the presence of the amino acid residues can be shown through an amino acid analysis of the hydrolysate of a pullulan sample. The results of such an analysis are presented in Table VII.10. The amounts of each residue are expressed in nmoles per mg of isolated material, where 1 mg represents ~5500 nmoles of glycosyl residues. The amounts of protein are relatively small. The residue present in the highest quantity is aspartate, although there are considerable amounts of glutamate, glycine, alanine and serine present. The quantities of those amino acids which were labelled in the amino acid pool of cell, arginine and lysine are comparatively smaller and may be in more rigid positions within the substructure, thus accounting for their absence in the pullulan spectrum. In addition, the aspartate may be labelled to a greater degree, depending on the relative usage of acetate during growth. There is a very high quantity of cysteine present, indicating that there are a relative abundance of disulphide bonds in the original material. For

Table VII.10

QUANTIFICATION OF ACID HYDROLYSIS
OF ISOLATED PULLULAN

<u>Amino Acid Residue</u>	<u>Amount</u> (nmoles per mg dry pullulan)
ASP	32.1
GLU	20.4
GLY	18.0
ALA	23.2
LYS	11.3
S-S linkages	41.4
THR	10.4
SER	18.3
PRO	4.6
VAL	7.7
MET	2.2
ILE	5.9
TYR	2.5
PHE	3.4
HIS	3.4
ARG	7.0

this reason, the amino acids are attributed to protein, rather than due to amino sugars within the pullulan polymer. Cysteine is readily available in the medium and is therefore unlabelled. Since tryptophan is degraded by acid hydrolysis, a separate determination of tryptophan was made. However, ornithine interferes with the tryptophan analysis and ornithine is present in small quantities. Tryptophan appears to be present but quantities are both small and not easily determined. The presence of β -carboxy aspartate cannot be confirmed by this analysis, since it is easily decarboxylated in acid medium (Christy et al., 1981), which accounts for its lack of detection until recently. The presence of asparagine also cannot be confirmed, since it too is degraded to aspartate upon hydrolysis. What the amino acid analysis does show is the predominance of aspartate derivatives within the pullulan structure that can be correlated with the spectral results.

The appearance of these labelled peaks associated with pullulan accounts for the metabolic fate of the rest of the acetate supplied into the medium. The results from GCMS indicated that not all the supplied acetate was incorporated into fatty acids, yet the amount of labelled acetate remaining in the medium after harvesting the cell mass was very small (as measured by NMR). The efficient incorporation of acetate into protein, predominantly aspartate residues, as is indicated by these results, accounts for the remaining labelled pool

that is not visible in the NMR spectrum. Thus there may be a significant amount of labelled material within the protein of the whole cell. The labelled aspartate is efficiently shunted into protein, and not pooled within the cell, but the labelling pattern appears in the pool of free threonine which does appear in the spectra of the cell. In agreement with the incorporation of only one labelled acetate unit into aspartate, the threonine label was observed only in the β and γ positions.

The presence of peptide or protein residues within isolated pullulan has not been previously reported. Characterization of the polymer has been limited to the nature of its sugar linkages. This is not unexpected since the amount of protein appears to be very small. However, this type of study shows how NMR analysis of specifically labelled material can be useful. Since the peptidyl material is isolated with the pullulan, it is likely bound by amino sugar linkages to the polymer backbone. The presence of a large amount of cysteine indicates that there may be a great deal of crosslinking by disulphide bonds, accounting for the relatively poor solubility at high concentration in water. The presence of strategically placed crosslinkages within an evenly repeating glycosyl sequence may contribute to the highly elastic and stable properties of pullulan.

The highly specific nature of the labelling indicates that it would be possible to deuterate the aspartate residue in the same manner from perdeuterated acetate, in order to study the motional properties of this small amount of protein within this polysaccharide by deuterium NMR. Further chemical characterization of this protein would be required in order to determine the role it plays in the metabolic determination of the organism. For the purposes of this study, however, the presence of the label in the residues of this protein show that there is active production of specific amino acids associated with growth under conditions of high nutrition, and that acetate is a preferred substrate. The complementarity of acetate and glucose in fulfilling the needs of the culture may account for higher cell yields on this combined substrate than on glucose alone. The labelling pattern observed is consistent with the hypothesis that the glyoxylate cycle is actively operating during the log phase of growth.

Discussion

It is apparent from the NMR spectra that pools of acetate and sugar in the growth medium have well-defined metabolic functions. The organism stores glucose for use in the synthesis of carbohydrate during periods of high glycolytic activity, and retains those reserves even under periods of

glucose shortage. It has the enzymatic ability to interconvert the various storage forms and to channel carbohydrate synthesis in response to the external environment. The relationships between the glucans produced by the organisms is well defined. The cell wall is almost exclusively β -glucan, while the intracellular and exocellular mobilized carbohydrate is exclusively α -glucan. The production of the two α -glucans, trehalose and pullulan, is complementary. There is high pullulan production during active growth in the yeast phase, while trehalose is associated with stationary phase culture or mycelia (Merdinger and Kohn, 1967). The production of glucan appears to be closely related to the carbon source, since pullulan is produced when glucose is easily available, and the pullulan may be used by the organism to eliminate competitive species, thus providing room for the rapidly increasing cell mass. Under conditions of desiccation or alternate carbon sources, pullulan production is minimized and trehalose is retained. We have seen that under conditions of high glucose turnover, glucose is maintained as a free pool within the cell, the form changing to trehalose when the medium becomes exhausted. Spectra also show that very little glycogen is produced, although glycogen synthetase activity has been observed in the species (Kikuchi et al., 1974).

Mannitol is also produced by the organism. Mannitol production in fungi is also associated with a glucose source, and there have been indications that trehalose and mannitol production are somehow linked (Lewis and Smith, 1967). Mannitol is a common polyol in mycelial fungi, while glycerol is more common in yeast. This may reflect the ease of morphological interconversion that is a characteristic of A. pullulans. In addition to playing a role as a soluble carbohydrate source, mannitol may also act as an osmotic regulator in the cytoplasm. The presence of a mannitol pool may account for the ability of this yeast to resist lysis under conditions that would be fatal to other yeasts. The reversible reduction of glucose to mannitol is mediated by NADPH, and thus may act as a regulator of coenzyme function (Lewis and Smith, 1967; Boonsaeng et al., 1976).

Under conditions of carbon deprivation, the sugar pool is maintained in the form reflecting the metabolic activity at the point of harvesting. Mannitol is also maintained. However, pullulan is no longer produced in order to conserve reserves. The biomass increases and budding continues at the expense of the storage triglycerides. This pool is reduced via β -oxidation to provide a source for continuing mitochondrial function. The starved cell spectra show that the fatty acids are largely incorporated into the amino acid pool, which increases in response to either reduced protein synthesis

or in an effort to maintain the correct osmotic pressure of the cytoplasm. The reduction of density of the cytoplasm upon starvation that was visible in Plate VII, reflects the decrease of metabolic activity and the loss of the triglyceride droplets. Amino acids also play a role in regulating osmotic pressure (Norton, 1979; Drainville and Gagnon, 1973), and the increase in amino acid content increases the cytoplasmic osmotic pressure. The increase in the volume of the major vacuole also aids this tendency, which hints that the amino acid pool may be largely stored in that vacuole. The use of triglyceride instead of sugar for metabolic function reflects the conserving nature that is a property of yeast in general. Whereas sugar requires energy to mobilize, the process by which a fatty acid chain is reduced by two carbon units not only provides the fuel for the citric acid cycle, but also releases an electron pair which travels through the respiratory chain resulting in the synthesis of two molecules of ATP (Sols et al., 1971). The following chapter shows that another storage pool is mobilized during starvation which also may be linked to a conserving of metabolic energy.

The labelling of the amino acid pool as well as the unlabelled spectra show that the predominant free amino acids present are arginine and lysine. Proline and histidine are present but to a lesser extent. It is well established in Neurospora, Candida and Saccharomyces that amino acids are

compartmentalized at the subcellular level (Nurse and Wiemken, 1974). The predominant pool which is slowly turning over is retained in vacuoles, while a small pool is retained in the cytoplasm, mobilized for protein synthesis. Arginine especially is sequestered in vacuoles in S. cerevisiae (Wiemken and Durr, 1974) and in osmotically sensitive vesicles in N. crassa (Weiss, 1973). Glutamate and aspartate are preferentially found in the cytoplasm. This compartmentalization prevents the bulk of the arginine pool from being catabolized or used in protein synthesis, thus there is only slight exchange between the vesicular and cytosolic pools (Subramanian et al., 1973). The vesicular pool is likely what is observed by NMR. Free arginine, ornithine, lysine and histidine pools have been detected in N. crassa, and there is evidence for functioning urea cycles in both Candida (Nurse and Wiemken, 1974) and N. crassa (Karlin et al., 1976). The function of the urea cycle may be to provide a cytoplasmic nitrogen source, especially since A. pullulans has been shown to have the ability to hydrolyse urea (Seeliger, 1956). The presence of lysine is due to its regulatory role in the biosynthesis of arginine, methionine and threonine, (de Robichon and Surdin-Kerjam, 1971), all of which can be found in cell extracts. Proline also functions as a regulator of ornithine biosynthesis, so that the presence of all three amino acids indicates that a sensitive feedback mechanism is in control

of the cellular concentrations. Histidine is often found as a free amino acid since it is a very effective regulator of enzyme activity (Metzler, 1980, p. 871). The NMR and amino acid analysis results show that the glutamate pool is greater than that of glutamine. Glutamate is not the dominant free pool. Much of the latter appears to exist in the form of small peptides, in contrast with bacteria, in which the osmoregulatory pool is composed of glutamate and proline (Measures, 1975), marine molluscs, in which the major amino acid species are glycine, taurine and betaine (Norton, 1979), and S. cerevisiae, which contains glutamate and arginine (Wiemken and Durr, 1974).

The results from labelled acetate suggest there is a significant compartmentalization of resources in the organism. The label from the acetate enters almost exclusively into mitochondrial reactions. Those processes which are cytosolic, such as glycolysis, are not observed to take part in the labelling. There appears to be little or slow exchange between the cytosolic and mitochondrial pools of certain species, especially pyruvate. The amino acids that are labelled receive their label within the processes of the Krebs' acid cycle, and those modifications that occur in the cytoplasm do not contribute further label. For example, aspartate easily crosses the mitochondrial membrane, but the labels seen in the aspartate derivatives, threonine and methionine, are only

those which derive from labelled aspartate. Citrulline also crosses the mitochondrial membrane, and the addition of CO_2 to form arginine is a cytosolic process. Consequently no label was observed on the ζC of arginine or on its derivative, urea. Another indication of strict compartmentalization is that in spite of the fact that the labelling pattern is observed three days (or six days, in the case of starved cells), after the label has been introduced there is virtually no scrambling of the label. The labels occur in the carbon positions predicted with a priori knowledge of their biosynthetic pathways. Scrambling of the labels in glutamate, due to the interaction of the citric acid cycle and gluconeogenesis, occur within hours of incubation with labelled precursors in yeast and rat liver cells (den Hollander et al., 1981). In addition to amino acids, the other pool which receives the acetate label is that of the fatty acids. In many organisms, the ACP process takes place in the cytoplasm. However, there is evidence that the conversion of acetate to acetyl CoA, as well as the β -oxidation process, take place in the peroxisomes or microbodies of the cell, for many organisms including yeast and Tetrahymena (Tolbert, 1981; Shrago and Elson, 1980). The glyoxylate pathway also takes place in the peroxisomes. Thus, there may be two or more pools of acetyl CoA, which may or may not be in equilibrium, but which are held within organelles. The reason for this compartmentalization of the labelled acetate may be purely energy

efficiency - whereas acetate can diffuse across subcellular membranes, glucose requires an expenditure of energy in order to cross a membrane or to break it down to acetyl CoA via pyruvate. The peroxisomes of yeast, like those of Tetrahymena, do not possess all the enzymes for the complete glyoxylate cycle, only those which produce malate from glyoxylate (malate synthase) and break down isocitrate to glyoxylate (isocitrate lyase); the reactions in the centre of the cycle of Figure VII.7. The malate and succinate diffuse back to the mitochondria to be metabolized by the enzymes of the Krebs's cycle (Tolbert, 1981). This mechanism and the labelling pattern are consistent with the addition of acetyl CoA from two pools, one mitochondrial and one peroxisomal. There is no evidence of cytosolic reactions involving acetyl CoA.

The intensities of the labelled peaks of the ^{13}C spectra of cells also allow estimation of the operation of various metabolic functions. In the initial growth on labelled acetate, much of the label has been incorporated into acyl chains, some has been incorporated into the most abundant of free amino acids, and the rest may well be incorporated highly into aspartate residues within protein, as evidenced by the pullulan spectra. Shulman and coworkers have shown that on the short time scale (about 1 hour) after administration of labelled acetate to S. cerevisiae, the label appears first in glutamate

and later in aspartate" (den Hollander et al., 1981). The time scale is too short to observe incorporation into lipids. However, the label is incorporated into both the α and β positions of aspartate equally. No incorporation was observed into arginine, which forms the major amino acid pool. The incorporation was observed only in those amino acids found in the cytoplasm (glutamate and aspartate) and which are immediate products of the Krebs's cycle. In A. pullulans as well, acetate is initially incorporated into aspartate and acetyl CoA (both of which can be peroxisomal in location), accounting for the highly specific labelling of the β C (in ^{13}C -2 acetate) and γ C (in ^{13}C -1 acetate) of aspartate that is used for protein synthesis. At the same time, a mitochondrial pool of acetyl CoA is operating in the Krebs's cycle to label the carbons deriving from the γ and δ C's of glutamate in lysine and arginine. Since glutamate is turning over fairly quickly, there is not much chance of glutamate becoming additionally labelled in the carboxyl and positions, since this involves diffusion of malate, labelled from the addition of acetyl CoA to glyoxylate, from the peroxisome into the mitochondria for the addition of a second unit of acetyl CoA. Much of the glyoxylate incorporated label leaves the mitochondria as aspartate for protein synthesis, instead of continuing around the Krebs's cycle. This accounts for both the lack of observation of β C's in the spectra of cells and

extracts and the seemingly more efficient labelling of the ϵ and δ positions of lysine and arginine respectively, than the α position of arginine and glutamate. The labelling pattern of proline in the starved cell spectra also reflects this trend, since the δC (from the ^{13}C -1 acetate spectrum) appears to be more efficiently labelled than the αC from the ^{13}C -2 acetate spectrum. The discrepancy between the observation of a pyruvate-derived labelling of the tryptophan residue of protein and the lack of label in other pyruvate-derived amino acids may be explained on the basis of two pools of pyruvate, for which there is precedent in liver and Tetrahymena (Shrago and Elson, 1980). Some of the highly labelled oxaloacetate from the glyoxylate cycle may form pyruvate which is used for cytosolic amino acid synthesis (recall that the remaining portions of the aromatic amino acids are derived from sugar catabolism), since this pyruvate pool is not required for glycogen or sugar synthesis. The other amino acids, such as alanine and leucine, may be derived from a pyruvate pool coming from the glyoxylate of the peroxisome. The efficient labelling pattern of the aspartate in the protein and the apparent lack of these latter glyoxylate-derived amino acids suggests that during the rapid growth of the early culture, the glyoxylate shunt dominates over the Krebs' acid cycle.

During starvation, the labelling pattern of the amino acid pool increases, due to slowed protein synthesis. Under

these circumstances the amino acid carbons which arise from the glyoxylate carbons, become labelled as intermediates make more than one pass about the Kreb's cycle of glyoxylate shunt before entering the free amino acid pool. The labelling pattern is approximately proportional to the relative amounts of the different amino acids. The labelling of glyoxylate is most evident from the appearance of the α C of glycine, and those of alanine, and leucine. The two carbons of glyoxylate can become labelled when doubly labelled citrate from the mitochondria return to the peroxisome and is broken down to succinate and glyoxylate. If this labelled glyoxylate then makes an additional path about the Kreb's cycle to glutamate, the β C's of the glutamate-derived amino acids are labelled, as well as the α C's of those derived from aspartate. These carbons can also become labelled through one and a half cycles around the entire Kreb's acid cycle.

The labelling pattern for the starved cells can be rationalized on the basis of three trends. The first is the slowing of the glyoxylate cycle and the greater shunting of glyoxylate itself into the amino acids which derive from it rather than into oxaloacetate. This is reflected by the appearance of glycine, alanine and leucine pools. The pathway is still effectively operating as indicated by the greater labelling of the α C than the β C of glutamate (the β C's of arginine and proline are not resolved), and the appearance of

more labelling in the peaks in general. The close proximity of β -oxidation and the reactions of the glyoxylate pathway make this the immediate use of resulting acetyl-CoA. The second trend is that more of the labelled oxaloacetate is shunted into the Krebs's cycle rather than into aspartate. This results in a stronger appearance of the acetyl-CoA unit from the glyoxylate pathway in the amino acids deriving from glutamate (the α C's) and in the β C's of aspartate, methionine and threonine. The demand for aspartate in protein has decreased and the intracellular pool has increased along with the appearance of methionine. The third trend is a reflection of the second and that is an increase in the role of the Krebs's acid cycle. Whereas the aspartate and threonine labels were exclusively in the β and γ positions after three days growth, the α and β positions of aspartate and threonine in the ^{13}C -2 acetate derived starved cell spectrum have almost equal intensity, as would be expected for more labelling via succinate. In addition to the slowing of metabolism, reflected in the greater retention of label around reaction of the Krebs's cycle, the control of the cycle may have switched to favour greater production of amino acids derived from glutamate (i.e., those which are osmoregulatory in function), over those derived from oxaloacetate.

In addition to the two incorporation sites in malate and citrate, there is evidence of labelling by the keto-chain

elongation process in both regular and starved cells. The effect appears in the efficient labelling of the α and carboxyl carbons of lysine (and leucine, although this amino acid is smaller in amount). It also appears in the labelling of β C of alanine and δ C's of leucine, where it is not nearly as efficient. These latter labels derive from the elongation of glyoxylate to pyruvate, whereas the lysine label results from direct addition to α -ketoglutarate. If this process were cytosolic, then additional scrambling of the labelled acetate would be observed in processes originating in the cytoplasm. Since this was not observed, the labelling pattern for lysine suggests the presence of a third acetyl CoA pool, which is somehow specific for α -ketoglutarate, or that this particular keto-chain elongation takes place within the mitochondria.

The labelling pattern of those metabolites derived from pyruvate shows that there is no reversible decarboxylation of pyruvate available to the organism. This formation of pyruvate directly from the addition of CO_2 to acetate requires the special enzyme pyruvate-ferredoxin oxidoreductase, which is normally found in strict anaerobes. However, the pyruvate derived from this source would lead to the observation of the α C's of alanine and leucine in the ^{13}C -1 acetate derived spectrum rather than the alternate label. The proline component in the starved cells appears to be more highly labelled

than the other components, when compared to the relative abundance determined by amino acid analysis. This may be due to the fact that, in yeast, the precursor of proline, glutamate semialdehyde, can be synthesized from ornithine as well as glutamate. Since the labelling pattern is the same from both sources, this feedback mechanism from labelled ornithine (and arginine) may reinforce the extent of labelling of the proline pool. Finally, the labelling pattern observed for lysine shows that it is synthesized by the α -amino adipate pathway from α -ketoglutarate, as opposed to the diaminopimelic pathway from aspartate. While this is reasonable, since the latter pathway operates for all organisms other than fungi, biosynthesis by this pathway would predict the observation of the ϵ C in the ^{13}C -2 acetate spectrum and the γ C in the ^{13}C -1 acetate spectrum. Thus, while this merely confirms that the organism behaves like a typical fungus, it points out that it is easy to spot unusual metabolic pathways using specific labelling procedures. This is apparent in the observation of the C-2 of histidine, since the retention of the carboxyl carbon from acetate within the histidine ring, as the only non-sugar derived carbon of the molecule, involves some relatively sophisticated biochemical rearrangements. The appearance of this one peak in the ^{13}C -1 spectrum is simple proof that the accepted mechanism is correct.

The sensitivity of NMR for pH and amino acid linkage also allows one to observe the form of amino acids within the intracellular pools. Thus, it was observed that the majority of glycine was present as a peptide of some sort, as well as some of the glutamate pool. It is also present exclusively in an amino linkage, since there is no peak indicative of a population of glycine as an amino terminal species (see Table VII.2 for GLY-GLY). It is interesting to note that, of the major amino acids that increase upon hydrolysis of the cell extract, the amount of peptidyl glycine is approximately equal to the total amount of peptidyl glytamate, aspartate and ornithine (i.e., 5 parts glycine to 2:2:1 of the other residues respectively). This suggests the presence of dipeptides in which glycine is preferentially the carboxyl terminal residue, for example GLU-GLY, ASP-GLY and ORN-GLY. Why these particular peptides might occur in this manner is unknown. They may be common unit structures of cellular proteins, which build up when protein synthesis is slowed. They may act as small peptide hormones, in the manner of glutathione, to aid in the regulation of specific cell functions. Perhaps glycine is more stable as a peptide, or can act more efficiently in an osmoregulatory role. More specific information concerning the metabolic fate of such structures could possibly be determined through the use of specifically labelled peptides or amino acids introduced in the medium.

The results of this simple study of the metabolic fate of acetate in the growth medium of A. pullulans show that the organism effectively husband its resources with a minimum of energy expenditure. The metabolism of labelled acetate can be interpreted in terms of varying rates of metabolism under different environmental conditions. Efficient compartmentalization and regulation of metabolic levels and storage forms allow it to survive under adverse conditions. Although the organism is predominantly in the yeast phase, the metabolism is typical of a mycelial fungus, which undoubtedly accounts for its durability.

CHAPTER VIII
METABOLIC STUDIES BY ^{31}P NMR

Introduction

The potential of high resolution phosphorus NMR in probing cytoplasmic processes was suggested in the introduction to this thesis. While metabolic studies by this method in mammalian tissue are important, in that they provide a basis for a new human diagnostic tool, they are beyond the scope of this study to review. The ^{31}P NMR literature on microorganisms is more limited and is well represented by the work of R.G. Shulman and coworkers. E. coli and yeast metabolism have been studied by monitoring the internal pH and metabolite levels under conditions of anaerobicity and glycolysis. Under aerobic conditions intracellular pH increases and is greater than external pH (Navon et al., 1977, 1979), consistent with the chemiosmotic hypothesis that protons are pumped out during respiration. For the same reason mitochondrial pH can be expected to be higher than cytoplasmic pH, under conditions of active metabolism (Cohen et al., 1978). In both prokaryotes and eukaryotes, the existence of the pH gradient can be correlated with the appearance of peaks in the spectra due to ATP and/or ADP, which indicate active respiration. The onset of glycolysis, stimulated by perfusion of cell suspensions with glucose,

also produces an increase in intracellular pH (Urgurbil et al., 1978).. In addition, the onset of a dormant phase is correlated with a marked decrease in intracellular pH (from pH=6.5 to 5.5-5.5 for yeast) in yeast (Barton et al., 1980) and bacteria (Setlow and Setlow, 1980), but not in amoeba (Deslaurier et al., 1980(b)).

In most studies, the intracellular pH is deduced from the chemical shift of the inorganic phosphate peak, which is very sensitive to pH within the range of pH=5.0 to 7.0. However, other metabolite peaks are also sensitive to pH, for example, sugar phosphates and ATP, and thus monitoring chemical shift as a function of pH provides a means of identifying peaks. Since spectral lines are often broadened due to the viscosity of the cytoplasm, spectra are taken of perchloric acid extracts of cells, which provide better resolved peaks. This method has been used to identify sugar phosphates in glycolytic mutants of yeast (Navon et al., 1979), thereby indicating that ^{31}P NMR may be an efficient way to characterize mutant strains.

From the first ^{31}P spectrum of yeast (Baker's yeast) (Salhany et al., 1975), all spectra of yeast have been dominated by a large peak attributable to polyphosphate - a linear polymer of inorganic phosphate. However, only very recently has any attempt been made to characterize the role of this metabolite (Gillies et al., 1981) by NMR. Phosphate

metabolism in yeast has been well studied by biochemical techniques, but is still not well understood. Older literature has many studies of the extraction and molecular weight characterization of polyphosphates, but since such procedures are long and tedious, there has been little interest in linear phosphates in the last five years. ^{31}P NMR presents a more efficient way of determining the presence of polyphosphate in a variety of organisms, over extraction and chromatography.

Polyphosphates and related compounds pyrophosphate (diphosphate) and metaphosphates (cyclic compounds) are found in varying amounts in many microorganisms, although they are commonly associated with yeast and fungi. There are small quantities as well in higher plants and animals, since polyphosphates have been isolated from insects (Niemierko, 1962), and mammalian tissue (Gabel and Thomas, 1970). By ^{31}P NMR, polyphosphates have been identified in amoeba (Deslauriers et al., 1980a), E. coli (Jacobsen and Cohen, 1980), Tetrahymena (Deslauriers et al., 1981), and in methanogenic bacteria (I. Ekiel, unpublished results).

The precise role of polyphosphate in metabolism is uncertain although experimental results give rise to three plausible theories (for reviews see Harold, 1966; Kulaev, 1975; Davis and Senior, 1973). The first is that the polymer acts as a storage form of phosphate for phosphorylation of high energy compounds such as ADP or NAD, especially during

phosphate deprivation. A polymeric form of storage reduces the osmotic pressure and pH effect over an ionic form, and thus polyphosphate may also aid in osmoregulation of the cell. In addition, polyphosphate binds strongly to ions as Mg^{2+} , which hints to an additional role in ion storage (Glonek et al., 1971). The enzymes involved in phosphate polymerization and reduction are sensitive to Mg^{2+} and to pH, suggesting a feedback mechanism (Felter et al., 1970; Afanasedva and Kulaev, 1973). This theory is supported by the existence of phosphatases in close proximity to the polyphosphates, which reduce it to phosphate (Kulaev, 1975), plus the fact that polyphosphate contents of cells increase when phosphate is abundant in the medium and decrease during phosphate deprivation. Furthermore, in some organisms there is no correlation between respiration rate and the rate of polyphosphate synthesis or utilization (Harold, 1962). Tracer studies have shown the transfer of label between poly- and nucleotide phosphate (Harold, 1960) as well as phospholipid (Winder and Denny, 1957).

The second theory is an extension of the first, in that the polyphosphate actively regulates the phosphorus economy of the cell. Support for this more active role comes from evidence for control of enzymes involved in polyphosphate metabolism by inorganic phosphate concentrations (Felter and Stahl, 1979; Kaltwasser, 1962), and the existence of the

"polyphosphate overplus" - the burst in polyphosphate synthesis immediately after reintroduction of phosphate following a phosphate starvation (Liss and Langen, 1962). This latter phenomenon could be seen as a mechanism for controlling the level of intracellular phosphate.

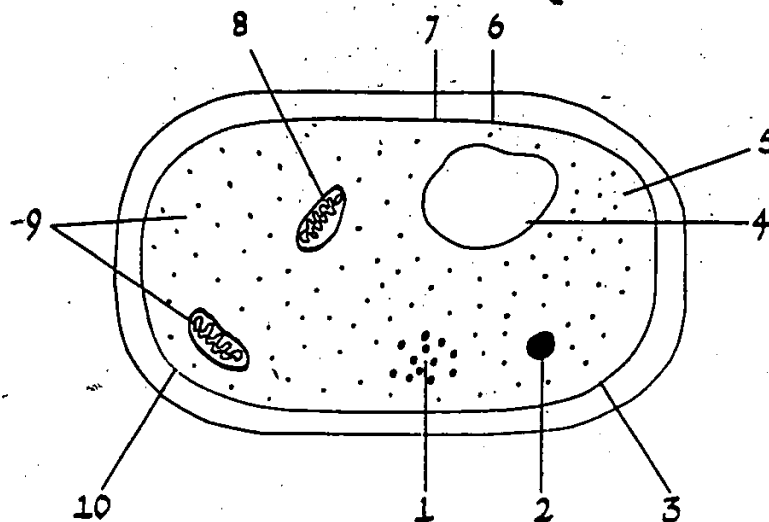
The third theory is the most interesting since it suggests an evolutionary role for the polymer. It is that polyphosphate acts directly as a phosphagen; that is, not only as a storage of phosphate but of phosphate energy as well. It has been put forward that this is an evolutionary precursor of ATP. Polyphosphates are kinetically stable, but thermodynamically unstable (have a large negative ΔG for hydrolysis). They are known to induce polymerization of other monomer units - nucleotides, peptides, sugars - as well as condense ATP, AMP, etc. from their precursors (Lehninger, 1970). Most of the condensing reactions from inorganic phosphate to pyro-, meta- and polyphosphate require only heating or cyanophosphates, and were thus likely part of the "pre-biotic soup". Evidence to support this hypothesis include reversible transfer between ATP and polyphosphate, and correlation of utilization of polyphosphate with high cellular metabolic demands (Hoffman-Ostenhof, 1962). Direct phosphorylation of glucose from polyphosphate with conservation of the phosphate group transfer potential has been observed in fungi (Kulaev, 1975). Additional evidence has been provided

by Felter and Stahl (1973), who isolated a soluble polyphosphate ADP-phosphotransferase from Baker's yeast and proposed that the enzyme worked essentially as a breakdown enzyme of polyphosphates, transferring phosphate to ADP to form ATP. Generally, evidence is somewhat negative in nature, in that the phosphagen hypothesis is based on evidence that polyphosphates build up under conditions of slowed growth, or when ATP content exceeds metabolic demands.

At present, the phosphagen hypothesis is well supported by Kulaev and coworkers, while the storage role is preferred by the lab of Harold and coworkers. It is conceivable that the evidence against phosphagenic activity could result from the genetic nature of the organism under study. It may be that as organisms evolve, their ability to synthesize phosphate polymers remains, while specific enzymes required for phosphagen activity are replaced with ATP-specific enzymes. Thus, in these organisms the polyphosphate acts only as a storage form. It would be interesting to correlate the results of previous studies with the type of organism used and the relative nucleotide phosphate levels in order to determine if the response is genetic in nature. Polyphosphate synthesis has also been linked with cell division in yeast (Kulaev et al., 1973; Spoerl and Looney, 1958), with the active transport of glucose in yeast (van Steveninck, 1969), as well as with polysaccharide synthesis (Kulaev et al., 1972).

If not directly a phosphagen; polyphosphate may act as a storage form for pyrophosphate, which has also been put forward as an evolutionary precursor to ATP as a source of phosphate bond energy. The existence of a specific phosphatase that produces pyrophosphate from polyphosphate has been shown (see Figure VIII.1). There have been many reports that pyrophosphate can act as a phosphorylating energy acceptor in the functioning of the electron transport chain (Kulaev, 1975). Originally considered only as a byproduct of metabolism, pyrophosphate accumulation has been correlated with decreasing polyphosphate and intense metabolic activity (Ermakova et al., 1981). The use of ATP as the phosphorylating vehicle ATPase, whereas pyrophosphate would require a pyrophosphatase - two different enzymes but with a similar function. It is possible that an organism possessing the genetic ability to synthesize both enzymes could alternate between pathways, as a sort of survival mechanism. It has been postulated that a change in fluidity of the mitochondrial membrane can cause the switch from ATP-producing phosphorylation to pyrophosphate-producing phosphorylation (Kulaev et al., 1980).

A summary of the cellular location of the constituents of phosphate metabolism, as found both in yeast and filamentous fungi is shown in Figure VIII.1. A classification of various chain lengths of polyphosphate is based historically on the response to different extraction procedures. Langen et al. (1962) has shown that long chain polyphosphates are synthesized



1. ACID SOLUBLE FRACTION OF POLYPHOSPHATE (P_4) IN VOLUTIN GRANULES (FUNGI).
2. SALT SOLUBLE FRACTION OF POLYPHOSPHATE (P_{20}) IN CELL NUCLEUS (FUNGI).
3. ALKALI SOLUBLE FRACTIONS OF POLYPHOSPHATE (P_{55} AND P_{260}) IN PLASMA MEMBRANE (FUNGI).
4. HIGH MOLECULAR WEIGHT POLYPHOSPHATE IN VACUOLE (YEAST).
5. LOW MOLECULAR WEIGHT POLYPHOSPHATE IN CYTOPLASM (YEAST).
6. POLYPHOSPHATE DEPOLYMERASE IN PLASMA MEMBRANE.

$$(P_n \rightarrow P_x + P_{n-x})$$
7. POLYPHOSPHATASE IN CYTOPLASMIC MEMBRANE.

$$(P_n \rightarrow P_{n-1} + P)$$
8. TRIPOLYPHOSPHATASE IN MITOCHONDRIA.

$$(PPP \rightleftharpoons PP + P)$$
9. PYROPHOSPHATASE IN MITOCHONDRIA AND CYTOPLASM.

$$(PP \rightleftharpoons 2P)$$
10. POLYPHOSPHATE KINASE IN CYTOPLASMIC MEMBRANE.

$$(P_n + ATP \rightleftharpoons ADP + P_{n+1})$$

Figure VIII.1 - Summary of cellular locations of the constituents of phosphate metabolism, as found in filamentous fungi (N. crassa) and yeast (S. cerevisiae)

directly by transfer of phosphate from ATP. Two fractions are formed, one of ~260 monomer units (P_{260}) and one of ~55 units (P_{55}). These are hydrolysed by depolymerase to give two more fractions, P_{20} and P_4 , which are located in different parts of the cell from the longer chain fragments. It was postulated that it was on these smaller chain fractions, rather than the longer ones, that polyphosphatases acted to produce inorganic phosphate, which could then be used to regenerate ATP. This was dubbed the "polyphosphate cycle". The shorter chain units are extractable with alkali and alkali plus salt, while the longer chains require strong acid to extract, perhaps with the addition of a detergent (SDS).

There appears to be some differences in location of polyphosphate fractions between yeast and filamentous fungi. Polyphosphates have been traditionally associated with volutin or metachromatic granules - small grains that give a characteristic colour when stained with basic dyes (Drews, 1962). These granular polyphosphates, in fungi, appear to constitute only the short chain fragments. The medium chain lengths (P_{20}) are associated with the nucleus, while the long chain fractions have been isolated with the plasma membrane. Other workers insist that the majority of polyphosphate, at least in yeast cells, is to be found in the vacuoles (Urech *et al.*, 1978), with a small concentration of shorter fragments in the cytoplasm (Indge, 1968). The ability of free polyphosphate to complex

with membrane fractions may account for the association between polyphosphate and plasma membrane. However, volutin granules have also been observed in some yeasts (Ebel, 1952). The hydrolytic enzymes of polyphosphate, both those which hydrolyse to shorter chain lengths and the polyphosphatase which hydrolyses one phosphate unit at a time, have been associated with the plasma membrane. Pyrophosphate is found in highest quantities in mitochondria, and thus it is not surprising that the hydrolytic enzymes specific for tripolyphosphate and pyrophosphate are found in the mitochondria or free in the cytoplasm. There appears to have been no studies that specifically locate these enzymes in yeast, other than by Stahl (1969), in which polyphosphate kinase (which catalyses the transfer of phosphate between ATP and polyphosphate) was associated with cell walls and membranes. This enzyme is sensitive to Mg^{2+} and ATP levels, and the reaction has been shown to be reversible, although not in vivo.

The general correlations between polyphosphate levels and metabolic behaviour of yeasts and fungi that are important to the following study are, in summary:

- (1) The buildup of polyphosphate levels are associated with high activity under conditions of generous nutrition.
- (2) A decrease in polyphosphate occurs under conditions of phosphorus deprivation or high activity when levels

of ATP are insufficient.

- (3) Polyphosphate levels are maintained under conditions of slowed growth with adequate nutrition.
- (4) Accumulation of polyphosphate has been shown to occur under conditions of carbon deprivation (Harold, 1960).

Experimental Conditions

Samples for NMR analysis were prepared by harvesting a 50 ml culture at 3000 x g for 10 min., rinsing once with sterile distilled water and suspending cells to a density of $1.5-2 \times 10^9$ cells per ml in 10 mM phosphate buffer, pH=5.5 in a NMR tube of 10 mm OD. Viability curves and the conditions of NMR spectral accumulation have been outlined in Chapter III.

Nystatin was added as a suspension in two different doses: 1 mg and 5 mg per 50 ml cultures, and cell suspensions were vortexed to ensure even distribution of antibiotic. Methyl mercury chloride was added as an aqueous solution to give a final concentration of 6×10^{-4} M.

Oxygenation of cell cultures was obtained by bubbling oxygen very slowly and evenly through the cell suspension from a glass micropipette, held in place in the NMR tube by a length of 1/16" plastic tubing. The bubbling rate through the tubing was controlled by a peristaltic pump. The back pressure for the pump was created by slowly bubbling oxygen

from a cylinder through water in a closed flask. Excess pressure buildup in the flask was avoided by venting the flask through glycerol. This setup maintained a low but even back pressure for the peristaltic pump. Small bubbles diffused through the cell suspension from the tip of the capillary placed near the bottom of the NMR tube. The bubbles dispersed without causing the cell suspension to bump. The bubbling rate was approximately one per second. Such treatment did not disturb the resolution of the NMR spectra. For consistency between oxygenated and non-oxygenated experiments, none of the samples were spun in the magnet. Linewidths were of the order of 20-80 Hz.

All spectra of each series were scaled to the highest intensity peak of the first spectrum. Linewidths, chemical shifts and peak heights were monitored as a function of time. Each spectrum of a series required 17 minutes for accumulation and the time scale refers to the midpoint in time of each spectrum. Time zero is then the point at which sample preparation was completed (i.e., suspension in the NMR tube plus addition of nystatin or MeHgCl).

Model polyphosphate compounds (Glass 65 and Sodium Phosphate Glass) were obtained from Sigma Chemical Co. The viability curve for methyl mercury treatment was done by Dr. J. Phipps of the Division of Biology, NRC.

Results

(a) Effect of Aerobicity

The typical phosphorus NMR spectrum of A. pullulans, freshly harvested from culture medium is shown in Figure VIII.2(a). The spectrum is very simple and is dominated by a large peak which can be assigned to polyphosphate. The additional peaks, as shown on the expanded vertical scale, can be assigned to inorganic phosphate (P_{ext} and P_{int}) as well as to the terminal and penultimate phosphate groups of polyphosphate ($P_{n-1}P$, and $P_{n-x}P_x$). ATP is present as indicated by the small peak at 10.5 ppm, and the even smaller neighbouring peak at ~12 ppm is due to UDPG. The broad peak downfield of inorganic phosphate is due to sugar phosphates, whose specific identities may be determined from an extract of the cell suspension (vide infra). Two factors make this spectrum much different from those of other yeast. The first is that the ATP levels are very low and the polyphosphate content is very high in comparison to S. cerevisiae (Navon et al., 1979) or Baker's yeast (Salhany et al., 1975). The second is that the polyphosphate appears to be, on average, of high molecular weight, from the intensity ratio of the peaks due to terminal polyphosphate and intermediate long chain groups. Navon et al. (1979) has estimated the average chain length of polyphosphate from S. cerevisiae to be ~12

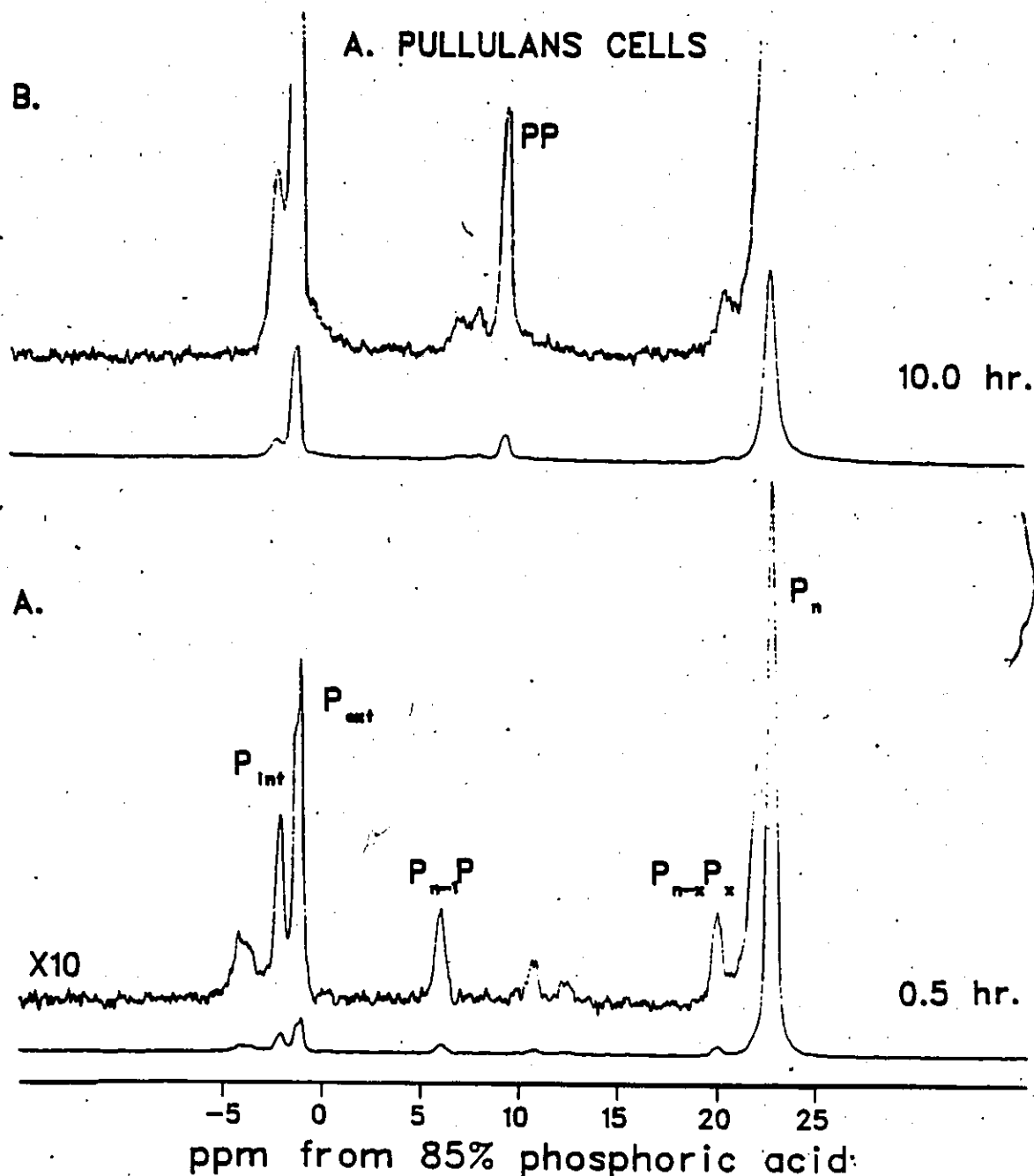


Figure VIII.2 - 121 MHz ^{31}P spectrum of cells of *A. pullulans* at 2×10^9 cells per ml (a) 0.5 hours and (b) 10.0 hours after harvesting. The spectra are a result of 2000 accumulations with a 45° pulse and 0.5 sec recycle time. The large peak at high field is attributable to polyphosphate. Smaller peaks that appear in the enlargement (x 10) of the spectrum are due to internal and external inorganic phosphate (P_{int} , P_{ext}), penultimate and terminal phosphate groups of polyphosphate (P_{n-1} , P , P_n , P_y) and ATP. The broad peak downfield of inorganic phosphate is due to sugar phosphate. Spectrum (b) shows the appearance of a new peak due to pyrophosphate (PP)

units, based on this same intensity ratio. For A. pullulans, the average length is approximately 70 units, and may be much longer. The fact that the polyphosphate is observed as a relatively narrow peak (60 Hz), indicates that the polymer is soluble and mobile, not held in rigid substructures such as volutin granules or membrane associated. Other rigid phosphorylated compounds such as RNA or DNA are not visible in the high resolution spectrum. This may be some support for the location of polyphosphate within the vacuoles as opposed to granules (Urech et al., 1978). The cellular polyphosphate may be expected to be at its maximum, since the cells are harvested at early stationary phase. Maximum polyphosphate content in S. cerevisiae occurs during late log phase, after which the polyphosphate appears to decrease (Katchman and Fetty, 1955).

As a function of time, the inorganic phosphate peaks increase, polyphosphate decreases and new peak appears that is assignable to pyrophosphate (PP, see Figure VIII.2(b)). There is no appearance of shorter fragments of polyphosphate which may be a result of general hydrolysis. This shows that the organism possesses the specific enzymes that hydrolyze polyphosphate to phosphate and to pyrophosphate. It is possible that the organism can form pyrophosphate from inorganic phosphate, but this would tend to reduce the intensity of the inorganic phosphate peak and would not be energy efficient.

Positive identification of the spectral peaks can be made by enhancing the resolution through an extract of the cell suspension. Figure VIII.3 is a perchloric acid extract of freshly harvested cells. The cells proved somewhat more difficult to extract than other organisms and it was necessary to sonicate the suspension in the presence of 35% perchloric acid in a cell disruptor in order to extract the metabolites adequately. Not all the polyphosphate is extracted by this process, which accounts for the reduced relative intensity of the polyphosphate peak. In addition, the release of phosphate enzymes during the extraction may cause some degradation of the polyphosphate pool.

The chemical shifts of the perchloric acid spectra are monitored as a function of pH, giving rise to the titration curves of Figure VIII.4. The titration curves were compared to those of Gadian et al., (1979), Navon et al., (1979), and Salhany et al., (1975), as well as standard solutions for identification. Those peaks that titrate strongly in the physiological pH region can be assigned to glucose 6-phosphate (A), fructose diphosphate (B) and fructose 6-phosphate (C). In the region of pH observed in the cell, all of these species form one broad peak, making it difficult to identify individual sugar phosphates by NMR. However, the presence of FDP and G6P in cell extracts were indicated by both ^{13}C NMR and paper chromatography. Inorganic phosphate also shows a characteristic

PERCHLORIC ACID EXTRACT pH=7.5

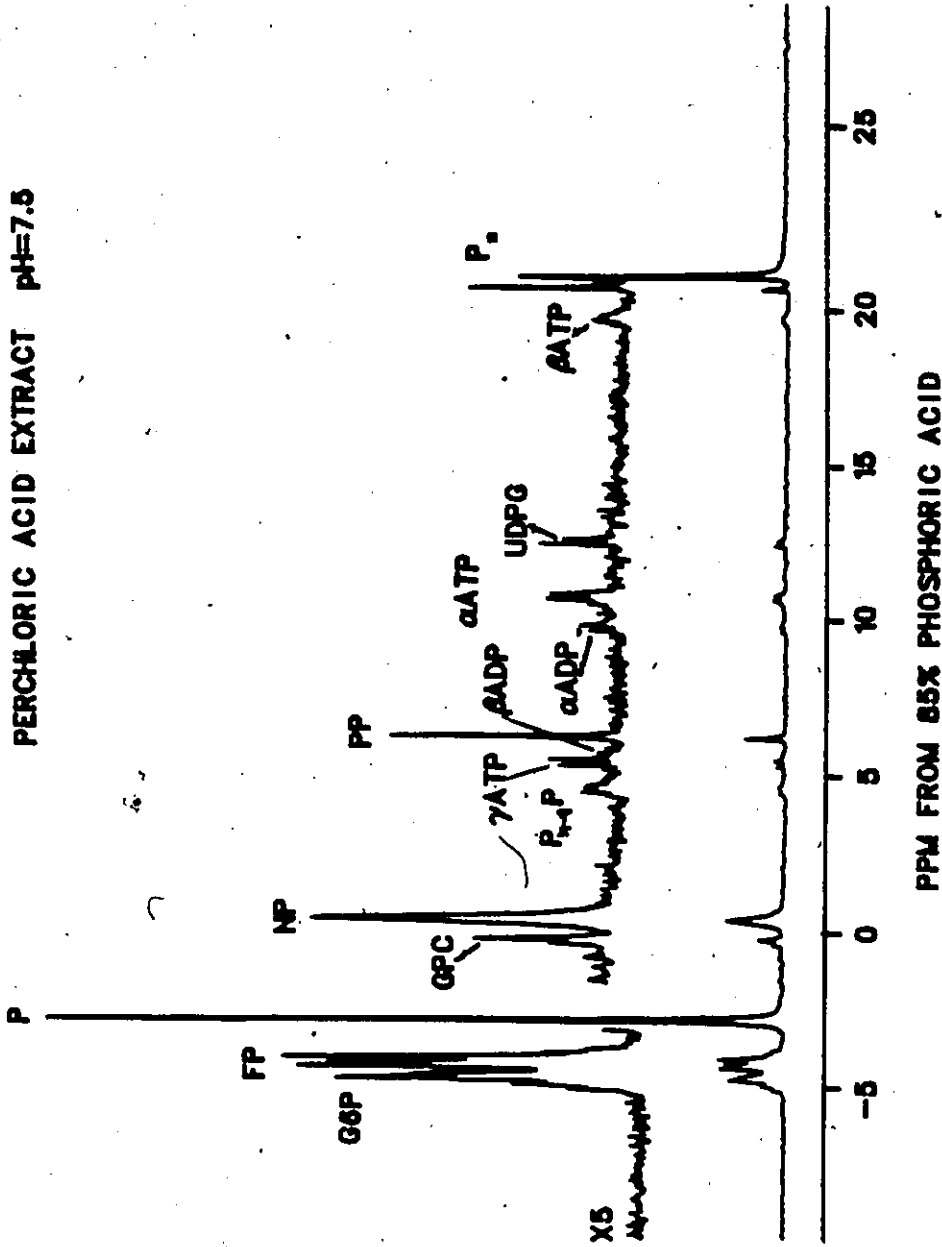


Figure VIII.3 - ^{31}P spectrum of perchloric acid extract of fresh cells. The peaks are assigned on the basis of the titration curves (Figure VIII.4). The enhanced resolution allows the observation of additional peaks hidden in the spectra of Figure VIII.2, as well as the unambiguous assignment of the existing peaks. The sugar phosphate region can be resolved to show the presence of both G6P and fructose phosphates (FP). In addition to the species observed in the previous figure, the presence of ADP, UDPG, nucleotide phosphates (NP) and glycerol-3-phosphatidyl choline (GPC) are confirmed.

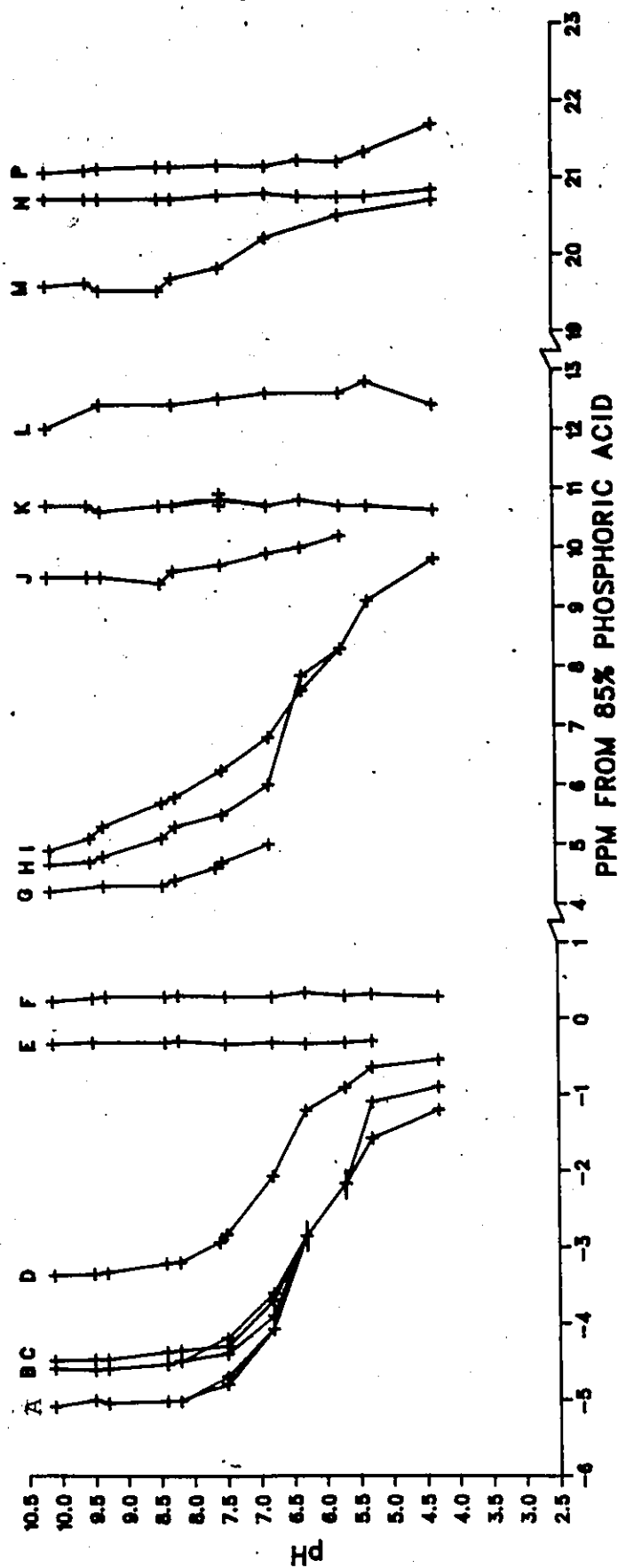


Figure VIII.4 - Titration curves for the spectrum of the previous figure. Spectral peaks are identified by comparison of titration curves to those of standards. Curves are identified as follows: A, G6P; B, FDP; C, F6P; D, inorganic phosphate; E, GPC; F, nucleotide phosphate; G, terminal polyphosphate; H, γ -ATP; I, pyrophosphate; J, α -ADP; K, α -ATP; L, UDPG; M, β -ATP; N, penultimate or smaller polyphosphate; P, polyphosphate

pH curve (D). In addition, the perchloric acid extract also shows the presence of the breakdown products of nucleic acid (NP, (F)) and phospholipid (GPC, (E)), which were not visible in the whole cell spectrum. Most lipidic phosphodiester have a slightly positive chemical shift (Navon et al., 1979). GPC would be expected to occur, since PC is the major phospholipid of the cell. The small shoulder on the GPC peak is consistent with GPE, which appears to be found in the same ratio to GPC as in phospholipid (1:2, respectively). The peak assigned to nucleotide phosphate is much wider (25 Hz) than the others (~7 Hz), indicating that it may be due to a polymer rather than a small molecule. It has been shown that RNA and DNA, when subjected to cold perchloric acid, give a characteristic peak at +0.3 ppm (R. Deslauriers and S. Poignant, unpublished). Since this peak occurs at 0.32 ppm, it is likely due to cellular genetic material. Both peaks would appear in the base of the inorganic phosphate peak at the pH of the cytoplasm, and are therefore not resolved in the cell spectrum. GPC and NP are also pH-independent, which allows for their easy identification. The terminal group of polyphosphate (G) has a very small pH dependence, which allows for distinction between it and pyrophosphate (I). The γ -phosphate of ATP (H) has a pH curve very similar to that of pyrophosphate and thus, at the pH of the cell, cannot be distinguished in the spectrum. α -ATP (K) and α -ADP (J),

however have only small pH dependences, as does UDPG (L). Whereas the nucleotide phosphates are seen as only a single broad peak in the whole cell spectrum, the resonance are resolved in the extract spectrum to show phosphorus-phosphorus couplings. The ADP/ATP ratio appears to be less than 0.5 in the extract. Since ADP and ATP are not resolved in whole cell spectra, the predominance of ATP is confirmed by the intensity of the β -ATP peak (M) relative to those of α -ATP (or α -ADP) and γ -ATP (or β -ADP). When resolved, this resonance is a characteristic triplet. In the extract two polyphosphate peaks are observed. These are not pH dependent. The intensity of the peak which could be ascribed to penultimate polyphosphate groups (N), is not consistent with that for terminal polyphosphate, indicating that the peak is due to polyphosphate of substantially shorter chain length. The lack of pH dependence shows that the chain must be greater than three units long, since the central group of tripolyphosphate has a slight pH dependence. That there is not a general distribution of peaks up to the highest chemical shifts indicates that there are either two pools of polyphosphate of different chain lengths within the cell, or that a specific depolymerase exists to fragment the longer polymer. This discrimination was not observed in whole cell spectra due to the breadth of the polyphosphate line (60-100 Hz), although occasionally a shoulder was observed on the side of the polyphosphate peak,

which was more intense than the terminal phosphate peak. The length of the long chain polyphosphate, as indicated by the ratio of the peak intensity to that of the terminal group shows that the average chain is 70-80 monomer units. Comparison with standard phosphate glasses show that the smaller polyphosphate is approximately 10 units long. Since a certain amount of enzymatic degradation occurs during a perchloric acid extract, this extract spectrum indicates the presence of specific enzymes to interconvert phosphate polymers that are 1, 2, 10 and 80 units long, and the lack of others that produce triphosphate or other chain lengths.

The chemical shift of the long chain polyphosphate occurs at lower field in the extract spectrum than in the whole cell spectrum. Since polyphosphate has no titration effect, this is due to association of the polyphosphate with ions within the cell, specifically Mg. The chemical shift of the extracted polyphosphate shows it to be essentially magnesium free, by comparison to pure polyphosphate solutions. This shows that the deionization with EDTA and Chelex were efficient. The difference in chemical shift observed in cell spectra (2 ppm) shows that the cellular polyphosphate is substantially ion-bound (Glonek et al., 1971; see section (e)).

The relative intensities of metabolic peaks of the cell suspension were monitored each half hour for a period of ~10 hours for all of the following experiments. The spectra were

accumulated under conditions in which the peaks due to polyphosphate and pyrophosphate were not saturated but the peak due to inorganic phosphate was saturated, due to the restrictions involved in balancing signal to noise, time resolution, and cell density. This does not allow for absolute measurement of cell concentrations or even of the ratio of polyphosphate to inorganic phosphate. However, it does allow for the determination of relative changes in intensity, since the extent of saturation does not change throughout the experiment (i.e., T_1 does not substantially change). The symbols used on all the metabolic intensity plots are listed in Figure VIII.5. These conventions are preserved for Figures VIII.6 to VIII.30. Only the peaks with substantial intensity were monitored, as ATP levels were too low to be accurately measured. However, the appearance and disappearance as a function of time were noted. While metabolic levels were monitored by peak height, any changes in linewidth were also noted and the relative line heights were correspondingly corrected by crude triangulation to eliminate anomalous intensity changes due to increasing linewidth.

Figure VIII.6 shows such a metabolic plot for the spectra shown in Figure VIII.2 which is then correlated with the viability curve for cells under the NMR conditions. The viability curve shows that, under anaerobic conditions and increased cell density (x25), viability begins to decrease

KEY TO SYMBOLS IN ^{31}P METABOLIC LEVEL FIGURES



POLYPHOSPHATE (P_n)



TERMINAL GROUP OF POLYPHOSPHATE (P_{n-1}P)



PYROPHOSPHATE (PP)



INORGANIC PHOSPHATE (P)



SUGAR PHOSPHATE (SP)



GLYCERO-3-PHOSPHATE (GPC)

Figure VIII.5 - Key to symbols used in time course plots of phosphate metabolite levels (Figures VIII.6 to VIII.30)

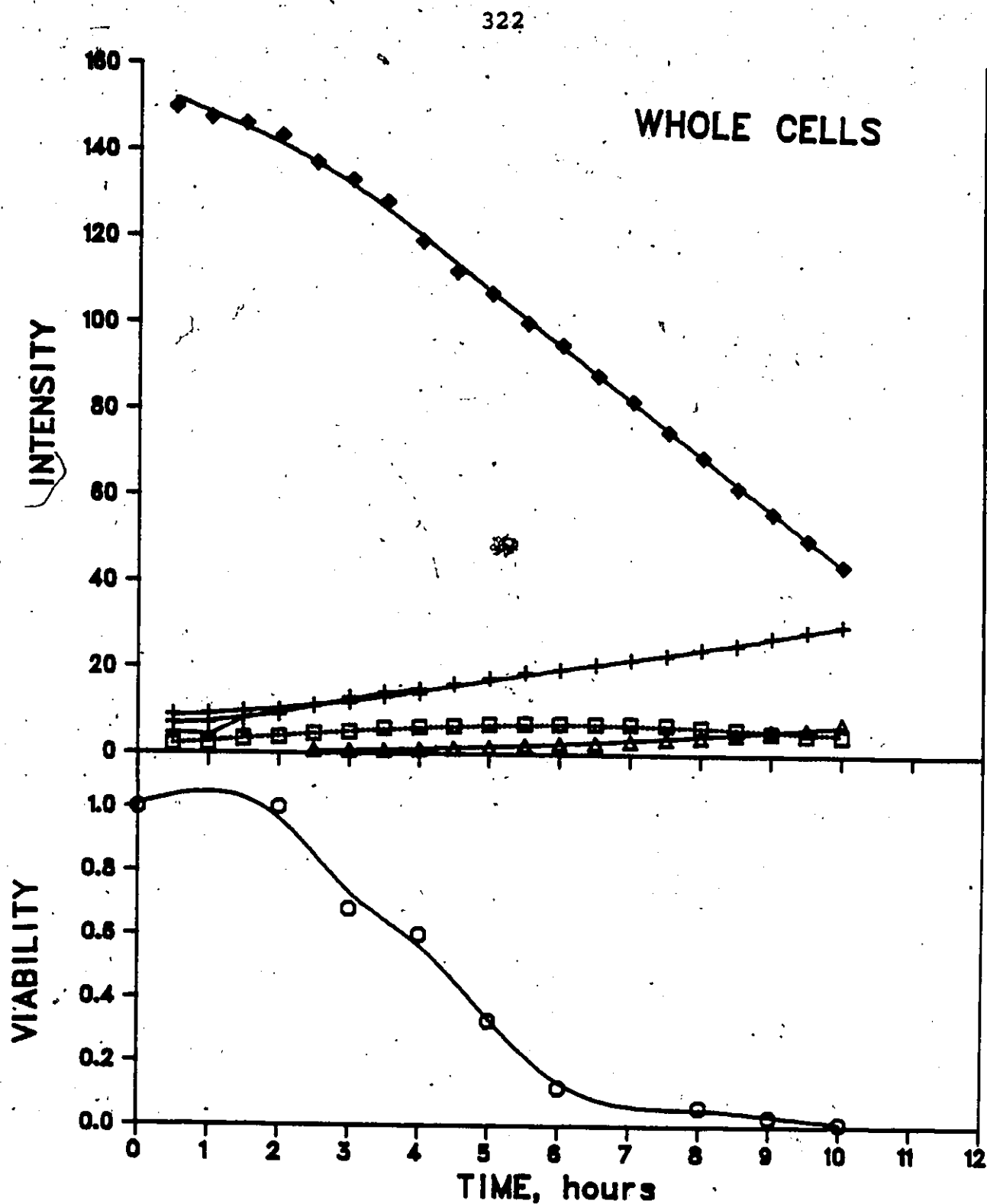


Figure VIII.6 - Relative intensity of spectral peaks of Figure VII.2 as a function of time. Metabolite intensities are correlated with the viability curve of the culture under the conditions of the NMR experiment

after 2 hours and slowly decays to complete loss of viability after 10 hours. A similar curve to this was observed for cells suspended at a higher cell density in D_2O , simulating conditions used for ^{13}C T_1 experiments (Chapter V). The polyphosphate content decreases more markedly after 2 hours, mirrored by an increasing pool of pyrophosphate and the loss of individual pools of inorganic phosphate, or the ability to maintain a pH gradient across the cytoplasmic membrane. Sugar phosphate pools appear to increase and then decrease slightly. The relative change in polyphosphate appears not to be adequately accounted for by the increase in the other visible phosphate pools indicating that there is a channeling of phosphate into other bound pools. Since, in this case, the inorganic phosphate peak has the same linewidth as the polyphosphate peak, the relative intensity changes are meaningful. The ATP and UDPG pools which are apparent at the beginning of the experiment disappear as the loss of viability begins (2 hours), concomitant with the appearance of pyrophosphate. The terminal polyphosphate peak remains at constant chemical shift, indicating that the polyphosphate is being hydrolyzed directly into inorganic phosphate and not randomly hydrolyzed into shorter chain lengths. The linewidth of the polyphosphate increases slightly during the course of the experiment, from 60 to 83 Hz accompanied by a slight (0.2 ppm) upfield shift, indicating an increasing



amount of ion binding. If the polyphosphate storage pool is also an ion pool, quickly decreasing polyphosphate will result in the remainder being more extensively ion-bound.

When cells were oxygenated, (Figure VIII.7), there was very little change in the spectrum over a period of ten hours. Low ATP and UDPG levels apparent in the initial spectrum were retained, and no pyrophosphate appeared. The polyphosphate degraded slowly but not completely. The greater relative intensity of the external inorganic phosphate peak may be due to slight differences in cell density or a slightly older culture. In addition, the cell may be actively pumping phosphate out of the cytoplasm to maintain correct cell concentrations. A pH gradient is maintained throughout the experiment, as indicated by the preservation of two inorganic phosphate peaks. However, only two phosphate pools are observed; instead of three found in the anaerobic spectrum. Sugar phosphate is low in concentration and appears to disappear after 6-8 hours. The polyphosphate peak linewidth and chemical shift remain constant, consistent with the maintenance of the pool concentration. The detailed time dependence of the inorganic phosphate and polyphosphate levels are correlated with the viability curve of the cell suspension in Figure VIII.8. Internal phosphate levels are maintained, while the slight decrease in polyphosphate is mirrored by a slight increase in external phosphate, indicating that the cell is

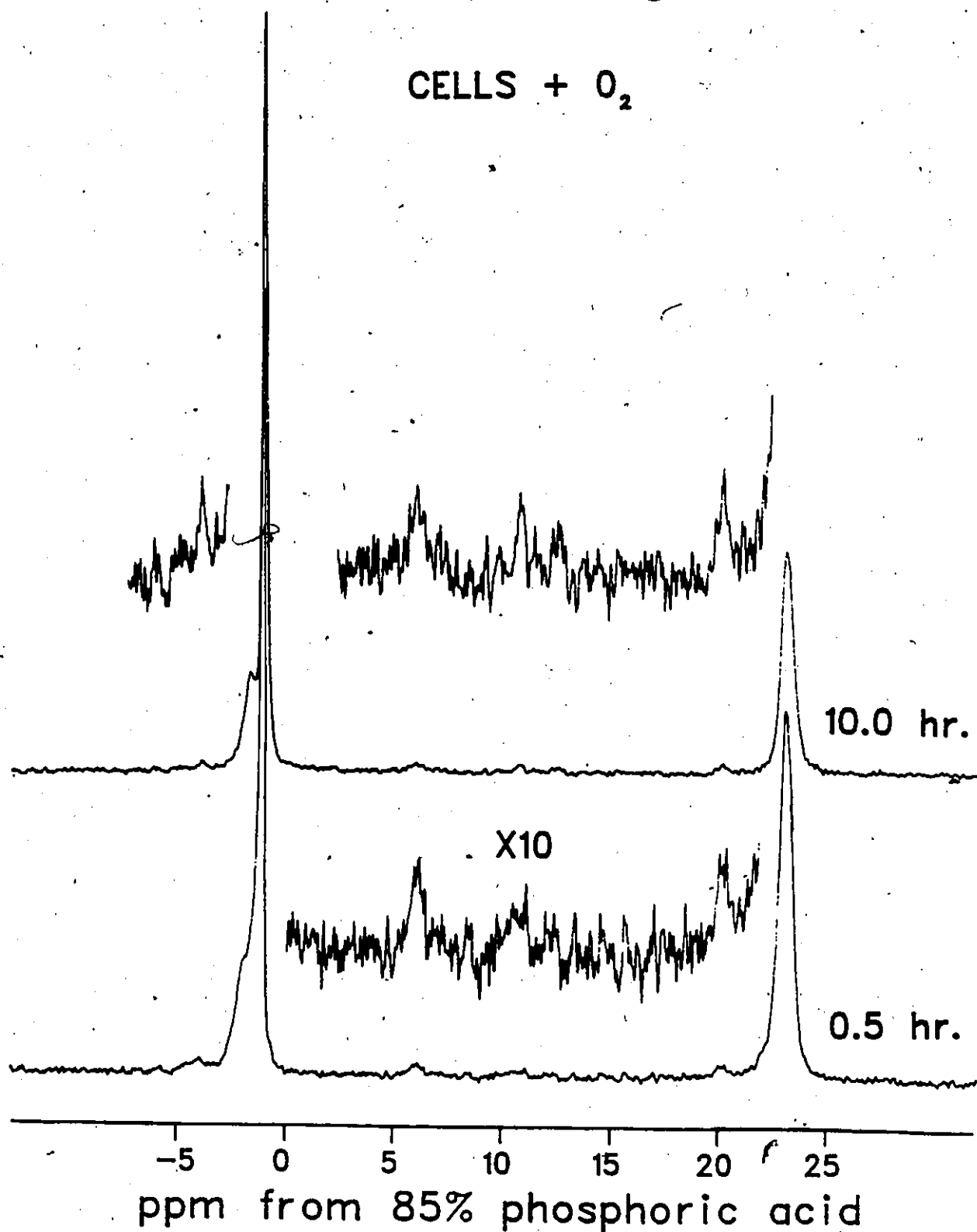


Figure VIII.7 - 121 MHz ³¹P spectrum of whole cells suspended at 2 x 10⁹ cells per ml and oxygenated. The spectra change very little in the course of 10 hours

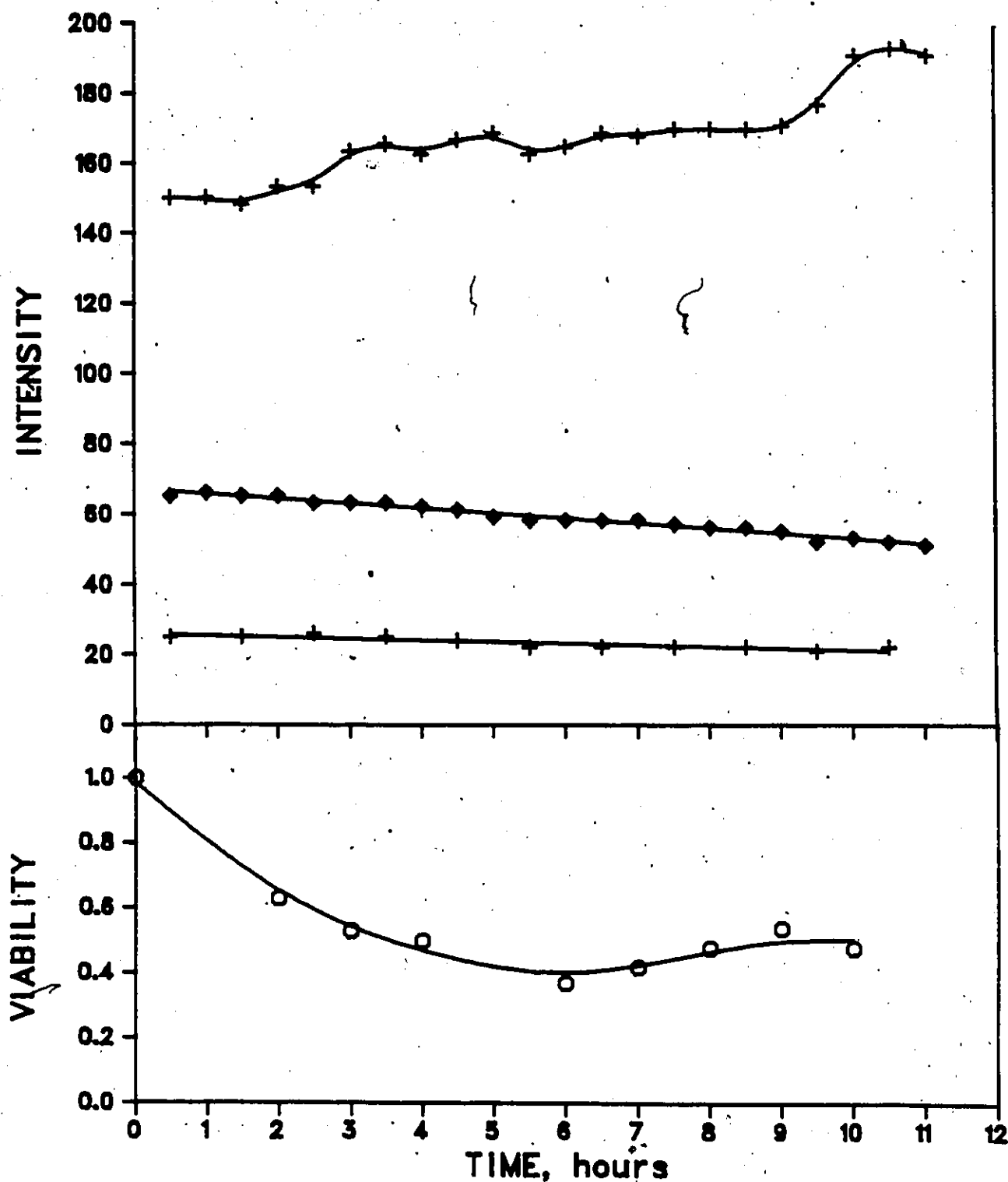
CELLS + O₂

Figure VIII.10 - Relative peak intensities for the aerated cell suspension as correlated with the viability curve. ATP levels are very small but remain through the course of the experiment

actively pumping out phosphate against the concentration gradient. Viability drops to ~50%, but is maintained at that level. Under conditions of adequate supplied phosphate, the cells do not increase their polyphosphate store, but retain it. This may be due to the limitations imposed by the high cell density. The cells may be retaining their resources under conditions of overcrowding and lack of adequate nutrition, but the presence of oxygen allows them to maintain their basal metabolic functions.

The pH behaviour for the anaerobic and aerobic cell suspensions is presented in Figure VIII.9. The chemical shift of the resolvable inorganic phosphate peaks were compared with the titration curve of a solution of 10 mM phosphate, in order to determine pH. Since the ionic strength of the cytoplasm was not taken into account, the absolute values of the pH are likely accurate to only 0.5 pH units. However, the relative pH differences are meaningful, since the effect of increasing ionic strength is to simply shift the entire titration curve slightly upfield. Therefore, the calculated pH is a minimum value. The effect of aeration of the culture is to not only maintain a pH gradient which is a minimum of 0.6 pH units across the cytoplasmic membrane, but to maintain an internal pH which is higher than that of the anaerobic cells. The pH curves of the aerobic culture (indicated by the squares in the figure, where the solid and dashed lines denote internal

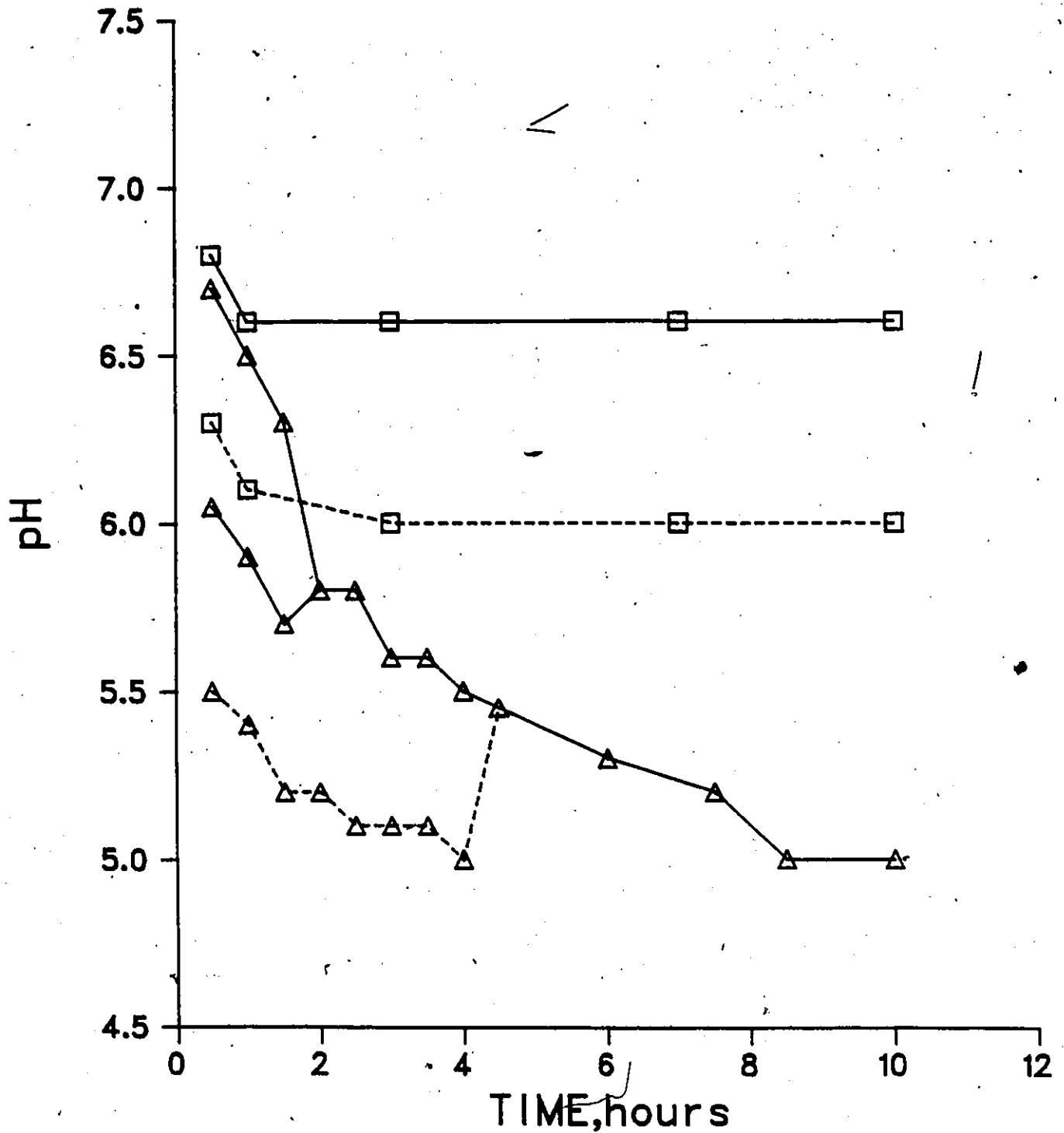


Figure VIII.9 - Time course of internal and external pH as measured from the chemical shift of inorganic phosphate for the anaerobic and aerobic cultures of Figures VIII.2 to VIII.8. The solid lines refer to internal phosphate pools and the dashed lines represent external pH. Triangles (Δ) and squares ($\square$) refer to the anaerobic and aerobic experiments respectively

and external pools, respectively), also decrease slightly with initial loss of viability and then level off, reflecting the viability curve. The anaerobic cell suspension (Δ) shows increased acidosis associated with cell death and the disappearance of all pH gradients by the time the culture has only 10% viability. The point at which the lowest and the highest pH pool (which may be the mitochondrial pool) have lost the ability to maintain a pH gradient may be correlated with the initial drop in viability, at ~2 hours.

Since it is difficult to ascertain whether the behaviour of the metabolite levels is due to active regulation by the cell or by simple enzymatic degradation due to loss of viability, the metabolite levels were monitored for a cell suspension which was not viable but not obviously lysed. This condition was fulfilled by using rehydrated lyophilized cells. Lyophilized cells were suspended in sterile phosphate buffer for one-half hour before adjusting the cell density and running the experiment in order to allow for adequate hydration. The anaerobic spectra are shown in Figure VIII.10. There is no evidence of inner and outer phosphate pools and the polyphosphate may have degraded somewhat on lyophilization. No ATP was observed although sugar phosphates were present, residual from the state of the culture when originally harvested. The spectra support the evidence for two pools of polyphosphate, as was indicated by the perchloric acid

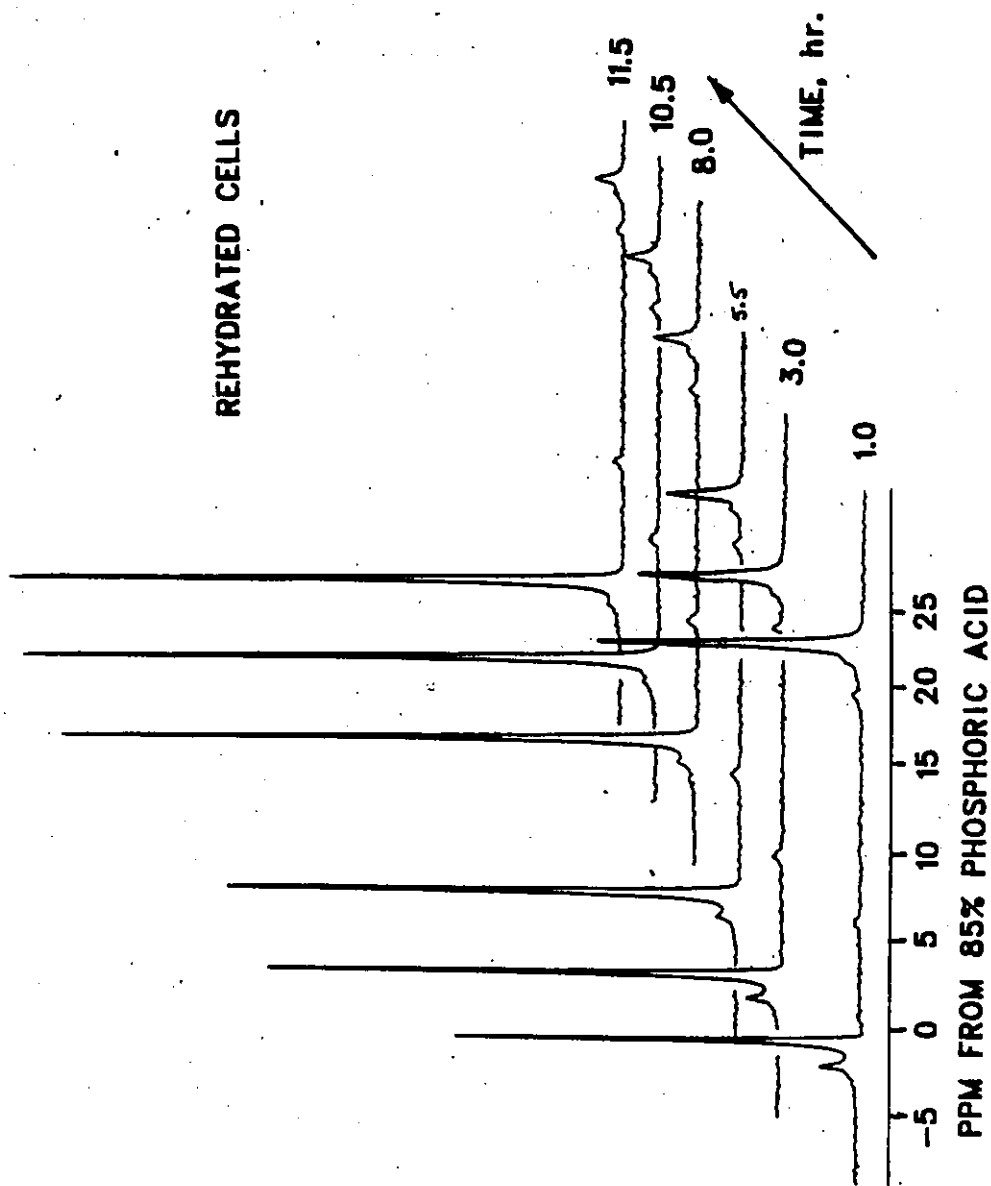


Figure VIII.10 -- Time course of spectra of rehydrated cells in 10 mM phosphate buffer. No differentiation between external and internal phosphate pools was observed. Polyphosphate and sugar phosphate pools decreased with time

extract. Next to the long chain polyphosphate peak at ~23 ppm, there is a peak due to a smaller chain length at 22 ppm, and the penultimate phosphate peak occurs at ~20 ppm. Line-widths remain constant during the time course and are somewhat narrower than those observed in viable culture (50 Hz for polyphosphate and 20 Hz for inorganic phosphate).

The polyphosphate degrades as a function of time, with very little change in the terminal group, indicating that the degradation is largely by hydrolysis of a single phosphate unit. The metabolite profile (Figure VIII.11), shows that the decrease in polyphosphate is mirrored by the increase in phosphate, as opposed to the precipitous drop observed for the anaerobic culture. The shape of the metabolite curves is similar to that of a first order reaction. The contribution of the sugar phosphate degradation to the inorganic phosphate pool is small. The viability curve shows that the suspension is at no time greater than 5% viable. Virtually no contamination was observed during viability measurements on lyophilized cells. Although lyophilized cells were successfully used to start inoculae for larger cultures, it appears that adequate nutrition is a requirement for growth from a desiccated state. The degradation pattern of polyphosphate here is due to lack of cellular regulation of enzymes and the resultant contact between the polyphosphate pool and the phosphatase pool within the cell. The relative increase in the

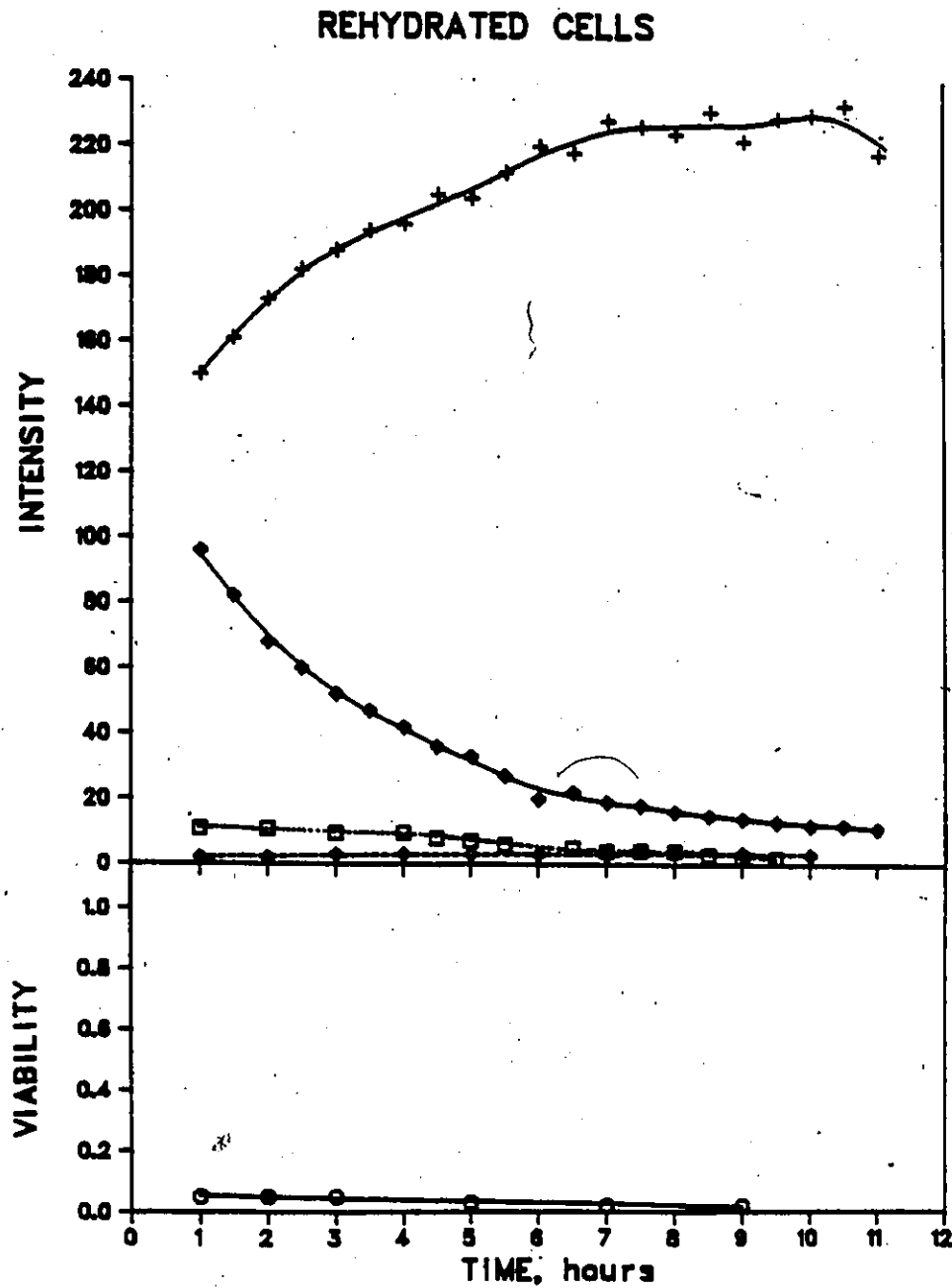


Figure VIII.11 - Time course of metabolite intensities for the spectra of the previous figure. Cells were not greater than 5% viable under these conditions. Changes observed are therefore due to simple enzymatic degradation in the absence of active respiration.

phosphate peak is the same as the relative decrease in polyphosphate, indicating that all of the polyphosphate is being converted to inorganic phosphate. If the polyphosphate pool is located in the central vacuole, this may indeed indicate that the polyphosphatase catalyzing this particular degradation is associated with the vacuolar membrane. Polyphosphatase has been associated with the cytoplasmic membrane in N. crassa (Kulaev et al., 1972(b)) and Baker's yeast (Souzu, 1967(b)). It has been shown that polyphosphatase is unaffected by freeze-thawing, which results in degradation of the polyphosphate to inorganic phosphate in Baker's yeast (Souzu, 1967(a)). It is possible that, like Baker's yeast, the degradation could occur through disruption of internal membranes by freezing, allowing the polyphosphate to reach the proximity of the cytoplasmic membrane. However, this would imply greater cellular damage on lyophilization than would be tolerated for the efficient growth that was observed for lyophilized cells when resuspended in minimal medium. The pH of the inorganic phosphate is measured by chemical shift to be that of the buffer (pH=5.5), indicating that the ionic strength effect due to ions of the cell is not significant. The pH remained constant throughout the experiment (see Figure VIII.22).

When a lyophilized suspension was aerated (Figure VIII.12, spectra not shown), the viability increased after a lag time

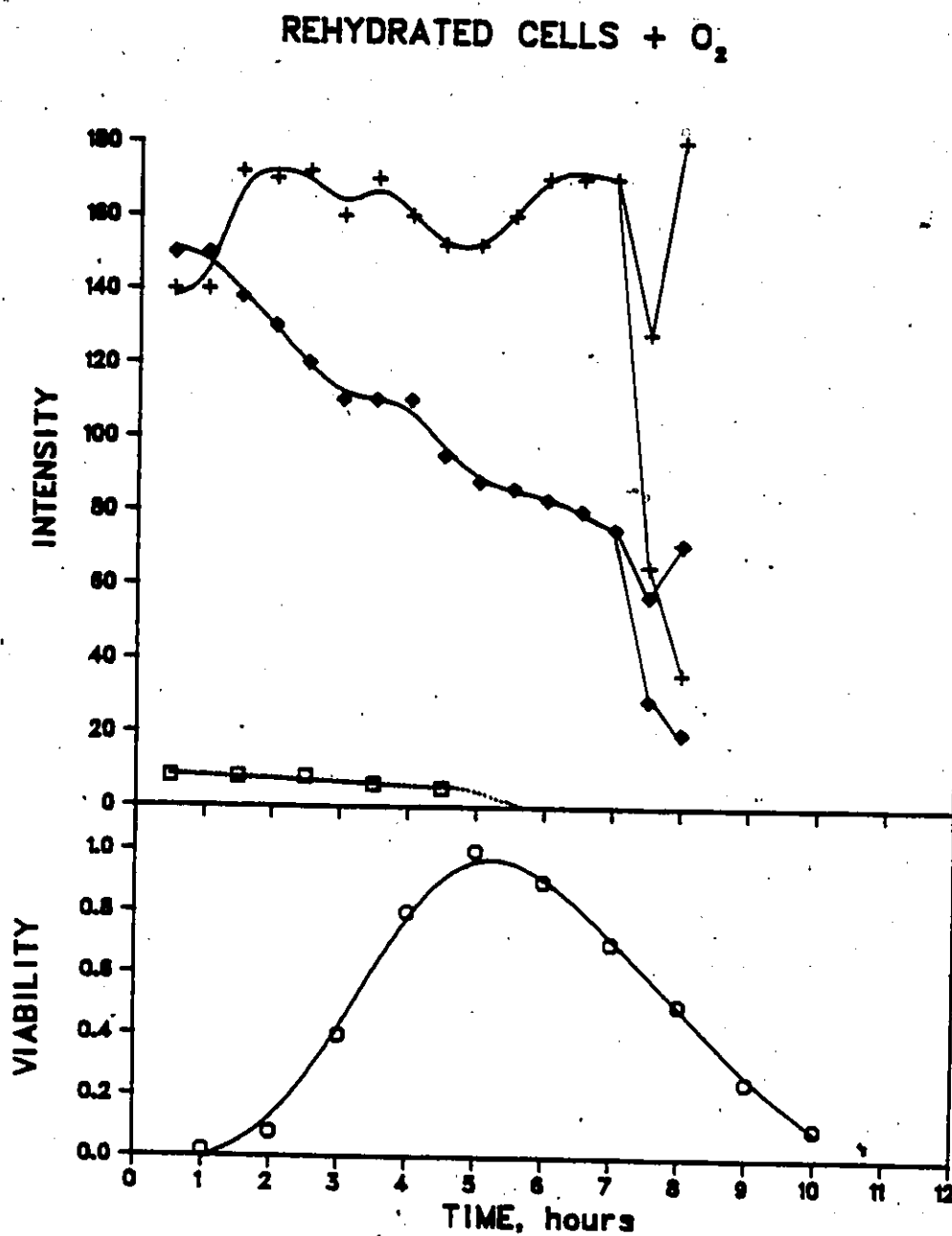


Figure VIII.12 - Time course of metabolite intensities of an aerated suspension of rehydrated cells. The resolution of two pools of inorganic phosphate and polyphosphate at ~7 hours may be correlated with the increased viability of the culture

and then decreased, due to lack of nutrients. This increase in viability is correlated with a more dramatic decrease in polyphosphate, the disappearance of sugar phosphate, and no dramatic increase in inorganic phosphate concentration. Levels of ATP were below the signal to noise. The phosphate released from the degradation of polyphosphate may enter into pools which are invisible on the NMR time-scale, i.e., RNA or DNA, for high activity accompanying the increase in viability. There is a slight increase in chemical shift of the polyphosphate (+0.2 ppm), indicating a greater extent of ion association. The two pools of polyphosphate are visible from the first spectrum, but as the culture begins to lose viability, an additional pool appears, on the high field side of the polyphosphate (23.5 ppm). Since this was observed on several occasions, interpretation of the nature of this new polyphosphate resonance is postponed until section (C). The inorganic phosphate resonance also splits into two peaks, reflecting the gain in viability. The pH of the external pool remains at the buffered pH, which although acidic, is not detrimental to normal fungi. As observed with the fresh culture, sugar phosphate levels were much lower in viable cells when oxygen was present.

(b) Effect of Starvation

Cell suspensions harvested after three days in starvation medium yielded a spectrum which showed no polyphosphate whatsoever (see Figure VIII.13). The spectrum shows no evidence

STARVED CELLS

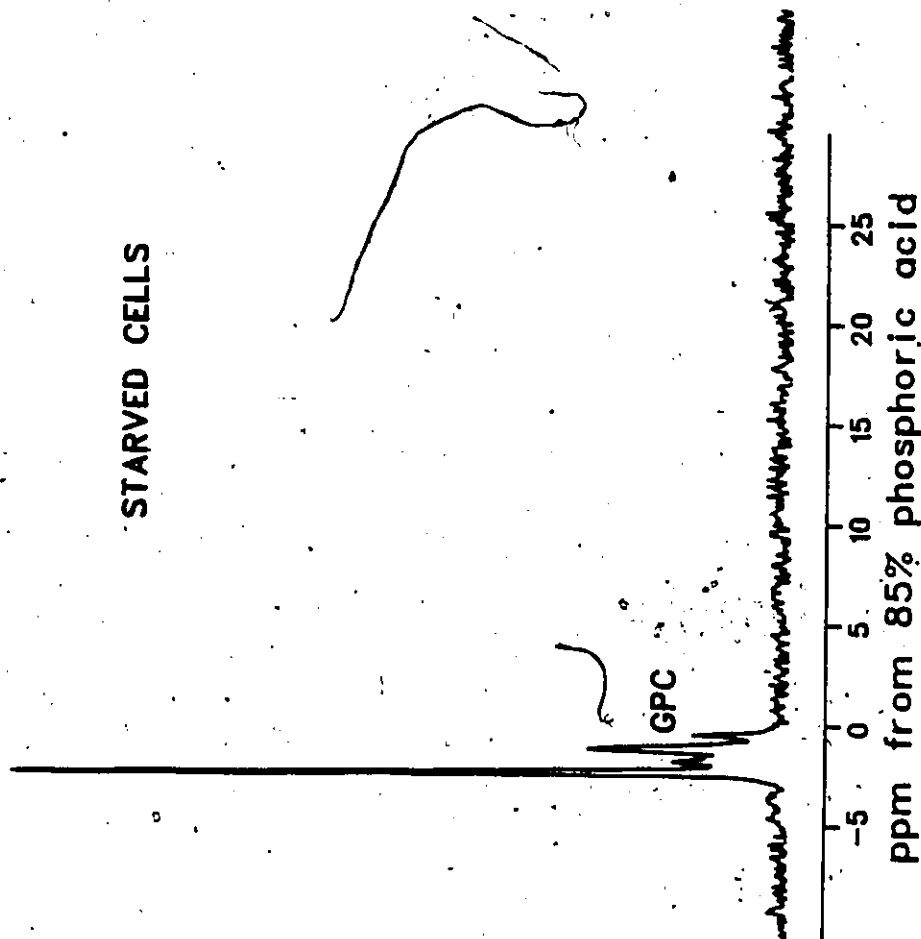


Figure 1.13 - ^{31}P 121 MHz spectrum of anaerobic cell suspension of starved cells, 0.25 hours after harvesting. ATP levels are very low and no polyphosphate peak is observed

for any polymeric forms of phosphate and ATP levels must be very low. The spectrum is dominated by peaks due to inorganic phosphate, of which three pools are resolvable, and a peak due to GPC, which appears much higher than for fresh cultures (Figure VIII.2). Thus, during carbon deprivation the cell appears to have utilized not only the triglyceride pool but the polyphosphate pool as well. The starvation medium contained the same concentration of inorganic phosphate as the original growth medium, and therefore the organism was not lacking a phosphorus source. This observation contradicts the theory that polyphosphate utilization reflects a phosphate deprivation.

In order to check that this interpretation is correct, and that the polyphosphate has not precipitated or otherwise become more rigid, so as to be not resolved in the NMR spectrum, a perchloric acid extract was performed. The titration curves of the observed peaks are shown in Figure VIII.14. The assignments of the titration curves are the same as for Figure VIII.3. No polyphosphate was observed in the extract spectrum. Sugar phosphates (A and B), ATP, (H, K, and M), and UDPG (L) were present, but ADP levels were barely resolvable. The ADP/ATP ratio was much lower than in the normal cells. This, combined with the lack of a peak due to degraded RNA and DNA in the extract spectrum, is indicative of the reduced metabolic activity of these

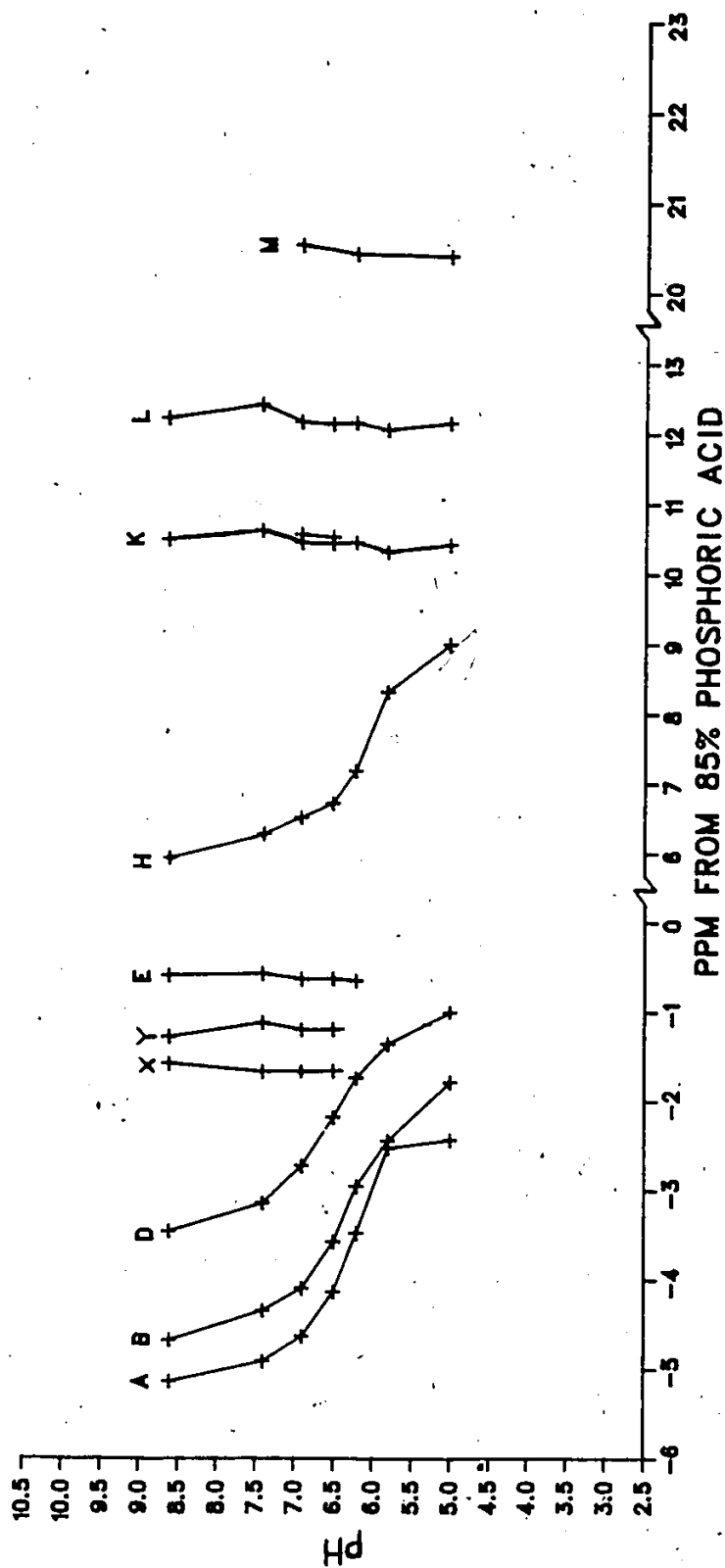


Figure VIII.14 - Titration curves of spectral peaks for a perchloric acid extract of starved cells. Identities of the curves are the same as for Figure VIII.4. Additional peaks were observed and are identified as GPS or GPI (X) and PGE (Y). ATP is present in low levels (curves H, K and M), too low to be observed in the spectrum of the intact cells (previous figure)

cells, which was suspected from the non-granular appearance of the cytoplasm (see Plate VII). The presence of inorganic phosphate (D) confirms the assignment of the cell spectra to only inorganic phosphate and GPC. In addition to the pH independent peak of GPC (E), two other pH independent peaks appear, due to GPE (Y) and either GPS or GPI (X). Confirmation of the latter peak was not obtained due to lack of suitable standards. However, it might be expected that these breakdown products of the major phospholipids of the cell would occur in the same ratio as in the phospholipid fraction, where PC, PE, PI and PS appear in order to decreasing amount. The amount of glycerophosphate is greater than in the fresh cell extract, likely due to significant breakdown of phospholipid by β -oxidation and reduced turnover of the lipid pool.

The viability curve and relative metabolic levels for the starved cell suspension are shown in Figure VIII.15. The most notable observation is that in spite of the deprivation of carbon for a period of three days, the culture is 100% viable. It also appears to be more able to withstand the increased cell density and anaerobic conditions imposed on it in the NMR tube than the fresh culture. Even after 10 hours, the culture is 40% viable. The husbanding of resources and the low metabolic activity must reduce the cell to a state similar to that found in spores. During the initial two hours, when viability is maintained, the level of sugar phosphate is

STARVED CELLS

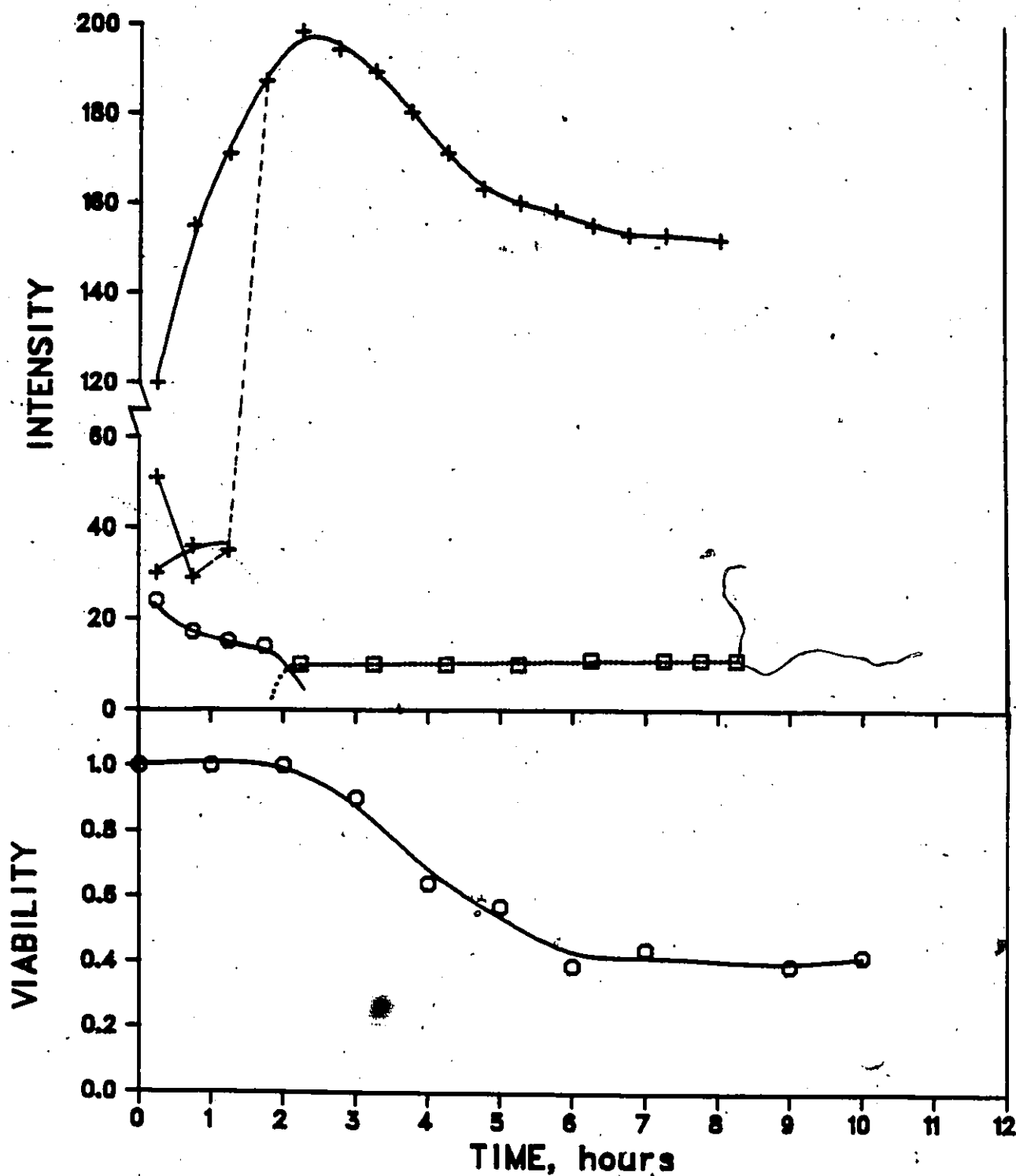


Figure VIII.15 - Time course of metabolite levels in the anaerobic starved cell spectrum, as correlated with the viability curve. The resolution of three inorganic phosphate pools disappeared after 2 hours, concomitant with the disappearance of GPC and appearance of a larger sugar phosphate pool

below observation and the total phosphate level is maintained. The sum of the intensities of all the compartments is approximately equal to the total phosphate observed when pH gradients are no longer discernible. At the point when the culture begins to lose viability, the pH gradients disappear, GPC disappears (likely hydrolyzed), and sugar phosphates appear, maintaining their level for the duration of the experiment. The reduction of the inorganic phosphate peak may be due to donation to the sugar phosphate or other invisible pools. The culture appears to be able to maintain relative viability without a pH gradient, or at least reduce that gradient below spectral resolution. The linewidth of the inorganic phosphate peak increases from 23 Hz to 40 Hz during hours 1 to 3, indicating that there are likely two pools of inorganic phosphate which are very close in pH, whose peaks overlap considerably. Since maintaining a pH gradient requires metabolic energy, this may be a mechanism by which the organism conserves its energy resources, and also be a reflection of the slowed metabolic activity. If the major phosphorylating agent in the cell is sugar phosphate, this may also be the reason why sugar resources in the starved cell are maintained, as was observed by ^{13}C NMR.

Aeration of the starved cell suspension (Figure VIII.16), produced no differences from the anaerobic situation other than the retention of pH gradients for longer periods of time, the retention of GPC for the duration of the experiment and

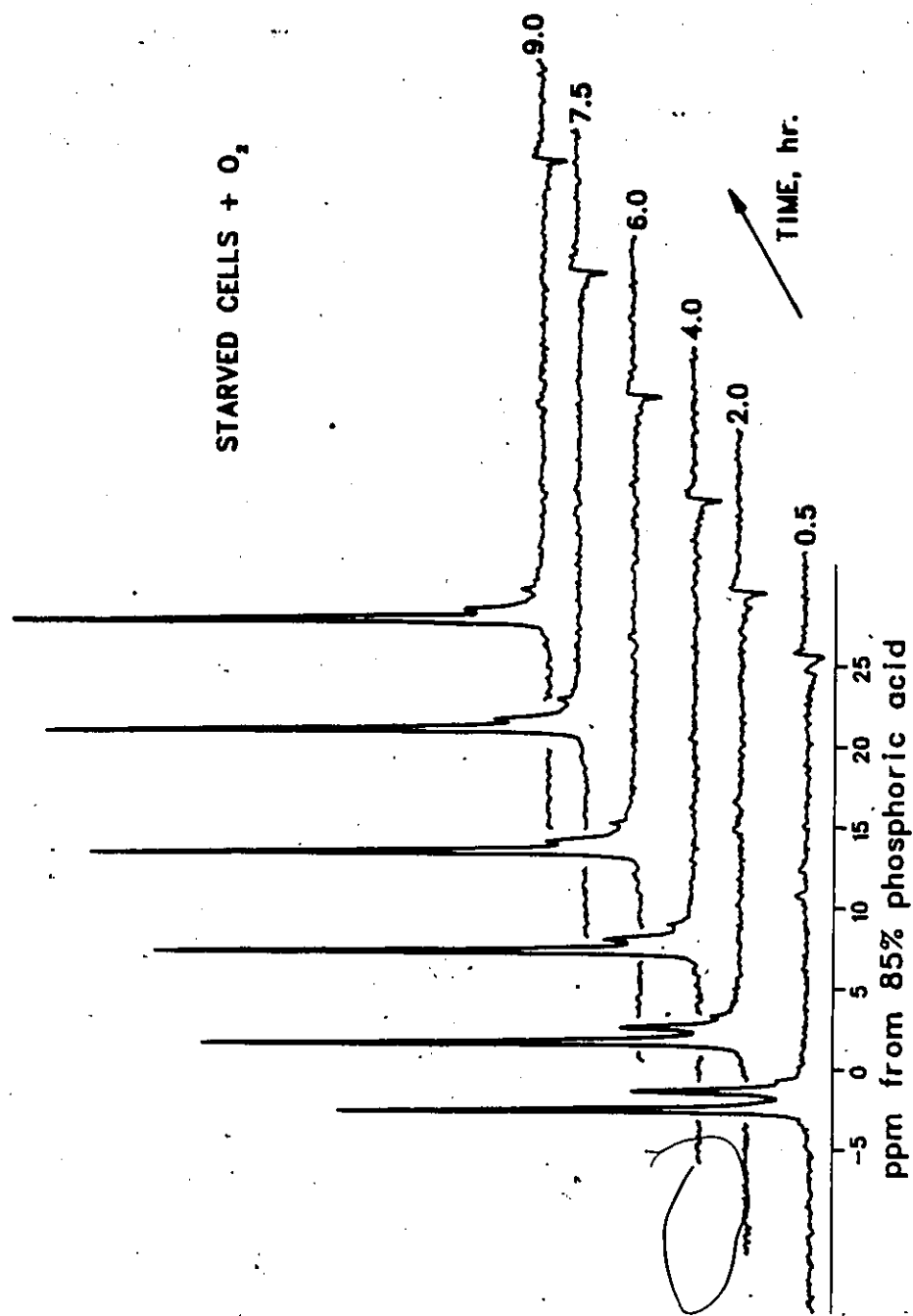


Figure VIII.16 - Time course of phosphorus spectra of an aerated suspension of starved cells. Small levels of ATP can be seen in the initial spectrum. The unphased peak at ~25 ppm is due to the transmitter

the lack of observation of sugar phosphates. Small levels of ATP and UDPG were visible in this case and persisted for ~9 hours. The linewidth of the inorganic phosphate remains ~30 Hz, indicating that the observed pools are unique and not overlapped as observed previously. The more intense of the two inorganic phosphate peaks is at higher pH, and is therefore assigned to the internal pool. The internal pool increases slightly at the expense of the external pool (Figure VIII.17), over the first 3 hours followed by maintenance of both pools. The metabolic profile also shows the maintenance of GPC. The other glycerophosphates are not resolved. The cell suspension can therefore maintain a phosphate concentration gradient across the cell wall as well as a pH gradient, although the concentration gradient appears to be independent of aeration and the pH gradient increases on aeration. This indicates that phosphate transport across the cytoplasmic membrane is controlled by more than simple diffusion. This is not a surprising result since phosphate uptake in yeast is known to be an energy consuming process and yeast have the ability to preserve large intracellular concentrations against a dilute aqueous medium (Rothstein, 1961). This ability is one of the factors which account for the preservation of viability in distilled water. A higher inorganic phosphate content may be an osmoregulatory device to counteract the reduced cytoplasmic density. By analogy

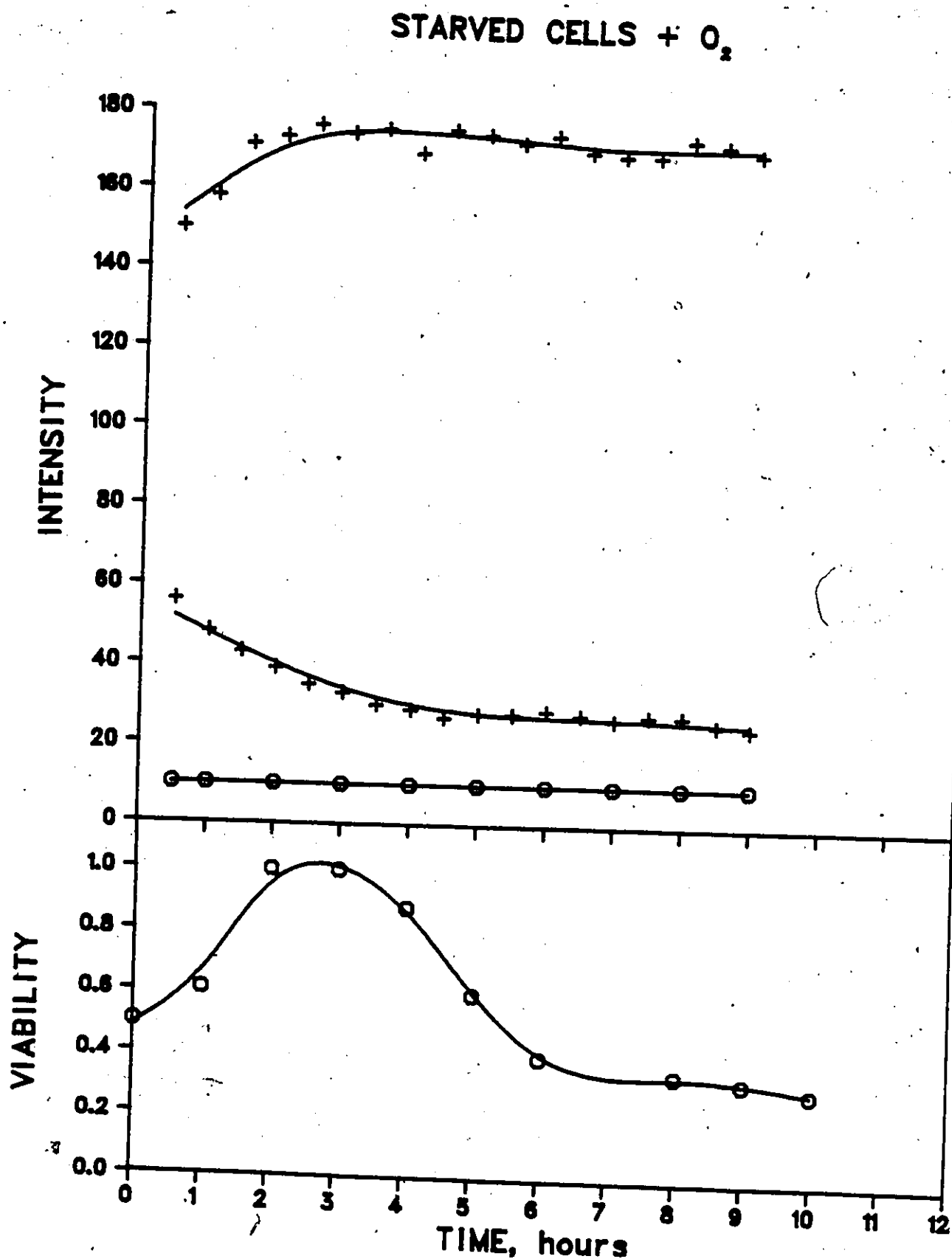


Figure VIII.17 - Metabolite levels as a function of time for inorganic phosphate and GPC for the spectra of the previous figure. Under oxygenation, the viability of the culture is enhanced and then falls off

with amino acids, the inorganic phosphate, if largely retained within the central vacuole, may combine with the enhanced acid pool to enlarge this vacuole, giving rise to the appearance of the cell as shown in Plate VII. In addition, the energy given off in hydrolysis of the polyphosphate may have been used by the cell in the utilization of its fatty acid stored.

The viability curve for the aerated cell suspension resembles that of the anaerobic one except for the increase in viability during the first 3 hours, which seems to be reflected by the small changes in inorganic phosphate pools. The effect of aeration on the starved culture is very similar to that of the lyophilized cells (Figure VIII.12). Since the viability curves are normalized to the highest plate count, the appearance of lower viability at times zero is anomalous. 100% viability for the anaerobic starved cell suspension represents one colony per observable cell, i.e., the plate count at time zero is that expected on the basis of cell density. The viability at time zero for the aerobic culture is 80-100% of that expected on the basis of cell density. Thus the increase in viability at hour 3 represents greater than 100% viability, likely due to faster replication stimulated by the presence of oxygen. Since the colonies tended to clump, it is possible that a single cell could give rise to more than one colony if the cell doubling time was much faster. Thus the viability curve for the aerated culture is

displaced above that for the anaerobic culture, and the viability is generally preserved throughout the experiment, as reflected by the NMR results.

The pH behaviour of the two starved cell suspensions is shown in Figure VIII.18. The intracellular pH of the aerobic culture is maintained at pH=6.6-6.8 ($\square$ --- $\square$), while the external pH climbs from the buffered value of 5.5 to 6.6. Since the buffer concentration is much less than the intracellular concentration, it is subject to pH changes imposed by products expelled by the cell. The suspension experimental determination of chemical shifts, since the presence of GPC, whose chemical shift is pH independent, provides an internal standard. The anaerobic starved cell suspension loses its pH gradient at the point when it begins to lose viability. Again the external pH (Δ --- Δ) rises from the original value to meet the more basic pH of the internal environment (pH=6.7). The behaviour is qualitatively the same as for the fresh cell suspension (Figure VIII.9), except that the increase in viability is reflected by a generally higher intracellular pH. The initial behaviour indicates that the starved cell pH, both cytoplasmic and mitochondrial, is somewhat higher than cells in early stationary phase. This is somewhat inconsistent with the seemingly slowed metabolism of the starved cell. For both bacteria and yeast, a slowed metabolism, as in dormancy, is characterized by a higher pH (Barton *et al.*, 1980), although

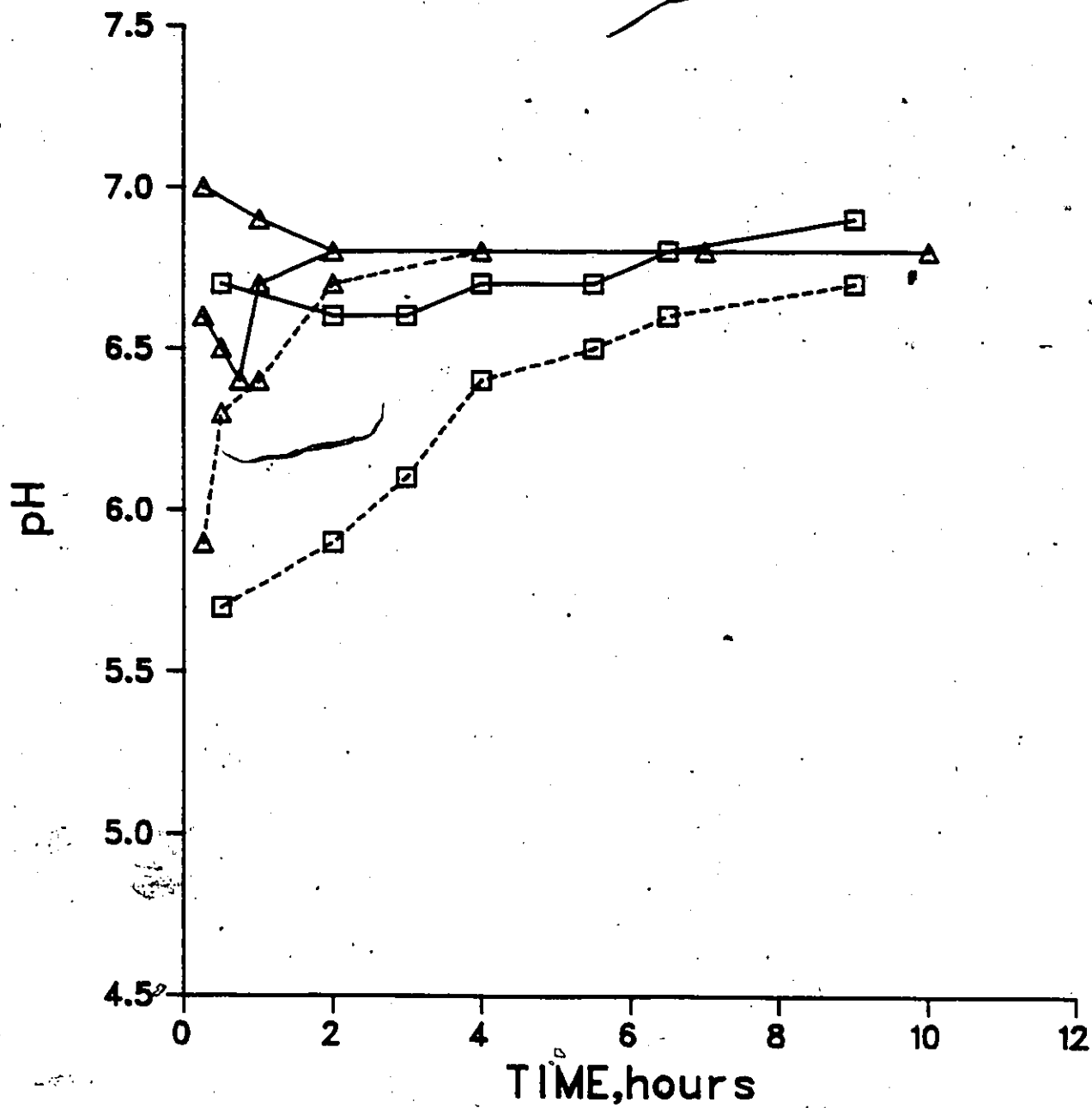


Figure VIII.18 - Time dependence of pH for anaerobic and aerobic starved cell suspensions. The codes are the same as for Figure VIII.9

is not true for Acanthamoeba castellanii (Deslauriers et al., 1980(b)). However, the maintenance of a higher pH, as well as the ability to maintain a phosphate gradient, in the face of high cell density, is consistent with the increased viability.

(c) Effect of Nystatin

Nystatin is a common antifungal polyene antibiotic whose action is mainly on the cytoplasmic membrane. The mechanism of action is thought to be based on a specific interaction with membrane sterols that results in a changed permeability of the membrane (Finkelstein and Holz, 1973; Bittman, 1979). The antibiotic also inhibits yeast glycolysis (Marini et al., 1961) and fermentation, perhaps by increasing the proton permeability of the plasma membrane (Palacios and Serrano, 1978).. The affinity of nystatin for sterols is greatest for ergosterol, the most common yeast sterol, and the sensitivity of an organism to nystatin may be correlated to the ergosterol content (Lampen et al., 1960). Resistance to nystatin is associated with high media concentrations of sterol (due to binding of nystatin by the free sterol (Lampen et al., 1960)), an ability to halt sterol synthesis such that the ergosterol content within the yeast is replaced by precursors of ergosterol (to reduce the binding capacity (Fryberg et al., 1974, 1975)), and modifications in the total fatty acid composition in yeast (Nagai et al., 1981).

The exact mechanism of action of nystatin is not yet clear. There is much evidence that the antibiotic causes leakage of cations through pores in the membrane which are formed from sterol-antibiotic complexes (Finkelstein and Holtz, 1973). Others believe that it affects membrane transport systems in which sterols play a role (Komor et al., 1974). It is true that nystatin binds irreversibly at the yeast cell wall, likely to ergosterol as the binding site (Lampen et al., 1962), and that the cytoplasmic membrane is the chief point of attack. Mitochondrial membranes contain very small amounts of sterol and are much less sensitive to polyene antibiotics than other membranes (Kinsky et al., 1965). Treatment of A. pullulans with nystatin produces visible lysis of the cytoplasmic membrane, especially at the bud scar, but internal structures remain intact (Plate VIII, Chapter III). This was the basis of the membrane preparation used for purposes of measuring T_1 's (Chapter V). The effect of nystatin is greatest at acidic pH, likely due to greater binding (Lampen et al., 1957). Thus, its lytic effects are maximized at the pH of the buffer used in these experiments (pH=5.5).

The effect of 5 mg of nystatin on the cells of 50 ml culture is shown in the phosphorus spectra of Figure VIII.19. The small amount of ATP visible in the initial spectrum immediately disappears, along with a dramatic decrease in polyphosphate. After 4 hours, there is only inorganic phosphate

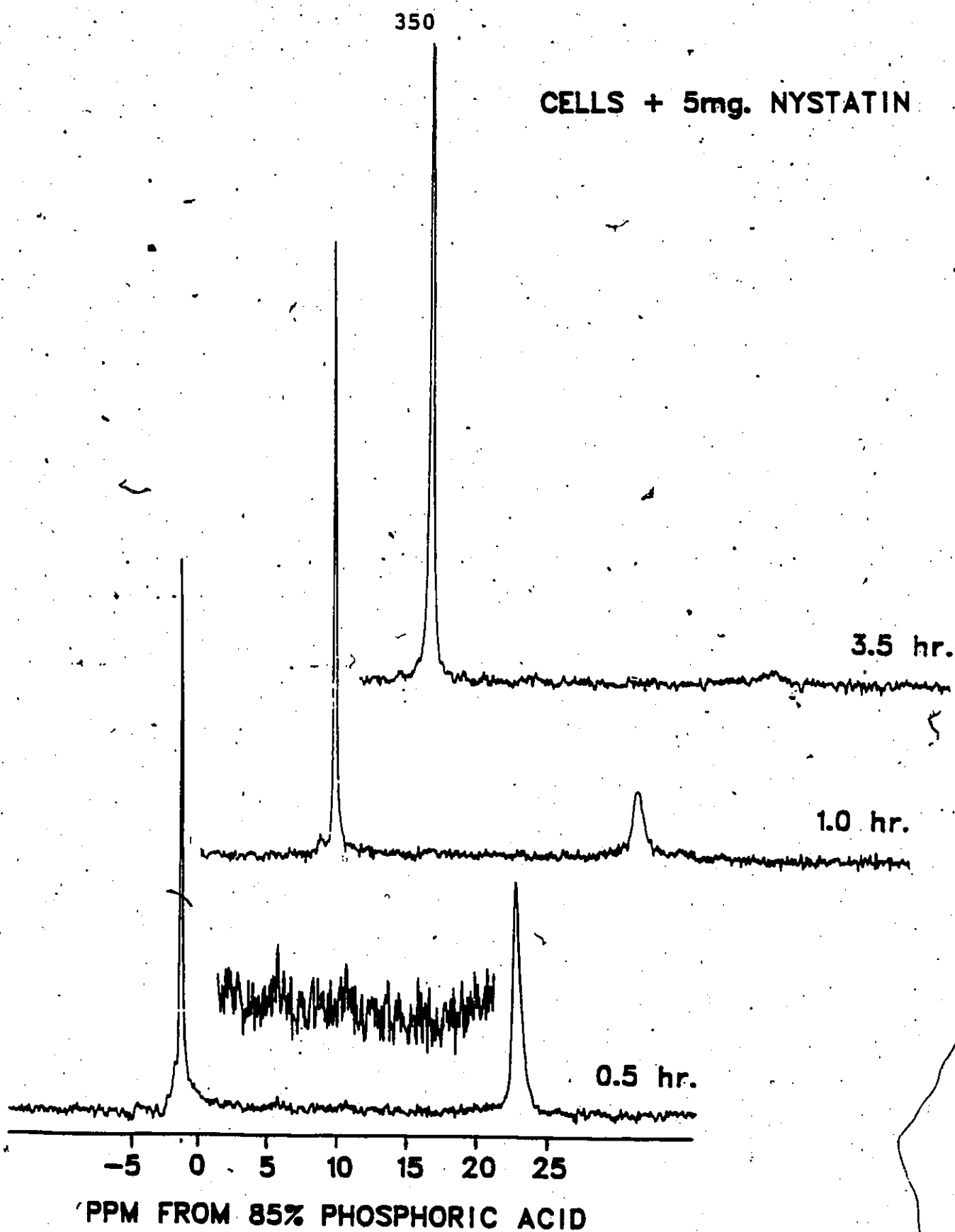


Figure VIII.19 -- Time course of phosphorus spectra for an anaerobic cell suspension treated with 5 mg nystatin

present. The brief hint at inorganic phosphate compartmentalization disappears after the first spectrum. There are no changes in linewidth throughout the experiment, the inorganic phosphate and polyphosphate lines remaining at 30 Hz and 80 Hz respectively. The detailed time course of the sugar phosphate, inorganic phosphate and polyphosphate are shown in Figure VIII.20. The viability of the cell suspension drops quickly during the first hour after treatment, and loss of viability is complete by four hours. This can be confirmed by phase contrast microscopy, since all cells appear lysed. This trend is reflected in the polyphosphate and inorganic phosphate intensity curves, which indicate that essentially all the polyphosphate is converted to inorganic phosphate. When the polyphosphate disappears, the level of inorganic phosphate remains constant. Sugar phosphates also disappear on lysis. The fast degradation of polyphosphate and the retention of narrow linewidth are spectral indications of lysis. This dosage of nystatin under anaerobic conditions appears to be immediately lethal.

If the dosage is reduced to 1 mg, the lytic effects are not as immediate, as shown in Figure VIII.21. The spectra are not shown in order to reduce the number of figures, but they show that in this case, the linewidths of both the inorganic phosphate and polyphosphate increase by ~5 Hz during the first 3 hours, while the culture remains relatively viable, indicating that there is minimal lysis during this

CELLS + 5mg. NYSTATIN

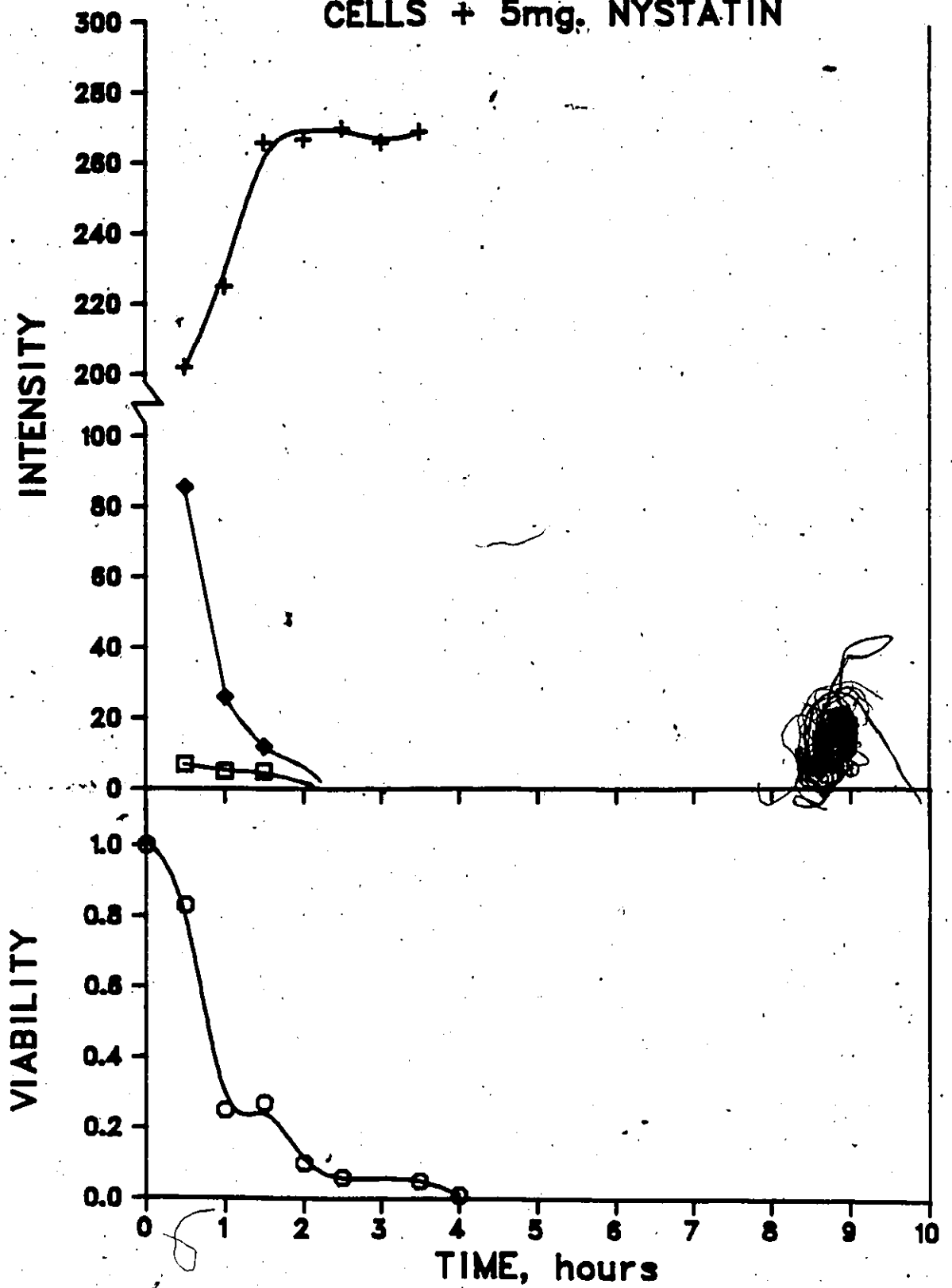


Figure VIII.20 - Metabolite levels as a function of time for the experiment of the previous figure, as correlated with the viability curve

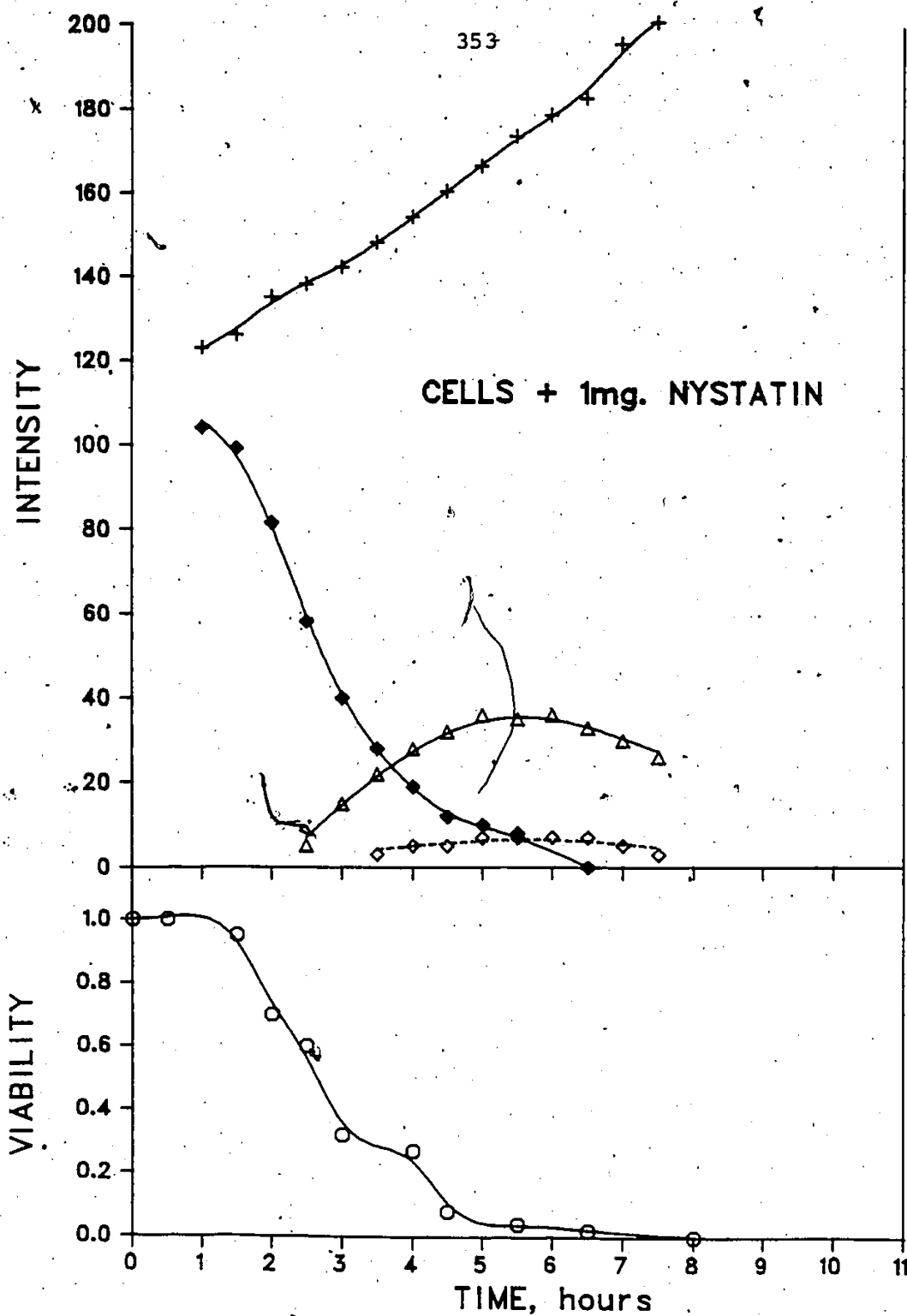


Figure VIII.21 - Time course of metabolite levels for an anaerobic cell suspension treated with only 1 mg nystatin (spectra not shown). The viability curve is not as precipitous as with the higher dosage, and pyrophosphate appears in the cell spectrum

period. The polyphosphate intensity drops precipitously during this initial time period, indicating that the polyphosphate is being broken down but at a greater rate than for resting cells or due to uncontrolled enzymatic action (shown by the rehydrated cell suspension). The culture loses viability over a greater period of time with this lower dosage, and the loss of viability is signaled by the appearance of pyrophosphate, which increases as the culture viability drops. The complete loss of viability signals the beginning of reduction of pyrophosphate due to enzymatic breakdown after complete lysis. The increases in the terminal phosphate peak shows that the polyphosphate chains are getting much shorter at low intensity. This does not appear to be consistent with the slight broadening of the polyphosphate peak, since it would be expected that shorter chains would be more mobile (i.e., would have longer T_1 's and narrower lines). However, this may indicate a greater extent of metal binding, an effect which reduces T_1 . The appearance of pyrophosphate appears to be associated with a gradual decrease in viability, as was observed for the anaerobic control experiment. Under greater dosage of nystatin, the culture loses viability before it has a chance to begin the production of pyrophosphate. This indicated that the appearance of pyrophosphate requires some environmental stimulus such as low intracellular pH in response to environmental distress, or lack of oxygen. The increase in

inorganic phosphate, does not merely reflect the decrease in polyphosphate as in the previous figure, but polyphosphate appears to be the donor to both pyrophosphate and inorganic phosphate pools. In spite of the fact that the culture remains viable for the initial 3 hours, there is no, or only a very small, pH gradient maintained with the external environment.

The pH behaviour for the two doses of nystatin is shown in Figure VIII.21, along with that for the rehydrated culture. The latter (☒) shows that the pH measured by NMR agrees with the electrode measurement. The pH adopted by the system under lower dosage of nystatin (V) is slightly higher than that with the higher dosage (Δ), perhaps reflecting less acidosis associated with less cell lysis. The culture appears to be able to tolerate low intracellular pH, since it has essentially no pH gradient while still maintaining viability for the first few hours after treatment. The lack of a pH gradient in the first spectrum may be due to a short time lag between treatment and starting the NMR experiment. The pH gradient for the higher dosage disappears after the first spectrum and the pH drops to a value slightly above that of the external buffer due to lysis and mixing of the cell contents with the dilute buffer.

Oxygenation of the cell suspension appears to increase the cell resistance to the antibiotic. Figure VIII.23 shows the spectra for an aerated culture treated with 5 mg of

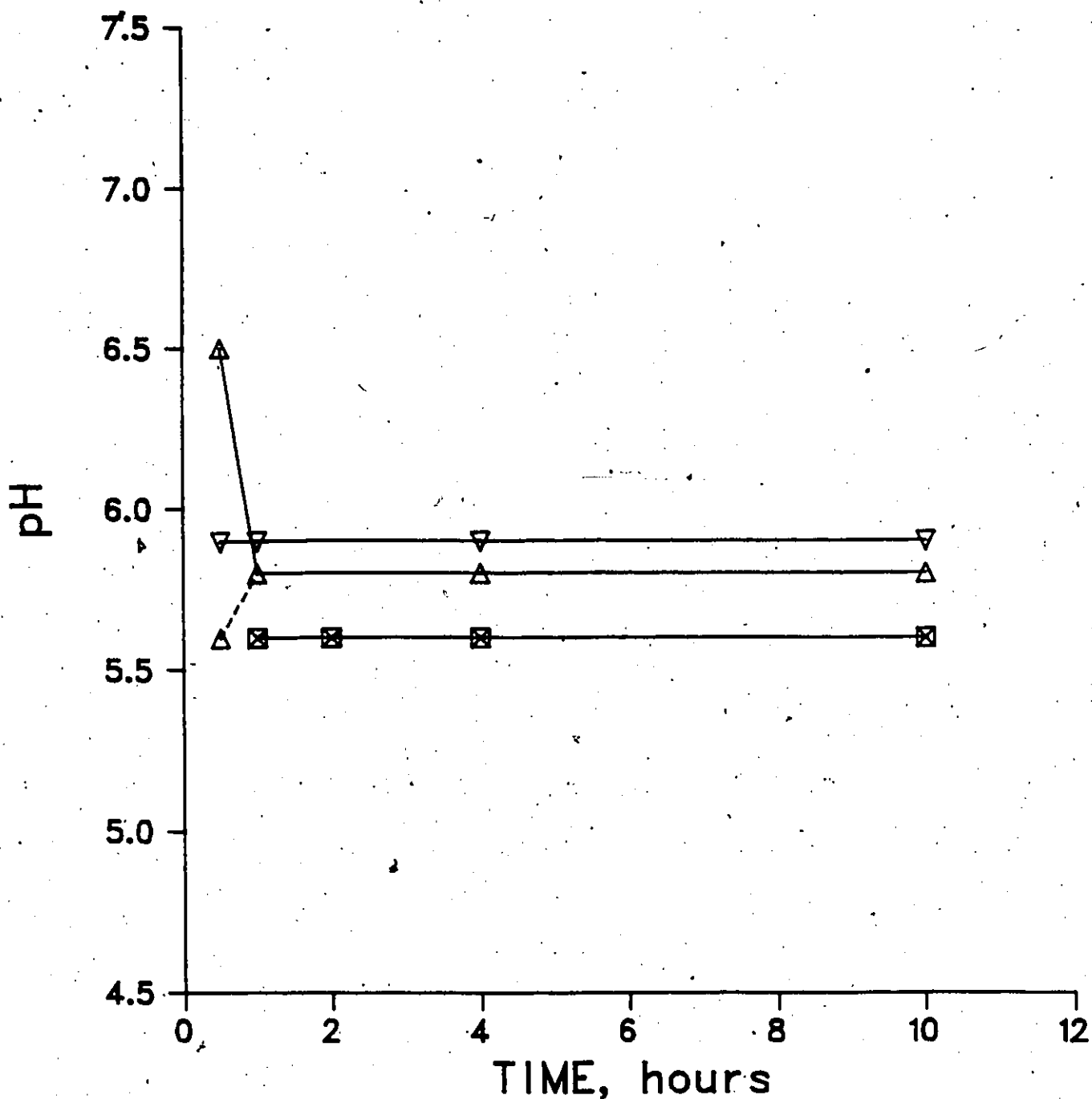


Figure VIII.22 - pH behaviour of the cell suspensions treated with 1 mg (▽) and 5 mg (Δ) nystatin, and rehydrated cells (⊠). A small pH difference between inner and outer pools was observed for the first spectrum of the 5 mg treated suspension, but was gone by the second. The lack of appearance of two pools for the lesser dosage may be due to a delay in starting the experiment. The rehydrated cells show that the measured pH is approximately as expected for the phosphate buffer

CELLS + 5mg. NYSTATIN + O₂

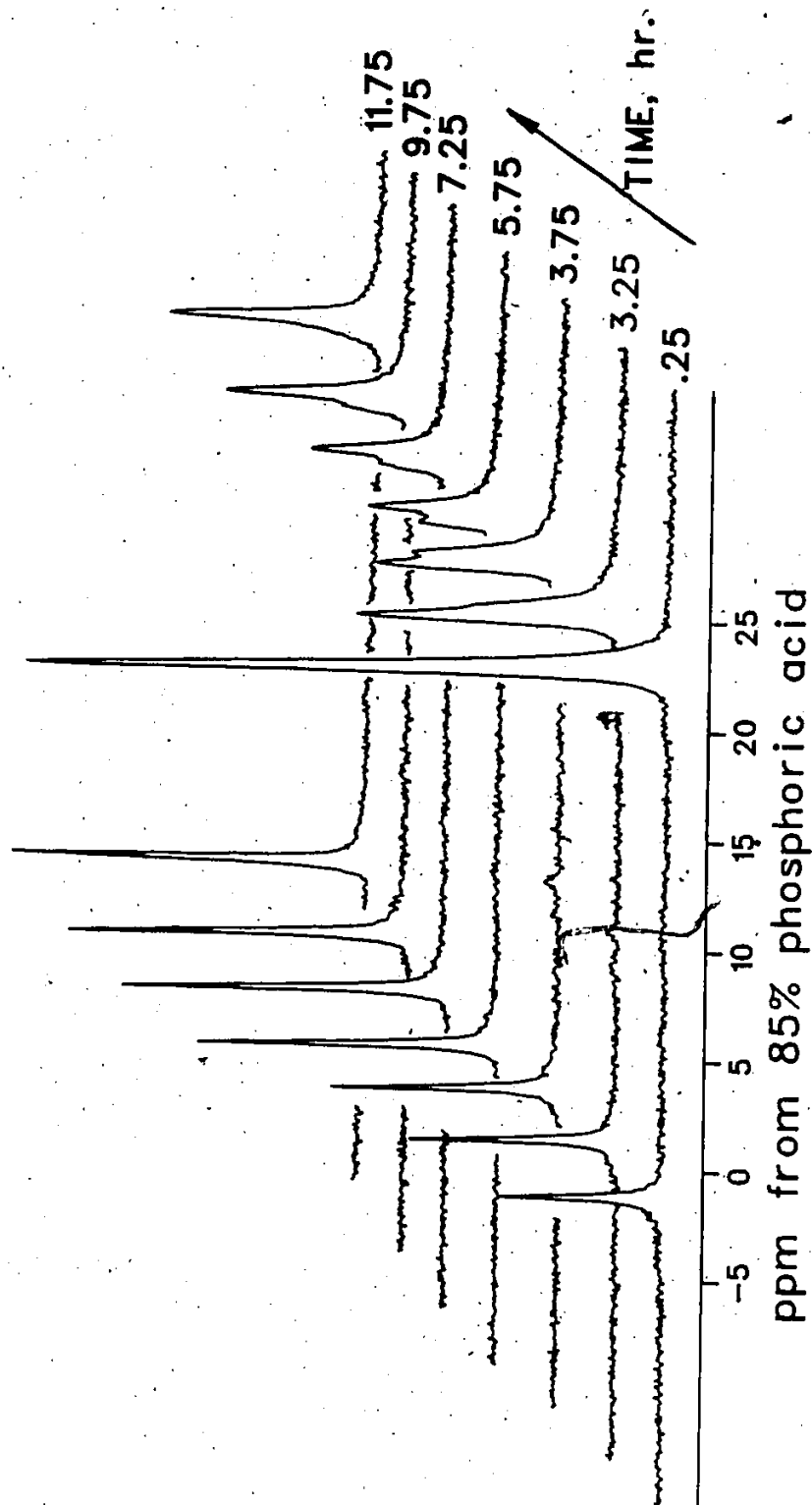


Figure VIII, 23 - Time course of spectra for an aerobic suspension of cells treated with 5 mg nystatin, showing the appearance of two pools of polyphosphate

nystatin. The polyphosphate peak decreases with time but does not disappear. A second polyphosphate peak at higher field appears at ~3 hours and remains for the duration of the experiment. The linewidths of the polyphosphate peaks do not change during the experiment. The second peak, which occurs at 23 ppm, may be due to a second polyphosphate pool of higher ionic binding, but cannot be due to a longer chain pool of polyphosphate, since the original polyphosphate pool, occurring at 22.5 ppm shows no sign of being reduced to shorter chain lengths (the peak due to terminal phosphate does not increase). For chains longer than ~20 units, the chemical shift of polyphosphate is no longer sensitive to the chain length. Greater ion binding is not consistent with the lytic action of nystatin, and indeed the extent of ion binding would have to be very great to produce a change in chemical shift of 0.5 ppm. This second pool grows in response to the decrease of the original pool so that the total polyphosphate content remains constant after ~4 hours. The inorganic phosphate pool increases slowly but shows no resolution of inner and outer pools.

The viability curve (Figure VIII.24) indicates that the effect of aeration is to tolerate the presence of the antibiotic for some time before the effect takes hold and viability drops in a precipitous manner as before. During the period of retained viability, the phosphate levels are preserved,

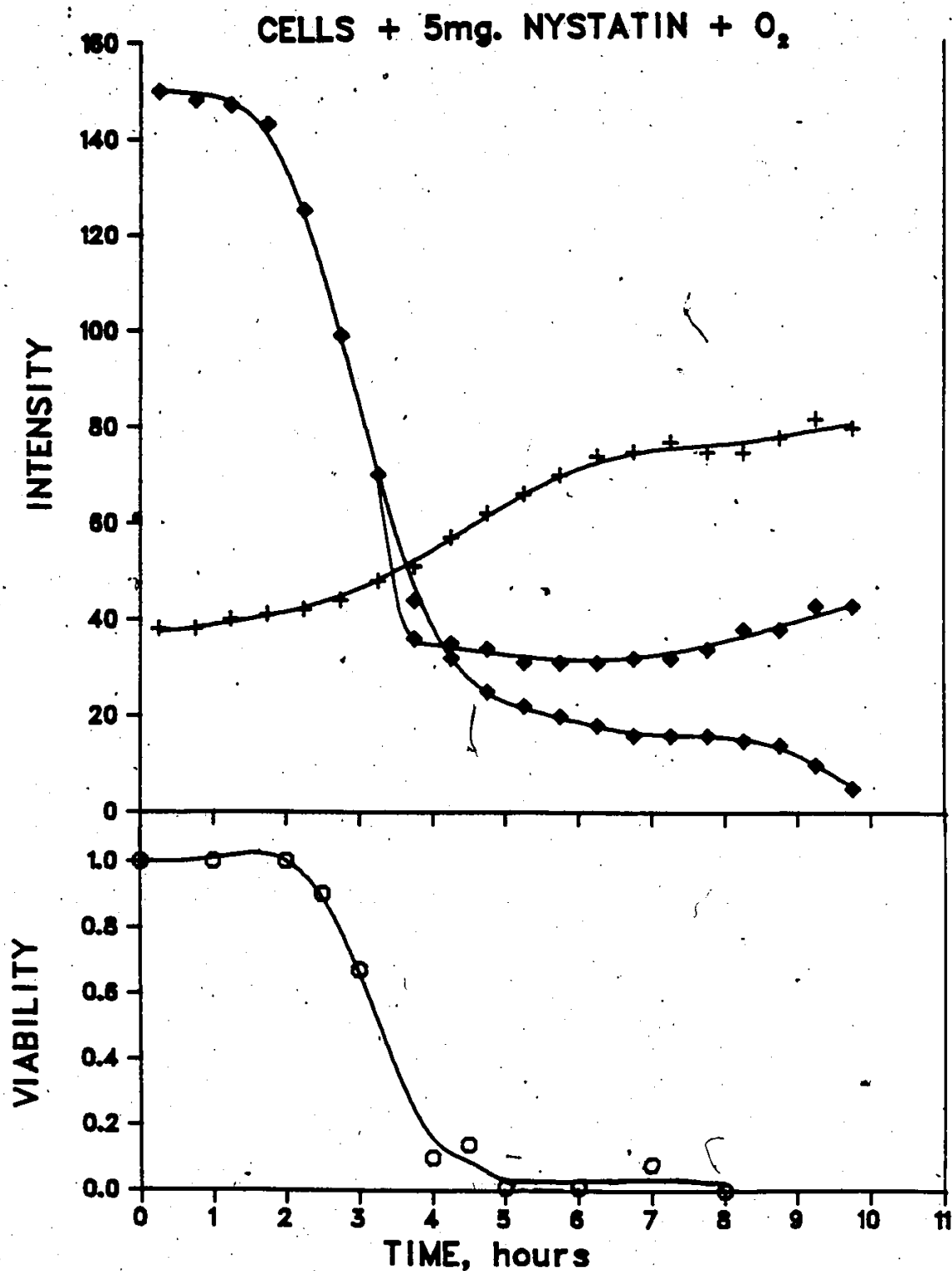


Figure VIII.24 - Viability, inorganic phosphate and polyphosphate curves for the experiment of the previous figure. The appearance of a second polyphosphate pool may be due to undegraded polyphosphate released into the medium by cell lysis

including low levels of ATP. At the point when loss of viability begins, there is a dramatic decrease in polyphosphate, the appearance of the second polyphosphate pool, and an increase in inorganic phosphate which is not sufficient to account for the decrease in polyphosphate. When viability is zero, levels again remain relatively constant. In this case, the loss of viability is too fast for the stimulation of pyrophosphate production. The polyphosphate does not completely disappear on complete loss of viability as was observed for the parallel anaerobic experiment. The presence of oxygen may slow the hydrolysis of polyphosphate by affecting the phosphatase that controls it, either directly or through a pH effect, since significant residual polyphosphate was observed for all aerated cell suspensions even after loss of viability.

The identity of the higher field polyphosphate pool is puzzling. The appearance may be correlated with the availability of oxygen and the presence of extensive lysis, since this occurred in the rehydrated aerobic cell suspension and to a very small extent under a lower dosage and aeration. The chemical shift is consistent only with hexametaphosphate, the cyclic phosphate of a six monomer unit chain. Hexametaphosphates can occur in isolated polyphosphate from microorganisms, and short chain polyphosphate have been shown to cyclize readily in the presence of Mg^{2+} and heat (Harold,

1966). The chemical shift of hexametaphosphate is relatively independent of the extent of ionic binding (Glonek et al., 1971), and titration of a standard phosphate glass mixture with Mg produces the observed chemical shift for hexametaphosphate, for a magnesium concentration which matches the chemical shift of the linear polyphosphate. It is likely that hexametaphosphate forms in the suspension from shorter polyphosphate released into the medium through lysis of the cells. However, the cyclization reaction appears to require the presence of oxygen. Further proof that this peak is not due to an additional pool of linear polyphosphate is provided by the fact that the T_1 's of the two polyphosphate peaks are approximately the same (see Section (E)). A significant increase in ion binding would be expected to result not only in a upfield shift, but also a drastic shortening in T_1 .

The initial and final spectra from an aerated cell suspension treated with the lower dosage (1 mg) of nystatin is shown in Figure VIII.25. In the same manner as for the anaerobic analogue, there is an appearance of pyrophosphate, accompanied by a significant shortening of the polyphosphate chain length, as witnessed by an increase of the terminal and penultimate peaks. As expected from other aerobic experiments, there is a pH gradient preserved across the cytoplasmic membrane for the duration of the experiment. The disappearance of the low levels of ATP and UDPG (seen in the

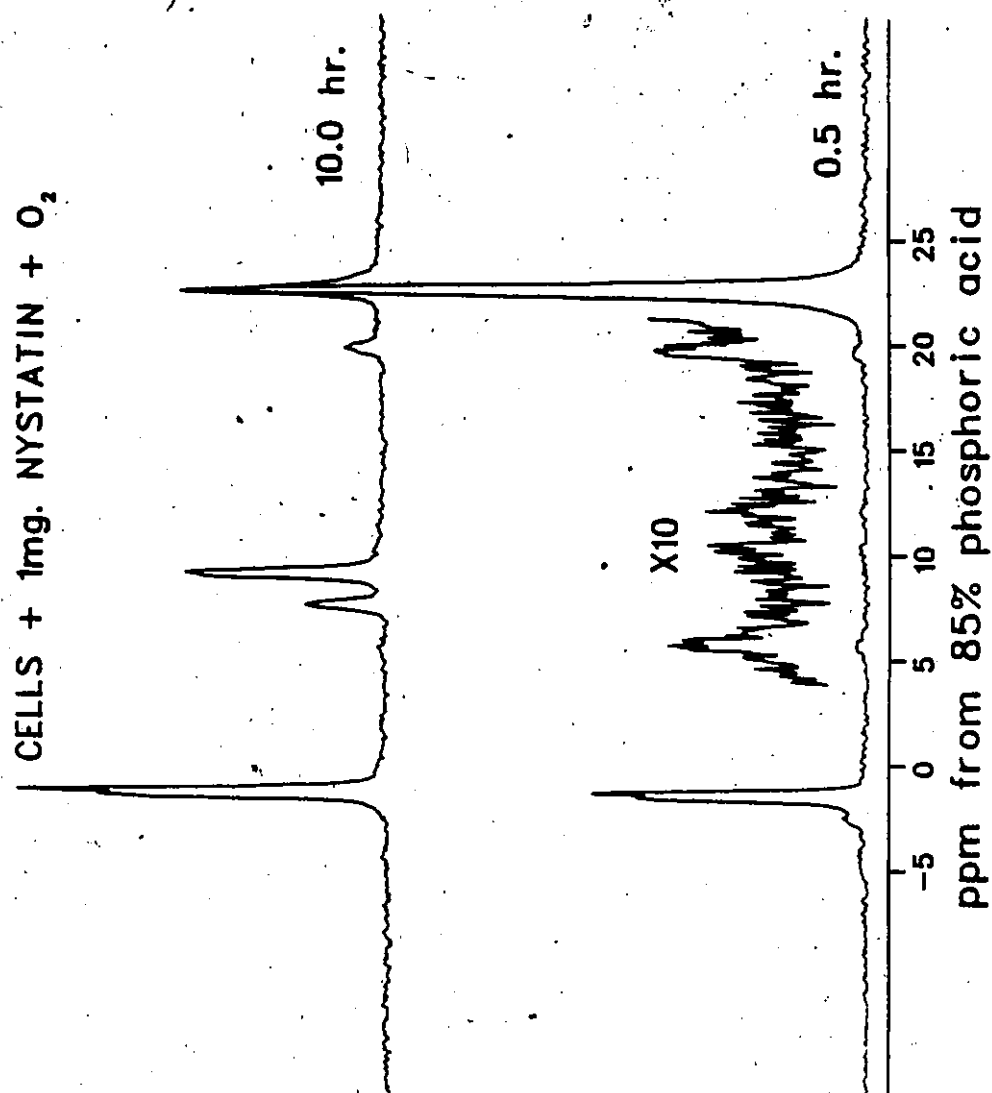


Figure VIII.25 - Initial and final spectra from an aerobic cell suspension treated with 1 mg nystatin. Pyrophosphate appears as for the anaerobic culture, and pH gradients appear to be preserved

enhanced sensitivity inset) is again concomitant with the appearance of the pyrophosphate peak. The production of pyrophosphate is therefore not the exclusive property of an anaerobic culture, but may have to do with coping with the additional stress caused by the nystatin. Sugar phosphates also disappear after 4 hours. Linewidths and chemical shifts do not change from their original values (70 Hz for polyphosphate, 30 Hz for inorganic phosphate), indicating that there was no change in the extent of Mg association. A small peak appears as a high field shoulder on the polyphosphate peak early in the experiment, in a similar position to that observed with higher dosage, but disappears after 6 hours, likely since there is only a small amount of lysis. The viability curve (Figure VIII.26), shows that aeration helps the cell suspension cope well with the presence of the antibiotic. The culture slowly loses viability over the time period, but is still 30% viable after 9 hours in the NMR tube, as compared to 50% for the untreated culture (Figure VIII.8). The inorganic phosphate pools increase slightly, preserving a concentration gradient that maintains higher external phosphate. The internal concentration remains almost constant. The dramatic decrease in polyphosphate is mirrored by the increase in pyrophosphate, indicating that there is a direct hydrolysis to form pyrophosphate. The small increase in polyphosphate between the first and second spectrum is not significant. The two lower curves show the increase in

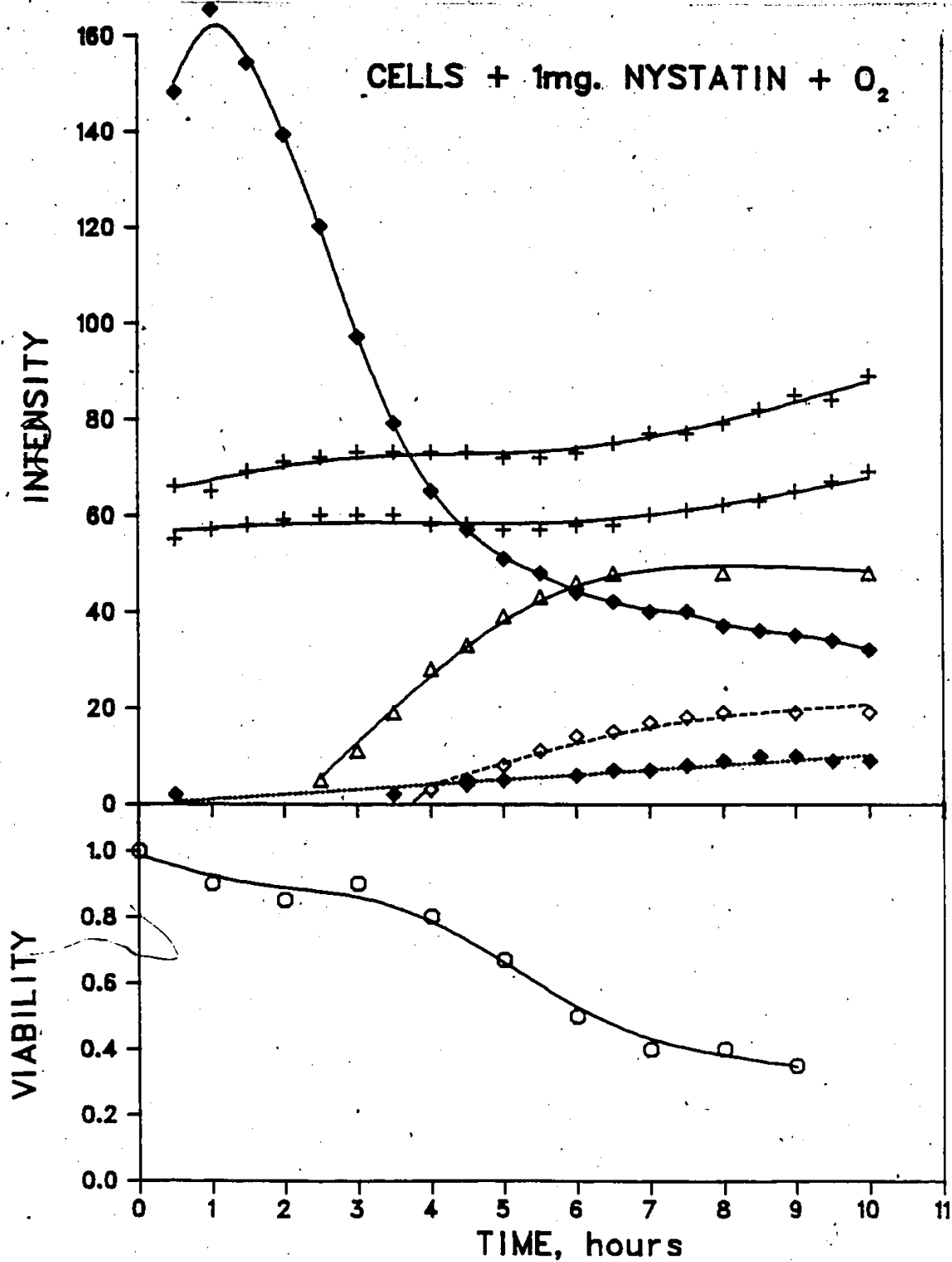


Figure VIII.26 - Time course of relative intensities of phosphorus-containing metabolites for aerobic culture treated with 1 mg nystatin. The viability curve shows that aeration helps the organism resist the effect of the nystatin. Decrease in polyphosphate and appearance of pyrophosphate can be correlated with the loss of viability

terminal and penultimate peaks. As before, pyrophosphate is likely produced in response to the stress of the nystatin, and under conditions where there is sufficient time to initiate its production. The dramatic shortening of the polyphosphate chains would be expected to accompany the hydrolysis of polyphosphate to pyrophosphate, since two monomer units are lost on each hydrolysis, reducing the average chain length more quickly than by hydrolysis of only one unit. Finally, the lack of change of linewidth allows us to compare the total amount of "visible" phosphate throughout the experiment, to check that there is no exchange between immobile and mobile phosphate pools. The sum of the peak intensities from all the phosphate peaks at the end of the experiment equals that at the beginning, confirming that we are observing direct exchange between phosphate pools.

The pH dependences of the aerobic nystatin treated cultures are shown in Figure VIII.27. There was no pH gradient observed for the higher dosage ($\square$) and there was no indication from the inorganic phosphate peak linewidth that this might be a superposition of two peaks, close in chemical shift. The pH drops slightly over the first two hours, during which the culture is viable, after which there is no further change. The increased acidosis may be due to enhanced metabolism in an attempt to repair the damage caused by the antibiotic, or simply due to the change in internal pH in

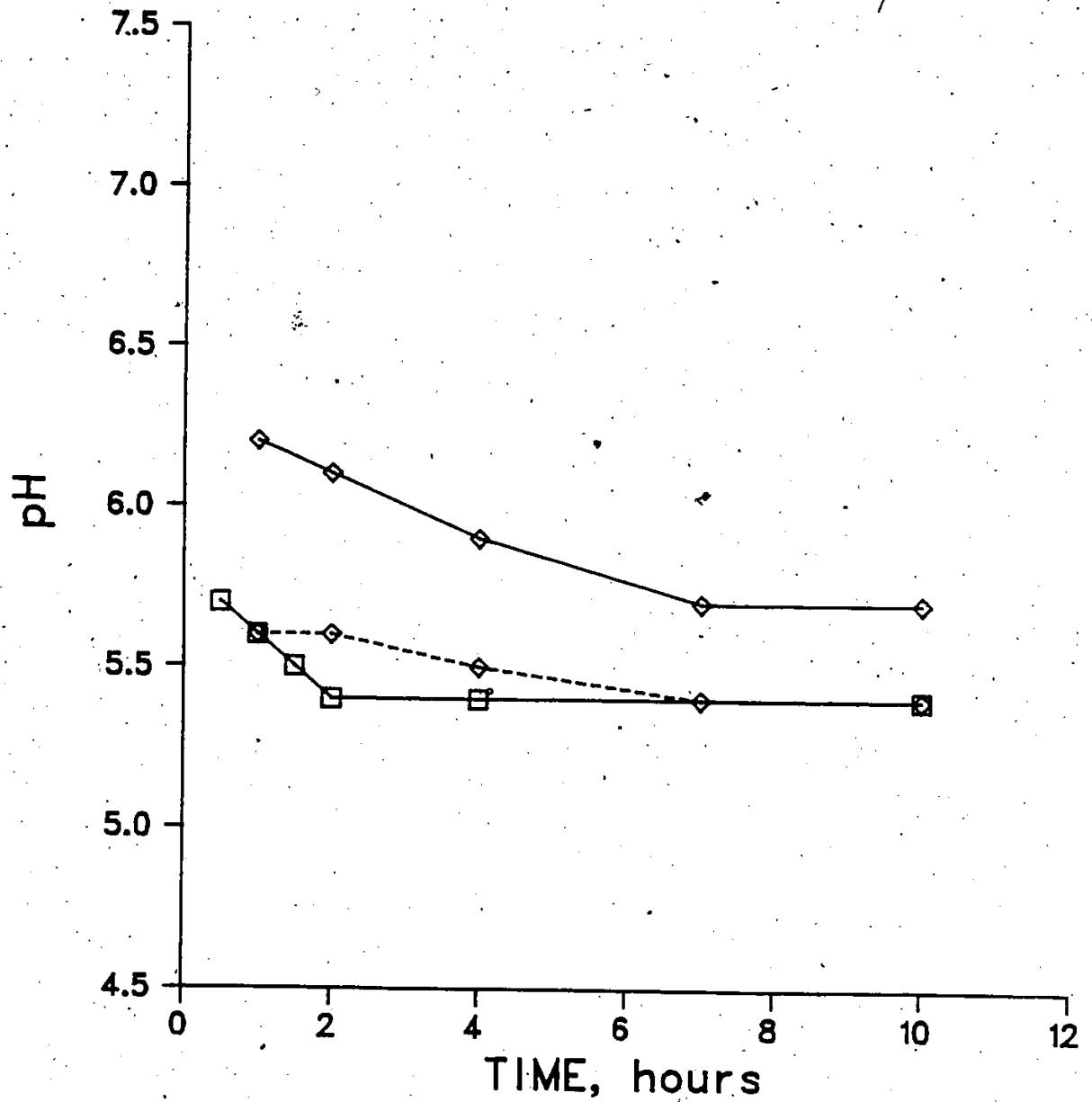


Figure VIII.27 - The dependence of pH for the experiments of Figures VIII.24 and VIII.26. No pH gradient was observed for the higher dosage of nystatin ($\square$), whereas inner and outer pools were preserved throughout for treatment with 1 mg nystatin ($\diamond$)

response to a stress. There was no indication of enhanced activity in the other phosphate pools during this initial time period. The external pH of the culture with lower nystatin dosage ($\diamond---\diamond$) is approximately the same as for the higher dose, which is that of the buffer, since the external phosphate concentration changes only slightly. The internal pH ($\diamond---\diamond$) is maintained about 0.5 pH units higher, although the pH decreases over the period of the experiment. Internal and external pH decrease in response to the viability curve. The decreasing internal pH may provide a signal for pyrophosphate production, although pyrophosphate was observed for anaerobic culture with no change in pH. It also may simply be in response to the decreasing external pH, a compensating effect due to the organism's ability to function at both high and low pH, which reduces the energy requirement of maintaining a large pH gradient.

(d) Effect of Methyl Mercury

The neurotoxic effects of methyl mercury poisoning in higher animals has been well documented (Takizawa, 1979). The mechanism of action, however, is not well understood. While, like all organo-metals, it is highly lipid soluble, the chief effect does not appear to be due to membrane disruption. Methyl mercury shows a strong affinity for thiol groups (Berg and Miles, 1978; Carty and Malone, 1979), hinting that the toxic effects result from the irreversible

binding to essential proteins, halting normal cell functions requiring these proteins. It has also been shown that methyl mercury induces genetic damage by directly breaking DNA strands, not simply by damaging the DNA repair enzymes (Yatscoff and Cummins, 1975). In microorganisms, the toxic effects are dose-dependent. Both the degree of viability, and the lag time before loss of viability begins, decrease with higher dosage, even though the uptake of methyl mercury is immediate and not dose dependent (J. Phipps, private communication). This evidence can be taken to imply that the cytoplasmic or plasma membrane levels of methyl mercury are not the toxic factor. Uptake is simply due to dissolution in membrane lipid and the fast establishment of equilibrium levels in the cytoplasm. A review of the essential features of toxic behaviour, namely the threshold behaviour, time lag before expression and the irreversibility of effects, has lead to the hypothesis the mechanism of action is initiated by the interaction of methyl mercury with mitochondrial DNA. If the dose is greater than genetic repair mechanisms can handle, then death is due to the resultant interruption of mitochondrial function (Miller and Miller, 1979).

Since the mechanism of action of methyl mercury is substantially different from that of nystatin, we have attempted to see if this difference would be reflected in the phosphorus NMR spectrum. The spectra of an anaerobic suspension of cells

treated with $\sim 6 \times 10^{-4}$ M MeHgCl (Figure VIII.28), show that there is greater preservation of cellular compartments than was previously observed. Three inorganic phosphate pools are observed (the external pool occurring at highest field), which are retained for 6 hours, after which only internal and external are seen. A concentration gradient favouring the inner pool appears to be maintained. The widths of the inorganic phosphate peaks remain constant during the experiment at 25 Hz. The disappearance of the third phosphate pool heralds the appearance of pyrophosphate, accompanied, as expected, by the shortening of the average polyphosphate chain length indicated by the increase in the terminal phosphate peak. However, two separate cellular compartments of pyrophosphate are apparent, due either to differences in pH or extent of ionic association. Comparison of the chemical shifts (9.3 and 9.6 ppm) to that of the extract titration curve estimates the pH of the two pyrophosphate environments to be 5.0-5.5 and 4.5-5.0 respectively, which agrees with the pH's estimated from the chemical shifts of the two inorganic phosphate peaks. Thus, the two pyrophosphate peaks can be assigned tentatively to external and cytoplasmic pools. Small levels of ATP are initially visible but disappear after 2 hours, while sugar phosphates persist for 7 hours.

The peak due to polyphosphate not only decreases with time but also broadens by a factor of 2 (from 44 to 100 Hz), accom-

CELLS + MeHgCl

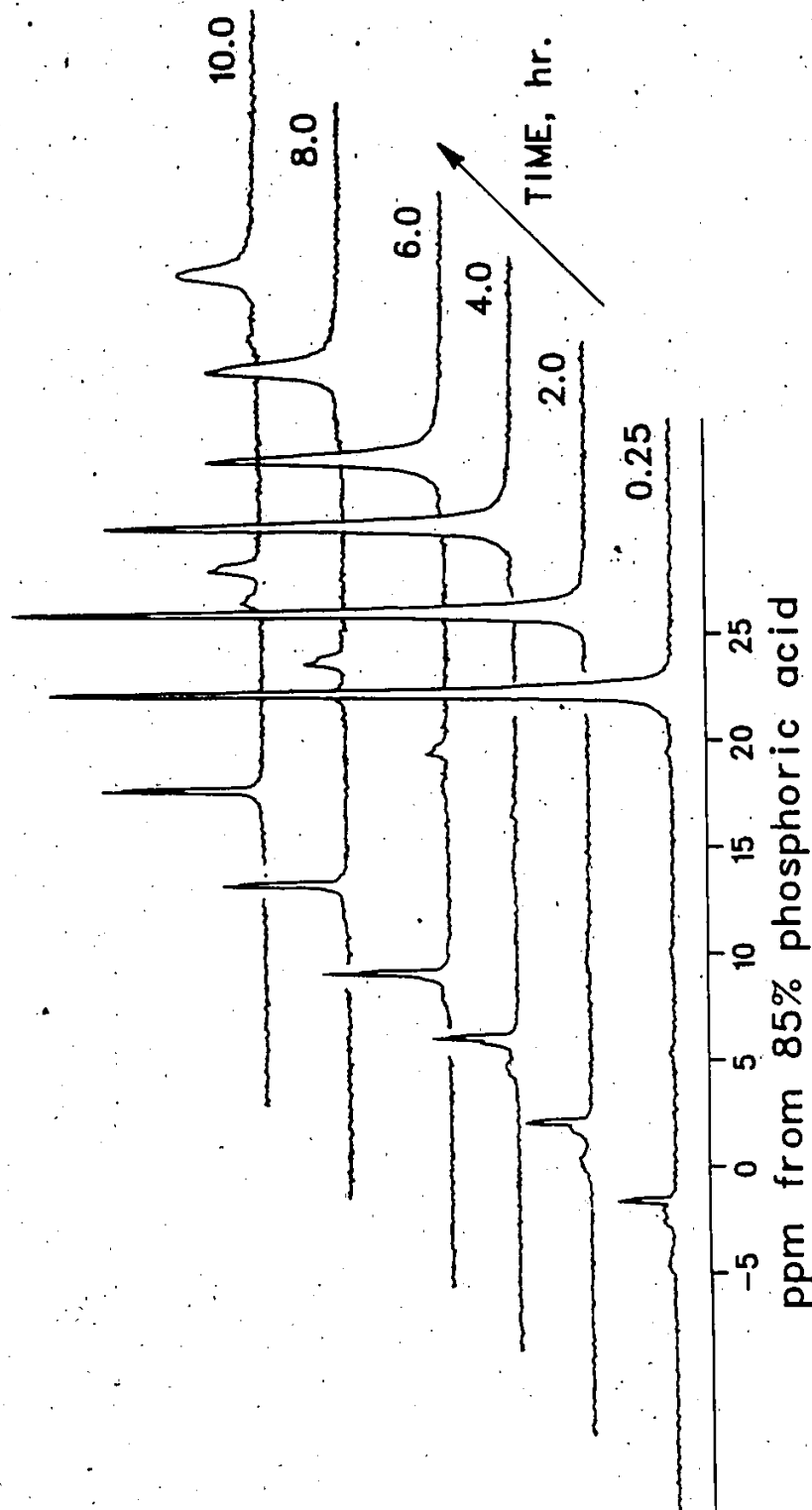


Figure VIII.28 - Time course of spectra for a cell suspension treated with 6×10^{-4} M MeHgCl. The cells do not appear to lyse, as indicated by the preservation of two pools of inorganic phosphate. The polyphosphate peak is reduced in size with time but also broadens. Pyrophosphate is produced after ~5-6 hours, and also appears to be in two pools

accompanied by a slow upfield shift of 0.6 ppm. This decrease in linewidth can result from two processes, either the reduction of the line to a series of superimposed lines resulting from a distribution of chain lengths; or a decrease in T_1 . The decrease in chain length implied by the increase in the terminal phosphate peak supports the former mechanism, although such shortening would not be accompanied by a resonance shift in the observed direction. Substantially shorter polyphosphates are observed at lower field. The effect is not due to the presence of an unresolved peak due to metaphosphate, as was found for the aerobic nystatin treated cultures, since this peak was observed even further upfield in those experiments. A decrease in relaxation time during the experiment could be a result of an increase in the extent of ionic association or an increase in the viscosity of the environment surrounding the polyphosphate. The decreasing chain length alone would imply greater mobility and an increase in T_1 .

Since the correlation between an increasing linewidth and a slight upfield shift was also observed to a less extent in the anaerobic control experiment, the polyphosphate T_1 of the methyl mercury-treated cells was monitored as a function of time in a separate experiment. The T_1 of polyphosphate in the cell was initially measured to be approximately 300 ms. Then changes in T_1 were roughly followed by monitoring any change in the null point (the point at which the intensity in an inversion recovery sequence is zero; at $t=0.69T_1$).

During the same time period as the linewidth increased, the T_1 decreased by a small amount (which is undetermined due to the nature of the experiment). There may, in addition, be a distribution of ionic environments leading to unresolvable peaks, accounting for the much greater increase in linewidth than decrease in T_1 . The effect is therefore assigned to changes in the degree of Mg binding in perhaps different cellular locations of polyphosphate, since the correlation between T_1 and Mg can be demonstrated (vide infra).

Figure VIII.29 reports the time dependence of the metabolite intensities for methyl mercury-treated cells. The intensity of the polyphosphate peak is corrected for the increase in linewidth. The dosage of methyl mercury was carefully chosen to give the illustrated viability curve. As was observed in other yeast varieties, the viability was a function of cell concentration. Larger doses produced complete loss of viability in a much shorter period of time, while lower ones did not produce complete loss of viability within a reasonable time for the NMR experiment (J. Phipps, private communication). Not all the polyphosphate is hydrolyzed after ten hours. However, the sum of the intensities due to the various pools is the same at the end of the experiment as at the beginning, indicating that we are observing all the interchangeable phosphate. Pyrophosphate is produced as the suspension begins to lose viability,

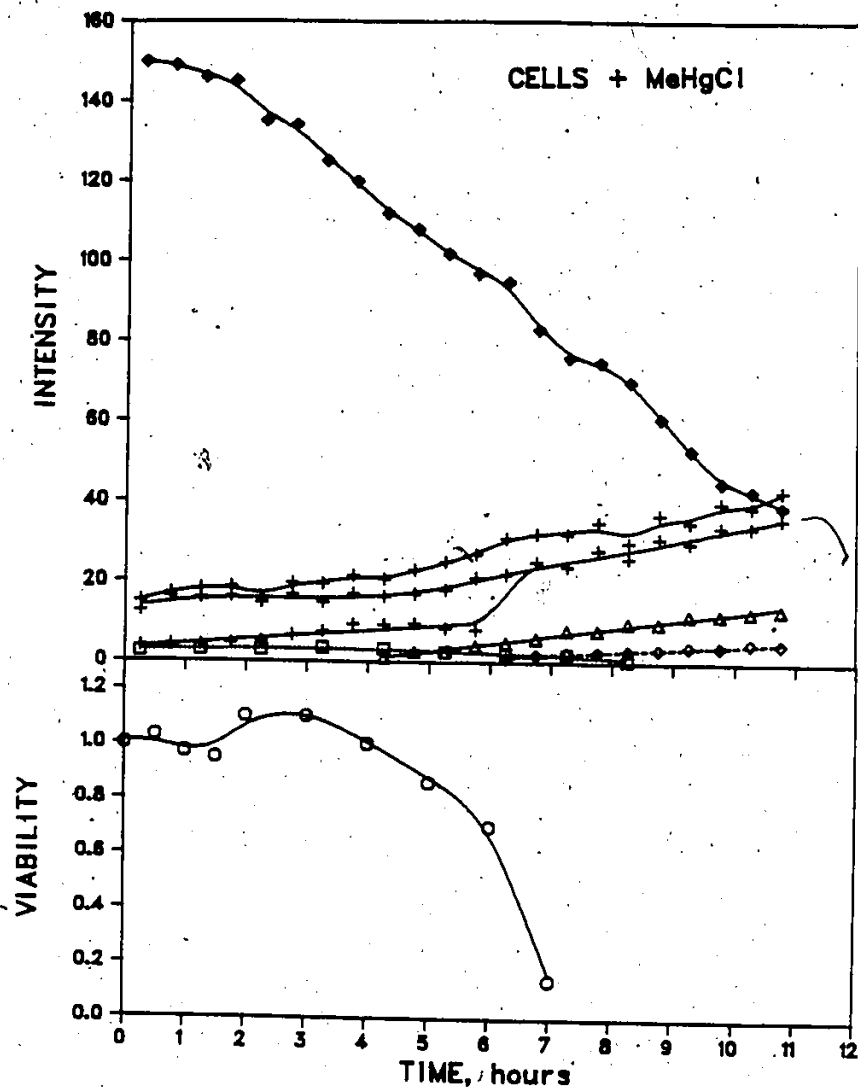


Figure VIII.29 - Time course of metabolites for methyl mercury-treated cells. The viability curve in this case represents a slightly aerated culture, as opposed to strictly anaerobic. The three inorganic phosphate pools are preserved far longer than for other treatments perhaps indicating very rapid respiration. The appearance of pyrophosphate is concomitant with the disappearance of one of the inorganic phosphate pools. The increase of the peak due to terminal polyphosphate, shows that the polyphosphate chain length is being reduced

although the reason for an increase after complete loss of viability is unclear. Perhaps due to lack of lysis of the cell, the enzyme responsible for pyrophosphate production is within the same cellular vicinity and does not lose activity. All inorganic phosphate pools increase with time, and the smaller, perhaps mitochondrial, pool disappears as the culture begins a steep drop in viability. It must be noted that this particular viability curve was not derived under conditions of strict anaerobicity, since the suspension was shaken during the time period. Thus, the time correlations with intensity changes may not be as accurate as for the previous experiments. This situation resulted from the toxic danger of the experiment and the need for extra precautions in deriving the viability curve. However, since the extent of aeration was not as great as for the aerated NMR experiment, the viability curve may be taken as a compromise between the two NMR situations. By analogy with the previous experiments, strict anaerobic conditions would produce loss of viability at somewhat shorter times, and oxygenation would aid the culture in coping with the toxic effects.

The intensity plots for the parallel oxygenated experiment are shown in Figure VIII.30. The spectra (not shown) are very similar to Figure VIII.28. The linewidth of the polyphosphate peak remains relatively narrow (60 Hz) after an initial decrease from 83 Hz, even though the chemical shift

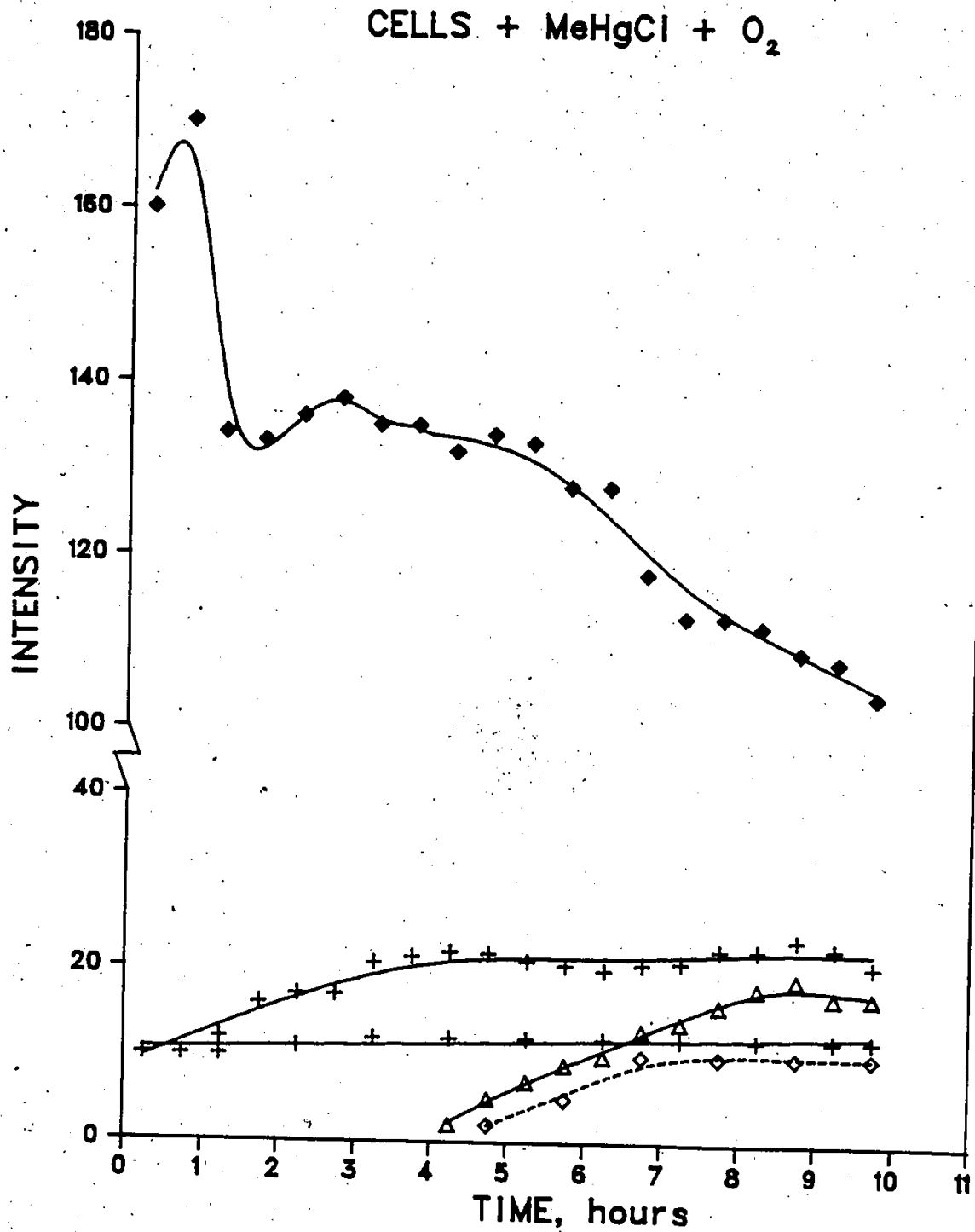


Figure VIII.30 - Time course for a similar experiment to that of the previous figure using oxygenated cell suspension. The change in polyphosphate levels at during the first hour may not be significant

moves upfield, again by 0.6 ppm. This indicates that while there is increased relative Mg binding, there is likely a contribution to the linewidth of viscosity of the polyphosphate environment. It was generally observed that the lines widened under anaerobicity, and this effect was not apparent when oxygen was provided. The presence of oxygen may reduce the tendency to increased intracellular viscosity by keeping the transport mechanisms in operation. The level of polyphosphate decreased, although not to the same extent as in the anaerobic experiment. The initial increase and subsequent decrease in level observed for the first two spectra is puzzling, although it in part may be due to correction of the line intensity due to the initial decrease in the polyphosphate linewidth. There is a plateau in the polyphosphate level during the period of retained viability (between 2-5 hours), followed by a decrease due to the introduction of pyrophosphate as loss of viability begins. The pyrophosphate content increases quickly, mirrored by the terminal phosphate peak, and then levels off. The inorganic phosphate pools remain at lower levels than in the aerobic experiment, and the suspension keeps the external pool at a constant and lower level than the internal pool. The initial decrease in polyphosphate is reflected in a slower increase in the internal pool, suggesting that it may be due to the initial reaction of the cell against methyl mercury. Here

again is proof that there is direct transfer between poly-, pyro-, and internal phosphate, since levels of different pools only change in response to each other: during viability between hours 2-5 all levels remain constant. As was observed for other anaerobic suspensions, the sugar phosphate disappears very quickly. Very low levels of ATP were consistent with other experiments, although the pH of the inner compartment matches that of the third (mitochondrial) pool in the anaerobic suspension. The viability here is likely greater after 7 hours than is illustrated in Figure VIII.29.

The pH effects illustrated in Figure VIII.31 show that both internal and external pH in both aerobic and anaerobic cases are much more acidic than their untreated analogues (Figure VIII.9)... In addition, the Δ pH appears to be higher. The identification of the different pools of the anaerobic experiment (Δ) is not clear, and therefore the external pool was chosen to be that which was more acidic and which began at the known external pH of 5.5. The cytoplasm remains at a pH below 5.5 after an initial drop, which is likely due to the internal concentration of methyl mercury. The smaller pool assigned to mitochondria drops in pH during the period of retained viability and disappears as loss of viability occurs. There is a Δ pH between mitochondria and cytoplasm of initially 1 pH unit, which decreases to 0.5 pH units over the first two hours. This differs from the control experiment

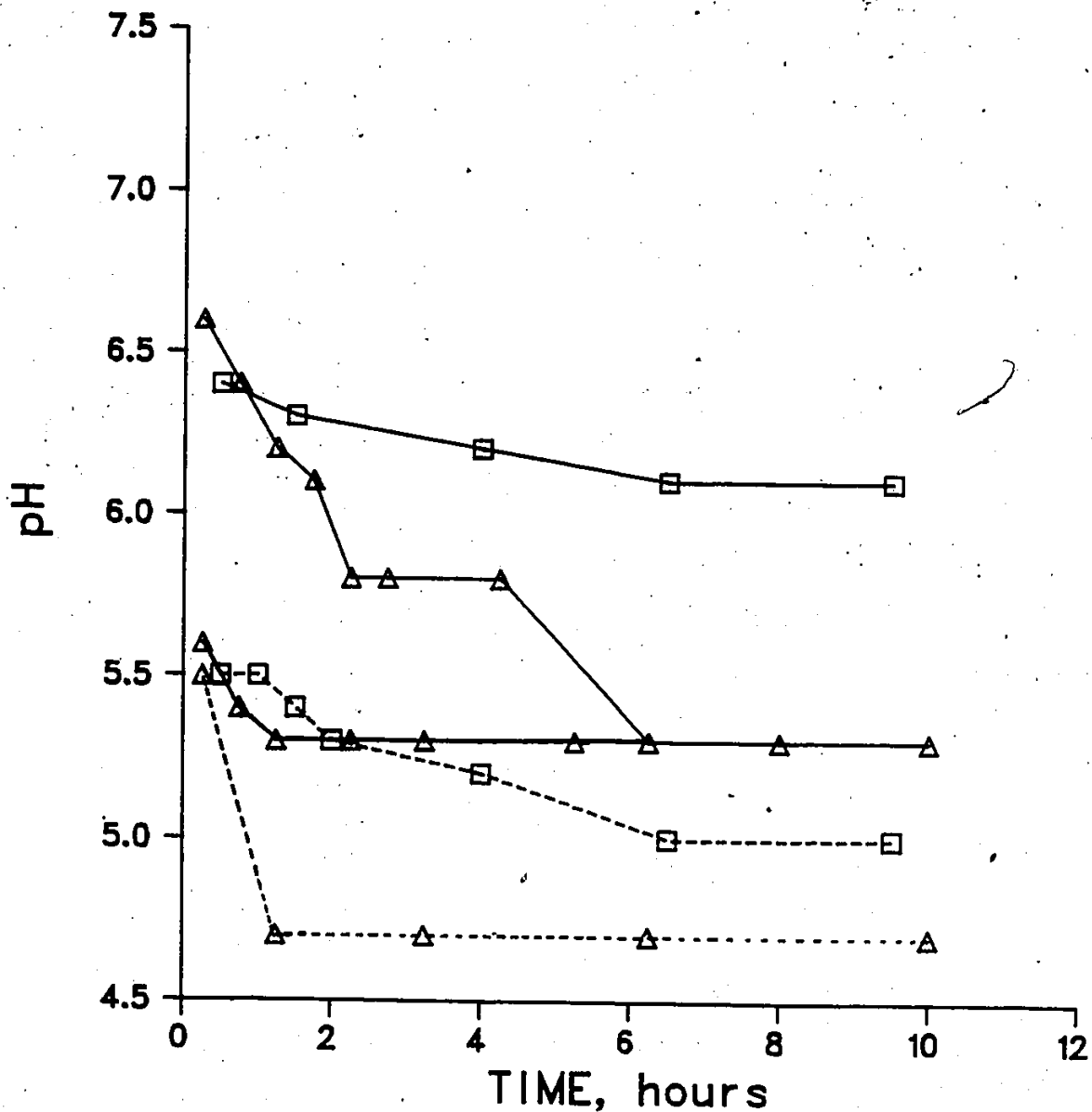


Figure VIII.31 - pH dependence as a function of time for aerobic ($\square$) and anaerobic (Δ) cell suspensions treated with MeHgCl . As before, dashed lines refer to outer pool and solid lines refer to the inner pool of phosphate. The two pools of highest intensity are assumed to be the external and cytoplasmic pools. One of these pools becomes quickly very acidic and the other more slowly so. The lower pH is not accurately determined ($\text{pH}=4.5 - 5.0$) since the titration curve becomes relatively insensitive to pH in this region

in which the corresponding ΔpH remained at 0.6 pH units, i.e., both mitochondria and cytoplasm dropped in pH in a parallel manner due to the effect of lack of oxygen. These enhanced proton gradients may indeed be due to greater mitochondrial activity in response to the methyl mercury. The external pH drops quickly to well below $\text{pH}=5.5$ due to protons being pumped out of the cell. During this time the external phosphate concentration changed very little. The external pH cannot be very accurately estimated, since below $\text{pH}=5$ the titration curve becomes relatively insensitive to pH. The value is certainly below 5.0 and above 4.5. The maintenance of pH gradients across the membrane even after cell death is simply due to lack of lysis of the cells. The cell wall must remain intact. Cell death is in some part due to lack of nutrition in the suspension, which is required as metabolic fuel to repair cellular damage. The ΔpH in the aerobic suspension ($\square$) is approximately 1 pH unit, as compared with 0.6 units observed in the control. This gradient is maintained and the oxygen provides for a more healthy internal pH, greater than 6.0. The mitochondrial pH is not resolved. The external pH decreases more than the internal pH in response to the methyl mercury. The chemical shift of the pyrophosphate also reflects this higher pH, and the slight increase in chemical shift (from 8.4-8.6) during the period of observation agrees with the illustrated slight decrease in internal pH. This

provides further evidence that the pyrophosphate is located in the cytoplasm.

(e) Effect of Magnesium (Mg^{2+})

In the previous discussion, changes in linewidth and chemical shift of the polyphosphate peak (which is not pH dependent) have been largely attributed to differences in the extent of bound Mg^{2+} . It is well known that Mg and Ca ions are implicated in the control of many enzymes responsible for transport and metabolism. It was also pointed out in the introduction to this chapter that Mg ions also affect many of the enzymes responsible for phosphate metabolism. In addition, it is the Mg-bound ATP which is the active phosphorylating nucleotide, rather than the ion-free form (Gupta and Yushok, 1980), so that ion bound phosphates may be associated with active metabolism. In order to check that this interpretation of the spectra was indeed valid, model polyphosphates were titrated with Mg salt and the linewidths, T_1 's and chemical shifts were followed.

The results of a rough titration of a polyphosphate glass consisting of 65 monomer units is shown in Table VIII.1(a). The ^{31}P T_1 in an ion-free solution is quite long and the resonance is much narrower than was observed in the cells. The chemical shift, however, is very close to that of polyphosphate extracted from the cells and passed through the

Chelex column. On addition of sufficient salt to provide 1 Mg ion per two phosphate units, the T_1 drops by a factor of 3.5, the linewidth increases by a factor of 3, and the chemical shift increases by 2.4 ppm. Further addition of Mg to give a 1:1 molar ratio, enhances these effects. The relationship between concentration and T_1 or chemical shift is definitely not linear. The effect on chemical shift is more pronounced at lower concentrations and on T_1 , at higher concentrations. The effect on linewidth, however, is approximately linear. The chemical shift effect for the variety of phosphates found in a polyphosphate mixture of average chain length equal to 15, is further illustrated in Table VIII.1(b). As has been already alluded to (Glonek *et al.*, 1971), metaphosphate is not as sensitive to the presence of Mg^{2+} as is the terminal unit of polyphosphate. The behaviour of pyrophosphate is similar to that of polyphosphate, but the effect is reduced (the change in shift is only 0.8 ppm, as opposed to 2.4 ppm, respectively).

The T_1 of polyphosphate observed in the cell (300 ms), includes the effects of differences in viscosity and conformation over and above that measured in solution. The magnitude of these effects is seen by comparison of the linewidth of the polyphosphate in the cell (50 Hz) to that in the extract (7 Hz) or in the pure solution (4 Hz). Taking this into account, the amount of Mg complexed with the polyphosphate

Table VIII.1

A) Titration of Polyphosphate Glass (P_{65})

Mole Ratio (Mg^{2+}/PO_4)	T_1 (sec)	$\Delta\epsilon_{1/2}$ (Hz)	δ (ppm)
0	2.17	4	21.1
0.5	0.62	11	23.5
1.0	0.13	20	23.9

B) Effect of Mg^{2+} on Chemical Shift in Polyphosphates (ppm)

	Mole Ratio (Mg^{2+}/PO_4)			
	<u>0</u>	<u>0.2</u>	<u>0.5</u>	<u>1.1</u>
Hexametaphosphate	23.3	23.5	24.3	24.7
Polyphosphate	21.1	22.5	23.5	23.9
Terminal Polyphosphate	7.5	7.6	8.5	8.9
Pyrophosphate	8.4	9.06	9.1	9.2

within the cell is approximately 0.5 moles/mole PO_4 . Estimation from the chemical shift of cellular polyphosphate gives a mole ratio of ~ 0.2 . Thus there is one Mg^{2+} ion per 2-5 phosphate monomer units in the cellular polyphosphate complex. The chemical shift estimation gives a smaller Mg content than the T_1 measurement due to the additional effects of viscosity and conformation on T_1 . It may be that the polyphosphate located in the vacuole is much more tightly coiled than when free and extended in solution, where motion is greater and relaxation times longer. Rough comparison of these results with those of Glonek et al. (1971) indicates that this represents a Mg concentration of approximately 20 mM in the cell storage material. Thus, the polyphosphate appears to be functioning efficiently as a storage vehicle for this metabolically important ion.

When estimating the relaxation time of the polyphosphate in the cell, it was observed that the T_1 of the inorganic phosphate was much longer than 300 ms, while the T_1 of the pyrophosphate was much shorter (~ 100 ms). The latter value is unusual for such a small molecule, suggesting that the pyrophosphate is also highly Mg-bound. It may also reflect different viscosities of the two locations in the cell where these phosphates are found. The pH effects indicate that the pyrophosphate is cytoplasmic while the polyphosphate is housed in the central vacuole. The chemical shifts, even though pH-dependent, also indicate that the number of ions per phosphate

may be somewhat greater than 0.2-0.5.

The amount of bound Mg observed in this way agrees well with that observed in other systems. The association of cations with polyphosphate has been observed in the bacterium M. lysodeikticus (Freidberg and Avigad, 1968), in which the major cation was Mg^{2+} and the mole ratio observed was approximately 1 ion per two phosphate monomers. The remaining cation content was mostly Ca^{2+} , which does not appear to have a large effect on the phosphate spectrum. Gupta and Yushok (1980) found that most of the intracellular Mg was preferentially bound and that 88% of the ATP content of intact tumor cells was complexed to Mg^{2+} . The ^{31}P NMR behaviour of Mg complexed to ATP has been studied (Bishop et al., 1981), indicating that binding constants may be correlated with the magnitude of the resonance shift observed. Titration of ATP and triphosphate show that the Mg complex with the latter is more stable, and that the most stable complex is that giving a mole ratio of 0.3 (i.e., 1 ion per three phosphate units). The effect of Mg titration on the central phosphate group is much greater (2.5-3.5 ppm) than on the terminal groups (0.7 ppm), in agreement with the above observation for polyphosphate and pyrophosphate. This does not, however, necessarily indicate preferential binding to the central positions (Jaffe and Cohn, 1978). Bishop et al. also show that the relaxation times are not linear with Mg

concentration, and that the T_1 of triphosphate is generally shorter, for the same Mg concentration, than that of ATP. The observed T_1 of polyphosphate for the same amount of Mg is somewhat shorter still, indicating that the relaxation time is dependent on more than simply the amount of Mg. Since the ion-free T_1 of triphosphate (2.6 s) agrees with that of polyphosphate (2.14 s), with the correct trend for differences in molecular size, this may be due to a contribution to T_1 from differing binding strengths in the three species. The conformation suggested for the complex of one ion per three phosphate units is one in which the phosphates are curled around the central ion. Extrapolation of this conformation to a polymer chain suggests the efficient coiling of the molecule. This would be effective in reducing oxygen-oxygen repulsions, and compacting a large amount of polyphosphate into a small area, as might be required for such a large concentration within the major vacuole of the cell. The strong association of pyrophosphate with Mg^{2+} , by analogy to ATP, may indicate that the bound species is an active phosphorylating agent, as has been suggested (Kulaev et al., 1980).

Discussion

The spectral results of the preceding experimental series cannot directly identify the specific enzyme controlled processes that are occurring. However, several correlations

have developed as a result of the various treatments of the cell suspension, which allow us to draw some conclusions concerning the phosphate metabolism of A. pullulans. In addition, it has been shown that while viability curves are sensitive to the amount of a toxic species, they are relatively insensitive to its mode of action. The latter can be probed by ^{31}P NMR.

The degradation pattern of the major phosphate metabolite, polyphosphate was dependent on the perturbing conditions. In general, polyphosphate utilization appears less under conditions of adequate oxygen supply than under anaerobic conditions. When the cell suspension was metabolically stable (i.e., untreated aerated suspension) no polyphosphate was consumed. Factors which increase the hydrolysis of polyphosphate are the presence of more than one perturbant or increased dosage of a single perturbant. Polyphosphate is the major source of phosphate for the cell, in spite of externally supplied phosphate. The presence of polyphosphate is not a prerequisite for viability, as shown by starved cells, but cells will retain their polyphosphate levels under conditions promoting viability. Degradation in the absence of viability, due to uncontrolled enzymatic action, is minimal. Thus, the observed hydrolysis of polyphosphate appears to be under cellular control. Degradation of polyphosphate is normally mirrored by increase in inorganic phosphate, except in cases where polyphosphate

appears to be a direct source of pyrophosphate. The end products of degradation therefore are only inorganic phosphate and pyrophosphate. This indicates the presence of the two specific phosphatases responsible for these reactions. In addition, the degradation pattern mirrors the viability curve, and drastic reduction of the polyphosphate pool accompanies sharp loss of viability.

In contrast to previous reports that polyphosphate pools decrease in response to phosphate deprivation, A. pullulans utilizes its polyphosphate in response to carbon deprivation. This latter observation has been reported only for the bacterium, M. lysodeikticus, in which volutin granules were observed to disappear after carbohydrate was removed from the medium (Friedberg and Avigad, 1968). The well-documented tendency in many organisms to reduce polyphosphate levels during stationary phase may simply be a reflection of this correlation. Under abundant carbon nutrition, high levels of polyphosphate are accumulated; when the medium is exhausted, polyphosphate is no longer accumulated and levels may decrease. The recent work of Shulman and coworkers (den Hollander et al., 1981(b)) also indicates that polyphosphate levels are dependent on glucose supply. Levels in S. cerevisiae are maintained on glucose feeding and reduced on gluconeogenic sources such as acetate and ethanol.

The direct transfer of polyphosphate to glucose to form G6P has been demonstrated in Aspergillus niger (Nishi, 1961). It has already been observed that glucose stores are maintained during carbon deprivation and that proteins and amino acids are derived largely from stored triglyceride (Chapter VIII). This glucose pool may be maintained as a source for glycolytic activity employing phosphate groups from phosphate. A reduction of polyphosphate stores was observed upon reintroduction of glucose to repressed cells of S. cerevisiae (Gillies et al., 1981). This was interpreted as a response to an immediate demand for inorganic phosphate that was not available in the medium. In the present experiment, since external phosphate in the starving medium is at the same concentration as for initial growth, this preferential use of polyphosphate indicates that it is not acting merely as a source of phosphate. While this is not proof of its role as a phosphagen, it does indicate that the polyphosphate pool plays an active role in the regulation of intracellular phosphate. The preferential use of polyphosphate over inorganic phosphate for phosphorylation is also an energy conserving strategy, since the latter must be transported against a concentration gradient. In addition, there was a correlation between higher levels of sugar phosphate and the rapid decrease of polyphosphate content. Under aerobic conditions, this decrease was not as drastic while sugar

phosphates disappeared more quickly. The relative magnitudes of change of the polyphosphate and sugar phosphate pools do not at first glance appear to be consistent with this mechanism. This is due, however, to the conditions of spectral accumulation, which are such that the peak intensities reflect the average quantity in each pool over a period of 17 minutes. Since the sugar phosphate pool is turning over steadily during glycolysis, the average amount of sugar phosphate present is much lower than the more static pools of inorganic phosphate and polyphosphate.

Two pools of polyphosphate were observed in the perchloric acid extract; one of long and the other of short chain length. There was no indication of changes in their relative amounts during the experiments. The shorter length may therefore serve as the direct donor of phosphate, while the longer is a more stable storage form. Metaphosphate appeared only under conditions of cell lysis and the possibility of polyphosphate release. This indicates that the presence of metaphosphate here as well as in other natural polyphosphate extractions (Harold, 1960) is due to spontaneous cyclization and not to a metabolic process.

Both spectral results and microscopic observation of starved and nystatin treated cultures indicates that the observed polyphosphate storage pool is probably located in the major vacuole of the cell. The relatively high resolution of the polyphosphate peak indicates a fairly mobile environment

that would not be found in volutin granules. ^{31}P NMR experiments on Tetrahymena (Deslauriers et al., 1981) indicate that volutin granules are not visible under high resolution conditions. The results of the previous chapter indicated that amino acids were also stored in the vacuoles. The increase in the amino acid pool during starvation may compensate for the loss of polyphosphate from the vacuole and aid in counteracting the higher cytoplasmic pressure from increased inorganic phosphate in the cytoplasm. The polyphosphate also acts as a storage vessel for Mg, and the conformation suggested by the observed stoichiometric ratio of Mg ions to phosphate groups is tightly coiled. This role in ion storage requires that polyphosphate be sequestered from the cytoplasm by a membrane which can control ion transport. The relatively high quantity of bound Mg in polyphosphate would imply a very high cytosolic Mg concentration if this polyphosphate were located in the cytoplasm. The storage of metal ions in a vacuolar space also aids in the regulation of the cytoplasmic osmotic pressure. Thus, the central vacuole appears to be the main larder of the yeast cell; only the triglyceride and glucose fractions are cytosolic. Such segregation of storage pools is likely a protective device against hostile environments. It may also indicate that the vacuolar membrane is active in regulating cytoplasmic concentrations of metabolites.

The low levels of ATP observed in this organism may correlate with its survival capabilities. If it has an ability to lower metabolic rates, available resources can be conserved, a trend which allows it to occur in the wide variety of habitats from which it has been isolated. In this series of experiments, the presence of ATP was correlated with lower levels of sugar phosphate, the absence of pyrophosphate and the presence of oxygen. The addition of a perturbation hastened the disappearance of ATP. Generally, observable ATP levels were associated with only 100% viability under aerated conditions. The extract spectrum shows that ATP levels are higher than ADP levels in freshly harvested cells, in agreement with the data for other yeasts (Navon et al., 1979). The possibility also exists that the ATP levels are supplemented with some other form of phosphorylating agent.

The addition of oxygen appeared to aid the cell suspension in coping with high cell density and the presence of toxic agents. This was reflected in higher ATP levels, lower inorganic phosphate levels, reduced degradation of polyphosphate, and a higher cytoplasmic pH. Similar correlations have been observed for S. cerevisiae (den Hollander et al., 1981(b)). Observable pH gradients were also maintained as well as a phosphate concentration gradient and a lower viscosity (as represented by narrower metabolite linewidths). Even under aerobic conditions, however, the internal pH appears to be

lower than that measured for other yeasts (den Hollander et al., 1981(b)), perhaps as a result of low glycolytic activity. This particular yeast may adjust its internal pH to more closely resemble its surroundings, which are relatively acidic (pH=5.5), whereas S. cerevisiae maintains a constant internal pH, even when the external conditions are changed from pH 7.2 to 5.2. Maintenance of a small pH gradient requires less net energy expenditure. The generally higher intracellular pH observed under aerobic conditions was not necessarily an indication of higher viability. The initial viability of the cell suspension was the same as under anaerobic conditions, where the cytoplasmic pH was much lower. However, aeration aided in the retention of viability. The lower cytoplasmic pH under anaerobic conditions is likely due to the accumulation of CO₂, as carbonic acid in the cytoplasm. Under aerobic conditions this factor can be controlled. This accumulation of acid leading to an intracellular pH with which the cell cannot cope is likely the cause of loss of viability in the control experiment.

Less compartmentalization of inorganic phosphate was also observed under aeration. The phosphate pool assigned to mitochondria appeared only in anaerobic cultures. The mitochondrial pool is resolved in this case due to the lower cytoplasmic pH. When oxygen is present, the mitochondrial pool is not resolved since the cytoplasmic pH is approximately

the same as the mitochondrial pH. While the cytoplasmic pH can be adjusted to its surroundings to minimize energy expenditure, the mitochondria likely require a less acidic environment to function. When the cytoplasmic pH is sufficiently high for adequate mitochondrial function, there is no need to maintain a pH gradient across the mitochondrial membrane. As was mentioned above, the initial viability was not dependent on the observed cytoplasmic pH, therefore the cells were as viable when this mitochondrial component was resolved as when it was not. However, a correlation was observed between loss of the mitochondrial component and the onset of loss of viability. Since the mitochondrial pH decreased during the initial viable period, this observation indicates that there is likely a minimal pH below which the mitochondria cannot function. The immediate cause of loss of viability was therefore probably the cessation of mitochondrial function. In those cell suspensions treated with nystatin, the loss of viability was due to loss of integrity, rather than mitochondrial disfunction, since the nystatin caused lysis of cellular membranes.

The sugar phosphate levels were relatively low as can be expected from the absence of glucose in the suspension medium (Navon et al., 1979). The observed levels are generated from the intracellular glucose storage pools under these conditions and therefore glycolytic activity is relatively low. It is not clear from these experiments whether glucose is phosphorylated

exclusively by transfer of a phosphate group from ATP or from polyphosphate or by a combination of the two mechanisms. In general, sugar phosphate levels were higher under anaerobic conditions, and almost unobservable under aerobic conditions. This is an opposite effect to that observed for ATP. This may in part be due to the higher internal pH under aerobic conditions. The sugar phosphate resonances from the different species are more spread out in chemical shift at higher pH, and individual levels are within spectral noise. However, the difference in resolution between the pH region of 5.5-6.5 is not sufficient to account for the complete disappearance of sugar phosphates under aerated conditions (see Figure VIII.4). No change in sugar phosphate levels was observed upon introduction of O_2 to wild type cells of *S. cerevisiae* (Navon et al., 1979), but a reduction was observed in a glycolytic mutant. An increase in sugar phosphate levels can be explained both in an increase in glycolytic activity or by a slower turnover in sugar phosphate. As was mentioned above, the hydrolysis of polyphosphate into inorganic phosphate was also enhanced under anaerobic conditions. Even in the absence of polyphosphate, inorganic phosphate levels are lower under aeration. This phenomenon can be explained by the Pasteur effect, (the slowing of glucose metabolism by oxygen) (Racker, 1965). Thus, higher sugar phosphate levels under anaerobicity are indicative of greater glycolytic activity by this mechanism.

The enhanced demand for phosphorylation of glucose under conditions where ATP levels are low may be filled by polyphosphate. In addition, there may be a pH effect, in that glucose may be more easily phosphorylated by polyphosphate at lower intracellular pH, while ATP is employed at higher pH. When no polyphosphate was available (i.e., starved cells), the sugar phosphate pool appeared to be maintained at the expense of inorganic phosphate (see Figure VIII.15). This was the only occasion that a decrease of inorganic phosphate was observed as a function of time. Although there appears to be no correlation between the levels of pyrophosphate and sugar phosphate, it is possible that G6P could be formed by transfer of a phosphate group from pyrophosphate. The enzyme which catalyzes the regeneration of glucose from G6P (glucose-6-phosphatase) also has the ability to transfer a phosphate group from pyrophosphate to sugar phosphate (White et al., 1978). However, since this property has been shown only for enzyme isolated from animal tissue, there is no evidence to indicate that this reaction can occur in yeast.

The appearance of pyrophosphate can be correlated with the disappearance of ATP under the influence of some stress factor. Anaerobicity alone is sufficient stress to induce pyrophosphate production, although production is observed under both anaerobic and aerobic conditions when the cell suspension was treated with low doses of nystatin or methyl

mercury. The appearance of the pyrophosphate resonance is not due simply to loss of viability, either under lytic (high doses of nystatin) or non-lytic (lyophilized) conditions, indicating that the observed pyrophosphate production is under cellular control. This resonance appears at the point that the cellular suspension begins to lose viability. Some lag time after the introduction of the perturbation (be it high cell density or a toxic agent) appears to be required to trigger its production. The maximum pyrophosphate was produced under conditions leading to a slowly decreasing viability curve rather than an immediate loss of viability. The pyrophosphate level increases over the period that viability is lost, perhaps in some response to enhanced metabolic rates required by repair mechanisms. There may be some factor, such as lowered cytoplasmic pH or decreasing ATP concentration, that is required to activate pyrophosphate production. This behaviour indicates that pyrophosphate may indeed act as a phosphagen: the cellular metabolism changing over to pyrophosphate from ATP as a phosphorylating agent when metabolic demand is very high. The complementarity of ATP and pyrophosphate production has been shown for rat liver mitochondria treated with various agents (Kulaev *et al.*, 1980). The triggering condition in this case was postulated to be the effect of the agent on the mitochondrial membrane. The great extent to which pyrophosphate is metal bound as observed from the chemical shift and T_1 of the resonance supports the phosphagen

hypothesis, by analogy to ATP. Since the activated forms of phosphorylating agents such as ATP are often metal-bound, the activity of pyrophosphate as a phosphorylating agent may also be influenced by the degree of metal binding. The activated form of ATP binds one Mg ion per molecule, indicating a conformational change over the inactive form (Bishop et al., 1981). A similar conformational change could also be introduced in pyrophosphate by the same stoichiometric ratio (i.e., a ratio of 0.5 mole Mg^{2+} /mole PO_4). Such a ratio is consistent with spectral observation.

The presence of pyrophosphate was in all cases accompanied by a marked shortening of the average chain length of polyphosphate. This effect was not observed when only inorganic phosphate was the product of polyphosphate hydrolysis. This indicates that the pyrophosphate may be formed directly from polyphosphate. Although the reverse reaction (synthesis of polyphosphate from pyrophosphate produced in nucleic acid synthesis) has been demonstrated for N. crassa (Kulaev, 1975), there has been no previous report of the hydrolysis of polyphosphate to pyrophosphate. The only hydrolytic enzymes that have been isolated are those which randomly hydrolyze the polyphosphate chain or specifically hydrolyze to inorganic phosphate. Since pyrophosphate can be synthesized without the presence of polyphosphate (either by hydrolysis of ATP to AMP or condensation of nucleotides to nucleic acids), it may be possible that the production of pyrophosphate and shortening

of the polyphosphate chain are two separate events. This implies that the appearance of pyrophosphate be accompanied by an increase in these other phosphate pools (i.e., AMP or nucleic acid phosphates). This was not observed. In addition, the production of pyrophosphate was accompanied by a dramatic decrease in polyphosphate levels, that could not be accounted for totally by the increase in inorganic phosphate. If the shortening of the polyphosphate chain length was predominantly a product of random hydrolysis by nonspecific phosphatases, there would be no decrease in the intensity of the polyphosphate resonance, since the total amount of phosphate contributing to the spectral intensity would remain the same. A drastic shortening of the average chain length (i.e., to chain lengths less than 10) would produce a slight downfield shift, which was not observed. Thus, there is strong evidence that pyrophosphate can be produced from polyphosphate in this organism as the metabolic conditions require. There may be some factor required to activate the specific polyphosphatase which produces pyrophosphate, although the identity of this factor is not evident from these experiments.

The approximate pH of the pyrophosphate environment as determined by the chemical shifts indicate that it is intracellular. Since the pyrophosphate resonance appears only under conditions in which no separate mitochondrial pH was

observed, it is not possible to further localize this pool to the mitochondria or cytoplasm, by this method. Pyrophosphate has previously been observed in mitochondria, and nuclei (Kulaev, 1975) as well as in cytoplasmic granules (Deslauriers et al., 1981). The appearance of two pools of pyrophosphate under treatment with methyl mercury supports this view, since the difference in chemical shifts of the pyrophosphate resonances reflects the pH difference across the plasma membrane as determined from the inorganic phosphate resonances. In the case of aerated cells treated with 1 mg nystatin, the chemical shift of the single pyrophosphate peak is indicative of the higher internal pH. The reason for only one observable pyrophosphate pool in the latter case, while two pools were observed in the former, is uncertain, but it may be related to differing viabilities (higher in the nystatin treatment) or modes of action of the two agents.

This series of experiments also demonstrates the ability of NMR to distinguish between various modes of action of toxic substances. The lytic action of nystatin, as observed by the immediate disappearance of a pH gradient accompanied by rapid hydrolysis of polyphosphate, correlates with what can be observed through the microscope. Methyl mercury chloride appears not to act through lysis even though this compound dissolves in the cytoplasmic membrane. It appears to enhance metabolic rates, as indicated by increased acidosis and a

more highly preserved mitochondrial pH gradient. While this evidence does not directly support the theory of mitochondrial action of methyl mercury, it does indicate that the mitochondria are somehow involved.

There are many additional experiments that could be performed in order to follow up some of the trends that were indicated in this chapter. Progressive toxicity of various agents could be followed by NMR under more highly controlled dosages. It would also be more relevant if the NMR and viability studies could be done on the same sample. This, however, is restricted by the latent loss of time resolution in the NMR experiment plus the requirement for sterile conditions to perform the viability study. In addition, the direct transfer of phosphate groups could be followed by NMR using the saturation transfer technique in the manner of Brown et al. (1977). This method would confirm the metabolic fate of polyphosphate units.

This study shows that both polyphosphate and pyrophosphate play a more active role in A. pullulans than simply as phosphate storage or metabolic byproduct. The high quantity of simple phosphate compounds found in this organism may account for its durability and may indicate that it is not as evolutionarily advanced as some other species of fungi. If the evolutionary theory of phosphate polymer function is correct, the disagreements observed in earlier literature as

to polyphosphate function are simply due to the species-specific nature of phosphate metabolism.

CHAPTER IX

ADDITIONAL EXPERIMENTS AND CONCLUSIONS

The following three preliminary studies were attempted during the course of this work. Due to the limitations, or to the properties of this organism, they were pursued no further. However, the preliminary results provide confirmatory evidence of properties of the organism that have been discussed in more detail in previous chapters and indicate the factors involved in the design of future experiments.

(a) ^{199}Hg NMR of Methyl Mercury Chloride (MeHgCl)

The ^{199}Hg nucleus (spin = 1/2) possess the advantages of relatively sharp NMR lines, a natural abundance of 17% and a very large chemical shift range of ~3000 ppm. The relative overall sensitivity is similar to ^{13}C (due to a lower gyromagnetic ratio), but there is no NOE enhancement since the CSA mechanism dominates the nuclear relaxation process (Sens et al., 1975). The relatively short T_1 's of mercury compounds in solution (for example, the T_1 of saturated HgCl_2 in ethanol is 1.4 sec (Maciel and Borzo, 1973)), compensate for this lack of sensitivity by allowing a rapid recycling rate.

Despite these advantages, ^{199}Hg NMR has not been one of the more popular "exotic" NMR nuclei to appear in the literature. From its magnetic properties, it appears to be an ideal tool to study the interactions of those mercury compounds which are environmental toxic hazards with biological systems. The ^{199}Hg nucleus gives rise to very narrow resonances (a few Hz) whose chemical shifts are highly sensitive to solvent (Sens et al., 1975; Borzo and Maciel, 1975) and pH (Sudmeier et al., 1978). The resonances of organomercury compounds will shift up to 100 ppm with solvent conditions. Methyl mercury salts are sensitive to pH over a range of ~150 ppm. However, when bound to a protein, that pH dependence is lost (Sudmeier et al., 1978). The toxic effects of methyl mercury are proposed to arise from enzyme inhibition and it is well known that organomercury compounds bind strongly to the sulphide groups in proteins. An additional advantage is that the resonances remain narrow when mercury compounds are irreversibly bound. This appears to be dependent upon the presence of ions. A pure mercury-protein complex exhibits a broad 400 Hz line, which sharpens and shifts upon the addition of salt (Sudmeier and Perkins, 1977). Binding of methyl mercury to glutathione from solutions of MeHgOH , resulted in a narrow resonance shifted 435 ppm downfield from that of aqueous MeHgOH (Sudmeier et al., 1975).

In theory, this property should permit the in situ observation of the different sites of methyl mercury in the living cell, which could be correlated with the viability curve and ^{31}P NMR results presented in Chapter VII. In practice, there are several spectral limitations. Resonances of a few Hz can be easily overlooked due to lack of digital resolution when employing the sweep widths required to look for resonances separated by orders of magnitude in shift. It may be necessary to use optimum sweep widths and scan adjacent spectral zones in separate spectra. Spectral intensity also becomes a problem when using the low concentrations that prove toxic to biological systems. The 6×10^{-4} M solution of MeHgCl used for the ^{31}P NMR studies is considerably more concentrated than is required for environmental toxic effects.

^{199}Hg was observed in a 15 mm broad band probe tuned to 53.6 MHz and using the 42-55 MHz transmitter normally used for deuterium. Solutions of 1.2 M $\text{Hg}(\text{Ac})_2$ in D_2O and 1.1 M HgCl_2 in ethanol/water were used as standards. The 90° pulse proved to be 52 μsec . The $\text{Hg}(\text{Ac})_2$ resonance was observed at 53.618047 MHz, while the HgCl_2 resonance occurred at 53.660085 MHz. The chemical shift of $\text{Hg}(\text{Ac})_2$ was concentration dependent. A 100-fold decrease in concentration (12 mM) shifted the resonance upfield by 9.65 ppm. A 10 mM aqueous solution of HgMeCl was observed at 53.697788 MHz.

A signal to noise ratio of 4:1 was observed after 500 accumulations using a 45° pulse and 0.5 sec recycle time. The observed frequencies are consistent with the resonance of HgCl_2 appearing 900 ppm downfield from 1.2 M $\text{Hg}(\text{Ac})_2$ and MeHgCl appearing 1550 ppm downfield from $\text{Hg}(\text{Ac})_2$, in agreement with the results of Borzo and Maciel (1975), who use $\text{Hg}(\text{Me})_2$ as a chemical shift zero.

A cellular suspension with the same concentration of MeHgCl showed an intensity reduction of a factor of two over the free solution, under the same spectral conditions. No other peaks were observed, even though the spectral window was sufficiently wide to permit observation of a bound peak ~265 ppm downfield, which was anticipated on the basis of the MeHg -glutathione complex (Sudmeier et al., 1978). This single crude experiment infers that a portion of the signal that is associated with the cellular uptake of mercury has been shifted outside the range of the spectral window or has been broadened beyond detection under high resolution conditions. The remaining signal intensity is due to residual aqueous MeHgCl , which is in equilibrium. It is likely that a significant portion of the mercury is dissolved in cellular lipid and would therefore resonate at a very different chemical shift; this effect being similar to that of changing the solvent conditions. In addition, applying the situation of the glutathione complex to the

various protein binding sites of the cell may not be valid, if chemical shifts are as sensitive to the immediate surroundings as the literature infers. Further studies using higher concentrations of cells and isotopically enriched methyl mercury could be used to investigate these trends.

The study shows that signal intensity will be a problem when using concentrations which are biologically relevant. Reduction of concentration from 10 mM to 6×10^{-4} M would require an increase in the accumulation time by a factor of 300 to obtain the same signal to noise ratio. For in situ studies, the methyl mercury should be isotopically enriched in order to obtain any time resolution in the experiment. In addition, parameters such as concentration and pH must be carefully controlled in order to obtain significant results. With these limitations in mind, ^{199}Hg NMR has a definite potential for probing intracellular location and specific interactions of organomercury compounds in cellular systems.

(b) The Fate of ^{13}C -1 Galactose in Cell Suspensions of *A. pullulans*

An attempt was made to follow the glycolytic pathway of a ^{13}C -labelled precursor in the yeast phase of *A. pullulans* by ^{13}C NMR, in the manner of den Hollander et al., (1979) where the fate of ^{13}C -1 glucose was followed in *S. cerevisiae*.

Galactose is readily utilized by A. pullulans as a sole carbon source, at approximately the same rate as glucose (Clarke and Wallace, 1958). This appears to be a property of many species of yeast (Sols et al., 1971), and is due to the presence of the enzymes which can first phosphorylate galactose to galactose-1-phosphate and then convert this to glucose-1-phosphate. A phosphoglucomutase then transfers the phosphate group to the C-6 position and normal glycolysis can occur. Galactose was employed for this initial experiment since the enriched material was readily available (synthesized courtesy of H. Jarrell).

10 mg of ^{13}C -1 galactose was added to a cell suspension from a 100 ml culture of A. pullulans (grown on unlabelled glucose medium), and 20 MHz ^{13}C spectra were recorded as a function of time. Trials were made under both aerobic and anaerobic conditions. Spectral conditions were such that only the resonances due to the enriched material were present. Two peaks appeared at 97.7 and 93.5 ppm due to the C-1(α) and C-1(β) positions of galactose respectively (Gorin and Mazurek, 1975), as well as a smaller peak at 64.4 ppm due to the C-1 position of the galactitol form, which was present in the starting material. Over a period of 20 hours (during which viability was lost under anaerobic conditions), there were no changes in the intensity of the galactose resonances, and no new peaks appeared. After this period, the cell

suspension was centrifuged, rinsed well and the spectrum rerun to determine if there had been significant absorption of galactose. The majority of the galactose was unabsorbed, remaining behind in the supernatant. The cell spectrum showed that a small fraction of galactose had been unchanged, but that the galactitol fraction was preferentially absorbed. The dominant resonance of the cell spectrum occurred at 64.4 ppm while the other C-1 resonances were much smaller. Uptake of the alditol form may require less energy than the pyranose form, in the membrane transport system of this yeast.

Since there is evidence from the ^{31}P spectra that the metabolism is slowed under the high cell density of the NMR tube and, from Chapter VII, that the organism appears to have the capability to optimize energy expenditure under deleterious conditions, this negative result is not surprising. Like glucose, galactose is absorbed by active transport, and the yeast prefers to use internal glucose storage pools over external sources for what glycolytic activity it requires under these high density conditions. In addition, the cells were harvested during the stationary phase when these storage pools are at a maximum. It is likely that the same result would be obtained with labelled glucose at this state of cell growth. However, it may be necessary to sensitize the culture by performing the initial growth on unlabelled galactose in order to trigger the synthesis of the enzymes

required to utilize galactose. While it would be interesting to correlate the changes in phosphate metabolism from ^{31}P NMR spectra with those in the ^{13}C spectra for a ^{13}C labelled precursor, for purposes of comparison to S. cerevisiae, the A. pullulans cellular system does not appear to lend itself easily to this particular sort of study.

(c) Growth of A. pullulans on Inositol

It was previously mentioned (Chapter IV) that an attempt was made to reduce the triglyceride content of the cell by supplementing the growth medium with inositol, only to discover that this yeast is one of the few that can utilize inositol as a sole carbon source. In general, the biomass was increased when the organism was cultured on a medium in which the glucose had been replaced by an equivalent amount of inositol but the acetate retained. The intracellular triglyceride content was reduced under these conditions but not as significantly as by the starving process (see Table IV.4). In addition, the C-18:2 acyl chain content was increased to the point that it represented 60% of the total fatty acid composition (Table IV.3). The morphology of the organism was modified to give a more uniform culture (Plate VI). Cultures were also supplemented with labelled acetate to observe any effect on the metabolism of the label or in the ^{13}C T_1 's (due to the change in fatty acid composition), from this change in carbon source.

The spectra of the labelled cell suspensions were identical to those observed in Figures V.2 to V.6 for growth on glucose. Both the fatty acid chains and the amino acid pool (of arginine and lysine) were labelled in the same manner and approximately to the same extent. The carbohydrate region of the spectrum showed that none of the label had entered the storage sugar pool. This region was compared to a spectrum of pure inositol which was assigned by comparison to standard spectra (Johnson and Jankowsky, 1972), to determine whether inositol, like glucose, was stored as a monosaccharide. The spectral resonances did not fit the inositol spectrum and could be completely assigned to trehalose and mannitol, present in approximately equal proportions. The presence of trehalose indicates that inositol is metabolized via the formation of G6P or UDPG, which are the direct precursors of trehalose (Metzler, 1977, p. 688). In microorganisms, inositol is manufactured from G6P to fulfill cellular requirements for phosphatidylinositol and polysaccharide synthesis. In mammalian systems, this reaction is not operative, which accounts for the role of inositol as an essential vitamin. In most microorganisms, the reaction is not reversible. A second pathway is also possible. Inositol can be utilized by some bacteria to form glucuronic acid, which then could form G6P via UDPG. However, this pathway involves several steps which have been demonstrated

to operate only in the opposite direction (i.e., G6P to UDPG to glucuronic acid). Therefore, it is likely that, in A. pullulans, the enzyme responsible for the process G6P inositol allows the reaction to occur in both directions; the control mechanism being the level of intracellular inositol. Under high inositol conditions, the G6P thus produced will be used for normal glycolysis. It appears that only glucose will be stored by the cell as a free monosaccharide. When other carbon sources are present, the more common fungal storage forms of trehalose and polyols are preferred. The appearance of mannitol under these conditions indicates that this polyol performs a specific function in cellular metabolism and is not simply a byproduct of abundant glucose in the medium.

As might be expected from the discussion of Chapter V, the ^{13}C T_1 profile of both cells and vesicles of the extracted lipid from inositol cultures differed very little from the curves of those grown on glucose. The motions that give rise to the relaxation are not sufficiently sensitive to this magnitude of change in the overall fatty acid composition, or the small reduction in triglyceride content. A spectrum of extracted lipid in CDCl_3 showed that the relative triglyceride content could be determined from the carbonyl region of the spectrum, as was suggested in Chapter V. The phospholipid resonance occurred at 177.9 ppm while a double

resonance (in the ratio 2:1, for the sn-1 and sn-2 positions of the glycerol backbone) occurred at 173.3 and 172.9 ppm. The intensity ratio of these two species was consistent with a TG/TL ratio of 1/3, which agrees well with the GC analysis.

While this type of "static" ^{13}C NMR experiment will not directly determine metabolic pathways, it does provide a method of identifying the metabolic end-products much more easily than by conventional radioactive labelling. Since the organism does not lend itself to studies of a more "dynamic" nature, i.e., following the label in situ, more detailed information on its metabolism could be obtained by monitoring metabolic end-products from growth on various carbon sources which have been spiked with isotopically enriched material. The ^{13}C label would then be retained by the carbohydrate fraction. It would also be of interest to study the path of the ^{13}C label during starvation for various initial carbon sources. Due to the effect of inositol on the fatty acid composition, the culture under these conditions represents an efficient and inexpensive method of producing relatively pure unsaturated phosphatidylcholine, both labelled and unlabelled. Growth on inositol did not appear to change the phospholipid headgroup composition to any extent. In retrospect, this may have been a better system to use for the study of bilayer dynamics, because of

the dominance of the C-18:2 fatty acid. The behaviour shown in this simple study is further evidence of the organism's ability to maintain a delicate balance of metabolic energy, which is a factor in its survival capabilities.

Conclusion

Nuclear Magnetic Resonance is an efficient tool for the investigation of both membrane and metabolic function. This study has illustrated the complementary information that can be derived from a multinuclear approach to these functions in an intact biological system. The major limitation to NMR, lack of sensitivity, can be used to advantage through bio-synthetic labelling to introduce a specificity that simplifies spectral interpretation. The time-dependent property of the NMR phenomenon provides an additional selectivity by allowing discrimination on the basis of motional properties by the choice of NMR parameters. The inherent properties of the NMR-active nucleus add a third type of specificity that permits, for example, the determination of intracellular pH and intracellular location by ^{31}P NMR. Using these three properties, the metabolic pathways of a ^{13}C -labelled substrate and the phosphate metabolism of many organisms can be studied by ^{13}C and ^{31}P NMR, respectively. In the ideal experiment, both nuclei can be monitored simultaneously through the use of a doubly-tuned probe. These studies can be easily extended to other classes of fungi or higher

eukaryotes in order to clarify the metabolic role of polyphosphate and pyrophosphate, study the variation in metabolic fate for a series of ^{13}C substrates, or determine the metabolic role of the various morphological forms in fungi. Further studies on A. pullulans could monitor the ^{13}C metabolism in a more dynamic way, for example, following starvation in situ or determining the factors involved in the production of pullulan. The monitoring of viability under NMR conditions is an important consideration when studying metabolic processes - one which has previously received little attention.

The application of NMR to eukaryotic organisms provides some complications, as this study points out. The structure of the natural membrane is quite fluid compared to that of prokaryotes and therefore the membrane properties of these systems cannot be easily studied in situ by ^{31}P and ^2H NMR. The use of solid state ^{31}P NMR techniques may permit the selection of the phospholipid component in the intact system, but the dilution of the phospholipid species requires conditions of greater sensitivity. The study of membrane dynamics by ^{13}C NMR, while useful for intramolecular comparisons, is not sufficiently sensitive to probe for specific group (protein, carbohydrate) functions or small changes between different organisms. However, with this consideration in mind, it is possible to compare the behaviour of lipids

in the intact, viable membrane to those in model systems or membrane preparations. The ability of A. pullulans to maintain some segregation of mitochondrial, peroxisomal and cytoplasmic function makes it an ideal source for the production of biosynthetically labelled lipid, which could be used directly or as a starting material for chemical modification.

The ability of the organism to utilize an extensive range of nitrogen, phosphorus and carbon sources, to adapt morphologically and metabolically to the immediate environment and to efficiently economize metabolic processes is responsible for the survival of A. pullulans under conditions that prove deleterious to many other species. The study of these processes relates immediately to the economically detrimental effects of the organism to paint and wood, as well as to the profitable aspect in the production of pullulan and control of its structural properties. The latter aspect is only one example of the industrial use of the products of fungal secondary metabolism. Many aspects of secondary metabolism are not yet well understood and the study of these metabolic pathways and their control mechanisms lends itself easily to the NMR approach.

SUMMARY

The primary hypothesis of this thesis was to determine the advantages and limitations of NMR in the understanding of several aspects of a little characterized organism in vivo. These aspects included membrane structure, cytoplasmic segregation of metabolic processes and phosphate metabolism.

A) Membrane Studies

Studies of the membrane components of A. pullulans were accomplished by selective biosynthetic incorporation of specifically labelled acetate into the fatty acid chains of the lipid moiety. The level of enrichment (20%) corresponded to the ratio of unlabelled glucose to labelled acetate in the original growth medium, and illustrated the efficient shunting of acetate into reactions of cellular bodies rather than glycolysis. The pattern of enrichment made it possible to follow the dynamics of the lipid chains via ^{13}C relaxation times and permitted the spectral resolution of most positions along the average fatty acid chain.

The relaxation profile of whole viable cells closely resembled that of model membranes (sonicated vesicles), indicating that the protein component does not appreciably affect chain dynamics on the time scale probed by ^{13}C NMR. Furthermore, the profile of viable cells was only marginally different from those from starved cells and two types of membrane preparations - these systems representing various stages of removal of the nonbilayer

(storage) lipid component. Thus the whole cell T_1 profile is indicative of a functioning membrane.

The qualitative aspects of the T_1 profile are preserved independent of the form of aggregation of the lipid. The contribution of nonbilayer lipid determines the position of the T_1 profile on the T_1 axis. Measurement of T_1 profiles at two magnetic fields, with the presumption that relaxation is predominantly dipolar, established that the T_1 's lie on the motional narrowing region of the rotational correlation function (assuming isotropic reorientation). The correlation times fall between 10^{-10} and 10^{-11} seconds, in agreement with results derived from ^2H relaxation times in model bilayer systems.

The chain dynamics were determined specifically in the region of the double bonds for the C-18:1 and C-18:2 chains. The steric hindrance due to the cis double bond system allows for greater motional freedom at carbons adjacent to allylic carbons as well as greater rotational freedom towards the end of the chain. This effect is enhanced when two double bonds are present, as was observed at C-11 and C-16, since these carbons could be resolved for the two chain species. The overall aspects of this more precisely determined T_1 profile are consistent with the model of restricted motional freedom from C-2 to C-13 (the region of concerted rotations of the acyl chain due to steric hindrance), with increasing rotational freedom at the centre of the bilayer (C-15 to C-18).

The fluid property of the highly unsaturated natural membrane

was further supported by evidence from ^2H NMR of the perdeuterated lipid species, which shows a large isotropic component and a pattern of quadrupolar splittings that compares well to results from specifically deuterated oleate membranes from A. laidlawii.

^{31}P CSA patterns of the isolated phospholipid show a high stability in bilayer structure with respect to temperature. This is likely due to the specific ratio of charged to zwitter-ionic phospholipids and this property may serve to compensate for the greater motional freedom of the acyl chains in stabilizing the membrane structure of this organism.

B) Metabolic Studies

The fate of the acetate label in carbon metabolism was determined from ^{13}C NMR through comparison of labelling patterns from initial growth in the labelled substrate and after carbon deprivation, the latter following utilization of storage labelled triglycerides. The labelling pattern showed a marked segregation between cytoplasmic processes and those of mitochondria and other cellular bodies. The major storage carbohydrates of this organism are glucose and trehalose, depending on the age of the culture, as well as varying amounts of mannitol. These carbohydrate reserves were retained during carbon deprivation.

During primary metabolism, the acetate label appeared in two major storage amino acids - lysine and arginine, via the citric acid cycle, in addition to lipid chains. Evidence from the isolation of pullulan indicated that some of the label enters protein, predominantly in aspartate residues. The labelling pattern of these amino acids was indicative of

substantial glyoxylate activity. The labelling pattern of amino acids during carbon deprivation was consistent with the slowing of protein synthesis (slow biomass increase), in that the amino acid pool becomes more fully labelled. The patterns are consistent with biochemical pathways that are known to exist in yeast. For example, lysine was shown to derive from glutarate rather than aspartate, and the ring structure of histidine was shown to retain only one carbon atom from acetate. Acetate from β -oxidation was shown to enter metabolism in three ways: directly via the citric acid cycle, by condensation with glyoxalate and by keto-chain elongation.

High resolution ^{31}P NMR studies of phosphate metabolism of the organism were performed under various perturbing conditions leading to loss of viability. Changes in cellular phosphates were observed to correlate with the overall viability curve of the culture under the conditions of the NMR tube. A minor hypothesis was to discern the role of polyphosphate and pyrophosphate in A. pullulans by NMR. The modes of action of nystatin and methyl mercury, which give rise to similar viability curves, could be discerned from differences in the NMR spectra.

This organism contains higher levels of polyphosphate and lower levels of ATP than have been observed for other yeasts. The behavior of the polyphosphate fraction is in contrast to other workers' observations in that polyphosphate reserves are depleted under conditions of carbon deprivation and in the

presence of adequate phosphate in the medium. Polyphosphate is also the major source of cellular phosphate in spite of externally supplied phosphate. Several correlations appeared during the course of this work which indicate that;

- a) polyphosphate is stored in vacuoles, possibly the central vacuole, rather than in volutin granules. Pyrophosphate appears to be cytosolic.
- b) polyphosphate is highly complexed with Mg^{2+} , serving an additional role in storage of cellular magnesium.
- c) polyphosphate plays a more active role in cellular metabolism than simply as a storage vessel for cellular phosphate, but there is no direct evidence that it acts as a phosphagen.
- d) it is apparent that pyrophosphate is, under certain conditions, derived directly from polyphosphate. Pyrophosphate may provide an alternative source to ATP for phosphate bond energy, under conditions of high intracellular pH or cellular stress.

This NMR study shows the advantages of NMR in determining cellular processes in vivo, under the simplifying conditions provided by the dynamic nature of this spectroscopic technique.

REFERENCES

- Abraham, A. The Principles of Nuclear Magnetism, Oxford University Press, London (1961).
- Afanasieva, T.P. and I.S. Kulaev. Biochim. Biophys. Acta, 321, 336 (1973).
- Andrew, E.R. Phil. Trans. R. Soc. London B, 289, 471 (1980).
- Angyal, S. and G.S. Bethell. Aust. J. Chem., 29, 1249 (1976).
- Armitage, I.M., H. Huber, D.H. Live, H. Pearson and J.D. Roberts. J. Magn. Res., 15, 142 (1974).
- Arnold, K., A. Lösche and K. Gawrisch. Biochim. Biophys. Acta, 645, 143 (1981).
- Avila, E.M., J.A. Hamilton, J. Harmony, A. Allerhand and E. Cordes. J. Biol. Chem., 253, 3983 (1978).
- Bailey, I.A., P.G. Gadian, P.M. Matthews, G.K. Radda and P.J. Seeley. FEBS Lett., 123(2), 315 (1981).
- Barton, J.K., J.A. den Hollander, T.M. Lee, A. MacLaughlin and R.G. Shulman. Proc. Natl. Acad. Sci. U.S.A., 77, 2470 (1980).
- Barton, P.G. Chem. Phys. Lipids, 14, 336 (1975).
- Basabe, J.R., C.A. Lee and R.L. Weiss. Analytical Biochem., 92, 356 (1979).
- Batchelor, J.G., H.J. Prestegard, R.J. Cushley and S.R. Lipsky. Biochim. Biophys. Res. Commun., 48, 70 (1972).
- Becker, E., J. Ferratti, R. Gupta and G. Weiss. J. Magn. Res., 37, 381 (1980).
- Berg, G.G. and E.F. Miles. Membrane Biochem., 2, 117 (1978).
- Bernassau, J. and F. Hyafil. J. Magn. Res., 40, 245 (1980).
- Birdsall, N.J.M., D.J. Ellen, A.G. Lee, J.C. Metcalfe and G.B. Warren. Biochim. Biophys. Acta, 380, 344 (1975).
- Bishop, E.O., S.J. Kimber, D. Orchard and B.E. Smith. Biochim. Biophys. Acta, 635, 63 (1981).

- Bittman, R. *Lipids*, 13(10), 686 (1979).
- Bligh, E.G. and W.J. Dyer. *Can. J. Biochem. Physiol.*, 37, 911 (1959).
- Bloom, M. *Can. J. Phys.*, 57, 2227 (1979).
- Bloom, M., H.J. Davis and A. MacKay. *Chem. Phys. Lett.*, 80, 198 (1981).
- Boonsaeng, V., P.A. Sullivan and M. Shepherd. *Can. J. Microbiol.*, 22, 808 (1976).
- Borzo, M. and G.E. Maciel. *J. Magn. Res.*, 19, 279 (1975).
- Brainard, J. and E. Cordes. *Biochemistry*, 20, 4607 (1981).
- Brainard, J. and A. Szabo. *Biochemistry*, 20, 4618 (1981).
- Brown, L.R. and K. Wüthrich. *Biochim. Biophys. Acta*, 468, 389 (1977).
- Brown, M.F. *J. Magn. Res.*, 35, 203 (1979).
- Brown, M.F. and J. Seelig. *Biochemistry*, 17, 381 (1978).
- Brown, M.F., J. Seelig and U. Häberlen. *J. Chem. Phys.*, 70, 5045 (1979).
- Brown, R.G. and B. Lindberg. *Acta Chem. Scand.*, 21, 2379 (1967).
- Brown, R.G., L.A. Hanic and M. Hsiao. *Can. J. Microbiol.*, 19, 163 (1973).
- Brown, T.R., K. Ugurbil and R.G. Shulman. *Proc. Natl. Acad. Sci. U.S.A.*, 74, 5551 (1977).
- Brown, T.R., J.A. den Hollander, R.G. Shulman and K. Ugurbil. In *Frontiers of Biological Energetics*, Vol. II (ed. P.H. Dutton, J.S. Leigh and A. Scarpa), p. 1365, Academic Press, New York (1978).
- Browning, J.L. and J. Seelig. *Biochemistry*, 19, 1262 (1980).
- Büldt, G. and R. Wohlgenuth. *J. Membrane Biol.*, 58, 81 (1981).
- Burnell, E., L. van Alphen, A. Verkleij and B. de Kruijff. *Biochim. Biophys. Acta*, 597, 492 (1980(a)).

- Burnell, E., L. van Alphen, A. Verkleij and B. de Kruijff. *Biochim. Biophys. Acta*, 597, 492 (1980(a)).
- Burnell, E., L. van Alphen, A. Verkleij and B. de Kruijff. *Biochem. Biophys. Acta*, 597, 518 (1980(b)).
- Burnell, E., P.R. Cullis and B. de Kruijff. *Biochim. Biophys. Acta*, 603, 63 (1980(c)).
- Bus, J., I. Sies and M. Lie Ken Jie. *Chem. Phys. Lipids*, 18, 130 (1977).
- Bussey, H., D. Saville, M.R. Chevallier and G.H. Rank. *Biochim. Biophys. Acta*, 553, 185 (1979).
- Canet, D. *J. Magn. Res.*, 23, 361 (1976).
- Canet, D., G.C. Levy and I.R. Peat. *J. Magn. Res.*, 18, 199 (1975).
- Carrington, A. and A. McLaughlin. *Introduction to Magnetic Resonance*. Harper and Row, New York (1967).
- Carty, A.J. and S.F. Malone. In *The Biogeochemistry of Mercury in the Environment*, Topics in Environmental Health, Vol. 3, Ch. 17. Elsevier North-Holland, New York (1979).
- Catley, B. *J. Gen. Microbiol.*, 78, 33 (1973).
- Catley, B.J. In *Microbiol Polysaccharides and Polysaccharases*, (ed. R.C. Berkeley, G.W. Gooday and D.C. Ellwood), Ch. 4, p. 69, Academic Press, New York (1979).
- Catley, B.J. and P.J. Kelly. *Biochem. Soc. Trans.*, 3, 1079 (1975).
- Cernakova, M., A. Kockova-Katochvilova, L. Sutý, J. Zemek and L. Kuniak. *Folia Microbiol.*, 25, 68 (1980).
- Challinor, S.W. and N.W.R. Daniels. *Nature*, 176, 1267 (1955).
- Chapman, D. *Quart. Rev. Biophys.*, 8, 185 (1975).
- Chapman, D., J.C. Gomez-Fernandez and F.M. Goni. *FEBS Lett.*, 98, 211 (1979).
- Christie, W.W. *Lipid Analysis*, Ch. 6, p. 158. Pergamon Press, Toronto (1973).
- Christl, M. and J.D. Roberts. *J. Amer. Chem. Soc.*, 94, 4565 (1972).

- Christy, M.R., R.M. Barkley, T.H. Koch, J.J. van Buskirk and W.M. Kirsch. *J. Amer. Chem. Soc.*, 103, 3935 (1981).
- Clarke, D.S. and R.H. Wallace. *Can. J. Microbiol.*, 4, 125 (1958).
- Cohen, S.M. and R.G. Shulman. *Phil. Trans. R. Soc. London B*, 289, 407 (1980).
- Cohen, S.M., S. Ogawa, H. Rottenberg, P. Glynn, T. Yamane, T.R. Brown and R.G. Shulman. *Nature*, 273, 554 (1978(a)).
- Cohen, S.M., S. Ogawa and R.G. Shulman. In *Frontiers of Biological Energetics*, Vol. II, (ed. P.L. Dutton, J. Leigh and A. Scarpa), p. 1357. Academic Press, New York (1978(b)).
- Cohen, S.M., R.G. Shulman and A.C. McLaughlin. *Proc. Natl. Acad. Sci. U.S.A.*, 76, 4808 (1979).
- Cohen, S.M., P. Glynn and R.G. Shulman. *Proc. Natl. Acad. Sci. U.S.A.*, 78, 60 (1981).
- Colson, P., H.J. Jennings and I.C.P. Smith. *J. Amer. Chem. Soc.*, 96, 8081 (1974).
- Colson, P., K.N. Slessor, H.J. Jennings and I.C.P. Smith. *Can. J. Chem.*, 53, 1030 (1975).
- Colson, P., H.C. Jarrell, B.L. Lamberts and I.C.P. Smith. *Carbohydrate Res.*, 71, 265 (1979).
- Cooke, W.B. *Mycopathol. Mycol.*, 12, 1 (1959).
- Cronan, J.E. and J.G. Batchelor. *Chem. Phys. Lipids*, 11, 196 (1973).
- Cullis, P.R. and B. de Kruijff. *Biochim. Biophys. Acta*, 513, 31 (1978(a)).
- Cullis, P.R. and B. de Kruijff. *Biochim. Biophys. Acta*, 507, 207 (1978(b)).
- Cullis, P.R. and B. de Kruijff. *Biochim. Biophys. Acta*, 559, 399 (1979).
- Cullis, P.R., P. van Dijck, B. de Kruijff and J. de Gier. *Biochim. Biophys. Acta*, 513, 21 (1978(a)).

- Cullis, P.R., A.J. Verkleij and P. Ververgaert. *Biochim. Biophys. Acta*, 513, 11 (1978(b)).
- Cullis, P.R., B. de Kruijff, M.J. Hope, R. Nayar and S.L. Schmid. *Can. J. Biochem.*, 58, 1092 (1980(a)).
- Cullis, P.R., B. de Kruijff, M.J. Hope, R. Nayar, A. Rietveld and A.J. Verkleij. *Biochim. Biophys. Acta*, 600, 625 (1980(b)).
- Damadian, R. *Phil. Trans. R. Soc. London B*, 289, 489 (1980).
- Daum, G., G. Gomerith and F. Paltauf. *Biochim. Biophys. Acta*, 573, 413 (1979).
- Davis, J.H., K.R. Jeffrey, M. Bloom, M.I. Valic and T.P. Higgs. *Chem. Phys. Lett.*, 42, 390 (1976).
- Davis, J.H., K.R. Jeffrey and M. Bloom. *J. Magn. Res.*, 29, 191 (1978).
- Davis, J.H., M. Bloom, K.W. Butler and I.C.P. Smith. *Biochim. Biophys. Acta*, 597, 477 (1980).
- Davoust, J., A. Bienvenue, P. Fellman and P.F. Devaux. *Biochim. Biophys. Acta*, 596, 28 (1980).
- Dawes, E.A. and P.J. Senior. In *Advances in Microbial Physiology*, (ed. A.H. Rose and D.W. Tempest), Vol. 10, 135 (1973).
- Deamer, D. and A.D. Bangham. *Biochim. Biophys. Acta*, 443, 629 (1976).
- Deese, A.J., E.A. Dratz, F.W. Dahlquist and M.R. Paddy. *Biochemistry*, 20, 6420 (1981).
- Degani, H., A. Danon and S.R. Caplan. *Biochemistry*, 19, 1626 (1980).
- de Kruijff, B., P.R. Cullis, G.K. Radda and R.E. Richards. *Biochim. Biophys. Acta*, 419, 411 (1976).
- de Kruijff, B., G.A. Morris and P.R. Cullis. *Biochim. Biophys. Acta*, 598, 206 (1980(a)).
- de Kruijff, B., A. Rietveld and P.R. Cullis. *Biochim. Biophys. Acta*, 600, 343 (1980(b)).

- de Kruijff, B., A. Verkleij, C. van Echteld, W. Gerritsen, C. Mombers, P. Noordam and J. de Gier. *Biochim. Biophys. Acta*, 555, 200 (1979).
- Demel, R.A. and B. de Kruijff. *Biochim. Biophys. Acta*, 457, 109 (1976).
- den Hollander, J.A., T.R. Brown, K. Ugurbil and R.G. Shulman. *Proc. Natl. Acad. Sci. U.S.A.*, 76, 6069 (1979).
- den Hollander, J.A., K.L. Behar and R.G. Shulman. *Proc. Natl. Acad. Sci. U.S.A.*, 78, 2693 (1981(a)).
- den Hollander, J.A., K. Ugurbil, T.R. Brown and R.G. Shulman. *Biochemistry*, 20, 5871 (1981(b)).
- Deslauriers, R., R. Walter and I.C.P. Smith. *Proc. Natl. Acad. Sci. U.S.A.*, 71, 265 (1974).
- Deslauriers, R., H. Jarrell, R. Byrd and I.C.P. Smith. *Biochem. Biophys. Res. Commun.*, 95, 1211 (1980(a)).
- Deslauriers, R., R.A. Byrd, H.C. Jarrell and I.C.P. Smith. *Eur. J. Biochem.*, 111, 369 (1980(b)).
- Deslauriers, R., J. Ekiel, R.A. Byrd, H.C. Jarrell and I.C.P. Smith. *Biochim. Biophys. Acta*, (1981), submitted.
- Dill, K. and A. Allerhand. *J. Amer. Chem. Soc.*, 101, 4378 (1979).
- Dittmer, J.D. and R.L. Lester. *J. Lipid Res.*, 5, 126 (1964).
- Doddrell, D., V. Glusko and A. Allerhand. *J. Chem. Phys.*, 56, 3683 (1972).
- Dominguez, A., M. Elorza, E. Santos, J.R. Villanueva and R. Sentandreu. *Antonie van Leeuwenhoek*, 44, 341 (1978).
- Drainville, G. and A. Gagnon. *Comp. Biochem. Physiol.*, 45A, 379 (1973).
- Drews, G. *Colloq. Intl. Centre Natl. Res. Sci. (Paris)*, 106, 533 (1962).
- Eakin, R.T., L.O. Morgan, C.T. Gregg and N.A. Matiwiyoff. *FEBS Lett.*, 28, 259 (1972).
- Ebel, J.B. *Bull. Ste. Chim. Biol.*, 34, 498 (1952).

- Elinov, N.P., N.A. Yurlova and T.P. Efimova. *Mikrobiologiya*, 44, 1051 (1975).
- Ermakova, S.A., S.E. Mansurova, T.S. Kelebina, E.S. Lobakova, I.O. Selyach and I.S. Kulsev. *Arch. Microbiol.*, 128, 394 (1981).
- Farrer, T.C. and E.D. Becker. *Pulse and Fourier Transform NMR*, Academic Press, New York, Ch. 4 (1971).
- Feeney, J., P. Partington and G.C.K. Roberts. *J. Magn. Res.*, 13, 268 (1974).
- Feigenson, G.W. and S.I. Chan. *J. Amer. Chem. Soc.*, 96, 1312 (1974).
- Felter, S., G. Dirheimer and J. Ebel. *Bull. Soc. Chim. Biol.*, 52, 433 (1970).
- Felter, S. and A.J.C. Stahl. *Bull. Soc. Chim. Biol.*, 52, 75 (1970).
- Felter, S. and A.J.C. Stahl. *Biochimie*, 55, 245 (1973).
- Finkelstein, A. and R. Holz. In *Membranes* (ed. G. Eisenmann), Vol. 2, Ch. 5, Marcel Dekker Inc., New York (1973).
- Folch, J., M. Lees and G.H. Sloan Stanley. *J. Biol. Chem.*, 226, 497 (1957).
- Freedman, M.H., J.R. Lyster, I.M. Chaikin and J.S. Cohen. *Eur. J. Biochem.*, 32, 215 (1973).
- Freeman, R., H.D.W. Hill and R. Kaptein. *J. Magn. Res.*, 7, 327 (1972).
- Friedberg, I. and G. Avigad. *J. Bacteriol.*, 96, 544 (1968).
- Fryberg, M., A.C. Oehlschlager and A.M. Unrau. *Archives Biochem. and Biophys.*, 160, 83 (1974).
- Ibid.*, 173, 171 (1975).
- Fukui, S., M. Mishina, S. Yanagawa and A. Tanaka. In *Yeasts and Yeast-like Microorganisms in Medical Science*, (ed. K. Iwata), p. 85 (1972).
- Gabel, N.W. and V. Thomas. *J. Neurochem.*, 18, 1229 (1971).

- Gadian, D.G., G.K. Radda, R.E. Richards and P.J. Seeley. In *Biological Applications in Magnetic Resonance*, (ed. R.G. Shulman), Academic Press, New York, Ch. 10 (1979).
- Gally, H.U., A. Seelig and J. Seelig. *Hoppe-Seyler's Z. Physiol. Chem.*, 357, 1447 (1976).
- Gally, H., G. Pluschke, P. Overath and J. Seelig. *Biochemistry*, 19, 1638 (1980).
- Gancedo, C., J.M. Gancedo and A. Sols. *Eur. J. Biochem.*, 5, 166 (1968).
- Gent, M.P.N. and J.H. Prestegard. *Biochem. Biophys. Res. Commun.*, 58, 549 (1974).
- Gerhards, R. and W. Dietrich. *J. Magn. Res.*, 23, 21 (1976).
- Gillies, R.J., K. Ugurbil, J.A. den Hollander and R.G. Shulman. *Proc. Natl. Acad. Sci. U.S.A.*, 78, 2125 (1981).
- Glasoe, P.K. *J. Phys. Chem.*, 64, 188 (1960).
- Glonek, T., M. Lunde, M. Mudgett and J.C. Myers. *Archives & Biochem. Biophys.*, 142, 508 (1971).
- Gorin, P.A. and M. Mazurek. *Can. J. Chem.*, 53, 1212 (1975).
- Gunstone, F.D., M.R. Pollard, C.M. Scrimgeour and H.S. Vedonayagam. *Chem. Phys. Lipids*, 18, 115 (1977).
- Gupta, R.K. and W.D. Yushok. *Proc. Natl. Acad. Sci. U.S.A.*, 77, 2487 (1980).
- Harold, F.M. *Biochim. Biophys. Acta*, 45, 172 (1960).
- Harold, F.M. *J. Bacteriol.*, 83, 1047 (1962).
- Harold, F.M. *Bacteriol. Rev.*, 30, 772 (1966).
- Harris, R.K. and R.H. Newman. *J. Magn. Res.*, 24, 449 (1976).
- Hartman, T.L. *Stain Technology*, 15, 23 (1940).
- Hauser, H., M.C. Phillips, B. Levine and R.J.P. Williams. *Eur. J. Biochem.*, 58, 133 (1975).
- Hawthorne, J.N. and D.A. White. *Vitamins and Hormones*, Vol. 33, (ed. P.L. Munson et al.), Academic Press, New York, p. 529 (1975).

Henderson, T.O., T. Glonek and T. Meyers. *Biochemistry*, 13, 623 (1974).

Hoffman-Ostenhof, O. *Colloq. Intl. Centre Nat. Res. Sci. (Paris)*, 106, 640 (1962).

Hollis, D.P. In *Biological Magnetic Resonance*, Vol. 2, (ed. L.J. Berliner and J. Reuben), Plenum Press, New York, p. 1 (1980).

Hope, M.J. and P.R. Cullis. *Biochem. Biophys. Res. Commun.*, 92, 846 (1980).

Howard, B. *Biochim. Biophys. Acta*, 488, 145 (1977).

Howarth, O.H. *Faraday Trans. II*, 75, 863 (1979).

Hunter, K. and A.H. Rose. In *The Yeasts*, Vol. 2, (ed. A.H. Rose and J.S. Harrison), Academic Press, New York, Ch. 6, p. 211 (1971).

Hutchison, H.T. and L.H. Hartwell. *J. Bacteriol.*, 94, 1697 (1967).

Inshenetskii, A.A. and T.F. Kondrateva. *Doklady Biol. Sci.*, 239, 149 (1978).

Indge, K.J. *J. Gen. Microbiol.*, 51, 447 (1968).

Irving, C.S. and A. Lapidot. *Biochim. Biophys. Acta*, 470, 251 (1977).

Jacob, G.S., J. Schaefer, E.O. Stejskal and R.A. McKay. *Biochem. Biophys. Res. Commun.*, 97, 1176 (1980).

Jacobs, R. and E. Oldfield. *Biochemistry*, 18, 3280 (1979).

Jacobsen, L. and J.S. Cohen. *TAMU*, 268, 25 (1981).

Jaffe, E.K. and M. Cohen. *Biochemistry*, 17, 652 (1978).

Jendrasiak, G.L. and T.N. Estep. *Chem. Phys. Lipids*, 18, 181 (1977).

Jennings, H. and I.C.P. Smith. *J. Amer. Chem. Soc.*, 95, 606 (1973).

Johnson, L.F. and W.C. Jankowski. *Carbon-13 NMR Spectra*, Wiley-Interscience, New York (1972).

- Joyce, A. and I.C.P. Smith. Microbiology, 5 (1979).
- Jung, G., G. Breitmaier, W. Voelter, T. Keller and G. Tanzer. Angew. Chem. Internat. Edit., 9, 894 (1970).
- Kainosko, K., P.A. Kroon, R. Lawaczeck, N.O. Petersen and S.I. Chan. Chem. Phys. Lipids, 21, 59 (1978).
- Kainosko, M. Tetrahedron Lett., 47, 4279 (1976).
- Kainosko, M., K. Ajisaka and H. Nakazawa. FEBS Lett., 80, 385 (1977).
- Kaltwasser, H. Arch. Microbiol., 41, 282 (1962).
- Karlin, J.N., B.J. Bowman and R.H. Davis. J. Biol. Chem., 251, 3948 (1976).
- Katchman, B.J. and W.O. Fetty. J. Bacteriol., 69, 607 (1955).
- Kates, M. Advances in Lipid Research, 2, 17 (1964).
- Kates, M. Techniques of Lipidology, North-Holland Pub. Co., Amsterdam (1972).
- Keough, K., E. Oldfield and D. Chapman. Chem. Phys. Lipids, 10, 37 (1973).
- Kikuchi, Y., Y. Sakano, R. Taguchi and T. Kobayashi. Agricult. Biol. Chem., 38(5), 1091 (1974).
- Kinskey, S.C., G.R. Gronau and M.M. Weber. Molec. Pharmacol., 1, 190 (1965).
- Kleinig, H. and C. Kopp. Planta., 139, 61 (1978).
- Kockova-Kratochvilova, A., M. Cernakova and E. Slavikova. Folia Microbiol., 25, 56 (1980).
- Koerner, T., Jr., L. Cary, N. Bhaccu and E. Younathen. Biochem. Biophys. Res. Commun., 51, 543 (1973).
- Koerner, T., Jr., R. Voll, L. Cary and E. Younathen. Biochem. Biophys. Res. Commun., 82, 1273 (1978).
- Komor, B., E. Komor and W. Tanner. J. Membrane Biol., 17, 231 (1974).
- Kulaev, I.S. Rev. Physiol. Biochem. Pharmacol., 73, 131 (1975).

- Kulaev, I.S., V.N. Vagubov and A.B. Tsiomenko. Doklady Akad. Nauk. SSSR, 206, 734 (1972(a)).
- Kulaev, I.S., G.I. Konoshenko and A.M. Umnov. Biochimia. 37, 227 (1972(b)).
- Kulaev, I.S., I.A. Krashenninilov and Y.A. Yyrsin. Mikrobiologiya, 42, 545 (1973).
- Kulaev, I.S., S.E. Mansurova, E.B. Burlakova and V.F. Daklovish. Biosystems, 12, 177 (1980).
- Kuswaka, S.C., M. Kates, G.D. Sprott and I.C.P. Smith. Biochim. Biophys. Acta, 664, 156 (1981).
- Lagaly, G., A. Weiss and E. Stüke. Biochim. Biophys. Acta, 470, 331 (1977).
- Lampin, J.O., E.R. Morgan and A.C. Slocum. J. Bacteriol., 74, 297 (1957).
- Lampin, J.O., P.M. Arnow and R.S. Safferman. J. Bacteriol., 80, 200 (1960).
- Lampin, J.O., P.M. Arnow, Z. Borowska and A.I. Laskin. J. Bacteriol., 84, 1152 (1962).
- Lancée-Hermkens, A.M.W. and B. de Kruijff. Biochim. Biophys. Acta, 470, 141 (1977).
- Langen, P., E. Liss and K. Köhmann. Colloq. Intl. Centre Natl. Res. Sci. (Paris), 106, 603 (1962).
- Lapidot, A. and C.S. Irving. Proc. Natl. Acad. Sci. U.S.A., 74, 1988 (1977).
- Lee, A.G., N.J.M. Birdsall and J.C. Metcalfe. In Methods in Membrane Biology, Vol. 2, (ed. E.D. Korn), Ch. 1, p. 1, Plenum Press, New York (1974).
- Lee, A.G., N.J.M. Birdsall, J.C. Metcalfe, G.B. Warren and G.C.K. Roberts. Proc. Royal Soc. B, 193, 253 (1976).
- Lehninger, A.L. Biochemistry, The Molecular Basis of Cell Structure and Function, Worth Pub. Inc., New York, p. 775 (1970).
- Levine, Y., N. Birdsall, A. Lee and J. Metcalfe. Biochemistry, 11, 1416 (1972).

- Levy, G.C. and I.R. Peat. *J. Magn. Res.*, 18, 500 (1975).
- Levy, G.C., M.P. Cordes, J.S. Lewis and D.E. Axelson. *J. Amer. Chem. Soc.*, 99, 5492 (1977).
- Lewin, L.M. *J. Gen. Microbiol.*, 41, 215 (1965).
- Liss, E. and P. Langen. *Archives Mikrobiol.*, 41, 383 (1962).
- Llinas, M., W. Meier and K. Wüthrich. *Biochim. Biophys. Acta*, 492, 1 (1977).
- London, E. and G.W. Feigenson. *J. Lipid Res.*, 20, 408 (1979).
- London, R.E. and J. Avitable. *J. Amer. Chem. Soc.*, 99, 7765 (1977).
- London, R.E., V.H. Kollman and N.A. Matwiyoff. *Biochemistry*, 14, 5492 (1975).
- Maciel, G.E. and M. Borzo. *J. Magn. Res.*, 10, 338 (1973).
- Mansch, H.H., H. Saito and I.C.P. Smith. *Prog. NMR Spectroscopy II*, 211 (1977).
- Marini, F., P. Arnow and J.O. Lampen. *J. Gen. Microbiol.*, 24, 51 (1961).
- Matsumaga, T., A. Okubo, S. Yamazaki and S. Toda. *Agric. Biol. Chem.*, 41, 2757 (1980).
- Measures, J.C. *Nature*, 257, 398 (1975).
- Merdinger, E. *Trans. Ill. State Acad. Sci.*, 57, 28 (1964).
- Merdinger, E. *J. Microbiol.*, 12, 206 (1966).
- Merdinger, E. *J. Bacteriol.*, 98, 1021 (1969).
- Merdinger, E. and P. Kohn. *Can. J. Microbiol.*, 13, 1126 (1967).
- Merdinger, E., P. Kohn and R.C. McClain. *Can. J. Microbiol.*, 14, 1021 (1968).
- Metcalf, J.C., N.J.M. Birdsall and A.G. Lee. *FEBS Lett.*, 21, 335 (1972).
- Metzler, D.E. *Biochemistry, The Chemical Reactions of Living Cells*, Academic Press, New York (1977).

- Miller, C.T. and D.R. Miller. In Effects of Mercury in the Canadian Environment, (ed. Associate Committee on Scientific Criteria for Environment Quality), Ch. 6, p. 106 (1979).
- Miller, K.W. and K.Y. Pang. *Nature*, 263, 253 (1976).
- Morris, G.A. *J. Magn. Res.*, 41, 185 (1980).
- Morris, G.A. and R. Freeman. *J. Magn. Res.*, 29, 433 (1978).
- Naga, J., S. Yokoe, M. Tanaka, H. Hibasami and T. Ikeda. *Lipids*, 16, 411 (1981).
- Nandini-Kishoe, S.G., Y. Kitajima and G.A. Thompson, Jr. *Biochim. Biophys. Acta*, 471, 157 (1977).
- Navon, G., S. Ogawa, R.G. Shulman and T. Yamane. *Proc. Natl. Acad. Sci. U.S.A.*, 74, 888 (1977).
- Navon, G., R. Shulman, T. Yamane, T.R. Eccleshall, K.B. Lam, Baronofsky and Marmur. *Biochemistry*, 18, 4487 (1979).
- Neff, R.J., S.A. Roy, W.F. Benton and M. Wilborn. In *Methods in Cell Physiology*, Vol. 1, (ed. D.M. Prescott), Academic Press, New York, Ch. 4 (1964).
- Nichol, C.P., J.H. Davis, G. Weeks and M. Bloom. *Biochemistry*, 19, 451 (1980).
- Niemierko, W. *Colloq. Intl. Centre Natl. Tech. Sci.*, 106, 615 (1962).
- Nishi, A. *J. Bacteriol.*, 81, 10 (1961).
- Norton, R.S. *Comp. Biochem. Physiol.*, 63B, 67 (1979).
- Norton, R.S. *Advances Magn. Res.*, in press (1980).
- Numez, H.A., T.E. Walker, R. Fuentes, J. O'Connor, A. Serrianni and R. Barker. *J. Biomol. Structure*, 6, 535 (1977).
- Nurse, P. and A. Wiemken. *J. Bacteriol.*, 117, 1108 (1974).
- Nwanze, C., R. Hanks, G. Dodds and O. Howarth. *Chem. Phys. Lipids*, 18, 267 (1977).
- Oldfield, E., M. Meadows, D. Rice and R. Jacobs. *Biochemistry*, 17, 2727 (1978).

- Ogino, T., Y. Arata and S. Fujiware. *Biochemistry*, 19, 3684 (1980).
- Ohsaka, A., K. Yoskikawa, S. Yamaya and T. Sugahara. *Physiol. Chem. and Physics*, 13, 159 (1981).
- O'Neill, T.B. and R.W. Drisko. In *Developments in Industrial Microbiology*, Vol. 21 (Proceedings of 36th General Meeting of the Society for Industrial Microbiology, Arlington, Virginia), p. 199 (1980).
- Owicki, J.C. and H.M. McConnell. *Proc. Natl. Acad. Sci. U.S.A.*, 76, 4750 (1979).
- Palacios, J. and R. Serrano. *FEBS Lett.*, 91, 198 (1978).
- Paltauf, F. and J.M. Johnson. *Biochim. Biophys. Acta*, 218, 424 (1970).
- Papahadjapoulos, D. and H.K. Kemelberg. *Prog. in Surface Sci.*, 4, 141 (1973).
- Paterson, S.J., K.W. Butler, P. Huong, J. Labelle, I.C.P. Smith and H. Schneider. *Biochim. Biophys. Acta*, 266, 597 (1972).
- Patton, G.M. and J.M. Lowenstein. *Biochemistry*, 18, 3186 (1979).
- Phaff, H.J., E.M. Mrak and O.B. Williams. *Mycologia*, 44, 431 (1952).
- Pitner, T.P. and J.F. Whidby. *Analytical Chem.*, 51, 2203 (1979).
- Plückthun, A. and E.A. Dennis. *J. Phys. Chem.*, 85, 678 (1981).
- Ponton, J., P. Regulez, J.B. Dominguez, F.M. Gôni and F. Urubura. *Current Microbiol.*, 3, 327 (1980).
- Racker, E. *Mechanisms in Bioenergetics*, Academic Press, New York, p. 253 (1965).
- Rajan, S., S. Kang, H. Gutowsky and E. Oldfield. *J. Biological Chem.*, 256, 1160 (1981).
- Ramós, S. and I. Garcia-Acha. *Trans. Brit. Mycol. Soc.*, 64, 129 (1975(a)).

- Ramos, S. and I. Garcia-Acha. *Archives Microbiol.*, 104, 271 (1975(b)).
- Ramos, S., I. Garcia-Acha and J.F. Peberdy. *Trans. Brit. Mycol. Soc.*, 64, 283 (1975).
- Rance, M., K.R. Jeffrey, A.P. Tulloch, K.W. Butler and I.C.P. Smith. *Biochim. Biophys. Acta*, 600, 245 (1980).
- Rattle, H.W.E. In *Annual Reports of NMR Spectroscopy*, Vol. 11A, (ed. G.A. Webb), p. 1 (1981).
- Richards, C.D. In *Biochemistry of Cell Walls and Membranes II*, Vol. 19, (ed. J.C. Metcalfe), Ch. 6, p. 159, (1978).
- Robichon-Szulmajster, H. and Y. Surdin-Kerjan. In *The Yeasts*, Vol. 2, (ed. A.H. Rose and J.S. Harrison), Ch. 9 (1971).
- Roomans, G.M., F. Blasco and G. Borst-Pauwels. *Biochim. Biophys. Acta*, 467, 65 (1977).
- Rose, A.H. In *Economic Microbiology*, Vol. 2, (ed. A.H. Rose), Academic Press, New York (1978).
- Rothstein, A. In *Membrane Transport and Metabolism*, (ed. A. Kleinzeller and A. Kotyk), p. 270 (1961).
- Salhany, J.M., T. Yamane, R.G. Shulman and S. Ogawa. *Proc. Natl. Acad. Sci. U.S.A.*, 72, 4966 (1975).
- Scarborough, G.A. *J. Biological Chem.*, 250, 1106 (1975).
- Schaefer, J., O.E. Stejskal and C.F. Beard. *Plant Physiol.*, 55, 1048 (1975).
- Schibeci, A., J. Rattray and D. Kidby. *Biochim. Biophys. Acta*, 311, 15 (1973).
- Schmidt, C.F., Y. Barenholz, C. Huong and T.E. Thompson. *Biochemistry*, 16, 3948 (1977).
- Schroepfer, G.J. and K. Bloch. *J. Biological Chem.*, 240, 54 (1965).
- Schwencke, J. and M. Nagy. *Methods in Cell Biology*, Vol. 20, (ed. D.M. Prescott), Academic Press, New York, Ch. 7 (1978).
- Scott, A.I. and R.L. Baxter. *Annual Rev. Biophys. Bioeng.*, 10, 151 (1981).

- Sears, B. J. Membrane Biol., 20, 59 (1975).
- Sears, B., R. Deckelbaum, M. Janiak, G. Shipley and D. Small. Biochemistry, 15, 4151 (1976).
- Seaston, A., C. Inkson and A.A. Eddy. Biochemistry J., 134, 1031 (1973).
- Seelig, A. and J. Seelig. Biochemistry, 16, 45 (1977).
- Seelig, J. Quart. Rev. Biophys., 10, 353 (1977).
- Seelig, J. Biochim. Biophys. Acta, 515, 105 (1978).
- Seelig, J. and W. Neiderberger. Biochemistry, 13, 1585 (1974).
- Seelig, J. and A. Seelig. Quart. Rev. Biophys., 13, 1 (1980).
- Seelig, J., A. Seelig and L. Tamm. In Lipid Protein Interactions, (ed. P. Jost and O.H. Griffith), in press (1981).
- Seelig, J. and N. Waespe-Sarcevic. Biochemistry, 17, 3310 (1978).
- Seeliger, H.P.R. J. Bacteriol., 72, 127 (1956).
- Sens, M.A., N.K. Wilson, P.D. Ellis and J.D. Odom. J. Magn. Res., 19, 323 (1975).
- Setlow, B. and P. Setlow. Proc. Natl. Acad. Sci. U.S.A., 77, 2474 (1980).
- Sevilla, M.J., P. Isusi, R. Gutierrez, L. Egea and F. Urubura. Trans. Brit. Mycol. Soc., 68, 300 (1977).
- Shaefer, J. and E.O. Stejskal. J. Amer. Oil Chem. Soc., 52, 366 (1975).
- Sharp, R.R. and E.P. Richards. Biochim. Biophys. Acta, 497, 14 (1977).
- Shulman, R.G., T.R. Brown, K. Ugurbil, S. Ogawa, S.M. Cohen and J.A. den Hollander. Science, 205, 160 (1979).
- Shrago, E. and C. Elson. In Biochemistry and Physiology of Protozoa, Vol. 3, (ed. M. Levondowsky and S. Hunter), Academic Press, New York, Ch. 8, p. 287 (1980).

- Singer, S.J. and G.L. Nicholson. *Science*, 175, 720 (1972).
- Singleton, W.S., M.S. Gray, M.L. Brown and J.L. White. *J. Amer. Oil Chem. Soc.*, 42, 53 (1965).
- Smith, I.C.P. and R. Deslauriers. In *Current Methods in Structural Biology*, (ed. D. Davies, W. Saenger and S. Danyluk), in press (1981).
- Smith, I.C.P., K.W. Butler, A.P. Tullock, J.H. Davis and M. Bloom. *FEBS Lett.*, 100, 57 (1979).
- Sols, A., G. Gancedo and D. Delafluento. In *The Yeasts*, Vol. 2, (ed. A.H. Rose and J.S. Harrison), Academic Press, New York, Ch. 7, p. 271 (1971).
- Souzu, H. *Archives Biochem. Biophys.*, 120, 338 (1972(a)).
- Souzu, H. *Archives Biochem. Biophys.*, 120, 343 (1972(b)).
- Sowa, W., A.C. Blackwood and G.A. Adams. *Can. J. Chem.*, 41, 2314 (1963).
- Spoerl, E. and D. Looney. *J. Bacteriol.*, 76, 63 (1958).
- Stahl, J.C. *Bull. Soc. Chim. Biol.*, 51, 1211 (1969).
- Stockton, G.W., C.F. Polnaszek, A.P. Tullock, F. Hasan and I.C.P. Smith. *Biochemistry*, 15, 954 (1976).
- Stoffel, W. and K. Bister. *Biochemistry*, 14, 2841 (1975).
- Stoffel, W., K. Bister, C. Schreiber and B. Tungyal. *Hoppe-Seyler's Z. Physiol. Chem.*, 357, 905 (1976).
- Stoffel, W., O. Zierenberg and H. Scheefers. *Hoppe-Seyler's Z. Physiol. Chem.*, 358, 865 (1977).
- Subramanian, K.N., R.L. Weiss and R.H. Davis. *J. Bacteriol.*, 115, 284 (1973).
- Sudmeier, J.L. and T.G. Perkins. *J. Amer. Chem. Soc.*, 99, 7732 (1977).
- Sudmeier, J.L., R.R. Birge and T.G. Perkins. *J. Magn. Res.*, 30, 491 (1978).
- Takizawa, Y. In *Topics in Environmental Health*, Vol. 3, (ed. J.O. Nriagu), Elsevier/North-Holland, New York, Ch. 14 (1979).

- Tancrede, P., R. Deslauriers, N.H. McGregor, E. Ralston, D. Sarontoxis, R.L. Somorjai and I.C.P. Smith. *Biochemistry*, 17, 2905 (1978).
- Tenforde, T.S. *Magnetic Field Effects on Biological Systems*, Plenum Press, New York (1979).
- Tolbert, N.E. *Annual Rev. Biochem.*, 50, 133 (1981).
- Ugurbil, K., A. Rottenberg, P. Glynn and R.G. Shulman. *Proc. Natl. Acad. Sci. U.S.A.*, 75, 2244 (1978).
- Ugurbil, K., R.G. Shulman and T.R. Brown. In *Biological Applications in Magnetic Resonance*, (ed. R.G. Shulman), Academic Press, New York, Ch. 11 (1979).
- Urech, K., M. Dürr, J. Boller and A. Wiemken. *Archives Microbiol.*, 116, 275 (1978).
- Usui, T., N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama and S. Seto. *J. Chem. Soc. Perkin I*, 2425 (1973).
- Van der Leeuw, Y.C.W. and G. Stulen. *J. Magn. Res.* 42, 434 (1981).
- Van Steveninck, J. *Archives Biochem. Biophys.*, 130, 244 (1969).
- Verkleij, A.J., C. Van Echteld, W. Gerritsen, P.R. Cullis and B. de Kruijff. *Biochim. Biophys. Acta*, 600, 620 (1980).
- Villaneuva, J.R. and I. Garcia-Acha. In *Methods in Microbiology*, Vol. 4, (ed. C. Booth), Ch. 24 (1971).
- Vold, R.L., R.R. Vold and D. Canet. *J. Chem. Phys.*, 66, 1202 (1977).
- Wakil, S.J. *Lipid Metabolism*, Academic Press, New York, p. 32 (1970).
- Warren, G.B., M.D. Houslay, J.C. Metcalfe and N.J.M. Birdsall. *Nature*, 255, 684 (1975).
- Watts, A., J. Davoust, D. Marsh and P.F. Devaux. *Biochim. Biophys. Acta*, 643, 673 (1981).
- Wieslander, A., A. Christiansson, L. Rilfours, A. Khan, L. Johansson and G. Lindblom. *FEBS Lett.*, 124, 273 (1981).

- Weiss, G., R. Gupta, J. Ferratti and E. Becker. *J. Magn. Res.*, 37, 369 (1980).
- Weiss, R.L. *J. Biological Chem.*, 248, 5409 (1973).
- Wennerström, H. and G. Lindblom. *Quart. Rev. Biophysics*, 10, 67 (1977).
- White, A., P. Handler, E.L. Smith, R.L. Hill and I.R. Lehmann. *Principles of Biochemistry*, McGraw Hill, New York, p. 434 (1978).
- White, R.H. *Biochemistry*, 19, 9 (1980).
- Wiemken, A. and M. Dürr. *Archives Microbiol.*, 101, 45 (1974).
- Wiley, W.R. In *Methods in Enzymology*, Vol. 31, Part A, (ed. S. Fleischer and L. Packer), p. 609 (1974).
- Williams, E., J.A. Hamilton, M.K. Jain, A. Allerhand, E.H. Cordes and S. Ochs. *Science*, 181, 869 (1973).
- Winder, F.G. and J.M. Denny. *J. Gen. Microbiol.*, 17, 573 (1957).
- Wittebort, R.J. and A. Szabo. *J. Chem. Phys.*, 69, 1722 (1978).
- Woessner, D.E. *J. Chem. Phys.*, 36, 1 (1962).
- Wohlgemuth, R., N. Waespe-Sarcevic and J. Seelig. *Biochemistry*, 19, 3315 (1980).
- Wright, D.A., D.E. Axelson and G.C. Levy. In *Topics in Carbon-13 NMR Spectroscopy*, Vol. 3, (ed. G.C. Levy), p. 101 (1979).
- Wüthrich, K. *NMR in Biological Research: Peptides and Proteins*, North-Holland, Amsterdam, Ch. 5 (1976).
- Wynne, E.S. and G.L. Gott. *J. Gen. Microbiol.*, 14, 512 (1956).
- Yang, S.Y., H.S. Gutowsky and E. Oldfield. *Biochemistry*, 18, 3268 (1979).
- Yatscoff, R. and J. Cummins. *Nature*, 257, 422 (1975).
- Yeagle, P. and R. Martin. *Biochem. Biophys. Res. Commun.*, 69, 775 (1976).

Yeagle, P., R.B. Martin, A.K. Lala, H.K. Lin and K. Bloch.
Proc. Natl. Acad. Sci. U.S.A., 74, 4924 (1977).

Yuen, S. Process Biochem., 9, 7 (1974).

Zabel, R.A. and F. Terracine. In Developments in Industrial Microbiology, Vol. 21, (Proceedings of 36th General Meeting of the Society for Industrial Microbiology, Arlington, Virginia), Ch. 18, p. 179 (1980).