SOME STUDIES ON LIPIDS

AND LIPASE ACTIVITIES IN

Streptomyces rimosus

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by
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ABBREVIATIONS

a	anteiso
BMECP	Bacterial Fatty Acid Methyl Ester CP
BNP	bis-nitrophenyl phosphate
C	carbon
CoA	Coenzyme A
CMC	critical micellar concentration
CV	coefficient of variation
Δ	cyclopropane ring
ΔA	change in absorbance
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetracetic acid
EU	Enzyme units
FAME	fatty acid methyl ester
FAS	Fatty Acid Synthetase
GC-MS	gas-chromatography-mass spectroscopy
GLC	gas-liquid chromatography
Glc	glucose
Gly	glycerol
GOD	glucose oxidase
HMM	Hobbs minimal medium
HPLC	high pressure liquid chromatography
i	iso
Man	mannitol
-Me	methyl group
m/e	mass to charge ratio
MGT	mean generation time
Mr	molecular weight
N	nitrogen
OD	optical density
-OH	hydroxyl group
ρnp	para-nitrophenyl
PC	phosphotidyl choline
PCA	perchloric acid
PE	phosphotidyl ethanolamine
PERID	peroxidase
PHB	polyhydroxybutyrate
PKS	Polyketide Synthase
PMDE	phosphotidyl dimethylethanolamine
PME	phosphotidyl methylethanolamine
PMSF	polymethylsulphonyl flouride
PPL	porcine pancreatic lipase
rso	rape seed oil
SBA	2-sulphonbenzoic cyclic anhydride
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM	soya mannitol
TEMED	Tetramethylethylenediamine

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SUMMARY

The aim of this study was to investigate lipids and lipid metabolism in *Streptomyces*. A number of aspects were studied: growth of *Streptomyces* on medium containing lipid as carbon source, the lipolytic activities present in *Streptomyces* and analysis of the fatty acid composition of *Streptomyces* grown under various nutritional conditions. In this study, particular attention was paid to the similarities between fatty acid metabolism and polyketide antibiotic production.

1. Growth of *Streptomyces*

(a) Development and refinement of standard growth media and culture conditions was undertaken to obtain growth in presence of triglycerides and polyoxyethylene esters. *Streptomyces coelicolor* A3(2)[1147] was the species initially chosen but was found to be unsuitable. The replacement organism, *Streptomyces rimosus* 4018, was found to satisfy the requirements of this study.

(b) In order to achieve reproducible results from standard growth experiments, a number of improvements had to be made to existing growth conditions for *Streptomyces*. The existing Hobbs Minimal Medium (HMM) was modified, cultures were stirred rather than shaken, and mycelia rather than spores, were used as an inoculum. These alterations allowed growth to be easily followed visually and accurately measured by optical density, DNA and protein concentrations. Reproducible results were obtained from *S. rimosus* cultures grown on modified HMM containing glucose or glycerol or mannitol as the carbon source.

(c) Growth of *S. rimosus* could not be detected when triglycerides were employed as the sole carbon source in modified HMM. Growth on modified HMM containing glucose and triglycerides could not be verified.

(d) The co-utilisation of polyoxyethylene esters and glucose as carbon sources in modified HMM by *S. rimosus* implied the presence of lipolytic enzymes.

2. Lipolytic activities present in *S. rimosus*

(a) The presence of at least two independent lipolytic activities, one intracellular and the other extracellular, were detected in *S. rimosus*. Characterisation studies of the two activities demonstrated a number of differences between them.

(b) Difficulties were encountered in determining the Mr of the lipolytic activities by a

number of techniques. However it was shown that the activities had very large Mr's.

(c) Inhibitor studies showed that both the extracellular and the intracellular activities were serine esterases/lipases.

3. Lipid composition of *Streptomyces*

(a) Comparison between the lipid composition of *S. rimosus*, *S. coelicolor* and *S. clavuligerus* demonstrated that the three different species were similar in composition. All three species contained high levels of branched-chain fatty acids and the triglyceride fraction of *S. rimosus* was particularly rich in these fatty acids.

(b) The use of GLC allowed the identification of the fatty acids present in *S. rimosus*. Major differences were found in the triglyceride fractions of oxytetracycline-producing and non-oxytetracycline-producing *S. rimosus*, particularly in the branched-chain fatty acid content. The use of GC-MS further characterised these differences.

(c) The nitrogen and phosphate limited growth conditions used in this study failed to promote lipid storage as triglyceride by *S. rimosus*. From this result and those mentioned in 3(b) it was assumed that under the conditions imposed in this study, the common precursors for the biosynthesis of polyketide antibiotics and branched-chain fatty acids were being used for oxytetracycline production and not being stored as the the branched-chain fatty acid components of triglyceride.

CHAPTER 1

INTRODUCTION

PAGE NUMBERING AS ORIGINAL

1.0 INTRODUCTION

The products of bacterial metabolism may be divided into two categories:

1. primary metabolites, the molecules and polymers of life including DNA, protein and lipid.

2. secondary metabolites such as antibiotics, pigments and similar molecules, apparently having little importance in the basic metabolism of the cell (Lechavalier, 1977).

The study of streptomycete primary metabolism has been somewhat neglected and the research carried out on *Streptomyces* has focused mainly on the identification and development of *Streptomyces* strains which produce economically and medically important secondary metabolites. An understanding of the *Streptomyces* basic physiology should facilitate the production of secondary metabolites as primary metabolism clearly affects the process of secondary metabolism. *Streptomyces* are capable of synthesising secondary metabolites by using either the same general enzymes used in primary metabolism or special synthetases, detectable under specific nutritional conditions, which will utilise primary metabolites as their substrates. The mutual regulation of, and similarities between fatty acid metabolism and polyketide antibiotic production provides evidence for, and a suitable model for studying, the relationship between primary and secondary metabolism in *Streptomyces*.

Little is known about the source of carbon for antibiotic production. Neutral lipids such as triglycerides have not been considered as storage polymers in bacteria because there is little evidence for their presence, whereas polyhydroxybutyrate (Dawes and Senior, 1973; Ranade and Vining, 1992) and glycogen (Preiss and Romeo, 1989) have been detected. However *Streptomyces* are not typical bacteria and the presence of triglycerides has been reported (Ballio *et al.*, 1965; Packter *et al.*, 1985; Metz *et al.*, 1988 and Novak *et al*, 1990). Triglycerides could be used as energy reserves and provide carbon units for antibiotic production or alternatively the storage of triglycerides could result in antibiotic precursors being diverted from antibiotic production. This

former phenomenon could be advantageous to the bacteria in that energy reserves are laid down for times of shortage. On the other hand if diversion of antibiotic precursors to triglyceride synthesis were to occur in industrial antibiotic producing strains, where the emphasis is on maximum antibiotic production, the process could be potentially wasteful.

1.1 THE GENUS *STREPTOMYCES*

Streptomyces are members of a large group of filamentous bacteria known as *Actinomycetes*. The genus *Streptomyces* includes a great number of species and varieties, which are gram-positive, spore forming, filamentous, obligate aerobes (Goodfellow and Cross, 1984). The name *Streptomyces* describes their unique morphology: The word means "chain fungus" (Chater, 1984). The life cycle (Fig. 1.1) and the growth habits are very different from bacteria outside the actinomycetes. In the soil and on solid media *Streptomyces* exhibits a mycelial fungus hyphae like cycle of morphological differentiation. Germination of dormant spores results in the production of germ tubes. Growth from these germ tubes results in the development of a branched mycelial network which attaches itself to a solid surface. From the mycelial network arise aerial hyphae which differentiate to form chains of spores, the natural resting and dispersal stage of the organism (Hopwood, 1988). *Streptomyces* spores (conidia), are not related in any way to the endospores of *Bacillus* and *Clostridium* since the streptomycete spores are produced simply by the formation of cross walls in the multinucleate aerial filaments followed by the separation of the individual cells directly into spores (Brock *et al.*, 1984). In the soil, the natural habitat of Streptomycetes, the purpose of this branched mycelial network is to penetrate and solubilise solid organic debris, by the action of extracellular hydrolytic enzymes (Losick and Shapiro, 1984). In fact the characteristic earthy odour of the soil is caused by the production of a series of streptomycete metabolites (sesquiterpenoid compounds) called geosmins (Brock *et al.*, 1984).

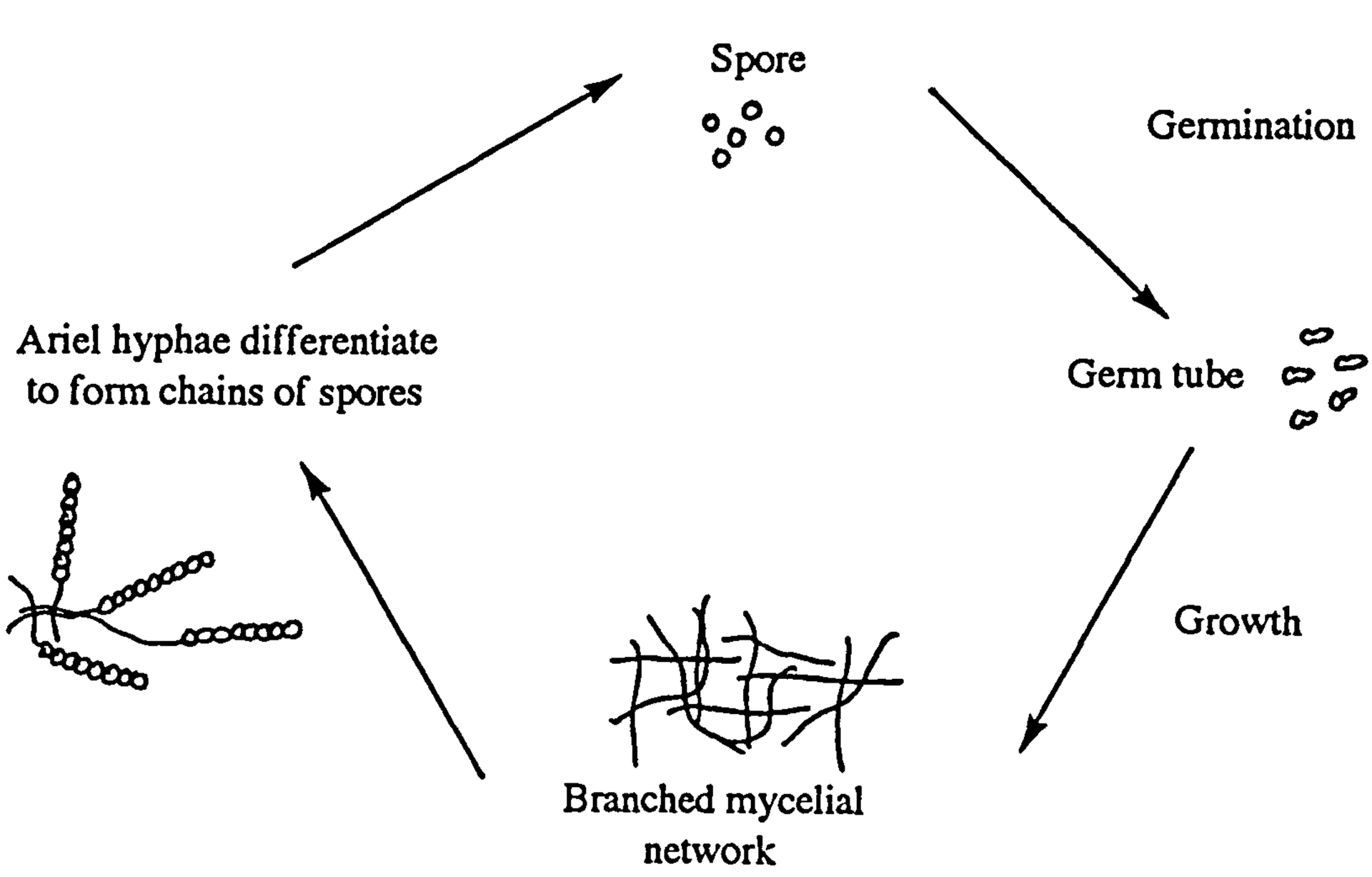

Fig. 1.1 Diagrammatic life cycle of *Streptomyces*.

The growth habit of *Streptomyces* is just one of the specialised features that set them apart from most bacteria. Perhaps the most striking property of the streptomycetes is the extent to which they produce antibiotics and indeed it is among members of the genus *Streptomyces*, that most of the antibiotics have been discovered (Brock *et al.*, 1984). More than fifty streptomycete antibiotics have found practical application in human and veterinary medicine, agriculture and industry. Some of the more common antibiotics synthesised by species of *Streptomyces* and used in medicine are listed in Table 1.1. Besides these commonly used, well known antibiotics there are a number of other interesting *streptomycete* products. *Streptomyces avermitilis* produces avermectins which have both antihelminthic and insecticidal activities. These avermectins are disaccharide derivatives of macrocyclic lactones whose exceptional antibiotic activity against parasites appears to be by interference with neurotransmission in many invertebrates. They have activity against both nematode and arthropod parasites in sheep, cattle, dogs, horses and pigs (Demain, 1984). *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus* both produce antibiotics which with modification have uses as a herbicide, known as bialophos. They are phosphothricin herbicides, where the phosphothricin interferes with the glutamine synthetase-glutamate synthase system of nitrogen assimilation resulting in ammonia accumulation which blocks photosynthesis and depletes glutamine in plant cells (Demain, 1984). *Streptomyces halstedii* produces a polyether which promotes growth of cattle and poultry, while the polyether-complex produced by *S. virginiae* promotes growth of pigs and poultry (Kurylowicz and Gyllenberg, 1984). *Streptomycete* secondary products are now also being investigated as anti-tumour agents. *S. pluricolorescens* produces a peptide which acts as an immunostimulant with anticancer activity (Kurylowicz and Gyllenberg, 1984). In addition to these uses a number of *Streptomycetes* produce enzyme inhibitors; *S. lavendulae* produces an esterase inhibitor, *S. violaceus* produces a protease inhibitor and *S. testaceus* produces a pepsin inhibitor. These examples demonstrate the wide variety of secondary products produced by *Streptomyces* and the diversity of their uses.

Table. 1.1 Some of the more common antibiotics synthesised by species of *Streptomyces* and their medical uses.

Chemical Class	Common Name	Produced by	Active Against
Aminoglycosides	Streptomycin	*S. griseus*	Most Gram -ve
	Spectinomycin	*Streptomyces spp.*	*M.tuberculosis*
			N.gonorrhoeae
	Neomycin		Broad spectrum
Tetracyclines	Tetracycline	*S. aureofaciens*	Broad spectrum, Gram +ve, Gram -ve, rickettsias and chlamydias
	Chlortetracycline	*S. aureofaciens*	As above
Macrolides	Erythromycin	*S. erythreus*	Most gram -ve, frequently used in place of penicillin,
	Clindamycin	*S. lincolensis*	Effective against obligate anaerobes
Polyenes	Nystatin	*S. noursei*	Fungi, especially *Candida* infections
	Amphocetin B	*S. nodosus*	Fungi
None	Chloramphenical	*S. venezuelae*	Broad spectrum; drug of choice for typhoid fever

1.1.2 *STREPTOMYCES RIMOSUS*

Streptomyces rimosus produces a commercially important, broad spectrum antibiotic known as oxytetracycline. Oxytetracycline is a member of the polyketide class of antibiotics, which includes, among others avermectin, monensin, erythromycin, and other tetracyclines such as 7-chloretetracycline. The oxyteracycline pathway of *S. rimosus* has been the subject of both biochemical and genetic analysis (Rhodes *et al.*, 1981). Fig. 1.2 outlines the proposed pathway from malonyl Coenzyme A to the final product (Binnie *et al.*, 1989). The basic unit used is derived from acetate.

1.1.3 *STREPTOMYCES COELICOLOR*

Streptomyces coelicolor is one of the most widely studied actinomycetes and is genetically the best characterised streptomycete (Hopwood *et al.*, 1973). This organism produces a range of secondary metabolites including actinorhodin, undecylprodigiosin, the calcium dependant antibiotic, and methylenomycin A (Hobbs *et al.*, 1992). Actinorhodin is another polyketide antibiotic derived from acetate. *S. coelicolor* A3(2) synthesises each half molecule of the dimeric polyketide antibiotic Actinorhodin (Act) from one acetyl and seven malonyl building units, catalysed by the Act polyketide synthase (Fig. 1.3) [Revill *et a.l*, 1995). Actinorhodin weakly inhibits the growth of some gram negative bacteria but its activity is very low (Wright and Hopwood, 1976).

1.2 LIPOLYTIC ENZYMES

Lipases or acylglycerol hydrolases [EC 3.1.1.3] (according to the Recommendations of the Nomenclature Committee of the International Union of Biochemistry), are water soluble enzymes which hydrolyse glycerol esters of long chain fatty acids (water insoluble) at oil/water interfaces. Lipases are different from esterases [EC 3.1.1.1 and 2] which hydrolyse esters of short chain fatty acids (water soluble). Brockerhoff and Jenson, (1974) provided a simplified definition where a lipase is defined to be an enzyme

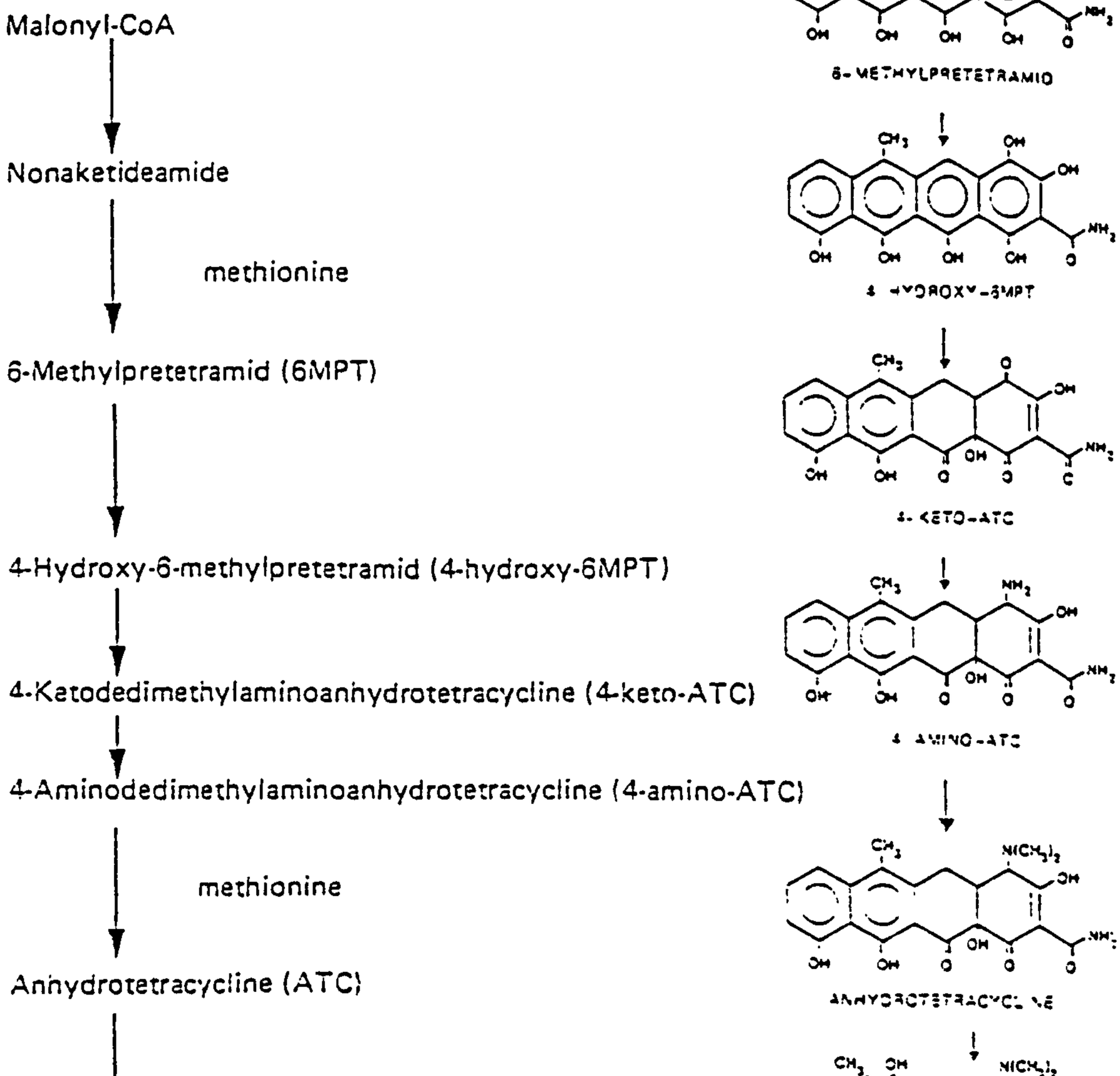

Fig. 1.2 The proposed pathway for oxytetracycline production, from malonyl CoA to the final product (Binnie *et al.*, 1989)

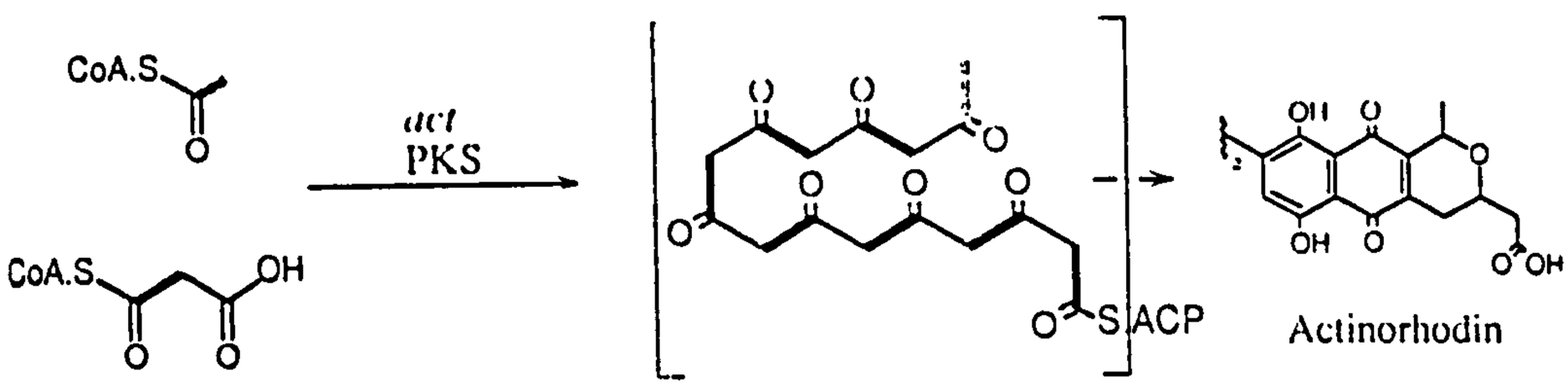

Fig. 1.3 The structure of the blue-pigmented polyketide, Actinorhodin produced by *S. coelicolor*. The Act PKS sythesises the full length polyketide chain (hypothetical intermediate shown in brackets) from one starter and seven malonyl extender residues in a manner resembling fatty acid biosynthesis. Subsequent modification of the polyketide chain yields Actinorhodin. Acetyl carrier protein (ACP) is the component of the Act PKS which serves to anchor the growing chain (Revill *et al*, 1995).

capable of hydrolysing esters of fatty acids containing twelve or more carbon atoms, and an esterase is an enzyme capable of hydrolysing fatty acid esters with less than twelve carbon atoms. Sarda and Desnuelle, (1958) demonstrated a fundamental difference between esterases and lipases, based upon their ability or inability to be activated by interfaces. Fig. 1.4 shows the difference between an esterase and a lipase. The esterase displays normal Michaelis-Menten kinetics unlike pancreatic lipase which displays almost no activity with the triglyceride substrate when it is in the monomeric state. When the solubility limit of triacetin is exceeded, there is a sharp increase in lipase activity with the substrate in emusified state. Apparently, the esterase is active only on molecularly dispersed substrate molecules. The lipase appeared to be a special class of esterase capable of hydrolysing substrate in insoluble form at high velocity.

1.2.1 ESTERASES

Esterases are widely distributed in vertebrate tissues, blood serum, insects, plants, citrus fruits, mycobacteria and fungi (Krisch, 1971). Esterases are frequently classified according to their behaviour towards organophosphorous compounds such as diethyl ρ-nitrophenyl phosphate. This system of classification was proposed by Aldridge (1953). A-esterases (E.C. 3.1.1.2) are not inhibited by organophosphorous compounds but hydrolyse them as substrates, while B-esterases (E.C.3.1.1.1) are inhibited stoichiometrically by organophosphates without hydrolysing them. The B-esterases have a serine residue at the active site and like lipases are classified as serine hydrolases. A third type of esterase (C-esterase), exists in pig kidney which is neither inhibited by organophosphorous compounds nor does it hydrolyse them (Bergman *et al.*, 1957).

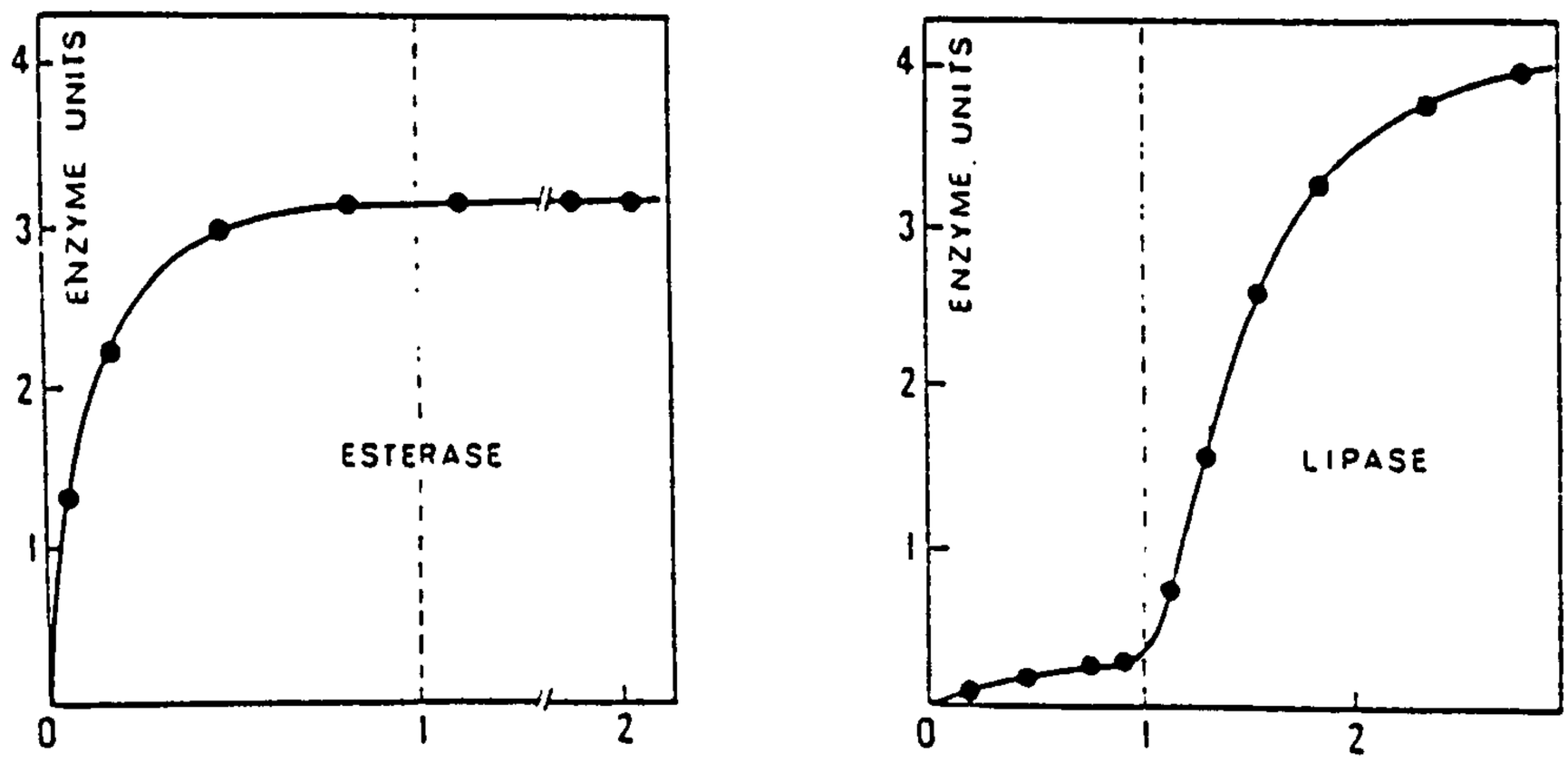

Fig. 1.4 Hydrolysis of triacetin by horse liver esterase and porcine pancreatic lipase. Reaction rates as a function of substrate concentration, which is expressed in multiples of saturation (Sarda and Desnuelle, 1958).

1.2.2 MICROBIAL ESTERASES

The presence of esterases has been found in a wide range of microorganisms. Rudek, (1978) found esterase activity in eight species of *Candida*, using the Tween detergents as water-soluble substrates. *Acinetobacter* O_{16} produces both an extracellular lipase and esterase (Breuil and Kushner, 1975). Eight strains of *Propionibacterium freudenreichii* subsp. *freudenreichii*, one of the most frequently used dairy proprionibacteria for Swiss-type cheese ripening, were found to contain esterase activities together with lipase activities (Dupuis *et al.*, 1993).

1.2.3 STREPTOMYCETE ESTERASES

Streptomyces scabies produces a heat stable extracellular esterase whose production is regulated by Zn^{2+} ions (Schottel *et al.*, 1992). This suberin-induced extracellular esterase has been isolated from *S. scabies* strains FL1 and PNT1. The secreted form of the esterase is a 36 kDa monomer (McQueen and Schottel, 1987). *Streptomyces scabies* is a Gram positive soil bacterium that causes common scab disease of potatoes and other underground vegetables. *S. scabies* enters through the lenticels or through wound sites which are covered in suberin (provides a mechanical barrier to pathogens) [Jones, 1987]. Suberin contains long-chain fatty acids, dicarboxylic acids, fatty alcohols and phenolic compounds (Kolattukudy, 1981). The extracellular esterase may be involved in breaking down the suberin, the insoluble waxy polyester forming a protective layer around aerial or subterranean parts of the plant (Kolattukudy, 1980). The zinc associated with the suberin is the critical component in the induction of the esterase.

1.2.4 LIPASES

Lipases are widely distributed in various animals, plants, and microorganisms. In animals mammalian lipases have received the most attention. There are three groups of lipases in mammals: those released by specialised organs, for example pancreatic lipase;

tissue lipases which have been found in heart, brain, muscle, arteries, kidney and adipose tissue; and milk lipases. Fish (Brockerhoff, 1966) and invertebrates (Patten and Quin, 1973) have also been found to contain lipases. Most of the studies on plant lipases have been carried out on seeds and fruit. In several seed species, including castor bean, corn, cotton, mustard and rape, the lipase is known to be associated with the membrane of the storage lipid bodies (Desnuelle, 1961) and are responsible for mobilising the reserve triacylglycerols for utilisation in the postgerminative growth of seeds. Recently microbial lipases (see Björkling *et al.*, 1991) have been subjected to considerable research with emphasis on their biotechnological applications. A large number of bacterial lipases have been screened for applications as medicines (digestive enzymes), food additives (flavour-modifying enzymes), clinical reagents (glycerol-hydrolysing enzymes) and cleaners (detergent additives). The usefulness of bacterial lipases in commerce and research stems from their physiological and physical properties. In particular, a large amount of purified lipase can be made available, the enzymes are generally more stable than animal or plant lipases and they have unique characteristics (Borgström and Brockman, 1984).

Lipases are surface-active enzymes because binding to emulsified triglyceride substrates markedly increases their activity towards dissolved substrates (Verger, 1984). This phenomenon is known as interfacial activation. Lipases may exhibit specificity for the reactions they catalyse and several specificities have been identified: substrate, positional, fatty acid, stereospecificity and a combination of these (Jenson *et al.*, 1983). Lipases have been classified as serine hydrolases owing to their inhibition by diethyl p-nitrophenyl phosphate (Brockerhoff and Jenson, 1974 and Verger, 1984). Lipases, sequenced to date share sequence homologies including a significant region, Gly-X-Ser-X-Gly, that is conserved in all. Three-dimensional studies carried out on human pancreatic lipase (Winkler *et al.*, 1990) and a lipase isolated from the fungus *Rhizomucor miehei* showed serine-histidine-aspartate triads at the active site which are characteristic of serine proteases. In both enzymes the active centres are buried beneath a short seven residue α-helix acting as a 'lid'. Studies carried out on the crystal structure

of lipase from *R.miehei* (Brzozowski *et al.*, 1991) have shown that the activation is achieved by the displacement of the lid structure whose movement exposes the catalytic groups and creates a non-polar surface which stabilises the contact between the enzyme and the lipid surface [Fig. 1.5 (Blow, 1991)].

1.2.5 MICROBIAL LIPASES

Lipases are widely distributed among bacteria, yeasts and fungi. Most of them are extracellular enzymes which are synthesised within the cell and secreted through the external wall into the culture medium. These extracellular enzymes may function as digestive enzymes, breaking down lipid micelles into fatty acids which can be transported into the cell. The literature contains many reports of extracellular lipase activity in a wide range of microbial species; *Geotrichum candidum* (Wouters, 1967), *Staphylococcus aureus* (Smeltzer *et al.*, 1992; Muraoka *et al.*, 1982; Tyski *et al.*, 1983), *Penicillum cambertii* (Isobe *et al.*, 1992), *Rhizomucor miehe*i (Brady *et a.*, 1990), *Bacillus subtilus* (Kennedy and Lennarz, 1979), *Pseudomonas aeruginosa* (Stuer *et al.*, 1986) and *Corynebacterium acnes* (Hassing, 1971).

Although most of the microbial lipases produced are extracellular there is evidence for the existence of intracellular lipases. An intracellular lipase from *Propionibacterium shermanii* (Otterholm *et al.*, 1970) has been purified and the lipases of *Brochothix thermosphacta* and *Lactobacillus curvatus* (Papon and Talon, 1989) were found in the soluble fraction of the cell. There was no evidence of secretion of these enzymes into the external culture medium. *Mycobacterium phlei* (Paznokas and Kaplan, 1977) contains a pool of triacylglycerols which has a rapid turnover in the late during early growth in liquid culture. An intracellular lipase was found to be responsible for both this turnover of the triacylglycerols and for the breakdown of the triacylglycerol pools on late stationary phase.

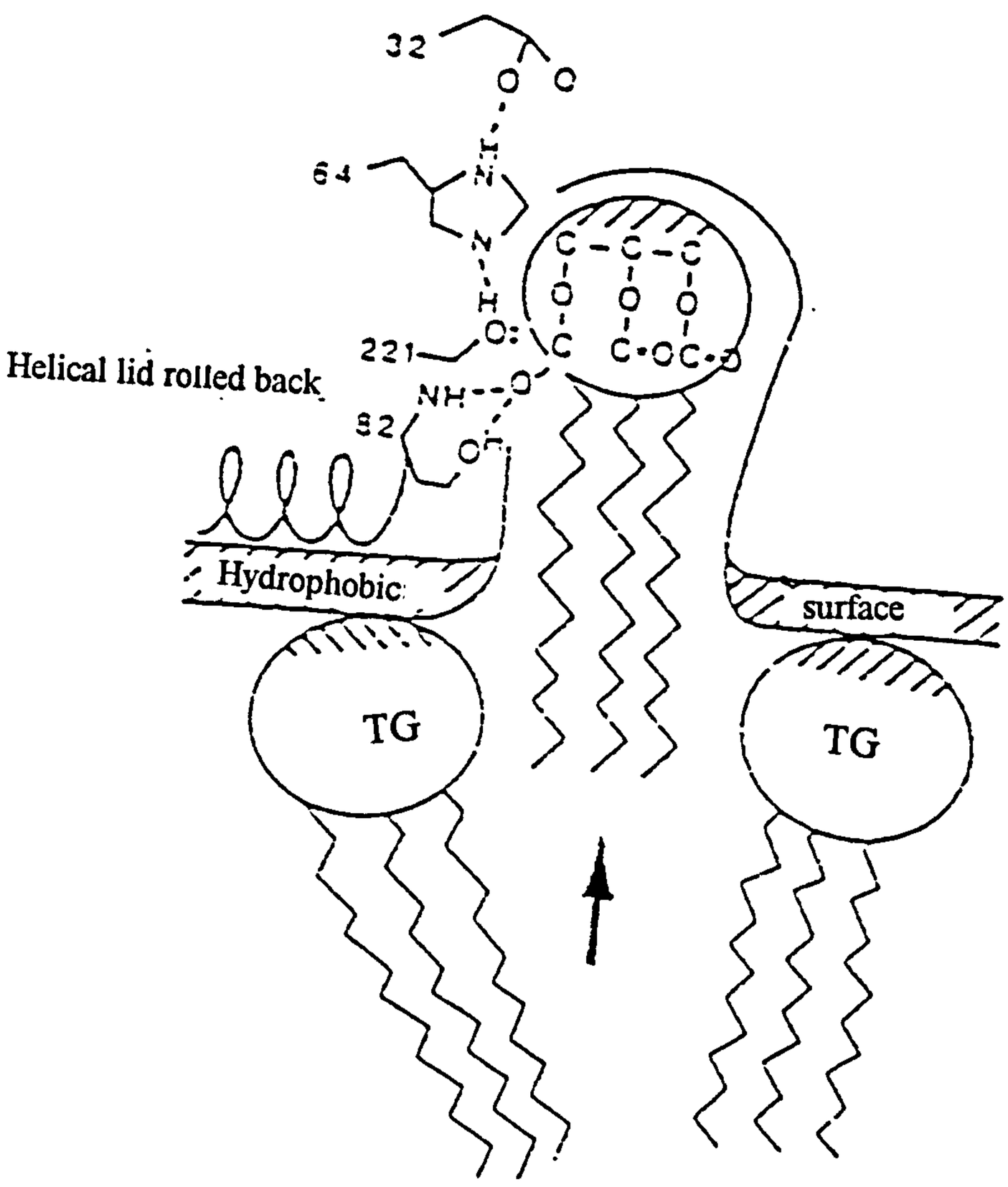

Fig. 1.5 Diagram of lipase as a interfacial substrate complex; (TG - triglyceride)

(Blow, 1991).

1.2.6 STREPTOMYCETE LIPASES

Streptomyces rely on the secretion of a wide variety of extracellular hydrolytic enzymes for their nutrition. However, until recently there has been little information about the production of lipases by *Streptomyces* strains, even though considerable data on proteases, carbohydrases and other enzymes has been accumulated (Peczynska-Czoch and Mordarski, 1988). A strain of *Streptomyces flavogriseus* (Mostafa and Ali, 1979) produces an extracellular lipase in the absence of a lipid substrate but growth in the presence of castor oil was found to further stimulate production. An oxytetracycline producing strain of *Streptomyces rimosus* (Engelbrecht and Mach, 1968) was found to produce an intracellular lipase which has been characterised. The industrial demand for highly active preparations of lipolytic enzymes has led to the discovery of high lipolytic activity in *Streptomyces* sp. PCM 27 and PCM 33, and *Streptomyces fradiae* NCIB 82 33 (Sztajer *et al.*, 1988). More recently, some extracellular lipases have been cloned. A gene (*lip*) encoding an extracellular lipase from *Streptomyces* sp M11 has been cloned, using *S. lividans* as the host (Perez *et al.*, 1993). A 28 kDa protein was secreted by *S. lividans* with a 48 amino acid signal sequence and a Gly-His-Ser-Met-Gly motif. The sequence for this lipase gene has been shown to have 82% sequence identity with an extracellular lipase encoding gene from *Streptomyces albus* G (Cruz *et al.*, 1994). These two sequences can be aligned with 33% identity to the sequence of *Lip* 1 from the Antarctic psychotroph *Moraxella* TA144 (Feller *et al.*, 1990). An alignment of the three sequences revealed amino acid substitutions which might be responsible for the greater thermal stability of the S*treptomyces* lipases. The presence of a *lip* gene family in several members of the *Streptomyces* genus including; *S. coelicolor* A3(2), *S. globisporus* ATCC 21553, *S. griseus* NRRL B-2682, *S. lividans* 66 and S. *parvullus* ATCC 12434 has been shown (Cruz *et al.*, 1994).

1.2.7 INDUCTION OF MICROBIAL LIPASES

The induction of lipase biosynthesis in microorganisms does not seem to obey any general rule. There are numerous reports in the literature that the inclusion of lipid substrates and related compounds induce the production of lipase. The extracellular enzyme from *Candida paralipolytica* has been reported to be induced by castor oil, triolein, cholesterol, sodium cholate or sodium deoxycholate (Ota *et al.*, 1968a). Similarly the lipase from *Candida cylindracea* is also enhanced by the addition of glycerides, free fatty acids, cholesterol and sodium cholate to the growth medium (Ota *et al.*, 1968b). Extracellular enzymes from *Galactomyces geotrichum* (Phillips and Pretorius, 1991) and *Pseudomonas* sp. strain ATCC 21808 (Kordel *et al.*, 1991) are induced when grown on media containing olive oil. Likewise the production of extracellular enzymes from *Geotrichum candidum* (Shimada *et al*, 1989) and *S. flavogriseus* (Mostafa and Ali, 1979) are induced when grown on soyabean oil and castor oil respectively. By contrast numerous lipases are produced in the absence of exogenous lipid substrates; *Rhizopus arrhizus* (Labourer and Labrousse, 1966), *Staphylococcus aureus* (Vadhera and Hamon, 1967), *Penicillum crustorum* (Oi *et al.*, 1967) and *Bacillus subtilis* (Kennedy and Lennarz, 1979). Indeed, the lipolytic activity of *Pseudomonas fragi* (Nashif and Nelson, 1953) has been shown to decrease upon addition of lipids to the culture medium. Tween detergents were found to stimulate the production of the lipolytic activities present in *Geotrichum candidum*, *Penicillum* species and *Fusarium solani* but inhibit lipolytic production by *Aspergillus niger* and *Aspergillus flavus* (Wouters, 1967).

1.3 THE LIPID CONTENT AND COMPOSITION OF MICROORGANISMS

The lipid content and composition of microorganisms are subjected to variations with environmental conditions (Rose, 1989). The constituents of the medium can play a part in determining the fatty acid composition. Switching from one carbon source to another can alter the fatty acyl composition of the microbial lipids. The most important factor in

the production of lipids by yeasts and fungi is the ratio of the concentrations of the carbon and nitrogen source (Ratledge, 1982). Physical factors such as temperature, which if lowered results in the degree of unsaturation increasing, can also influence lipid content and composition.

1.3.1 MICROBIAL FATTY ACIDS

Fatty acids occur in nearly all living organisms as the important predominant constituents of lipids (Kaneda, 1977). In bacterial cells, fatty acids occur mainly in cell membranes as acyl constituents of phospholipids (Kaneda, 1991). Bacterial fatty acids can be divided into two major families. One is the straight chain (normal) fatty acid family common in higher plants and animals (Lechavalier, 1977), which are synthesised from acetyl Coenzyme A as the primer and malonyl CoA as the chain extender. The other is the branched chain fatty acid family, which includes iso, anteiso and ω-alicyclic fatty acids with or without a substitution (unsaturation and hydroxylation). These fatty acids are synthesised in certain bacteria from iso, anteiso or cyclic primers and malonyl CoA.

1.3.2 STRAIGHT CHAIN FATTY ACIDS

The biosynthesis of straight-chain fatty acids begins with the condensation of an acetyl unit (starter) and a malonyl unit (extender) to yield a four carbon intermediate. In order to remove the keto group from the β-carbon of each malonate unit, a cycle of three reactions takes place: ketoreduction to a hydroxyl, followed by dehydration to give an enoyl group, and then further reduction to give an alkyl group. The addition of two carbon units and the cycle of reduction, dehydration and reduction continues until the chain reaches it's final length (see section 1.4.1, Fig. 1.8). In *E.coli* , as well as in higher plants, the enzyme which catalyses this reaction is a dissociable multienzyme complex known as Type II Fatty Acid Snthetase (FAS).

1.3.3 BRANCHED-CHAIN FATTY ACIDS

The occurrence of branched-chain fatty acids in bacteria is not nearly as common as that of the straight chain fatty acid family (for review see Kaneda, 1991). Some species which possess branched chain fatty acids are: *Staphylococcus*, *Bacillus* and *Arthrobacter* (gram-negative bacteria); *Legionella*, *Flavobacterium* and *Bacteroides* (gram-positive bacteria); *Cytophaga* and *Myxococcus* (prosthecate and gliding bacteria); and *Streptomyces* (actinomycete bacteria). Kaneda and Smith (1980) showed that branched chain acids when produced, generally account for 90-95% of the cellular fatty acids, implying that these organisms possess a branched fatty acid synthase only. A few species of *Bacillus* produce straight chain fatty acids (20-33% of total fatty acids) and it has been suggested that these species may possess a distinct straight chain fatty acid synthesising system as well as a branched chain synthase.

The majority of branched chain fatty acids are mono-methyl substituted acids, which are divided into two distinct series, the iso-series and the anteiso-series (Gurr and James, 1980) [see Fig. 1.7]. The mechanism of chain extension for the synthesis of branched chain fatty acids is very similar to that of straight chain fatty acids. The difference occurs in their respective primers but in all cases malonyl CoA can also functions as the chain extender. Fig. 1.6 shows a proposed pathway for the synthesis of branched chain fatty acids in *B. subtilis* (Kaneda, 1991): Part A is the synthesis of branched chain fatty acids from short-chain carboxylic acids (particularly isobutyric, isovaleric and 2-methyl butyric); while B represents the synthesis of branched chain fatty acids from branched chain α-keto acids. α-keto acids are formed from the degradation of amino acids by the removal of their α-amino groups by transamination. The type of the initial amino acid (Rezanka *et al.*, 1987) is crucial for the starter unit of fatty acids. The types of branched chain fatty acids from valine, leucine and isoleucine are shown in Fig. 1.7

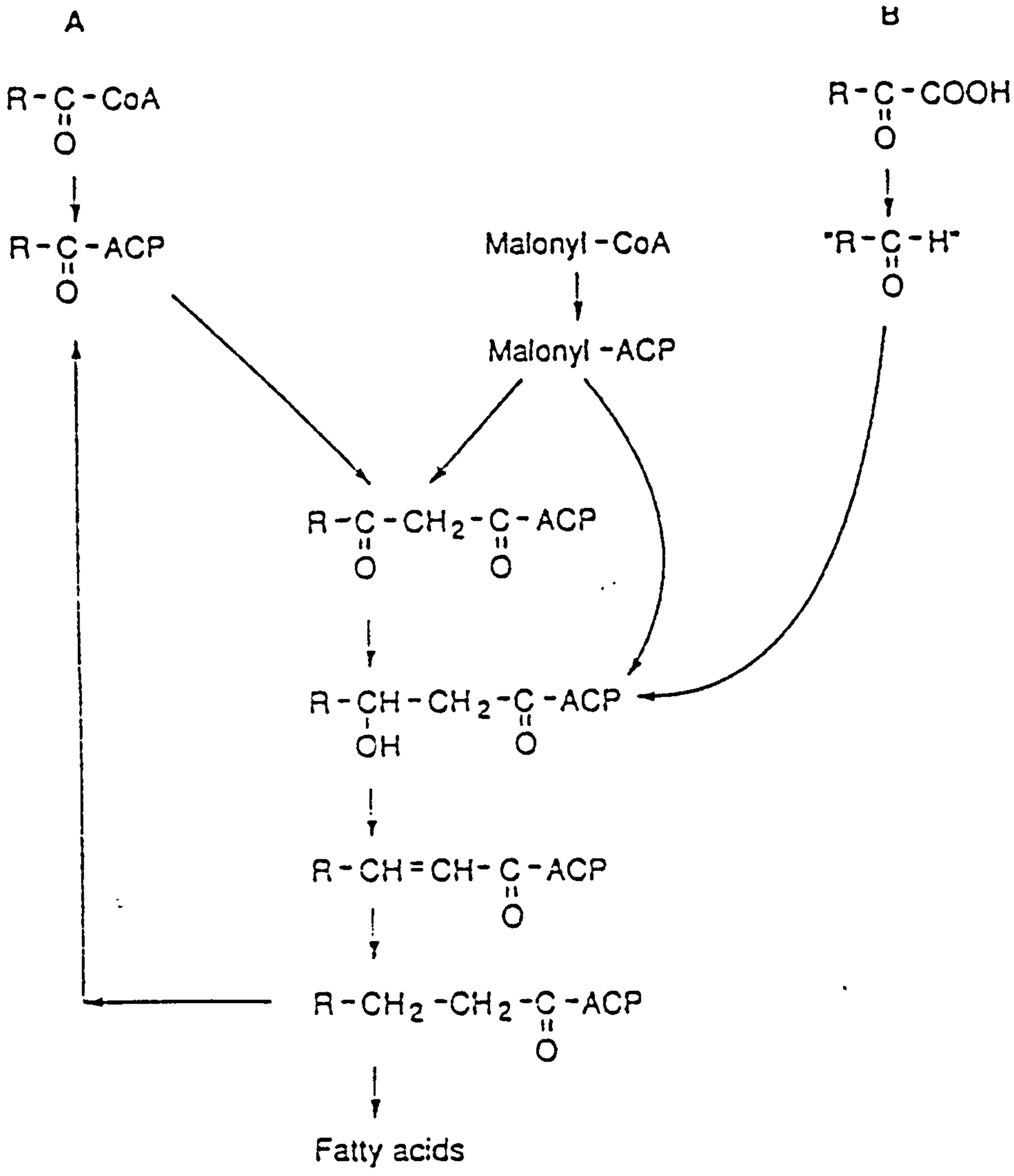

Fig. 1.6 Pathways of branched-chain fatty acid synthesis in *B. subtilis* and other organisms, which possess branched-chain fatty acids as the major cellular fatty acids. A, Pathway of the synthesis from branched-chain acyl-CoA ester as a primer; B, the other pathway of the synthesis from branched-chain α-keto acid as a primer source.

Amino acid	Starter	Type
Valine	CH_3 \ CH—CO— / CH_3	even, iso
Leucine	CH_3 \ CH— CH_2CO— / CH_3	odd, iso
Isoleucine	CH_3CH_2 \ CH—CO— / CH_3	odd, anteiso

Fig. 1.7 The types of branched-chain fatty acids that are derived from valine, leucine and isoleucine (Rezanka *et al*, 1987).

The role of branched chain fatty acids appears to be the maintenance of fluidity in the membranes of branched-chain fatty acid producers. Fluidity in straight chain fatty acid producers is achieved by the inclusion of monounsaturated fatty acids into the membrane bilayer. Branched-chain fatty acid producers generally do not produce unsaturated fatty acids during their growth and anteiso 15:0 plays a similar role to unsaturated fatty acids in branched chain fatty acid producers (Kaneda and Smith, 1980). The branched chain fatty acids are preferentially incorporated into the 2-position of phospholipids (Kaneda, 1972a and 1972b) and usually have lower melting points than the corresponding straight chain acids (Gurr and James, 1980). It has been suggested that branched chains could be more loosely packed than straight chains, and consequently could have a lower crystalline state transition temperature (Tm) [Haest *et al.*, 1974). The fluidity of membranes is closely related to both the average melting point and the Tm of their respective fatty acid compositions. Analysis of the phase transition of chemically synthesised diacylphosphatidylcholine samples showed that the phase transition temperatures of a normal acid and an iso-acid were significantly different with the branched fatty acids having a Tm of up to 28°C lower than the corresponding saturated acid.

1.3.4 STREPTOMYCETE FATTY ACIDS

Streptomycetes share a common fatty acid pattern in which saturated 14:0, 15:0 and 16:0 iso acids and the saturated 15:0 and 17:0 anteiso acids are the most prominent (Ballio and Barcelona, 1965). Recently conclusive evidence for the presence of a Type II FAS in *Streptomyces erythreus* has been produced (Hale *et al.*, 1987) Table 1.2 summarises the major fatty acids present in five streptomycetes. These five species display a typical streptomycete fatty acid spectrum. The fatty acids range from iso14:0 to 18:0 and include a high percentage of branched chain fatty acids and a low percentage of unsaturated fatty acids. Occasionally some unusual fatty acids are found: *S.coelicolor* contains a small amount of 17:0Δ (cis-9-10-methylenehexadecanoate) and *Streptomyces* R61 contains 10-Me-16:0, 10-Me-17:0 and 10-Me-18:0

Table. 1.2 A summary of the major fatty acids present in five streptomycetes; *Streptomyces* R61 (Brown and Cho, 1985), *S. griseus* (Verma and Khuller, 1981), *S. antibioticus* (Zuneda et al, 1984), *S. coelicolor* (Ballio and Barcellona, 1968), *S. viridrochromogenes* (Shim and Kim, 1993). * - phospholipid fraction.

Fatty Acid	*Str.* *	*S. griseus* *	*S.antibioticus* *	*S. coelicolor* Total lipids	*S. viridrochromogenes* Total lipids
i14:0	2.6	3.2	3.2	1	3.3
n14:0	0.2	5.9	5.2	6	1
i15:0	1			12.4	9.6
a15:0	1.3	23.6	27.9	26.3	16.8
n15:0	0.3			1.2	2.2
n16:1	0.6				2.1
i16:0	48	30.4	6.9	22.2	21.4
n16:1	3.5	7.4		10.5	5
n16:0	2	11.3	19.4		11.7
10-Me-16:0	3.7				
n17:1	0.1			5.8	
i17:0	2.2			11.2	4.3
a17:0	5.4	4.7	28.2	0.6	
n17:0	0.1	4.2	3.5	1.9	3.5
n17:0	2.9				10.5
10-Me-17:0	3.9				
i18:1	2.2	7			
n18:1	5	1.4			0.2
n18:0	0.7	0.8	6.3		
10-Me-18:0	15				
Branched chain	84	69	66	79	55
Straight chain	3	22	34	18	16
Unsaturated	13	9	0	0	7

(Brown and Cho, 1985). A direct link exists between the pathways of straight-chain and branched-chain fatty acid metabolism in streptomycetes. A novel enzyme activity was discovered in streptomycetes (Brendelberger *et al.*, 1988) that catalyses the reversible rearrangement of isobutryl CoA into butryl CoA (Reynolds *et al.*, 1988). This enzyme allows the products of fatty acid catabolism to be channelled into methyl malonyl CoA via the oxidation of isobutyrl CoA.

1.3.5 MICROBIAL PHOSPHOLIPIDS

Bacterial phospholipids occur primarily in association with membranes, playing a vital role in structure and function (Vance and Vance, 1985). Phosphatidyl ethanolamine (PE) is found in high concentrations in many bacteria but is particularly common among Gram-negative bacteria and actinomycetes. The N-methylated derivatives of PE, phosphatidyl methylethanolamine (PME) and phosphatidyl dimethylethanolamine (PDME) are not common compounds and when they do occur seem to be limited to Gram-negative or actinomycetes. Phoshatidyl serine (PS) is restricted to Gram-negative bacteria and phosphatidyl choline (PC) is rarely reported in Gram-positive and actinomycetes.

1.3.6 STREPTOMYCETE PHOSPHOLIPIDS

The prevalent phospholipids of the streptomycetes are *bis*-phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidyl-*myo*-inositol mannosides (Verma and Khuller, 1982; Kataska and Nojima, 1967). In general the streptomycetes have a high *bis*-phosphatidyl glycerol content which can sometimes be more than 65% of the total cell lipids (Batrakov and Bergelson, 1978). In addition to these some strains of the genus *Streptomyces* are believed to contain phosphatidyl glycerol (Kovalchuk *et al.*, 1973) and *Streptomyces canosus* has been reported to contain phosphatidyl choline. The phosphatidyl ethanolamine of *Streptomyces soiyaensis* was found to give a double spot on a thin-layer chromatogram. The upper PE spot contained only non-

hydroxylated fatty acids and the lower one contained hydroxy fatty acids in addition to non-hydroxylated fatty acids (Kawanami *et al.*, 1969). A very unusual phospholipid was isolated from the cells of *Streptomyces olivaceus*, which accounted for 20% of the total lipids, and was characterised as phosphatidyl-2,3-butanediol (Konova *et al.*, 1978).

1.3.7 ENERGY RESERVES IN MICROORGANISMS

The criterion for energy storage function in microorganisms consists of three main points (Dawes and Senior, 1973);

1. That the compound is intracellularly accumulated under conditions when the supply of energy from exogenous source is in excess of that required by the cell for growth and related processes at that particular moment in time.

2. That the compound is utilised when the supply of energy from exogenous sources is no longer sufficient for the optimal maintenance of the cell, either for growth and division, or for maintenance of viability and other processes.

3. That the compound is degraded to produce energy in a form utilisable by the cell and that is, in fact, utilised for some purpose which gives the cell a biological advantage in the struggle for existence.

Many bacteria can accumulate polymers that are believed to function as energy reserves. These compounds can accumulate during growth or at the end of growth phase and are degraded to provide energy to be utilised as a source of carbon that is no longer available from the media or environment (Preiss and Romeo., 1989). Three main classes of compound have usually been considered as possible storage compounds in microorganisms, namely polysaccharides, lipids (including poly-β-hydroxybutyrate) and polyphosphates (Dawes and Senoir, 1973 and Ranade and Vining, 1992).

1.3.8 LIPID ACCUMULATION IN MICROORGANISMS

Microorganisms nearly always contain lipids but a few species produce lipids in abundant amounts. These microorganisms are classed as oleaginous, the definition of which is any microorganism which accumulates lipid at 25-70% of the dried biomass, when cultured under optimal conditions (Ratledge, 1982). The lipid which is accumulated in these organisms is mainly triglyceride. Triglyceride biosynthesis is almost entirely confined to eukaryotic organisms e.g. algae, yeasts and molds. An oleaginous bacterium has been reported which accumulates high levels of triglyceride. Wayman *et al.*, (1984) found that *Arthrobacter* AK19, grown in medium with a high C:N ratio produces up to 80% of it's biomass as lipid of which 90% is triglyceride.

The key to lipid accumulation is to allow the nitrogen supplied to the culture to become exhausted within 24-48 h and to have a carbon source in excess. Lipid accumulation by deprivation of vitamins, phosphate, magnesium or iron has been reported.

1.3.9 LIPID RESERVES IN STREPTOMYCES

Streptomyces grown under certain nutritional conditions may form triglycerides as storage products which could be used as a source of C_2 units for antibiotic production, in suitable organisms. The storage of lipids could also prove to be disadvantageous, in industrial strains, by diverting fatty acids from antibiotic production. The relative importance of lipid reserves in actinomycetes seems not to have been widely investigated. Packer *et al.*, (1985) suggested that the storage of triglycerides was a possibility in streptomyces, when they discovered that triglycerides constituted over 50% of the total lipids in the vegetative mycelium of *S.coelicolor* A3(2).

Ranade and Vining, (1992) carried out investigations into the lipid reserves in *Streptomyces venezuelae*, which produces chloramphenicol. The polymer polyhydroxybutyrate (PHB) was found to be formed during biomass accumulation.

However it did not represent a long-term storage material as it was degraded in stationary phase regardless of whether the carbon energy source was in excess. The period of most intense chloramphenicol production was not correlated with lipid mobilisation, and the loss of intracellular PHB, during this period, could not have met the biosynthetic demand for acetoacetyl CoA required for antibiotic biosynthesis. As PHB accumulation preceded antibiotic production, the two processes were not competing for common precursors. Therefore the lipid reserves were neither competing with antibiotic biosynthesis or playing a role in providing a precursor for antibiotic biosynthesis in *Streptomyces venezuelae*.

A quantitative and temporal correlation was found between the production of avermectins and lipids in *Streptomyces avermitilis* grown in defined media (Novak *et al.*, 1990). Avermectins are polyketides and are synthesised from the same precursors as fatty acids. A significant increase in the synthesis of avermectins occurs during stationary phase and the synthesis of both forms stop simultaneously with the depletion of glucose from the medium. The lipid fraction contains mostly triglycerides: iso-even and odd, anteiso-odd and straight-chain fatty acids in the range of C_{14} to C_{18} (Metz *et al.*, 1988). The fact that triglycerides and avermectins are synthesised simultaneously indicates that these two biosynthetic pathways may have to compete for a limited supply of common precursors (2-methylbutyrate, propionate and acetate etc.). Further investigations carried out by Rezanka, Mikova and Jurkova (1992) involved growing *S. avermitilis* in the presence of inhibitors of fatty acid biosynthesis. The production of total lipids was inhibited and the cellular content of individual types of fatty acids was altered by these compounds. The resulting decrease in anteiso-acids was accompanied by the increased production of type 'a' avermectins (having a *s*-butyl group in the side chain). From this it was concluded that 2-methylbutyrl CoA was being diverted from the biosynthetic pathway of fatty acids to that of avermectins.

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1.4 POLYKETIDES

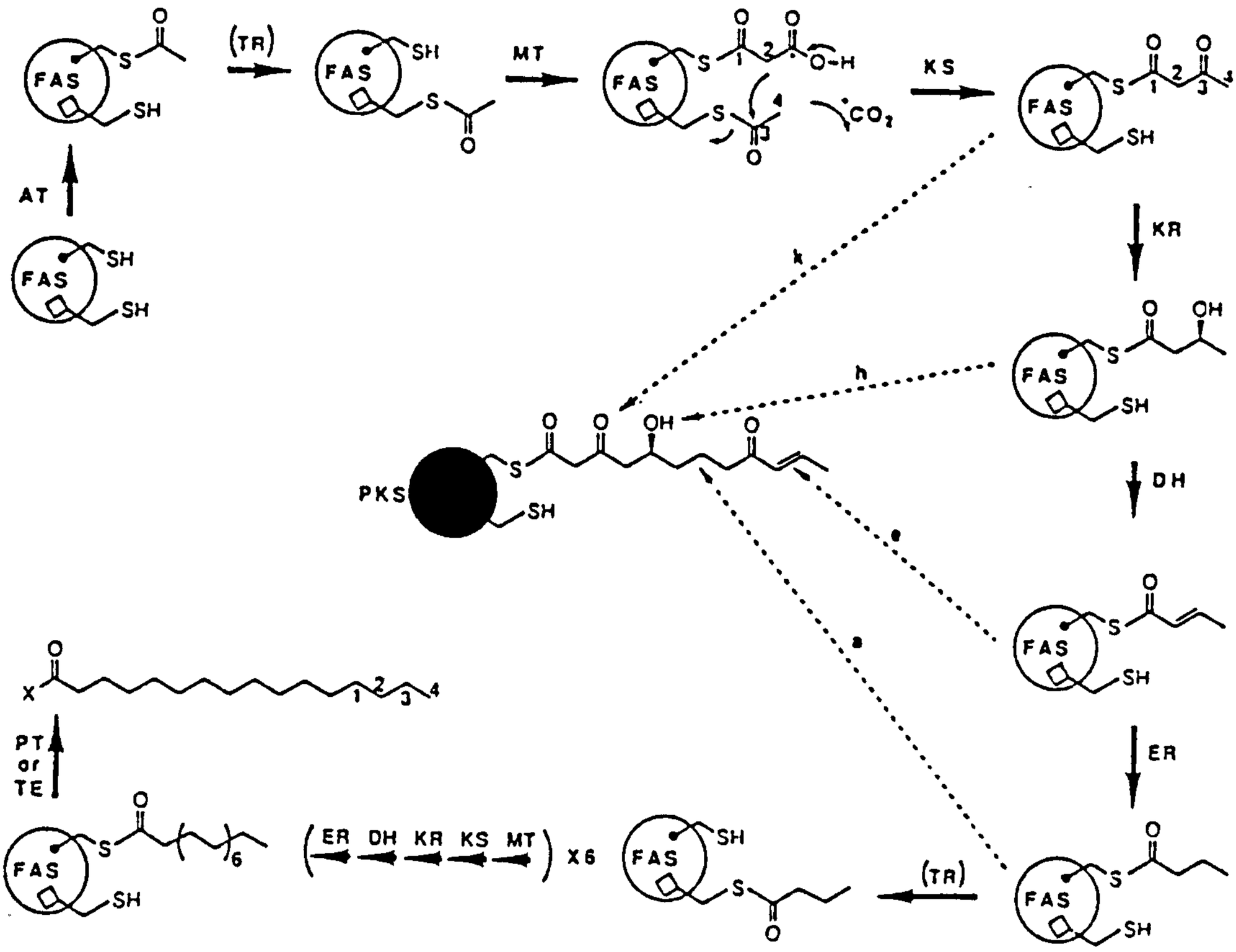
The polyketide family of natural products are made by bacteria (mainly actinomycetes), fungi and plants from simple fatty acids. Most of the important polyketide secondary metabolites produced by actinomycetes are antibiotics, such as tetracyclines, macrolides, polyethers and many others. The important fungal and plant products include aflatoxins, flavour compounds and pigments (Sherman, 1989).

1.4.1 POLYKETIDE BIOSYNTHESIS

The pathways of fatty acid biosynthesis and polyketide biosynthesis share many common features (Hopwood and Sherman, 1990; Sherman, 1989; Hutchison *et al.*, 1988). The similarities between these two biosynthetic pathways demonstrate another of the examples of the links between primary and secondary metabolism. Secondary metabolites are special types of metabolites produced, only by certain taxonomic groups, by using the general enzymes of primary metabolism or special synthetases produced by specific cells under specific nutritional conditions (Martin and Demain, 1980).

The synthesis of a polyketide antibiotic, during microbial metabolism follows the same basic pattern as fatty acid biosynthesis with the successive addition of carbon containing units to build a chain. Fig. 1.8 shows a schematic representation of fatty acid and polyketide biosynthesis. However polyketide biosynthesis is potentially more complex. The enzyme which catalyses polyketide biosynthesis is known as Polyketide Synthetase (PKS). Polyketide synthetase is not restrained to using acetates as starter units and malonates as extenders but can choose from a much wider range of possibilities including propionate and butyrate units. The cycle of three reactions that occurs after the initial condensation reaction in fatty acid biosynthesis is often omitted or curtailed, at all or some points in polyketide biosynthesis, giving either keto, hydroxyl, enoyl or alkyl groups. The occurrence of keto groups at many of the alternate carbon atoms of the chain gives rise to the name "polyketide". Studies carried out on PKS genes from the

Fig. 1.8 Schematic representation of fatty acid and polyketide biosythesis. The circle labelled FAS or PKS represents the fatty acid or polyketide synthase, carrying two thiol groups, one on the β-ketoacyl synthase (condensing enzyme) (•) and the other on the acyl carrier protein (◇). The reaction steps are labelled: AT, acetyl transferase; (TR), acyl transfer reaction, not ambiguously assigned to a specific enzyme component; MT, malonyl transferase; KS, β-ketoacyl synthase; KR, ketoreductase; DH, dehydrase; RE, enoyl reductase; PT, or TE, palmityl transferase or thioesterase, involved in chain termination to produce palmityl CoA (X = CoA) or free palmitic acid (X =OH) respectively. 1, 2, 3, 4 designate carbon atoms of malonate and acetate that contribute to chain building, while the asterisk labels the carbon of malonate that is eliminated as CO_2. k, h, e, a, represent the various possibilities that follow each condensation step to give keto, hydroxyl, enoyl or alkyl functionality at specific points in the product of polyketide synthase.


FAS
SH
(TR)
MT
KS
AT
PKS
KR
DH
ER
PT
or
TE
(ER DH KR KS MT) X6
(TR)

tetracenomycin C pathway in *Streptomyces glaucescens* and PKS enzymes from the tylosin pathway in *Streptomyces fradiae* have shown that polyketide biosynthesis involves a multienzyme complex similar to Type II FAS, rather than a multifunctional protein (Hutchison *et al*, 1988). Recently, significant progress has been made in the cloning and analysis of PKS genes from members of the genus *Streptomyces* and related bacteria (Hopwood and Sherman, 1990; Katz and Donadio, 1993). This includes type I (multifunctional) and type II (multicomponent) PKS systems. The type I PKSs specify the construction of macrolide antibiotics and can utilise a range of different precursor starter and extender unit carboxyl coenzyme A esters for carbon chain assembly (Cortes *et al.*, 1990; Donadio *et a.l*, 1991; Katz and Donadio, 1993).

The diversity in the final polyketide structure owes itself to the biosynthetic pathway, with PKS being much more highly programmed and facing many more choices than FAS does (Hopwood and Sherman, 1990). The second carbon atom donated by each unit to the growing polyketide chain can vary according to it's origin: a hydrogen in the case of acetate, methyl or ethyl groups for proprionate or butyrate and others for the rare more complex building units. For example, the synthesis of avermectins by *Streptomyces avermectilis* incorporates, in addition to acetic acid and propionic acids, isobutyric and S (+)-2-methylbutyric acids as part of the antibiotic structure (Hafner *et al.*, 1991). This side-chain variation coupled with the possible fate of the keto groups and the total chain length accounts for the endless variety of chemical forms of the polyketides.

The similarity of polyketide biosynthesis with that of fatty acids is illustrated by the inhibition of the biosynthesis of polyketides by cerulenin. Cerulenin, (2S) (3R) 2,3-epoxy-4-oxo-7,10-dodecadienoylamide, is a lipid biosynthesis inhibiting antibiotic produced by the fungus *Cephalosporium caerulens* (D'Agnolo, 1973 and Omura, 1976). This antibiotic inhibits lipid biosynthesis by binding irreversibly to a cysteine residue at the active site of β-ketoacyl carrier protein synthetase, the enzyme which catalyses the condensation reaction of fatty acid biosynthesis (Funabashi *et al.*, 1989). Synthesis of many polyketides is also inhibited by cerulenin and it has been suggested

that cerulenin inhibits the condensation step in polyketide biosynthesis (Hitunen and Söderhäll, 1992). Lecomycin, tetracycline and cycloheximide are just a few of the polyketides whose production has been shown to be inhibited by cerulenin (Omura, 1976).

1.5 AIMS

The aims of this study were to investigate several aspects of lipids and lipid metabolism in *Streptomyces*: including growth on lipids as energy sources, the lipolytic enzymes present, the fatty acid composition of *Streptomyces* grown under different nutritional conditions, to investigate the conditions required to promote lipid storage and the subsequent effects on polyketide antibiotic production, if any. *S. rimosus* was the organism on which most of the work was carried out on, but *S.coelicolor* and *S. clavuligerus* were also included in the lipid analysis studies.

CHAPTER 2

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 MATERIALS

All chemicals used were of the best quality available and were obtained from BDH Chemicals Ltd., Poole, Dorset, UK. except for those listed below:

Tris was obtained from Boehringer Corp., Lewes, Sussex, UK. Glucose and ammonium sulphate were obtained from Formachem (Research International) Ltd., Strathaven, Midlothian, UK. Acetic acid, acrylamide, N, N'methylene-bis-acrylamide, NaCl, SDS and sucrose were obtained from FSA Laboratory Supplies, Loughborough, Leics., UK. Ethanol was from James Burroughs (FAD) Ltd, Witham, Essex, UK. EDTA was obtained from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, Leics., UK. Yeast extract, Bacto Peptone and Emerson agar were obtained from Difco Laboratories Detroit, Michigan, USA. Silica gel H type 60, Merck was obtained from MacFarlane Robson Ltd, Burnfield Ave, Thornliebank, Glasgow, UK. BSA (plasma albumin, fraction V) used as a standard in protein estimations was obtained from Wilfred Smith Ltd., Gemini House, High St., Edgeware, Middlesex, UK. Malt extract was obtained from Scott's, Byres Road, Glasgow. Olive oil was obtained from Safeway, Byres Road, Glasgow. Soya bean flour and rape seed oil were both gifts from Beecham Pharmaceuticals. Junlon PW110 was obtained from Honeywell and Stein Ltd., Surrey.

The following chemicals listed below were all obtained from Sigma Chemical Co., Poole, Dorset, England: Coomassie Brilliant Blue G250, MOPS, Tween 20, Tween 40, Triton X-100, all ρ-nitrophenyl esters, Fast blue salt RR, PMSF, iodoacetamide, iodoacetate, mercuric chloride, 2', 7'-dichlorofluorescein and salmon sperm DNA.

2.2 MICROORGANISMS

The microorganisms used in this study; *Streptomyces coelicolor* A3(2) [1147](Glasgow definition), *Streptomyces rimosus* 4018, and *Streptomyces clavuligerus* ATCC 27064 were kindly donated by Dr. I.S. Hunter, Department of Genetics, University of Glasgow, Glasgow G12 8QQ.

2.3 MEDIA

2.3.1 Minimal media

a) Hobbs Minimal Medium (HMM)

This was the minimal medium, used initially to allow growth of *Streptomycetes* under defined conditions (developed by Hobbs *et al.*, 1989). The medium has two component solutions. Solution 1 consists of 6.25 g NaCl, 6.25 g Na_2SO_4, 2.5 g KH_2PO_4, 1.5 g Tris buffer and 1.875 g Junlon. Solution 1 was boiled to dissolve the Junlon, adjusted to pH 7.2 with 1 M NaOH , made up to a final volume of 1 litre and sterilised by autoclaving. Solution 2 was autoclaved separately and added to solution 1 prior to inoculation. Solution 2 consists of 20 g glucose, 10 g $MgSO_4.7H_2O$ and 0.1 g $ZnSO_4$, dissolved in 100 ml of distilled water.

A filter-sterilised trace salts solution was prepared. This trace salts solution consists of 8.775 g $FeCl_3$, 2.040 g $ZnCl_2$, 1.015 g $MnCl_2.4H_2O$, 0.425 g NaI, 0.310 g H_3BO_3, 0.238 g $CaCl_2$ and 0.242 g $Na_2MoO_4.2H_2O$ in a total volume of 1 l of distilled water.

Complete HMM was prepared by mixing eight parts Solution 1 with one part Solution 2 and one part (200 ml) sterile sodium nitrate. To this final medium the trace salts solution was added to a final concentration of 0.1% (v/v)of the original trace salts solution.

b) Modified HMM

This medium is the same as HMM except that it does not contain Junlon, the $MgSO_4.7H_2O$ concentration is reduced four fold to 0.25 gl^{-1}, and MOPS, at 40 mM, is added to improve the ability of the medium to accommodate acid formation. This modified HMM was the standard defined growth medium used in culturing *S. rimosus*. The standard carbon source is 10 mM glucose but this is replaced by 20 mM glycerol or 10 mM mannitol in certain experiments. The nitrogen source used in this modified HMM was 20 mM $(NH_4)_2SO_4$ which was autoclaved separately and added in place of sodium nitrate.

2.3.2 Complex media

a) Soya Mannitol Agar (SM)

This was used as a general plating medium for *Streptomyces*, particularly for the production of spores and the preparation of stock cultures. It consists of 20 g mannitol, 20 g soya bean flour, 16 g agar, dissolved in 1 l of tap water.

b) Emerson Agar

This agar was inoculated with appropriately diluted samples from cultures for the determination of colony forming units. Emerson agar was prepared by dissolving 41.5 g of this agar in 1 l of distilled water and sterilised by autoclaving.

c) Yeast extract-Malt extract (YEME)

This was used as a general purpose complex medium. It consists of 3 g yeast extract, 5 g Bacto Peptone, 3 g malt extract, 10 g glucose and 340 g sucrose dissolved in 1 l of distilled water. The pH of the medium was not adjusted but had a value of 6.8.

2.4 PRODUCTION OF STREPTOMYCES SPORES

a) Preparation of spore suspensions and storage

The protocol described below is a modified version of that described by Hopwood *et al*, (1985). A boiling tube containing a slant of Soya Mannitol agar (produced by pouring 15 ml of molten agar into the tube and allowing it to solidify with the tube held at 5° from the horizontal) was inoculated with 150 µl of a spore or mycelial fragment suspension and incubated at 30°C. After 10 days the surface of the culture was covered in a mass of dark grey (*S. coelicolor*) or yellow (*S. rimosus*) spores. Spores could then be harvested for immediate use or stored at -20°C indefinitely. The spores were harvested by adding 5 ml of distilled water to the slant and resuspending the spores by rubbing the surface of the slant with a sterilized loop. When the surface had been scraped clean of spores, the spore suspension was decanted into a sterile universal. The slant was rinsed with an additional 5 ml of distilled water to remove any remaining spores. This spore suspension (100 µl) was used to inoculate soya mannitol agar plates which were incubated for 10 days at 30°C. These plates were harvested in a similar way to the slants. The resulting spore suspensions were pooled and concentrated by centrifugation in sterile centrifuge tubes, at 3000 rpm using a Beckman Model TJ-6 Centrifuge (Beckman Instruments Inc., Palo Alto, California 94304.). The spore suspensions were either stored in sterile distilled water at 4°C, and used within 2 weeks, or frozen at -20°C after the addition of sucrose to 20% (w/v).

b) Spore Counts

Colony forming units were determined by plating suitably diluted spore suspensions on Emerson agar plates. Known values of dilutions of spores in sterile water in the range of 1:10 to $1:10^9$ were added to molten Emerson agar, mixed and poured into petri dishes. Counts of the number of colonies in the dishes were made after incubation at 30°C for 5 days. The concentration of spores in the spore suspension was calculated by multiplying the number of colonies by the appropriate dilution factor.

2.5 GROWTH OF BACTERIA

Cultures were always incubated at 30°C. Liquid cultures were grown in a range of flasks (250 ml-10 l), depending on the purpose of the experiment, either on an orbital shaker, at180 rpm, or using a magnetic stirrer, at 300 rpm. Flasks containing 4 l of medium (medium was mixed using a magnetic stirrer) were supplied with a supply of air to ensure adequate aeration.

2.6 TURBIDITY MEASUREMENTS

Bacterial growth was monitored by measuring the absorbance of the culture or a dilution of the culture at 450 nm (in the presence of Junlon) or 500 nm (in the absence of Junlon) in a 1 cm light path cuvette using a LKB Ultrospec II (LKB Instruments Ltd., L.K.B. Engineering Co. Ltd., Stoke Poges.). Samples with an optical density of more than 0.5 were diluted with distilled water.

2.7 HARVESTING AND STORAGE OF BACTERIA

Harvesting of bacteria was carried out at 4°C. Volumes of culture up to 50 ml were harvested by centrifugation at 10 000 rpm for 20 min in M.S.E. Highspeed 18 Centrifuge (M.S.E. Ltd., London, U.K.). Larger volumes were harvested at 5000 rpm for 30 min in an M.S.E. 6 L Centrifuge. Supernatants and pellets were either used immediately or stored at -20°C.

2.8 ULTRASONIC DISRUPTION OF BACTERIA

1 g wet weight of pellet was suspended in 5 ml of ice cold 50 mM KH_2PO_4, pH 7.0 buffer and sonicated, using a Lucas Dawe Ultrasonics Soniprobe Type 7534A (Lucas Dawe Ultrasonics Ltd., Concord Road, Western Avenue, London, W3 05D.) at 60 W,

on a 30% duty cycle for 5 min. During sonication, the sample was maintained at 0°C by immersion of the sample holder in an ice water slurry.

2.9 PROCEDURE USED TO INDUCE LIPASE ACTIVITY

The development of the standard procedure used in this study to induce lipase activity in *S. rimosus* is described in 4.6.2. This method involved growing cells from an initial spore density of $6x10^9$ spores ml^{-1}, in 1 l of YEME, and then resuspension of 1 g of the harvested pellet in 100 ml of modified HMM, containing10 mM glucose as the carbon source (Fig. 2.1). After 48 h incubation at 30 °C, with shaking, cultures were harvested and crude extracts containing intracellular and extracellular lipase activities prepared according to Method 2.10.

2.10 PREPARATION OF CRUDE EXTRACTS CONTAINING INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITY

Intracellular lipase was assayed in cell extracts. After harvesting the cultures and preparing pellets as described in Methods 2.7, the extracts were prepared by sonication (Methods 2.8). The sonicated samples were spun in 1.5 ml Eppendorf tubes at 11 000 g for 5 min in a microcentrifuge. The supernatant from these samples, which contained greater than 90% (determined by assaying) of the lipase activity, was either used immediately, concentrated (Methods 2.12) or stored at -20°C until required. To assay the extracellular lipase the supernatants from the harvested cultures were concentrated, and either used immediately or stored at -20°C.

2.11 PREPARATION OF DIALYSIS TUBING

Dialysis tubing was prepared by boiling for 10 min in 1% (w/v) EDTA, then rinsed and boiled in distilled water three times for 10 min each time and then stored in 20% (v/v) ethanol until required.

Fig. 2.1 PROCEDURE USED TO INDUCE LIPASE ACTIVITY

This procedure involved growing *S.rimosus* in 1 l volumes of YEME and then resuspending 1 g aliquots of the harvested biomass into 100 ml of modified HMM, utilising 10 mM glucose as the carbon source. After 48 h incubation with shaking at 30°C, these cultures were harvested and crude extracts containing intracellular and extracellular lipase activities were prepared according to Methods 2.10.

S.rimosus (YEME)

↓ Harvest 5000 rpm for 30 min
(ref Methods 2.7)

Resuspend 1 g in 100 ml
HMM containing 10 mM Glc.

↓ Harvest after 48 h
(3000 g for 30 min)
(ref Methods 2.7)

↙ ↘

culture supernatant — pellet

↓ ↓

concentrate
(ref Methods 2.12) — resuspend in 5 ml
50 mM KH2PO4, pH 7.0
sonication (ref Methods 2.8)

↓ ↓

assay for lipase
(ref Methods 2.31) — spin in microcentrifuge
(11 000 g for 5 min)
(ref Methods 2.10)

↓

concentrate supernatant
(ref Methods 2.12)

↓

assay for lipase
(ref Methods 2.31)

2.12 CONCENTRATION OF CRUDE EXTRACTS CONTAINING INTRACELLULAR AND EXTRACELLULAR ACTIVITY

Concentration of crude extracts was carried out by placing either 500 ml of supernatant or 50 ml of pellet extract in dialysis tubing surrounded by CM cellulose and leaving at 4°C, for 48 h after which time approximately 5 ml of material was left.

2.13 DIALYSIS OF CRUDE EXTRACTS

Before samples were run on polyacrylamide gels or on columns, they were dialysed overnight against 10 mM KH_2PO_4 pH 7.0 at 4°C.

2.14 ULTRACENTRIFUGATION OF CRUDE EXTRACTS TO DETERMINE THE DISTRIBUTION OF LIPASE ACTIVITY

Aliquots of sonicated biomass and concentrated supernatant were spun using a Type 65 rotor in a Beckman Model L5-65 ultracentrifige for 2.5 h at 40,000 rpm. The resulting pellet and supernatant fractions were assayed for lipase activity (Methods 2.31), to determine the distribution of lipase activity.

2. 15 pH MEASUREMENT

The pH measurements of all solutions were carried out using a direct reading pH meter (Model 7010, EIL Ltd., Cumbernauld, Glasgow G67 IAG.) connected to a combined glass electrode (Type 224, Probion Ltd., Glenrothes, Fife KY6 3AE.).

2.16 STERILISATION

2.16.1 Moist Heat

Media and all other solutions were sterilised by autoclaving at 5 psi and 109°C for the times established by Fewson (unpublished results) which ensure that materials are exposed to high temperatures for the minium length of time required for complete sterilisation. Efficiency of sterilisation was verified by using Browne's tubes Type 1 (A. Browne Ltd.,Chancery Street, Leicester).

2.16.2 Dry Heat

Glassware and glass pipettes were packed in sterilisation bags (DRG, Hospital Supplies, Dixon Road, Bristol B54 5QY) and sterilized in an oven at 160°C for 1.75 h. Each sterilisation was checked by including a Browne's tube (Type 3).

2.16.3 Ethylene Oxide

Plastic pipettes were sterilised with ethylene oxide. They were packed in sterilisation bags and exposed to ethylene oxide (Anprolene) for 12 h in a sterilising box (AN 74) [H.W. Anderson Products Ltd., Clacton-on Sea, Essex.]. Sterilisation was verified by an Anprolene exposure indicator (AN 85) or by a Steritest unit (AN 80). All bags of pipettes were aired in a fume cupboard for at least 24 h prior to use, to remove any residual ethylene oxide.

2.16.4 Filter Sterilisation

Volatile or heat labile compounds in large volumes of solution were filtered through Millex-HV sterile filters (Millipore UK, Watford, UK.) into pre-sterilised glassware.

Small volumes were sterilised using Nalgene disposible sterile filter units with a 0.22 μm pore size (Sybron Corporation, Rochester, N.Y., U.S.A.).

2.17 PROTEIN ESTIMATION

Protein concentrations of cell extracts etc. were determined using the method of Bradford (1976) using bovine serine albumin to construct standard curves.

Protein concentration in column fractions was determined by measuring their A_{280} using an LKB Ultrospec II.

2.18 DNA ESTIMATION

The method used was a modification of that described by Burton (1956). The reagent was a prepared by mixing 100 ml of glacial acetic acid with 10 μl of paraldehyde and then adding 4 g of diphenylamine. 2 ml of the reagent and 250 μl of 5 M perchloric acid (PCA) were added to 1 ml of culture broth. This solution was mixed by vortexing and the tubes were covered with parafilm and left at 30°C. After 18-24 h the absorbance of the sample supernatants was measured at 600 nm (LKB Ultrospec II). DNA concentration was expressed in units of μg ml^{-1} by comparison with a standard solution of salmon sperm DNA. The standard solution of DNA was made by dissolving 20 mg DNA in 100 ml of 0.5 M PCA, and this was stored at 4°C.

2.19 GLUCOSE ASSAY

Glucose was assayed using the GOD-PERID kit (Boehringer Mannheim GmbH Diagnostica, Boehringer Mannheim France SA, 38240 Meylan.) which is based on the method of Werner *et al.*, (1970). The test kit contains glucose oxidase (GOD) and peroxidase (PERID). In the assay, these two enzymes act sequentially to form a green, coloured complex, measurable at 610 nm.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS*}\ \text{(pale yellow)} \xrightarrow{\text{PERID}} \text{oxidised ABTS (green)} + H_2O$$

* di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)

The reagents, 1 (glucose standard, 0.505 m mol l^{-1}) and 2 (buffer/ enzymes/ chromogen) were prepared according to the manufacturers directions and stored at 4°C. 200 µl volumes of culture or dilutions of culture were mixed with 5 ml of reagent 2 and incubated at 37°C for 15 min. A standard containing 200 µl of reagent 1 and a blank containing 200 µl of distilled water were also incubated at 37°C for 15 minutes. A linear relationship exists between absorbance and glucose concentration. The concentration of glucose in culture samples were calculated using the following calculation:

$$\text{Glucose mM} = \frac{\text{Asample}}{\text{Astandard}} \times 0.505$$

2.20 AMMONIA ASSAY

This assay was used to give an estimate of nitrogen concentration in culture media. Ion chromatography at low pH using a Waters HPLC (712 WISP autoinjector, 431 conductivity detector) [Waters International, High Wycombe, Bucks., UK.] was used to detect the ammonium cation. The mobile phase (4 mM nitric acid) was prepared by adding 0.507 ml concentrated nitric acid (70% v/v) to 2000 ml deionised water. A cation standard was prepared by adding 0.254 g sodium chloride, 0.594 g ammonium chloride and 0.760 g potassium chloride to 100 ml of deionised water. To prepare a working standard the cation standard was diluted 1:20 with deionised water. This gave 5 ppm (0.208 mM) sodium, 10 ppm (0.554 mM) ammonium and 20 ppm (0.512 mM) potassium. The conductivity detector was set to 0.01µS sensitivity range and the column (Shodex IC Y-521 [Phenomenex Ltd.]) equilibrated with mobile phase until a stable baseline was achieved (flow rate 1 ml/min, 40°C). 0.05 ml samples were injected

with any necessary dilutions. Ammonia concentrations were calculated by comparing the samples with the known standard solution.

2.21 INORGANIC PHOSPHATE ASSAY

A mixture of three parts 0.045% (w/v) Malachite Green hydrochloride to one part 4.2% (w/v) Ammonium molybdate in 5 M HCL was prepared and left at room temperature for 30 minutes. To each 30 ml of this solution 100 µl of Triton N-101 was added. 850 µl of the resulting reagent was added to 50 µl of culture samples. After 1 minute 100 µl of 34% (w/v) citric acid was added to each tube, which was then vortexed and incubated at room temperature for 60 minutes. In parallel with the samples triplicate standards (0.2 mM mono-potassium dihydrogen orthophosphate) and blanks (distilled water) were processed. The absorbance of the samples was measured at 645 nm. A linear relationship exists between absorbance and phosphate concentration. The concentration of inorganic phosphate in the culture samples was calculated using the following calculation:

$$\text{Phosphate mM} = \frac{\text{Asample}}{\text{Astandard}} \times 0.2$$

2.22 TOTAL LIPID ASSAY

Lipid was assayed using a commercial kit (Boehringer Mannheim). The method involves a sulphophosphovanillin reaction in which unsaturated lipids react with sulphuric acid, phosphoric acid and vanillin to form a pink coloured complex, measurable at 530 nm (Zöllner and Krisch, 1962) [LKB Ultrospec II]. 2 ml of sulphuric acid was added to 50 µl of sample or to 50 µl of lipid standard (10 g l^{-1}), mixed and boiled for 10 min, then cooled. 100 µl was then added to 2.5 ml of phosphoric acid / vanillin colour reagent, mixed and incubated at 20-25°C, for 30 min. A blank consisting of 100 µl of sulphuric acid and 2.5 ml of phosphoric acid / vanillin colour reagent was

used to zero the spectrophotometer. A linear relationship between absorbance and the lipid concentration exists The concentration (c) of the total lipids in the sample is calculated using the following calculation:

$$c\ (g\ l^{-1}) = 10 \times \frac{A\text{sample}}{A\text{ standard}}$$

2.23 LIPID EXTRACTION AND PURIFICATION

Lipids were extracted using a chloroform/ methanol mixture (2:1, v/v) as described by Folch *et al.*, (1957). *Streptomyces* pellets were mixed with 20 volumes of chloroform/methanol (2:1, v/v). This suspension was homogenised using a Ultra-Turrax (Janke and Kuukel, Staufen, iBr, Germany.) and then filtered through Whatman No.1 filter paper (Whatman International Ltd., Maidstone, England.) into a stoppered glass flask. The filter paper and the original flask were washed with a further 50 ml of chloroform/methanol , to remove any remaining lipid. A quarter of the total extract volume of 0.88% w/v KCl in water was added, mixed thoroughly and allowed to stand for at least 30 min. The upper layer containing methanol and water was removed carefully by aspiration, and the volume was recorded. The same volume of methanol/water (1:1, v/v) was added, mixed and again the upper layer was removed. This step was repeated a further time and then the solvent was evaporated from the chloroform layer containing the extracted lipids, as described in Methods 2.27. Lipid samples were either stored in a small volume of chloroform (5-10 ml) at -20°C or analysed immediately.

2.24 THIN LAYER CHROMATOGRAPHY (T.L.C.)

The thin layer plates used were 20 x 20cm glass plates with a 0.5 mm layer of silica gel H (type 60). They were made using a Quickfit spreader and plate holder (Quickfit Instruments, Corning Ltd., Laboratory Division, Stone, Staffordshire ST15 OBG). 50 g of Silica Gel H60 (Merck, Damstadt, Germany.) was slurried with 110 ml of distilled

water and spread over five plates with the spreader gap set at 0.5 mm and left overnight to set. The plates were activated before use by heating at 105°C for a minimum of 2 h.

The samples (10-50 µl) were applied 2-3 cm up from the bottom of the plate with a 25 µl Hamilton syringe. 20 µl standard solutions (10 mg ml $^{-1}$in chloroform) of triglyceride, cetyl alcohol and oleic acid were run along side the samples to help identify spots on the chromatogram.

2.25 DEVELOPMENT OF THE CHROMATOGRAM

The thin layer chromatograms were developed at room temperature in an ascending direction in Shandon TLC tanks (Shandon Southern Instruments Ltd., Frimley Road, Camberley, Surrey.) containing the solvent mixture, petroleum ether 40-60°C / diethyl ether / formic acid (150/50/1, by vol.).

2.26 DETECTION OF LIPIDS ON TLC PLATE

Two methods were used to visualise lipids on TLC plates. The first involved placing dried TLC plates in an iodine tank which allowed the lipids to appear as brown spots. The second involved visualising the lipids in ultra-violet after the chromatogram had been sprayed with a solution of 0.1% (w/v) 2', 7'-dichloroflourescein in 95% ethanol when the lipids appeared as bright-green fluorescent spots. The areas containing fatty acids were carefully scraped off the plate and their methyl esters were prepared (Methods 2.27) for further analysis by GLC (Methods 2 29).

2.27 PREPARATION OF FATTY ACID METHYL ESTERS

The silica gel containing the separated fatty acids was transferred to a scew-capped pyrex tube and heated with 2 ml of 2% (v/v) sulphuric acid in dry methanol for 3 h at 70°C in a heating block (Ori-Block OB-3, Techne Ltd., Cambridge). The tube was

cooled and 4 ml of diethyl ether and then 5 ml of water were added. The contents of the tube were mixed thoroughly after each addition and the diethyl ether layer was removed into a stoppered tube and 5 ml of sodium carbonate (0.05% w/v) was added to neutralise any remaining sulphuric acid. The ether layer was removed and dried over anhydrous sodium sulphate. The solvent was evaporated off as described in Methods 2.28.

2.28 EVAPORATION OF SOLVENTS

All organic solvents were evaporated in a fume cupboard. Large volumes of solvents were removed from extracted lipids *in vacuo* under nitrogen (oxygen free) at 40°C. Small volumes (<10 ml) were transferred to conical glass tubes and heated at 40°C on a heating block. A stream of oxygen free nitrogen was directed on to the surface of the solution to aid evaporation and prevent oxidation of the lipids.

2.29 FATTY ACID ANALYSIS

After the evaporation of the solvent, the methyl esters of fatty acids prepared were dissolved in ~5 µl of toluene. The sample (0.1-0.4 µl) was injected into a Perkin Elmer 8420 Capillary Gas Chromatograph (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire) using a 1 µl syringe (Hamilton Co., Reno, Nevada. 89510, U.S.A.). The G.L.C was equipped with an SPB-1 fused silica capillary column (length 30 m, internal diameter 0.25 mm) coated with a stationary phase (0.25 µm thickness). The instrument was operated in the split mode (split ratio, 1:100). The temperature programme that gave the best resolution of the fatty acid methyl esters was one with an oven temperature of 150°C, held for 10 min and then rising at 4°C min^{-1} to 250°C with a final hold of 10 min. The injector and detector were set at 250°C and 280°C respectively. The carrier gas (oxygen free nitrogen) was passed through an oxygen trap (Alltech Associates Inc., New Street, Camforth, Lancashire LA5 9BX.) at a flow rate through the column of 1.5 ml min^{-1}. The instrument was attached to an integrator,

which produced a table of retention times and calculated peak areas. Individual fatty acids present in samples were identified by comparisons with standards; Bacterial Acid Methyl Esters CP Mix (Supelco, Sigma Aldrich Co Ltd., Fancy Road, Poole, Dorset, England).

2.30 GC-MS

This work was undertaken by Dr. R.A. Anderson in the Department of Forensic Medicine and Science of the University of Glasgow. The analysis of fatty acid methyl esters was carried out using a VG Analytical model 70-250S gas chromatograph-mass spectrometer fitted with a Chromopack CP-SIL 5CB 25 m x 0.32 mm (Chromack Ltd.). The MS source temperature was 240°C and the electron ionization potential was 35 eV.

2.30.1 Interpretation of mass spectra of fatty acid methyl esters

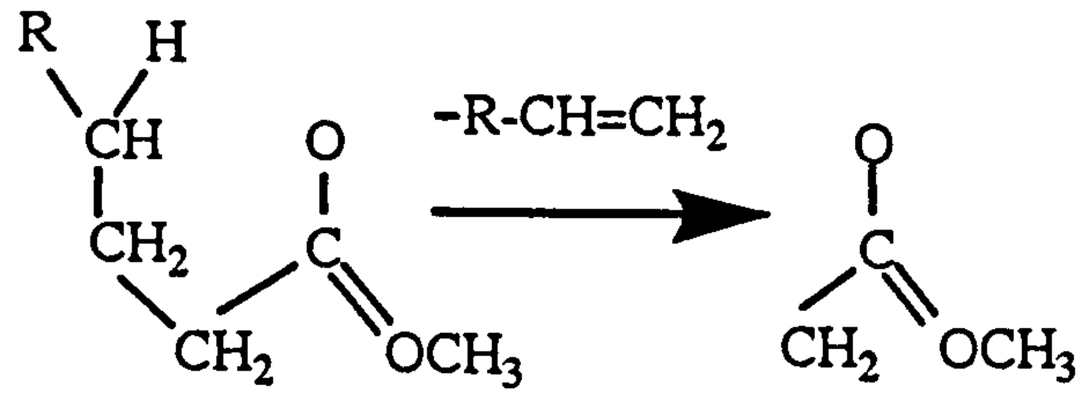
Mass spectra are obtained by bombarding the molecules of a compound in the vapour phase at low pressure with electrons of low energy. If the electrons have sufficient energy to cause ionisation the molecular or parent ion is formed:

$$M + e^- \rightarrow M^+ + 2e^-$$

Long chain saturated methyl esters are easily identified and characterised by this prominent molecular ion (M^+). This 'parent peak', has the highest m/e (mass to charge) ratio and provides the value of the molecular weight of the compound to within one unit. At greater electron energies fragment ions are produced which can be used to identify the type of fatty acid methyl esters (Chapman, 1965).

The guidelines for identifying only the types of fatty acid methyl esters that exist in the standard Fatty acid methyl ester CP mix (Method 2.29) are given below. For saturated fatty acid methyl esters, there were structurally significant ions at m/e M-31 (loss of methanol) and M-43 (loss of three CH2 groups and one H group), together with a series of ions of the general formula $CH_3O_2C.(CH_2)n^+$. The base peak at m/e = 74 is known as

the 'McLafferty rearrangement ion' is formed in a rearrangement reaction after cleavage of the parent molecule beta to the carboxyl group:



This characteristic McLafferty rearrangement ion allows fatty acid methyl esters to be recognised (Walker, 1972 and Christie, 1982 and 1989). Fig. 2.2 shows a mass spectrum for methyl stearate with its identifying peaks (Ryage and Stenhagen, 1960)

Mass spectra of methyl esters of unsaturated fatty acids differ from those of saturated compounds. Characteristic peaks occur at m/e = M-31 (loss of a methoxy radical) or M-32, m/e = M-74 (loss of the McLafferty rearrangement ion) and m/e = M-116 (Christie, 1989). Methyl esters of branched-chain fatty acids have mass spectra that are superficially similar to those of corresponding straight chain compounds. The hardest branches to identify are the iso and anteiso branches which commonly occur in *Streptomycetes*. However it is possible to distinguish between them. Branched chain methyl esters tend to have a peak at m/e = M-29 rather than M-31. Iso-branched chain methyl esters tend to have a small peak at m/e = M-65 and a very small doublet peak occurring at m/ e= M-50 and M-56. Anteiso-branched chain methyl esters can have small peaks at m/e = M-61, M-60 and M-79 (Christie, 1989). Hydroxy fatty acid methyl esters tend not to show a molecular ion peak but their molecular weight can be obtained from the peak at m/e = M-50 (loss of water plus methanol -[18+32]). This peak is significant in height except for 2-hydroxy esters which have a significant peak at m/e = M-59 which is formed due to the easy cleavage of the bond between carbon atoms 1 and 2 . Hydroxy acids can also have characteristic peaks at m/e 90 and 103 (Chapman, 1965). Cyclopropane esters are not readily distinguished from mono-unsaturated esters with similar numbers of carbon atoms by MS, apparently because on ionisation the cyclopropane ring opens to form such a monoenoic compound (Christie, 1982).

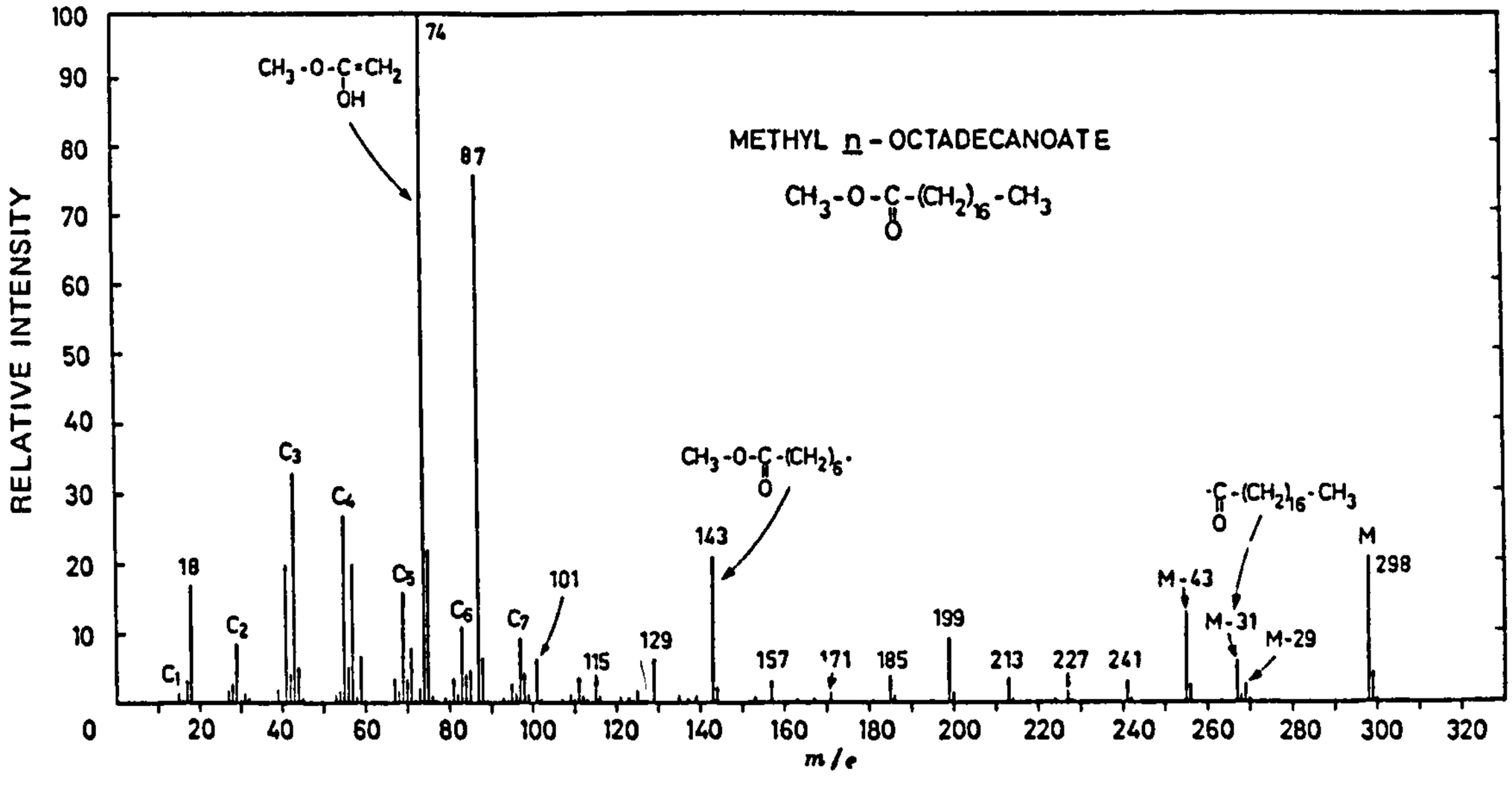


Fig. 2.2 Mass spectrum of methyl stearate (180°C, Dempster-type instrment, 70eV, 200 °C vapour inlet) [Ryage and Stenhagen, 1960].

2.31 LIPASE ASSAY

The routine assay used to detect lipase activity employed ρ-nitrophenyl palmitate as a substrate (Paznokas and Kaplan, 1977). ρ-nitrophenyl palmitate was dissolved in 1 ml of dimethyl formamide and then dispersed in 50 mM Tris-HCl, pH 8.5 containing 1% (v/v) Triton X-100, to give a final substrate concentration of 3.2 mM. This solution was heated above 60°C to ensure complete solubilisation of the substrate. Hydrolysis of the substrate ester bond by lipase results in the release of ρ-nitrophenol which is measured spectrophotometrically at 400 nm. Routinely the assay was carried out at 27°C with 100 µl of crude extract and 900 µl of substrate solution.

2.32 DETERMINATION OF KINETIC CONSTANTS

Kinetic data was analysed by the Lineweaver-Burk method using the Enzpack computer program (Williams, 1985).

Three different methods of preparing the substrate were employed when determining the kinetic constants of the lipase in both the intracellular and extracellular crude extracts:

a) Method 1

This method involved dissolving a range of concentrations of ρ-nitrophenyl palmitate (0.11 mM-6.4 mM) in 50mM KH_2PO_4, pH 8.5 buffer containing 0.9% Triton X-100.

b) Method 2

This method involved preparing ρ-nitrophenyl palmitate at different concentrations in assay buffer containing a corresponding range of Triton X-100 concentrations. In each assay the ρ-nitrophenyl palmitate to Triton X-100 ratio remained constant (1:1).

c) Method 3

This method involved dissolving a stock solution (6.4 mM) of ρ-nitrophenyl palmitate made up in assay buffer, containing 0.9% Triton X-100, in assay buffer containing 0.9%

Triton X-100, but no ρ-nitrophenyl palmitate, to give a range of ρ-nitrophenyl palmitate concentrations.

2.33 POLY ACRYLAMIDE GEL ELECTROPHORESIS

2.33.1 SDS-PAGE (discontinuous system)

The SDS-PAGE system used was that of Laemmli (1970) using a discontinuous Tris-glycine buffer system.

2.33.1.1 Preparation of gels

Stock solutions

Solution A : 3 M Tris-HCl, pH 8.5, 0.25% (v/v) TEMED

Solution B : (Running Buffer): 25 mM Tris (base), 192 mM glycine, pH 8.8, 0.1% (w/v) SDS

Solution C : 28% (w/v) acrylamide, 0.735% (w/v) N,N' methylene bisacrylamide (deionized with amberlite [0.1%, w/v] and filtered)

Solution C : 0.1 M Tris-HCl, pH 6.8. 0.8% (w/v) SDS, 0.25% (v/v) TEMED

Solution E : 20% (w/v) SDS

2.33.1.2 Gel plates

Glass plates (9.5 cm x 20 cm x 0.15 cm) were washed with Decon-90 to remove any silicone grease. The plates were assembled with 1.5 mm Teflon spacers and placed in a home-made gel making cast.

2.33.1.3 Separating gel (10% [w/v] acrylamide)

The main separating gel was prepared by mixing, 25 ml solution A, 71.5 ml solution C, 1 ml solution E and 99 ml distilled water (final volume 197.5 ml). The solution was degassed for 2 min in a 500 ml side arm flask with a vacuum pump. Solid ammonium persulphate (150 mg) was added to initiate polymerisation and the mixture was degassed for a further 2 min. Then it was poured into four plates to approximately 1.5 cm below the top edge of the plates. The edge of the gel was smoothed by adding a thin-layer of propan-2-ol on the top of the gel with a pasteur pipette immediately after pouring. After the gel was set, the top of the gel was washed with distilled water to remove the propan-2-ol and dried with a filter paper before pouring the stacking gel.

2.33.1.4 Stacking gel (5.2% [w/v] acrylamide)

The stacking gel was prepared by mixing 10 ml solution D, 17.5 ml solution C and 55 ml distilled water (final volume 82.5 ml). Polymerisation was initiated by adding 150 mg of solid ammonium persulphate, the mixture was degassed for 2 min and poured on the top of the separating gel. Teflon combs (4 x 18 track) were placed in the stacking gels before polymerisation had started.

2.33.1.5 Preparation of samples and electrophoretic conditions

Stock solutions

Solution F (Tracker dye) : To 10 ml of 0.5 M Tris-HCl, pH 8.8 were added 1.5 g SDS, 8 g sucrose and 100 mg Pyronin Y

Solution G : 0.2 M DTT

The samples were prepared by mixing protein (40 µl), 25 µl of solution G and 10 µl of solution F together in a 1.5 ml Eppendorf tube. The amount of protein per track was 10-50 µg. The samples were heated to 100°C in a boiling water bath for 2 min and then

loaded on to the gel. The samples were electrophoresed at 80 mA per gel until the dye front was approximately 0.2 cm up from the bottom of the gel. The gel was kept cool during electrophoresis by circulating ice cold water through the electrophoresis chamber.

2.33.2 Non-denaturing gels

2.33.2.1 Preparation of gels

Stock solutions

Solution A : same as for denaturing gel system (Methods 2.33.1.1)

Solution B : (Running buffer) : 25 mM Tris (base) and 192 mM glycine, pH 8.5

Solution C : same as for denaturing gel system (Methods 2.33.1.1)

The gel (5% [w/v]) were prepared by mixing 25 ml solution A, 36 ml solution C and 137 ml distilled water. Polymerisation was initiated by the addition of 150 mg solid ammonium persulphate. The solution was degassed for 2 min and poured into the casting box containing four plates. Teflon combs (4 x 18 track) were used to make wells for sample application.

2.33.2.2 Sample preparation and electrophoresis conditions

The samples containing 10-50 µg protein were mixed with 10 µl of 0.05% (w/v) Bromophenol Blue in 10% (v/v) glycerol. The samples were loaded on the gel and electrophoresed at 40 mA per gel until the dye front was approximately 0.2 cm up from the bottom of the gel. The gel was kept cool during electrophoresis by circulating ice cold water (4°C) through the electrophoresis chamber.

2.34 STAINING OF GELS

2.34.1 Protein staining

The gels were stained by immersing in 0.1% (w/v) Coomassie Brilliant Blue G 250 in methanol/ acetic acid/ H_2O (10:10:80, by vol.) for about 2 h at 60°C. Destaining was carried out using several changes of destain solution (methanol/ acetic acid/ H_2O [10:10:80, by vol.]) until bands were judged sufficiently differentiated.

2.34.2 Activity staining with β-naphthyl acetate

Activity staining of native gels was performed directly, but SDS containing gels were first washed by agitating for 2h on a shaker at slow speed in two changes of 500 ml of Tris-HCl, pH 8.5 at 23°C. The activity stain contained 0.2% (w/v) β-naphthyl acetate, 0.1% (w/v) Fast blue salt RR in 50 mM Tris-HCl, pH 8.5. The gels were kept in 100 ml of the staining solution at 37°C until stained bands developed (approximately 10 min). If there was any evidence of a coloured precipitate forming in the staining solution, it was replaced with fresh solution.

2.35 DETERMINATION OF Mr

a) HPLC

500 µl samples of concentrated culture supernatant were run on a PU 4100 Liquid Chromatograph HPLC (Phillips Ltd., Cambridge, UK.) using a TSK G2000 SWXL silica base column, at room temperature. The mobile phase was 50 mM KH_2PO_4 buffer, pH 6.5 with a flow rate of 500 µl min^{-1}. Protein was detected using a 820-FP Spectroflourimeter detecting tryptophan fluorescence at 290 nm/ 348 nm. 500 µl fractions were collected every minute and assayed for lipase activity (see Methods 2.31). Molecular weight standards were used to calibrate the column and Fig. 2.3 gives the molecular weight standard curve obtained.

b) AcA 34 Gel Filtration Column

3-4 ml of concentrated culture supernatant were run on a AcA 34 Gel Filtration Column, at 0°C. The running buffer used was 50 mM KH_2PO_4 buffer, pH 7.5 and the flow rate was 20 ml h^{-1}. 5.3 ml fractions were collected every 15 minutes and assayed for lipase activity (see Methods 2.31). Protein concentration was followed at 280 nm using an LKB Ultrospec II. The column was calibrated using standards of known molecular weight and Fig. 2.4 gives the molecular weight standard curve obtained.

2.36 GLASSWARE

Glassware was washed by immersion in a solution of approximately 1% Haemosol [Alfred Cox (Surgical) Ltd., Coulsdon, Surrey, UK.] according to the manufacturers instructions, and then rinsed thoroughly with tap water and then distilled water Glassware used in the inorganic phosphate assays was washed in a boiling solution of 10% (v/v) nitric acid for 30 minutes, then rinsed thoroughly in distilled water.

2.37 PHOTOGRAPHY

Photography was carried out by the members of staff at the Medical Illustration Unit, University of Glasgow.

2.38 SAFETY

Bacterial cultures were killed by autoclaving and all spillages of live bacteria were swabbed with 30% (v/v) propan-1-ol.

All other safety precautions followed, were as described in the University of Glasgow Safety Handbook (1992). From 1990 COSHH assessments were completed for all procedures involving potential hazards.

Fig. 2.3 DETERMINATION OF Mr

(a) HPLC

The molecular weight standard curve, used to calibrate the HPLC column (Methods 2.35), was constructed using the following standards:

Standard	Mr (KDa)
Tryptophan	0.204
RNase	13.7
Bovine serum albumin	66
Bovine serum albumin x 2	136

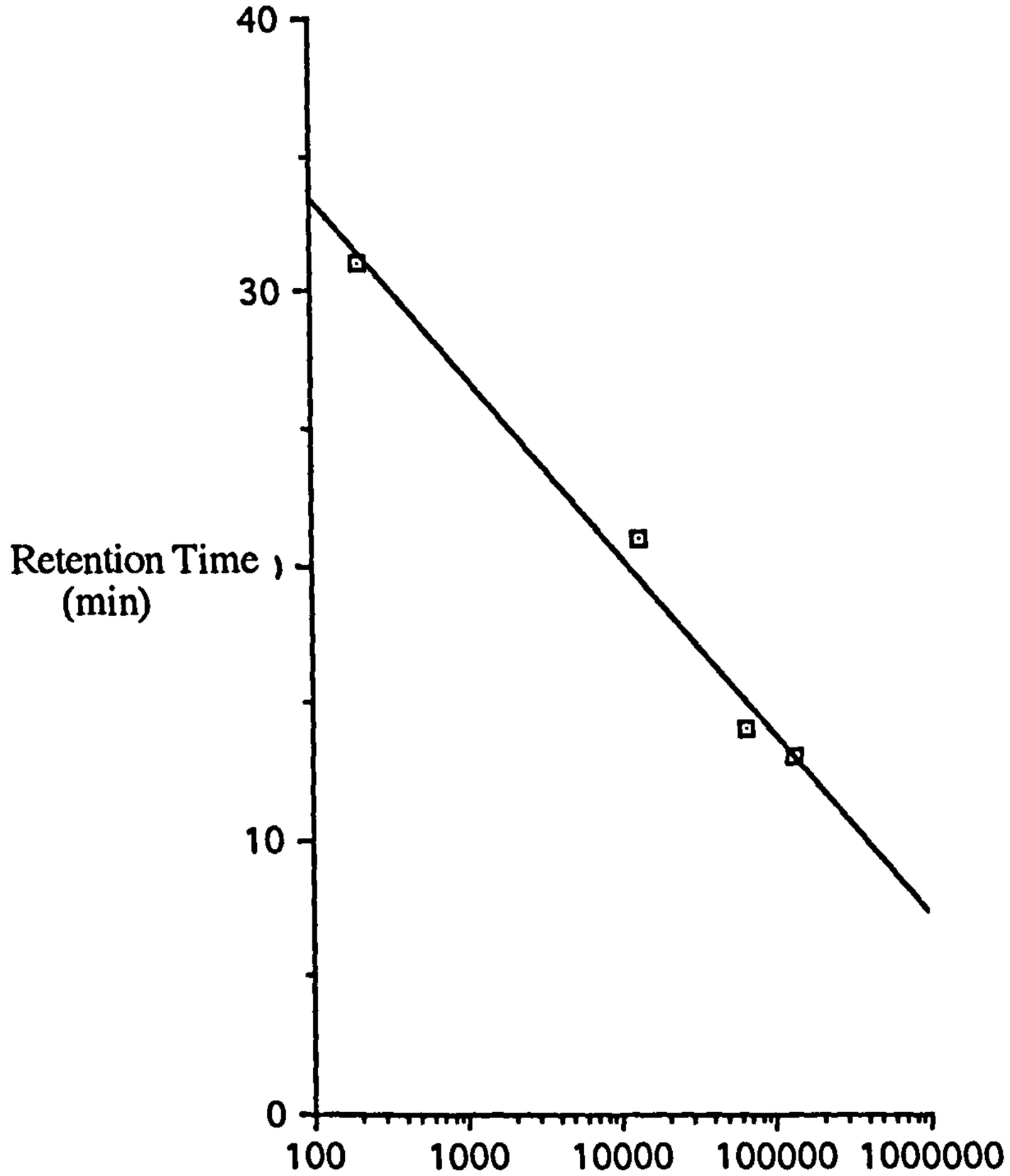
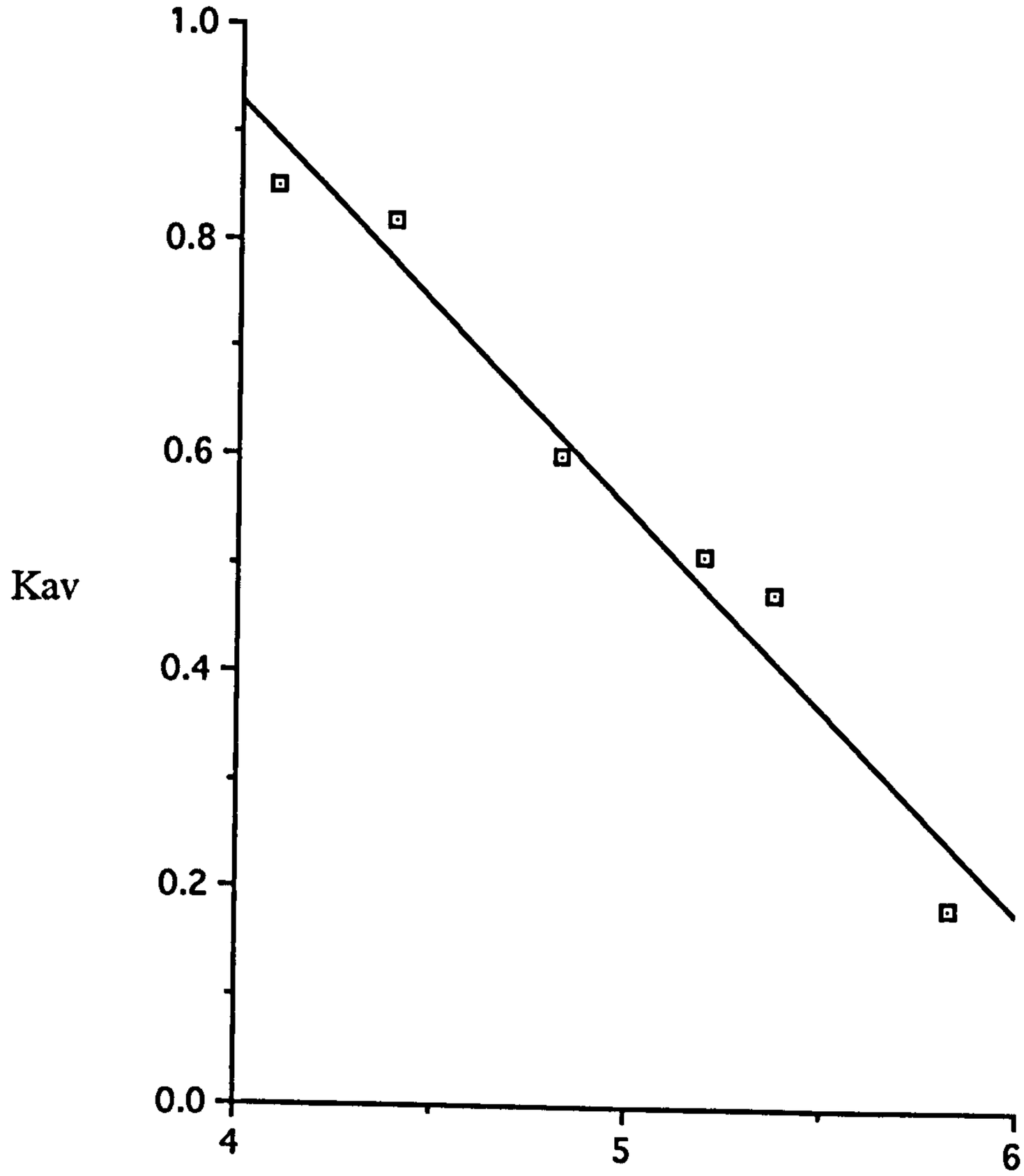

40
30
Retention Time
(min)
10
0
100
1000
10000
100000
1000000
Log Mr

Fig. 2.4 DETERMINATION OF Mr

(b) ACA 34 GEL FILTRATION COLUMN

The molecular weight standard curve, used to calibrate the ACA 34 Gel Filtration Column [Methods 2.35 (b)], was constructed using the following standards:

Standard	Mr (KDa)
Thyroglobulin	669
Catalase	240
Aldolase	158
Bovine serum albumin	67
Chymotrypsin A	25
Cytochrome C	12.5


1.0
0.8
0.6
0.4
0.2
0.0
Kav
4
5
6
Log Mr

2.39 STATISTICAL ANALYSIS

Data is presented as means ± standard deviation (SD). The SD was calculated from the formula:

$$SD = \sqrt{\sum (x-m)^2 / (n-1)}$$

where m = arithmetic mean of n observations

x = an observed value

n = number of observations

The significance of differences between means was determined using Student's t test. The value of 't' was calculated using:

$$\text{'t'} = \frac{m_1 - m_2}{\sqrt{(SEM_1)^2 + (SEM_2)^2}}$$

where m_1 and m_2 are means of two groups of observations. The SEM was calculated from the formula:

$$SEM = \frac{SD}{\sqrt{n}}$$

The probability (P) that the difference between two means was significant was obtained from the value of 't' and the number of degrees of freedom (n-1) by reference to a probability table. Differences were considered significant when P is < 0.05.

CHAPTER 3

GROWTH OF *STREPTOMYCES*

RESULTS AND DISCUSSION

3.0 GROWTH OF *STREPTOMYCES*

3.1 INTRODUCTION

Before undertaking studies on lipid metabolism in *Streptomyces,* it was important that reproducible results were obtained from standard growth experiments. Only then could the carbon energy source be supplemented or replaced by lipids, and the enzymes involved in lipid metabolism studied. Unfortunately one of the major problems in studying *Streptomyces* is their filamentous growth habit which results in difficulties in both determining growth rate and obtaining homogenous samples of biomass.

In shaken liquid cultures, *Streptomyces* grow in pellet form. These pellets are composed of densely interwoven hyphae and are generally spherical in shape (Hobbs *et al.*, 1989). Lawton *et al.*, (1989) studied the morphology of forty-nine *Streptomyces* strains in liquid shake cultures and observed a great variety of morphological forms including compact pellets, spiky or fluffy pellets, oblong pellets, multi-pellets and flakes. Growth of cultures containing pellets usually follows cube root kinetics because nutrients can only diffuse into large (> 2mm in diameter) pellets fast enough to maintain growth of the peripheral shell of the pellet (Marshall and Alexander, 1960). Thus, cells at the centre of the pellets become nutrient limited and as the pellet increases in size, growth is eventually confined to a shell of limited thickness at the surface of the sphere. This filamentous growth habit makes it difficult to study *Streptomyces* as it results in cultures containing pellets composed of mycelia of various ages, nutritional status and stages of development (Doull and Vining, 1989). In addition to difficulty in determining growth rates, problems arise in studying secondary metabolism as the switch from primary to secondary metabolism is often triggered by nutrient limitation.

A number of methods have been employed in achieving dispersed growth in cultures of *Streptomycetes*. The diffuse growth of certain selected strains was achieved by the addition of polyethylene glycol (PEG) and the use of stainless steel springs in the culture flasks (Hodgson, 1982). High concentrations of PEG increased the viscosity of the medium and this was thought to aid dispersal. This is similar to the use of high concentrations of sucrose, to aid dispersion, in the complex YEME medium (Hopwood *et al.*, 1985). Doull and Vining, (1989) found that the inclusion of glass beads in the culture flask caused better dispersal of the mycelium and avoided the foaming which sometimes occurred with springs. A problem resulting from the use of dispersal agents is the possible cell wall damage, caused by either the abrasive or shearing action of these agents. This could lead to difficulties when determining whether enzymes are intracellular or extracellular, because of leakage through damaged cell walls. This phenomenon of pellet formation is also seen in liquid cultures of fungi, the other major group of filamentous microorganisms. Well dispersed growth in fungi has been achieved by the incorporation of high molecular weight polyanions into their growth media (Trinci, 1983 and Jones *et al.*, 1988). Not only did dispersed growth allow easier determinations of growth rates but it also affected product formation. *Aspergillus niger* grows faster and produces more amylase as filamentous dispersed mycelia than when grown as pellets. Therefore, more recently the problem of pellet formation in *Streptomyces* has been overcome by the incorporation of a high molecular weight polyanion into the growth media (Hobbs *et al.*, 1989). A number of polymers were studied for their ability to produce dispersed growth in *S. coelicolor* and the most efficient was a negatively charged, polyacrylate known as Junlon PW110 (normally used as a paint thinner). It is thought that Junlon is absorbed to the surface of spores and hyphae and acts by causing electrostatic repulsion between the spores and thus preventing aggregation of spores and the subsequent clumping of mycelia in the growing culture. The subsequent reduction in pellet size and clumping resulted in an overall increase in biomass yield and product formation in *S. coelicolor* (Hobbs *et al.*, 1989).

Growth rates of unicellular microbes are usually determined by measurements of culture turbidity and cell counts but these methods do not prove accurate enough when following the growth rates of filamentous microorganisms. An alternative to these methods are gravimetric determinations but these are laborious and lack accuracy at low concentrations. In many fermentations of filamentous microorganisms biomass dry weight continues to slowly increase even after the cessation of growth (Martin and Demain, 1980). Non-replicatory growth usually results from the accumulation of reserve materials which can form up to 50% of the dry weight at the end of fermentation. Biomass dry weight is therefore a poor measurement of true growth in *Streptomyces.* One of the best parameters with which to measure true replicatory growth is the increase in deoxyribonucleic acid (Martin and McDaniel, 1975). However, Flowers and Williams, (1977) showed that growth rates of filamentous actinomycetes could be accurately determined from turbidity measurements, providing that reasonably dispersed growth occurred, by selecting an appropriate medium, culture conditions and inoculum size.

Therefore the aims of this chapter are to explore the selection of an appropriate medium, culture conditions, organism and inoculum size and to demonstrate that reproducible results could be achieved from standard growth experiments.

One of the most economic points in the fermentative production of antibiotics is the raw material used in the formulation of the fermentative media (Abouzeid and Baeshina, 1992). Growth on lipids, either as sole carbon source or as a supplement to glucose, was attempted. The use of triglycerides as substrates did not prove successful and therefore the water- soluble Tweens were looked at as possible substrates.

3.2 APPROPRIATE MEDIUM

The majority of the media used for growing *Streptomyces* have been formulated during investigations of antibiotic production and these were not necessarily the most appropriate media for the use in the study of the biochemical properties of the organism. The medium used in this study was based on the media developed by Hobbs *et al.*, (1989) to enable them to study the effects of nutrient limitation on the transition from primary to secondary metabolism in *Streptomyces.* A number of alterations were made to this medium including; switching the nitrogen source from nitrate to ammonium, the introduction of MOPS buffer for pH control and a reduction in the amount of magnesium added.

3.2.1 NITROGEN SOURCE

HMM, as originally formulated, contains sodium nitrate as the sole nitrogen source. *S.coelicolor* 1147 grows more rapidly in media containing ammonia than in media with nitrate as sole nitrogen source, but actinorhodin production is extremely sensitive to inhibition or repression by ammonium (Hobbs *et al.*, 1989b). As HMM was designed for the purpose of studying aspects of secondary metabolism, sodium nitrate was employed as the nitrogen source to allow the production of actinorhodin. In this study the production of actinorhodin was not necessary. The production of biomass was important, therefore three nitrogen sources; ammonium sulphate, ammonium nitrate and sodium nitrate were looked at for their ability to support good growth of *S. coelicolor* 1147 and *S. rimosus* 4018. Three concentrations of each nitrogen source were used; 20 mM, 2 mM and 0.2 mM. The medium used was HMM containing Junlon and the cultures were inoculated with fresh spores. Cultures were grown in 250 ml conical flasks containing 100 ml medium at 30°C on an orbital shaker. Growth was followed by measuring optical density at 450 nm (Figs. 3.1 and 3.2.). These experiments were never intended to be more than quantitative. They were to demonstrate which of the chosen nitrogen sources and at which concentration

would allow cells to grow to a yield sufficient for biochemical analysis on the biomass generated at a reasonable growth rate. It is clear from these results that the most effective nitrogen source for growth of *S. rimosus* proved to be 20 mM ammonium sulphate, which produced a culture with a reasonable doubling time (~5.5 h) and a good yield of biomass (OD of ~6 at 450 nm)[Fig. 3.1 (a), Table 3.1). Neither ammonium nitrate (Fig. 3.1 [b], Table 3.1) nor sodium nitrate (Fig. 3.1 [c], Table 3.1) gave as good results. Ammonium nitrate supported a comparable growth rate but the yield was poorer, while the sodium nitrate permitted both a slow growth rate and a poor yield (Table 3.1). The data also indicated that the lower concentrations of ammonium sulphate might be imposing a nitrogen limitation on the culture and this was considered undesirable at this stage in the development of a good growth medium. Despite the presence of Junlon, the growth curves obtained from *S. coelicolor* were erratic and difficult to follow. *S. coelicolor* showed very poor growth with doubling times of seventeen hours or more and absorbances, at 450 nm, reaching values of less than 1.6 (Table 3.2). Nevertheless the results were similar to those of *S. rimosus* with the presence of ammonia resulting in a more rapid growth rate and higher yields. As a consequence of these results the nitrogen source chosen for subsequent experiments was 20 mM ammonium sulphate. The optical densities decreased at the end of growth and this was thought to be as a result of changes in the structure of the pellets. Even with the alternative nitrogen source the conditions for growth and the results achieved were far from ideal.

3.2.2 BUFFERING SYSTEM

In the original HMM, the buffers employed were Tris (pKa 8.1) and phosphate which buffered the increase in pH due to the utilisation of nitrate which in effect leads to the production of base. The use of a different nitrogen source, ammonium sulphate, resulted in a decrease in pH from 7.2 to 6.1 in the course of growth due to the production of acid. This pH decrease was worrying because the growth of *S. rimosus* might be affected by the

ten fold change in hydrogen ion concentrations. In addition to this the acidic conditions could also be detrimental to the production of lipolytic enzymes. A number of buffers were investigated to determine their suitability in counteracting this decrease and MOPS (pKa 7.2) was chosen as it has been shown with both *Escherichia coli* and *Erwinia carotovora* to be effective at reducing the pH change due to acid production and has little effect on growth [Dr. I.D.Hamilton, unpublished results]. Cultures were grown in one litre side-arm flasks, containing 800 ml medium, at 30°C, on a stirrer. 5 ml samples were taken at various time intervals and DNA concentration (μg/ml) and pH were measured. Fig. 3.3 shows the growth (followed by DNA concentration) and pH changes in *S. rimosus* cultures grown on modified HMM containing different concentrations of MOPS. Although in all cases the cells pH falls as the cells grow the rate of decline is reduced by the addition of MOPS. The addition of 10 mM MOPS prevented the pH from decreasing below pH 6.6 but the addition of 40 mM MOPS proved even more effective at buffering with the pH remaining around pH 7.0. There was no detrimental effect on the growth rate and the final yields of *S. rimosus* resulting from the inclusion of MOPS buffer.

3.2.3 MAGNESIUM ION CONCENTRATION

One of the problems of the original HMM was the precipitation of ions which occurred shortly after stirring of the medium began. The resulting cloudy solution caused problems as it was difficult to follow growth spectrophotometrically. This problem was overcome by reducing the magnesium salt concentration, from 4 mM to 1 mM. This alteration had no detrimental effect on the growth rate and final yield of *S. rimosus*.

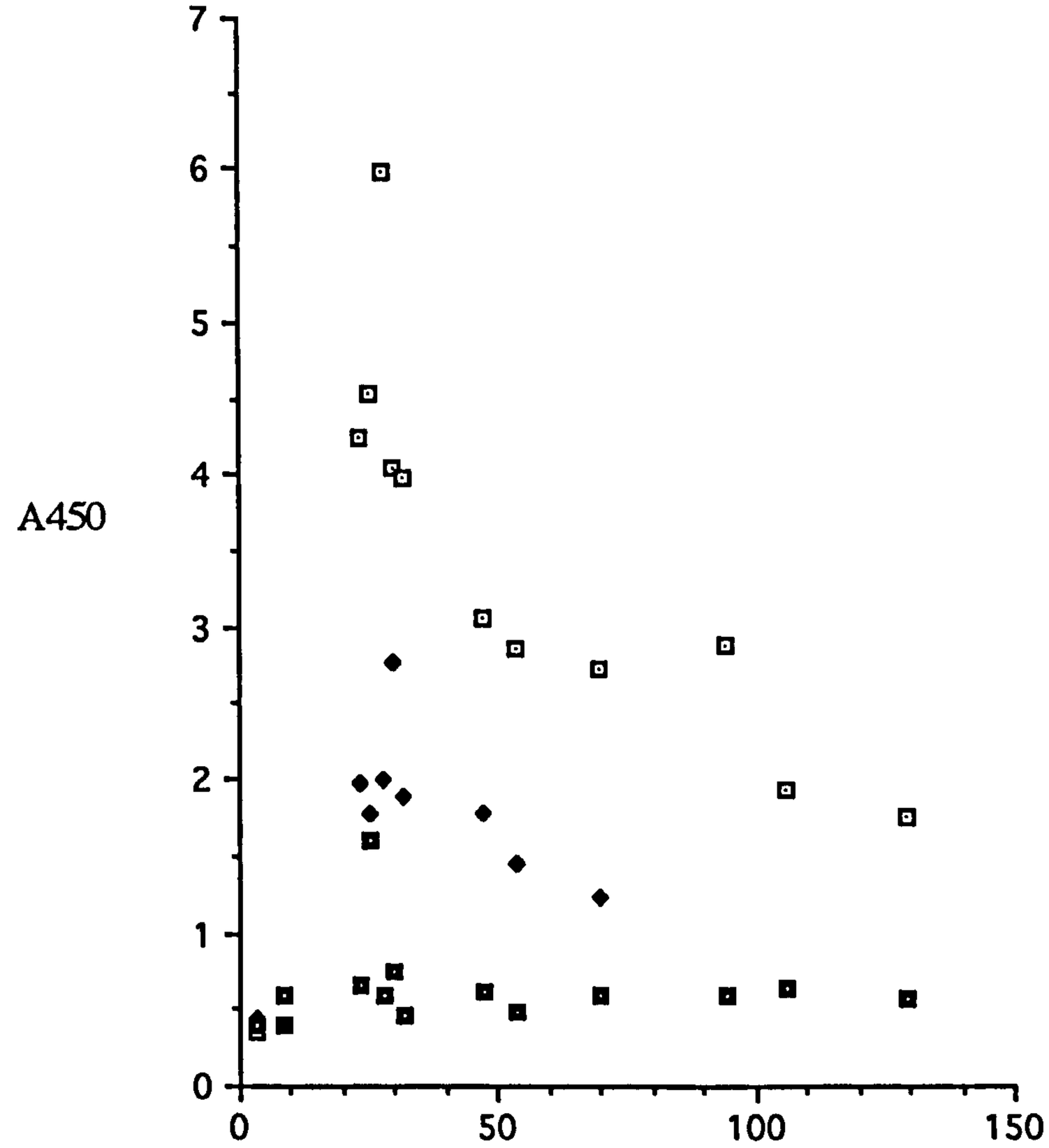
Fig. 3.1 THE EFFECT OF NITROGEN SOURCE ON *S. RIMOSUS*

Effect of the nitrogen source on cultures of *S.rimosus* grown in HMM, containing Junlon. Cultures were grown in 250 ml conical flasks, containing 100 ml of medium, at 30°C on an orbital shaker. Growth was followed by measuring optical density at 450 nm (Methods 2.6). Three nitrogen sources were examined:

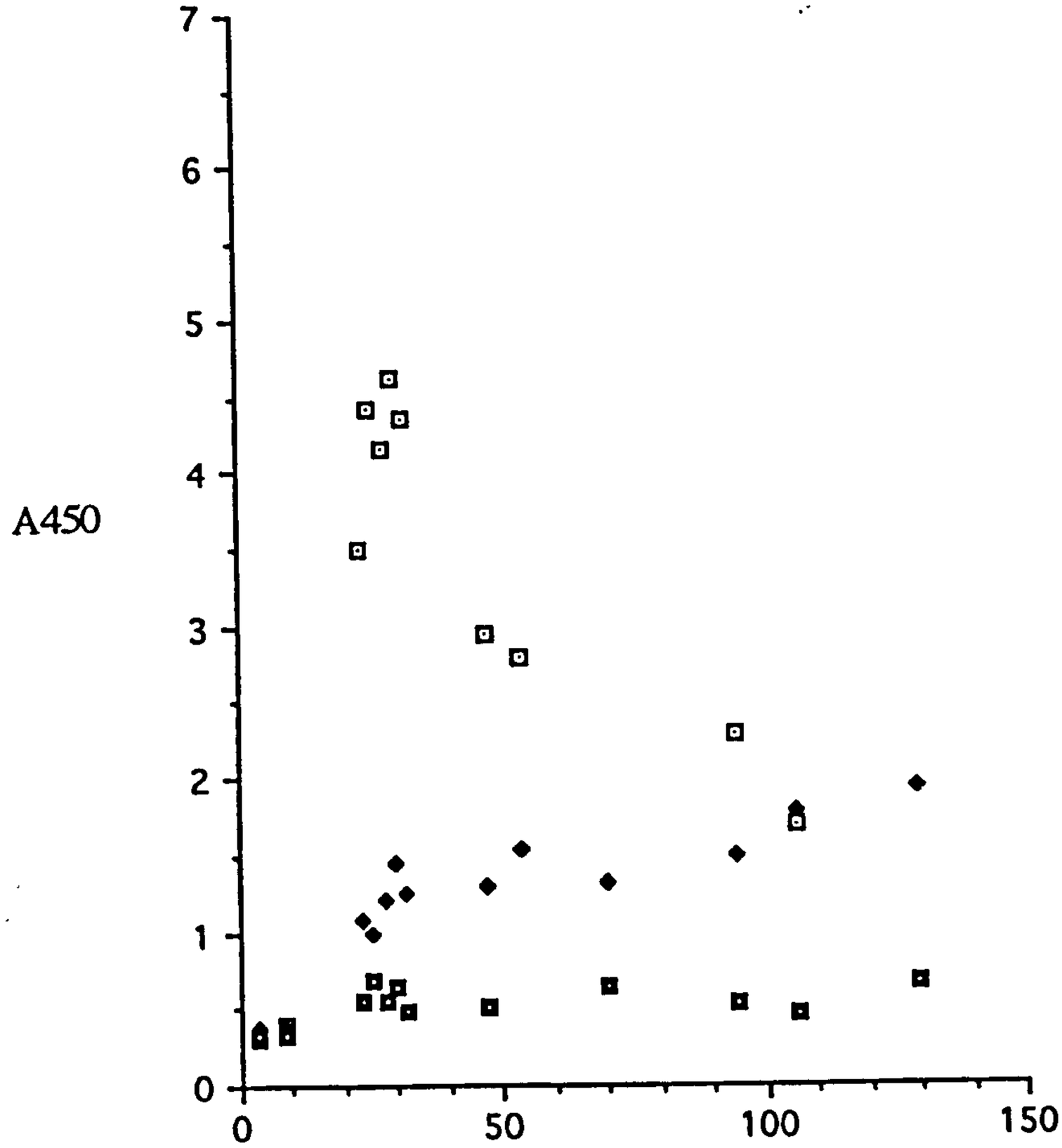
a) Ammonium sulphate: 20 mM 2 mM 0.2 mM

b) Ammonium nitrate: 20 mM 2 mM 0.2 mM

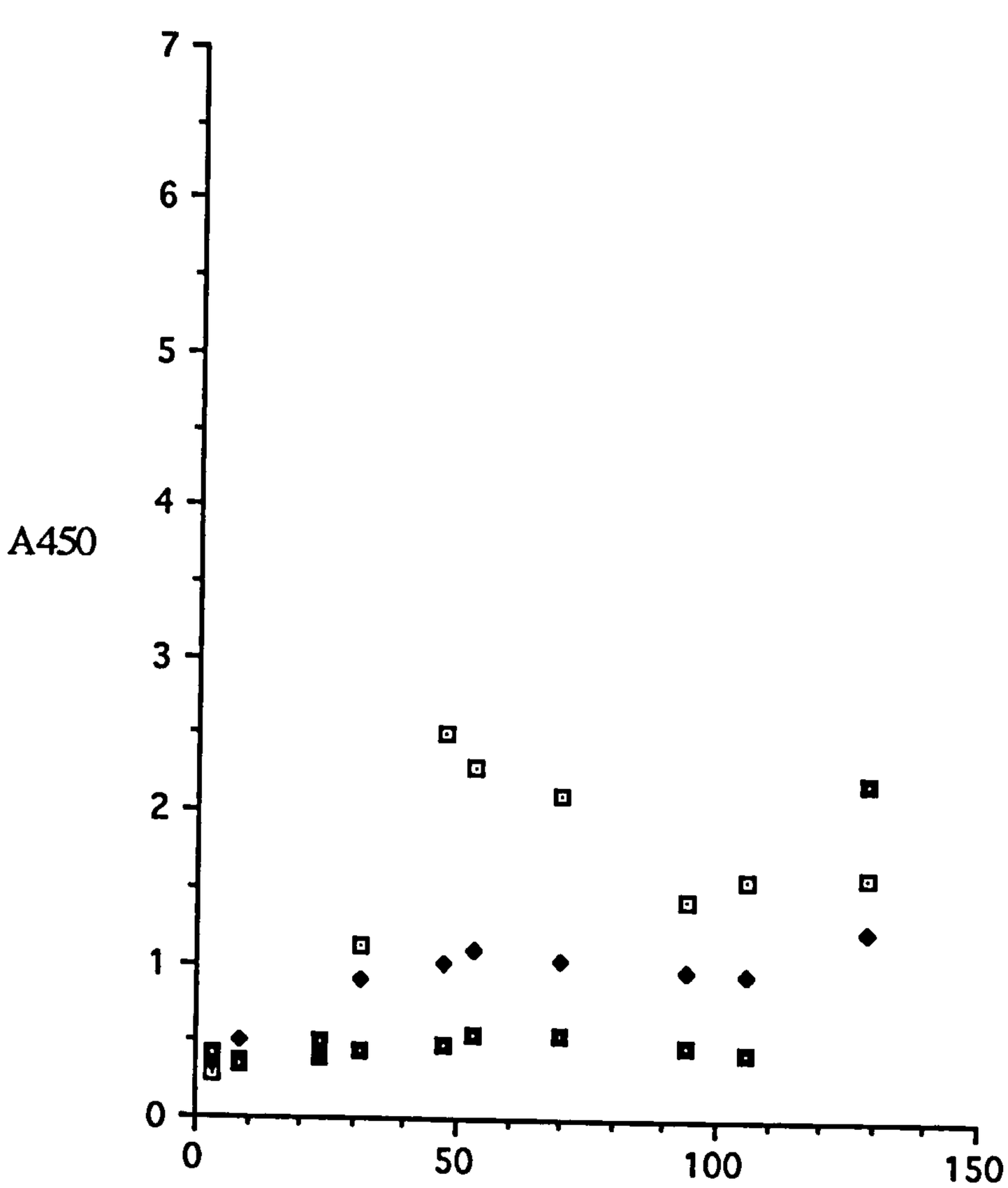
c) Sodium nitrate: 20 mM 2 mM 0.2 mM



(a)



(b)

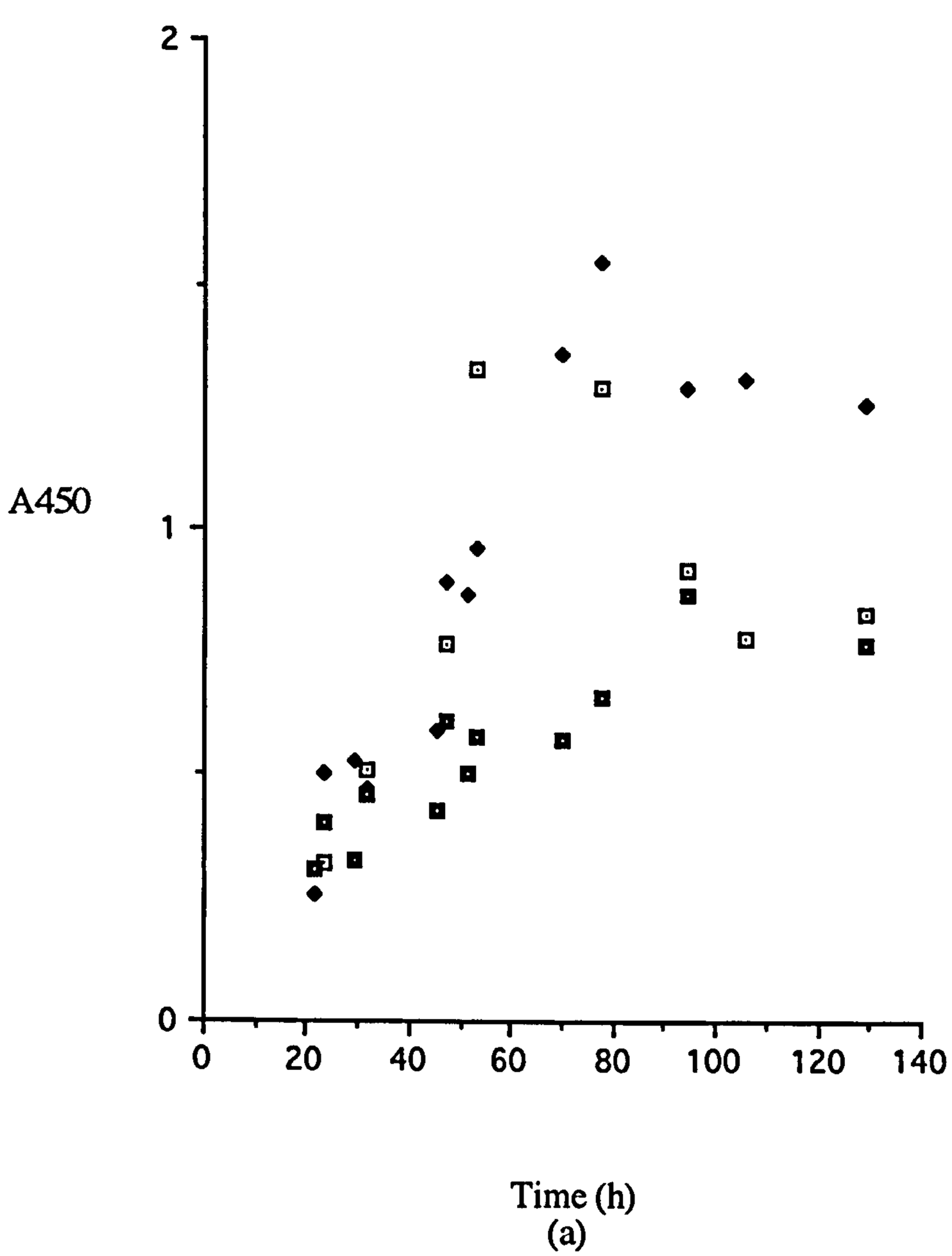


(c)

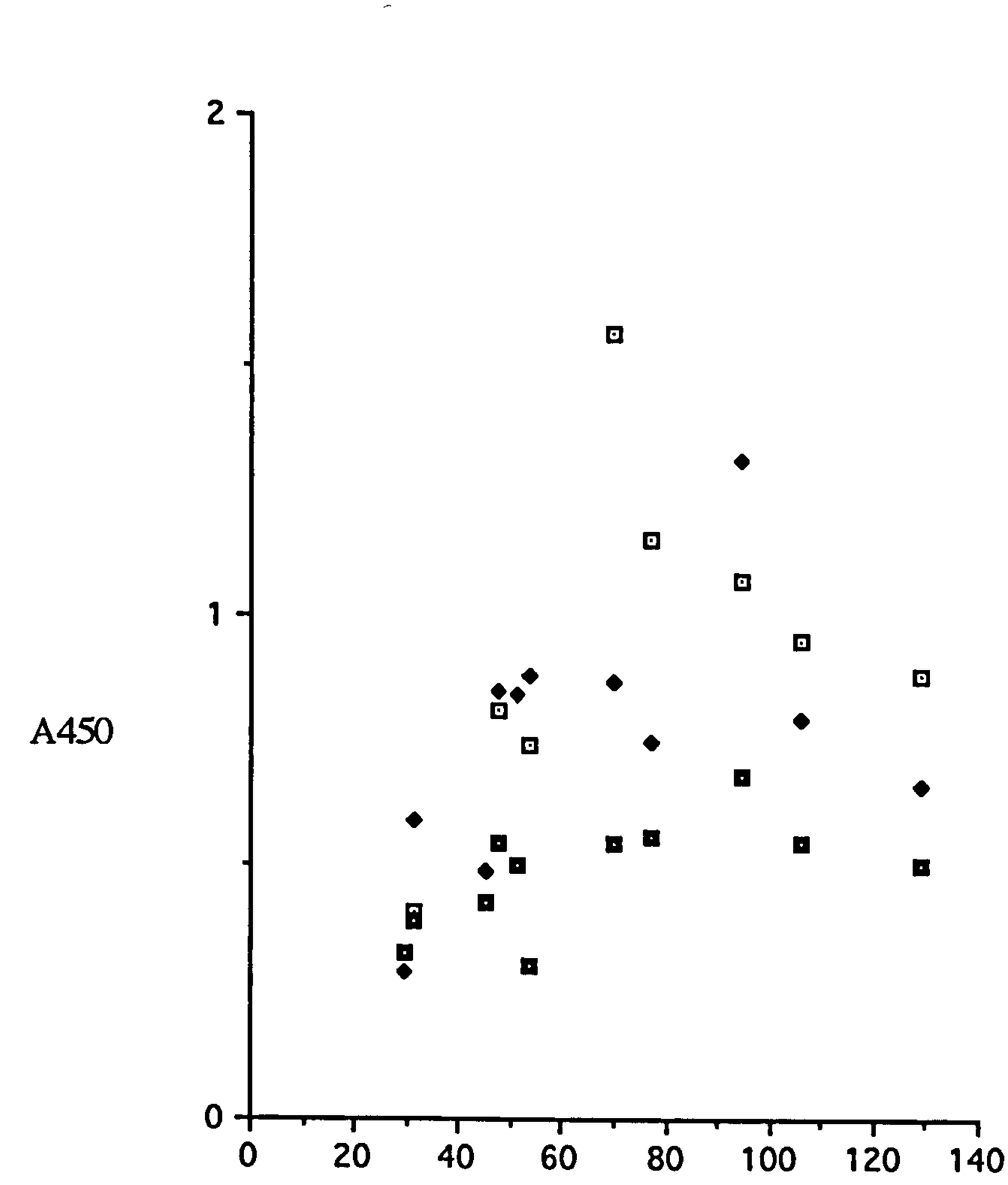
Fig. 3.2 THE EFFECT OF NITROGEN SOURCE ON *S. COELICOLOR*

Effect of the nitrogen source on cultures of *S.coelicolor* grown in HMM, containing Junlon. Cultures were grown in 250 ml flasks, containing 100 ml of medium, at 30°C on an orbital shaker. Growth was followed by measuring optical density at 450 nm (Methods 2.6). Three nitrogen sources were examined:

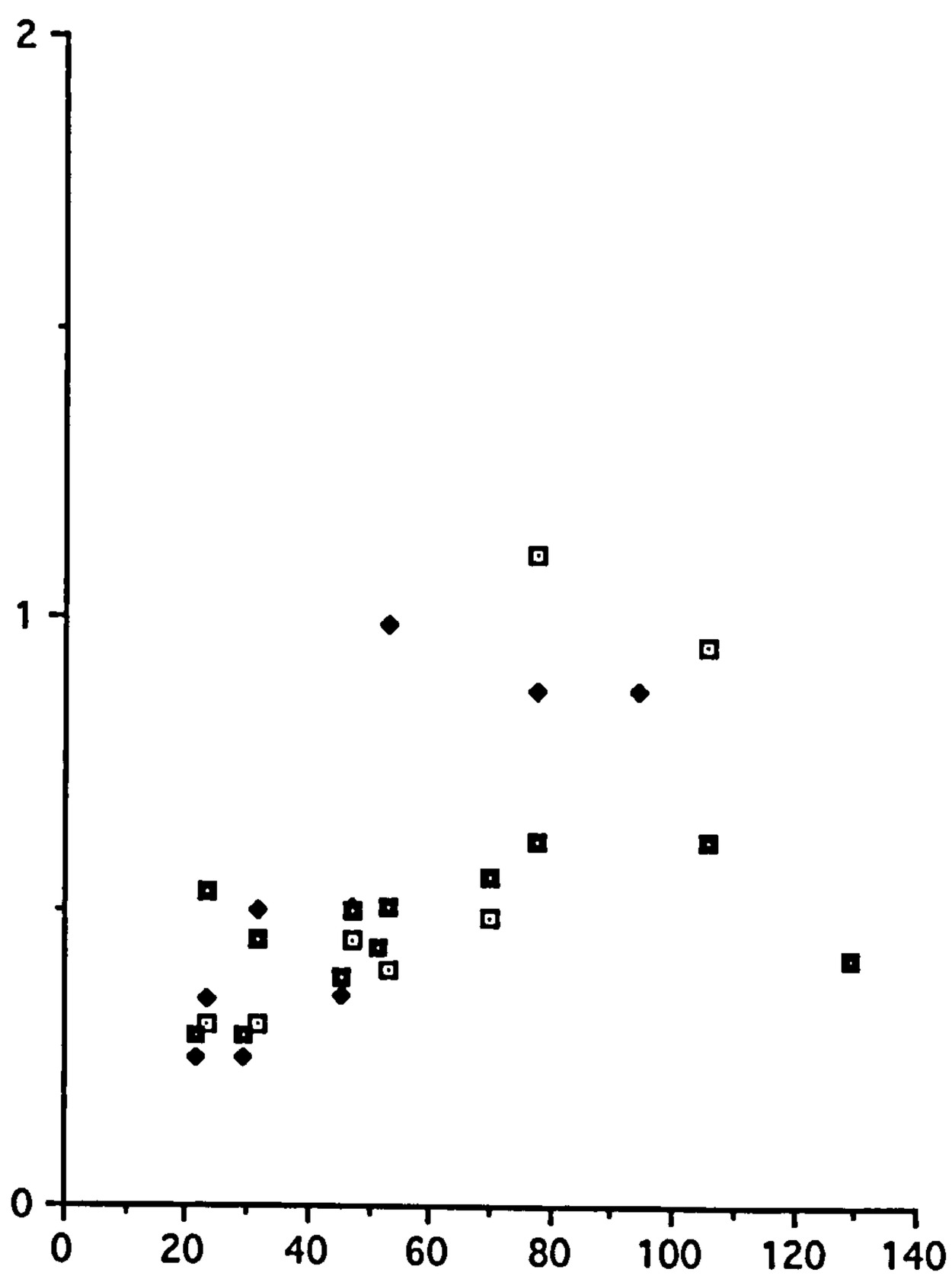
a) Ammonium sulphate	20 mM	2 mM	0.2 mM
b) Ammonium nitrate	20 mM	2 mM	0.2 mM
c) Sodium nitrate	20 mM	2 mM	0.2 mM



(a)



(b)



(c)

Table 3.1 THE EFFECT OF NITROGEN SOURCE ON *S. RIMOSUS*

Effect of the nitrogen source on cultures of *S.rimosus* grown in HMM, containing Junlon [ref. Fig. 3.1 a), b) and c).

a) Mean Generation Time (h)

b) Maximum Optical Density, measured at 450 nm (Methods 2.6).

Nitrogen source	M.G.T. (h) 20mM	2mM	0.2mM
a) Ammonium sulphate	5.5	13.5	>50.0
b) Ammonium nitrate	6	11.5	24.5
c) Sodium nitrate	22.5	37.5	>50.0

(a)

Nitrogen source	Maximum A450 20mM	2mM	0.2mM
a) Ammonium sulphate	5.98	2.78	1.61
b) Ammonium nitrate	4.62	1.54	0.68
c) Sodium nitrate	2.52	1.11	0.56

(b)

Table 3.2 THE EFFECT OF NITROGEN SOURCE ON *S. COELICOLOR*

Effect of the nitrogen source on cultures of *S.coelicolor* grown in in HMM, containing Junlon [ref. Fig. 3.2 a), b) and c).

a) Mean Generation Time (h)

b) Maximum Optical Density, measured at 450 nm (Methods 2.6).

Nitrogen source	M.G.T. (h) 20mM	2mM	0.2mM
a) Ammonium sulphate	20.5	17.5	>50
b) Ammonium nitrate	20	20.5	37.5
c) Sodium nitrate	>50	>50	>50

(a)

Nitrogen source	Maximum A450 20mM	2mM	0.2mM
a) Ammonium sulphate	1.33	1.55	0.87
b) Ammonium nitrate	1.11	0.99	0.62
c) Sodium nitrate	1.56	1.31	0.68

(b)

Fig. 3.3 THE EFFECT OF MOPS BUFFER

The effect of MOPS buffer on growth and pH of cultures of *S.rimosus,* when added to modified HMM. Cultures were grown in 1 litre side-arm flasks, containing 800 ml of medium, at 30°C on a stirrer at 300 rpm. Growth was followed by measuring DNA concentration using Burtons reagent (Methods 2.17). pH was measured according to Methods 2.14.

a) Tris buffer control	Growth curve:	Time (h) v DNA (µg/ml)	
	pH curve:	Time (h) v pH	
b) 10 mM MOPS	Growth curve:	Time (h) v DNA (µg/ml)	
	pH curve:	Time (h) v pH	
c) 40 mM MOPS	Growth curve:	Time (h) v DNA (µg/ml)	
	pH curve:	Time (h) v pH	

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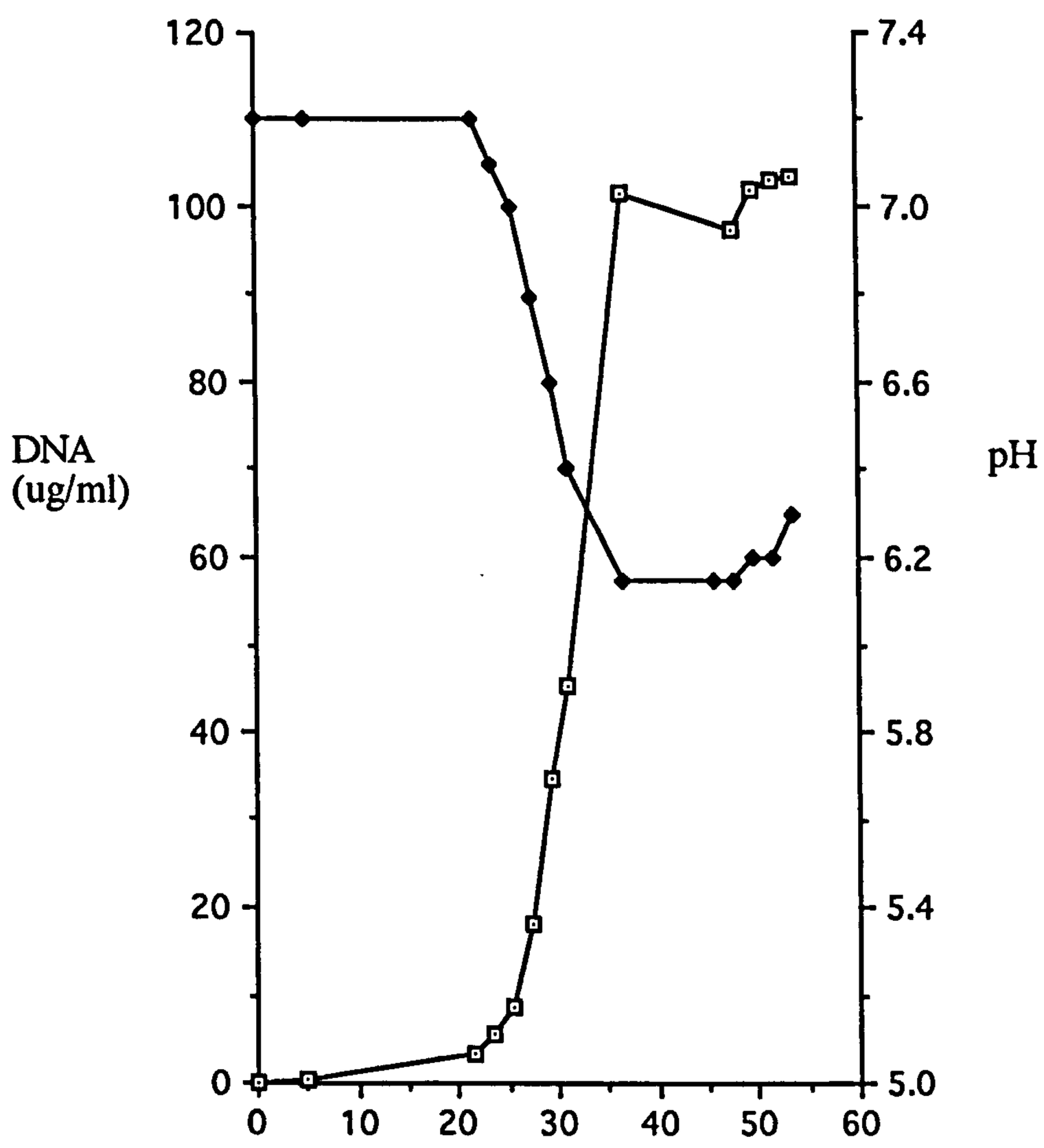
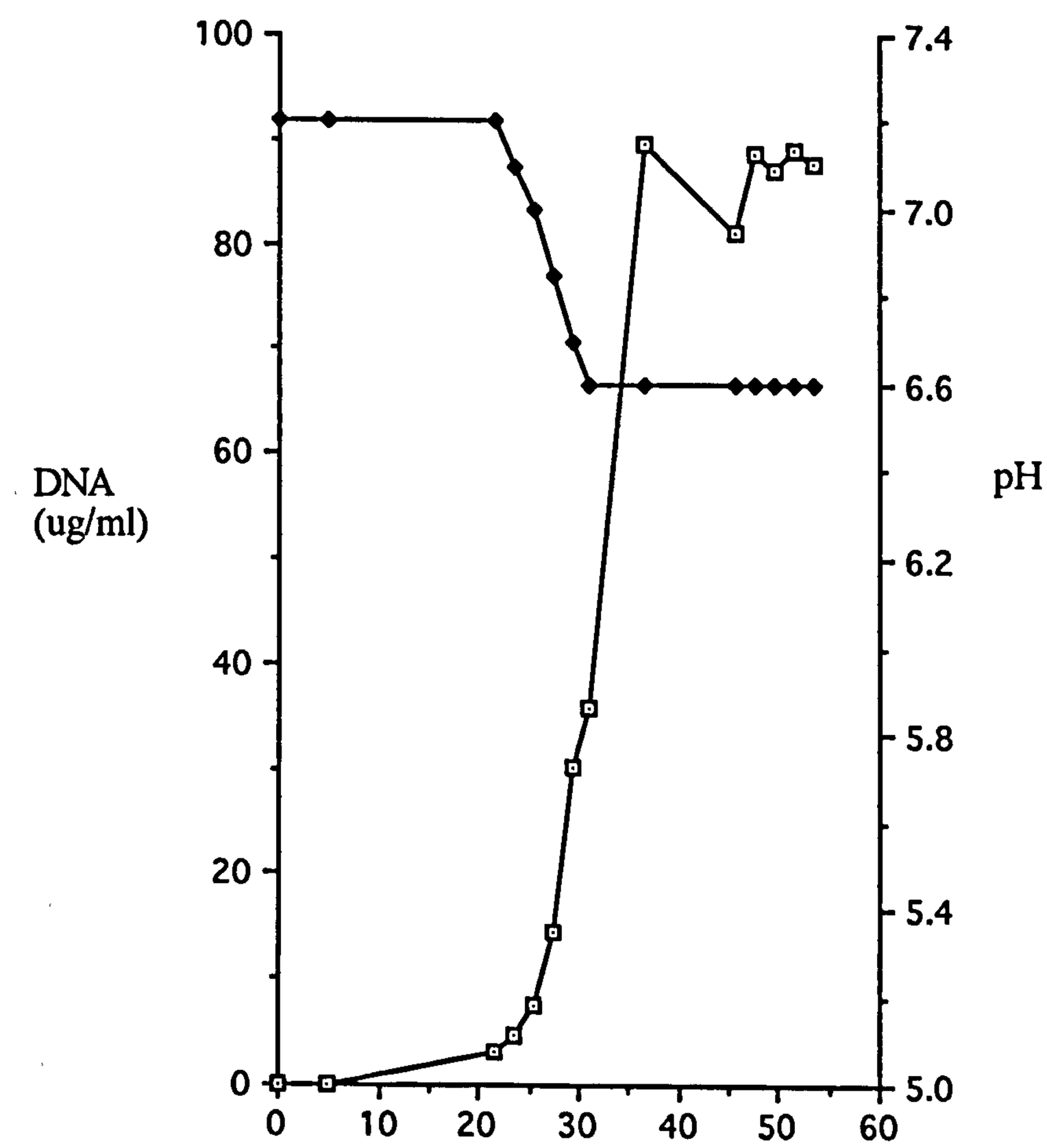
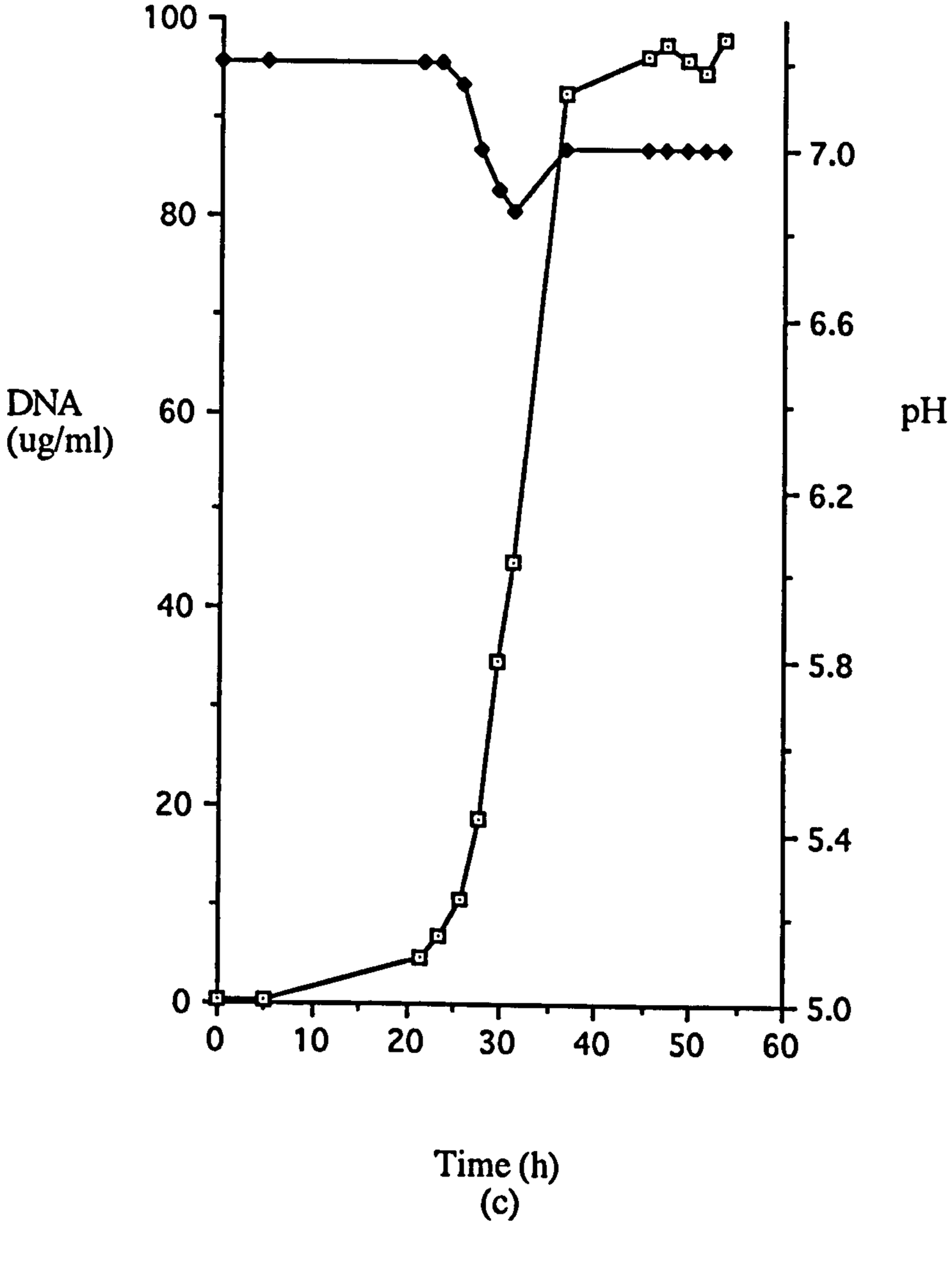

120
100
80
60
40
20
0
DNA
(ug/ml)
7.4
7.0
6.6
6.2
5.8
5.4
5.0
pH
0
10
20
30
40
50
60
Time (h)

(a)


100
80
60
40
20
0
DNA
(ug/ml)
7.4
7.0
6.6
6.2
5.8
5.4
5.0
pH
0
10
20
30
40
50
60
Time (h)

(b)


100
80
60
40
20
0
DNA
(ug/ml)
7.0
6.6
6.2
5.8
5.4
5.0
pH
0
10
20
30
40
50
60
Time (h)

(c)

3.2.4 INCLUSION OF JUNLON IN THE GROWTH MEDIA

At the beginning of this study, Junlon was routinely used as a dispersal agent in growth experiments as it is virtually impossible to follow growth by optical density in cultures containing pellets. Unfortunately it is impossible to wash the *Streptomyces* biomass completely free of Junlon. This proved to be a problem when extracting lipids from the bacterial pellets as the presence of Junlon resulted in an artificial increase in wet weight. Typically 1 to 2 g of biomass were harvested from cultures grown in HMM, without Junlon present. But in the presence of Junlon, pellets were harvested weighing up to 30 g. The volume of solvents added, when extracting lipids, is dependant on the wet weight of the bacterial pellet. Therefore this increased weight of the pellets, containing Junlon, meant that impractical volumes of solvents would have to be used. A further disadvantage is that mycelia harvested from media containing Junlon cannot be stored frozen because the pellets have a sickly, glue like consistency once thawed. It was therefore decided not to include Junlon in the growth media. An additional factor which was taken into account in making this decision was the possible complication of assaying intracellular and extracellular lipase activities due to the presence of Junlon. Thus an alternative method of obtaining dispersed growth had to be found (see section 3.2.6).

3.2.5 SELECTION OF ORGANISM

Streptomyces coelicolor A3(2) is one of the most widely studied actinomycetes because it is genetically the best characterised *Streptomycete* (Hobbs *et al.*, 1989). Hence it was decided to use *S. coelicolor 1147* (Glasgow definition) which is an isogenic strain derived from the wild type of *S. coelicolor* A3(2). However, despite being the organism of choice for genetic experiments the physiology of the organism remains relatively poorly defined (Hobbs *et al.*, 1989b). Progress has been slow and this is probably due to the difficulties in culturing *S. coelicolor* in chemically defined media. It was decided that *S. coelicolor* was

not a suitable organism for this study because of its poor growth rates, low biomass yields and the need for dispersants to achieve homogenous growth. Therefore an alternative species was chosen, which was an oxytetracycline producing strain of *Streptomyces.* Fig. 3.4 compares growth curves from *S. coelicolor* (a) and *S. rimosus* (b) grown under identical conditions. Cultures were grown in one litre side-arm flasks in 800 ml of HMM, containing 1.5 g l^{-1} Junlon, with 20 mM ammonium sulphate as nitrogen source, at 30°C, on a stirrer. Cultures were inoculated with fresh spores; 2 ml and 1 ml inoculums of 8 x 10^6 spores ml^{-1} for *S. rimosus* and 14 x 10^6 spores ml^{-1} in the case of *S. coelicolor*. The results shown in Fig. 3.4 demonstrate that *S. rimosus* was a more suitable candidate for further study. *S. rimosus* reaches stationary phase within 25 h, compared with the 60 h that it takes for *S.coelicolor* to reach stationary phase. *S .rimosus* grew with a MGT of 7 h and reached a maximum absorbance of 8.0 compared with a MGT of 25 h and a maximum absorbance of 1.8 for *S. coelicolor.* Further the final yield of biomass for *S.rimosus* is independent of the size of the inoculum suggesting that particle number or size is not important when this species is used. An additional reason for selecting *S. rimosus* is that there are reports of lipid utilisation by this species (Orlova, 1961 and 1962), and therefore in addition to analysing the lipid content of the organism, the utilisation of lipids could be studied.·

3.2.6 CULTURE CONDITIONS

Comparisons were made between *S. rimosus* cultures grown, in modified HMM in shaken and stirred flasks. The shaken cultures were grown in 500 ml conical flasks containing 200 ml of media, at 30°C, on an orbital shaker, at 180 rpm. Similarly the stirred cultures were grown in 500 ml flasks containing 200 ml of media, at 30°C, on a magnetic stirrer, at 300 rpm. Samples were taken from both and cultures slides were prepared and examined under a Leitz Ortholux II microscope, with camera attachment, using Kodak ASA 400 film (Leica, UK.). Fig 3.5 shows the photographs of these slides; (a) shaken cultures and (b) stirred cultures.

Fig. 3.4 GROWTH CURVES FROM *S. RIMOSUS* AND *S. COELICOLOR*

Growth curves followed by optical density, measured at 450 nm (Methods 2.6).

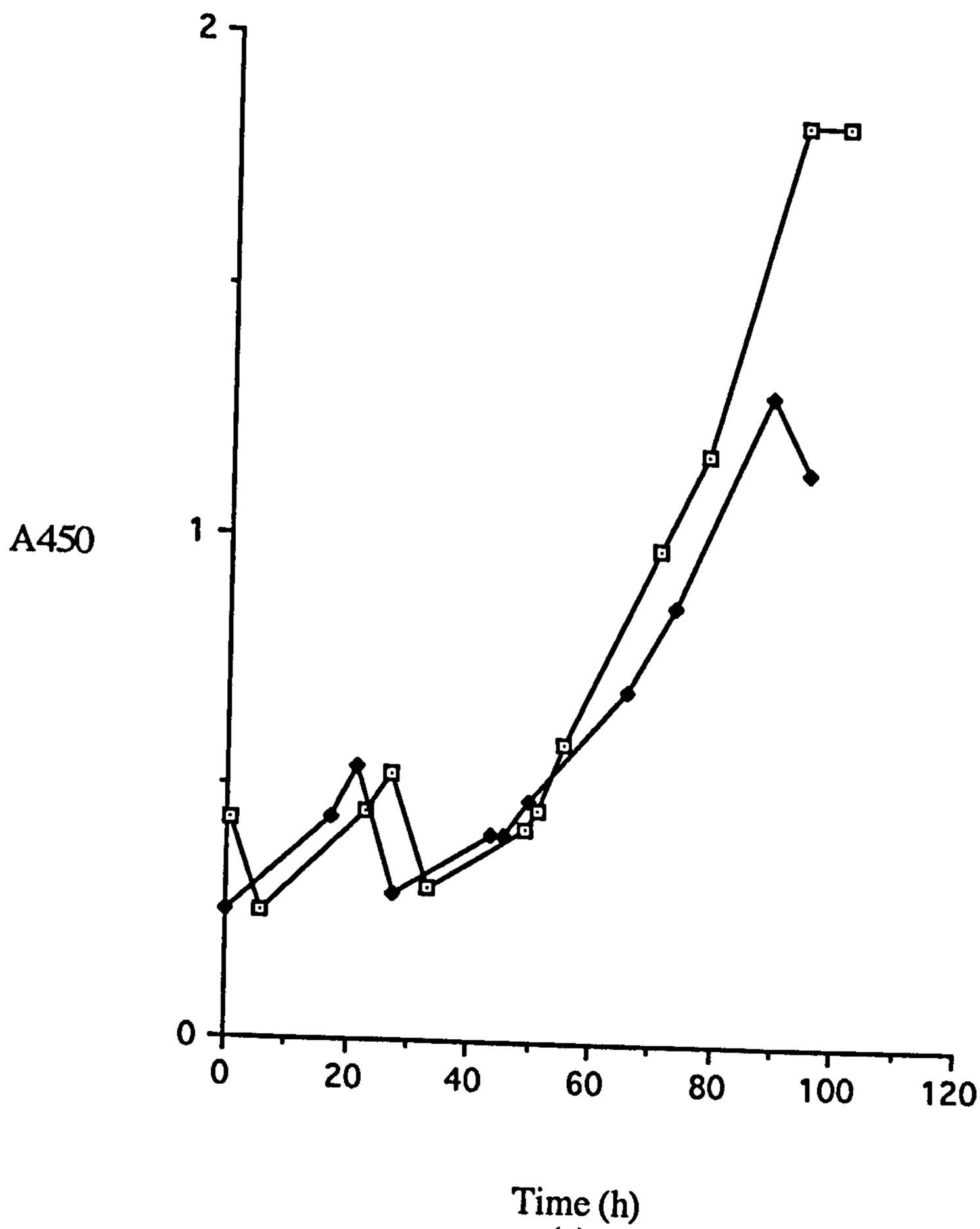
Cultures were grown in one litre side-arm flasks in 800 ml of HMM, containing 1.5 gl^{-1} Junlon, with 20 mM ammonium sulphate as nitrogen source, at 30°C.

(a) *S.coelicolor* 1 ml inoculum

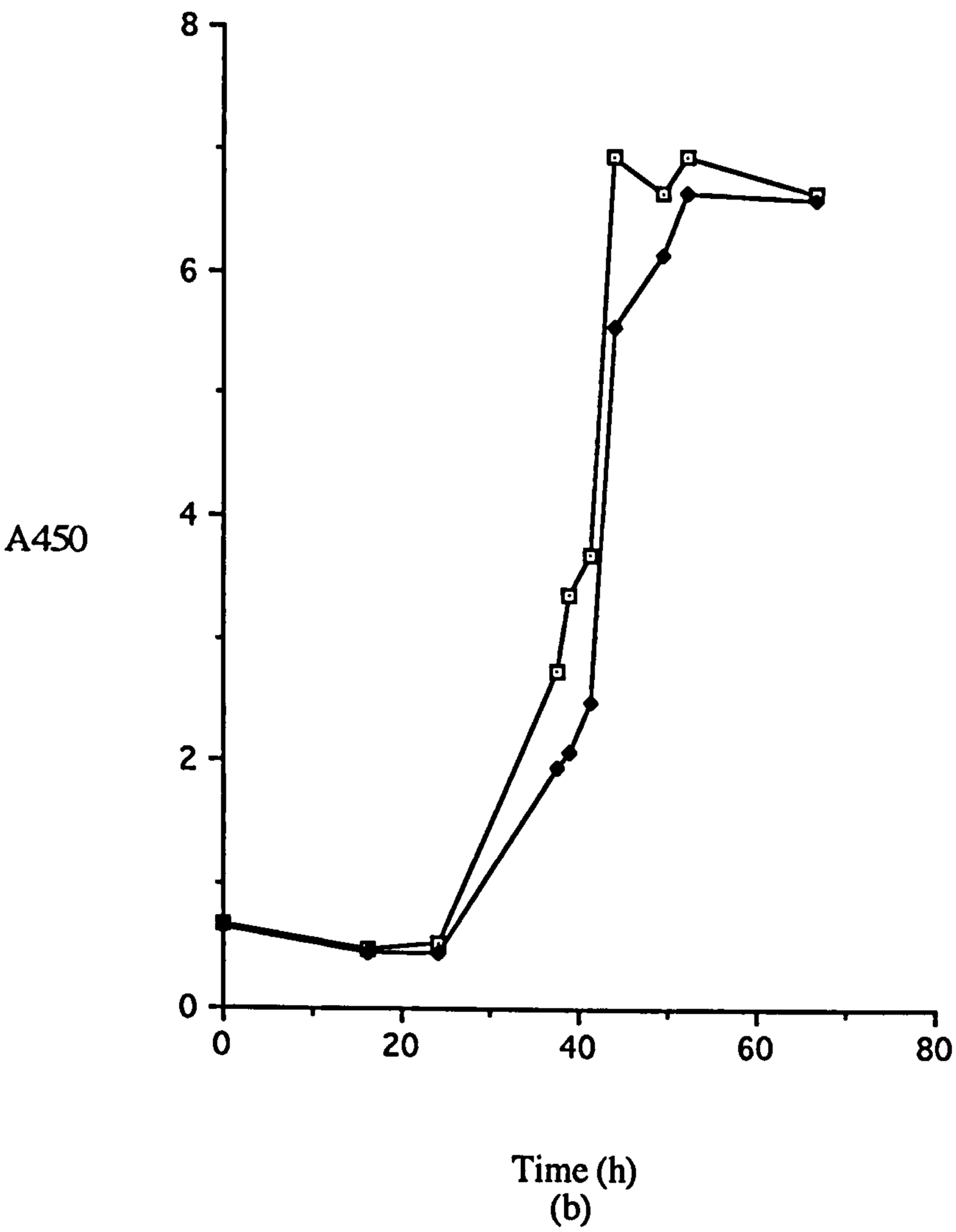
2 ml inoculum

(b) *S.rimosus* 1 ml inoculum

2 ml inoculum



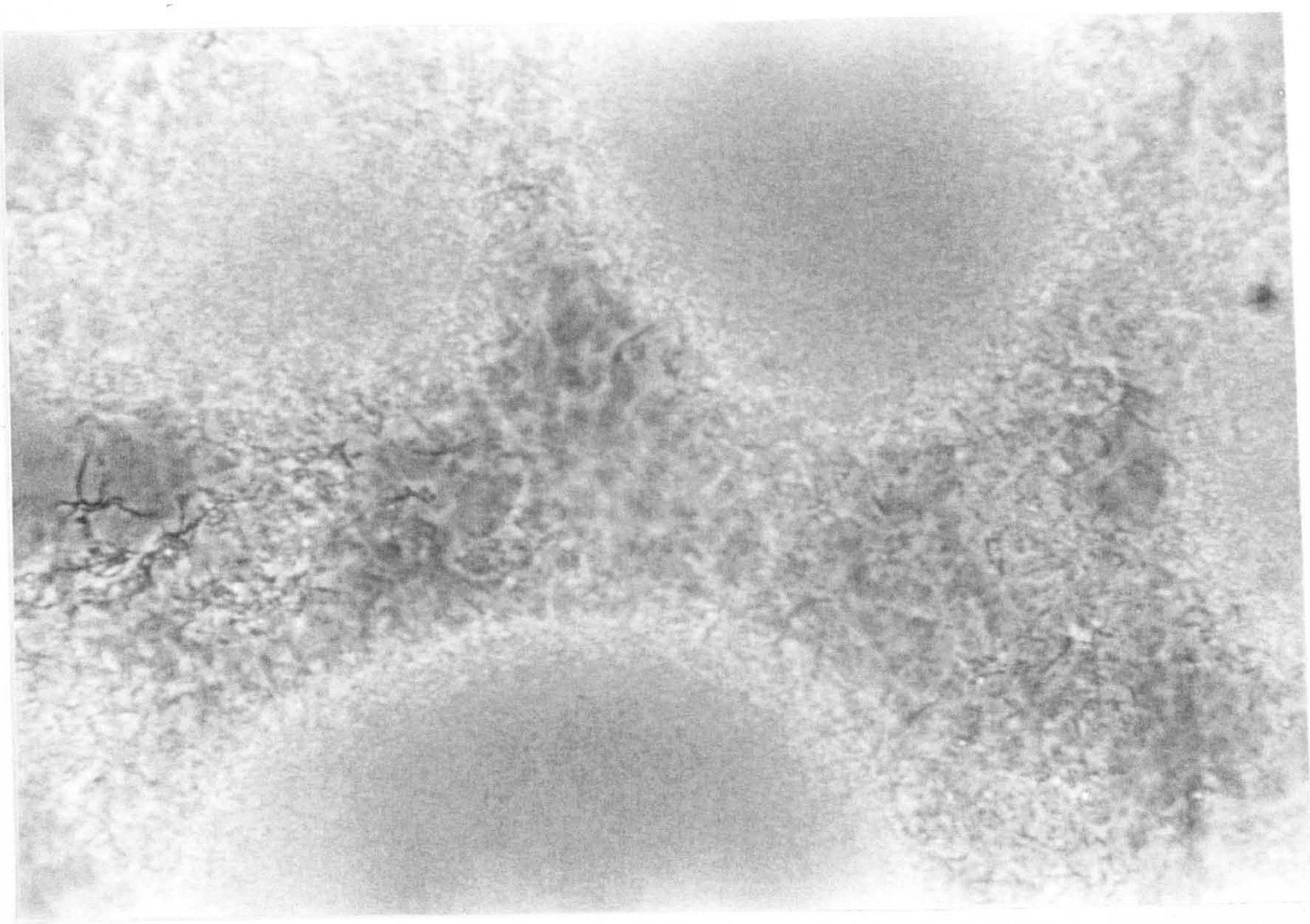
(a)



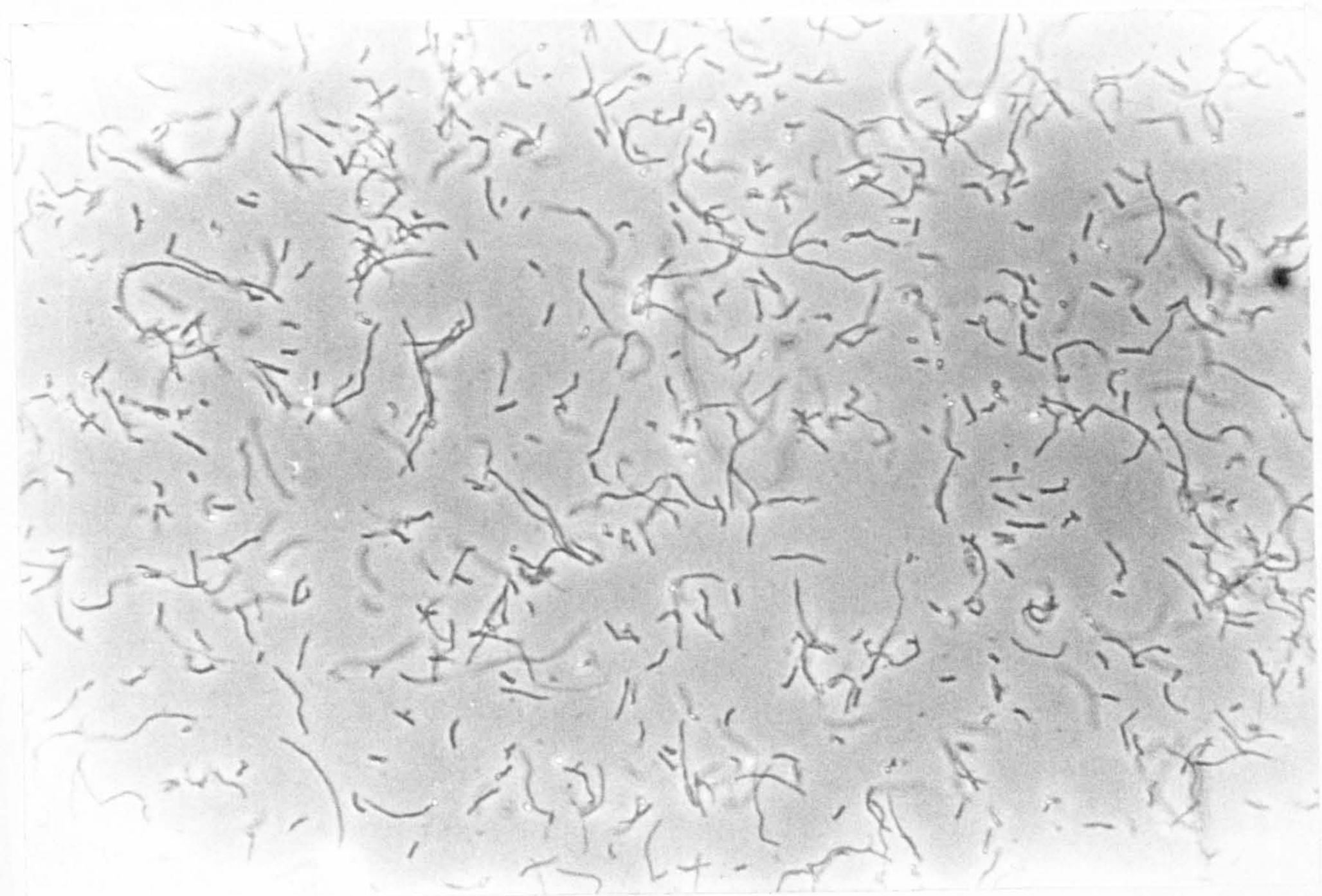
(b)

Fig. 3.5 PHOTOGRAPHS OF *S. RIMOSUS* GROWN EITHER SHAKEN OR STIRRED

S.rimosus cultures were grown in 500 ml conical flasks, containing 200 ml of modified HMM at 30°C, either on an orbital shaker, at 180 rpm, or on a magnetic stirrer at 300 rpm. Samples were taken from both and culture slides were prepared and examined under a Leitz Ortholux II microscope.



shaken



stirred

3.2.7 INOCULUM

The spore concentration in inocula can have a considerable influence on the morphology of actinomycetes in liquid culture (Lawton *et al.*, 1989). This also proves to be true for fungi, the other major group of filamentous organisms (Metz and Kossen, 1977). Lawton *et al.*, (1989) found that a high concentration of spores tends to produce a dispersed form of growth whilst a low concentration normally results in pellet formation. Young *et al.*, (1987) reported that tetracycline production, in addition to morphology, was affected by inoculum size in *S. aureofaciens* cultures.

Originally fresh spores with an inoculum size of 10^6 spores ml^{-1} were used as an inoculum as this is the recommended protocol for studying actinorhodin production by *S. coelicolor* (Hobbs *et al.*, 1989b). However it was found to be more convenient to inoculate growth flasks with an aliquot of a stock mycelia grown in modified HMM, from a stored stock solution of spores, prior to the actual growth experiment. This proved a reliable method with the results being of greater reproducibility as each flask received an equal and identical inoculum. Using mycelia as an inoculum also meant it was easier to decrease the growth time by controlling the size of the inoculum, and allowed the elimination of the long lag phase. Referring to Fig. 3.4, which shows growth curves from *S. coelicolor* and *S. rimosus*, it can be seen the use of spores as an inoculum can lead to lag phases of between 20 and 30 h. Typically, 100 µl of a stock solution of spores were used to inoculate a flask containing 200 ml of modified HMM and 10 mM glucose. After approximately 3 days, or when logarithmic growth had ended, the culture would reach an optical density of between 2 and 3, measured at 500 nm. Between 5 and 10 ml of this "stock" culture would be used as an inoculum for growth experiments, as a starting optical density of between 0.1 and 0.2 was found to be suitable for most experiments.

3.3 GROWTH OF *S. RIMOSUS* UNDER STANDARD GROWTH CONDITIONS

Cultures were grown in 500 ml conical flasks containing 200 ml of modified HMM, at 30°C, on a magnetic stirrer. The carbon sources used were as follows; 10 mM glucose, 20 mM glycerol or 10 mM mannitol. Succinate and acetate were also tested as potential carbon sources but were not able to support growth of *S.rimosus* under these conditions. Cultures were inoculated with equal volumes of mycelia from a stock culture grown on a medium based on the same carbon source on identical medium. Growth was followed by optical density measured at 500 nm, protein concentration and DNA concentration. Typical growth curves for glucose, glycerol and mannitol are shown in Figs. 3.6, 3.7 and 3.8. These figures show growth curves followed by optical density (a), DNA concentration (b) and protein concentration (c). The growth curves followed by DNA and protein concentration follow the same basic trends as those followed by optical density. This implies that the cultures are sufficiently dispersed and therefore it is acceptable to follow growth, under these conditions, by optical density. These growth experiments were repeated a number of times and a summary of the results obtained are shown in Tables 3.3 (a), (b) and (c). Table 3(a) gives the figures for MGT (h). The figures for a particular carbon source vary slightly from experiment to experiment but are always fairly constant within a set of experiments. The mean MGT for all three of the substrates is very similar with the values falling between 3.4 and 3.8 hours. Tables 3 (b) and (c) give the figures for the DNA:OD and protein:OD ratios. Statistical analysis on this data is discussed in 3.6.1.

3.4 GROWTH OF *S. RIMOSUS* ON STANDARD DEFINED GROWTH MEDIA CONTAINING TRIGLYCERIDES AS CARBON SOURCES

Once repeatable results had been achieved with standard growth experiments numerous attempts were made to culture *S. rimosus* in modified HMM containing either olive oil or rape seed oil as sole carbon source. None of these attempts proved successful, with no

growth being observed either by protein or DNA estimations (it was not possible to follow growth by optical density as media containing the oils formed milky emulsions). Typical results from one of these experiments are shown in Fig. 3.9. Cultures of *S. rimosus* were grown in 250 ml conical flasks, containing 100 ml of medium with 0.5% (v/v) rape seed oil as sole carbon source, at 30°C, on an orbital shaker. Growth was followed by measuring DNA concentration and no significant increase was detected. The pH of the cultures also showed no significant alteration.

Attempts were also made to culture *S. rimosus* in standard defined growth media containing glucose as a carbon source supplemented with oil. *S. rimosus* was cultured in 1 litre side-arm flasks, containing 800 ml of modified HMM with 10 mM glucose and 0.5% (v/v) rape seed oil, at 30°C on a magnetic stirrer, at 70 rpm. Cultures were inoculated with fresh spores. Fig. 3.10 compares a control growth curve (a) containing only glucose as carbon source and (b) supplemented with rape seed oil. In both cases growth ends shortly after 30 h and the MGTs are not significantly different. The culture supplemented with rape seed oil reaches a slightly higher final concentration of DNA, but taken by itself this piece of evidence cannot be taken as an indication of lipid utilisation.

One of the methods to determine whether lipid has been utilised by growing cultures would be to measure the disappearance of the lipid. To do this a Boehringer Mannheim Total Lipid test kit was used. Unfortunately problems were encountered in handling culture samples containing lipid. It was found that samples couldn't be frozen after sampling as this resulted in inaccuracies in determining the concentration of lipid in the sample. A 1 litre side- arm flask containing 800 ml of H_2O and 4 ml of olive oil (4.55 g l^{-1}) was stirred until an emulsion formed. Five 10 ml samples were taken and stored at -20°C for 24 h. Similarly five 1 ml samples were taken and added to 9 ml of chloroform /methanol (1:1) and left at room temperature for 24 h. The samples were assayed in triplicate using the test kit. The results are shown in Table 3.4 (a) -20°C samples and (b) room temperature samples.

Extraction of the lipid into chloroform/ methanol immediately the samples were taken, gave a mean value closer to the correct value but there was still a significant standard deviation. On thawing the -20°C samples the lipid came out of emulsion and as a result stuck to the sides of the container. This resulted in lower than expected values. Reproducibility within the assay was also investigated and if a positive displacement pipette was used at every stage the standard deviation was reduced. Table 3.5 gives figures for the test kit standard (A530) assayed without using a positive displacement pipette (a) and (b) using a positive displacement pipette at every stage. Despite these improvements in handling the samples and in assay procedure results were still insufficiently reproducible to allow accurate measurements of lipid concentration.

3.5 GROWTH OF *S.RIMOSUS* ON STANDARD DEFINED GROWTH MEDIA SUPPLEMENTED WITH POLYOXYETHYLENE ESTERS

Due to the difficulties encountered with growth on media containing or supplemented with triglycerides it was decided to look at the water-soluble Tweens (polyoxyethylene esters) as possible substrates. In particular the detergents Tween 20 (polyoxyethylene sorbitan monolaurate) and Tween 40 (polyoxyethylene sorbitan monopalmitate) were studied for their effect on the growth of *S. rimosus*.

Preliminary experiments indicated that *S. rimosus* did not grow when Tweens were the sole carbon source present. Cultures were grown in 200 ml of modified HMM, with either 0.1% (v/v) or 0.5% (v/v) Tween 20 and either 0.1% (v/v) or 0.5% (v/v) Tween 40 as carbon sources (in 500 ml conical flasks, at 30°C) on a stirrer. Fig. 3.11 shows the growth curves, followed by optical density measured at 500 nm. No growth was observed until 10 mM glucose was added to the cultures after 98 h. It was interesting that each of the cultures containing the 0.5% concentrations of both Tween 20 and Tween 40 reached twice the final optical density of the corresponding cultures containing 0.1% of the detergent. This may

imply that the Tweens are being utilised and supplementing glucose as a carbon source but demonstrated that glucose is required to be present before growth can take place. An additional point to notice is that the cultures containing Tween 20 have a longer lag phase than those containing Tween 40, before growth is observed upon glucose addition.

Subsequent experiments, including glucose controls and wider ranges of Tween concentrations were carried out. Cultures were grown under the same conditions as the preliminary experiment. Fig. 3.12 a) and b) show the growth curves of *S. rimosus*, followed by optical density of 500 nm and DNA concentration respectively, grown in modified HMM and 10 mM glucose supplemented with either one of 0.1%, 0.5%, 1% or 2% (v/v) Tween 40. Again the final optical densities of the cultures were higher with increasing Tween concentration, implying that the Tween was being utilised for growth. This was confirmed by analysis of DNA concentration which also increased with increasing Tween concentration. The DNA curves followed the same basic pattern as the optical density curves although they are not identical in shape. This could be explained by the fact that fewer of the samples were analysed for DNA content. Growth on glucose ended before growth in cultures supplemented with 0.5%, 1%, and 2% (v/v) Tween 40. The growth curves of the glucose control and the culture supplemented with 0.1% (v/v) Tween 40 are similar, with cessation of growth occurring simultaneously and both curves fall off with increasing time. After the initial logarithmic growth, the cultures supplemented with 0.5%, 1% and 2% (v/v) Tween 40 continued to grow at a slower rate for a further 20 h. Growth then levelled off at greater than twice the optical density and DNA values of the glucose control. Fig. 3.13 a) and b) show the growth curves of *S.rimosus,* followed by optical density measured at 500 nm and DNA concentration respectively, grown in modified HMM and 10 mM glucose supplemented with 0.1%, 0.5%, 1%, or 2% (v/v) Tween 20. The results were similar to those achieved with Tween 40. Again the glucose control and the culture supplemented with 0.1% (v/v) Tween 20 finished growth before the cultures containing the higher concentrations of detergent, which continued growing slowly after the

initial logarithmic growth phase. The culture containing 2% (v/v) Tween 20 appeared to be still growing after 41 h while the other cultures appeared to have ceased growing. The cultures containing Tween 20 again had a longer lag phase than the glucose control taking 16 h to begin logarithmic growth compared to 10 h with the glucose control. The MGTs for the cultures containing the Tweens are shown in Table. 3.6. They are slightly higher than those from the glucose control.

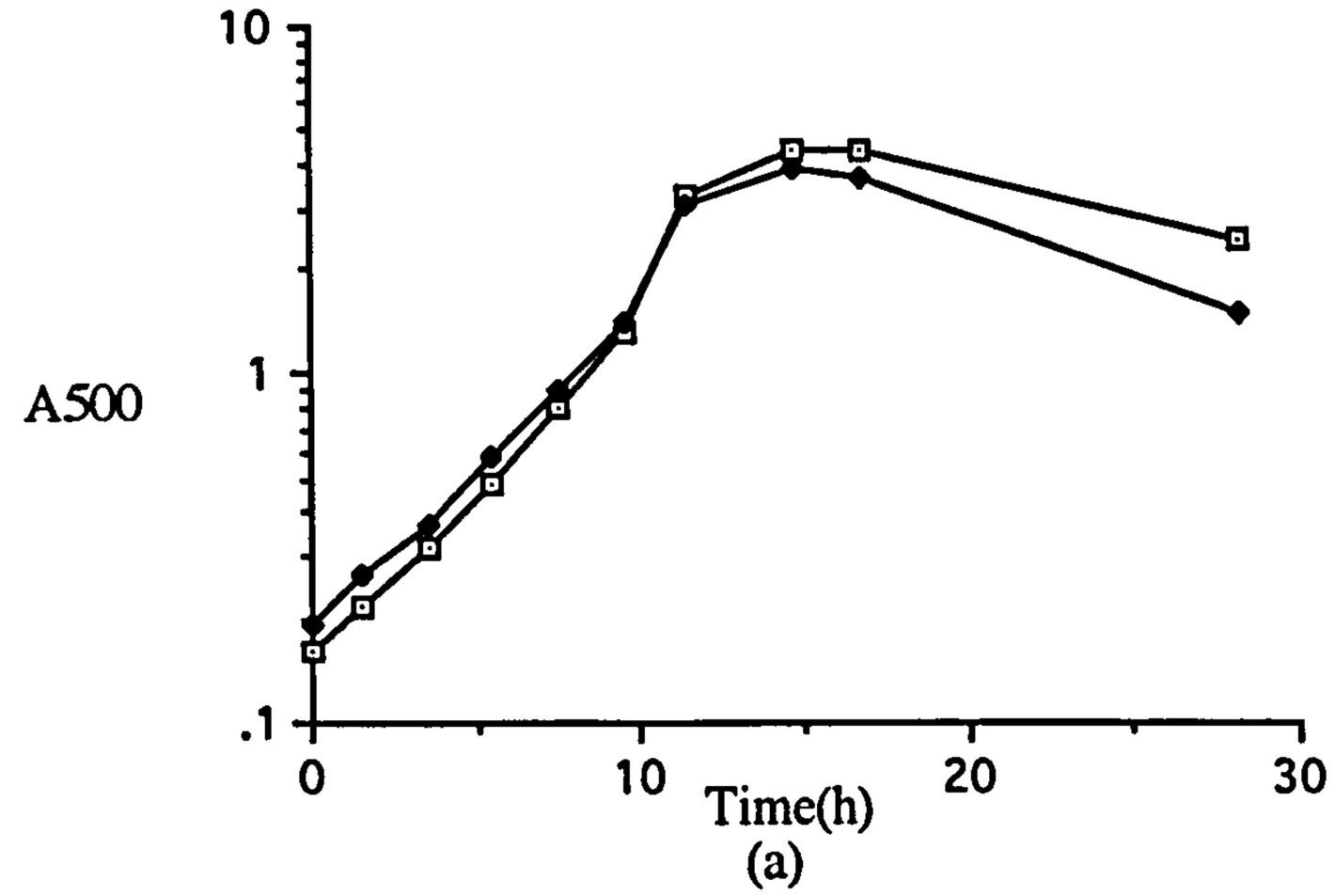
Fig. 3.6 GROWTH OF *S.RIMOSUS* IN MODIFIED HMM CONTAINING GLUCOSE AS THE CARBON SOURCE

Growth of *S.rimosus* under standard growth conditions, in modified HMM containing 10 mM glucose as carbon source. Growth was followed by;

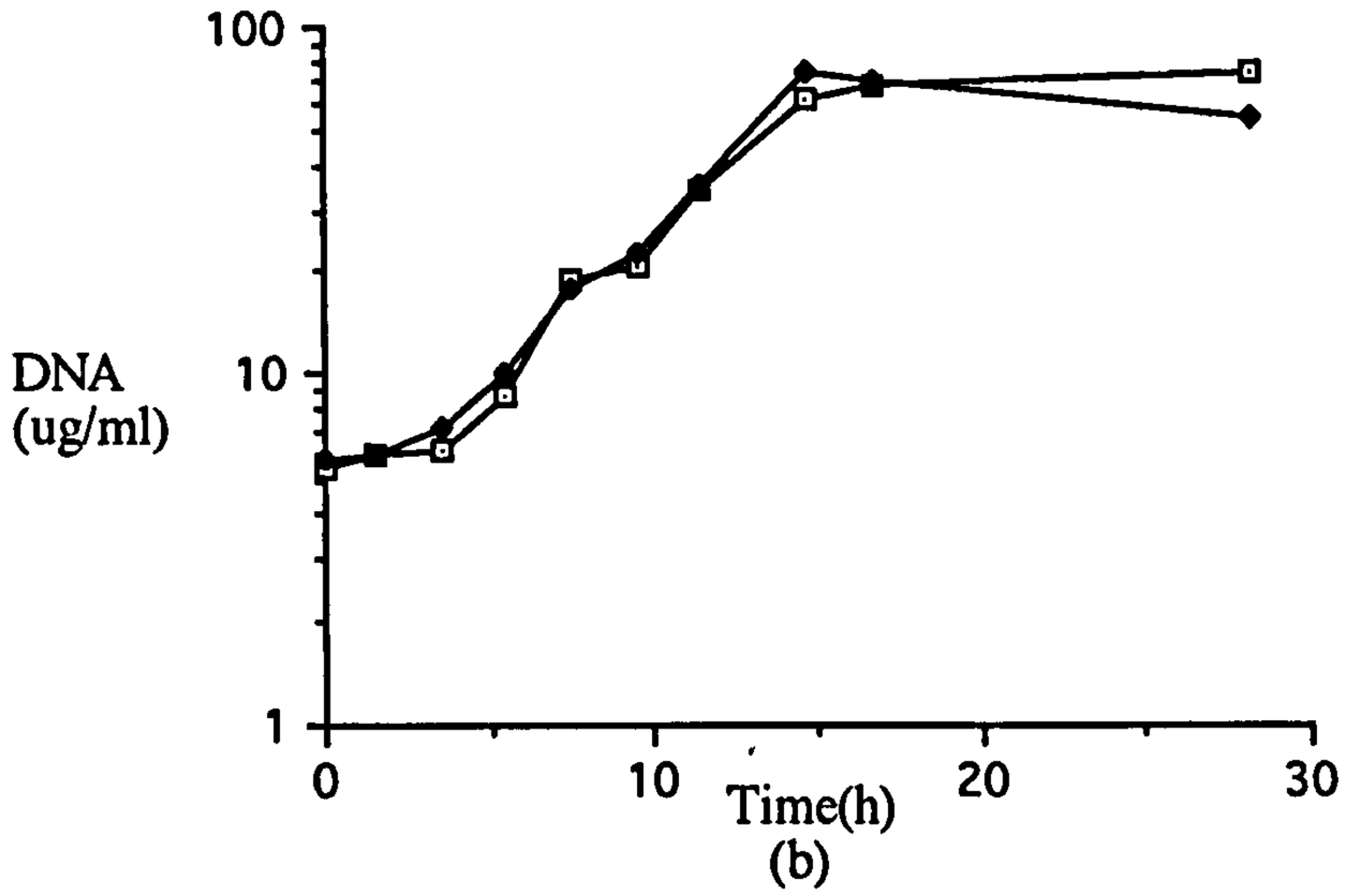
(a) Optical density, measured at 500 nm (Methods 2.6).

(b) DNA concentration (μg/ml) (Methods 2.18).

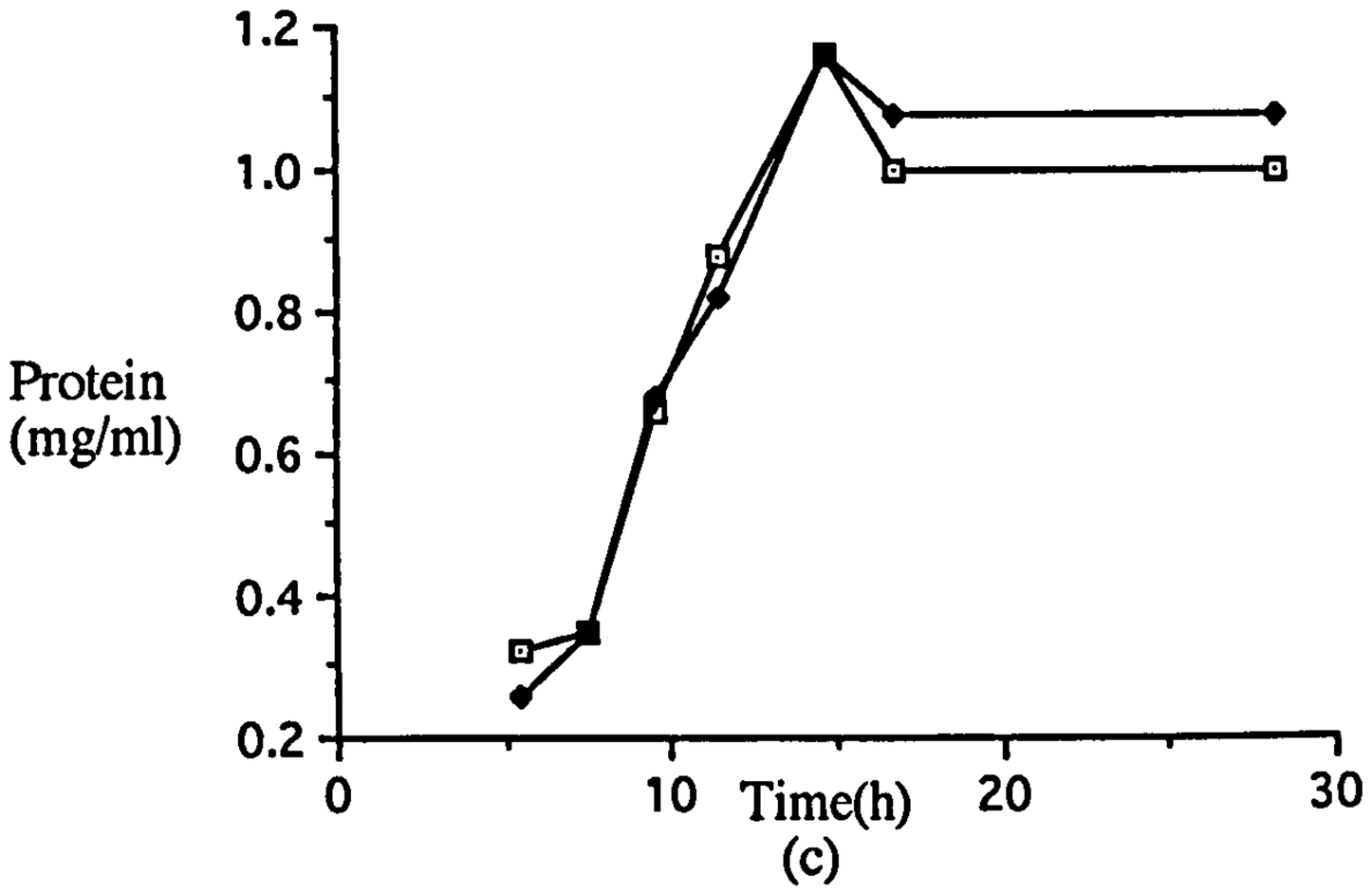
(c) Protein concentration (mg/ml) (Methods 2.17).


A500
10
1
.1
0
10
20
30
Time(h)

(a)


DNA
(ug/ml)
100
10
1
0
10
20
30
Time(h)

(b)


Protein
(mg/ml)
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
Time(h)

(c)

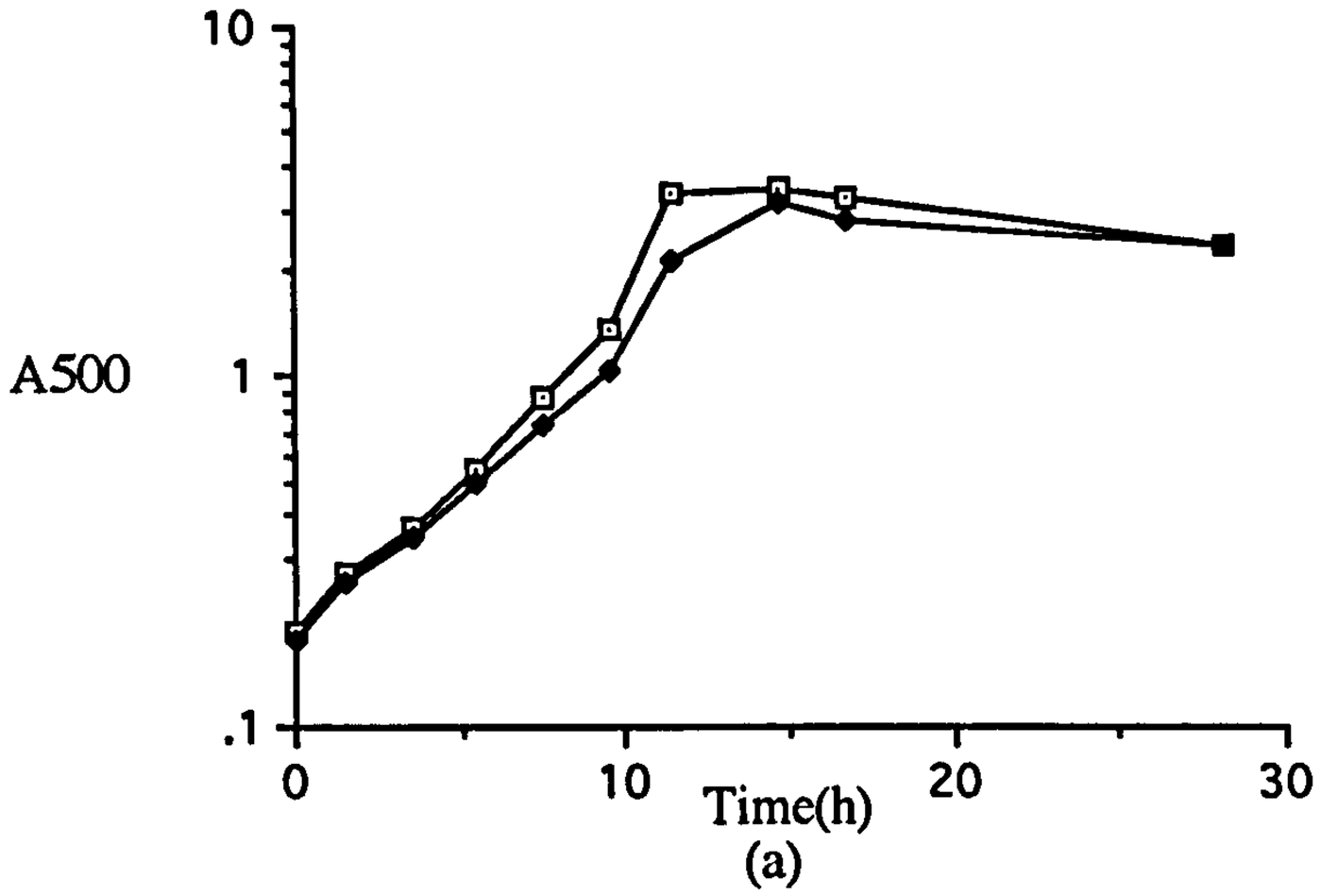
Fig. 3.7 GROWTH OF *S. RIMOSUS* IN MODIFIED HMM CONTAINING GLYCEROL AS THE CARBON SOURCE

Growth of *S.rimosus* under standard growth conditions, in modified HMM containing 20 mM glycerol. Growth was followed by;

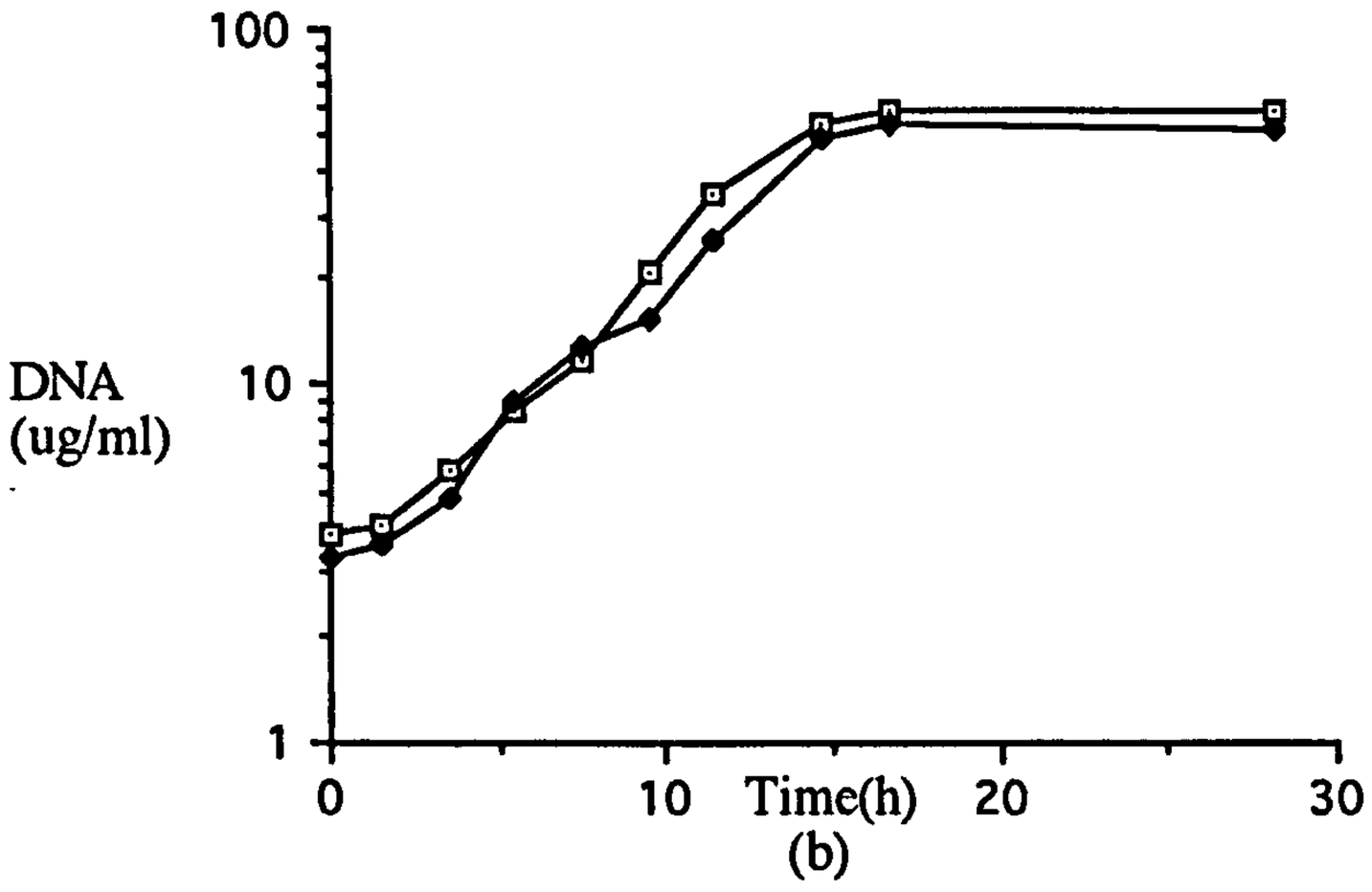
(a) Optical density, measured at 500 nm (Methods 2.6).

(b) DNA concentration (µg/ml) (Methods 2.18).

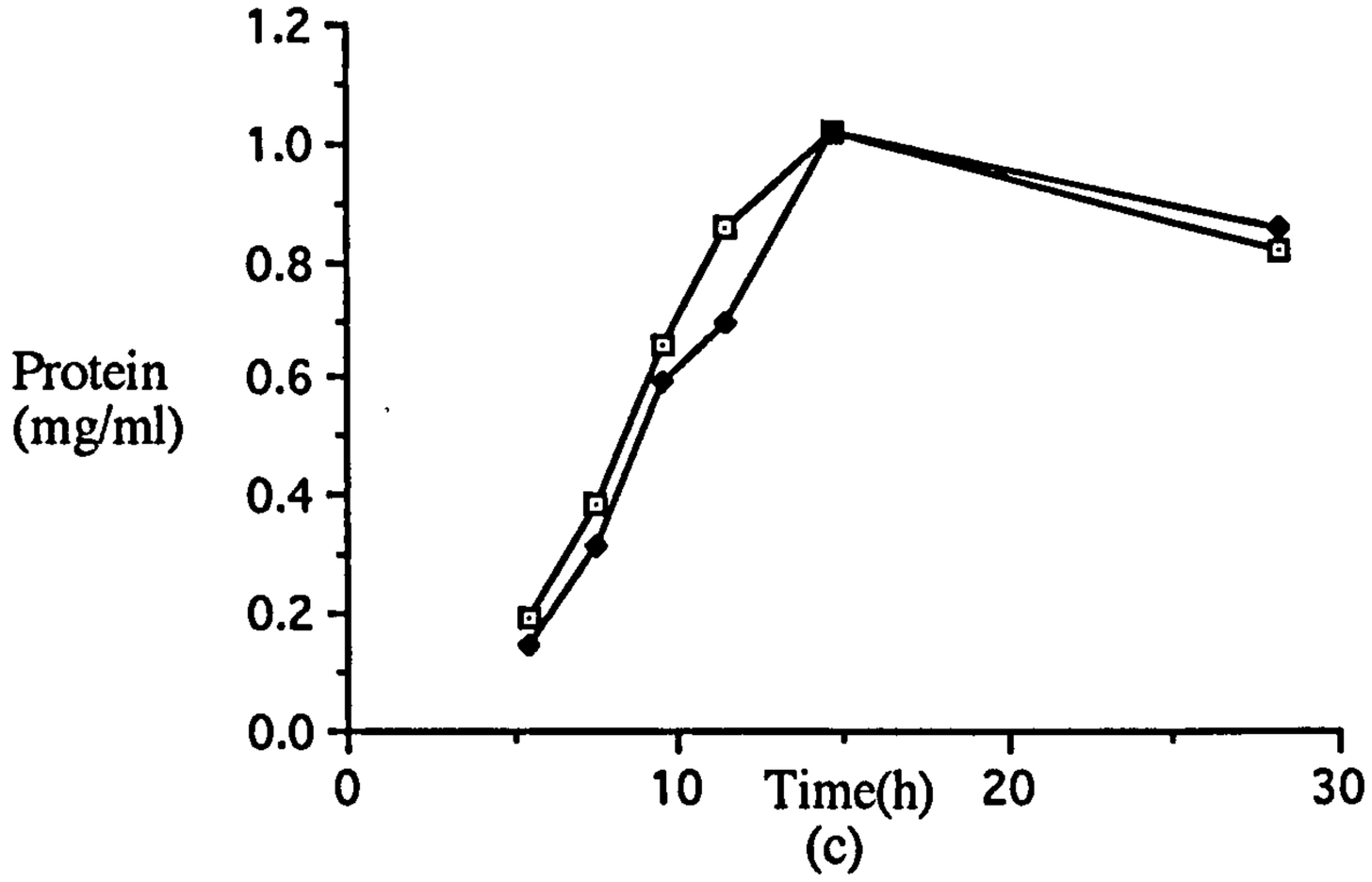
(c) Protein concentration (mg/ml) (Methods 2.17).


10
A500
1
.1
0
10
20
30
Time(h)

(a)


100
DNA
(ug/ml)
10
1
0
10
Time(h)
20
30

(b)


1.2
1.0
0.8
Protein
(mg/ml)
0.6
0.4
0.2
0.0
0
10
Time(h)
20
30

(c)

Fig. 3.8 GROWTH OF *S. RIMOSUS* IN MODIFIED HMM CONTAINING MANNITOL AS THE CARBON SOURCE

Growth of *S.rimosus* under standard growth conditions, in modified HMM containing 10 mM mannitol. Growth was followed by;

(a) Optical density, measured at 500 nm (Methods 2.6).

(b) DNA concentration (µg/ml) (Methods 2.18).

(c) Protein concentration (mg/ml) (Methods 2.17).

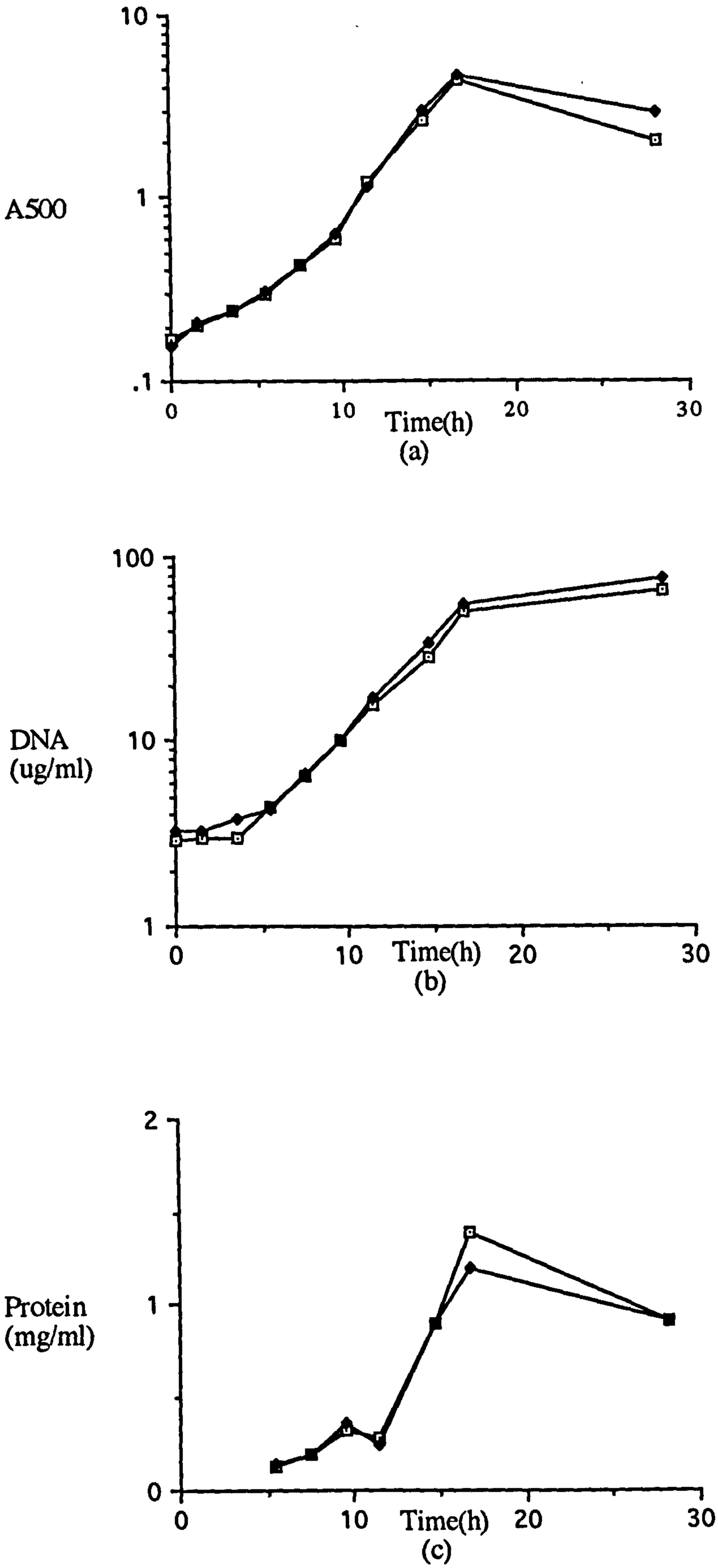

A500
10
1
.1
0
10
20
30
Time(h)
(a)
DNA
(ug/ml)
100
10
1
0
10
20
30
Time(h)
(b)
Protein
(mg/ml)
2
1
0
0
10
20
30
Time(h)
(c)

Table 3.3 SUMMARY OF RESULTS FROM GROWTH EXPERIMENTS WITH EITHER GLUCOSE, GLYCEROL OR MANNITOL PRESENT AS THE CARBON SOURCE IN MODIFIED HMM

These tables provide a summary of the results obtained in repeated standard growth experiments [(a), (b), and (c)]. Cultures of *S.rimosus* were grown in modified HMM containing either 10 mM glucose, 20 mM glycerol or 10 mM mannitol.

(a) MGT (h)

(b) DNA : OD

(c) Protein : OD

Carbon Source	Mean Generation Time (h)				Mean
	(a)	(b)	(c)	(d)	
Glucose	4	3	2.8	3.2	3.38±0.76
	5	3	2.8	3.2	
Glycerol	4	4	2.8	3.4	3.52±0.58
	4	4	2.6	3.4	
Mannitol	5	4.2	3.8	2.9	3.82±0.78
	5	3	3.8	2.9	

(a)

Carbon source	DNA / OD				Mean
	(a)	(b)	(c)	(d)	
Glucose	1:25.19	1:16.34	1:17.42	1:14.00	18.73±3.52
	1:20.96	1:16.20	1:20.69	1:19.04	
Glycerol	1:37.55	1:15.38	1:13.59	1:15.51	19.46±8.40
	1:26.89	1:15.81	1:15.25	1:15.73	
Mannitol	1:20.13	1:13.66	1:25.00	1:10.99	16.98±5.94
	1:15.02	1:13.40	1:26.00	1:11.67	

(b)

Carbon source	Protein / OD		Mean
	(c)	(d)	
Glucose	2.32:1	3.78:1	3.02±0.66
	2.66:1	3.34:1	
Glycerol	3.59:1	3.38:1	3.53±0.30
	3.92:1	3.24:1	
Mannitol	3.67:1	2.92:1	2.99±0.49
	2.50:1	2.87:1	

(c)

Fig. 3.9 GROWTH OF *S. RIMOSUS* IN MODIFIED HMM CONTAINING 0.5% RAPE SEED OIL AS THE CARBON SOURCE

Cultures of *S.rimosus* were grown in 250 ml conical flasks, containing 100 ml of modified HMM with 0.5% (v/v) rape seed oil as sole carbon source, at 30°C on a shaker at 300 rpm. Growth was followed by measuring DNA concentration (Methods 2.18).

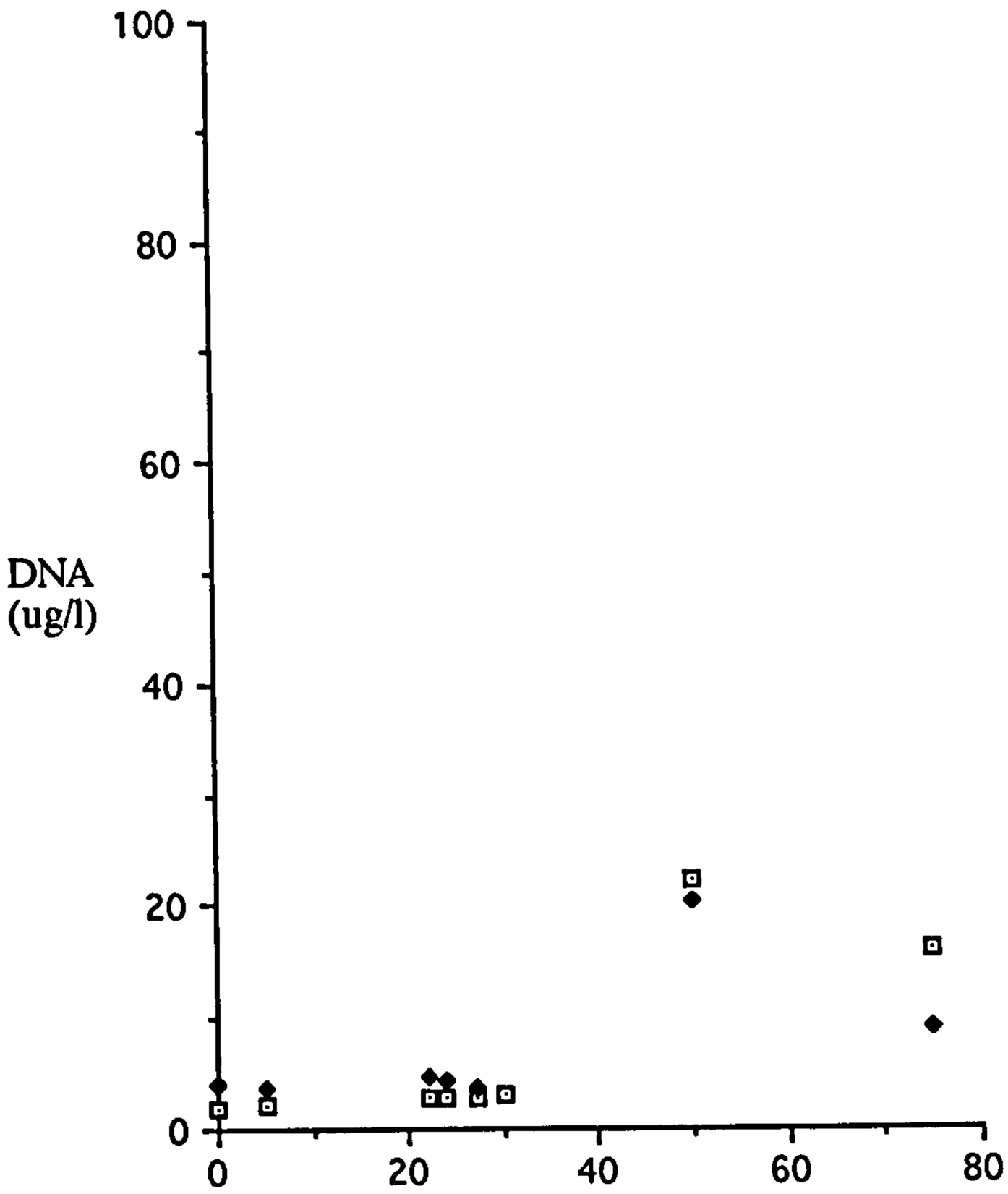
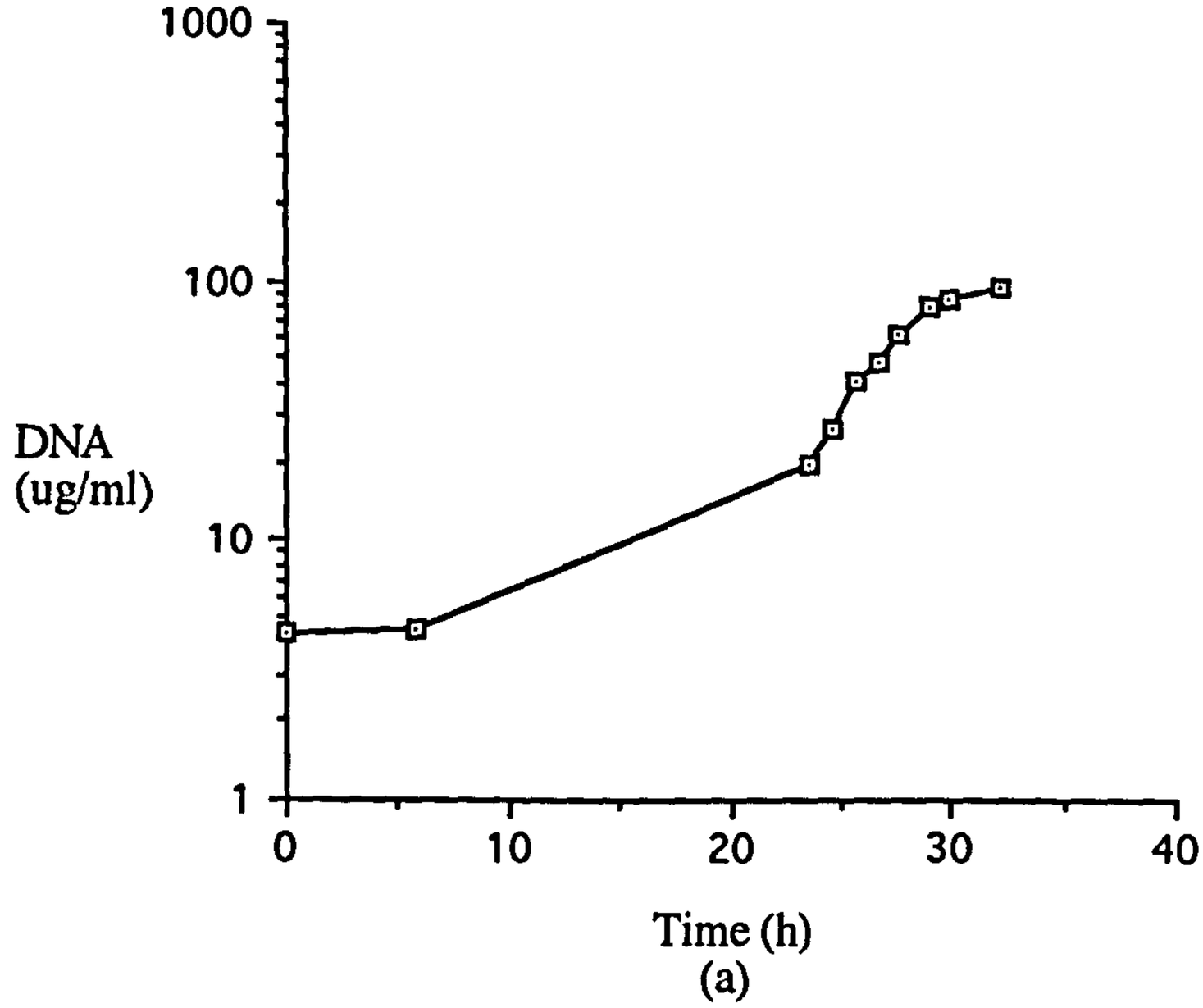

100
80
60
40
20
0
DNA
(ug/l)
0
20
40
60
80
Time (h)

Fig. 3.10 GROWTH OF *S. RIMOSUS* IN MODIFIED HMM CONTAINING GLUCOSE AS THE CARBON SOURCE, SUPPLEMENTED WITH 0.5% RAPE SEED OIL

Cultures of *S.rimosus* were grown under standard conditions, inmodified HMM containing 10 mM glucose supplemented by 0.5% (v/v) rape seed oil. Growth was followed by measuring DNA concentration (Methods 2.18).

(a) glucose control

(b) glucose + 0.5% (v/v) rape seed oil


1000
100
10
1
DNA
(ug/ml)
0
10
20
30
40
Time (h)
(a)

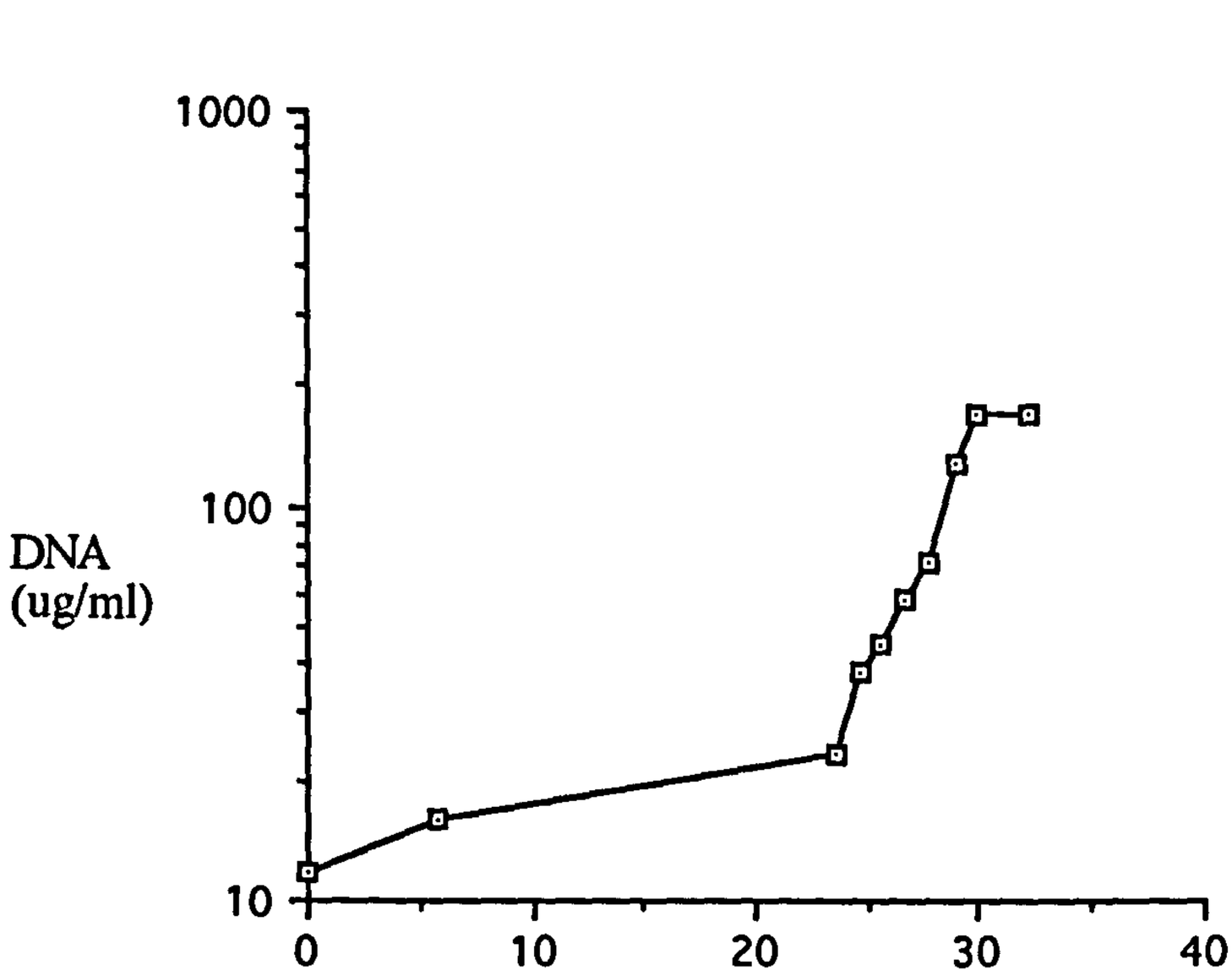

1000
100
10
DNA
(ug/ml)
0
10
20
30
40
Time (h)
(b)

Table 3.4 TOTAL LIPID ASSAYS ON FROZEN AND ROOM TEMPERATURE STORED SAMPLES

Comparison of total lipids assay on samples which have been (a) frozen at -20°C for 24 h and (b) stored at room temperature in chloroform/ methanol (1:1, v/v). A 1 litre side-arm flask containing 800 ml H_2O and 4 ml of olive oil (4.55 g l^{-1}) was stirred until an emulsion formed. Five 10 ml samples were taken and stored at -20°C for 24 h. Similarly five 1 ml samples were taken and added to 9 ml of chloroform/ methanol (1:1, v/v) and left at room temperature for 24 h. Samples were assayed in triplicate using a Boehringer Mannheim Total Lipid test kit (Methods 2.22).

Frozen samples	Lipid concentration (g/l)			Mean
1	0.66	0.85	1.64	1.05±0.52
2	0.42	1.15	1.21	0.92±0.44
3	0.2	2	2.17	1.45±1.09
4	0.59	1.21	1.35	1.05±0.40
5	0.63	2.07		1.35±1.02
Mean for data set				1.15±0.64
CV for data set				55%

Chloroform/methanol samples	Lipid concentration (g/l)			Mean
1	4.55		4.55	4.55
2	5.37	2.89	5.37	4.54±1.43
3	4.55	3.31	6.19	4.68±1.44
4	4.13	4.55	7.43	5.37±1.80
5	4.13	3.31	7.02	4.82±1.95
Mean for data set				4.81±1.35
CV for data set				28%

Table 3.5 TOTAL LIPID ASSAY EXECUTED WITH AND WITHOUT THE USE OF A POSITIVE DISPLACEMENT PIPETTE

Figures for the lipid test kit standard (Methods 2.22) assayed:

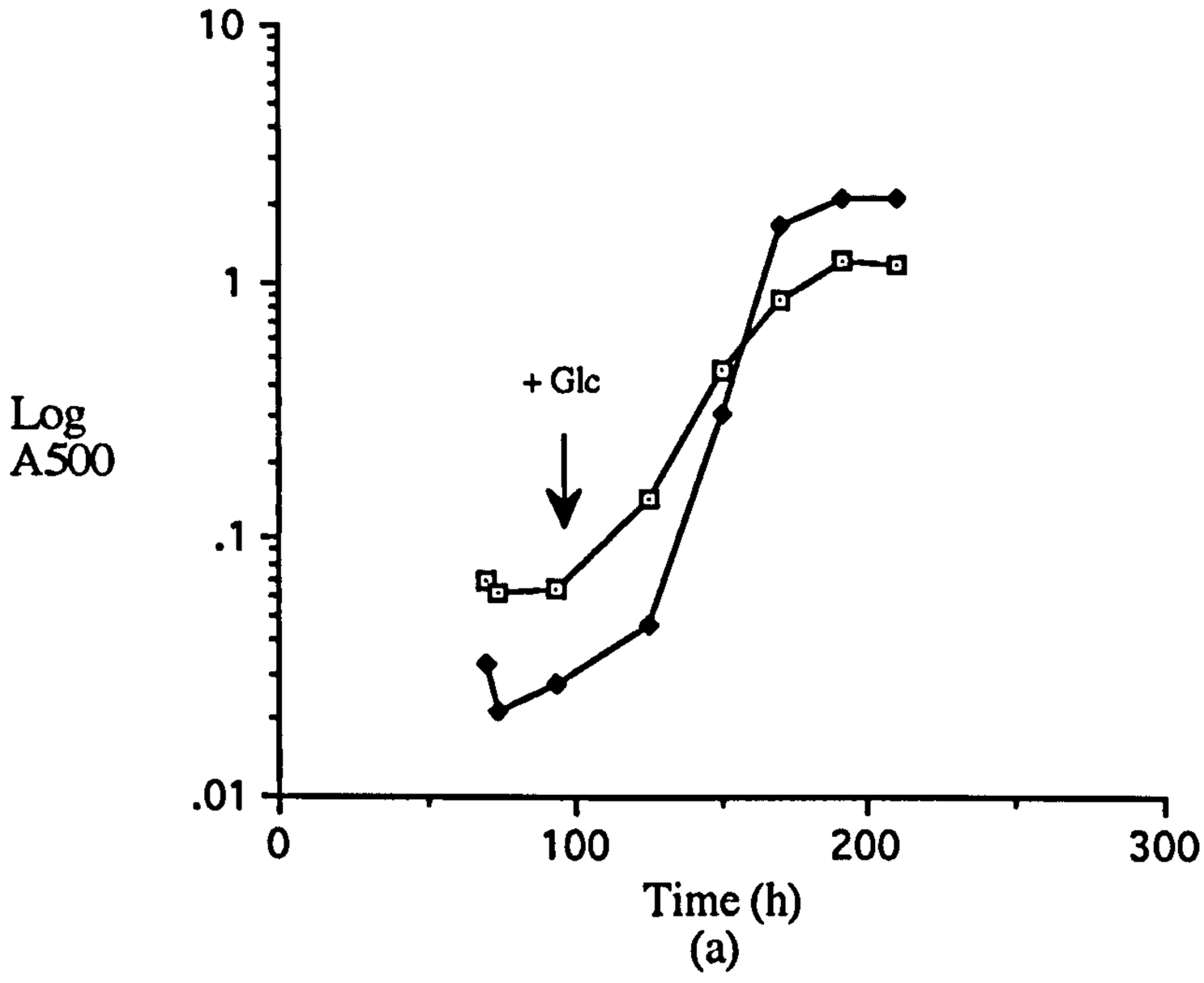
(a) without positive displacement pipette.

(b) using a positive displacement pipette at every stage.

Samples	A530	
	(a)	(b)
1	0.606	0.52
2	0.461	0.458
3	0.54	0.492
4	0.532	0.516
5	0.486	0.5
Mean	0.525±0.056	0.497±0.025
CV	11%	5%

Fig. 3.11 GROWTH OF *S. RIMOSUS* IN MODIFIED HMM CONTAINING EITHER TWEEN 20 OR TWEEN 40 AS THE CARBON SOURCE SUPPLEMENTED WITH GLUCOSE

Cultures of *S.rimosus* were grown in 200 ml of modified HMM with either (a) 0.1%⊡, 0.5%—◆— (v/v) Tween 20 or (b) 0.1%—⊡—, 0.5%—◆— (v/v) Tween 40 as carbon sources, in 500 ml conical flasks, at 30°C, on a magnetic stirrer at 300 rpm. Growth was followed by measuring optical density at 500 nm (Methods 2.6). After 98 h the cultures were supplemented with 10 mM glucose.


10
1
.1
.01
Log
A500
+ Glc
0
100
200
300
Time (h)

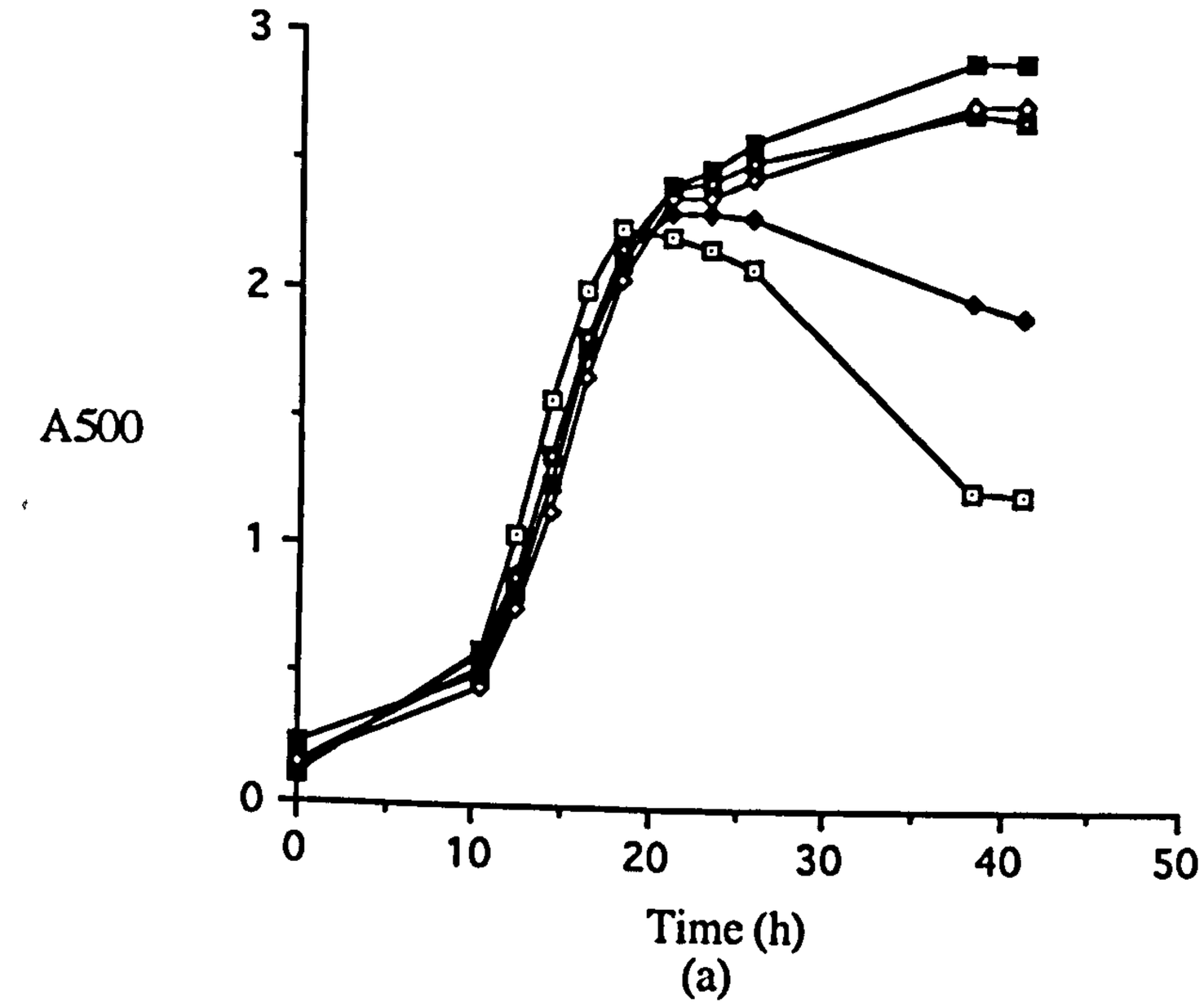
(a)

10
1
.1
Log
A500
+ Glc
0
100
200
300
Time (h)

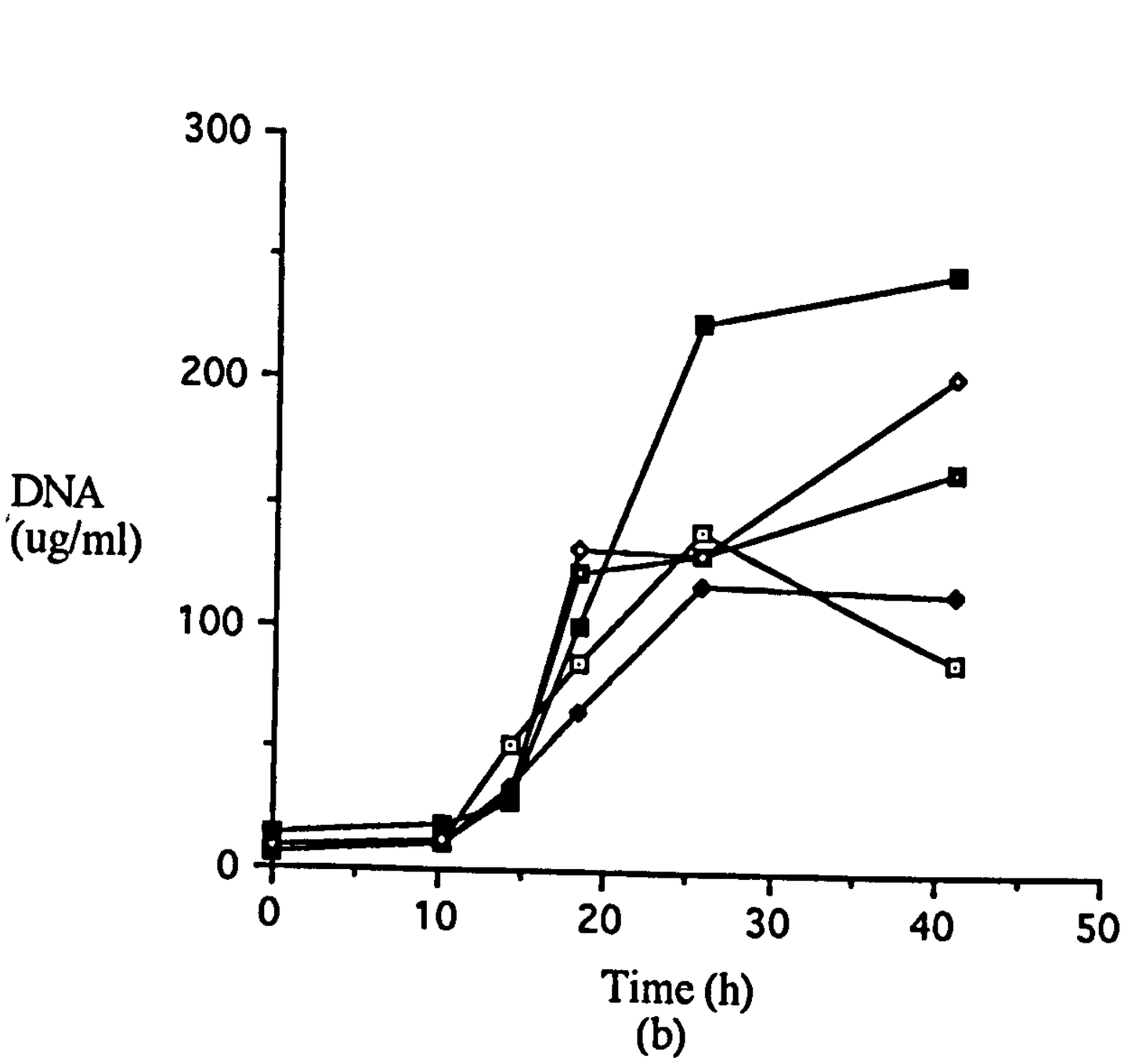
(b)

Fig. 3.12 GROWTH OF *S. RIMOSUS* IN MODIFIED HMM CONTAINING GLUCOSE AS THE CARBON SOURCE SUPPLEMENTED WITH TWEEN 40

Growth curves of *S.rimosus*, followed by measuring optical density at 500 nm [Methods 2.6] (a) and DNA concentration [Methods 2.18] (b), grown in modified HMM and 10 mM glucose supplemented with 0.1%, 0.5%, 1% or 2% (v/v) Tween 40.


A500
3
2
1
0
0
10
20
30
40
50
Time (h)

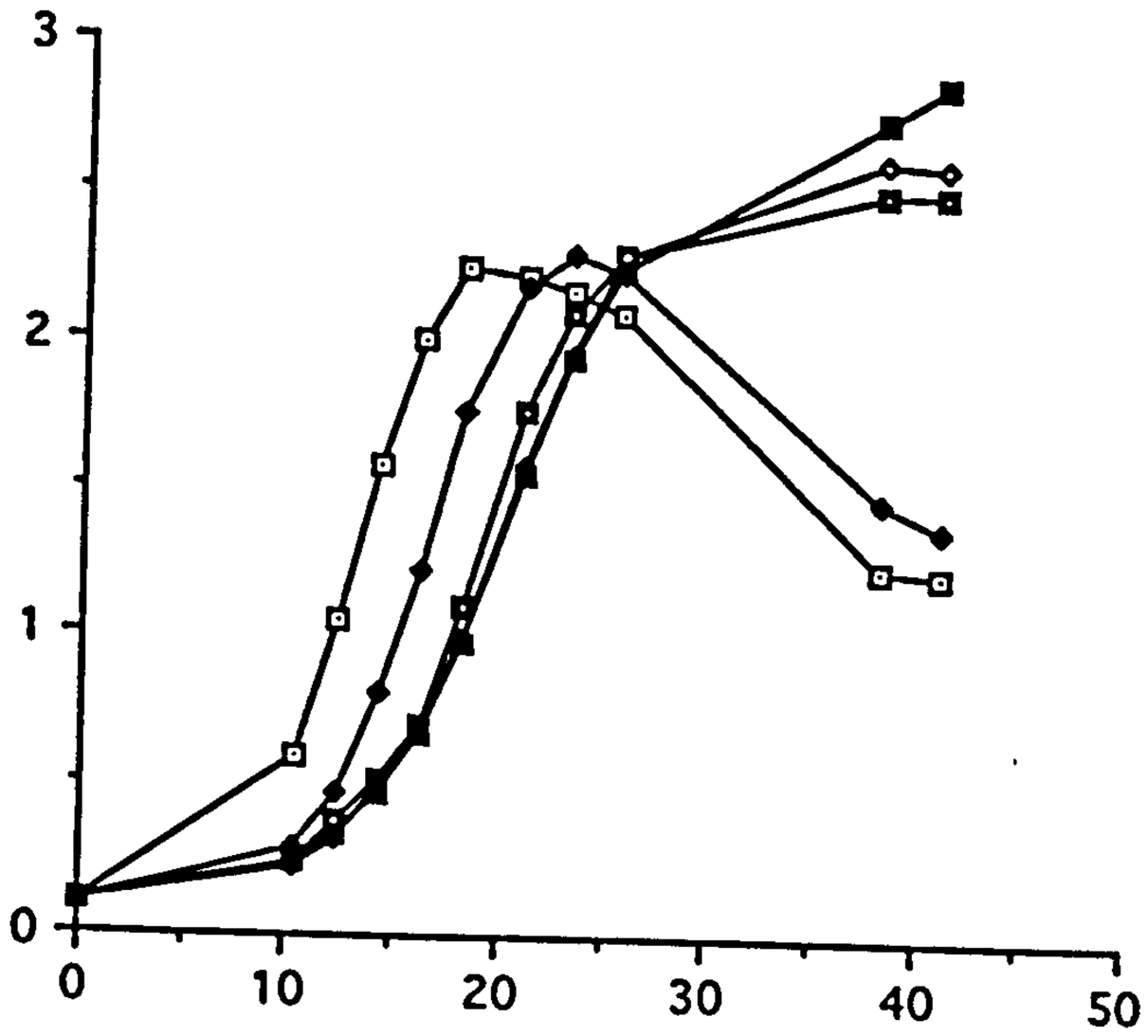
(a)


DNA
(ug/ml)
300
200
100
0
0
10
20
30
40
50
Time (h)

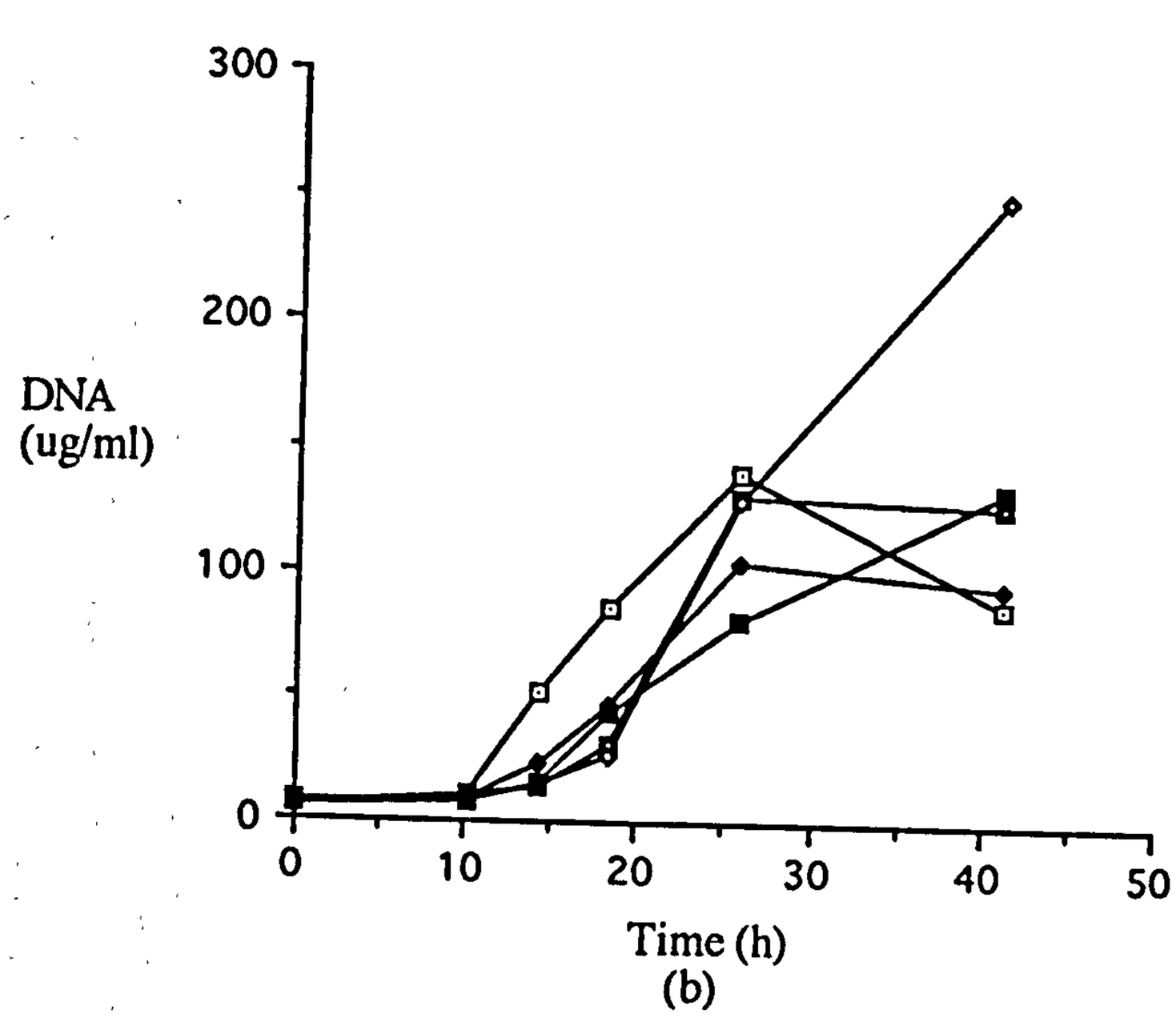
(b)

Fig 3.13 GROWTH OF *S. RIMOSUS* IN MODIFIED HMM CONTAINING GLUCOSE AS THE CARBON SOURCE SUPPLEMENTED WITH TWEEN 20

Growth curves of *S.rimosus*, followed by measuring optical density at 500 nm [Methods 2.6] (a) and DNA concentration [Methods 2.18] (b), grown in modified HMM and 10 mM glucose supplemented with 0.1%, 0.5%, 1% or 2% (v/v) Tween 20


A500
3
2
1
0
0
10
20
30
40
50
Time (h)

(a)


DNA
(ug/ml)
300
200
100
0
0
10
20
30
40
50
Time (h)

(b)

Table 3.6 MEAN GENERATION TIMES FOR *S. RIMOSUS* GROWN IN MODIFIED HMM CONTAINING GLUCOSE AS THE CARBON SOURCE SUPPLEMENTED WITH EITHER TWEEN 20 OR TWEEN 40

MGTs from growth curves of *S.rimosus*, grown in modified HMM and 10 mM glucose supplemented with 0.1%, 0.5%, 1% or 2% (v/v) Tween 20 and Tween 40.

CULTURE CONDITIONS	M.G.T.'S (h)
Glucose control	4.4
Glc / 0.1% Tween 20	5.2
Glc / 0.5% Tween 20	5
Glc / 1.0% Tween 20	4.8
Glc / 2.0% Tween 20	5.4
Glc / 0.1% Tween 40	4.6
Glc / 0.5% Tween 40	4.6
Glc / 1.0% Tween 40	5.2
Glc / 2.0% Tween 40	5

3.6 DISCUSSION

3.6.1 THE ACHIEVEMENT OF REPRODUCIBLE RESULTS FROM STANDARD GROWTH EXPERIMENTS

The growth experiments carried out in 3.2.1, where a suitable nitrogen source was selected, demonstrate a number of the problems encountered at the beginning of this study. The high doubling times of both organisms, especially *S. coelicolor*, were far from ideal. Inoculating flasks with spores did not result in particularly repeatable results and sometimes the lag phase, before growth began, could last for as short a time as 2 h or as long as 24 h. It was difficult to follow growth over long periods of time particularly when it was not known how long the lag phase will last. Another problem with slow growing organisms is that the risk of contamination is increased the longer the growth experiments continue. A faster growing organism with a shorter MGT could be introduced at one of the sampling points and use all the nutrients before the *streptomycete* had a chance to begin growing.

A number of alterations to the existing HMM resulted in reproducible results being achieved from standard growth experiments with *S. rimosus*, provided mycelia were used as an inoculum and the cultures were grown in flasks stirred with a magnetic stirrer, to aid dispersion. The use of 20 mM ammonium sulphate as a nitrogen source allowed increased growth rates and higher yields in both *S. rimosus* and *S. coelicolor*. Unfortunately the use of this nitrogen source caused a decrease in pH levels. It was thought that this would affect the growth of *Streptomyces* and may be detrimental to the production of lipolytic enzymes, which favour neutral pHs. The inclusion of 40 mM MOPS in this medium proved to be effective at buffering the pH at neutral levels. The reduction of the magnesium ion concentration allowed the visual determination of growth because the medium no longer was cloudy, when stirred. The exclusion of Junlon, as a dispersal agent, meant that it was easier to visually and spectrophotometrically follow growth and in addition to this the

medium was now more suitable for studying lipid storage and lipolytic enzyme production. *S. coelicolor* was abandoned as an organism to study as it proved to be unsuitable for the purposes of this investigation with it's poor growth rates, low biomass yields and the need to include polymers for dispersion. *S. rimosus* proved to be a more suitable organism to study, not only because of its better growth qualities, but there is also evidence of lipid utilisation and lipolytic enzyme production. Stirring cultures of *S. rimosus* proved sufficient to provide dispersed growth which could be followed by optical density. The use of mycelia instead of spores as an inoculum proved a more reliable method of inoculating cultures, with the lag phase being restricted to under 2 h or eliminated altogether.

Using this improved growth system, diffuse growth in liquid medium was achieved with an exponential increase in absorbance (at 500 nm) being obtained. The results from the growth of *S. rimosus* under these standard growth conditions, using either glucose, glycerol or mannitol as a carbon source , demonstrate that the growth rates of the filamentous actinomycete *S. rimosus* could be determined accurately from turbidity measurements without the addition of polymers to the culture medium. Growth rates of *Streptomycetes* determined by turbidimetric methods (Flowers and Williams, 1977) ranged from 1 h to 2.8 h in a complex medium. The growth rates that have been determined for *S.rimosus* here range from 2.8 h to 4.2 h, but taking into account that in this case growth was on a defined medium and the differences that will occur between species, the MGTs are fairly similar. Student t tests were carried out on the values obtained for; MGT; DNA:OD and protein:OD ratios and there were no significant differences between growth on glucose, glycerol and mannitol with the probability being greater than 0.1 in all cases. The measurement of growth by following optical density, DNA concentration ($\mu g\ ml^{-1}$) and protein concentration ($mg\ ml^{-1}$) provided growth curves that followed the same basic pattern with the cessation of growth occurring at the same time. The measurement of absorbance at 500 nm was useful as a method of following growth during the experiment,

while the DNA and protein measurements could provide additional information after growth had finished.

3.6.2 GROWTH OF *S.RIMOSUS* ON STANDARD DEFINED GROWTH MEDIA CONTAINING TRIGLYCERIDES AS CARBON SOURCES

Date-seed lipids and date-seed hydrolysate were investigated as carbon and nitrogen sources in the fermentative medium for the formation of oxytetracycline by *Streptomyces rimosus* (Abouzeid and Baeshina, 1992). Date seed lipids in a concentration of 70 mg ml^{-1} were found to be a good carbon source and gave higher titres for the antibiotic than glucose. Animal and vegetable oils have previously been used in oxytetracycline production (Mancy-Courtillet et al, 1959). Peanut oil can be substituted for sucrose in oxytetracycline production (Borensztajin and Wolf, 1955). Engelbrecht and Mach, (1967) found that vegetable oils and fatty acids were better than glucose for oxytetracycline production. However, no growth of *S. rimosus* could be detected in HMM containing triglycerides as the sole carbon source. Evidence of additional growth when triglycerides were used to supplement glucose, as the carbon source, was found. In the presence of both rape seed oil and glucose a higher final concentration of DNA, than was present in the glucose control, was detected. This could have been as a result of experimental variation in the values for DNA concentration or alternatively this could have been due to the presence of the additional carbon source.

To determine whether lipid was being utilised as a carbon source it was decided to measure the disappearance of lipid from the culture medium. A magnetic stirrer, at 300 rpm, was sufficient for the lipid to be dispersed in an emulsion, in the culture medium. Normally, during a growth experiment individual samples were frozen to be processed at a later date. Unfortunately the analysis of thawed, frozen samples gave inaccurate values with a high variation between individual results. A coefficient of variation (CV) was calculated for the

fourteen samples analysed and this was 55%. A students 't' test was carried out to compare the frozen samples data with the chloroform/methanol samples data. A P value of < 0.001 was obtained which means that the difference between the method of storing the samples is very significant. Storing the samples in chloroform/methanol, until they could be processed, gave a mean which was closer to the correct value and a coefficient of variation of 28%. It is not unusual in field experiments for the coefficient of variation to reach 20%, but in laboratory studies something like V = 5-10% should be attainable (Clarke, 1971). Both methods have very high values for CV. However the value for the chloroform/ methanol method is half that of the freezing method, but despite this improvement it is still unacceptable. Many individual samples would have to be taken at each time point in order to determine an accurate value for lipid concentration and this would not be practical for a number of reasons. It is desirable to carry out sampling from growth flasks in as short a time and as few times as possible, in order to reduce the risk of contamination. In addition to this taking a large number of samples at each time point, would decrease the volume of medium in the growth flasks such that growth would be affected.

Sampling and storage were responsible for the great majority of errors introduced. However, it was decided to determine whether handling of the samples during the actual lipid assay could introduce errors. It was thought that a positive displacement pipette would be a more accurate method, than Gilson pipettes, of handling samples containing lipid. Using a positive displacement pipette in the assay reduces the coefficient of variation from 11% to 5%. However, considering the errors that occur early on in the whole process, in sample sampling and storage, this result is purely academic.

3.6.3 GROWTH OF *S.RIMOSUS* ON STANDARD DEFINED GROWTH MEDIA SUPPLEMENTED WITH POLYOXYETHYLENE ESTERS

Concentrations of 0.5% (v/v) Tween 20 and 40, and above, lead to an additional, slower phase of growth that does not occur in the glucose control or with 0.1% (v/v) Tween. This would imply that the Tween is being utilised as a carbon source. However the presence of glucose is required before growth can begin. Growth on Tween implies the presence of extracellular lipolytic enzymes, which would have to enter the culture medium and hydrolyse the Tween into polyoxyethylene-sorbitan and fatty acid. These cultures were further analysed for their lipolytic enzyme content and this is dealt with in section 4.6.2.

CHAPTER 4

LIPOLYTIC ENZYMES

RESULTS AND DISCUSSION

4.0 LIPOLYTIC ENZYMES FROM *S. RIMOSUS*

4.1 INTRODUCTION

One of the aims of this project was to grow *S.rimosus*, in liquid culture, utilising triglycerides as a carbon source. As the initial event in the utilisation of triglycerides as an energy source is hydrolysis by lipase, the presence of lipolytic activity in cultures of *S.rimosus* was assessed. Many lipases from microorganisms, animals and plants have been purified and their properties investigated but unfortunately there is little information about lipid degradation by the *Streptomycetes*, directly relevant to growth in the presence of triglyceride. Microbial lipases have a wide range of biotechnological applications (Björkling *et al.*, 1991), and those produced by the streptomycetes might add enzymes with novel characteristics. Understanding the mechanisms that regulate lipase synthesis in these organisms might also help in improving the utilization of different oils used as carbon sources in many industrial fermentations. The majority of the work carried out on microbial lipases has concentrated on fungi, many species of which provide commercially useful lipases. Most of these are inducible extracellular enzymes, which are synthesised within the cell, exported to the external surface and secreted into the culture medium (Shabtei and Daya-Mishne, 1992). Recently the industrial demand for highly active preparations of lipases has stimulated the search for new enzyme sources. As a consequence of this recent demand three lipases have been found with high activity; from *Streptomyces* sp. PCM 33, *Streptomyces* sp. PCM 27 and *Streptomyces fradiae* NCiB 82 33 (Sztajer *et al.*, 1988). A strain of *S.rimosus* (IAW 116) was included in this study but no lipolytic activity could be detected. However, Engelbrecht and Mach, (1968) isolated and characterised an extracellular lipase from *S.rimosus* and there are reports of *S.rimosus* strains being able to utilise lipids as energy sources (Orlova, 1961 and 1962), which would imply the presence of lipases.

The routine assay used in these studies to detect lipase activity was a photometric assay employing the chromogenic substrate ρ-nitrophenyl palmitate. Initially the presence of

an intracellular lipolytic activity was detected, at low levels (0.02 EU mg protein^{-1}), in cultures of *S.rimosus* grown in YEME medium . The successful choice of growth conditions which gave increased intracellular lipolytic activity also resulted in the detection of an additional extracellular lipolytic activity in the culture supernatant. This chapter deals with a characterisation of these two enzyme activities, in crude extracts, and a comparison of their properties. In addition to this, the effect of supplementing the growth medium with polyoxyethylene esters on lipase production was studied, in an attempt to overcome the practical problems of growth on insoluble lipid substrates.

4.2 CHARACTERISATION OF THE INTRACELLULAR LIPASE ACTIVITY

Using the standard lipase assay (Methods 2.31), whole washed cells from *S.rimosus* were assayed. No lipolytic activity could be detected until the cells were broken by sonication. Samples were sonicated according to Methods 2.8 and spun according to Methods 2.10. The resulting supernatant was assayed for lipase activity

The production of ρ-nitrophenol from ρ-nitrophenyl palmitate by the action of the enzyme was linear from 0 to 4 minutes under standard conditions (Fig. 4.1). Therefore the enzyme activity was always calculated within this period. The rate of production of ρ-nitrophenol was proportional to the amount of protein up to 2.5 mg protein per assay (Fig. 4.2). Therefore protein concentrations within this range were used in the assay.

4.2.1 THE EFFECT OF pH

The pH of the standard ρ-nitrophenyl palmitate assay was varied over the range pH 6.5 - 10.0 by using a variety of 50 mM buffers at different pH values. The maximum intracellular lipase activity was found to occur at pH 8.5 [Fig. 4.3(a)].

4.2.2 THE EFFECT OF ASSAY TEMPERATURE

The assay temperature of the standard assay was varied from 19°C to 65°C to determine an optimum temperature for the lipase assay. The maximum temperature used was limited to 65°C, the Kraft point for Triton X-100 (the detergent used in the assay mix) [Houslay and Stanley, 1984]. The maximum enzyme activity for the intracellular lipase was observed at 45°C with a second smaller activity peak detected at 60°C [Fig. 4.4(a)]. As this result seemed unusual the procedure was repeated five times to confirm it's authenticity.

The melting point of ρ-nitrophenyl palmitate is between 65°C and 66°C which is very close to the maximum activity peaks found at 60°C. Because of the previous unexpected result, the experiment was repeated with alternative substrates, to determine whether the melting point of the substrate was affecting the activity. The shorter chain ρ -nitrophenyl myristate, with a melting point of 51°C was used in exactly the same experiment as above. Again the intracellular enzyme was found to produce two peaks corresponding to maximum activity, one at 45°C and the other at 60°C (Fig. 4.5). The longer chain ρ-nitrophenyl stearate was also used in a similar experiment but it was found that this substrate came out of solution above 41°C so no conclusive results could be obtained using this substrate [Fig. 4.6(a)]. The ρ-nitrophenyl stearate behaved differently from the other two substrates used, despite being used at the same concentration.

4.2.3 THERMAL STABILITY

The effect of temperature on the stability of the lipase activity was measured over a period of 90 min, by incubating extracts of the enzyme at various temperatures. After 90 min incubation at 50°C, 60°C, 70°C and 90°C, the intracellular enzyme lost 7%, 53%, 79% and 92% of its activity respectively [Fig. 4.7(a)]. The intracellular enzyme

lost activity within 20 minutes of incubation at 90°C. Incubating denatured samples on ice for up to 30 min did not allow the recovery of enzyme activity.

4.2.4 THE EFFECT OF THE ADDITION OF METAL IONS

The effect of a range of metal ions on the intracellular lipase activity was investigated by adding solutions of metal salts to give a final concentration of 10 μM in the standard lipase assay. Table 4.1 gives a list of the metal salts added and the results achieved. Fe^{2+} and Fe^{3+} ions inhibited the activity by 70%. Another metal ion that proved to be a potent inhibitor was Zn^{2+}, with the intracellular enzyme losing approximately 30% of its activity. With the exception of Ca^{2+} ions, which gave an increase of approximately 20% in intracellular activity, the other ions tested had very minor effects. The addition of the metal chelator, EDTA resulted in a slight inhibition with a decrease of less than 12%.

4.2.5 THE EFFECT OF SUBSTRATE CHAIN LENGTH

The standard lipase assay was carried out as described in Methods 2.31 with the exception that the standard ρ-nitrophenyl palmitate substrate was replaced, at the same concentration, by a number of alternatives: ρ-nitrophenyl acetate (2), ρ-nitrophenyl butyrate (4), ρ-nitrophenyl caprylate (8), ρ-nitrophenyl caprate (10), ρ-nitrophenyl laurate (12), ρ-nitrophenyl myristate (14) and ρ-nitrophenyl stearate (18), [number of carbons shown in brackets]. Fig. 4.8(a) gives the results obtained when the intracellular activity was assayed using this range of substrates. The intracellular crude extract showed two major peaks of activity occurring with the four carbon substrate, ρ-nitrophenyl butyrate and the ten carbon substrate, ρ-nitrophenyl caprate. The intracellular enzyme showed very little activity with the acetate ester.

4.3 CHARACTERISATION OF THE EXTRACELLULAR LIPASE ACTIVITY

An extracellular lipase activity was found in the culture supernatant. This activity was characterised and compared with the intracellular activity. In order for this to be carried out the extracellular enzyme activity was concentrated (Methods 2.12), resulting in a ten fold concentration. The methods employed in characterising the extracellular enzyme activity were identical to those used in studying the intracellular activity.

4.3.1 THE EFFECT OF pH

The pH of the standard assay was varied using the same buffers as used in the studies on the intracellular enzyme (see section 4.2.1). The maximum extracellular lipase activity was found to occur at pH 8.0 [Fig. 4.3(b)].

4.3.2 THE EFFECT OF ASSAY TEMPERATURE

These studies were carried out using exactly the same method as used for the intracellular enzyme. The maximum enzyme activity for the extracellular lipase was found to be 60°C [Fig. 4.4(b)] under the specific conditions of these experiments. No peak of activity was detected at 45°C.

Temperature studies were carried out as in section 4.2.2, with ρ-nitrophenyl myristate replacing ρ-nitrophenyl palmitate in the standard lipase assay. As with ρ-nitrophenyl palmitate the extracellular enzyme reached a maximum activity at 60°C [Fig. 4.5(b)]. No conclusive results could be obtained using ρ-nitrophenyl stearate as a substrate in the assay (see section 4.2.2) [Fig. 4.6].

4.3.3 THERMAL STABILITY

After 90 min incubation, at 50°C, 60°C, 70°C and 90°C, the extracellular activity lost 5%, 50%, 55% and 97% of it's activity respectively [Fig. 4.7(b)]. These results were similar to those obtained for the intracellular activity, except that the extracellular was somewhat more stable at 70°C.

4.3.4 THE EFFECT OF THE ADDITION OF METAL IONS

The effect of a range of metal ions on the extracellular lipase activity was investigated by adding solutions of metal salts to give a final concentration of 10 μM in the standard lipase assay (Table 4.1). In contrast to the intracellular activity Zn^{2+} ions had the largest effect on the extracellular activity with a 50% decrease in activity occurring. Fe^{3+} and Fe^{2+} ions gave a smaller reduction (less than 20%), differing from their effect on the intracellular where an inhibition of 70% occurred. None of the other ions tested gave significant increases or decreases in activity. The addition of EDTA resulted in a slight inhibition with a decrease in activity of less than 20%, which was similar to the effect seen on the intracellular activity.

4.3.5 THE EFFECT OF SUBSTRATE CHAIN LENGTH

The standard lipase assay was carried out as described in Methods 2.31 with the exception that the standard ρ-nitrophenyl palmitate substrate was replaced by a number of alternatives: ρ-nitrophenyl acetate (2), ρ-nitrophenyl butyrate (4), ρ-nitrophenyl caprylate (8), ρ-nitrophenyl caprate (10), ρ-nitrophenyl laurate (12), ρ-nitrophenyl myristate (14) and ρ-nitrophenyl stearate (18), [number of carbons shown in brackets] [Fig. 4.8(b)]. The extracellular crude extract gave one major peak of activity corresponding to the twelve carbon substrate ρ-nitrophenyl laurate, in contrast to the intracellular extract which gave two major peaks of activity occurring with the four

Fig.4.1 THE EFFECT OF TIME ON THE STANDARD LIPASE ASSAY

This figure demonstrates the effect of time on the standard lipase assay (Methods 2.31). Lipase activity was measured over a period of 4 min and the results are expressed in EU.

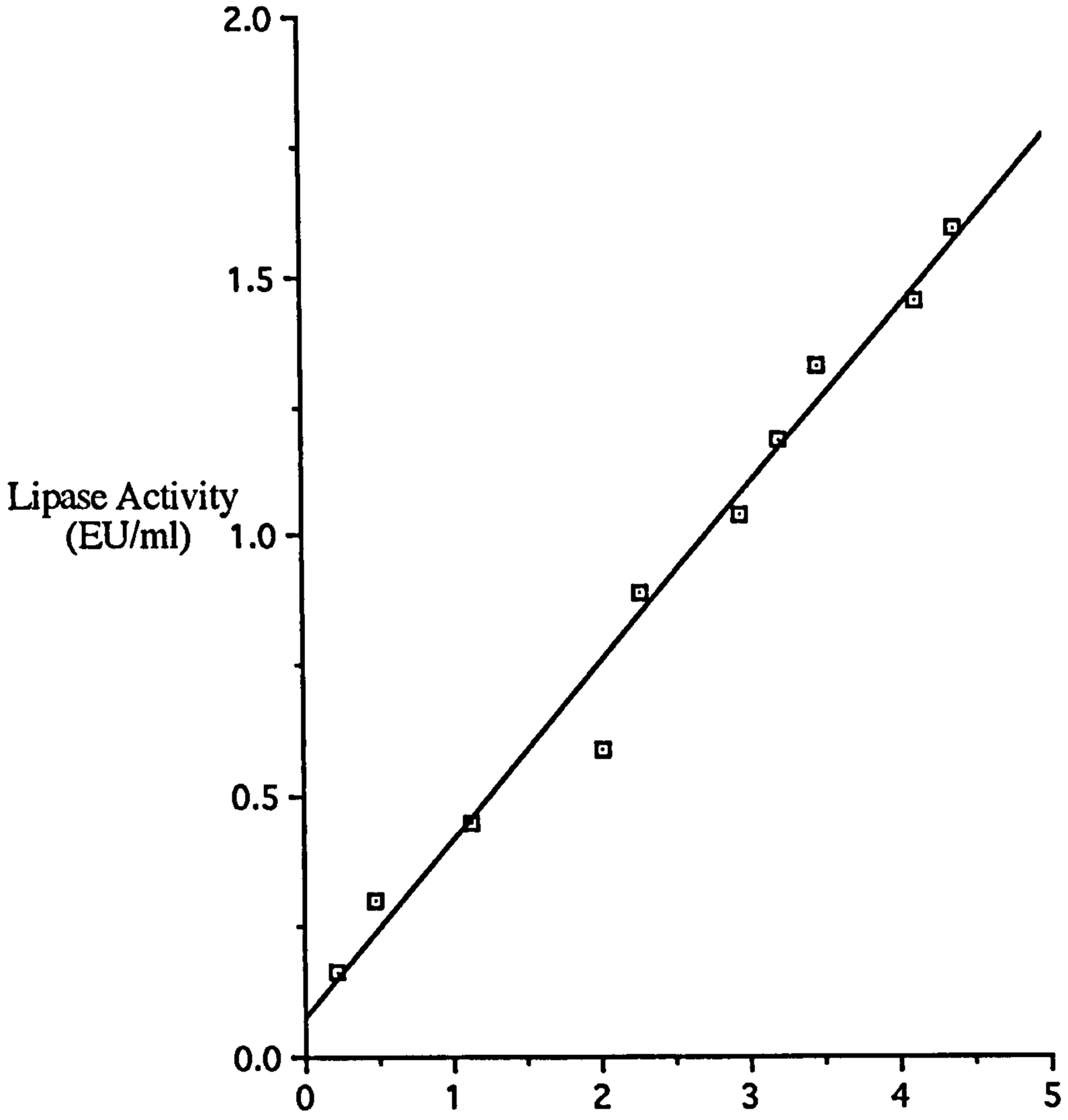

2.0
1.5
1.0
0.5
0.0
Lipase Activity
(EU/ml)
0
1
2
3
4
5
Time (min)

Fig.4.2 THE EFFECT OF PROTEIN CONCENTRATION ON THE STANDARD LIPASE ASSAY

The effect of protein concentration on the standard ρ-nitrophenyl palmitate assay (Methods 2.31), used in the detection of lipase activity, was measured using a range of protein concentrations. Lipase activity is expressed in EU.

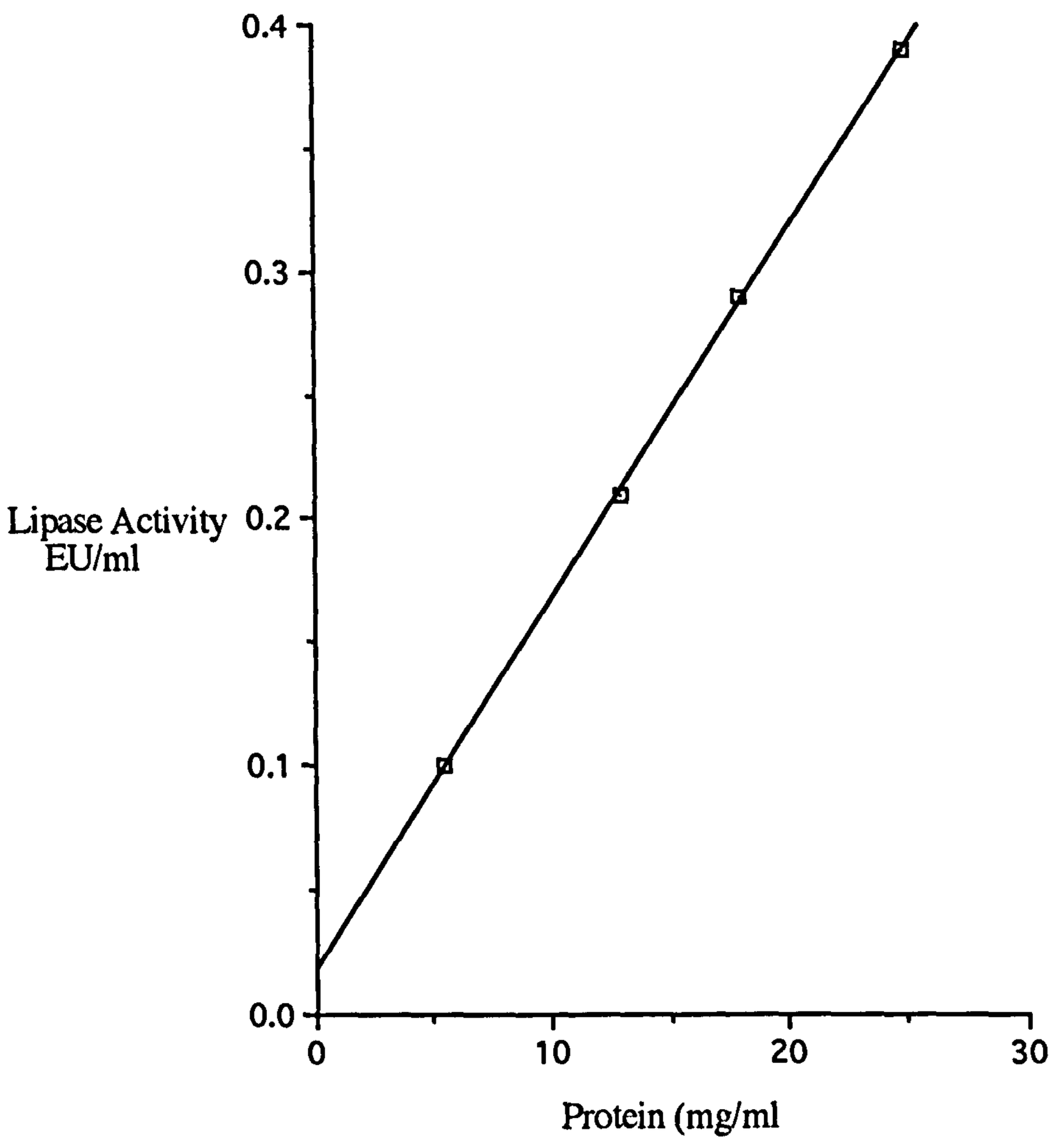
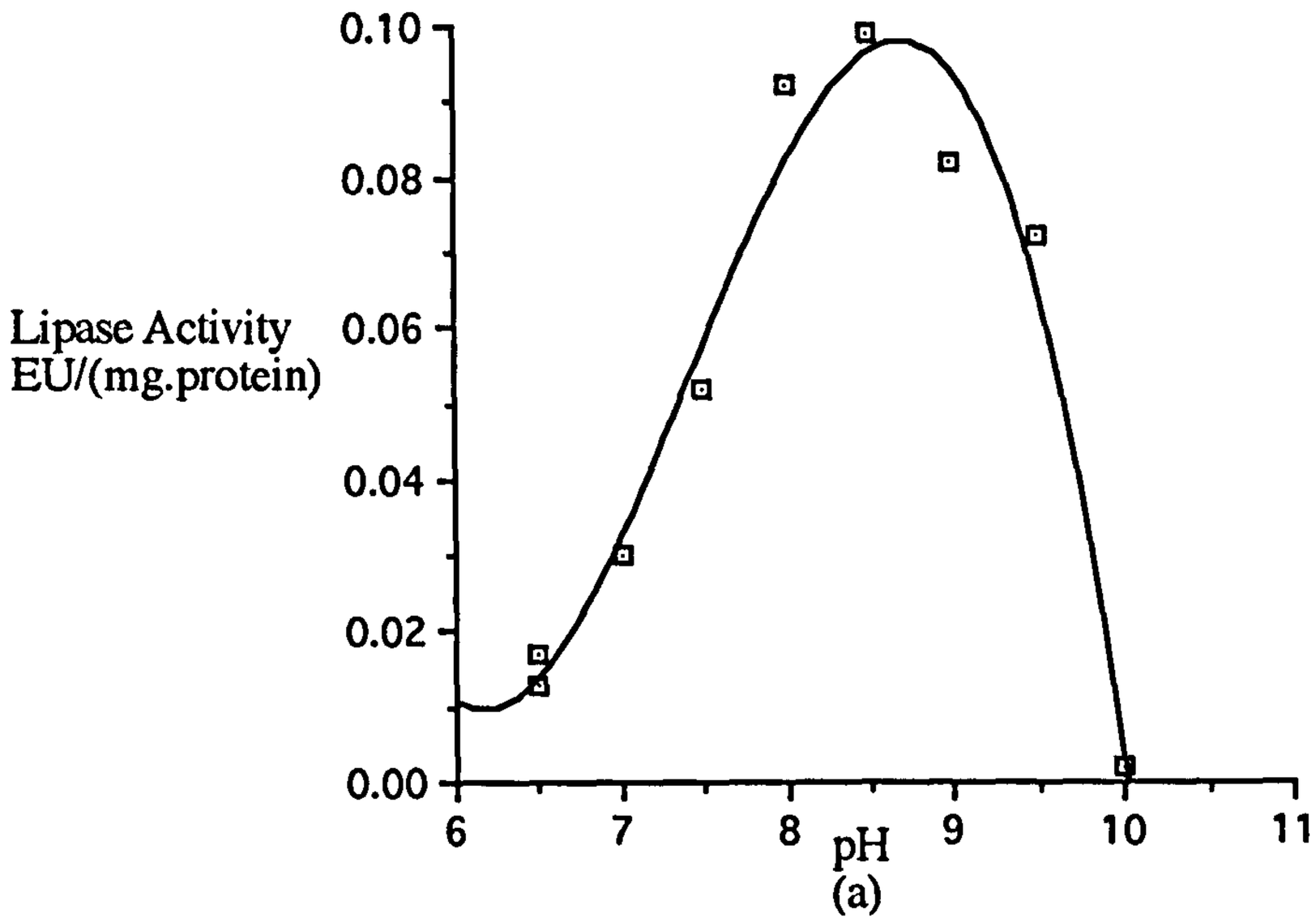

Lipase Activity
EU/ml
0.4
0.3
0.2
0.1
0.0
0
10
20
30
Protein (mg/ml

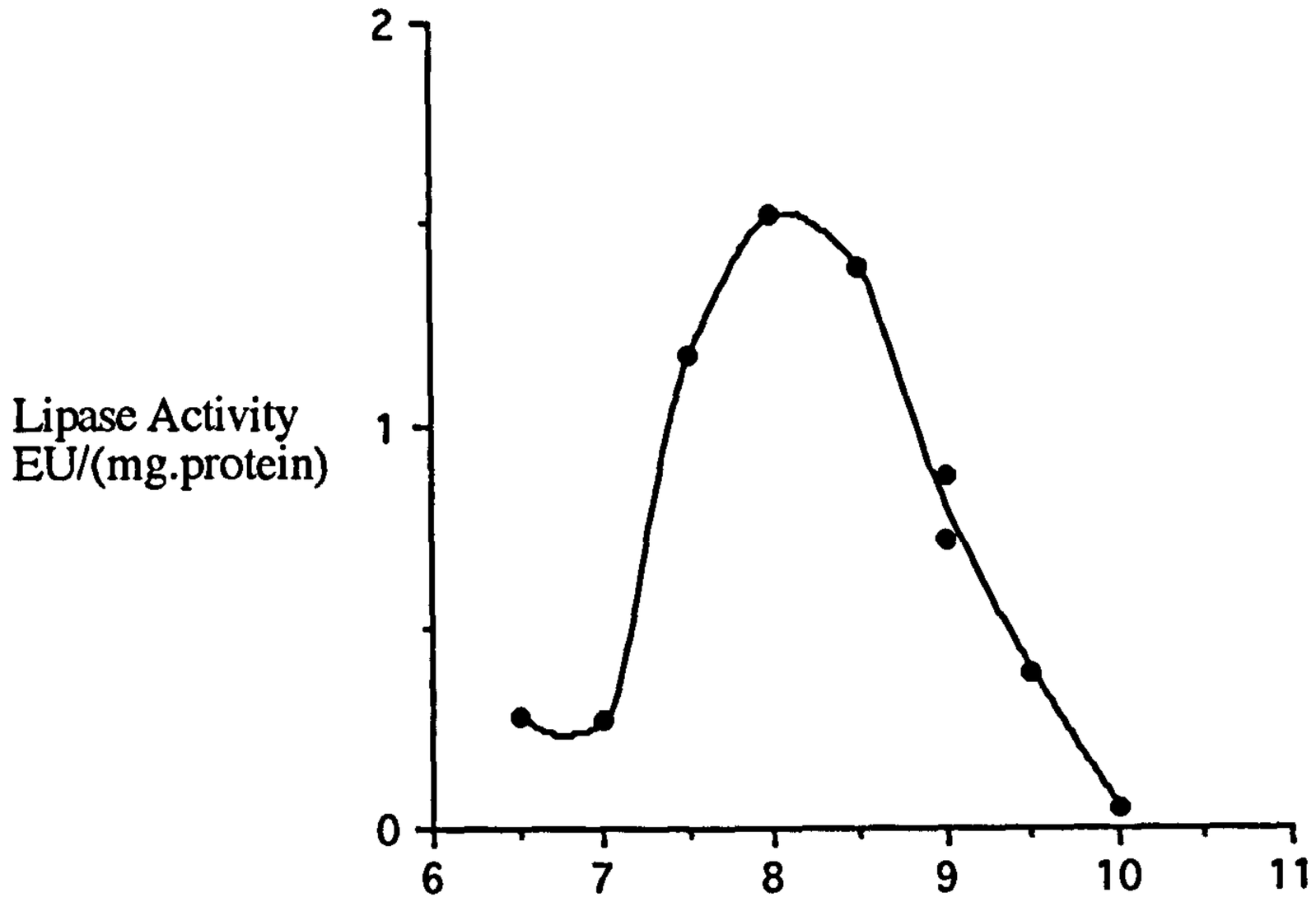
Fig.4.3 THE EFFECT OF pH ON INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

The effect of pH on (a) intracellular lipase activity and (b) extracellular lipase activity. The lipase activity was determined using the standard ρ-nitrophenyl palmitate assay (Methods 2.31) with a range of 50 mM buffers at different pH values (listed below). The lipase activity is expressed as EU mg protein^{-1}

Buffer	pH
Mes - NaOH	6.5
Phosphate - NaOH	6.5, 7.0, 7.5
Tris - HCl	8.0, 8.5, 9.0
Tetrasodiumpyrophosphate	9.0, 9.5, 10.0


Lipase Activity
EU/(mg.protein)
0.10
0.08
0.06
0.04
0.02
0.00
6
7
8
9
10
11
pH

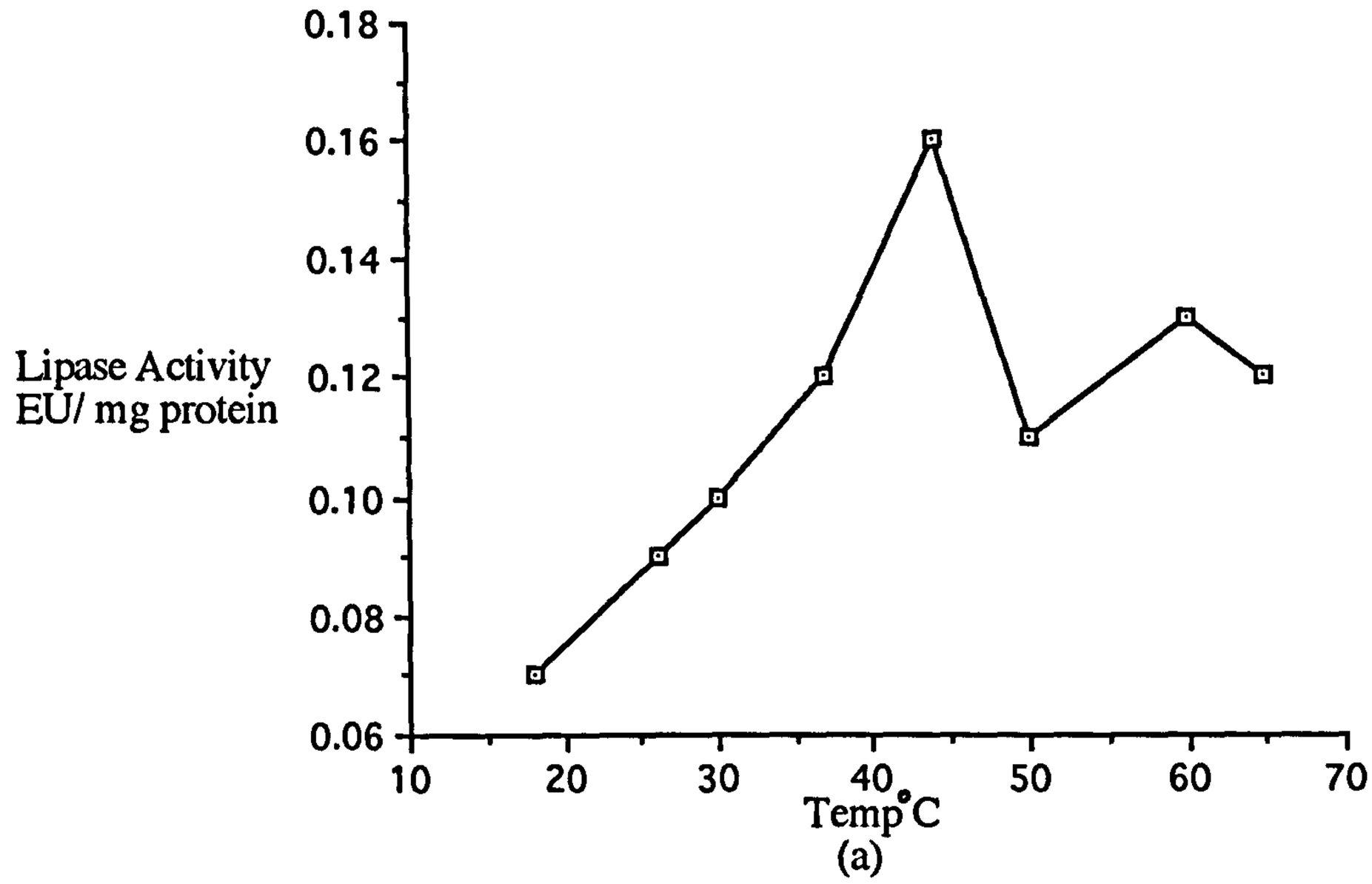
(a)


Lipase Activity
EU/(mg.protein)
2
1
0
6
7
8
9
10
11
pH

(b)

Fig.4.4 THE EFFECT OF ASSAY TEMPERATURE ON INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

The effect of assay temperature on (a) intracellular lipase activity and (b) extracellular lipase activity. The lipase activity was determined using the standard ρ-nitrophenyl palmitate substrate (Methods 2.31), incubated at a range of assay temperatures (19°C - 65°C). Lipase activity is expressed as EU mg protein^{-1}.


0.18
0.16
0.14
0.12
0.10
0.08
0.06
Lipase Activity
EU/ mg protein
10
20
30
40
50
60
70
Temp°C
(a)

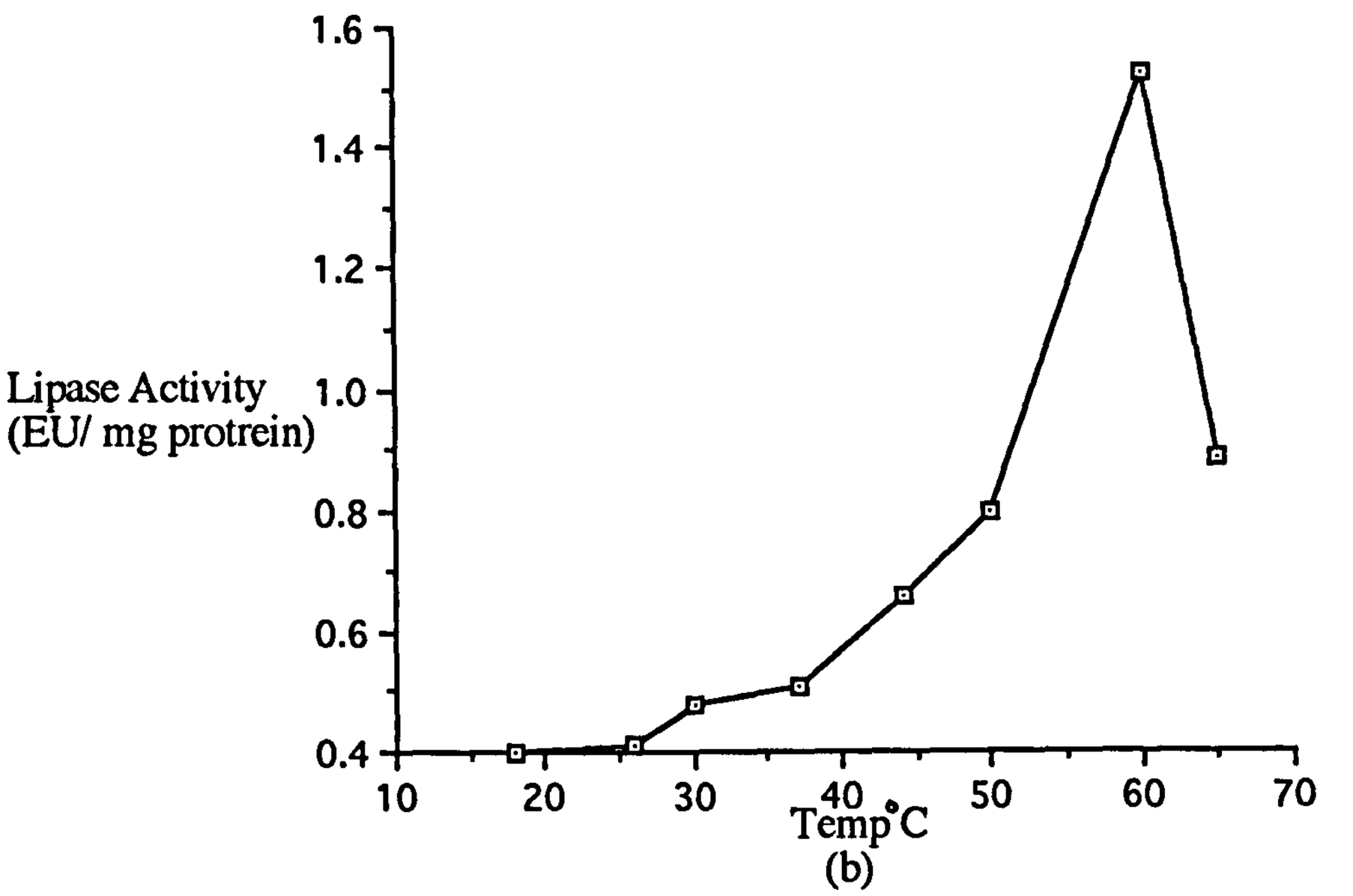

1.6
1.4
1.2
1.0
0.8
0.6
0.4
Lipase Activity
(EU/ mg protrein)
10
20
30
40
50
60
70
Temp°C
(b)

Fig.4.5 THE EFFECT OF ASSAY TEMPERATURE ON LIPASE ACTIVITY USING AN ALTERNATIVE SUBSTRATE IN THE STANDARD ASSAY

The effect of assay temperature on intracellular (-□) and extracellular (-●-) lipase activities using ρ-nitrophenyl myristate as the substrate, incubated at a range of assay temperatures in the standard lipase assay (Methods 2.31). Lipase activity is expressed as μmoles min^{-1}.

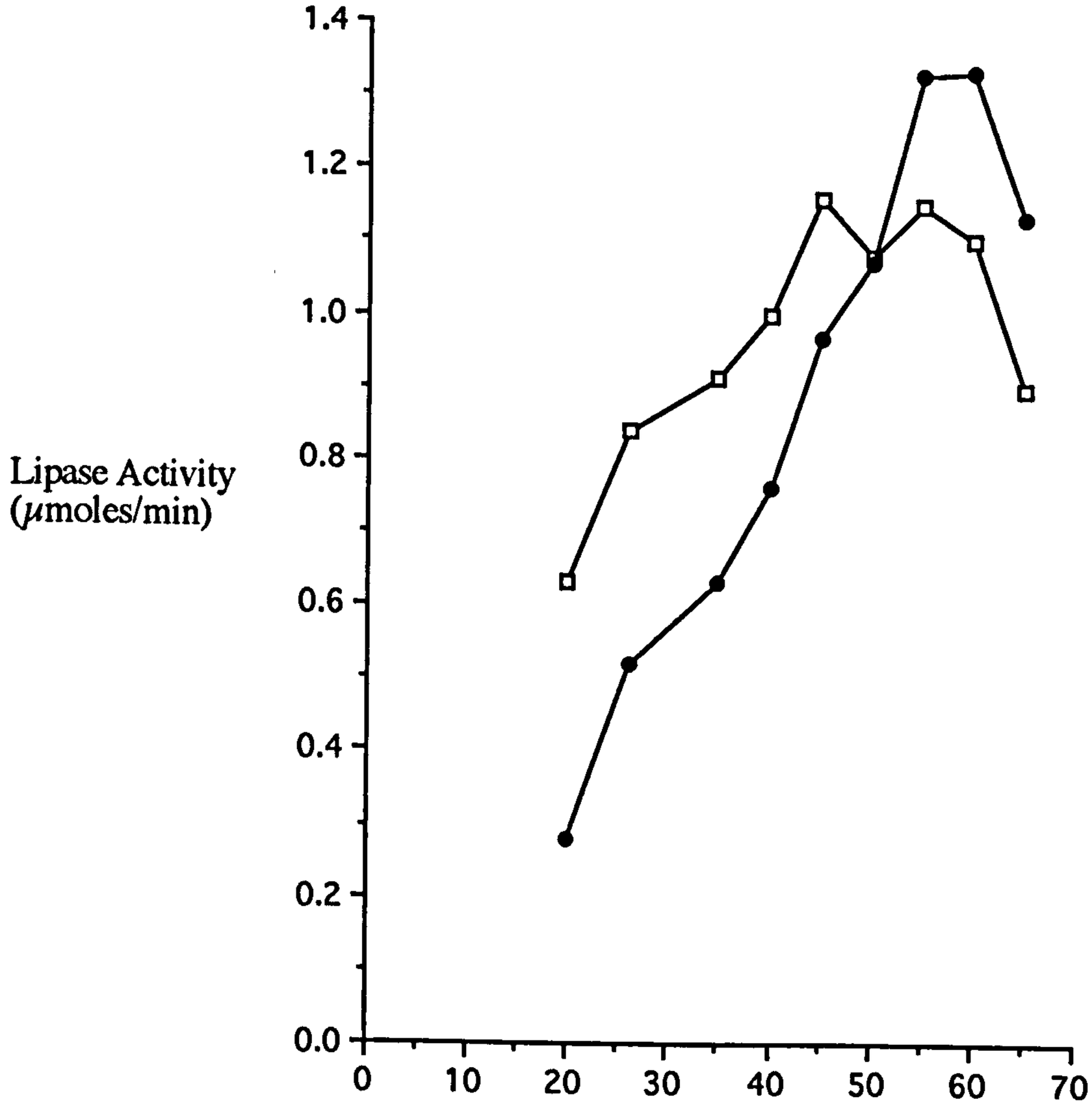
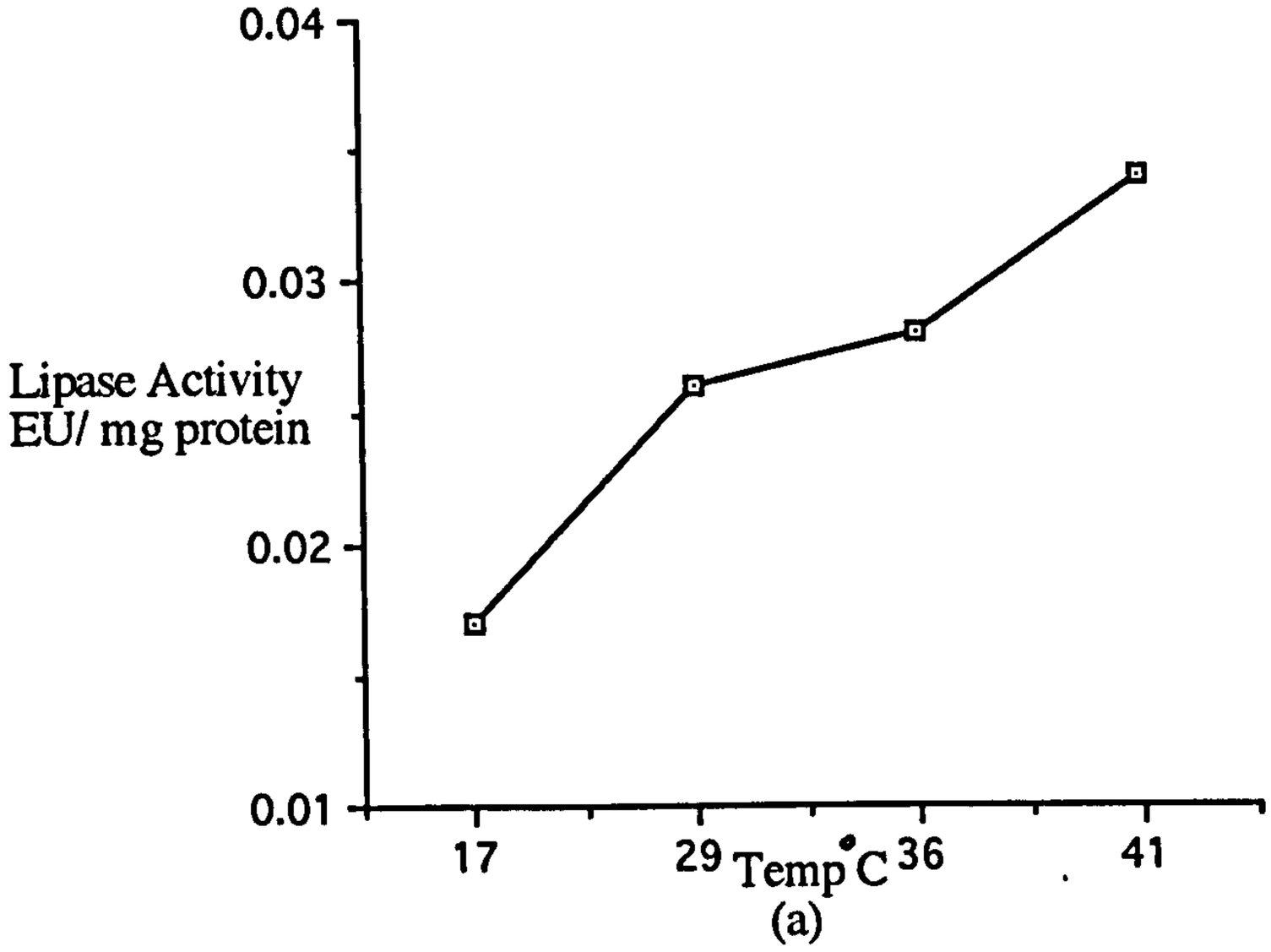
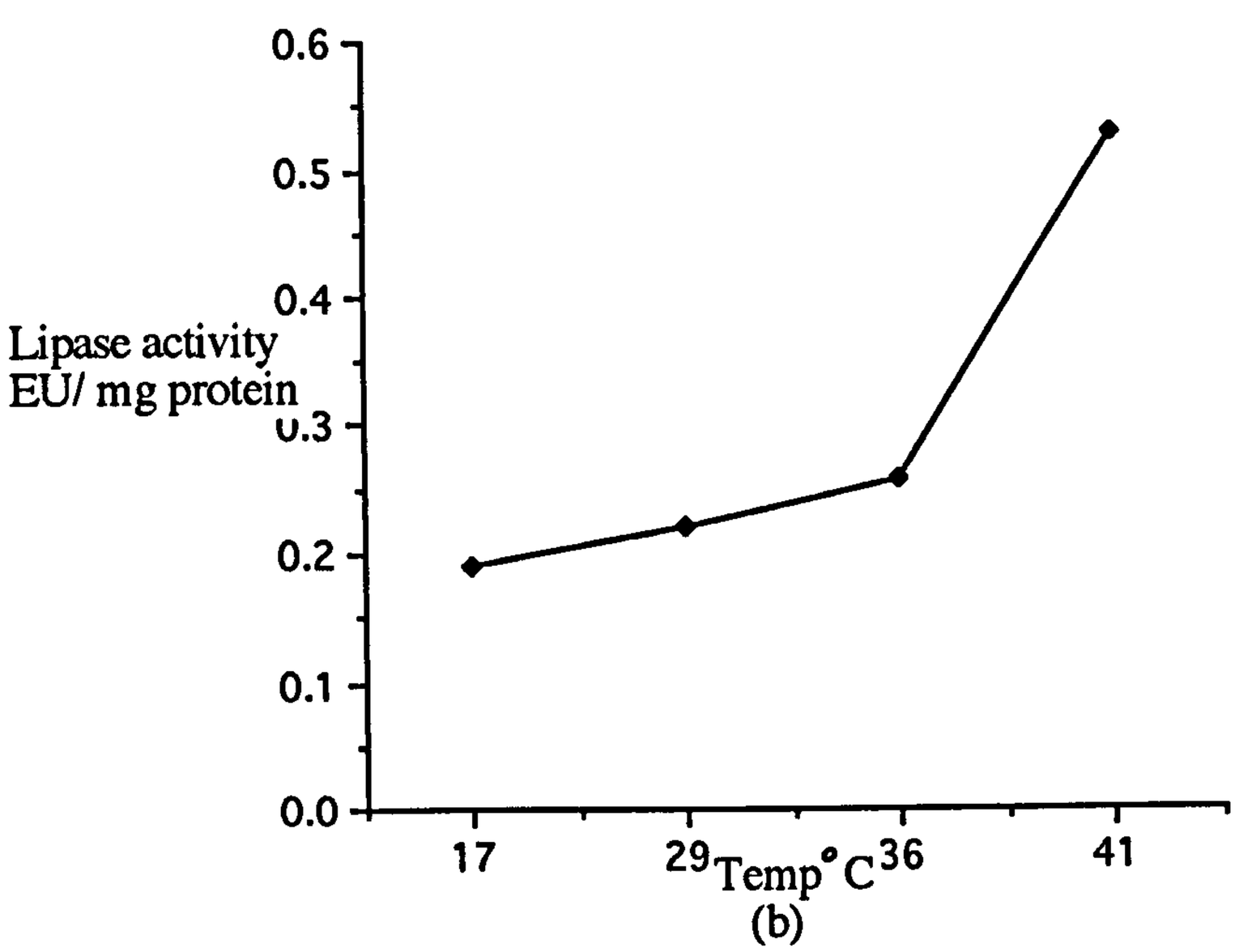

Lipase Activity
(μmoles/min)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Temp°C

Fig.4.6 THE EFFECT OF ASSAY TEMPERATURE ON LIPASE ACTIVITY USING ρ-NITROPHENYL STEARATE IN THE STANDARD ASSAY

The effect of assay temperature on the intracellular (a) and extracellular (b) lipase activities using ρ-nitrophenyl stearate in the standard assay (Methods 2.31), incubated at a range of assay temperature. Lipase activity is expressed in EU mg protein^{-1}.


0.04
0.03
Lipase Activity
EU/ mg protein
0.02
0.01
17
29
Temp°C
36
41

(a)


0.6
0.5
0.4
Lipase activity
EU/ mg protein
0.3
0.2
0.1
0.0
17
29
Temp°C
36
41

(b)

Fig.4.7 THERMAL STABILITY OF THE INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

Extracts of both enzymes were incubated at four different temperatures; 50°C, 60°C, 70°C and 90°C. Samples were taken over a period of 90 min and assayed for lipase activity (Methods 2.31) which is expressed as EU mg protein^{-1}.

(a) Thermal stability of the intracellular enzyme

50°C

60°C

70°C

90°C

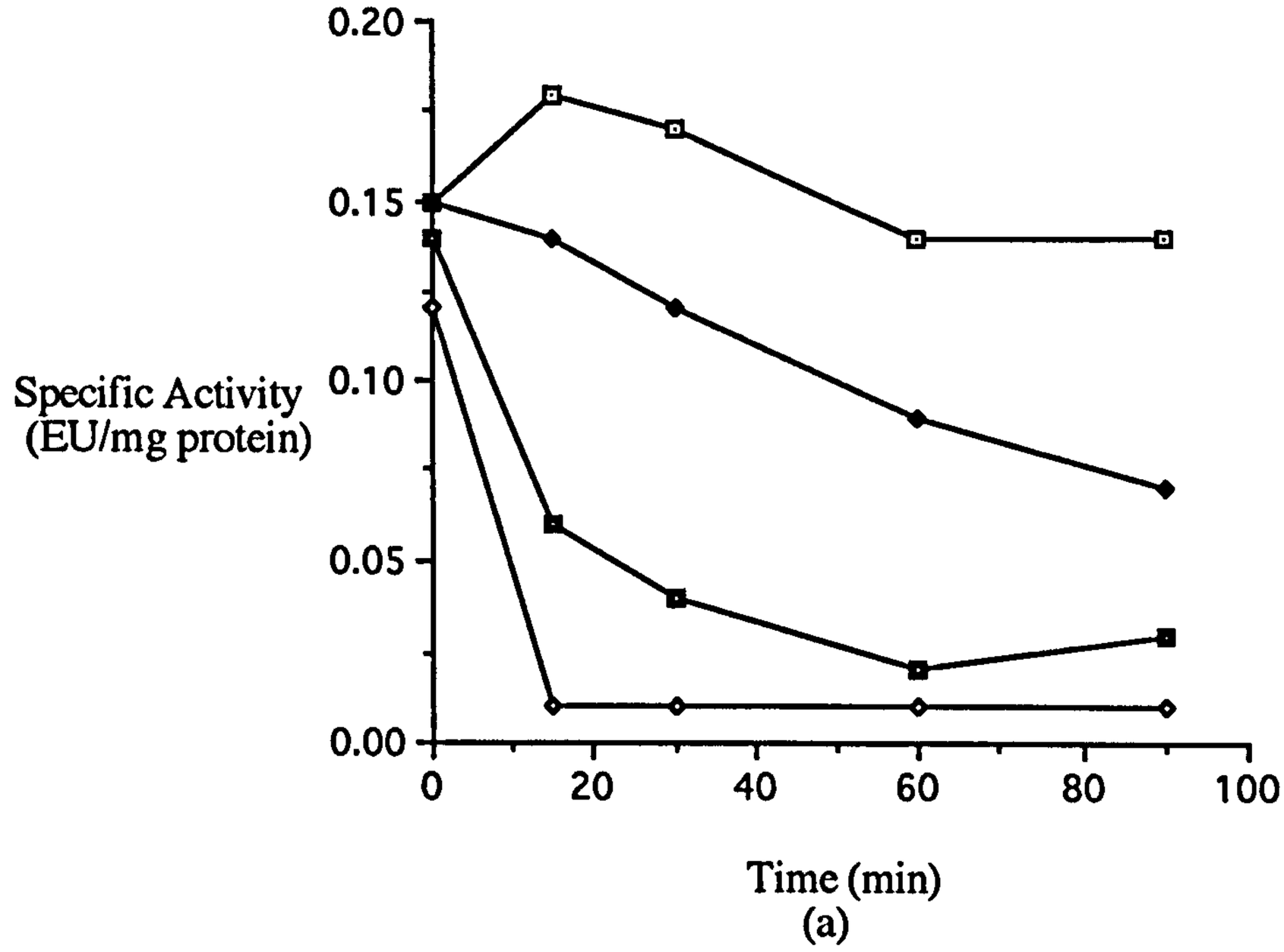
(b) Thermal stability of the extracellular enzyme

50°C

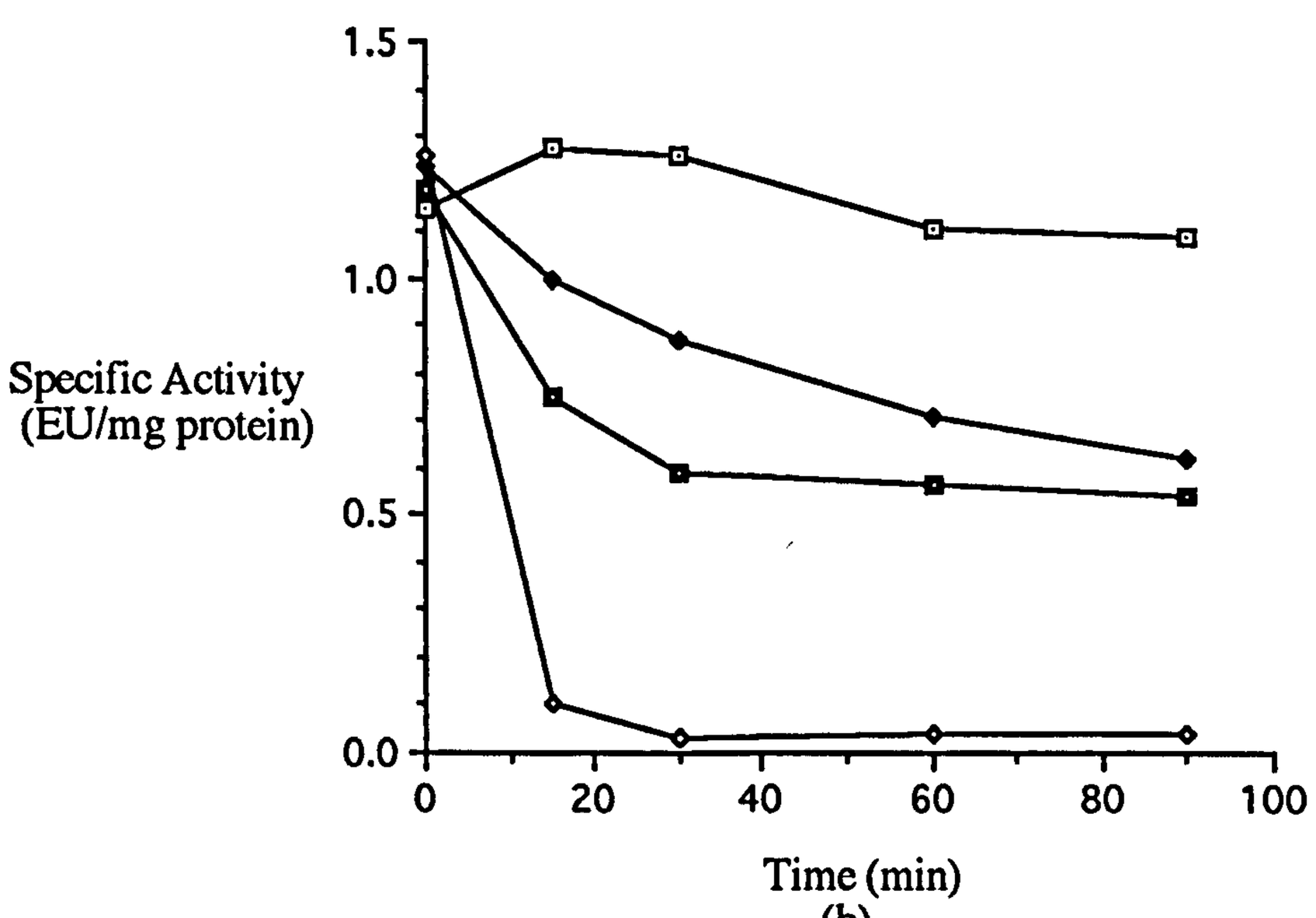
60°C

70°C

90°C


0.20
0.15
0.10
0.05
0.00
Specific Activity
(EU/mg protein)
0
20
40
60
80
100
Time (min)

(a)


1.5
1.0
0.5
0.0
Specific Activity
(EU/mg protein)
0
20
40
60
80
100
Time (min)

(b)

Table 4.1 THE EFFECT OF THE ADDITION OF METAL SALTS ON THE INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

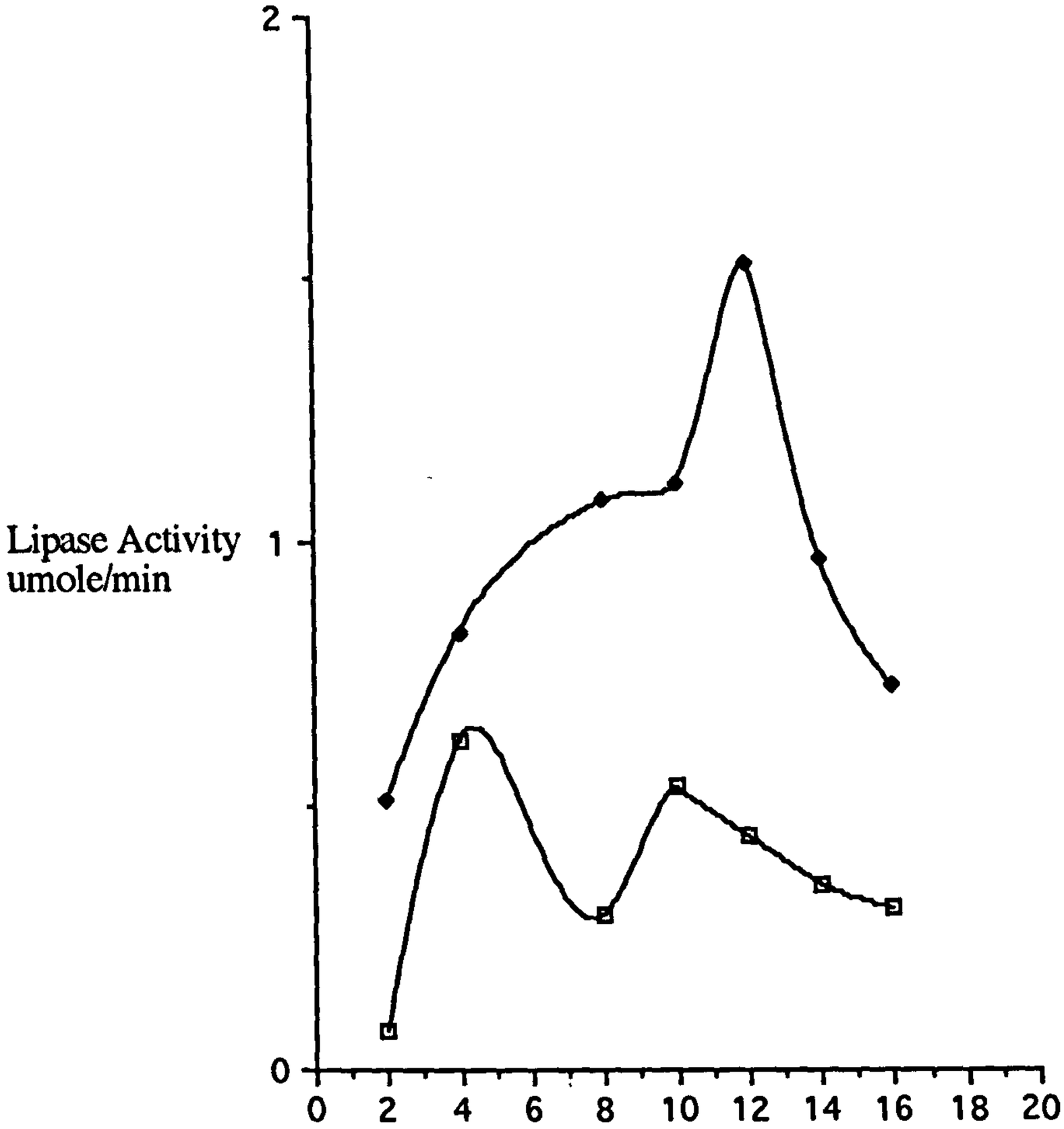
The effect of a range of metal ions on the two lipase activities was investigated by adding solutions of metals to give a final concentration of 10 μM in the standard lipase assay (Methods 2.31). The metal salts are listed and their effects are expressed in terms of percentage inhibition or activation.

Metal salt	Intracellular activity		Extracellular activity	
	Activation %	Inhibition %	Activation %	Inhibition %
Ferric sulphate		69		12
Ferrous chloride		78		17
Cobalt (III) sulphate	8			9
Cobalt chloride	14			8
Calcium chloride	17		7	
Calcium nitrate	19		4	
Magnesium acetate	9		9	
Magnesium sulphate	7			0
Magnesium chloride	10			7
Zinc sulphate		31		50
Zinc acetate		26		44
Potassium nitrate		4		8
Potassium sulphate	2			0
Potassium acetate		14	7	
Barium chloride		3		8
Manganese sulphate	5			14
EDTA		12		19

Fig.4.8 THE EFFECT OF ALTERING THE SUBSTRATE CHAIN LENGTH IN THE STANDARD LIPASE ASSAY

The standard lipase assay was carried out as described in Methods 2.31, with the exception that the ρ-nitrophenyl substrate was replaced by a number of alternatives with a different fatty acid chain length [listed below]. Results are expressed as μmoles $min.^{-1}$, (a) intracellular activity ▫ and (b) extracellular activity • .

Substrate	Carbon chain length
ρnp acetate	2
ρnp butyrate	4
ρnp caprylate	8
ρnp caprate	10
ρnp laurate	12
ρnp myristate	14
ρnp palmitate	16
ρnp stearate	18


Lipase Activity
umole/min
2
1
0
0
2
4
6
8
10
12
14
16
18
20
No of carbons

carbon substrate, ρ-nitrophenyl butyrate and the ten carbon substrate, ρ-nitrophenyl caprate. The extracellular enzyme showed activity with the acetate ester.

4.4 PHYSICAL PROPERTIES OF THE LIPASE ACTIVITIES

4.4.1 ATTEMPTS AT DETERMINATION OF MOLECULAR WEIGHT

4.4.1.1 DETERMINATION OF Mr USING HPLC

Concentrated culture supernatant was analysed by HPLC to determine the Mr for the extracellular enzyme [Methods 2. 32(a)]. Protein concentration was measured and lipase activity was assayed. Fig. 4.9(a) shows the protein profile and Fig. 4.9(b) shows the two peaks of lipase activity which were detected at 12 and 22 min. The first activity peak corresponded to the void volume of the column with an approximate Mr of 200 KDa or greater and the second peak corresponds to a Mr of approximately 4500 Da. This second peak, which has an Mr of value too small to be a protein, maybe as a result of interactions between the activity and the column and thus does not present an accurate Mr.

4.4.1.2 DETERMINATION OF Mr USING AN ACA 34 GEL FILTRATION COLUMN

Concentrated culture supernatant was used to determine the Mr, for the extracellular enzyme, by gel filtration [Methods 2.32(b)]. Fractions were analysed for protein concentration and assayed for lipase activity. Two protein peaks were detected at; 58 ml and 148 ml [Fig. 4.10(a)]. The lipase activity detected appeared in the void volume with an approximate Mr of 2000 KDa or greater (Fig. 4.10(b)). Interaction between the lipase and other components of the extract, which artificially increased the Mr to a value much larger than the protein, were suspected. 1% Triton X-100 (v/v) was added to both the samples and the running buffer in an attempt to prevent these interactions, but unfortunately no

lipase activity could be detected in any of the fractions collected from the column, including the void volume, under these conditions.

4.4.2 ULTRACENTRIFUGATION OF CRUDE EXTRACTS CONTAINING LIPASE ACTIVITY

Both the extracellular and intracellular lipase preparations were spun at 40 K (Methods 2.14) and the resulting pellets and supernatants were assayed for activity using either ρ-nitrophenyl palmitate, ρ-nitrophenyl caprate and ρ-nitrophenyl butyrate as substrates in the standard lipase assay. The purpose of this experiment was to compare the distribution of the two enzyme activities between the resulting pellet and supernatant fractions. Without exception, the majority of the activity was found in the pellet from the spin at 40 K for both the intracellular and extracellular lipase preparations. The results, expressed in Specific Activity (EU mg protein^{-1}) are given in Table 4.2. This experiment reinforces the idea that the enzymes have a large Mr as the data suggests that the enzymes are not soluble enzymes, but particulate enzyme activities.

4.5 EFFECTS ON LIPASE ACTIVITIES

4.5.1 THE EFFECT OF INHIBITORS ON THE INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES.

4.5.1.1 THE EFFECT OF REAGENTS WHICH REACT WITH AN ACTIVE SITE SERINE ON LIPASE ACTIVITY

1 ml volumes of extracts of intracellular and extracellular enzymes were incubated, on ice, with 500 µl of PMSF, dissolved in 2 - propanol / 50 mM KH_2PO_4 pH 7.5 (2:1) (Turini *et al.*, (1969) to give final concentrations of 1, 2, 5, or 10 mM. Enzyme activity was measured from aliquots taken at various time intervals over a period of 120 min. A control experiment was carried out which involved the addition of 500 µl of 50 mM

KH_2PO_4 buffer/ propanol in the absence of inhibitor. Fig. 4.11 shows the effects of the addition of PMSF on the intracellular (a) and extracellular (b) lipase activity. PMSF does not inhibit the lipase at 1 mM concentrations. Increasing concentrations of PMSF resulted in increasing amounts of inhibition for both the intracellular and extracellular lipase activities. PMSF appears to have a more gradual effect on the intracellular lipase activity than the extracellular enzyme lost most activity in the first 15 min incubation than in the remaining 105 min, where very little activity was lost.

This experiment was repeated using ρ-nitrophenyl butyrate as a replacement for ρ-nitrophenyl palmitate in the standard lipase assay. This experiment was undertaken because of the results obtained in section 4.2.4 as the intracellular enzyme had a maximum activity peak corresponding to ρ-nitrophenyl butyrate. The activity measured with this substrate was not affected by incubation with PMSF. The results from this experiment are given in Fig. 4.11(c).

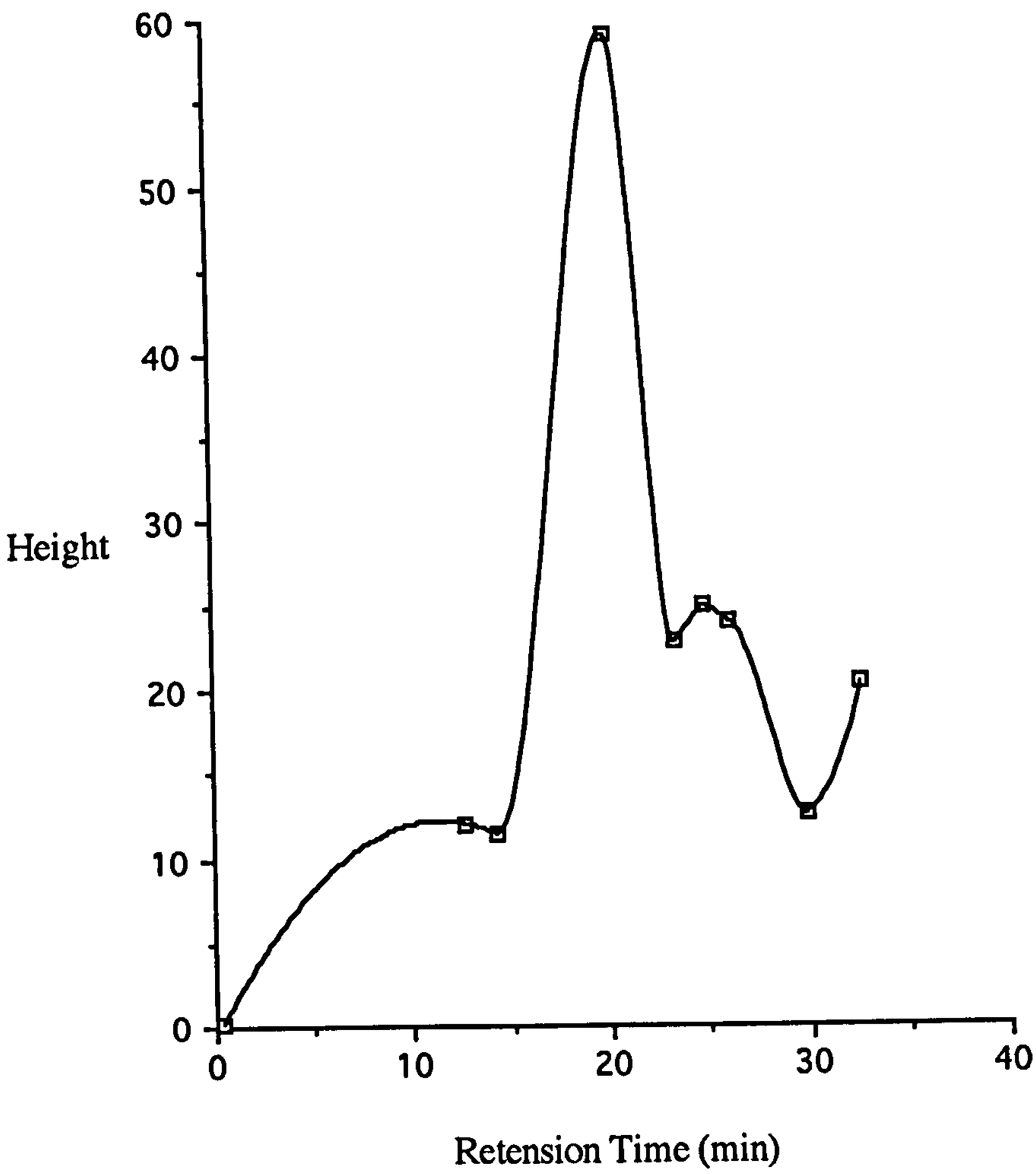
1 ml volumes of extracts of the intracellular enzyme were incubated, on ice, with 500 µl of bis-p-nitrophenyl phosphate, dissolved in 50 mM KH_2PO_4 buffer pH 7.5 to give final concentrations of 1 and 5 mM. Again the enzyme activity was measured from aliquots taken at time intervals over a period of 90 min. Fig. 4.11(d) demonstrates that there was no effect on intracellular lipase activity due to incubation with bis-ρ-nitrophenyl phosphate.

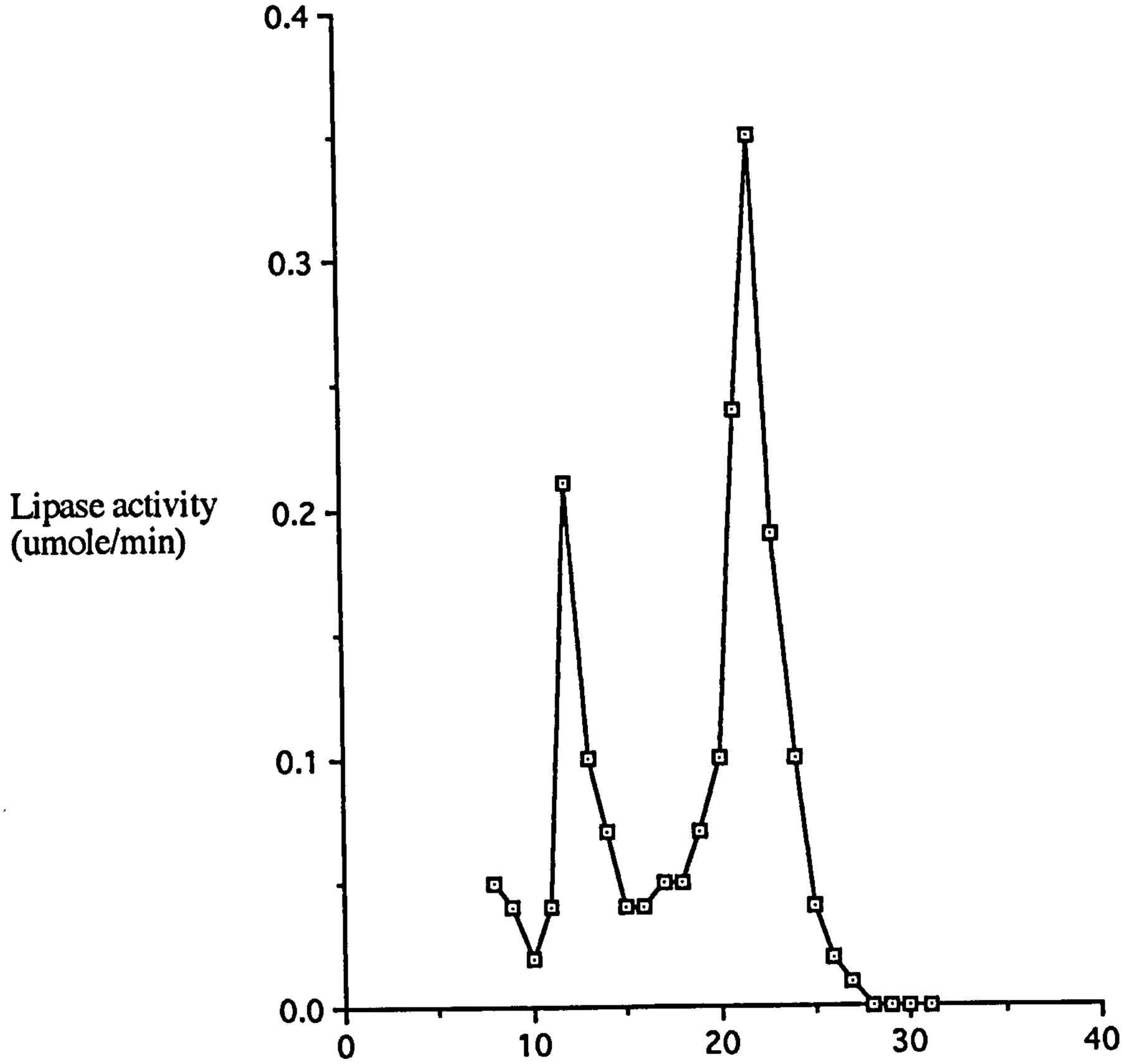
4.5.1.2 THE EFFECTS OF SULPHYDRYL REAGENTS ON LIPASE ACTIVITY

The effect of incubation with mercuric chloride and iodoacetamide on the intracellular lipase activity is shown in Fig. 4.12. 1 ml volumes of enzyme extracts were incubated, on ice, with 500 µl of each inhibitor, dissolved in 50 mM KH_2PO_4 buffer pH 7.5 to give a final concentration of 1 mM. Enzyme activity was measured from aliquots taken at

Fig.4.9 DETERMINATION OF MOLECULAR WEIGHT USING HPLC

Concentrated supernatant was run on an HPLC column [Methods 2.35(a)]. Protein concentration was measured (a) and lipase activity was assayed (b) using the standard lipase assay (Methods 2.31). Lipase activity is expressed as μmoles min^{-1}.

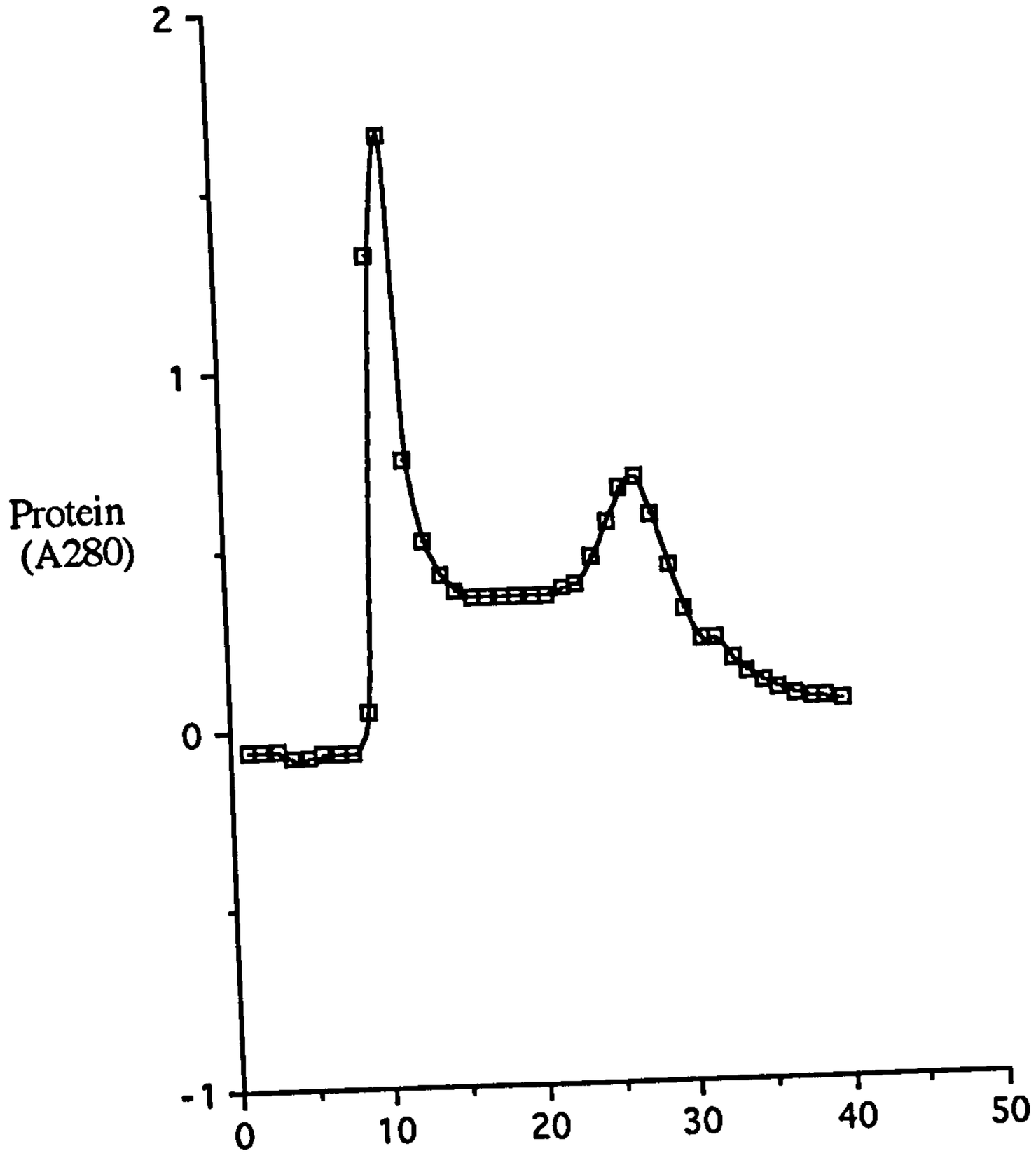

Height
60
50
40
30
20
10
0
0
10
20
30
40
Retension Time (min)


Lipase activity
(umole/min)
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
Fraction no.

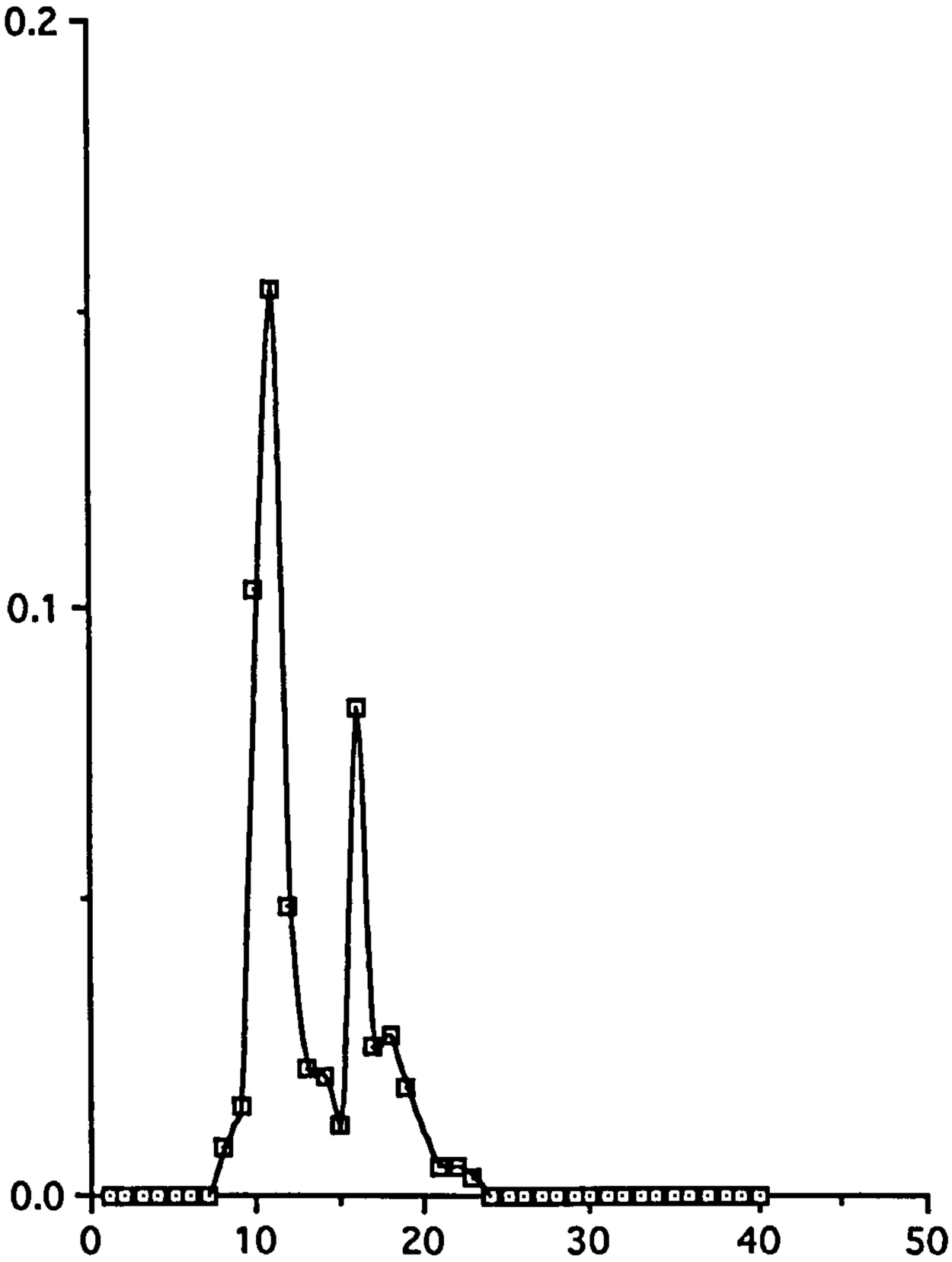
(b)

Fig.4.10 DETERMINATION OF MOLECULAR WEIGHT USING AN ACA 34 GEL FILTRATION COLUMN

Concentrated culture supernatant was run on an ACA 34 gel filtration column [Methods 2.35(b)]. Protein concentration was measured (Methods 2.17) (a) and lipase activity (EU) (b) was assayed using the standard lipase assay (Methods 2.31).



(a)


0.2
0.1
0.0
Enzyme Activity
(umoles/min)
0
10
20
30
40
50
Fraction no.

(b)

Table 4.2 ULTRACENTRIFUGATION OF CRUDE EXTRACTS CONTAINING LIPASE ACTIVITY

Both the intracellular and extracellular lipase preparations were spun at 40 K (Methods 2.14) and the resulting pellets and supernatants were assayed for activity using either ρ-nitrophenyl palmitate, ρ- nitrophenyl caprate or ρ-nitrophenyl butyrate as substrates in the standard lipase assay (Methods 2.31). The results are expressed in terms of specific activity (EU mg protein^{-1}).

Substrate	Fraction	Lipase Activity EU/mg protein
pnp palmitate	sonicated pellet	0.072
	40K pellet	0.1
	40K supernatant	0.02
	supernatant	0.131
	40K pellet	0.306
	40K supernatant	0.021
pnp caprate	sonicated pellet	0.074
	40K pellet	0.111
	40K supernatant	0.018
	supernatant	0.143
	40K pellet	0.489
	40K supernatant	0.169
pnp butyrate	sonicated pellet	0.386
	40K pellet	0.423
	40K supernatant	0.22
	supernatant	0.408
	40K pellet	1.117
	40K supernatant	0.217

Fig.4.11 THE EFFECT OF REAGENTS WHICH REACT WITH AN ACTIVE SITE SERINE

Intracellular (a) and extracellular (b) lipase preparations were incubated,on ice, with the serine reactive agents PMSF and *bis*-nitrophenyl phosphate at the concentrations indicated (see section 4.5.1.1). Aliquots removed from the incubations were assayed in the standard lipase assay (Methods 2.31) using either ρ- nitrophenyl palmitate or ρ-nitrophenyl butyrate as substrates.

4.11 (a)Effect of PMSF concentrations on intracellular lipase activity with ρ- nitrophenyl palmitate as substrate.

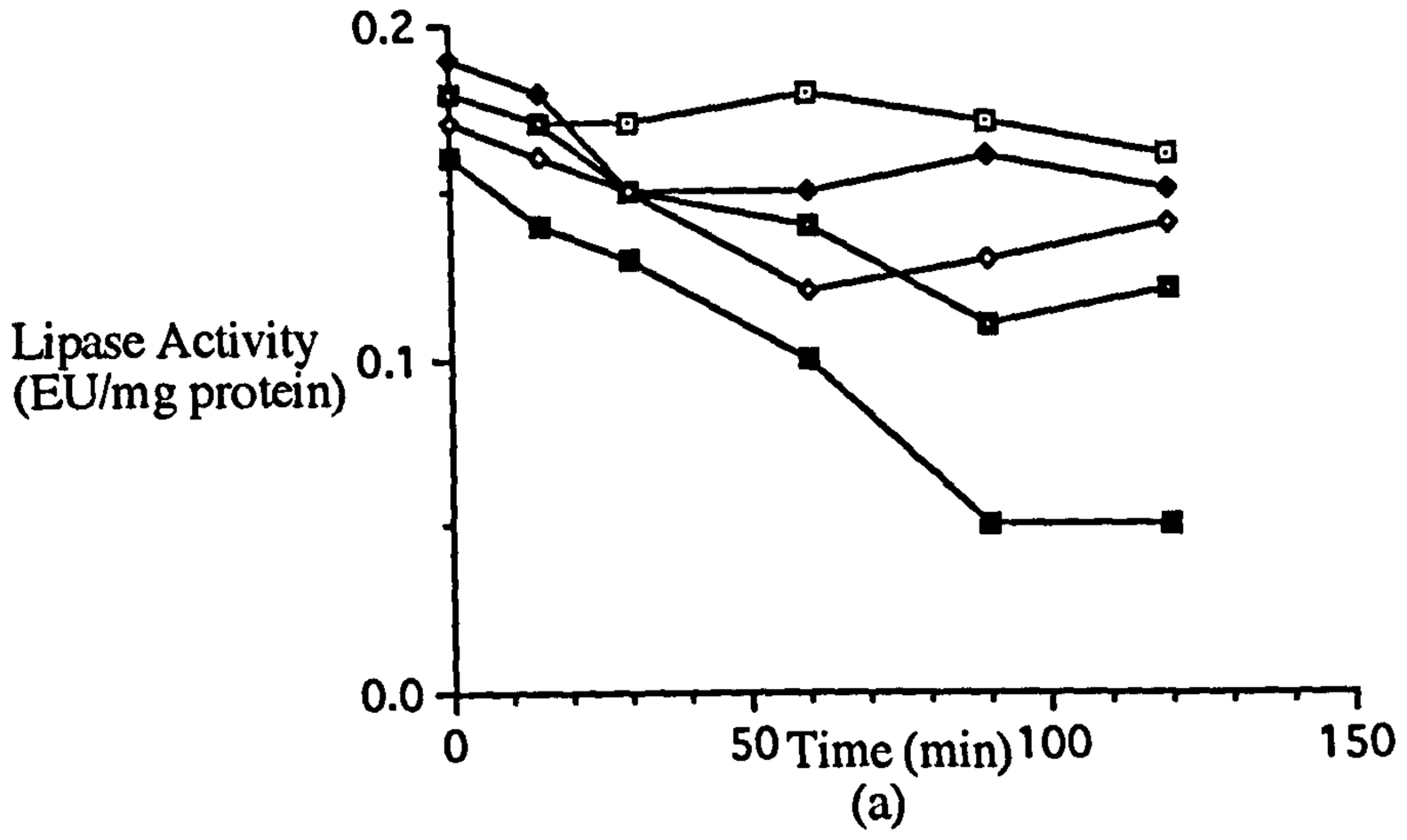
Concentration of PMSF	Symbol
Control	
1 mM	
2 mM	
5 mM	
10 mM	

4.11 (b)Effect of PMSF concentrations on extracellular lipase activity with ρ-nitrophenyl palmitate as substrate. Symbols as above.

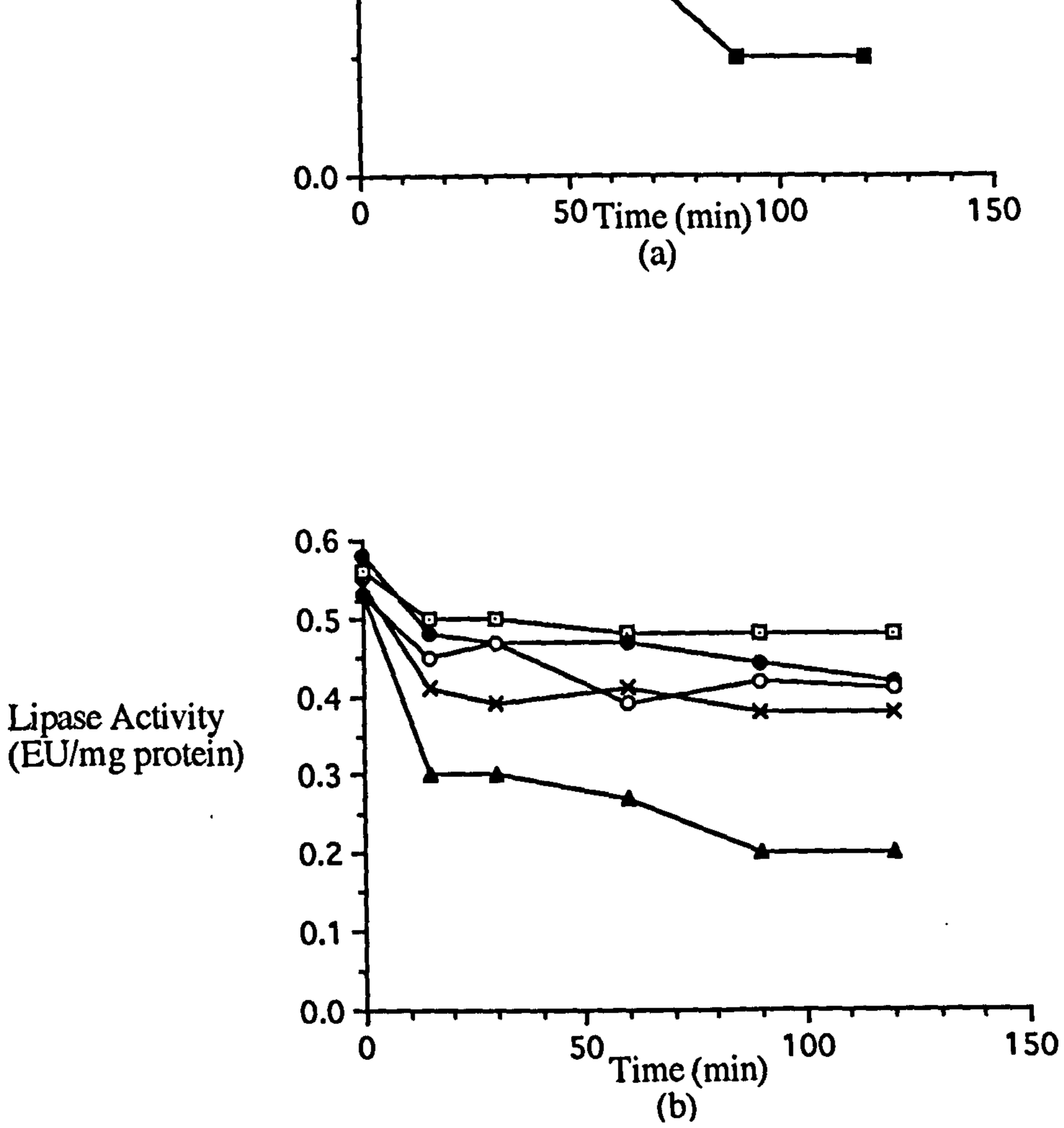
4.11 (c)Effect of PMSF on intracellular lipase activity with ρ-nitrophenyl butyrate as substrate. Control 1mM PMSF

4.11(d) Effect of bis-nitrophenyl phosphate intracellular lipase activity.

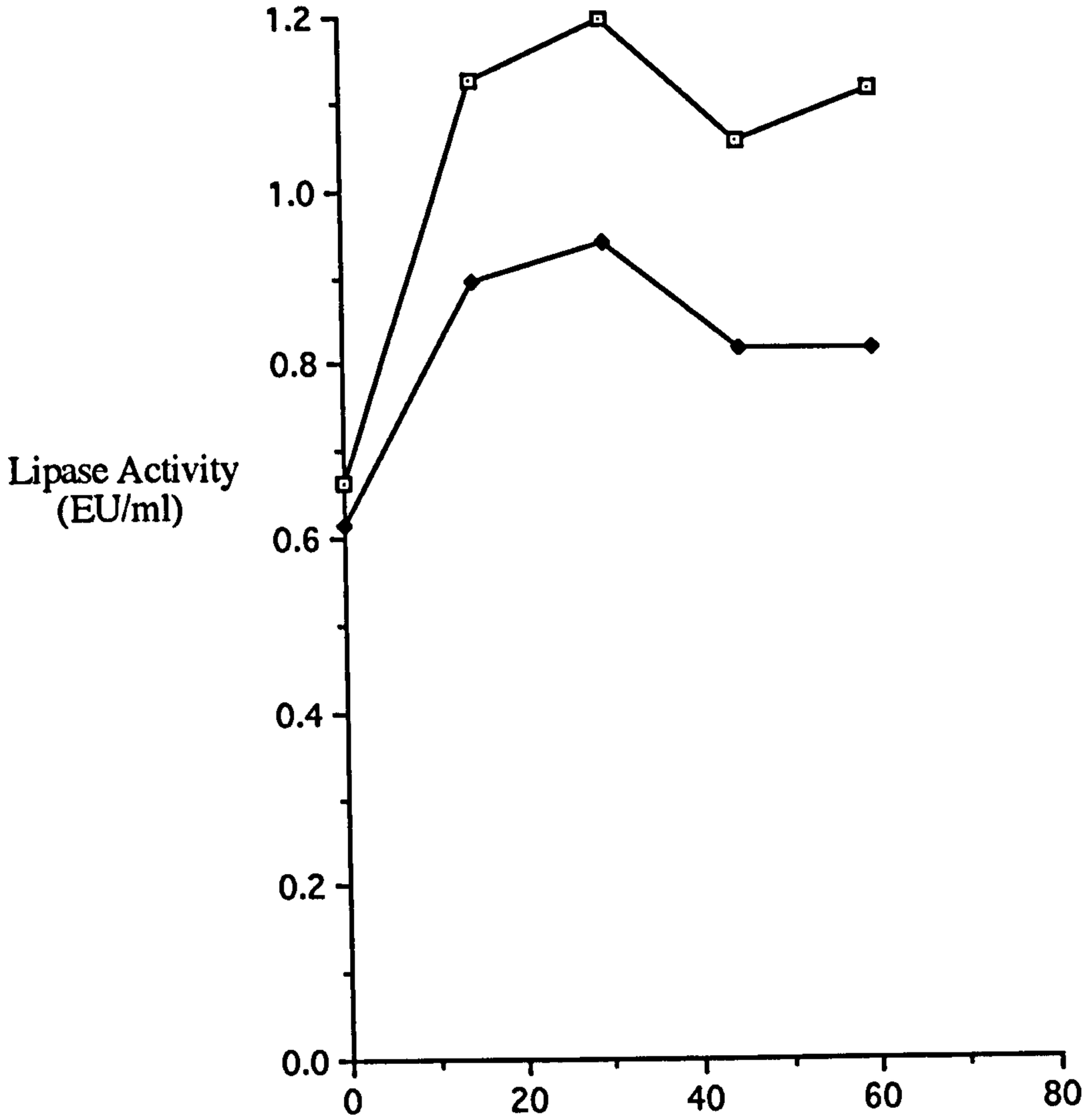
Concentration of BNP	Symbol
Control	
1 mM	
5 mM	


0.2
0.1
0.0
Lipase Activity
(EU/mg protein)
0
50
Time (min)
100
150

(a)


0.6
0.5
0.4
0.3
0.2
0.1
0.0
Lipase Activity
(EU/mg protein)
0
50
Time (min)
100
150

(b)



(c)

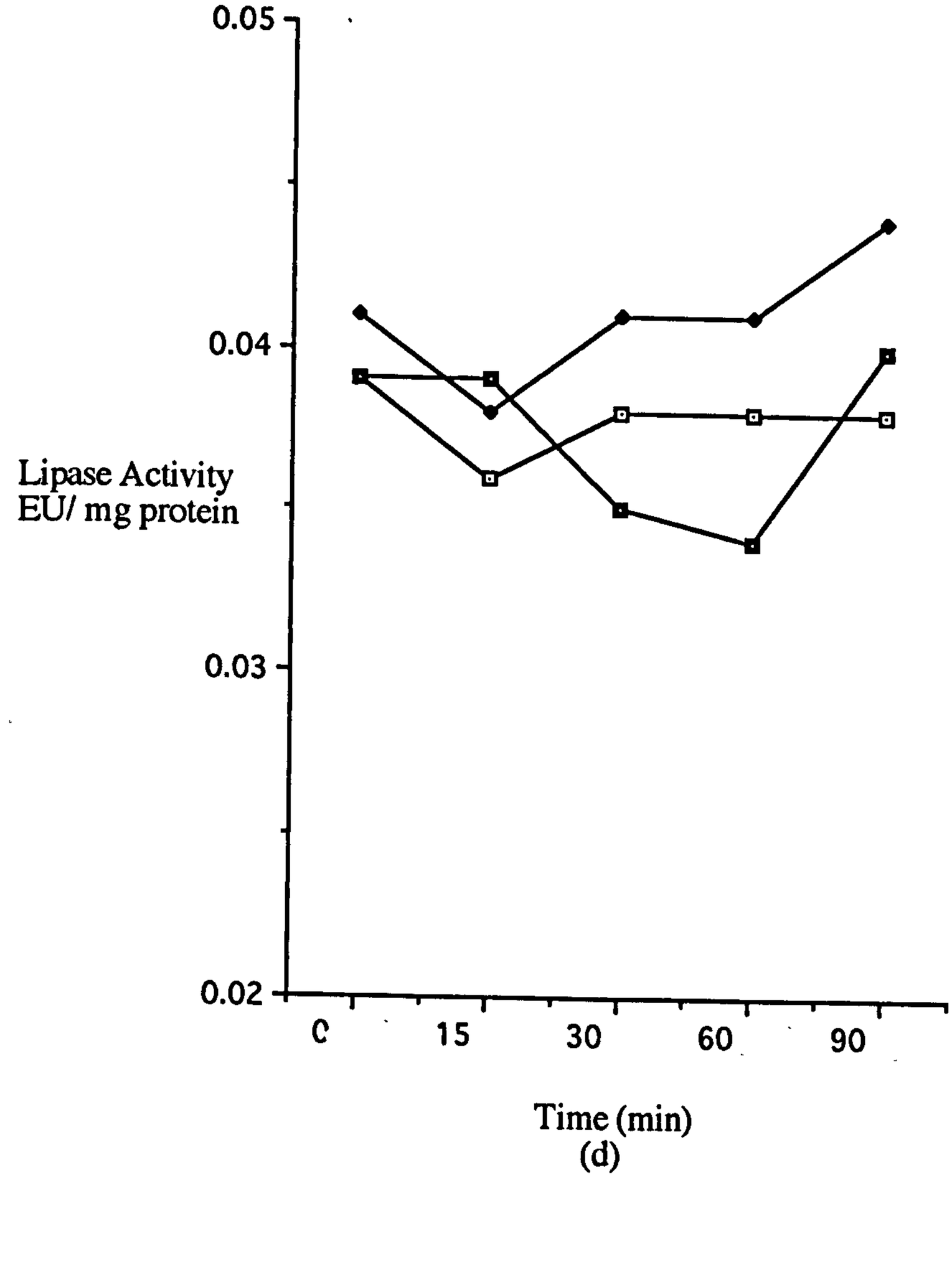
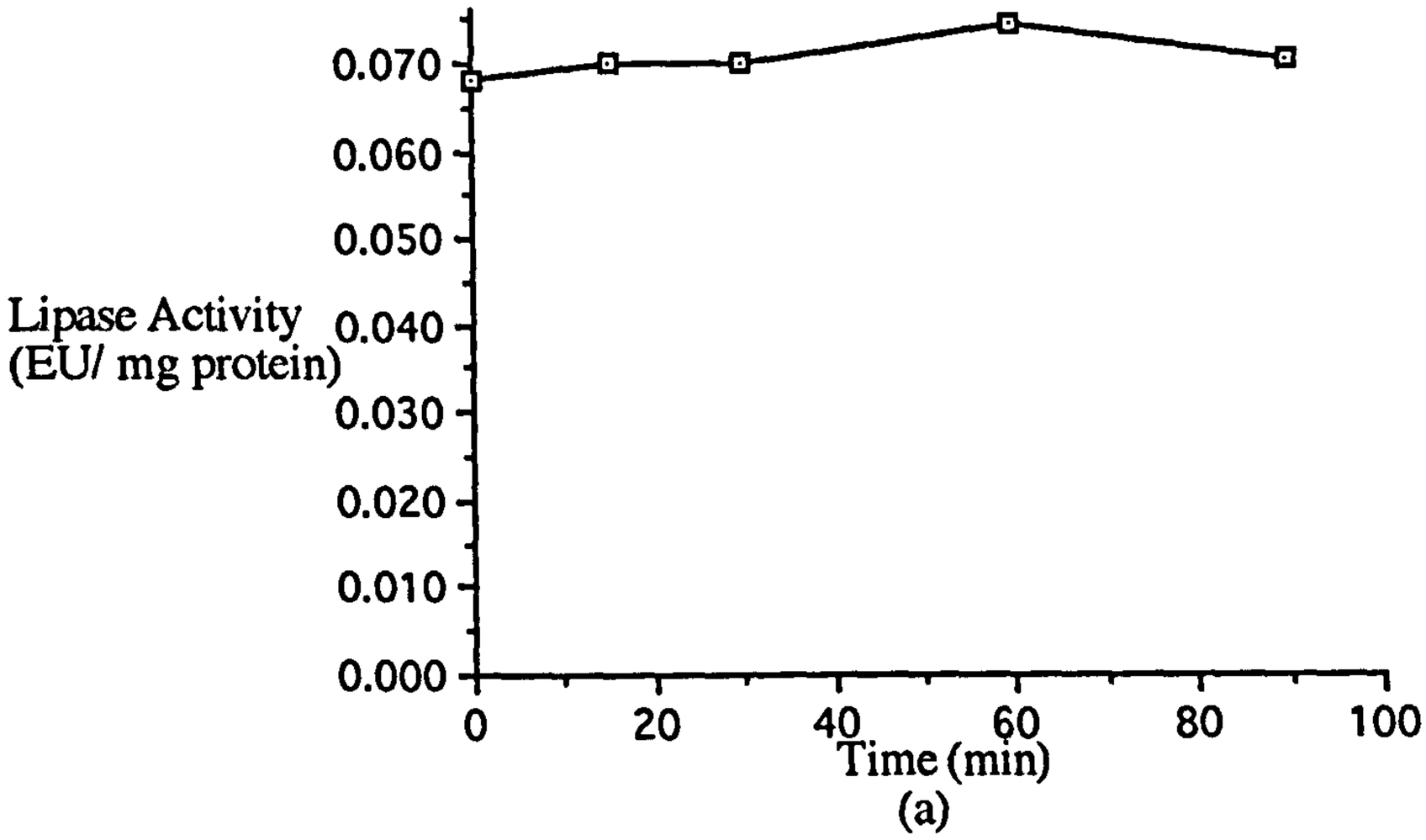
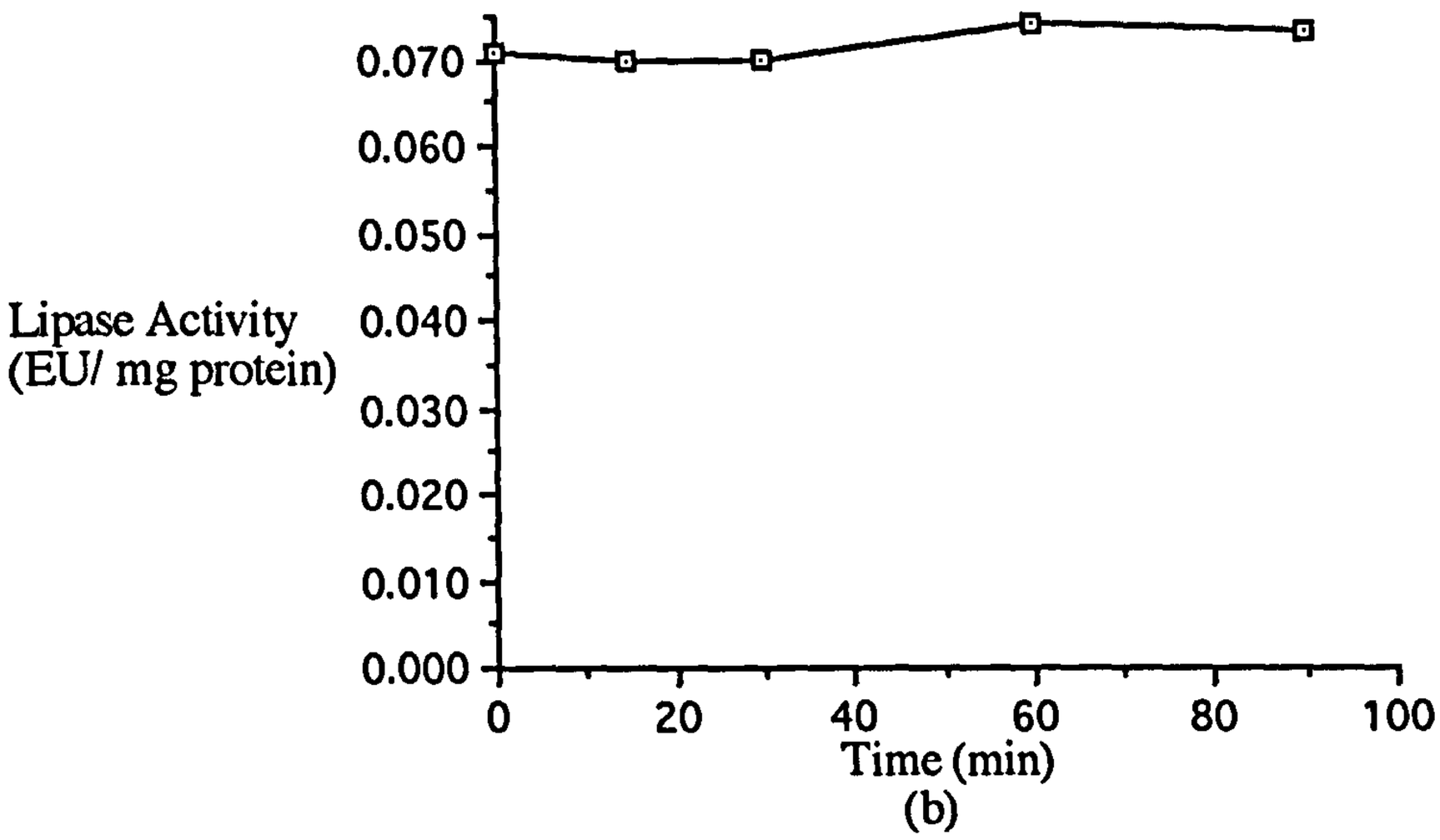

0.05
0.04
0.03
0.02
Lipase Activity
EU/ mg protein
0
15
30
60
90
Time (min)
(d)

Fig.4.12 THE EFFECTS OF SULPHYDRYL REAGENTS ON LIPASE ACTIVITY

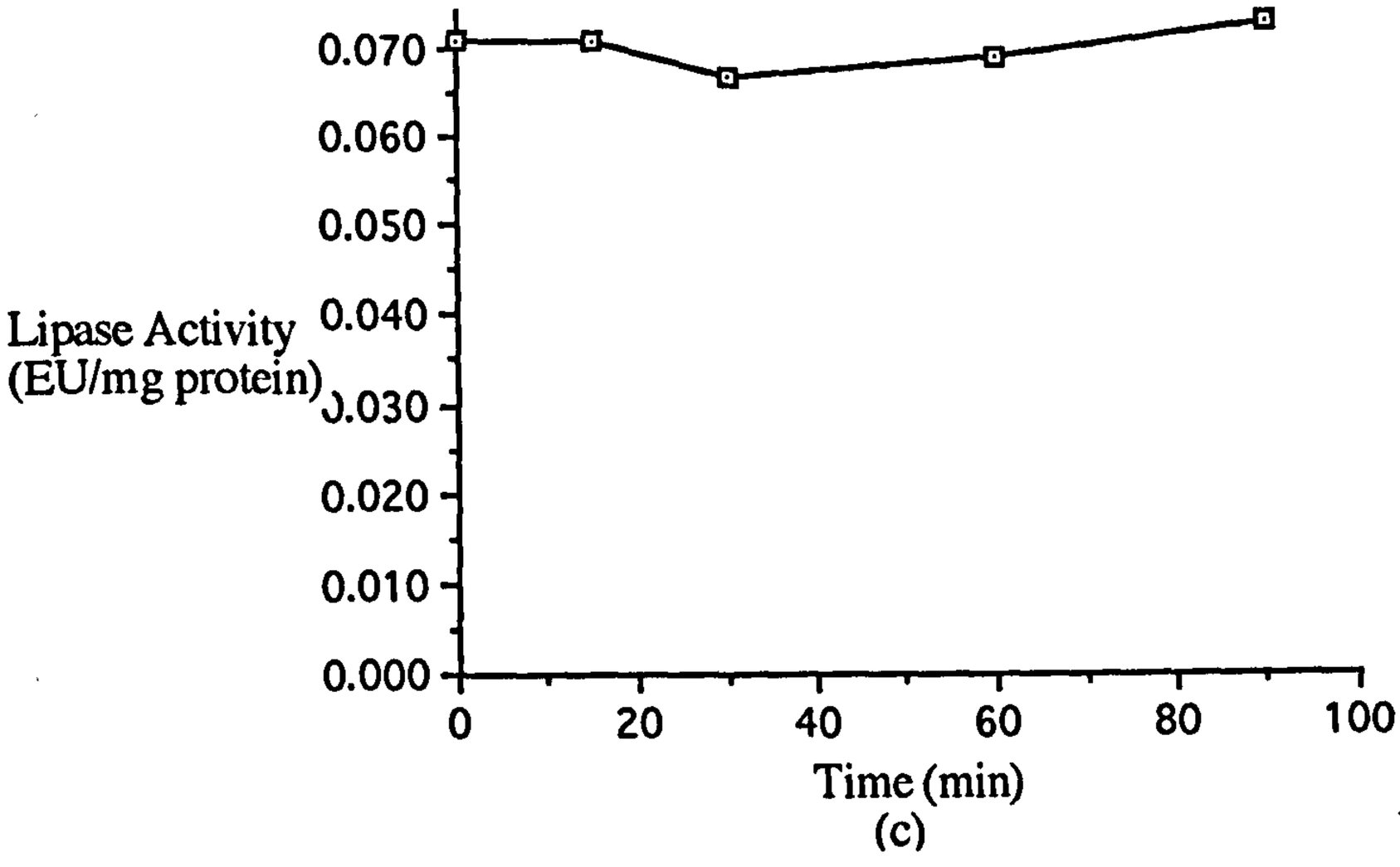
The effect of incubation with mercuric chloride (a) and iodoacetamide (b) on the intracellular lipase activity (see section 4.5.1.2 for method). An assay buffer control was included in this study (c). Lipase activities are expressed in EU mg protein^{-1}.


0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
Lipase Activity
(EU/ mg protein)
0
20
40
60
80
100
Time (min)

(a)


0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
Lipase Activity
(EU/ mg protein)
0
20
40
60
80
100
Time (min)

(b)


0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
Lipase Activity
(EU/mg protein)
0
20
40
60
80
100
Time (min)

(c)

time intervals over a period of 90 min. The sulphydryl reagents had no significant effect on lipase activity when incubated with extracts on ice.

4.5.2 DETERMINATION OF Km's FROM INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

Three different methods (see Methods 2.32) of changing the concentration of substrate in the assay were used to determine the Km of the lipases for ρ-nitrophenyl palmitate.. These three methods were as follows;

Method 1; The surface concentration of ρ-nitrophenyl palmitate in the Triton micelles was varied but the concentration of Triton/ substrate micelles remained constant.

Method 2; A constant ρ-nitrophenyl palmitate to Triton X-100 ratio was maintained in the micelles. Therefore, the surface concentration of ρ-nitrophenyl palmitate in the Triton micelles was constant and the concentration of substrate was varied by varying the concentration of micelles in the assay.

Method 3; The surface concentration of ρ-nitrophenyl palmitate in the micelles and the total concentration of micelles was constant but the substrate concentration was varied by replacing substrate containing micelles with micelles containing no substrate.

Assays were carried out according to Methods 2.31 and Kms were calculated according to Methods 2.32. Examples of Lineweaver-Burk plots for both the intracellular and extracellular activities are shown in Fig. 4.13. These figures demonstrate that although assays were carried out using crude extracts good correlation coefficients could be obtained. Table 4.3 gives the Kms determined by the three methods. The Kms vary according to the method used in preparing the assay substrate. For all three methods the Kms for the intracellular and extracellular activities are different. An interesting point to note is that using Method 3 to prepare the substrate gave the best fit lines when constructing the Lineweaver-Burk plots.

4.5.3 THE EFFECT OF TRITON X-100 CONCENTRATION ON Km

From the Kms determined it can be seen that Method 3 gave the highest Km's for both the intracellular and extracellular lipase activities. This was further investigated and Method 3 was repeated using a range of Triton X-100 concentrations. The results are shown in Table 4.4. Increasing the number of empty triton micelles, as a consequence of increasing the concentration of Triton X-100, results in an increase in Km for both the intracellular and extracellular lipase activities. Interestingly the Vmax doesn't alter significantly when compared to the increase in Kms.

4.5.4 THE EFFECT OF THE PRESENCE OF RAPE SEED OIL ON LIPASE ACTIVITY

To investigate the effect of rape seed oil on lipase activities, *S.rimosus* was grown in YEME and 1 g of washed biomass was resuspended in 100 ml of HMM, containing 10 mM glucose as a carbon source. After 48 h incubation, the cultures were harvested and the biomass was split into two equal portions. One portion was resuspended in 100 ml of HMM, containing no carbon source, while the remainder was resuspended in 100 ml of HMM, containing either 0.5% (v/v) rape seed oil (rso) or 0.1% (v/v) rape seed oil. After 1 h incubation, these cultures were harvested and the biomass was assayed for lipase activity. Table 4.5 gives the lipase activities, obtained from these experiments, expressed in terms of EU ml^{-1}. As can be seen from this table there is no definite pattern to the results. Incubation in the presence of 0.5% (v/v) rape seed oil gave an increased lipase activity in two flasks (Flasks 1 and 2) but a decrease in the third flask (Flask 3). Incubation in the presence of 0.1% (v/v) rape seed oil (Flasks 4, 5 and 6) showed no significant change in lipase activity.

4.5.5 THE EFFECT OF RAPE SEED OIL ON THE Km FOR ρ-NITROPHENYL PALMITATE, IN THE STANDARD LIPASE ASSAY

The effect of rape seed oil on the standard lipase assay, was investigated using the ρ-nitrophenyl palmitate substrate. As method 3 gave the best fit Lineweaver-Burk plots this was the method used in preparing the substrates for assay. A stock solution of 6.4 mM ρ-nitrophenyl palmitate, containing 1.8% (v/v) Triton X-100, was prepared and diluted with assay buffer, again containing 1.8% (v/v) Triton X-100, to give a range of ρ-nitrophenyl palmitate concentrations. Preparations of both the intracellular lipase and the extracellular lipase were used and the Km's were determined according to Methods 2.32. Similarly a stock solution of ρ-nitrophenyl palmitate was prepared but this was diluted with assay buffer containing 1.6 mM rape seed oil (highest suitable concentration which would dissolve) and 1.8% (v/v) Triton X-100. Identical intracellular and extracellular lipase preparations were used and the Kms were determined as before. Table 4.6 gives the Kms and Vmaxs obtained. The Kms for both enzyme activities are higher when rape seed oil is present.

4.6 INDUCTION OF LIPOLYTIC ACTIVITY

4.6.1 THE DEVELOPMENT OF A PROCEDURE TO INDUCE INCREASED LIPASE ACTIVITIES IN CULTURES OF *S.RIMOSUS*

One of the obvious methods to induce increased lipase activity would be to supplement the minimal growth media with triglycerides. Unfortunately, owing to the difficulties encountered in growing *S.rimosus* in the presence of triglycerides an alternative method of exploring the effect of rape seed oil on biomass lipase activity had to be found. Experiments were carried out whereby pellets, harvested from *S.rimosus* cultures grown in YEME medium, were resuspended in HMM containing different combinations of glucose and rape seed oil. These cultures were harvested at time intervals, up to 48 h, and samples of biomass and concentrated supernatant were assayed for lipase activity.

Concentrating the supernatant produced a level of lipase activity that could be reliably detected. Figs. 4.13(a) and (b) show the lipase activities obtained from these experiments. 1 g wet weight samples of YEME biomass were resuspended in HMM to which rape seed oil, at two concentrations, in the presence and absence of 10 mM glucose was added (see legend to Fig. 4.14). The largest increase, in both intracellular and extracellular lipase activity, was achieved when the biomass was resuspended in HMM, containing 10 mM glucose as the carbon source. The intracellular specific activity increased by a factor of twenty over 48 h while the extracellular specific activity increased by a factor of nine. The next highest activities were observed when glucose was supplemented with rape seed oil, followed by the cultures supplemented with rape seed oil alone. The control cultures containing no carbon source, apart from any remaining residues carried over from the YEME, showed no significant change in lipase activity. Thus the procedure of growing cultures in YEME and then resuspending the harvested biomass in HMM, containing 10 mM glucose, for 48 h was used (Methods 2.9) whenever higher levels of lipase activity were required.

4.6.2 THE EFFECT ON LIPASE ACTIVITIES IN CULTURES OF *S.RIMOSUS* GROWN ON STANDARD GROWTH MEDIA SUPPLEMENTED WITH POLYOXYETHYLENE ESTERS

S.rimosus cultures were grown on the standard growth media supplemented with either Tween 20 or Tween 40 (see section 3.5). The results obtained implied that *S.rimosus* was able to utilise both Tween 20 (Fig. 3.13) and Tween 40 (Fig. 3.12) as growth substrates, and would require the presence of an ester-hydrolysing enzyme or enzymes. Cultures were harvested at the end of growth in the presence of these detergents and both the pellets and supernatants were assayed for lipase activity using the standard ρ-nitrophenyl palmitate assay. The results shown in Table 4.7 are the lipase activities from three separate growth experiments.

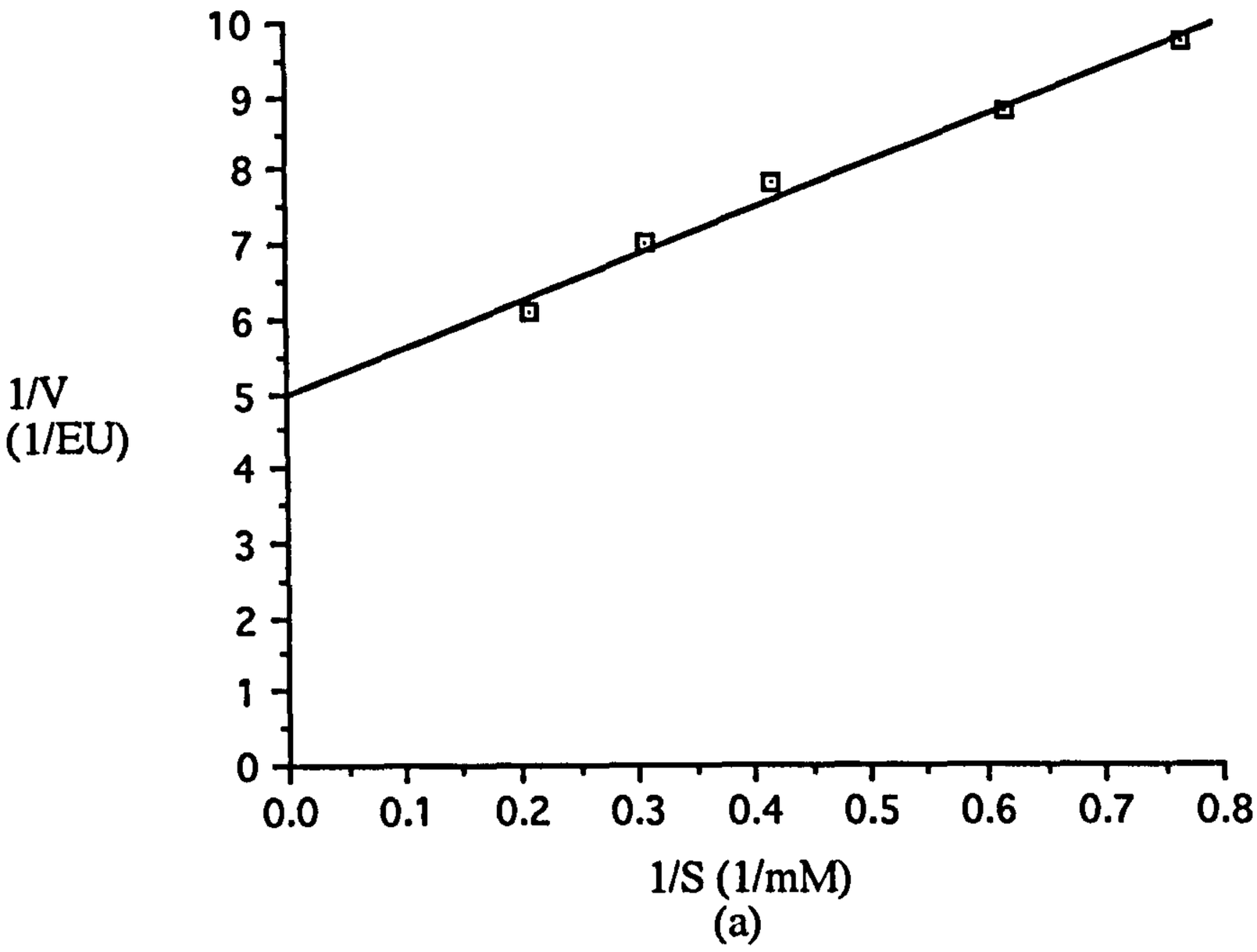
In the first series of experiments (see (a), Table 4.7) when the cells were harvested 4 h after the end of growth in the glucose control, the glucose control has a higher intracellular activity than those from the cultures containing Tween. However the extracellular lipase activities from the Tween containing cultures are higher than those from the glucose control. In the other experiments (see (b) and (c), Table 4.7) the intracellular and extracellular lipase activities from the cultures containing Tween are generally higher than those from the glucose control. The figures for the lipase activities from the three sets of experiments do not show identical trends but there are a number of possible explanations for this. In experiment (a) the cultures were harvested shortly after the cessation of growth in the glucose control. In the subsequent experiments, (b) and (c), the cultures were not harvested until 24 hours after the cessation of growth in the glucose control. This allowed an additional period of slower growth, after the initial logarithmic phase, in the presence of Tween. The cultures containing Tween that were allowed to undergo this second phase of growth generally had higher lipase activities than the glucose control

4.6.2.1 THE EFFECT ON LIPASE ACTIVITY OF THE ADDITION OF TWEEN DETERGENTS TO THE STANDARD LIPASE ASSAY

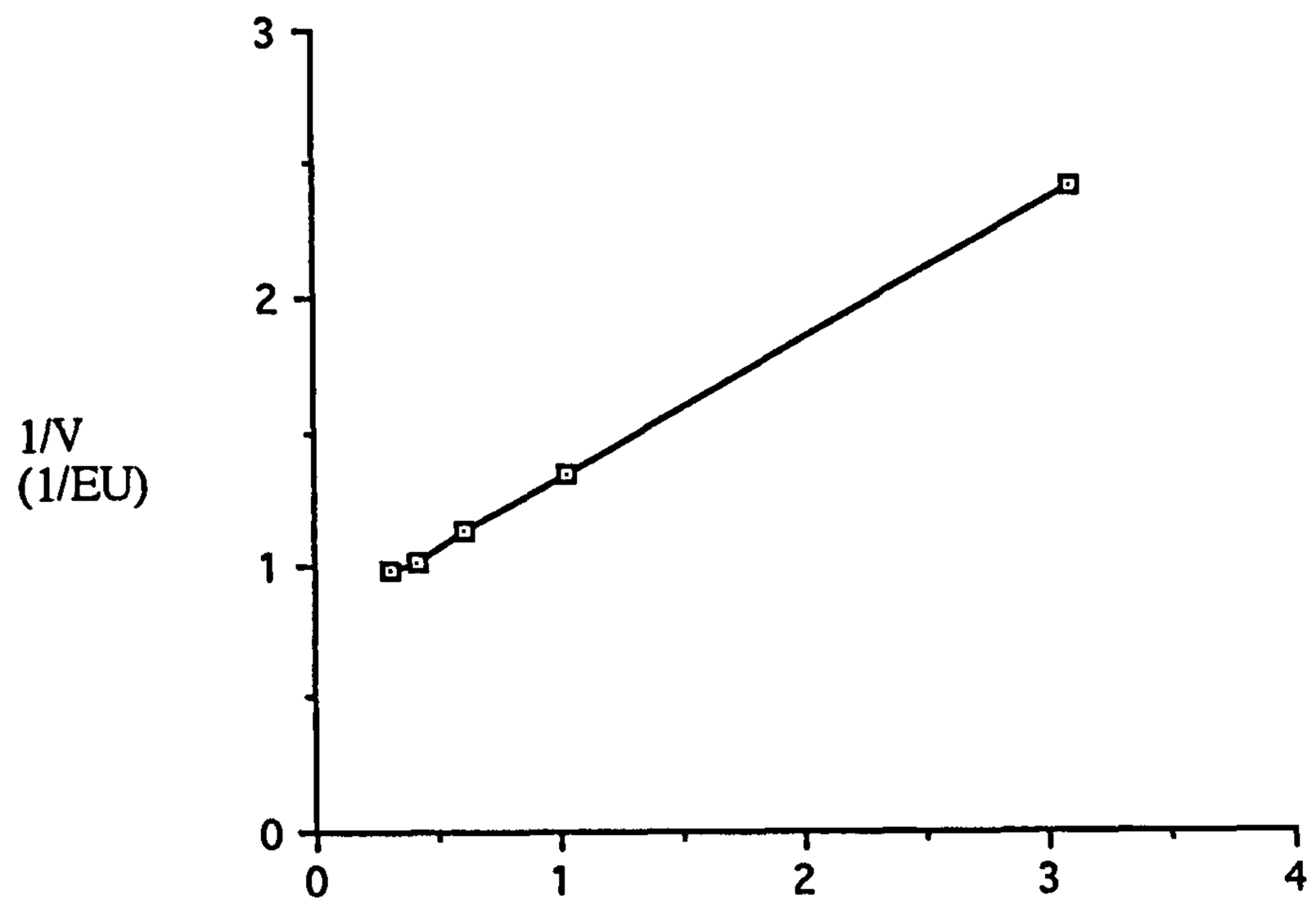
In addition to an effect on the production of lipase by *S.rimosus*, both Tween 20 and Tween 40 could have an effect on the assay of enzyme activity. Since preparations of lipase may contain the detergent this effect must be assessed. Samples, harvested from *S.rimosus* cultures grown up on standard defined media containing 10 mM glucose were prepared to assay their lipase activity. Table 4.8 demonstrates the effect of the addition of 2% (v/v) Tween 20 and 2% (v/v) Tween 40, to the standard lipase assay, on intracellular lipase activities. The presence of 2% detergent (maximum concentration used in the growth experiments) results in a decrease in lipase activity. The presence of 2% (v/v) Tween 20 results in an average reduction in lipase activity of 27% when compared to that of the control assay. The presence of 2% (v/v) Tween 40 results in an

Fig.4.13 LINEWEAVER-BURKE PLOTS FOR THE INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

Examples of typical Lineweaver-Burk plots for both the intracellular (a) and extracellular (b) lipase activities. Lipase assays were carried out according to Methods 2.31


10
9
8
7
6
5
4
3
2
1
0
1/V
(1/EU)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
1/S (1/mM)

(a)


3
2
1
0
1/V
(1/EU)
0
1
2
3
4
1/S (1/mM)

(b)

Table 4.3 DETERMINATION OF Kms FROM THE INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

Three different methods of preparing the substrate were employed (see Methods 2.32), in the standard ρ-nitrophenyl palmitate assay (Methods 2.31). Kms were calculated according to Methods 2.32. The Kms for the three methods are expressed in mM. The three different methods changed the substrate concentration as follows:

Method 1; The surface concentration of ρ-nitrophenyl palmitate in the Triton micelles was varied but the concentration of Triton/ substrate micelles remained constant.

Method 2; A constant ρ-nitrophenyl palmitate to Triton X-100 ratio was maintained in the micelles. Therefore, the surface concentration of ρ-nitrophenyl palmitate in the Triton micelles was constant and the concentration of substrate was varied by varying the concentration of micelles in the assay.

Method 3; The surface concentration of ρ-nitrophenyl palmitate in the micelles and the total concentration of micelles was constant but the substrate concentration was varied by replacing substrate containing micelles with micelles containing no substrate.

Enzyme	Km (mM)		
	Method 1	Method 2	Method 3
Intracellular	0.651	0.177	2.500
Extracellular	0.406	0.268	0.531

Table 4.4 THE EFFECT OF TRITON X-100 CONCENTRATION ON Km

The effect of increasing the Triton X-100 concentration, in the standard lipase assay (Methods 2.31), on theKm values of the intracellular and extracellular lipase activities Km. The substrate used in the assay was prepared according to Method 3 (Methods 2.32). The Kms are expressed in mM.

[Triton X-100]	Intra. enzyme		Extra. enzyme	
	Km (mM)	Vmax	Km (mM)	Vmax
0.90%	0.672	0.470	0.384	1.28
1.40%	1.480	0.600	0.531	1.67
1.80%	2.46	0.714	0.684	1.71
2.50%	3.38	0.805		
3.00%	5.34	0.880		
3.60%			1.61	1.96

Table 4.5 THE EFFECT OF THE PRESENCE OF RAPE SEED OIL ON LIPASE ACTIVITY

S.rimosus was grown in YEME and 1 g wet weight of biomass was resuspended in 100 ml of HMM, containing 10 mM Glc as a carbon source. After 48 h incubation, the cultures were harvested and the biomass was split into two equal portions. One half of the biomass was resuspended in 100 ml of HMM with no carbon source, while the other half was resuspended in 100 ml of HMM, containing either 0.5% rso or 0.1% rso. After1 h incubation the cultures were harvested and the biomass was assayed for lipase activity (Methods 2.31). Lipase activities are expressed in terms of EU.

Flask No.	Incubation	Lipase Activity
1	no oil	0.011
	0.5% rso	0.044
2	no oil	0.014
	0.5% rso	0.026
3	no oil	0.033
	0.5% rso	0.005
4	no oil	0.011
	0.1% oil	0.010
5	no oil	0.009
	0.1% rso	0.014
6	no oil	0.007
	0.1% rso	0.008

Table 4.6 THE EFFECT OF RAPE SEED OIL ON THE Km FOR ρ-NITROPHENYL PALMITATE, IN THE STANDARD LIPASE ASSAY

The effect of rape seed oil on the standard lipase assay, was investigated using the ρ-nitrophenyl palmitate substrate. As method 3 gave the best fit Lineweaver-Burk plots this was the method used in preparing the substrates for assay. A stock solution of 6.4 mM ρ-nitrophenyl palmitate, containing 1.8% (v/v) Triton X-100, was prepared and diluted with assay buffer, again containing 1.8% (v/v) Triton X-100, to give a range of ρ -nitrophenyl palmitate concentrations. Preparations of both the intracellular lipase and the extracellular lipase were used and the Kms were determined according to Methods 2.32. Similarly a stock solution of ρ-nitrophenyl palmitate was prepared but this was diluted with assay buffer containing 1.6 mM rape seed oil and 1.8% (v/v) Triton X-100.

Lipase Activity	Km (mM)	
	+ rso	− rso
Intracellular	0.804	0.326
Extracellular	0.78	0.643

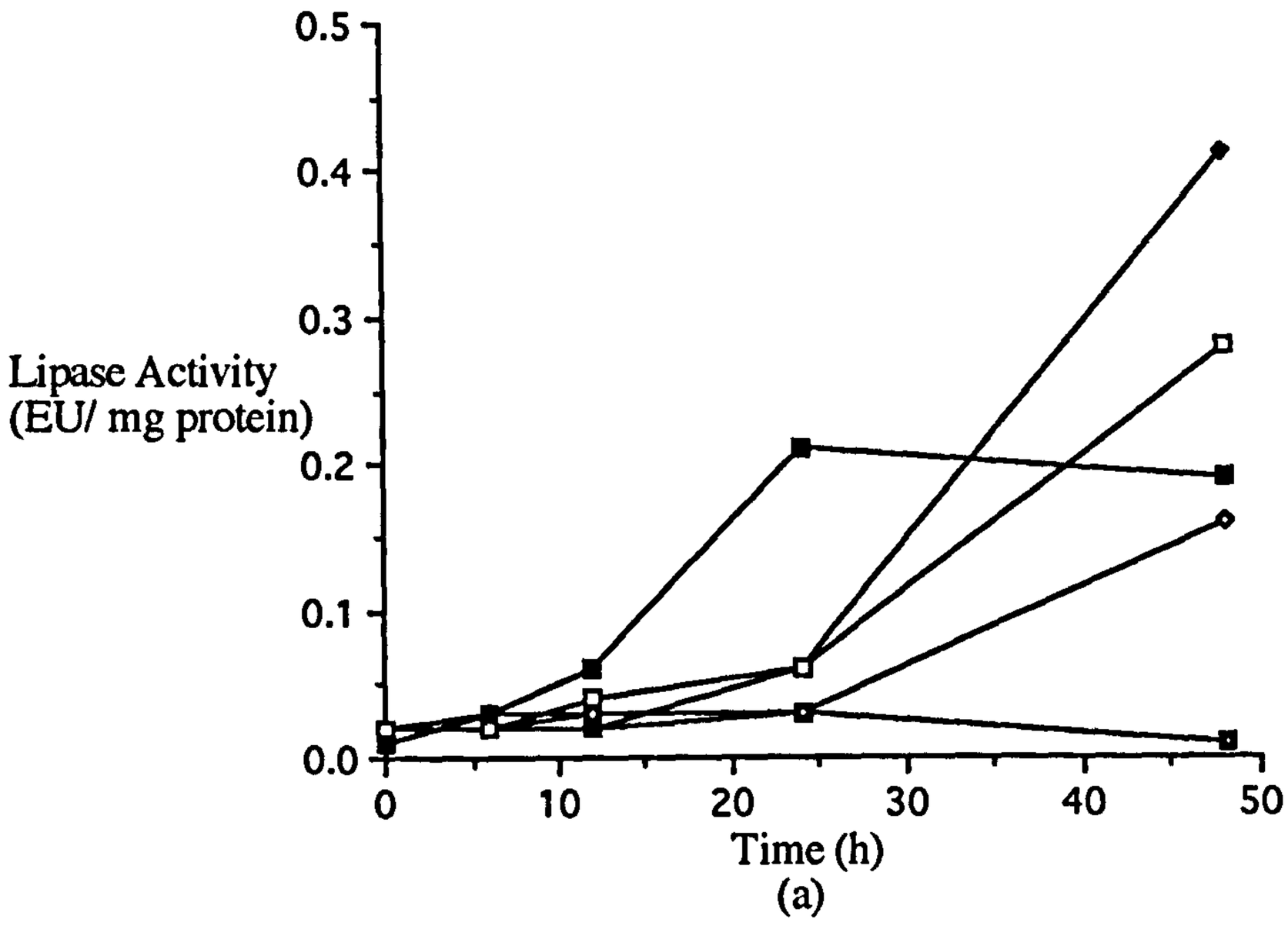
Fig.4.14 THE DEVELOPMENT OF A PROCEDURE TO INDUCE INCREASED LIPASE ACTIVITIES IN CULTURES OF *S.RIMOSUS*

1 g wet weight of *S.rimosus* biomass, obtained from cultures grown in YEME, was resuspended in 100 ml of HMM, containing either; no carbon source, 10 mM Glc, 10 mM Glc and 0.1% rape seed oil,10 mM Glc and 0.5% rape seed oil, 0.1% rape seed oil or 0.5% rape seed oil. Cultures were harvested at various time intervals, up to48 h, and the biomass and supernatant were assayed for lipase activity (Methods 2.31). Lipase activity is expressed as EU mg protein^{-1}.

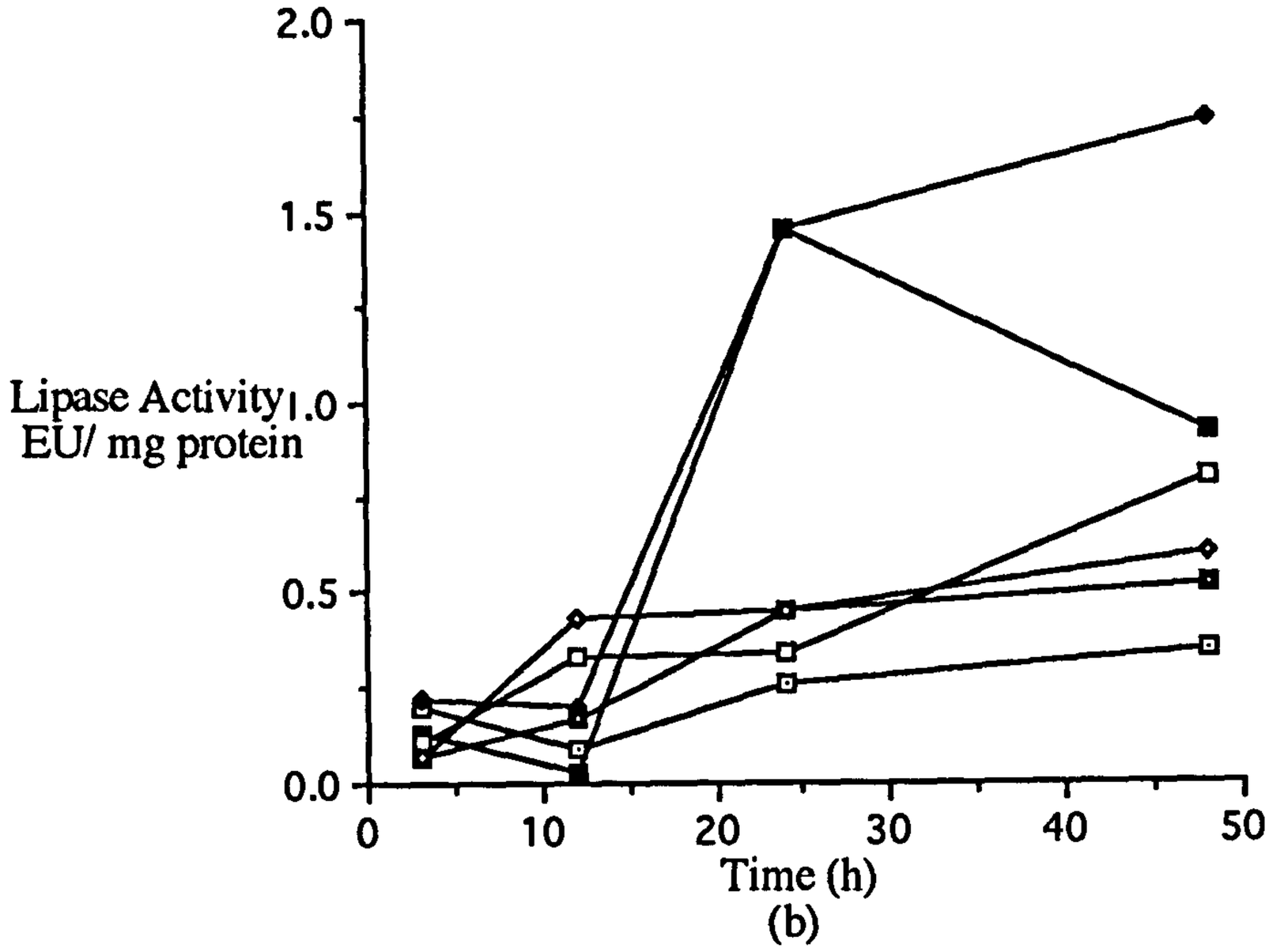
(a) Intracellular lipase activity

(b) extracellular lipase activity

- 10 mM Glc
- 10 mM Glc and 0.5% rso
- 10 mM Glc and 0.1% rso
- 0.5% rso
- 0.1% rso
- no carbon source


0.5
0.4
0.3
0.2
0.1
0.0
Lipase Activity
(EU/ mg protein)
0
10
20
30
40
50
Time (h)

(a)


2.0
1.5
1.0
0.5
0.0
Lipase Activity
EU/ mg protein
0
10
20
30
40
50
Time (h)

(b)

Table 4.7 THE EFFECT ON LIPASE ACTIVITIES IN CULTURES OF *S.RIMOSUS* GROWN ON STANDARD GROWTH MEDIA SUPPLEMENTED WITH POLYOXYETHYLENE ESTERS

Lipase activities determined for pellets (intracellular lipase activities) and supernatants (extracellular lipase activities) harvested from cultures of *S.rimosus*, grown in the standard defined growth media supplemented with either; 0.1%, 0.5%, 1.0%, and 2.0% v/v Tween 20 or Tween 40. The results were taken from three separate growth experiments:

(a) Cultures containing 10 mM glucose, harvested 4 h after the cessation of growth in the glucose control flask.

(b) Cultures containing 10 mM glucose, harvested 24 h after the cessation of growth in the glucose control flask.

(c) Cultures containing 40 mM glucose, harvested 24 h after the cessation of growth in the glucose control flask.

CULTURE CONDITIONS	INTRACELLULAR LIPASE ACTIVITIES EU/ mg protein			EXTRACELLULAR LIPASE ACTIVITIES EU/mg protein		
	(a)	(b)	(c)	(a)	(b)	(c)
Glucose control	0.47	0.09	0.17	0.02	0.15	0.07
Glc / 0.1% Tween 20	0.14	0.15	0.25	0.10	0.24	0.36
Glc / 0.5% Tween 20	0.17	0.16	0.47	0.46	0.29	1.02
Glc / 1.0% Tween 20	0.22	0.15	0.32	0.7	0.37	1.02
Glc / 2.0% Tween 20	0.17	0.18	0.22	0.36	0.74	0.42
Glc / 0.1% Tween 40	0.29	0.13	0.15	0.07	0.14	0.59
Glc / 0.5% Tween 40	0.41	0.15	0.37	0.07	0.19	0.82
Glc / 1.0% Tween 40	0.24	0.12	0.32	0.08	0.45	1.01
Glc / 2.0% Tween 40	0.22	0.31	0.26	0.32	1.26	0.29

Table 4.8 THE EFFECT ON LIPASE ACTIVITY OF THE ADDITION OF TWEEN DETERGENTS TO THE STANDARD LIPASE ASSAY

The effect of the addition of 2.0% v/v Tween 20 and 2.0% v/v Tween 40, on intracellular lipase activity, to the standard ρ-nitrophenyl palmitate assay (Methods 2.31). Lipase activities are expressed in EU ml^{-1}.

Assay	Lipase Activity EU/ml assayed	% Activity
Control	0.594	100
2% Tween 20	0.445	75
2% Tween 20	0.428	72
Control	0.523	100
2% Tween 40	0.192	37
2% Tween40	0.183	35

even greater reduction with an average 64% decrease in lipase activity when compared with the control assay.

4.7 DISCUSSION

4.7.1 DETECTION AND ASSAY OF LIPASES

For the purposes of this thesis the assay routinely employed utilised ρ-nitrophenyl palmitate as a substrate because this was a simple, quick and reliable assay. Unfortunately this substrate does not meet the definition of a true lipase assay. In order for the true definition of a lipase to be met hydrolysis of long-chain acylglycerols at an oil-water interface must take place (Brockerhoff and Jenson, (1974). Trioleolylglycerol (18:1-18:1-18:1) is an ideal substrate for lipases. If it is hydrolysed in an emulsion by an enzyme, the definition of a lipase is fulfilled (Jenson, 1983). However olive oil is a good alternative as it is inexpensive and contains 70% 18:1.

A number of "true" lipase assays were attempted using olive oil, in an emulsion, as the substrate; that of Muraoka *et al.*, (1982) and Kordel *et al.*, (1991) where changes in pH due to the production of fatty acids were monitored, and Smeltzer *et al.*, (1992) where an olive oil emulsion was stabilised by the addition of a low-gelling temperature agarose and the optical density at 450 nm was followed as there should be a decrease due to the clearing of the emulsion. Tributyrin was employed as a substrate in the double agar technique where the appearance of clear zones around colonies indicates a lipolytic enzyme activity (Fryer *et al.*, 1967). The colorimetric procedure described by Dunscombe, (1963) utilising the production of copper soaps for the determination of long-chain fatty acids in chloroform solution was also attempted. None of these true lipase assays were successful. A possible explanation for the failure of these assays is that in crude extracts the lipase activity was very weak and the activity may have gone unnoticed. Extremely sensitive methods of detection should perhaps have been applied,

for example the use of radioactivity or gas-liquid chromatography to detect free fatty acids.

4.7.2 THE EXISTENCE OF INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

The intracellular lipolytic activity was detected only when the cells were broken by sonication which implies that this activity is a true intracellular activity. In addition an extracellular lipase activity was detected in supernatants from cultures which had originally been grown in complex medium (YEME) and then resuspended in minimal medium containing various carbon sources. The induction of extracellular lipase activity mirrored the induction of intracellular lipase activity. If the intracellular lipase was an intermediate in the secretion of the extracellular lipase, it could be expected that the appearance of the new extracellular lipase activity would be paralleled by the loss of an equivalent amount of lipase activity from inside the cell, if the rate of synthesis was less than the rate of secretion. This has been demonstrated in *Bacillus subtilis* CMK33 which contains a membrane-bound intracellular lipase. Under appropriate conditions, in protoplasts, this intracellular activity decreases as the extracellular lipase activity increases (Kennedy and Lennarz, 1979). *Acinetobacter* O16 produces an esterase which at first is cell-bound and then significant amounts appear in the external medium in late growth. This extracellular enzyme appears as the cell-bound enzyme disappears implying that the enzyme is secreted through the membrane (Breuil and Kushner, 1975). This is not the case for the two lipolytic activities that I have detected in *S.rimosus*. The intracellular activity does not decrease as the extracellular activity increases, which indicates that the rate of synthesis is greater than the rate of secretion.

Another possibility is that the extracellular enzyme is the result of cell lysis or leakage. To determine whether this was happening it would be necessary to follow optical density as this parameter would decrease if lysis was occurring. Unfortunately the induction experiments were carried out in shake cultures, with no method of dispersion attempted,

therefore it was not possible to follow the optical density of the culture. Again as the intracellular lipase activity does not decrease there is no reason to suspect that the extracellular activity is as a result of the intracellular enzyme being released into the culture medium as a result of cell lysis. It is unlikely that cell lysis explains the appearance of the extracellular activity as evidence summarised in Table 4. 9 shows that the intracellular and extracellular activities do have different properties.

4.7.3 COMPARISON BETWEEN THE TWO ACTIVITIES USING THE STANDARD LIPASE ASSAY

To confirm that the intracellular and extracellular activities were indeed separate a number of comparisons were made between them. The standard ρ-nitrophenyl palmitate assay was suitable as the aim of this set of experiments was only to compare the two activities, not define them as true lipase activities.

Most bacterial lipases exhibit pH optima in the neutral pH range (Brogström and Brockman, 1984; Papon *et al.*, 1989). For example Pseudomonas lipase is optimally active at pH 7.0 (Suigura *et al.*, 1977) and Streptococcus lipase at pH 6.0-8.0 (Chander *et al.*, 1979). However exceptions can be expected; *Mucor pusillus* has an optimum pH range of 5.0-6.0 (Brockerhoff and Jenson, 1974). Because of the adaptable nature of bacteria, the pH dependencies of activity may depend on culture conditions (Brogström and Brockman, 1984).

The lipase activities in this study have slightly different pH optima, with the intracellular enzyme having a pH optimum of 8.5 and the extracellular enzyme being optimally active at pH 8.0. Considering that these studies were carried out on crude extracts of the two lipase activities I do not think that these differences are significant. The extracellular lipase isolated and characterised from *Streptomyces rimosus* by Engelbrecht and Mach, (1968) had a pH optimum of pH 5.0. This is a very different result from the one that I have obtained. The culture conditions and the substrate (olive oil) used by Engelbrecht

and Mach are different from those that I have used but despite this I do not think that these differences are sufficient to explain such a large difference.

Lipolytic enzymes may be active over a wide temperature range, for example some microbial lipases act at -20°C (Alford and Pierce, 1961) and *Staphylococcus aureus* 226 produces an extracellular lipase with a temperature optimum of 60°C. However, most bacterial lipases have been reported to be most active within a temperature range of 30°C to 40°C (Papon *et al.*, 1989).

The optimum temperature for the intracellular enzyme activity in the ρ-nitrophenyl palmitate assay was found to occur principally at 45°C, however a small additional peak of maximal activity was detected at 60°C. This second peak corresponds to the optimum temperature for the extracellular lipase activity, which could imply that the extracellular enzyme is a secreted enzyme as some of this activity is present within the cell, thus resulting in the detection of two peaks of maximal activity for the intracellular enzyme.

The melting point of ρ-nitrophenyl palmitate is between 65°C and 66°C which is very close to the maximum activity peak found at 60°C. To determine whether the melting point of the substrate was affecting the activity determination, an alternative substrate was used in the standard assay. The shorter chain ρ-nitrophenyl myristate, with a melting point of 51°C , was used. The use of this alternative substrate gave exactly the same results as obtained with ρ-nitrophenyl palmitate. From this it can be concluded that the melting points of the substrates in this experiment do not determine the maximum optimum temperature found using standard assay conditions.

Generally enzymes are heat labile, but one of the most significant properties of lipases is their marked stability to heat (Nashif and Nelson, 1953). An extreme example of just how heat stable a bacterial lipase can be is provided by a lipase isolated from *Achromobacter lipolyticum*. This lipase lost only 47% of it's activity when incubated at

71°C for 180 minutes, and in order for complete inactivation to take place incubation at 99°C for 40 minutes was required (Kahn *et al.*, 1967). A more typical example is seen for the extracellular lipase isolated from *Galactomyces geotrichum* whereby complete inactivation takes place with incubation at 56°C for 35 min. Preliminary experiments indicated that both the intracellular and extracellular activities examined in this study were extremely thermostable. Incubation of extracts containing either extracellular or intracellular enzyme activity at temperatures up to and including 50°C resulted in little loss of activity. Both enzymes were completely inactivated by incubations at 90°C within 25 minutes. The extracellular enzyme appears to be the more thermostable of the two. At 70°C it loses only 50% of it's activity compared to the intracellular enzyme which loses 79% of it's activity. The extracellular lipase isolated from *S.rimosus* by Engelbrecht and Mach, (1968) was inactivated by temperatures of 60°C, confirming that the activity examined here is quite different from that previously reported.

Tyski, (1983) found that when an extracellular lipase isolated from *Staphylococci* was incubated with a substrate at 70°C for 30 minutes, there was no drastic loss of activity. This appears to be as a result of substrate stabilisation whereby interaction of a protein molecule by a hydrophobic substrate protects the enzyme from irreversible denaturation. Borgström and Brockman, (1984) suggest that because of the propensity of lipases to adsorb to the lipid-water interfaces, increased stability in the presence of substrate is probably due to this immobilisation of the enzyme.

A variety of metal ions are known to interact with a large number of proteins, with some of them being implicated as physiological regulators of enzymatic activity (Nigaki *et al*, 1992). The effect of metal ions on bacterial lipases does not appear to obey any particular hard and fast rule, although Ca^{2+} and Ba^{2+} ions tend to increase activity. Ca^{2+} ions increase the activity of lipases isolated from *Anaerovibrio lipolytica* (Henderson, 1971), *Staphylococcus aureus* (O'Leary and Weld, 1964) and *Streptomyces rimosus* (Engelbrecht and Mach, (1968). Desnuelle, (1961) suggested that this increase in activity is due to the removal of fatty acids from the active site as insoluble salts.

Suprisingly Ca^{2+} ions only resulted in a slight increase in activity for the extracellular and intracellular activities, but perhaps this can be explained by the fact that a triglyceride was not employed as the substrate. Engelbrecht and Mach also found that their lipase activity was increased by the addition of Fe^{3+} and K^{+} ions. In this study Fe^{3+} and Fe^{2+} ions inhibited both the intracellular and extracellular lipase activities, which agrees with the findings of Sugiura and Isobe, (1974) and Lu and Liska, (1969) for *Chromobacterium viscosum* and *Pseudomonas fragi*. Zn^{2+} strongly inhibits both the intracellular and extracellular activities. This was also found to be the case for *Anaerobica lipolyticum* (Henderson, (1971) and *Chromobacterium viscosum* (Sugiura and Isobe, 1974). However, the extracellular activity is far less sensitive to Fe ions and exhibits a greater sensitivity towards Zn^{2+} ions.

Lipases can be classified according to the types of reactions catalysed or their specificity (IUPAC-IUB Commission on Enzyme Nomenclature). Specificity is a comparative difference in rates of catalysis of certain reactions. The specificity of lipases is controlled by the molecular properties of the enzyme, structure of the substrate and factors affecting the binding of the enzyme to the substrate. The types of lipase specificity have been identified as: substrate, positional, fatty acid and a combination of these three (Jenson *et al.*, 1983).

In this study an attempt was made to determine the chain-length specificity (refers to the fatty acid part of the ester) of the two enzyme activities, using a range of ρ-nitrophenyl esters. The intracellular enzyme has two major peaks of activity occurring at the four carbon substrate ρ-nitrophenyl butyrate and at the ten carbon substrate ρ-nitrophenyl caprate, while the extracellular enzyme has one major peak of activity corresponding to the twelve carbon substrate ρ-nitrophenyl laurate. The fact that the intracellular enzyme has two major peaks of activity is similar to the findings of the temperature optimum studies. However the explanation for these two peaks of activity with different substrates is not obvious. The maximum activity peak corresponding to ρ-nitrophenyl butyrate would suggest the presence of an esterase activity. The second peak for the

intracellular enzyme does not exactly correspond to the peak for the extracellular enzyme. If this intracellular activity is a pre-secreted form of the extracellular enzyme, some modification could take place during or after secretion which could alter the enzyme's substrate specificity, which could explain the differences between the second intracellular peak and the extracellular peak. Alternatively the intracellular enzyme could have dual substrate specificity, with affinity for both esterase and lipase substrates. Both crude extracts and various preparations obtained during purification of a lipase from *Proprionibacterium shermanii* were found to obtain a weak esterase activity (Otterholm *et al.*, 1970). However it was concluded that this small esterase activity was due to an activity of the lipase itself. Similar dual substrate properties have been reported for other lipases; from *Aspergillus niger* (Iwai *et al.*, 1964), from *Rhizopus delemar* (Fukumoto *et al.*, 1964) and from *Pseudomonas fragi* (Lu and Liska, 1969). A simpler explanation could be that the different specificities for the higher chain length substrate are due to the fact that the experiments were carried out in crude extracts and some external factor present could be interfering with substrate binding. Keeping in mind the evidence from the temperature optimum studies I think that it is more likely that there are two separate lipolytic enzymes present in the intracellular crude extracts, possibly an esterase and a lipase. *Propionibacterium freudenreichi* subsp. *freudenreichi* has been shown to contain both esterase and lipase activities (Dupuis *et al.*, 1993). Acinetobacter O16 produces an extracellular lipase and a cell-bound esterase which is secreted into the external medium late in growth (Breuil and Kushner, 1975)

PMSF was the only inhibitor found to affect the enzyme activities. Turini *et al.*, (1969) found that the serine proteases, Chymotrypsin and Trypsin were completely inhibited by incubation with 1 mM PMSF for 30 minutes. In this study only the higher concentrations of PMSF (10 mM and 20 mM) have a significant effect on either the intracellular or extracellular lipase activities. This phenomenon could be due to the fact that experiments were carried out using crude extracts. Moulin *et al.*, (1989) found that larger concentrations of the inhibitor 2-sulphobenzoic cyclic anhydride (SBA) were required to inhibit crude preparations of porcine pancreatic lipase (PPL). Their data also

suggested that the inactivation of PPL by SBA occurred preferentially at an oil / water interface and not in the aqueous phase. Gargouri *et al.*, (1991) also demonstrated that the inactivation of pancreatic and gastric lipase occurs preferentially at an oil / water interface.

The use of sulphydryl reagents had no effect on either of the enzyme activities although this was expected as it is rare for these reagents to affect lipase activities. However gastric lipases have been reported to be inactivated by sulphydryl reagents (Gargouri *et al.*, 1988) which resulted in the modification of one sulphydryl group which was shown to be essential for the enzymatic activity (Gargouri *et al.*, 1989).

4.7.4 MOLECULAR WEIGHT DETERMINATION

Generally microbial lipases have molecular weights which fall into a 20-70 kDa range, although there are exceptions. *Geotrichum candidum* produces two forms of lipase (I and II) which have molecular weights of 64 and 66 kDa (Sugihara *et al.*, 1990). *Chromobacterium viscosum* produces a lipase with a MW of 27kDa (Sugiura and Isobe, 1994), while *Staphylococcus aureus* 226 produces a lipase with a MW of 34 kDa (Muraoka *et al.*, 1982).

Both the use of HPLC and a standard gel filtration column to determine the MW of the extracellular enzyme proved unsuccessful. The main reason for the failure was that crude extracts were used in these studies. Aggregates of more than one enzyme or aggregates resulting from interactions with lipids or other proteins could result in large particles which would pass through the column and appear in the initial peak. An extracellular lipase secreted by *Pseudomonas aeruginosa* was found to be present as high molecular weight aggregates consisting of proteins and lipopolysaccharide (Stuer *et al.*, 1986).

The molecular weights of bacterial lipases have generally been determined by gel filtration techniques and by SDS-PAGE. Samples of crude extracts of the extracellular enzyme were run on native polyacrylamide gel electrophoresis and stained for activity using β-napthyl acetate. Activity was detected as a red coloured band. Unfortunately this red band appeared just under the well in the stacker gel. This failure to run on native gels could be due to the formation of large aggregates. The activity stained band on the native gels was excised and treated with SDS and then run on SDS-PAGE. The first attempt proved successful with the detection of four separate bands but unfortunately this could not be repeated.

The ultracentrifugation experiments agree with the findings of the Mr determination studies, in that both enzymes were of a sufficiently high Mr to remain insoluble.

4.7.5 KINETIC STUDIES ON THE INTRACELLULAR AND EXTRACELLULAR LIPASE ACTIVITIES

Conventional enzyme kinetics has been developed for reactions where the enzyme and the substrate are water soluble. The enzymatic hydrolysis of a lipid is different because the enzyme is water soluble and the substrates are not. The enzyme-substrate interaction takes place at a lipid-water interface and is therefore two-dimensional in character. In enzyme reactions that follow Michaelis-Menton kinetics substrate concentration is always expressed in moles l^{-1}, but the definition and measurement of substrate concentration in interfacial enzyme kinetic studies constitutes a major problem (Verger and de Haas, 1967).

Emulsified lipid substrates tend to minimise contact with water and favour the formation of rather large emulsion droplets covered by a single layer of lipid molecules one molecule thick. Benzonana and Desnuelle, (1965) demonstrated that calculating Km values for lipases using substrates in the form of emulsions, can be meaningless. They demonstrated that the physical parameters of the emulsion, such as size of the emulsion

droplet can strongly influence the value of Km. If the substrate concentration was expressed as area/volume rather weight/volume the Lineweaver-Burk plots comparing rates of lipolysis of coarse and fine emulsions of substrate could provide identical Km values. This demonstrated that one of the consequences of the interfacial nature of lipolysis relates more directly to the surface excesses of enzyme and substrates rather than the overall concentrations in the reaction.. Fig. 4.15 gives a schematic representation of a lipolytic reaction (Borgström and Brockman, 1984), demonstrating that the partitioning of reactants and products to and from the surface. The main difference between this and simple Michaelis-Menton kinetics is the independence of enzyme adsorption to the interface and catalysis in the interfacial plane. Verger and de Haas, (1976) had more serious reservations to determining interfacial Km values using emulsions as substrates for lipolysis. They felt that, in practice, it was not easy to determine accurately the surface area of emulsion droplets and that when detergents were needed to stabilise the emulsion, the calculations of Km in area/volume are questionable because the interfacial area occupied by the detergent is unknown. However differences in particle size and distribution from one preparation resulting in alterations in the Km value can be overcome by performing all the experiments on the same substrate preparation (Borgstrom and Brockman, 1984).

A lipid-water interface that can be characterised in physiochemical terms better than emulsions is the micellar solution (Verger and de Haas, 1976). Because micelles are generally smaller than emulsions, they exhibit relative optical clarity (Carey and Small, 1971). This allows convenient assay of enzymatic activity with chromogenic substrates (Momsen and Brockman, 1977). At the critical micellar concentration (CMC), the limit of molecular solubility is reached and aggregation of the molecules into spherical or rod-shaped particles (micelles) occurs. The lipid molecules in the micelle are in rapid equilibrium with the molecules in free solution. The classical definition of Km in concentration units should be able to be applied to micellar systems. In the micelle every lipid molecule is present at the interface and able, in principal to interact with the enzyme molecule. Using the known size of the micelle, one could calculate the surface

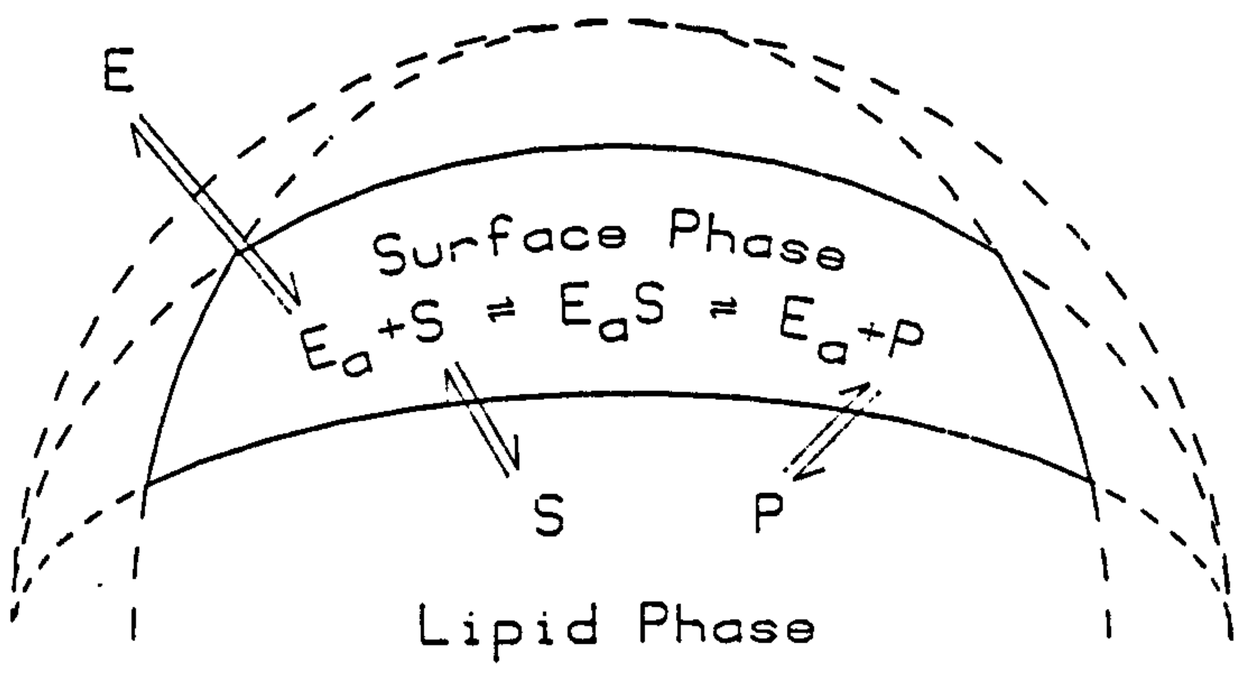


Fig. 4.15 Diagrammatic representation of a lipolytic reaction (Borgström and Brockman, 1984) E, enzyme in solution; Ea, adsorbed enzyme; S, substrate; P, products.

area of the lipid water interface, and this should be directly proportional to the lipid concentration. Care must be taken when using micellar solutions to ensure that the concentrations of substrate are higher than the CMC. Micellization is a continuous process and therefore a small percentage of the lipid exists as micelles below the CMC (Entressangles and Desnuelle, 1968).

The method of lipid dispersion used in this study involved the addition of a neutral detergent, Triton X-100 to the chromogenic substrate ρ-nitrophenyl palmitate to give an optically clear solution of mixed micelles. Mixed micelles are reported to be rapidly attacked by most lipolytic enzymes and linear kinetics are often obtained because of the removal of the degradation products from the lipid water interface (Mattson and Volpenheim, 1966; Benzonana and Desnuelle, 1968). However the addition of one further component to the already existing enzyme-substrate-water system might complicate kinetic analysis.

The concentrations of Triton X-100 used in these kinetic studies was always higher than the CMC (0.2 mM) [Houslay and Stanley, 1984]. Thus it is reasonable to assume that practically all of the Triton molecules are in the form of micelles Individual comparisons between the intracellular and extracellular Km values were carried out using the same substrate preparations, to avoid differences between separate substrate preparations. Method 3. of preparing the mixed micelles gave the highest Km values for both the intracellular and extracellular activities. The increase in Km could be as a result of increasing the number of micelles which resemble pure Triton micelles. The enzyme might bind to pure Triton micelles which do not contain substrate and therefore delay hydrolysis. This explanation was favoured by Kaplan and Tang, (1971) when studying a triglyceride lipase. Experimental evidence for the coexistence of pure detergent micelles and mixed phospholipid micelles has been provided by Dervichian, (1968) for a bile salt-phosphatidylcholine system and Yedgar *et al.*, (1974) for a sphingomyelin-Triton X-100 system. Alternatively, increasing the Triton X-100 concentration could provide a competitive inhibition by effectively lowering the concentration of lipid

substrate in the mixed micelle. If the enzyme did not bind to the mixed micelle, but rather only to the substrate in the mixed micelle, then additional Triton could cause a decrease in activity if it were to change the characteristics of the mixed micelle so as to place the substrate in a less optimal physical arrangement in the mixed micelle. The latter two hypotheses were postulated by Dennis, (1973). Further studies carried out using Method 3 (Methods 2.32) as the method of preparing mixed micelles with increasing concentrations of Triton X-100 at a constant ρ-nitrophenyl palmitate concentration provide further evidence for a competitive inhibition by Triton X-100. Method 2. of preparing mixed micelles gave the lowest values for Km, when comparing the three methods used. An explanation for this could be that even though the total number of micelles is decreasing, the micelles present should all contain substrate molecules as the ratio between Triton and substrate is kept constant. Method 1. gives micelles which have a decreasing number of substrate molecules on the surface of the micelle, because the Triton concentration remains constant and only the substrate concentration is decreasing. These findings demonstrate the importance of the consistent preparation of the substrate, to ensure that substrate presentation is the same, when wishing to significantly and directly compare one set of experimental data to another. The Kms obtained for the intracellular and extracellular activities were consistently different, independent of the method of substrate preparation.

4.7.6 THE EFFECT OF THE PRESENCE OF RAPE SEED OIL ON LIPASE ACTIVITY

In determining the procedure which would allow the induction of increased lipase activities, in cultures of *S.rimosus*, it was necessary to consider what effects the presence of rape seed oil, if any, would have on these activities. Surprisingly, it was found that the presence of rape seed oil did not induce the greatest increase in lipase production. Glucose was required in addition to rape seed oil before there was a significant increase in lipase activity, but even then this induced activity was not as high as that produced by glucose alone. This led to the belief that possibly the rape seed oil

was interfering in some part of the assay process. The lipase preparations, prepared from biomass which had been resuspended in media containing rape seed oil, may contain some residues of the oil. Thus rape seed oil could be interfering with the standard lipase assay by competing with the ρ-nitrophenyl substrate. Alternatively the lipase could be binding to rape seed oil which is removed during the normal process of harvesting, which would result in lower than expected activities.

The results obtained demonstrate the difficulties involved in handling lipids. It is impossible to predict how much of the rape seed oil will be carried over to the lipase preparations and therefore whether it is going to interfere with the assay. Measurements of protein concentrations showed that there was not a significant loss of protein from the cultures resuspended in media containing rape seed oil. This excludes the possibility of lipase being removed together with the rape seed oil , during harvesting. This experiment does not answer the question of whether the rape seed oil is competing with the ρ-nitrophenyl substrate in the standard lipase assay. The effect of rape seed oil on the lipase affinity for the ρ-nitrophenyl palmitate substrate was investigated, by studying the effect that the presence of rape seed oil had on the Km for ρ-nitrophenyl palmitate. The results indicated that, even with the rape seed oil present at a quarter of the ρ-nitrophenyl palmitate concentration, the presence of rape seed oil does affect the affinity of both the intracellular and extracellular enzymes for the ρ-nitrophenyl substrate.

4.7.7 THE EFFECT ON LIPASE ACTIVITIES IN CULTURES OF *S.RIMOSUS* GROWN ON STANDARD GROWTH MEDIA SUPPLEMENTED WITH POLYOXYETHYLENE ESTERS

A lipase preparation from *Aspergillus niger* was found to be capable of hydrolysing Tween 80 (Wouters, 1967). However, as most natural substrates for lipases are insoluble glycerides, it is possible that some lipases are inactive towards soluble esters like Tween. For example pancreatic lipase acts exclusively at lipid water interfaces and does not attack soluble esters (Desnuelle, 1961). A stimulation of the production of

extracellular lipase activity from *Geotrichum candidum*, *Penicillium spec.*, and *Fusarium solani* is seen in experiments carried out by Wouters, (1967) although this stimulation is only observed in media with concentrations of below 0.5% Tween. The influence of Tweens on the production of lipase is not the same for all microorganisms as they inhibit production by *Aspergillus niger* and *Aspergillus flavus.* Other detergents were tested and it was found that lipase production was only stimulated in media with detergents containing an ester group. The addition of Tween 20 to cultures of *Acinetobacter* had no effect on lipase production, while the addition of Tween 80 resulted in an inhibition (Breuil and Kushner, 1975).The effect of supplementing cultures of *S.rimosus* with Tween is that a second phase of growth occurs and in this period there appears to be an increased production of both the intracellular and extracellular lipases. . If Tween stimulates the production of the lipase or the additional slower phase of growth allows further production of the lipase this could explain why the cultures containing Tween in experiments (a) had lower lipase activities as they were harvested at the end of the first phase of growth. There are a few discrepancies in the results and these could be due to the presence of Tween interfering with ρ-nitrophenyl palmitate assay.

A possible explanation for the decrease in lipase activity, with the presence of Tween in the standard assay, is that surface-active compounds like Tweens generally attach themselves to the water-lipid interface where the reaction takes place. Therefore the inhibition could be competitive with the Tweens containing an ester group with which the lipase could combine with rather than the ρ-nitrophenyl palmitate. This result also compares with the findings of the kinetic studies, using Method 3 has the method of preparing the substrate for the assay The lipase of *Geotrichum candidum* was found to be inhibited by different detergents and emulsifiers such as Tweens, gum arabic and other non-ionic surfactants (Wouters, 1967). Considering that the presence of both Tween 20 and 40, in the standard assay, result in a decrease in lipolytic activity, the results obtained from the induction of lipolytic activity by Tween may be artificially

lowered. The crude extract preparations will contain some Tween and therefore this could result in contamination of the standard assay.

4.7.8 CONCLUSIONS AND SUMMARY

The induction experiments would suggest that there were two lipolytic activities. From the comparisons made between the intracellular and extracellular activities (Table 4.9) it can be determined that the intracellular and extracellular activites are indeed due to the presence of independent lipolytic enzymes. The extracellular activity has a greater temperature optimum, exhibits a greater thermal stability, responds differently to Fe ions and Zn^{2+} ions, has a higher substrate chain length specificity, and behaves differently kinetically using a number of methods of substrate preparation. Therefore the two activities have a number of different properties.

The temperature optimum experiment implies that the extracellular activity is a secreted enzyme as some of its activity is present in the intracellular extracts. The substrate chain length experiment would also imply this and in addition would suggest the presence of an intracellular esterase activity. With hindsight a useful experiment to undertake would be to carry out substrate chain length specificity studies at 45°C and 60°C. This would help to determine whether the 45°C peak corresponds to the four carbon chain length substrate maximum and whether the ten-twelve carbon chain length maximum corresponds to the 60°C peak.

Unfortunately inhibitor studies proved inconclusive. In order for these to be effectively carried out it would be desirable to use purified lipolytic enzyme and a true lipase assay employing a triglyceride as a substrate. This is also true for the molecular weight studies which were probably unsuccessful due to the presence of large aggregates. Kinetic studies on purified lipase would enable comparisons between Vmaxs and additionally comparisons could be made between different preparations of enzyme at constant protein concentrations. However, the aims of this study were not to purify a lipase but

to look at lipid metabolism as a whole in *Streptomyces*. Future work purifying the lipolytic enzymes present in *Streptomyces rimosus* would prove interesting and useful in light of the present search for biotechnologically useful lipases and prove helpful in improving the utilisation of oils in industrial streptomycete fermentations.

Table 4.9 SUMMARY OF THE PROPERTIES OF THE INTRACELLULAR AND EXTRACELLULAR ACTIVITIES

This table gives a summary of the properties of the intracellular and extracellular activities found in crude extracts of *S. rimosus* 4018 (see sections 4.2-4.5).

Studies	Intracellular activity	Extracellular activity	Difference
pH Optimum	8	8.5	?
Temp. Optimum	45 C, 60 C	60 C	yes
Temp. stability		> stability at 70 C	yes
Metal ions		< sensitive to Fe ions >sensitivity to Zn ions	yes
Substrate chain length specificity	4, 10	12	yes
Inhibitors			inconclusive
Mr studies			inconclusive
Ultracentrifugation studies			inconclusive
Kinetic studies	Different Kms		yes

CHAPTER 5

LIPIDS PRESENT IN *STREPTOMYCES*

RESULTS AND DISCUSSION

5.0 LIPID AND FATTY ACID COMPOSITION OF *STREPTOMYCES* SPECIES

5.1 INTRODUCTION

The relationship between primary and secondary metabolism can be investigated by studying the supply of common precursors to fatty acid biosynthesis and polyketide antibiotic biosynthesis. In this chapter, analyses were carried out to determine the fatty acid composition of lipid fractions extracted from *Streptomyces* sp.. Particular emphasis was placed on the identification of branched chain fatty acids which are typical of *Streptomycetes*. The importance of the branched-chain fatty acid content is explained by the fact that they share common precursors with polyketide antibiotics. A change in the content or composition of the branched-chain fatty acids could be indicative of a diversion of these common precursors from antibiotic production to lipid storage or vice versa. Conditions which were expected to promote antibiotic production and/ or lipid storage were investigated for their potential to change the branched-chain fatty acid content.

A comparison was made between the lipid profiles obtained from three species of *Streptomyces*; *S. coelicolor*, *S. rimosus* and *S. clavuligerus*. *S. rimosus* and *S.coelicolor* produce the polyketide antibiotics oxytetracycline and actinorhodin respectively, while *S. clavuligerus* does not produce a polyketide antibiotic but does produce several β-lactam compounds, including clavulanic acid, penicillin N, deoxycephalosporin C and cephamycin C (Jenson, 1986). Efforts were made to identify any differences between the fatty acid profiles and to determine the branched-chain fatty acid composition (results from gas-liquid chromatography studies are expressed as the % by weight of the total fatty acid methyl esters detected) of the lipid fractions with a view to identifying possible sites for the storage or diversion of polyketide antibiotic precursors. A quantitative and temporal correlation was found between the production of avermectins and lipids in *Streptomyces avermitilis* grown in defined media (Novak *et al.*, 1990). The fact that triglycerides and avermectins were synthesised simultaneously indicated that

these two biosynthetic pathways may compete for a limited supply of common precursors (2-methylbutyrate, propionate and acetate etc.). The lipid fraction was found to contain mostly triglycerides: iso-even and odd, anteiso-odd and straight-chain fatty acids in the range of C_{14} to C_{18} (Metz *et al.*, 1988). Further studies carried out by Rezanka *et al*, (1992) indicated that 2-methylbutyrl CoA was a common precursor for both fatty acid and avermectin biosynthesis

The accumulation of lipids in nitrogen-limited cultures is typical of oleaginous microorganisms which tend to be eukaryotes e.g. algae, yeasts and moulds which accumulate lipid mainly in the form of triglycerides. An oleaginous microorganism may be defined as one that has the potential to accumulate lipid at 25 to 70% of the dried biomass, when cultured under optimal conditions (Ratledge, 1982). In these organisms under conditions where there is an excess of carbon and a deficit of nitrogen, protein and nucleic acid synthesis are curtailed. However, lipid synthesis continues at an undiminished rate and thus lipid builds up with respect to the remaining biomass. Of particular relevance to this project are reports that certain species of *Streptomycete* accumulate lipids. Packter *et al*, (1985) reported that *Streptomyces coelicolor* grown under certain conditions had 50% of its total lipid content as triglycerides. Of even greater interest was that *Streptomyces avermitilis* was found to produce a lipid rich fraction in conjunction with the polyketide antibiotic avermectin, when grown under conditions where a high carbon/ nitrogen ratio was present (Novak *et al*, 1990). Evidence of lipid storage was investigated by culturing *S. rimosus* under conditions expected to produce this phenomena, one where a high carbon/ nitrogen ratio was present and the second where a high carbon/ phosphate ratio existed.

5.2 IDENTIFICATION OF STANDARD FATTY ACIDS BY GAS-LIQUID CHROMATOGRAPHY ANALYSIS USING RETENTION TIMES

To identify fatty acids, using GLC the retention times were compared with those of known standards (Methods 2.29). Fig. 5.1 is a typical GLC trace of this standard

Bacterial Acid Methyl Ester CP Mix (see Methods 2.29). Table 5.1 lists the retention times for the twenty six fatty acids present in this standard. Retention times are listed for analysis carried out; on the same day, on consecutive days, a week apart and a month apart. Analysis carried out on consecutive days are very close. Analysis carried out over a week can vary enough to cause confusion if a sample was compared with a standard from a different week. Despite this, analysing the data generated over a month the coefficient of variants generally remained within an acceptable limit of below 5%. However because of the similarity of the retention times for iso and anteiso fatty acids a standard was run every day that analyses were carried out.

5.3 THE LIPID AND FATTY ACID COMPOSITION OF *S. RIMOSUS*, *S. COELICOLOR* AND *S. CLAVULIGERUS*

A preliminary study of the lipid profiles obtained from *S. rimosus*, *S. coelicolor* and *S. clavuligerus* was undertaken using thin-layer chromatography and gas-liquid chromatography. *S. rimosus* and *S. coelicolor* were grown in YEME and *S. clavuligerus* was grown in minimal medium supplemented with 20 gl^{-1} maltodextran until stationary phase was reached. Pellets were harvested (Methods 2.7) and the lipid was extracted (Methods 2.23). Whole lipid samples were run on thin-layer chromatography plates (Methods 2.24) using petroleum ether 40-60°C/ diethyl ether/ formic acid (150:50:1, v/v/v). Fig. 5.2 gives a typical example of one of the thin-layer chromatography plates obtained. Arrows indicate which spots were further analysed using gas-liquid chromatography. All three streptomycetes contained spots corresponding to triglycerides and di or monoglycerides, when compared with a standard mixture. Both *S. coelicolor* and *S. rimosus* contained spots which ran with the same Rf value as the standard free fatty acids , but *S. clavuligerus* did not. In addition, the TLC profile from *S.coelicolor* contained a spot which ran with the same Rf value as the fatty alcohol standard. The spot which remained at the origin of the chromatogram was made up of non-polar lipid, including phospholipids. For the remainder of this thesis the lipid extracted from this spot is reffered to as the phospholipid containing

fraction. The triglyceride and free fatty acid TLC spots were converted into methyl esters and characterised by comparing their retention times with those of known standards using gas-liquid chromatography. Table 5.2 lists the percentages (expressed as % by weight of the total fatty acid methyl esters detected) of the main fatty acids present in the whole lipid and triglyceride fractions from *S. coelicolor*, *S. rimosus* and *S. clavuligerus*, and the free fatty acid fraction from *S. coelicolor* and *S. rimosus*.

Characteristically of *Streptomycetes*, all three species contained a high percentage of branched-chain fatty acids, particularly *S. rimosus* which was found to contain approximately 50% branched-chain fatty acids. Generally, saturated fatty acids were found to be more predominant than unsaturated fatty acids. Although similar fatty acid profiles were obtained for each organism, there were differences between the individual fatty acid compositions. *S. coelicolor* had no iso (represented by 'i' before the fatty acid) fatty acids present, only anteiso (represented by 'a' before the fatty acid) branched-chain fatty acids appear. *S. coelicolor* also had a high percentage of a twenty carbon straight chain fatty acid (20:0) present which does not appear in the other two species. *S. rimosus* did not contain any 18 carbon chain length fatty acids which was unusual. *S. clavuligerus* was found to have a cyclopropane fatty acid consisting of sixteen carbons in a straight chain and one methyl containing cylopropane ring (17:0Δ) present in the triglyceride fraction. Cyclopropane fatty acid synthesis occurs primarily as bacterial cultures enter stationary phase (Vance and Vance, 1985). The cultures of *S. rimosus* and *S. coelicolor* might not have been far enough into stationary phase for the production of cyclopropane fatty acids to occur.

5.4 A COMPARISON BETWEEN THE FATTY ACID PROFILES FROM *S. RIMOSUS* GROWN ON A MINIMAL MEDIUM AND A COMPLEX MEDIUM

S. rimosus was cultured on the complex medium, YEME and on the minimal medium, modified HMM. A comparison was made between the fatty acid profiles of the biomass harvested from these two culture conditions, using GC-MS. Table 5.3 (a) and (b) lists the percentages of the main fatty acids, determined by GLC, present in the four fractions

Fig. 5.1 GLC TRACE OF BACTERIAL ACID METHYL ESTER CP MIX

The GLC trace shows the individual peaks obtained when 0.4 µl of the standard Bacterial Acid Methyl Ester CP Mix, containing twenty six standard fatty acid methyl esters, was analysed by GLC (Methods 2.29). The retention times (Table 5.1) obtained for these standards were used to identify samples of fatty acids. A sample of this standard mix was always run on the same day as unkown samples. The individual peaks are numbered from one to twenty six and correspond to the following fatty acids:

Peak no.	Fatty acid
1	11:0
2	2-OH 10:0
3	12:0
4	13:0
5	2-OH 12:0
6	3-OH 13:0
7	14:0
8	i15:0
9	a15:0
10	15:0
11	2-OH 14:0
12	3-OH 14:0
13	i16:0
14	$16:1^{9}$
15	16:0
16	i17:0
17	17:0Δ
18	17:0
19	2-OH 16:0
20	$18:2^{9,12}$
21	$18:1^{9'}$
22	$18:1^{9}$, $18:1^{11}$
23	18:0
24	19:0Δ
25	19:0
26	20:0

The symbols used to define types of fatty acids are: i - iso fatty acid, a - anteiso fatty acid, 2-OH - fatty acid containing a methyl group on carbon 2, 3-OH as before except the hydroxyl group is on carbon 3, ^{9,11,12} denote the position of the double bonds and Δ denotes a fatty acid containing a cyclopropane ring.

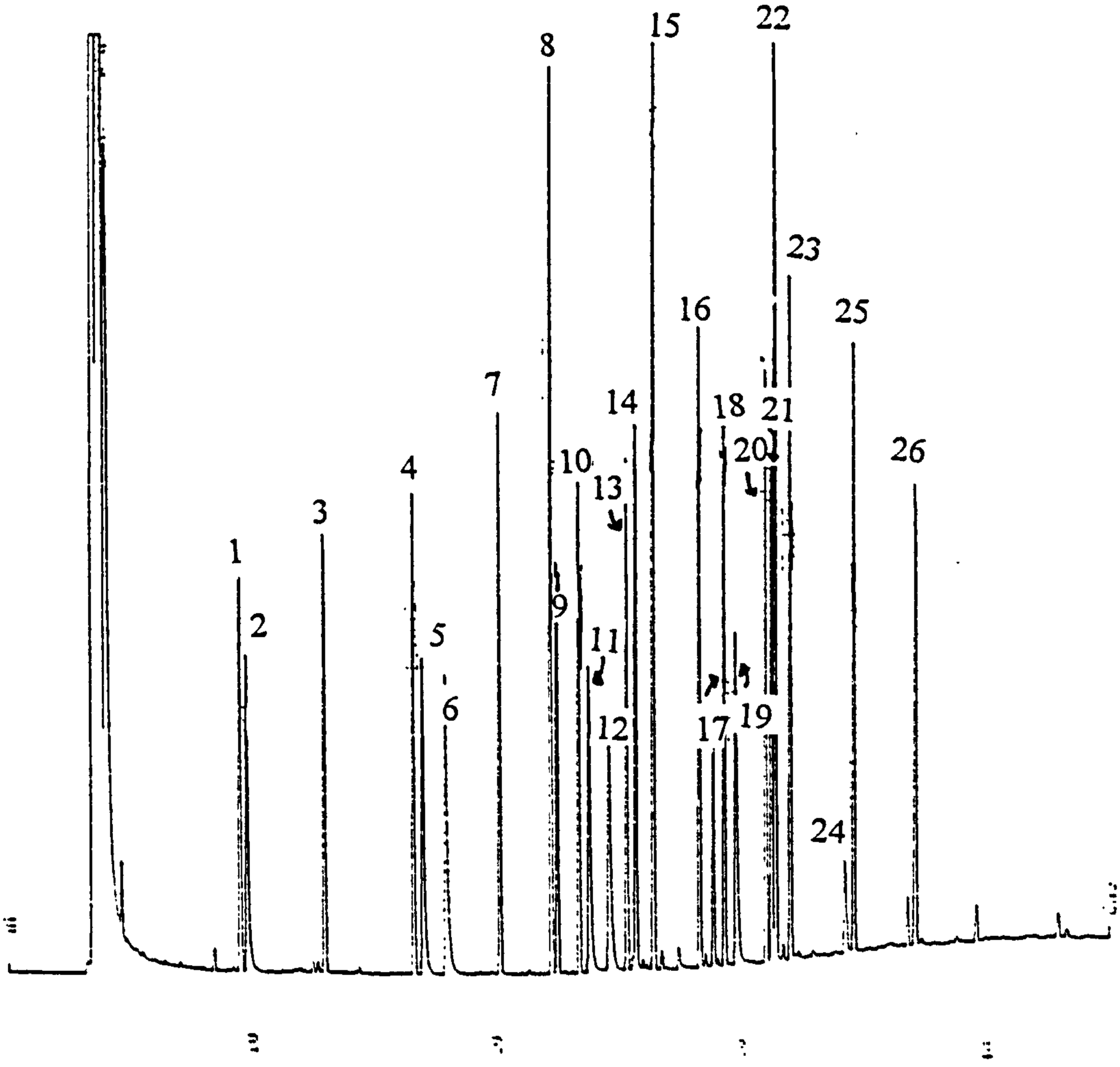

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Table 5.1 THE PERCENTAGES OF THE MAIN FATTY ACIDS PRESENT IN THE LIPID FRACTIONS PRESENT IN *S. COELICOLOR*, *S. RIMOSUS* AND *S. CLAVULIGERUS*

The percentages of the main fatty acids present in *S. coelicolor*, *S. rimosus* and *S. clavuligerus* whole lipid, triglyceride and free fatty acid fractions are given in this table.. The fatty acids were converted into fatty acid methyl esters (Methods 2.27) and the identification is by comparison of retention times with those of known standards (Methods 2.29). For a definition of the symbols used to describe the fatty acids see the legend to Fig. 5.1.

Peak no.	Fatty acid	Day no.									Mean SD	CV
		1	8(a)	8(b)	12	18	30	31	32	45		%
		Retention times (min)										
1	11:0	9.31	9.95	9.95	9.54	9.36	9.55	9.63	9.90	9.31	9.55±0.32	3.3
2	2-OH10:0	9.60	10.24	10.24	9.83	9.65	9.82	9.91	9.88	9.60	9.86±0.24	2.5
3	12:0	12.88	13.39	13.39	12.97	12.84	12.97	13.06	13.02	12.78	13.03±0.22	1.7
4	13:0	16.65	17.04	17.04	16.61	16.52	16.62	16.70	16.66	16.47	16.70±.020	1.2
5	2-OH12:0	17.04	17.42	17.42	16.99	16.90	16.99	17.07	17.04	16.85	17.08±0.20	1.2
6	3-OH12:0	18.00	18.37	18.37	17.94	17.84	17.93	18.00	17.96	17.80	18.02±0.21	1.2
7	14:0	20.28	20.57	20.57	20.16	20.09	20.16	20.32	21.09	20.03	20.36±0.33	1.6
8	i15:0	22.47	22.72	22.72	22.30	22.26	22.32	22.39	22.35	22.19	22.41±0.19	0.8
9	a15:0	22.73	22.97	22.97	22.56	22.51	22.57	22.64	22.59	22.44	22.66±0.19	0.8
10	15:0	23.65	23.37	23.37	23.46	23.42	23.4	23.54	23.50	23.35	23.44±0.08	0.3
11	2-OH14:0	24.09	24.32	24.32	23.90	23.84	23.91	23.94	23.93	23.77	24.00±0.20	0.8
12	3-OH14:0	24.96	25.15	25.15	24.73	24.64	24.70	24.78	24.74	24.59	24.83±0.21	0.8
13	i16:0	25.66	25.86	25.86	25.46	25.42	25.46	25.53	25.48	25.35	25.56±0.19	0.7
14	16:1^{9}	26.56	26.24	26.24	25.82	25.79	25.84	25.9	25.86	25.73	26.00±0.28	1
15	16:0	26.75	26.94	26.94	26.52	26.51	26.55	26.61	26.56	26.44	26.65±0.19	0.7
16	i17:0	28.62	28.79	28.79	28.38	28.35	28.4	28.45	28.41	28.28	28.50±0.19	0.7
17	17:0Δ	29.17	29.79	29.79	29.37	28.91	28.94	29.00	28.95	28.83	19.19±0.37	1.3
18	17:0	29.62	30.27	30.27	29.85	29.35	29.52	29.45	29.4	29.28	29.67±0.38	1.3
19	2-OH16:0	30.12	31.48	31.48	31.06	29.82	29.86	29.91	29.87	29.73	30.37±0.74	2.4
20	18:2$^{\omega 12}$	31.33	31.71	31.71	31.45	31.04	31.08	31.13	31.08	30.97	31.28±0.29	0.9
21	18:1^{9}	31.56	31.87	31.87	32.04	31.27	31.31	31.36	31.32	31.20	31.53±0.31	1
22	18:1^{6}, 18:1^{11}	32.11	32.44	32.44	32.90	31.43	31.47	31.52	31.48	31.36	31.91±0.58	1.8
23	18:0	32.30	33.44	33.49	34.18	32.02	32.05	32.30	32.07	31.94	32.62±0.84	2.6
24	19:0Δ	34.15	34.94	34.95	34.53	34.52	34.20	34.25	34.20	34.08	34.42±0.33	1
25	19:0	34.81	35.60	35.60	35.43	35.70	34.55	34.61	34.56	34.44	35.03±0.53	1.5
26	20:0	37.76	37.52	37.52	37.04	37.03	37.03	37.12	37.05	37.03	37.23±0.28	0.8

Table 5.2 THE PERCENTAGES OF THE MAIN FATTY ACIDS PRESENT IN THE LIPID FRACTIONS PRESENT IN *S. COELICOLOR*, *S. RIMOSUS* AND *S. CLAVULIGERUS*

The percentages of the main fatty acids present in *S. coelicolor*, *S. rimosus* and *S. clavuligerus* whole lipid, triglyceride and free fatty acid fractions are given in this table.. The fatty acids were converted into fatty acid methyl esters (Methods 2.27) and the identification is by comparison of retention times with those of known standards (Methods 2.29). For a definition of the symbols used to describe the fatty acids see the legend to Fig. 5.1.

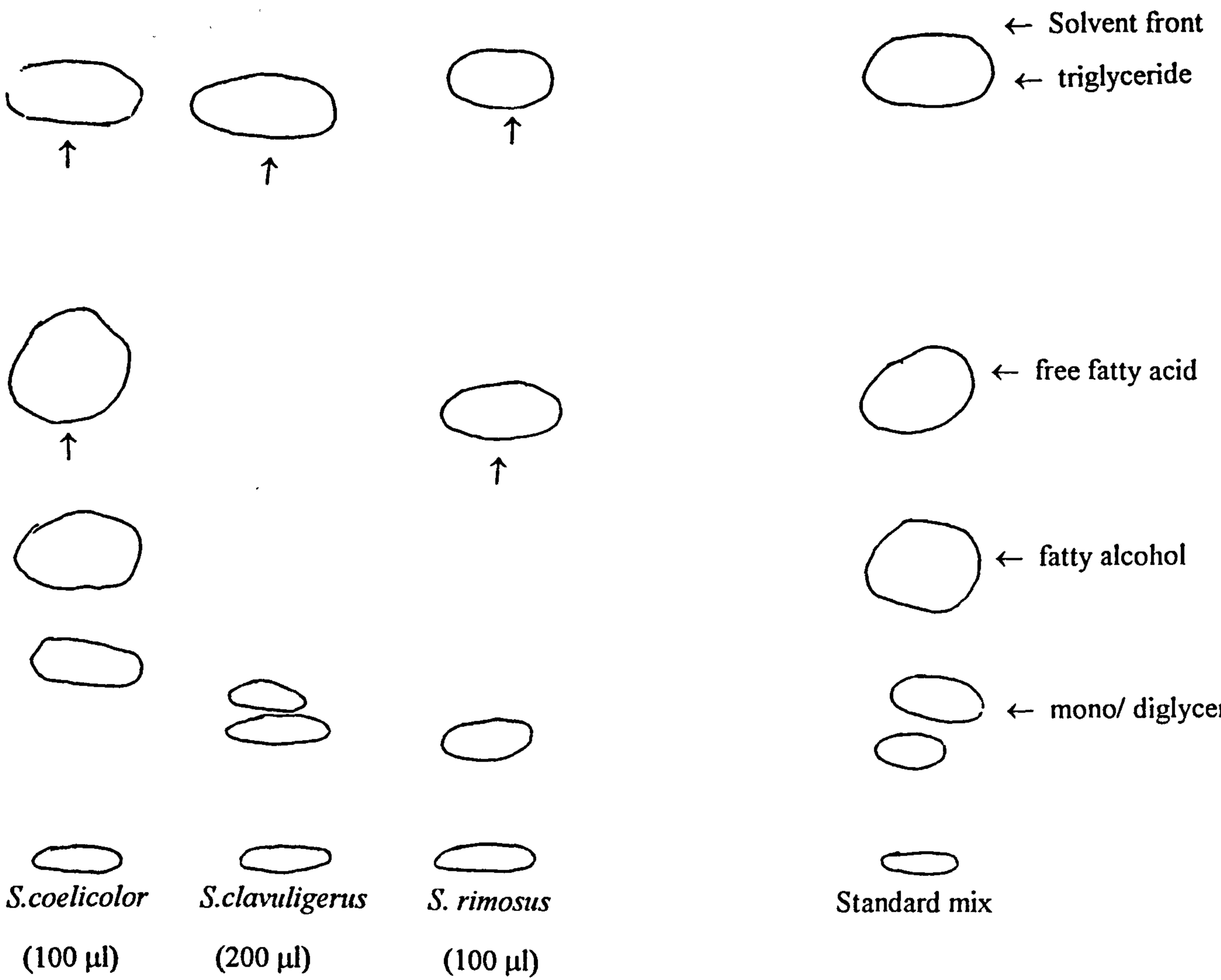

← Solvent front
← triglyceride
← free fatty acid
← fatty alcohol
← mono/ diglyceride
S.coelicolor
(100 µl)
S.clavuligerus
(200 µl)
S. rimosus
(100 µl)
Standard mix

Fig. 5.2 ANALYSIS OF THE LIPID COMPOSITIONS OF *S. COELICOLOR*, *S. RIMOSUS* AND *S. CLAVULIGERUS* USING THIN-LAYER CHROMATOGRAPHY

A typical example of a thin-layer chromatogram plate of lipids extracted from *S. coelicolor*, *S. rimosus* and *S. clavuligerus* (Methods 2.23). The solvent used was petroleum ether 40-60°C/ diethyl ether/ formic acid (150:50:1 [v/v/v]). Lipids were located by placing the thin-layer plate in a tank of iodine vapour. A standard mix containing a fatty acid, a fatty alcohol and a triglyceride (Methods 2.24) was run adjacent to the samples, to allow identification.

Fatty acid	*S. coelicolor* WL %	*S. coelicolor* TG %	*S. coelicolor* FFA %	*S. clavuligerus* WL %	*S. clavuligerus* TG %	*S. rimosus* WL %	*S. rimosus* TG %	*S. rimosus* FFA %
14:1		9.7			6.9			
14:0						7.8	5.7	
i14:0								
15:1	7.7							
15:0						5.2		
a15:0	22.8	8.8	18.1	9.2	5.3	17.6	18.4	9.7
i15:0				5.0		6.0	13.1	9.6
16:1'				17.1	7.4			
16:0		8.4		11.0	14.4	16.0	12.2	8.9
i16:0				8.6		15.6		28.8
17:1	9.2	16.1	6.2			15.0	13.4	22.3
17:0							14.3	
i17:0						9.6	9.1	
17:0Δ					14.3			
18:1'	6.2		5.7		10.4			
18:0				6.1	5.0			
19:0	8.5		7.3					
20:0	17.3		20.8					
Branched	22.8	8.8	18.1	22.8	5.3	48.8	40.8	38.1
Saturated	22.8	17.2	46.2	39.9	39.0	72.1	58.5	57.0
Unsaturated	23.1	25.8	11.9	17.1	24.7	15.0	13.4	22.3
Odd no. chain	48.2	24.9	31.6	14.2	19.6	48.2	54.0	41.6
Even no. chain	23.5	18.1	26.5	42.8	44.1	39.4	17.9	37.7

(whole lipid, phospholipid, triglyceride and free fatty acid) extracted from the YEME biomass and in the three fractions (whole lipid, phospholipid containing and triglyceride) extracted from the modified HMM biomass. The most abundant fatty acid present in the whole lipid fractions from both the YEME biomass and the modified HMM biomass was i16:0. The profiles for the whole lipid were in fact very similar with i15:0, a15:0, i17:0 and 16:0 appearing in both, at relatively the same high proportions. This was also the case for the phospholipid fractions. However, a major difference is that 17:0Δ (straight chain sixteen carbon chain length plus a cyclopropane ring) was present at a high percentage in the YEME grown biomass but did not appear in the modified HMM grown biomass. i16:0 remained the most abundant fatty acid in the phospholipid containing fraction of the modified HMM grown biomass. A peak ocurred at approximately 44 min at very high concentrations in the phospholipid containing fraction from the YEME grown biomass, but its identity as a fatty acid was not confirmed (see GC-MS analysis, section 5.4). Similarly this unidentified peak at approximately 44 min was the most abundant component present in the triglyceride fraction from both types of biomass. A major difference was seen in the triglyceride fractions extracted from the two growth media. The triglyceride fraction from the YEME grown biomass contained the same major fatty acids as the whole lipid and phospholipid fraction from both types of biomass. However, the triglyceride fraction from the antibiotic producing *S. rimosus*, grown on modified HMM contained i17:0 as its only branched-chain fatty acid and was made up predominantly of longer chain fatty acids (> 17 carbons), most of which remain unidentified.

Whole lipid fatty acid methyl esters from both the YEME biomass and the modified HMM biomass were analysed by GC-MS. Fig. 5.4 shows the GC trace obtained for the Bacterial Acid Methyl Ester CP Mix. Peaks from this trace were further analysed by MS. Fig 5.5 shows the individual mass spectra obtained for each of the twenty six standard fatty acid methyl esters in this mix. Table 5.4 summarises the characteristic identifying peaks (highlighted in the spectra) for the standards (interpretation of MS spectra is covered in Methods 2.30.1). The fatty acids in the two samples were

characterised by comparisons with those in the standard mix. The GC trace for the whole lipid fatty acid methyl esters from the YEME grown biomass is shown in Fig. 5.6 and the individual peaks on this trace were further analysed by MS (individual mass spectra are shown in Fig. 5.7). Table 5.5 summarises the fatty acids present in the whole lipid, obtained from YEME grown *S. rimosus*, and their identifying mass spectrum peaks. The GC trace for the whole lipid fatty acid methyl esters from the modified HMM grown biomass is shown in Fig. 5.8 and the individual peaks on this trace were further analysed by MS (individual mass spectra are shown in Fig. 5.9). Table 5.6 summarises the fatty acids present in the whole lipid, obtained from the modified HMM grown *S. rimosus*, and their identifying mass spectrum peaks.

The main fatty acids present in the whole lipid samples obtained from the YEME biomass and the modified HMM biomass identified by GC-MS were found to be similar. Both samples contained the following fatty acids as major constituents; 15:0, i15:0, 16:0, i16:0 and a17:0. A major difference between the two samples was that the whole lipid sample obtained from the biomass grown on modified HMM contained 2-OH 15:0 (straight chain fatty acid with fifteen carbons and a hydroxyl group on carbon 2) and 2-OH 16:0 as a major constituents. The GC-MS did not identify a fatty acid present in the YEME grown biomass corresponding to 17:0Δ, as identified by GLC analysis. However, a fatty acid corresponding to a methyl branched saturated 17 carbon chain fatty acid was identified by GC-MS. This methyl 17:0 could possibly result in a peak with a retention time corresponding to that of 17:0Δ when analysed by GLC. However, 19:0Δ was identified as a minor component in the YEME grown biomass. Ther were no fatty acids detected with a chain length greater than twenty carbons in the YEME biomass and twenty one carbons in the modified HMM biomass.

Closer analysis of the mass spectra enables further characterisation of the fatty acids present. Peak identification number 341 (major) and peak identification number 364 (minor) both correspond to 14:0. There are no large differences between the spectra. However, the base peak for 341 is at m/e = 199 and for 346 it occurs at m/e = 87. The

peak at m/e = 199 is a characteristically intense peak resulting from the loss of an oxygen containing ion of the type;

$$[(CH_2)_nCOOCH_3]^+$$

where in this case n = 10 (Walker, 1972). The peak occurring at m/e = 87 is also an oxygen containing ion of this type where n = 2. A more intense peak at m/e = 111 can be seen in the mass spectrum from 341 which could correspond to loss of a methanol. This could be interpreted as a methyl branch on carbon 8. Looking even more closely at the mass spectrum from 341 there is a peak present at m/e M-29 which is only marginally less intense than the peak at m/e M-31. A peak occurring at M-29 implies that this is the spectrum of a methyl branched fatty acid methyl ester. Therefore it is possible that peak identification number 341 corresponds to 8-methyl 14:0 Two major peaks (identification numbers 479 and 484) were found to correspond to 2-OH 15:0. Both spectra contain peaks at m/e = 90 and m/e = 103 which can be explained as a result of the rearranged ions at m/e = 74 and m/e = 85 for saturated methyl esters. In addition the mass spectra contain a peak at m/e M-59. The presence of these three peaks is indicative of a hydroxy fatty acid with the hydroxyl group at position 2. Looking closer at the spectra there are three differing peaks in the central region of the mass spectra of these two fatty acids; 479 has peaks at 199, 179 and 168, while 484 has peaks at 193, 183, and 165. These differences indicate that there is a structural difference between 479 and 484 because there are different ion fragments generated. Peak with the identification numbers 513 and 520 both correspond to 17:1 (straight chain fatty acid of seventeen carbons in length with one double bond) with characteristic peaks occurring at m/e M-32 and m/e M-116. I would suggest that the presence of two separate peaks both corresponding to 17:1 is due to them having the double bond in a different position. Unfortunately it is not possible to deduce the position of the double bond from a mass spectrum (Walker, 1972). This could again be the case for the two peaks with the identification numbers 592 and 598 which both correspond to 18:1. The peak with identification number 915 corresponds to a branched chain 21:0 fatty acid, but from the spectrum it was difficult to determine where the branch was.

Table 5.3 THE FATTY ACID PROFILES OBTAINED FROM *S. RIMOSUS* GROWN ON EITHER YEME OR MODIFIED HMM

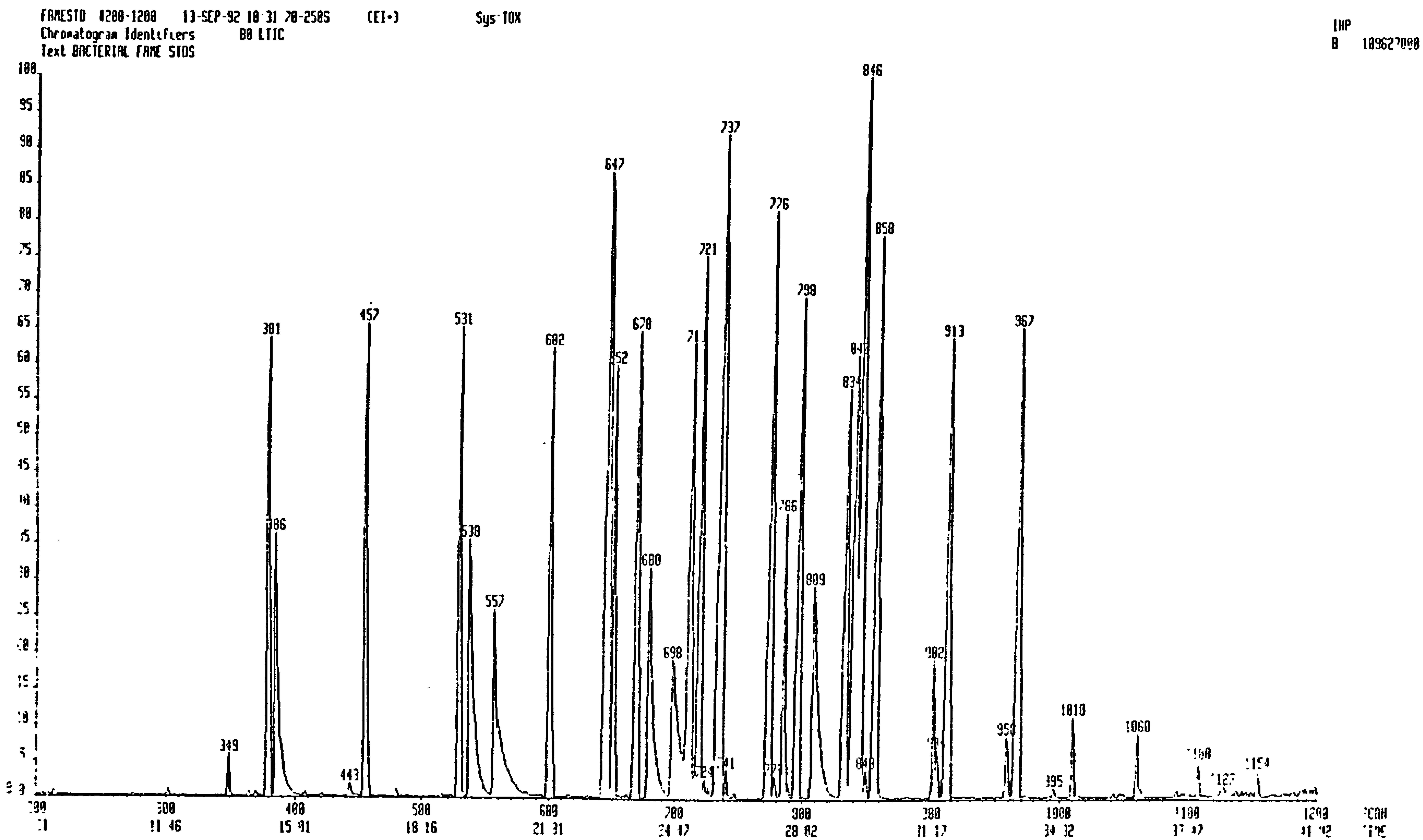
S.rimosus was grown (Methods 2.5) on the complex medium, YEME (Methods 2.3.2[c]) and on the minimal medium, modified HMM (Methods 2.3.1[b]). The percentages (results are expressed as % by weight of weight of total fatty acid esters) of the main fatty acids present in the extracted lipid fractions, determined by gas-liquid chromatography (Methods 2.23-2.29) are listed for the YEME grown biomass (a) and the modified HMM grown biomass (b). The use of the symbol '*' denotes that the peak was of scale and therefore was not included in the total weight of the fatty acid methyl esters.

WL	%	PL	%	TG	%	FFA	%
i16:0	17.7	43.78	*	43.82	*	43.07	*
17:0Δ	15.4	19.36	11.2	i16:0	10.6	20:0	33.1
i15:0	15.3	a15:0	9.7	i15:0	10.4	i16:0	11.3
a15:0	12.9	i16:0	8.6	17:0Δ	9.8	19:0Δ	8.5
i17:0	12.0	i17:0	7.2	a15:0	9.4	28.28	8.1
16:0	8.6	16:0	6.0	33.03	7.3	16:1^{9}	6.8
17:0	2.6	28.41	5.8	20:0	6.2	12.86	6.1
15:0	2.5	i15:0	5.2	16:0	6.0	22.19	5.9
18:2$^{9/12}$	2.0	17:0	4.7	i17:0	5.3	18:1^{9}	5.8
18.954	2.0	18:1^{9}	4.0	39.68	4.6	i15:0	3.4

(a) YEME

WL	%	PL	%	TG	%
i16:0	25.8	i16:0	26	43.97	19.2
a15:0	11.5	i17:0	9.2	18:0	9.3
i15:0	10	a15:0	8.6	i17:0	9.2
16:0	9.7	43.38	8.5	19:0	8.2
i17:0	9.2	16:0	7.6	36.67	7.4
18:1^{9}	6.0	26.24	5.8	40.69	5.5
22.34	5.7	i15:0	5.4	19:0Δ	5.2
26.18	5.1	18:0	3.7	38.45	4.6
18:0	4.4	16:1^{9}	3.5	42.35	4.1
16:1^{9}	3.3	18:1^{9}	2.5	2-OH 14:0	3.8

(b) modified HMM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Chromatogram Identifiers 00 LTIC
Text BACTERIAL FAME STDS
IHP
8 10962?000
349
381
386
443
457
531
538
557
602
647
652
670
680
698
721
737
776
798
809
834
846
858
902
913
950
967
1010
1060
SCAN
TIME

Fig. 5.5 INDIVIDUAL MASS SPECTRA FOR THE TWENTY SIX FATTY ACID METHYL ESTERS IN THE BACTERIAL ACID METHYL ESTERS CP MIX

The individual MS spectra (Methods 2.30) for each of the twenty six fatty acid methyl esters contained in the standard mix are as follows:

(a) 11:0
(b) 2-OH 10:0
(c) 12:0
(d) 13:0
(e) 2-OH 12:0
(f) 3-OH 13:0
(g) 14:0
(h) i15:0
(i) a15:0
(j) 15:0
(k) 2-OH 14:0
(l) 3-OH 14:0
(m) i16:0
(n) $16:1^{9}$
(o) 16:0
(p) i17:0
(q) 17:0Δ
(r) 17:0
(s) 2-OH 16:0
(t) $18:2^{9,12}$
(u) $18:1^{9'}$
(v) $18:1^{9}$, $18:1^{11}$
(w) 18:0
(x) 19:0Δ
(y) 19:0
(z) 20:0

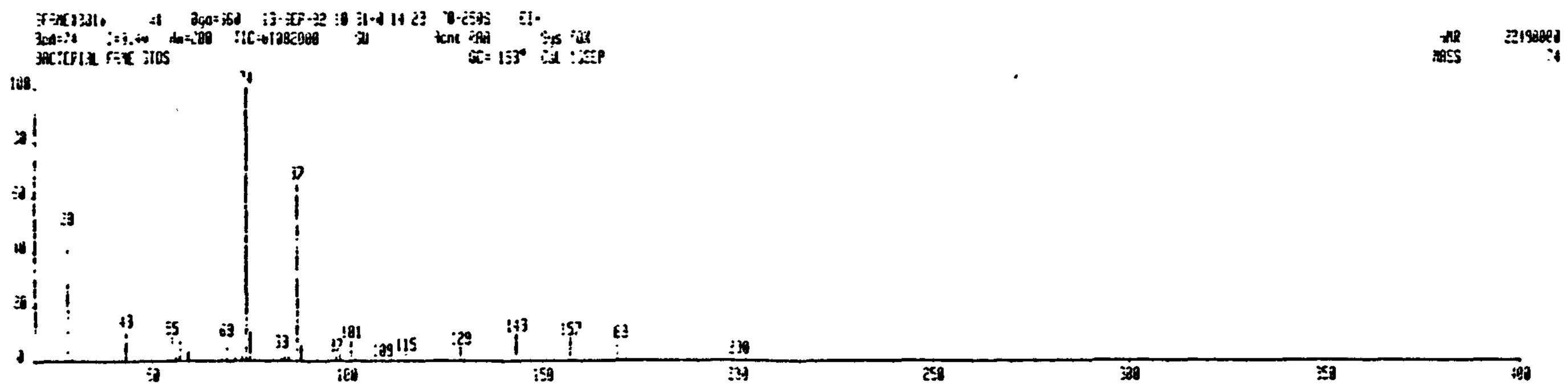
The characteristic features are summarised in Table 5.4.

Fig. 5.5 INDIVIDUAL MASS SPECTRA FOR THE TWENTY SIX FATTY ACID METHYL ESTERS IN THE BACTERIAL ACID METHYL ESTERS CP MIX

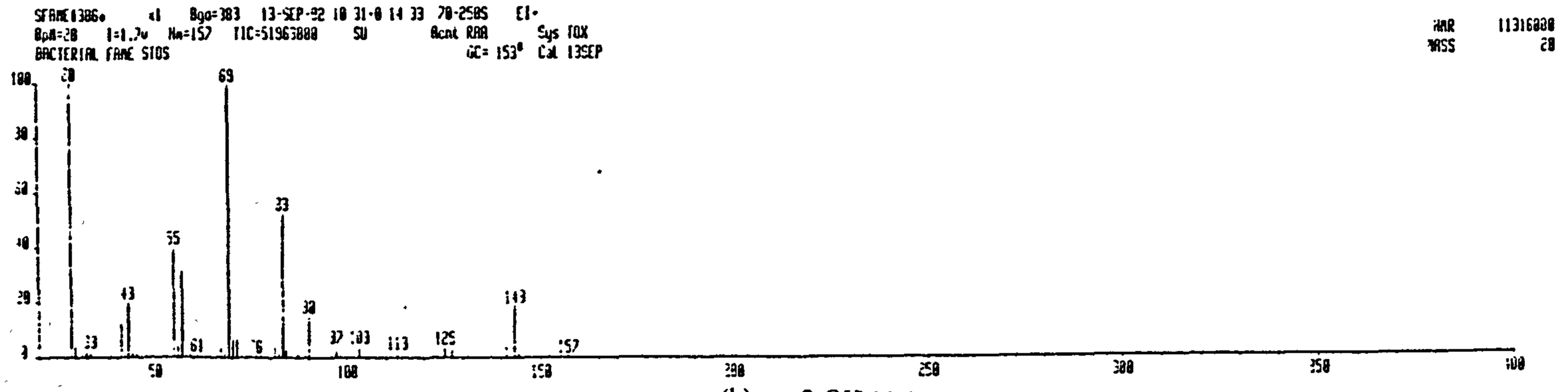
The individual MS spectra (Methods 2.30) for each of the twenty six fatty acid methyl esters contained in the standard mix are as follows:

(a) 11:0
(b) 2-OH 10:0
(c) 12:0
(d) 13:0
(e) 2-OH 12:0
(f) 3-OH 13:0
(g) 14:0
(h) i15:0
(i) a15:0
(j) 15:0
(k) 2-OH 14:0
(l) 3-OH 14:0
(m) i16:0
(n) $16:1^{9}$
(o) 16:0
(p) i17:0
(q) 17:0Δ
(r) 17:0
(s) 2-OH 16:0
(t) $18:2^{9,12}$
(u) $18:1^{9'}$
(v) $18:1^{9}$, $18:1^{11}$
(w) 18:0
(x) 19:0Δ
(y) 19:0
(z) 20:0

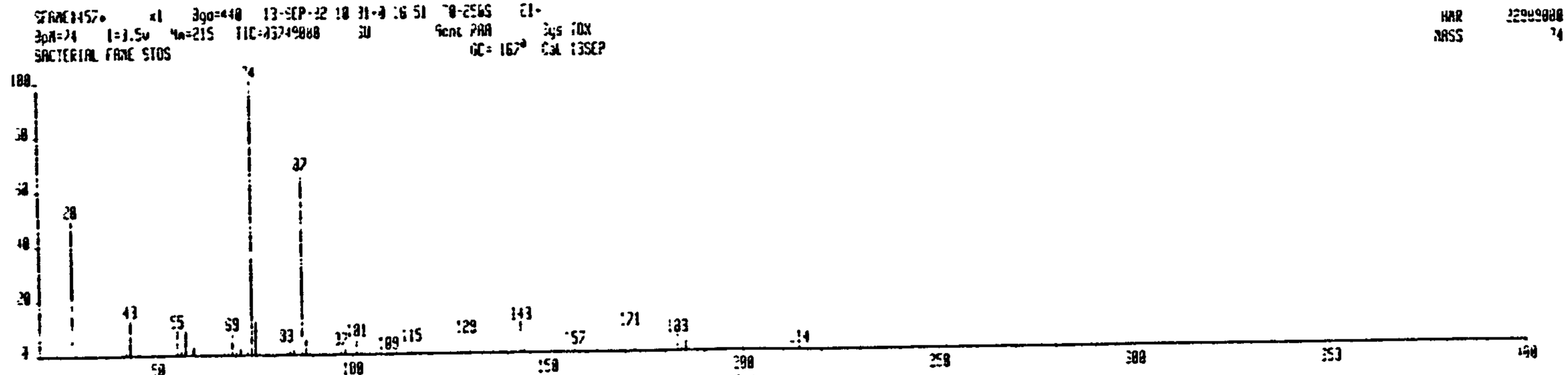
The characteristic features are summarised in Table 5.3.



(a) 11:0



(b) 2-OH 10:0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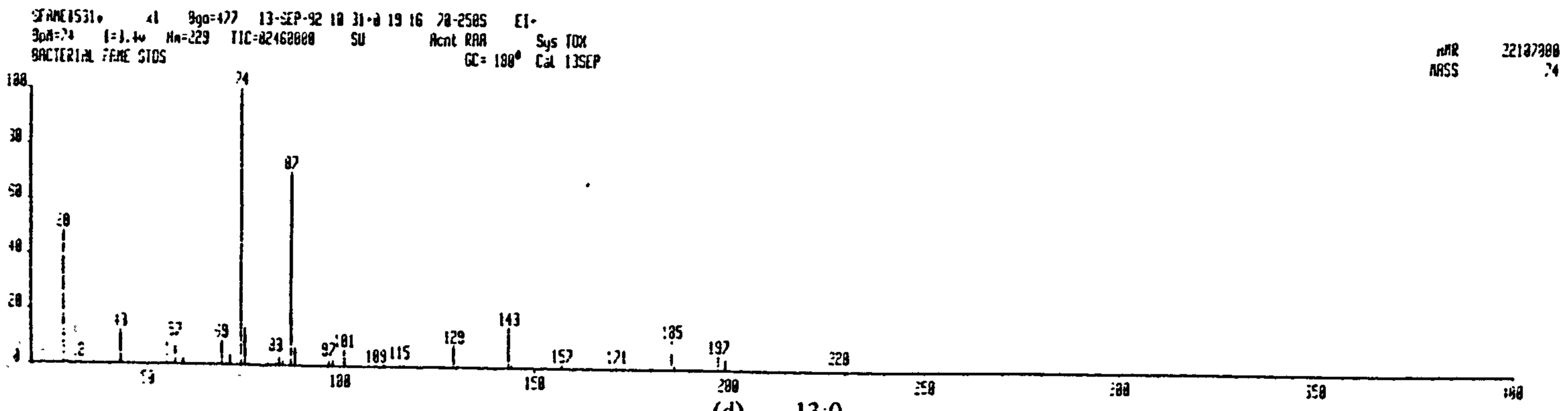
RAA 22949888
RASS 74
74
87
28
43
55
59
83
97
101
109
115
129
143
157
171
183
197
214
50
100
150
200
250
300
350
400

(c) 12:0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BACTERIAL FAME STDS GC= 180° Cal 13SEP
nMR 22107980
MASS 74
74
87
28
43
57
59
83
97
101
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115
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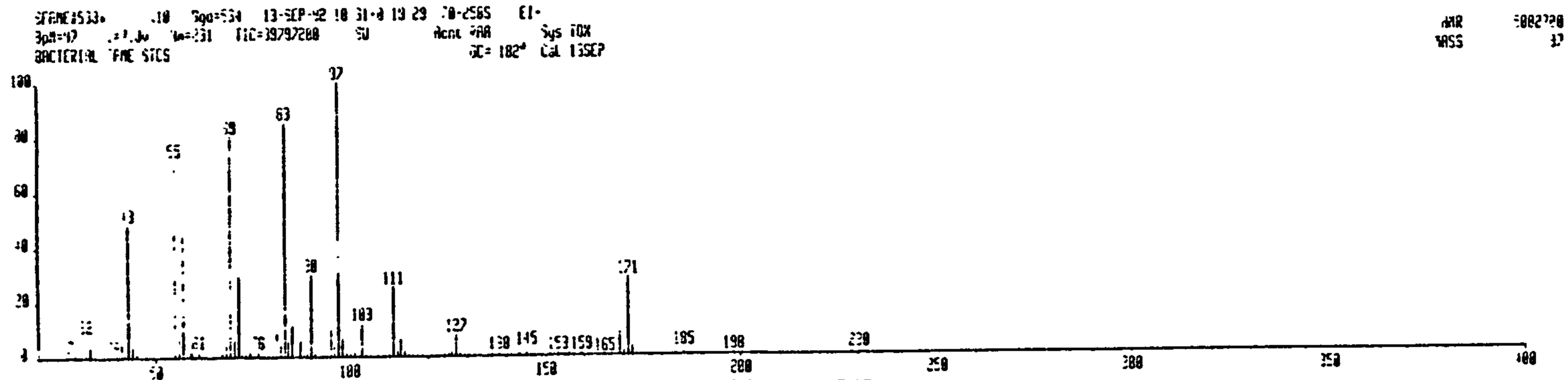
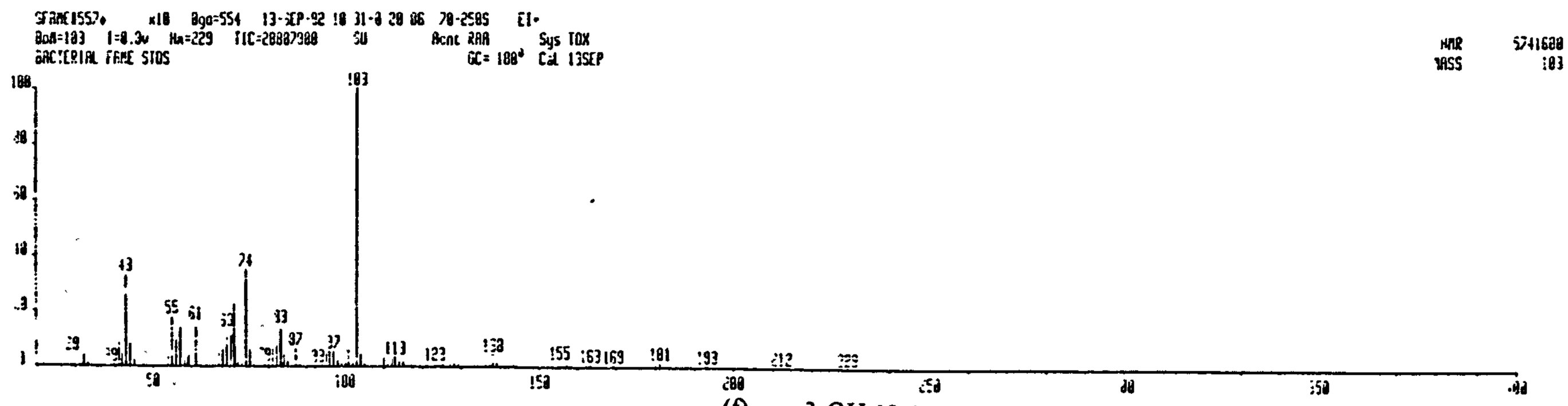
(d) 13:0



(e) 2-OH 12:0



(f) 3-OH 13:0


FAME4602. x1 Bgd=596 13-SEP-92 10 31-0 21 34 70-250S EI+
Bpk=74 I=3.2v Mw=243 TIC=7821200 SU Acnt 7AA Sys TOX
BACTERIAL FAME STDS GC= 189° Cal 13SEP
MAR 12767200
MASS 74
74
57
43
87
99
83
97
101
115
129
143
157
171
185
199
211
242
50
100
150
200
250
300
350
400

(g) 14:0


FAME4647. x1 Bgd=629 13-SEP-92 10 31-0 23 02 70-250S EI+
Bpk=74 I=3.7v Mw=257 TIC=18708600 SU Acnt RAA Sys TOX
BACTERIAL FAME STDS GC= 198° Cal 13SEP
MAR 24518000
MASS 74
74
97
43
57
69
83
101
111
121
129
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157
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199
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100
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300
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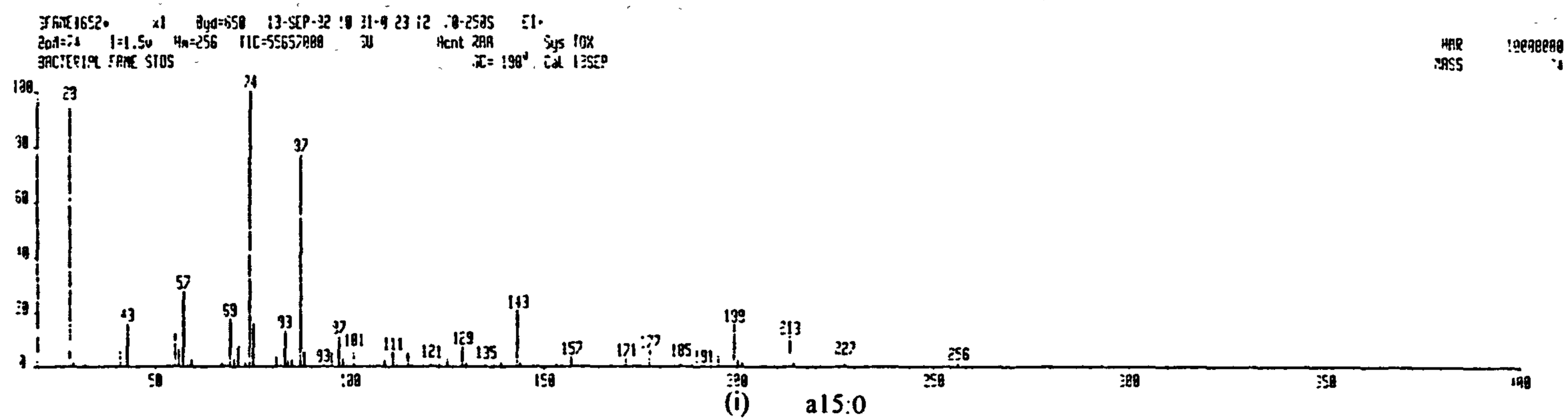
400

(h) i15:0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BACTERIAL FAME STDS GC= 190° Cal 13SEP
MAR 10000000
MASS 74
100
80
60
40
20
0
74
29
87
57
43
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143
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150
200
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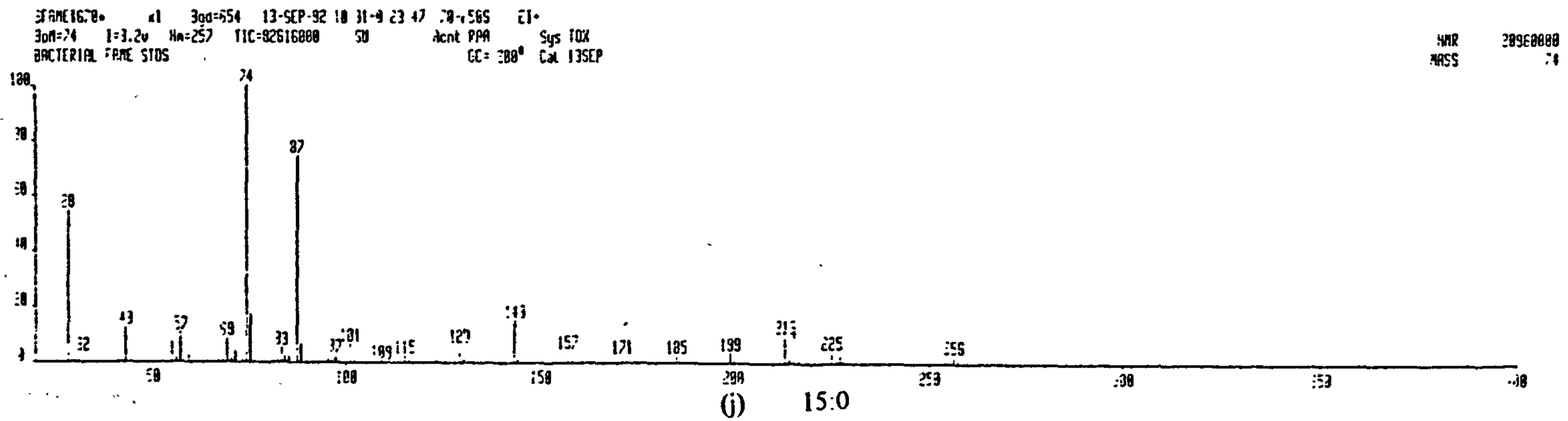
400

(i) a15:0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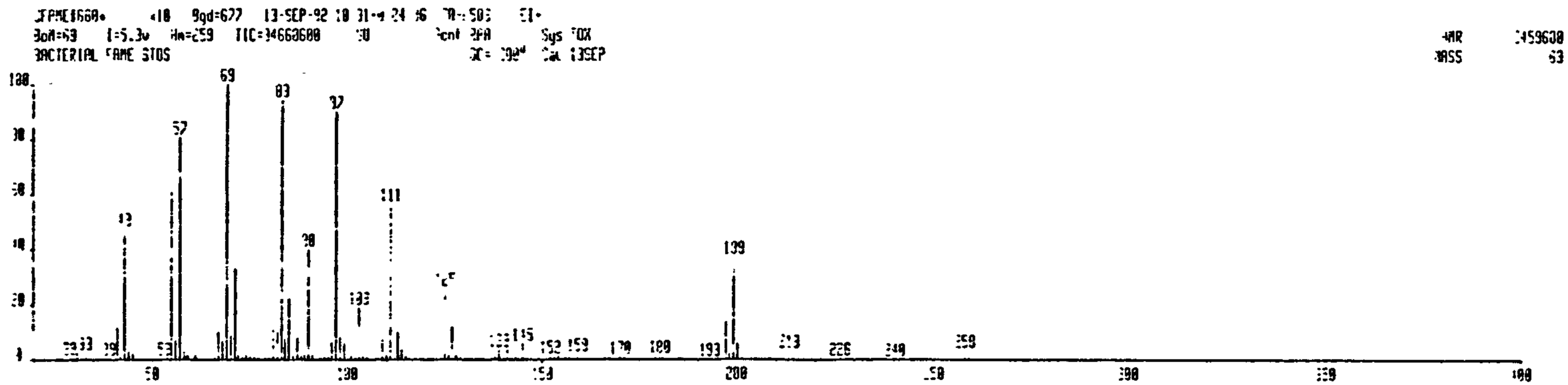
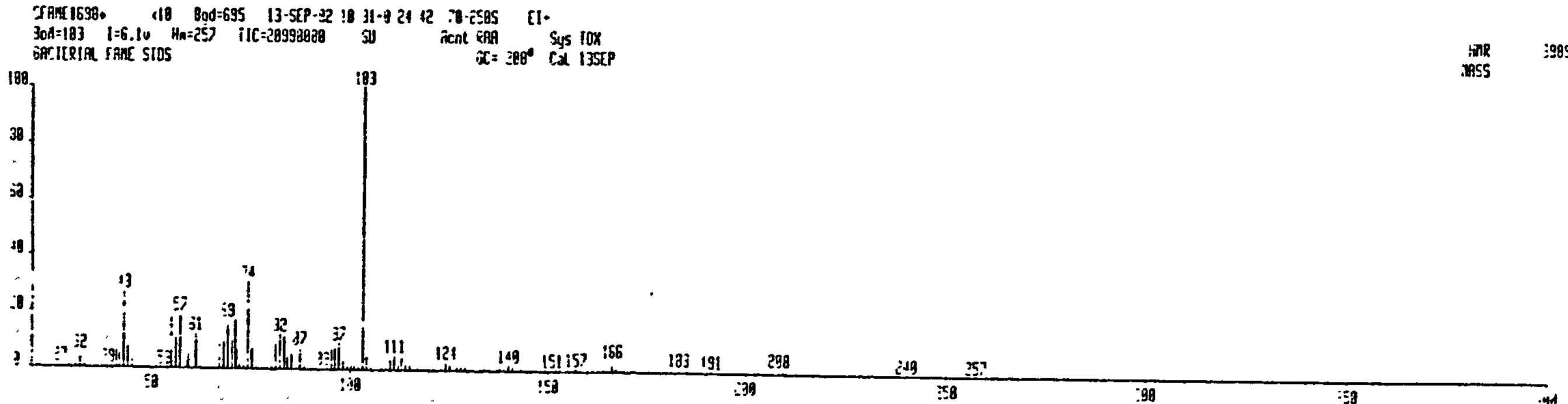
BACTERIAL FAME STDS GC= 200° Cal 13SEP
MAR 209E0000
MASS 74
100
80
60
40
20
0
74
87
28
43
57
59
143
199
213
225
256
50
100
150
200
250
300
350
400

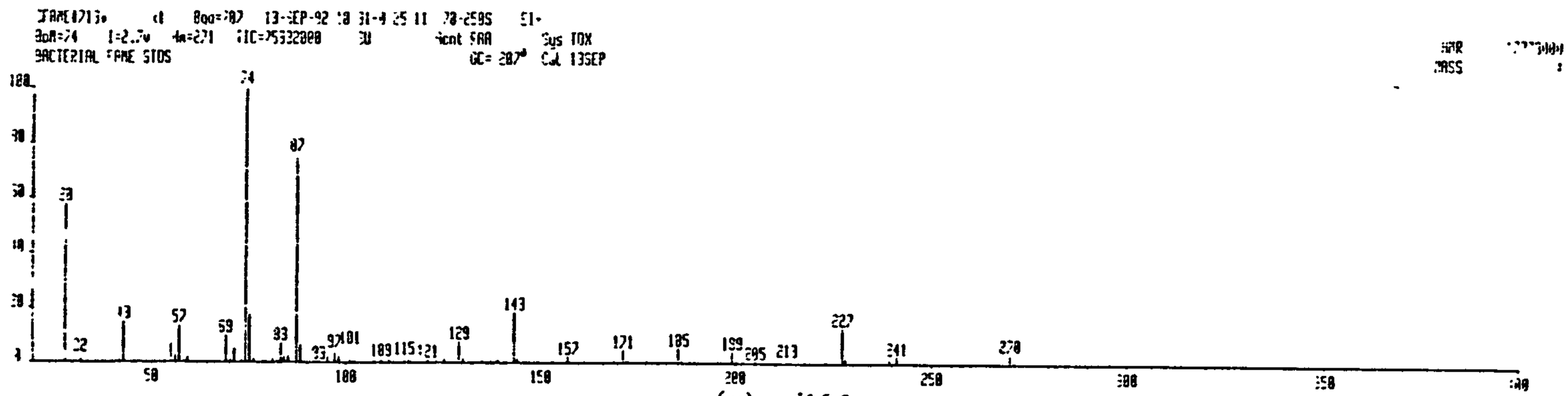
(j) 15:0



(k) 2-OH 14:0



(l) 3-OH 14.0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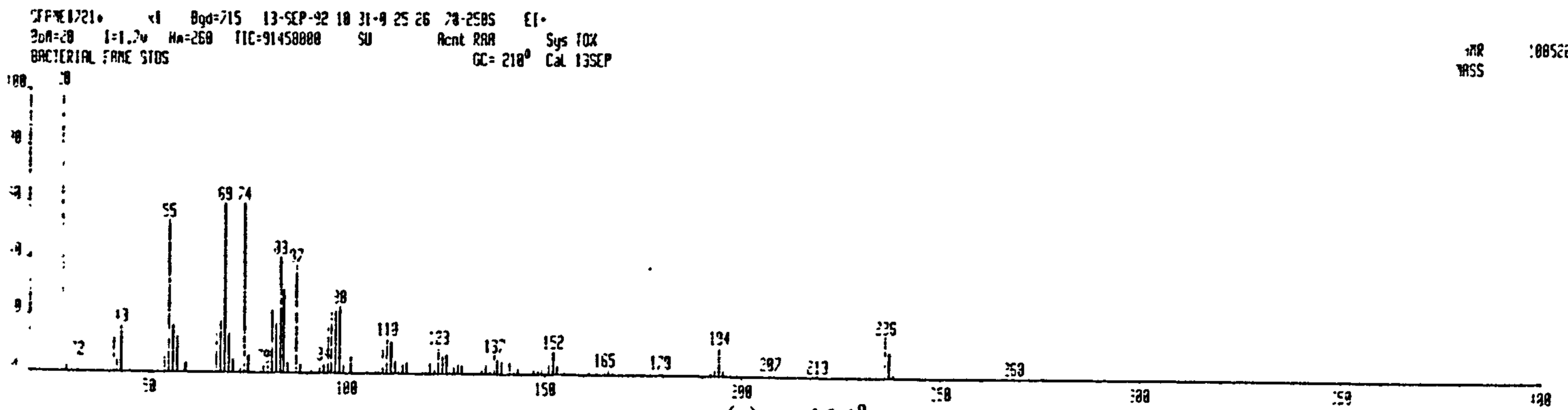
74
87
43
57
59
93
143
129
157
171
185
199
227
241
270
50
100
150
200
250
300
350
400

(m) i16:0


BACTERIAL FAME STDS
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69
74
83
97
111
123
137
152
165
179
194
207
213
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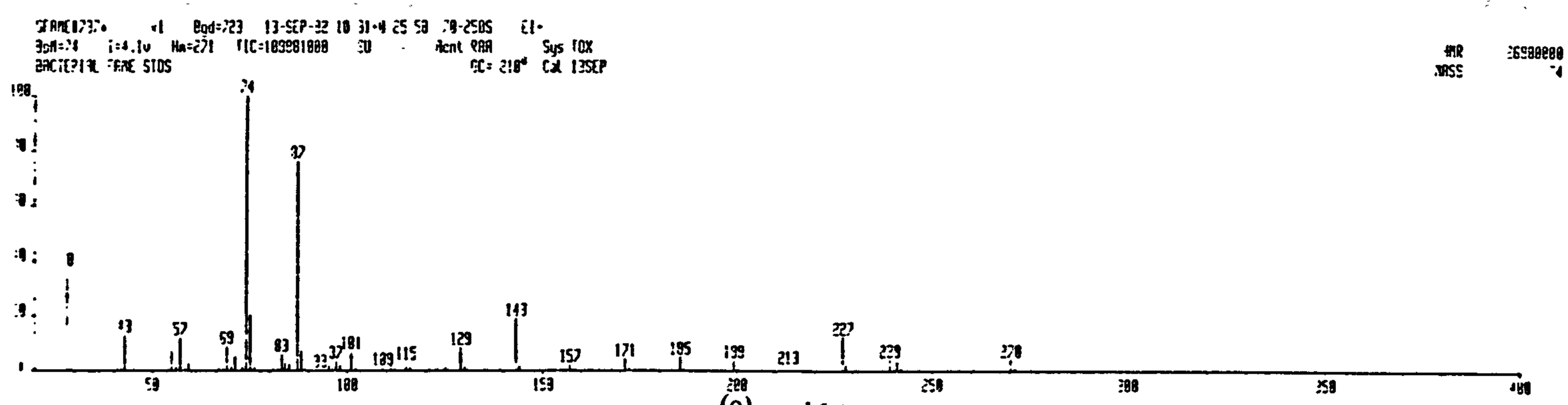
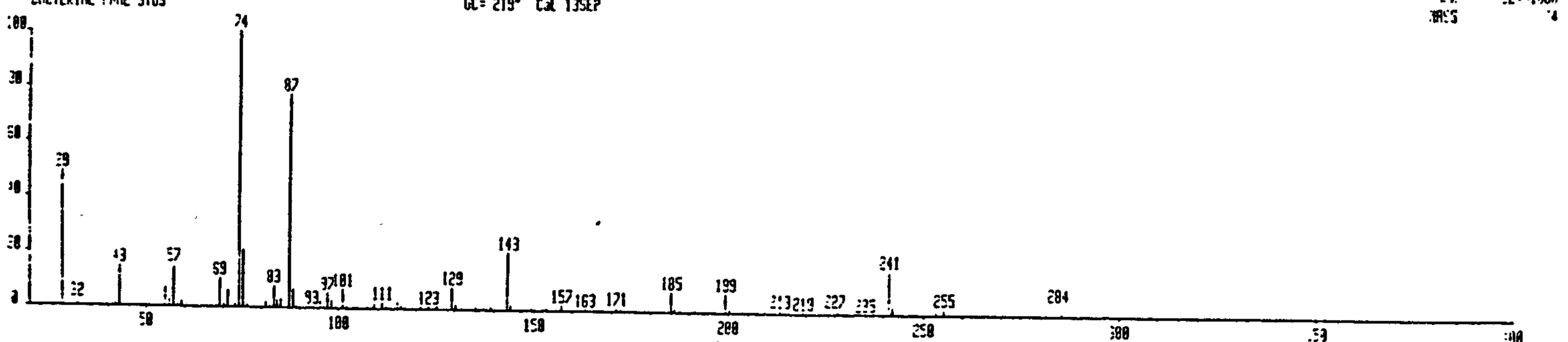
150
200
250
300
350
400

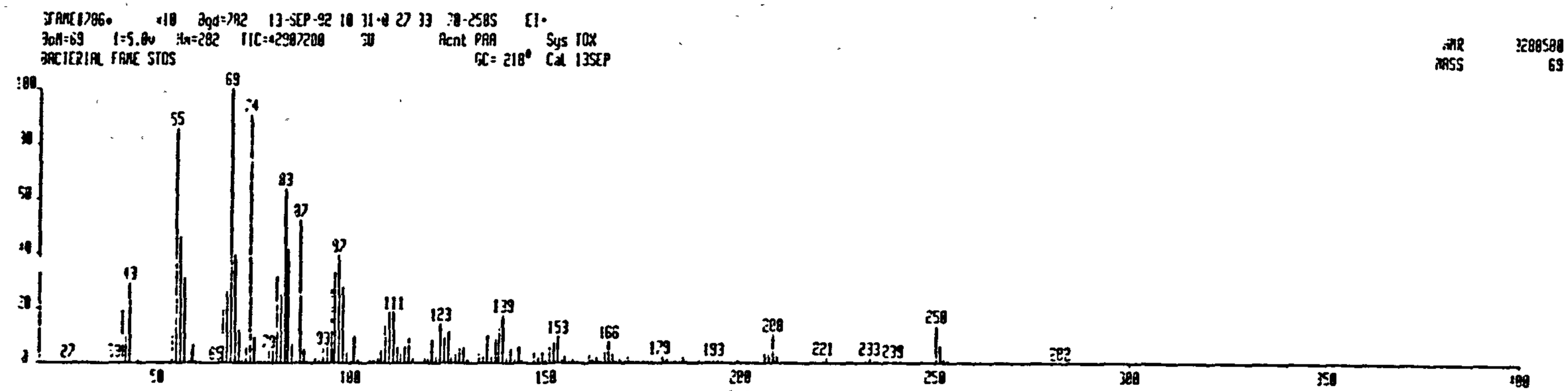
(n) $16:1^{9}$


BACTERIAL FAME STDS
74
87
43
57
59
83
97
101
129
143
157
171
185
199
213
227
241
255
284
50
100
150
200
250
300
350
400

(o) 16:0


BACTERIAL FAME STDS
74
87
43
57
59
83
97
101
111
123
129
143
157
163
171
185
199
213
219
227
241
255
284
50
100
150
200
250
300
350
400

(p) i17:0


69
74
55
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97
92
43
111
123
139
153
166
179
193
208
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233239
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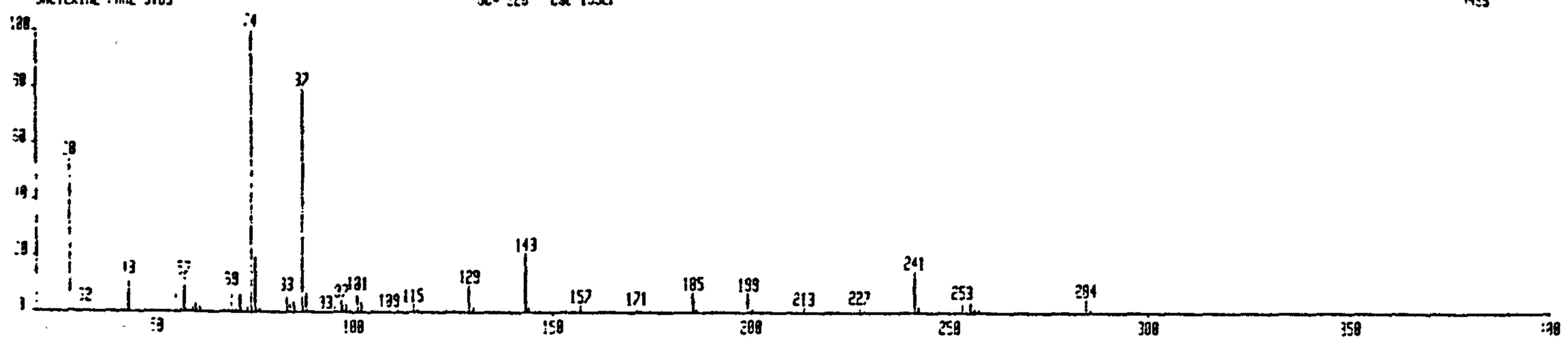
27
69
100
150
200
250
300
350
400

(q) 17:0Δ


74
37
18
143
241
43
57
69
33
129
157
171
185
199
213
227
253
284
50
100
150
200
250
300
350
400

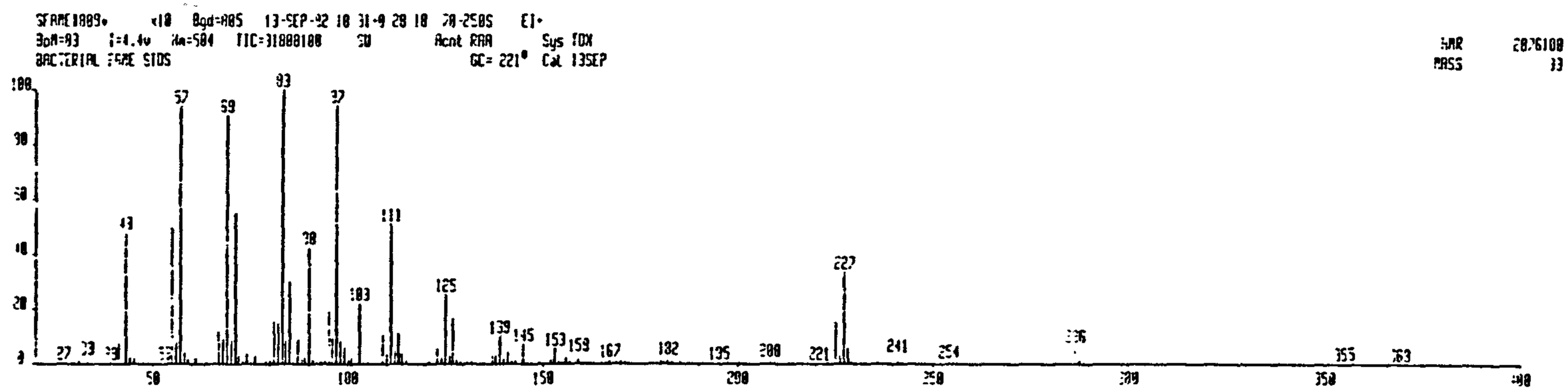
(r) 17:0


Acnt PAA
Sys TOX
BACTERIAL FAME SIDS
GC= 218°
Cal 13SEP
MASS
69
74
55
83
97
43
111
123
139
153
166
179
193
208
221
233
239
259
282
27
50
100
150
200
250
300
350

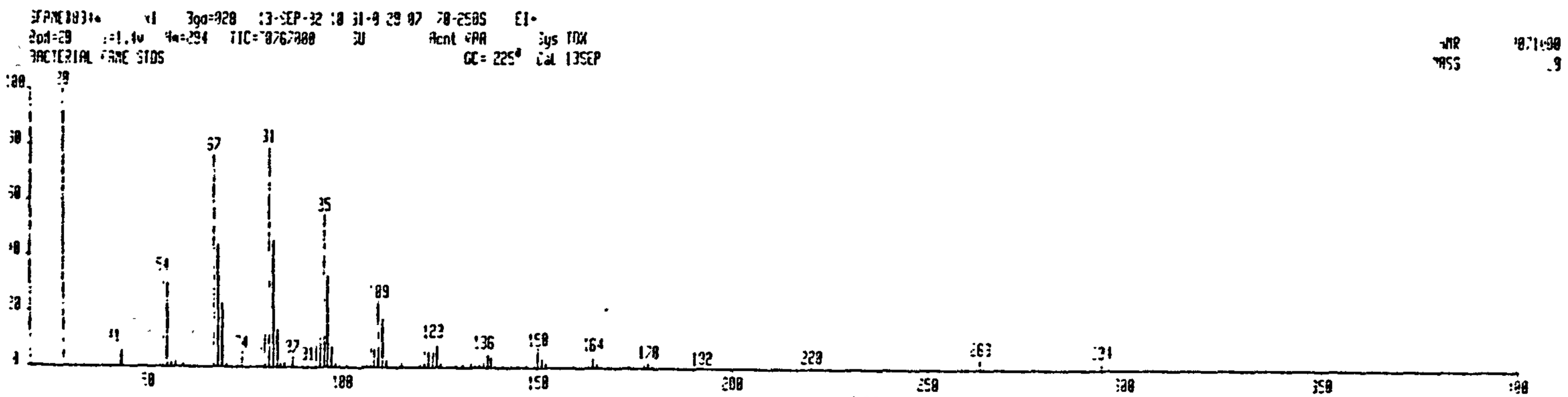
(q) 17:0Δ


Acnt PAA
Sys TOX
BACTERIAL FAME SIDS
GC= 229°
Cal 13SEP
MASS
74
87
143
241
129
157
171
185
199
213
227
253
294
50
100
150
200
250
300
350

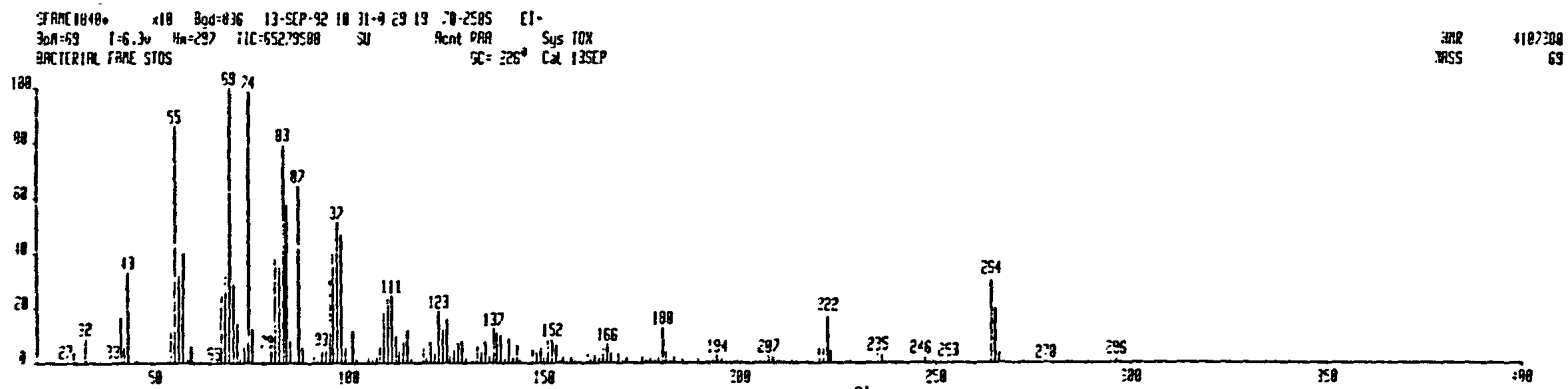
(r) 17:0



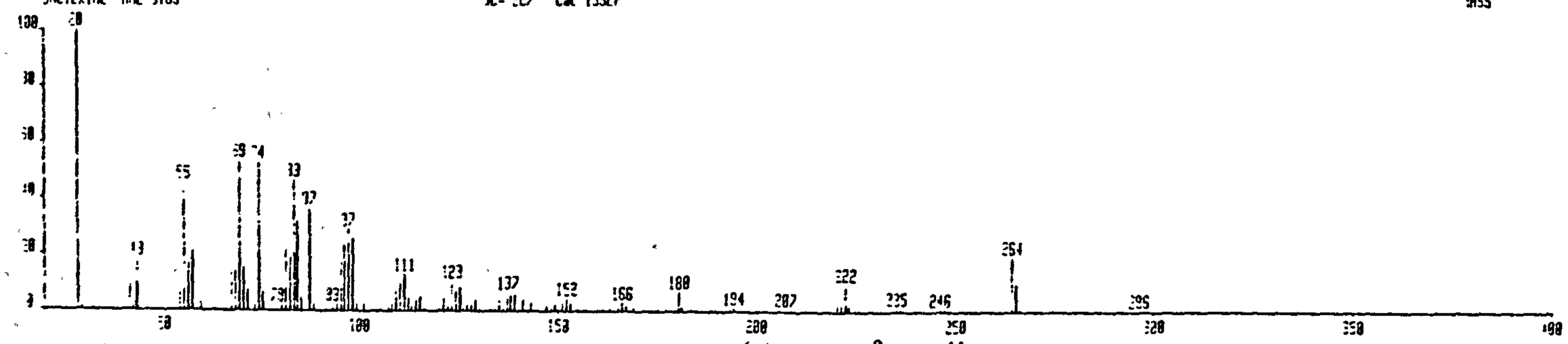
(s) 2-OH 16:0



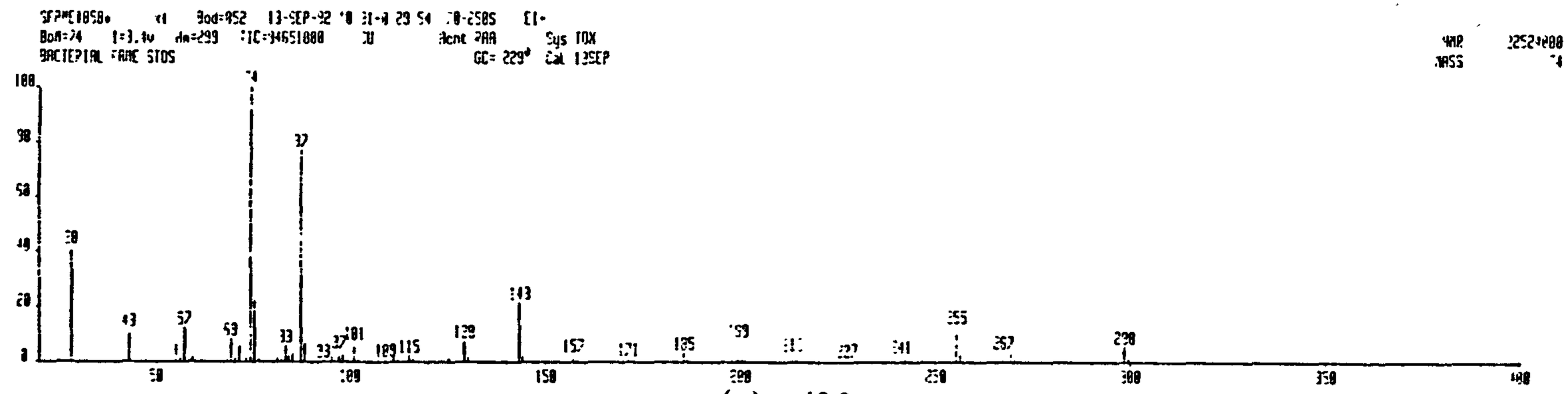
(t) $18{:}2^{9,12}$



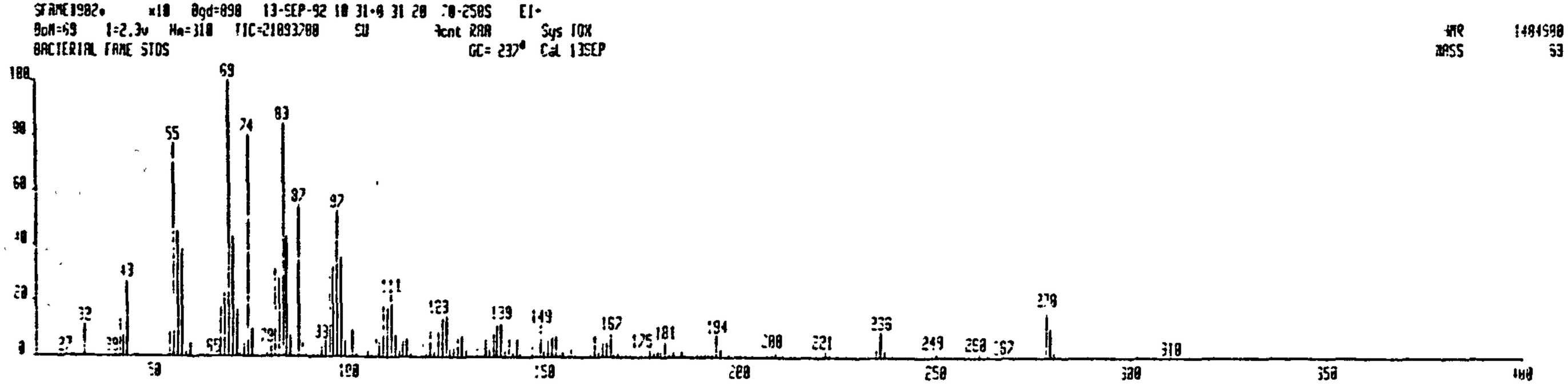
(u) $18:1^{9}$



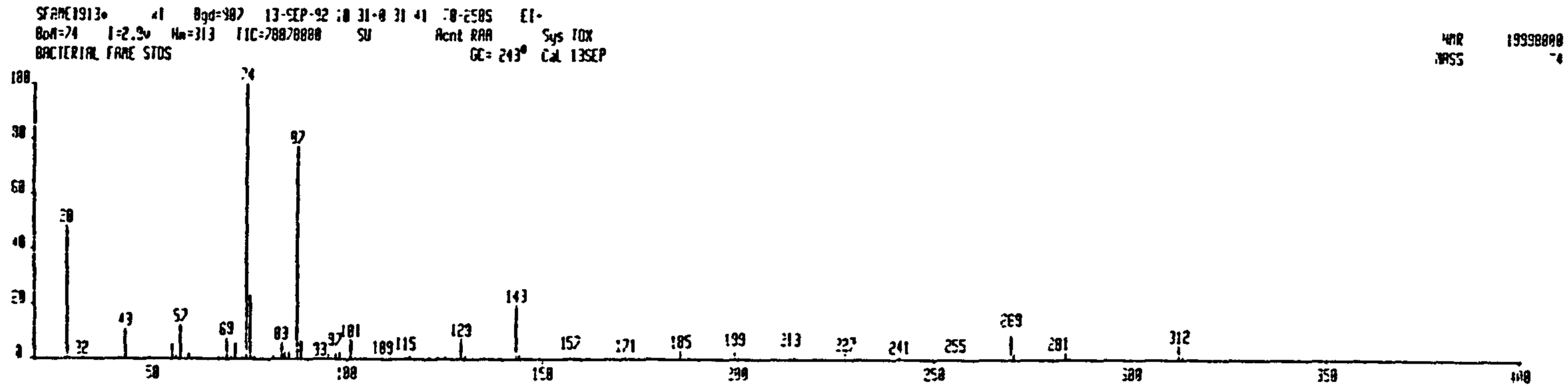
(v) $18:1^{9}$, $18:1^{11}$


SFAME1858• x1 Bgd=852 13-SEP-92 18 31•0 29 54 70-2585 EI•
BoM=74 I=3.1v Hm=299 TIC=34651888 38 Acnt 2AA Sys TOX
BACTERIAL FAME STDS GC= 229° Cal 13SEP
MAR 1252•888
MASS 74
100
98
58
48
28
0
74
87
38
43
57
63
33
81
109
115
129
143
157
171
185
199
213
227
241
255
269
298
50
100
150
200
250
300
350
400

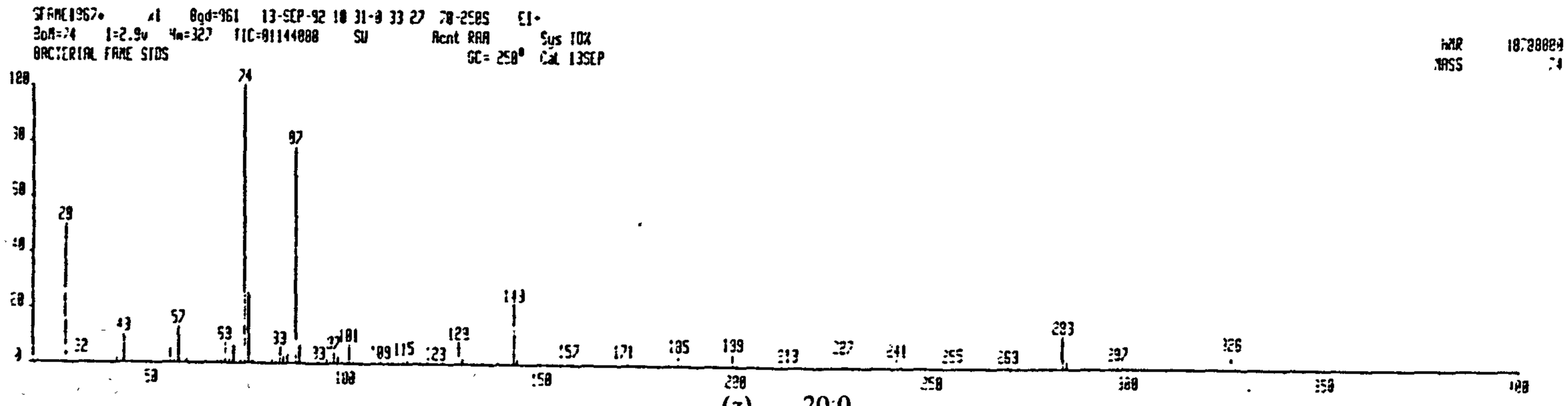
(w) 18:0


SFAME1982• x10 Bgd=898 13-SEP-92 18 31•0 31 28 70-2585 EI•
BoM=69 I=2.3v Hm=318 TIC=21893788 SU Acnt 2AA Sys TOX
BACTERIAL FAME STDS GC= 237° Cal 13SEP
MAR 1484598
MASS 69
100
98
68
48
28
0
69
74
83
55
87
97
43
111
123
139
149
167
181
194
208
221
236
249
258
270
310
50
100
150
200
250
300
350
400

(x) 19:0Δ


SFAME1913• #1 Bgd=987 13-SEP-92 10 31-0 31 41 70-2505 EI•
BoM=74 I=2.9v Hm=313 TIC=78878888 SU Acnt RAA Sys TOX
BACTERIAL FAME STDS GC= 243° Cal 13SEP
MAR 19998888
MASS 74
100
80
60
40
20
0
74
97
29
43
57
69
83
143
269
312
50
100
150
200
250
300
350
400

(y) 19:0


SFAME1967• #1 Bgd=961 13-SEP-92 10 31-0 33 27 70-2505 EI•
BoM=74 I=2.9v Hm=327 TIC=81144888 SU Acnt RAA Sys TOX
BACTERIAL FAME STDS GC= 250° Cal 13SEP
MAR 18738888
MASS 74
100
80
60
40
20
0
74
97
29
43
57
143
283
326
50
100
150
200
250
300
350
400

(z) 20:0

Table 5.4 SUMMARY OF THE CHARACTERISTIC PEAKS FOUND IN THE MASS SPECTRA OF THE TWENTY SIX FATTY ACID METHYL ESTERS IN THE BACTERIAL ACID METHYL ESTERS CP MIX

The characteristic peaks on the mass spectra (Fig. 5.5), obtained by GC-MS (Methods 2.30), which identify each individual fatty acid methyl ester in the standard mix are summarised in this table. Peaks were identified using the criteria described in Methods 2.30.1. The peak numbers refer to the GC trace for this standard mix (Fig. 5.4). Where the symbol '>' is used it means that one peak is greater in height than another and where the symbol '=' is used it means that the peaks are of equal height.

Peak no.	Fatty acid	M+	Characteristic peaks						
381	11:0	200	74	M-31	M-43				
386	2-OH 10:0	202	69	90	M-59				
457	12:0	214	74	M-31	M-43	M-57			
531	13:0	228	74	M-31	M-43	M-57			
538	2-OH 12:0	230	69	90	M-59				
557	3-OH 12:0	230	74>69	103					
602	14:0	242	74	M-31	M-43	M-57			
647	i15:0	256	74	M-29	M-43	M-57	M-65		
652	a15:0	256	74	M-29	M-43	M-57	M-79		
670	15:0	256	74	M-31	M-43	M-57			
680	2-OH 14:0	258	69	90	M-59				
698	3-OH 14:0	258	74>69	103	M-50				
713	i16:0	270	74	M-29	M-43	M-57	M-65		
721	16:1^{9}	268	69=74	55	M-32	M-74	M-116		
737	16:0	270	74	M-31	M-43	M-57			
776	i17:0	284	74	M-29	M-43	M-57	M-65		
786	17:0Δ	282	69>74	55	M-32	M-43			
798	17:0	284	74	M-31	M-43	M-57			
809	2-OH 16:0	286	69	90	103	M-59			
834	18:2^{9,12}	294	67>74	54	M-31	M-74	M-116		
842	18:1^{9}	296	69>74	55	M-31	M-32	M-43	M-74	M-116
846	18:1^{9}, 18:1^{11}	296	69=74	55	M-31	M-32	M-74	M-116	
858	18:0	296	74	M-31	M-43	M-57			
902	19:0Δ	310	69=74	55	M-32	M-43			
913	19:0	312	74	M-31	M-43	M-57			
967	20:0	326	74	M-29*	M-43	M-57			

Fig. 5.6 GC TRACE OF THE WHOLE LIPID FATTY ACID METHYL ESTERS EXTRACTED FROM *S.RIMOSUS* GROWN ON YEME MEDIUM

S. rimosus was grown according to Methods 2.5 in YEME [Methods 2.3.2 (c)]. The whole lipid fatty methyl ester (see Methods 2.23-2.27 for preparation) was run on GC-MS (Methods 2.30). This is the GC trace from which the individual peaks were taken for further analysis by MS (see Fig. 5.7). Each individual peak (corresponds to a fatty acid) was assigned a peak number automatically by the machine.

DRYHAH1 81-1168 31-JUL-92 12:29 70-250S (EI+) Sys:RAA2

IHP
0 4579556864

Chromatogram Identifiers 80 LTIC
Text:COMPLEX MEDIUM WHOLE LIPID

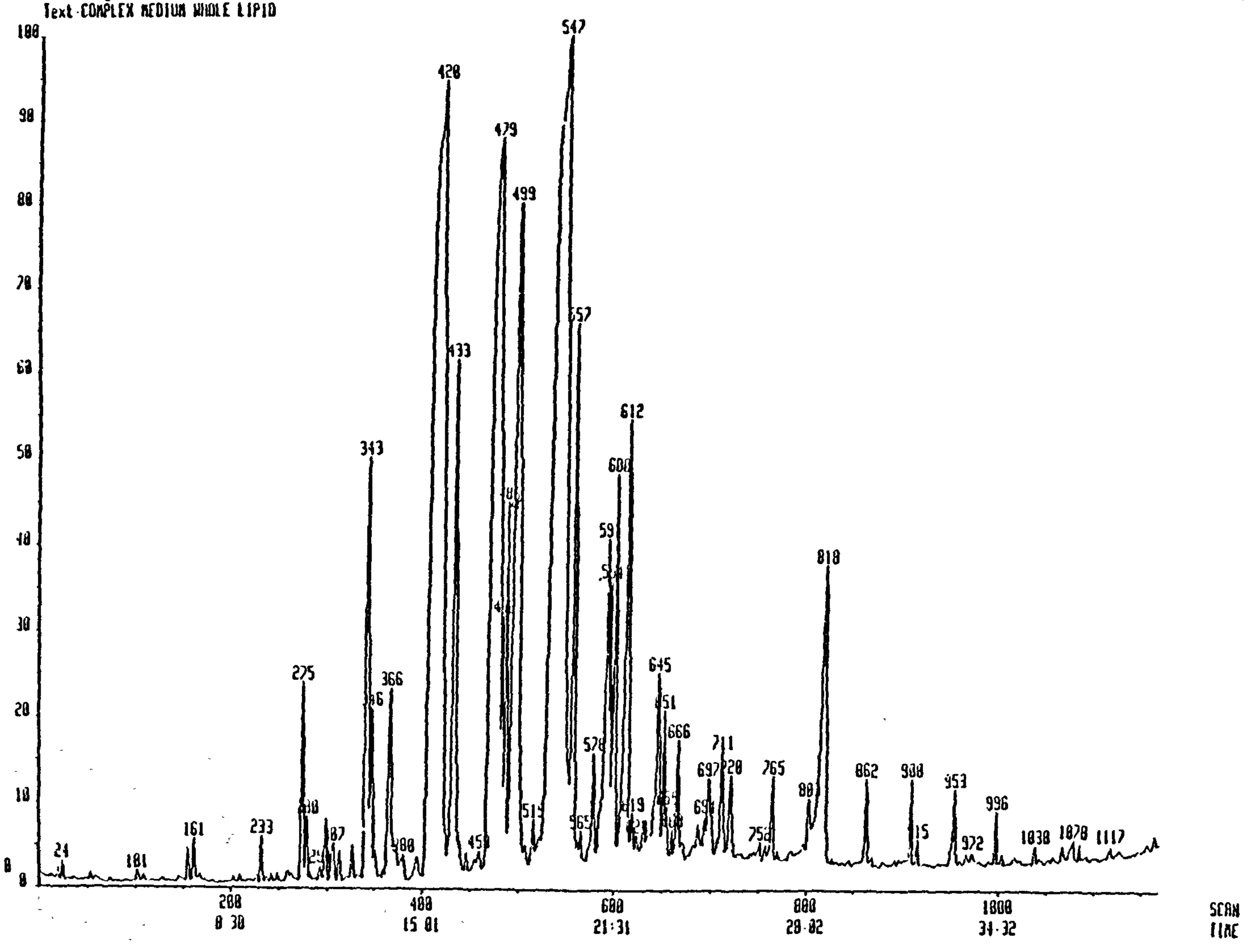
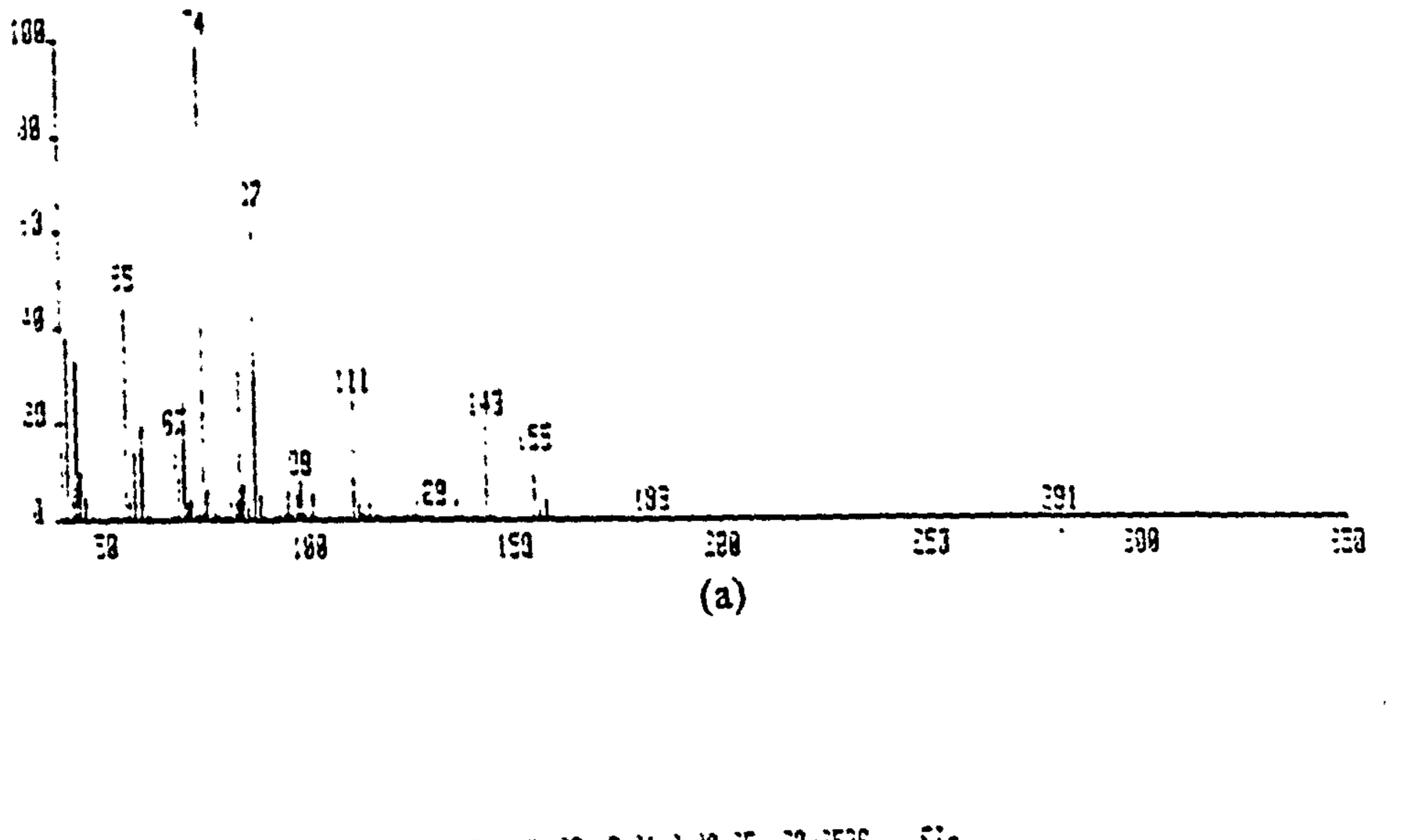


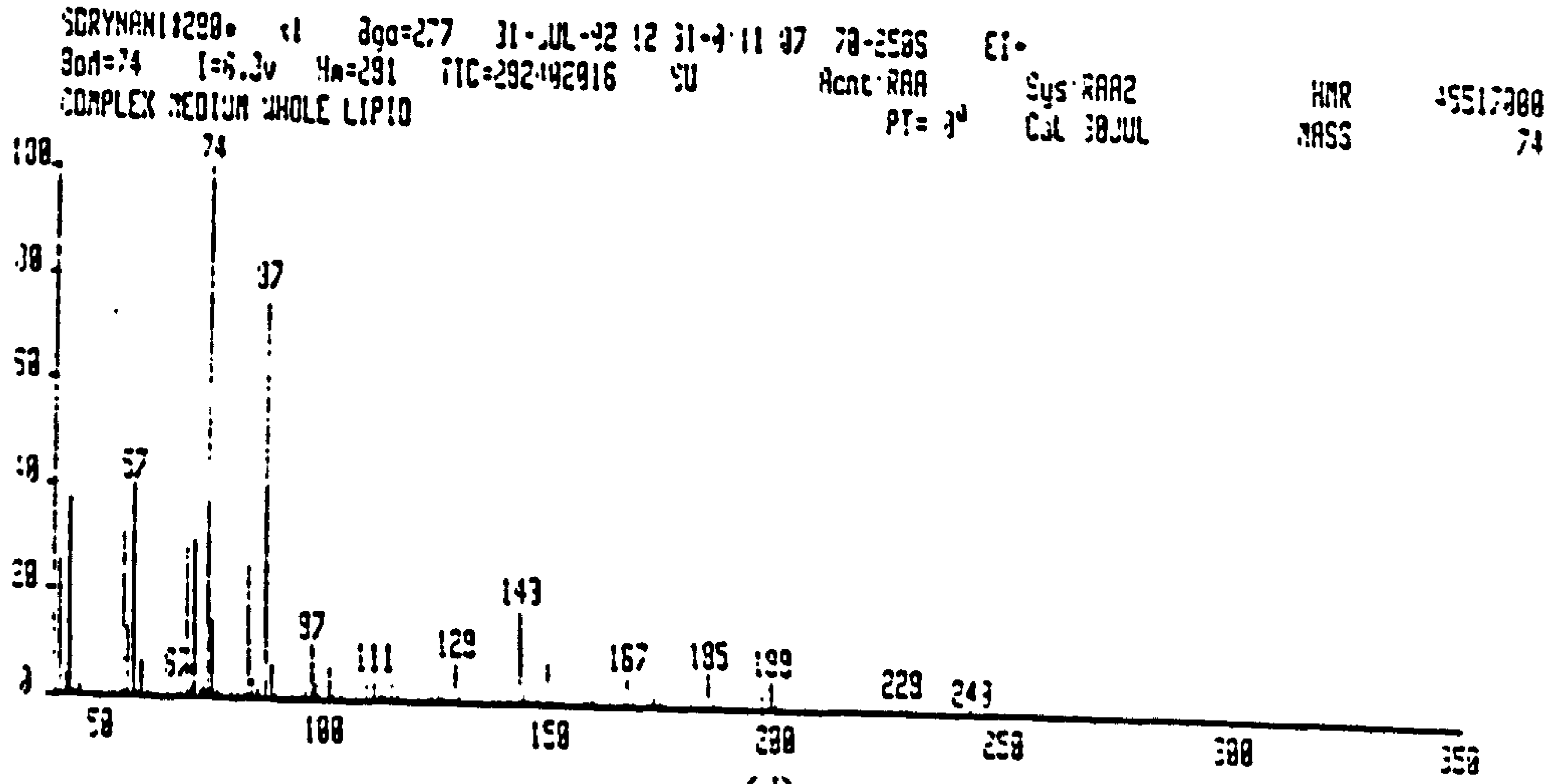
Fig. 5.7 INDIVIDUAL MASS SPECTRA FOR THE WHOLE LIPID FATTY ACID METHYL ESTERS FROM *S. RIMOSUS* CULTURED IN YEME

S.rimosus was cultured in YEME (Methods 2.3.2 (c) and 2.5). Whole lipid fatty acid methyl esters from the biomass were prepared and run on GC-MS (Methods 2.23-2.30). Individual peaks from the GC trace were further analysed by MS (Methods 2.30). The peak numbers correspond to the GC trace (see Fig. 5.6). The mass spectra for each peak are shown as follows:

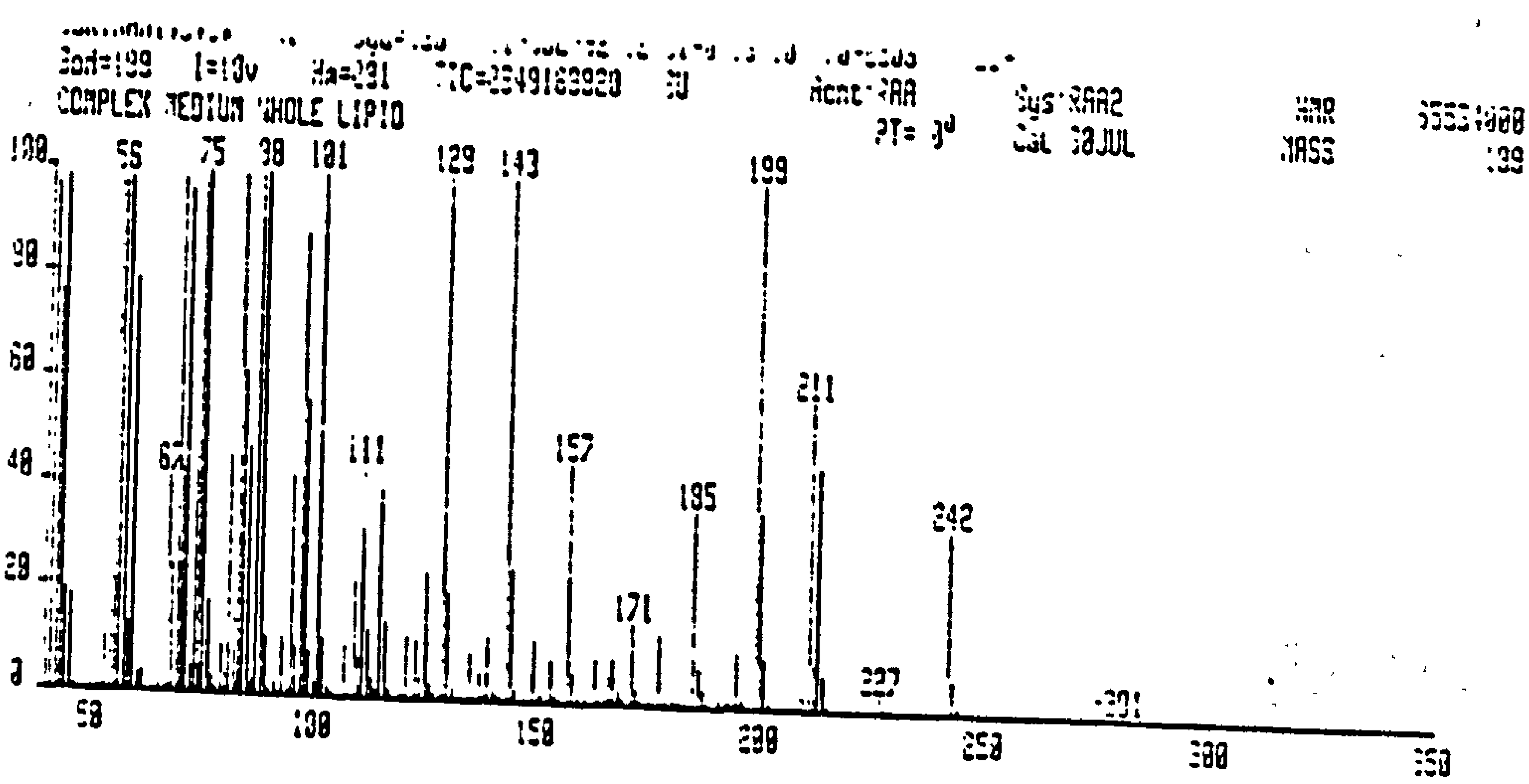
Peak no.

(a) 161
(b) 233
(c) 275
(d) 280
(e) 343
(f) 366
(g) 410
(h) 416
(i) 433
(j) 479
(k) 494
(l) 499
(m) 532
(n) 547
(o) 578
(p) 591
(q) 594
(r) 612
(s) 651
(t) 655
(u) 720

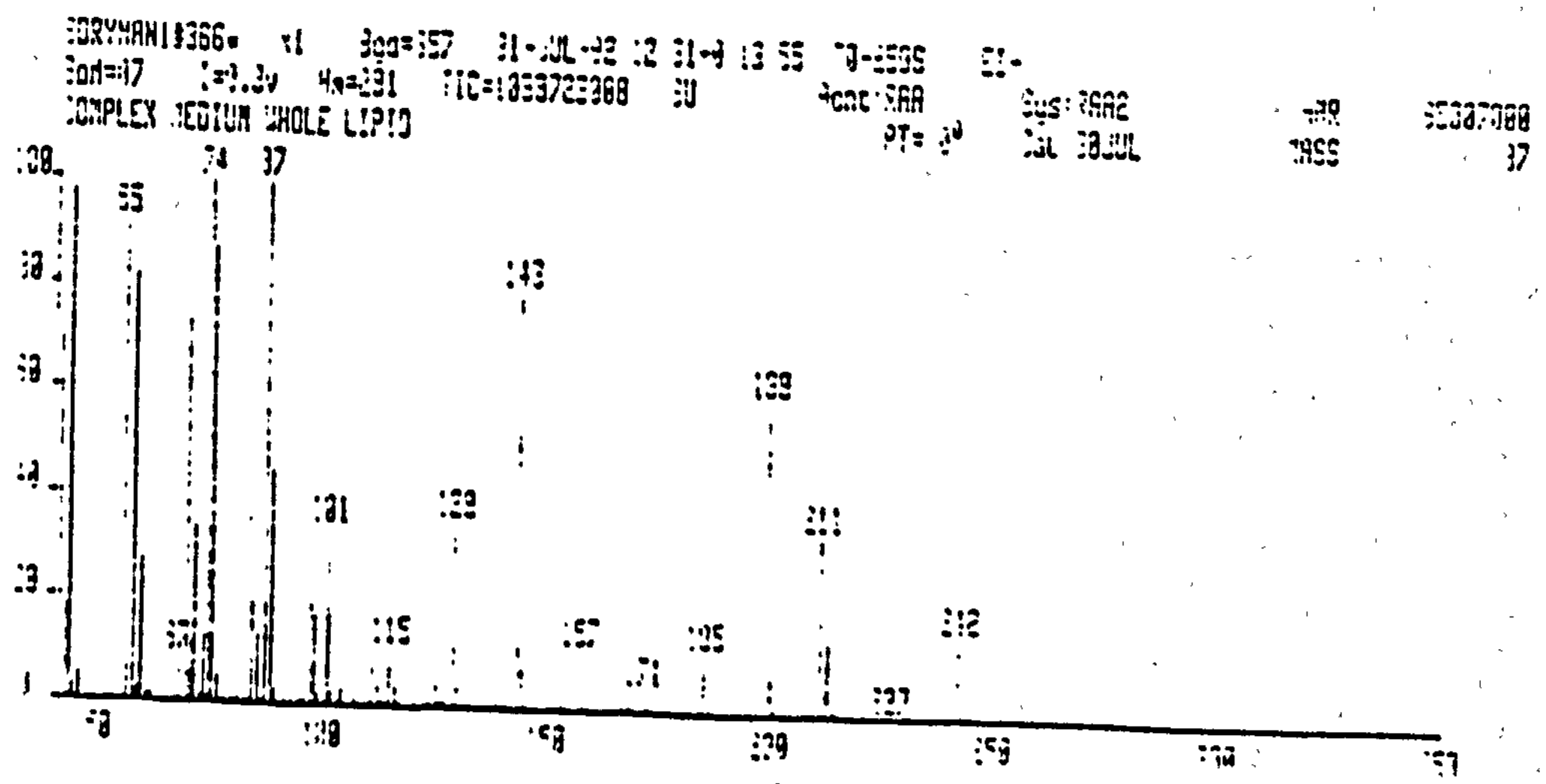

74
37
55
54
39
111
143
155
29.
193
391
(a)
74
37
55
64
181
115
129
143
157
171
183
214
391
(b)
74
37
55
64
181
111
129
143
153
165
197
213
229
231
(c)


COMPLEX MEDIUM WHOLE LIPID
Acnt:RAA
Sys:RAA2
HMR
MASS
74
57
97
111
129
143
167
195
229
243
50
100
150
200
250
300
350

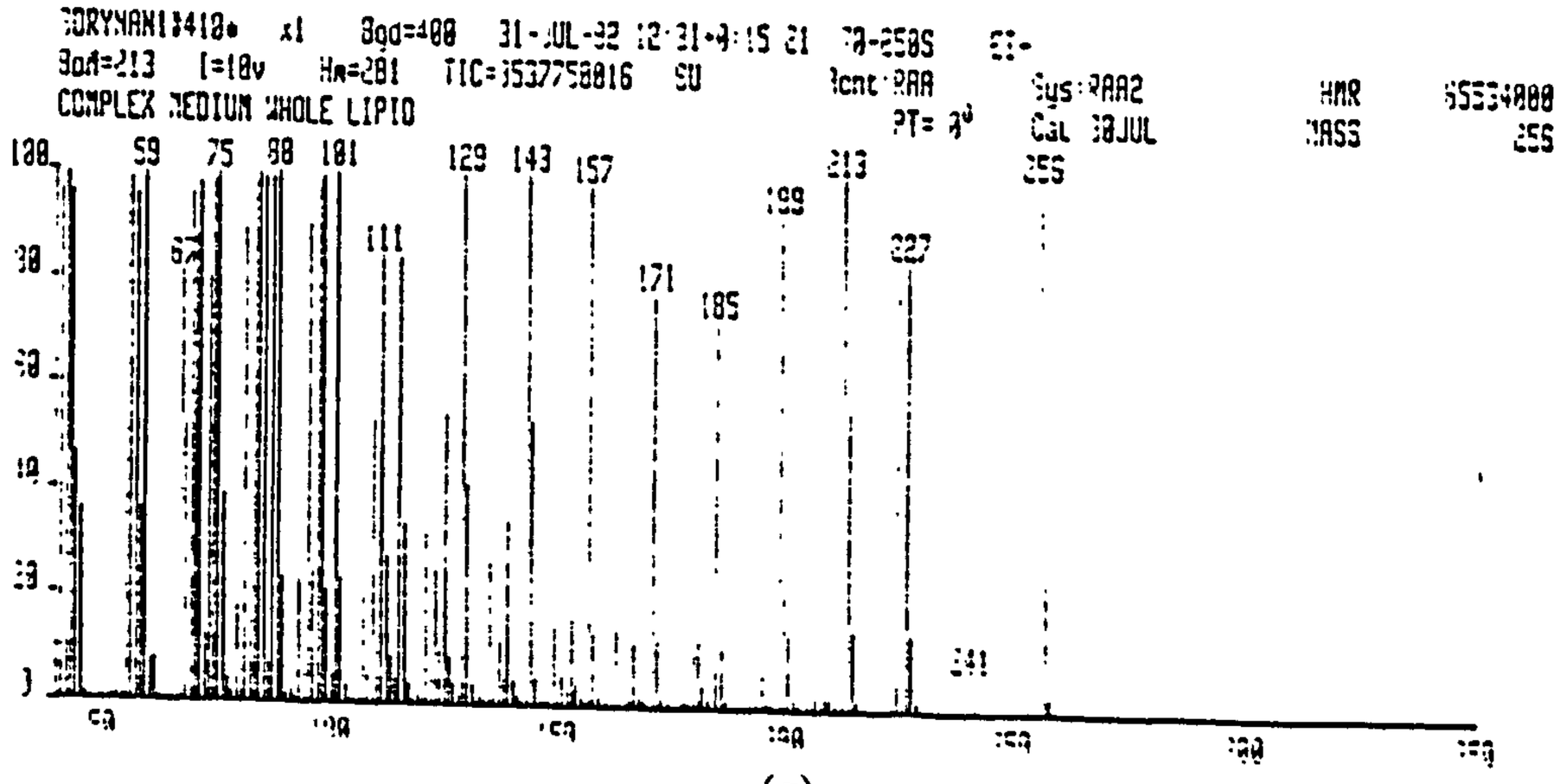
(d)


COMPLEX MEDIUM WHOLE LIPID
Acnt:RAA
Sys:RAA2
Cal 30JUL
MASS
55
75
101
129
143
199
211
157
195
171
242
50
100
150
200
250
300
350

(e)


COMPLEX MEDIUM WHOLE LIPID
74
87

(f)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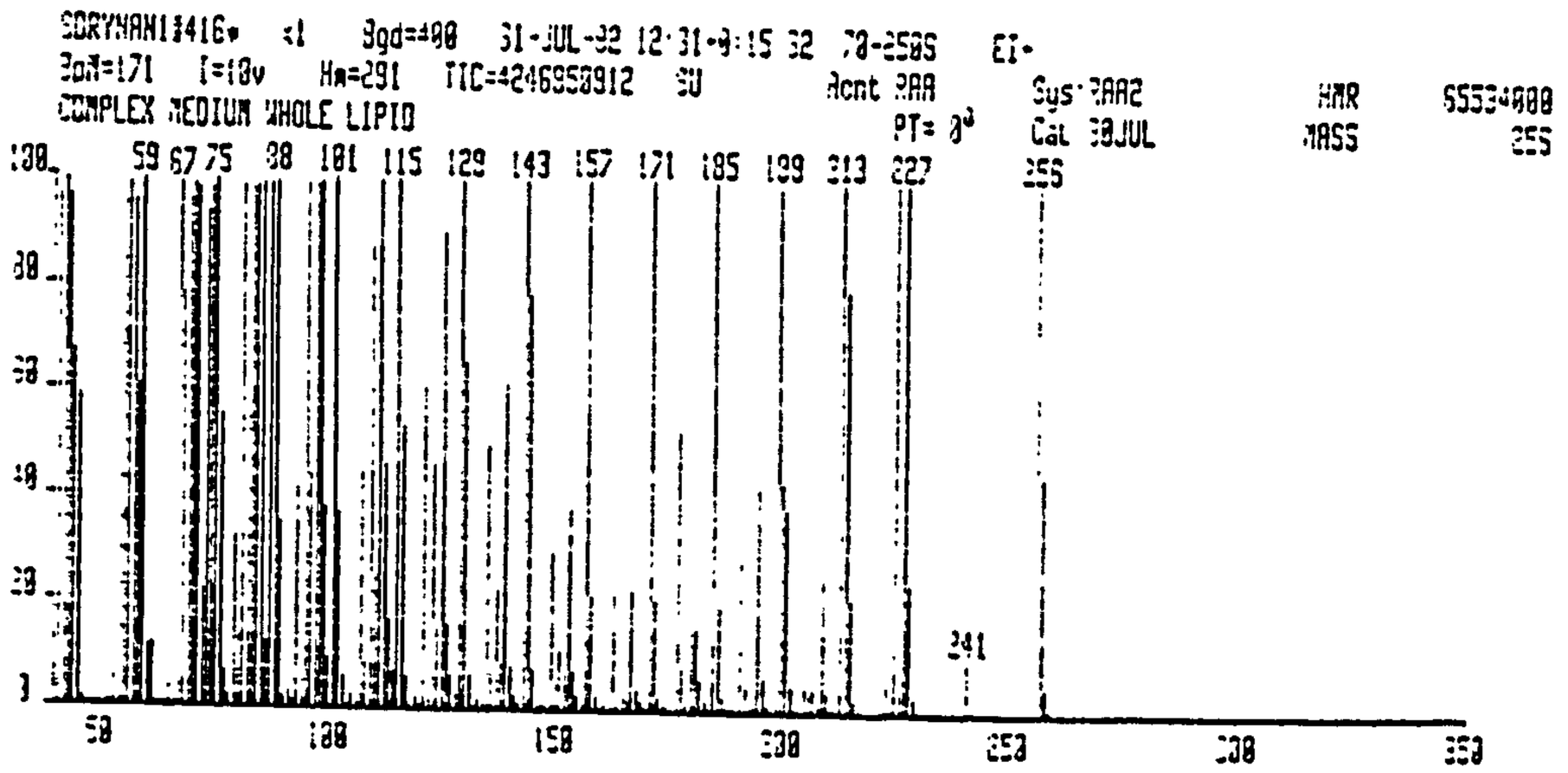
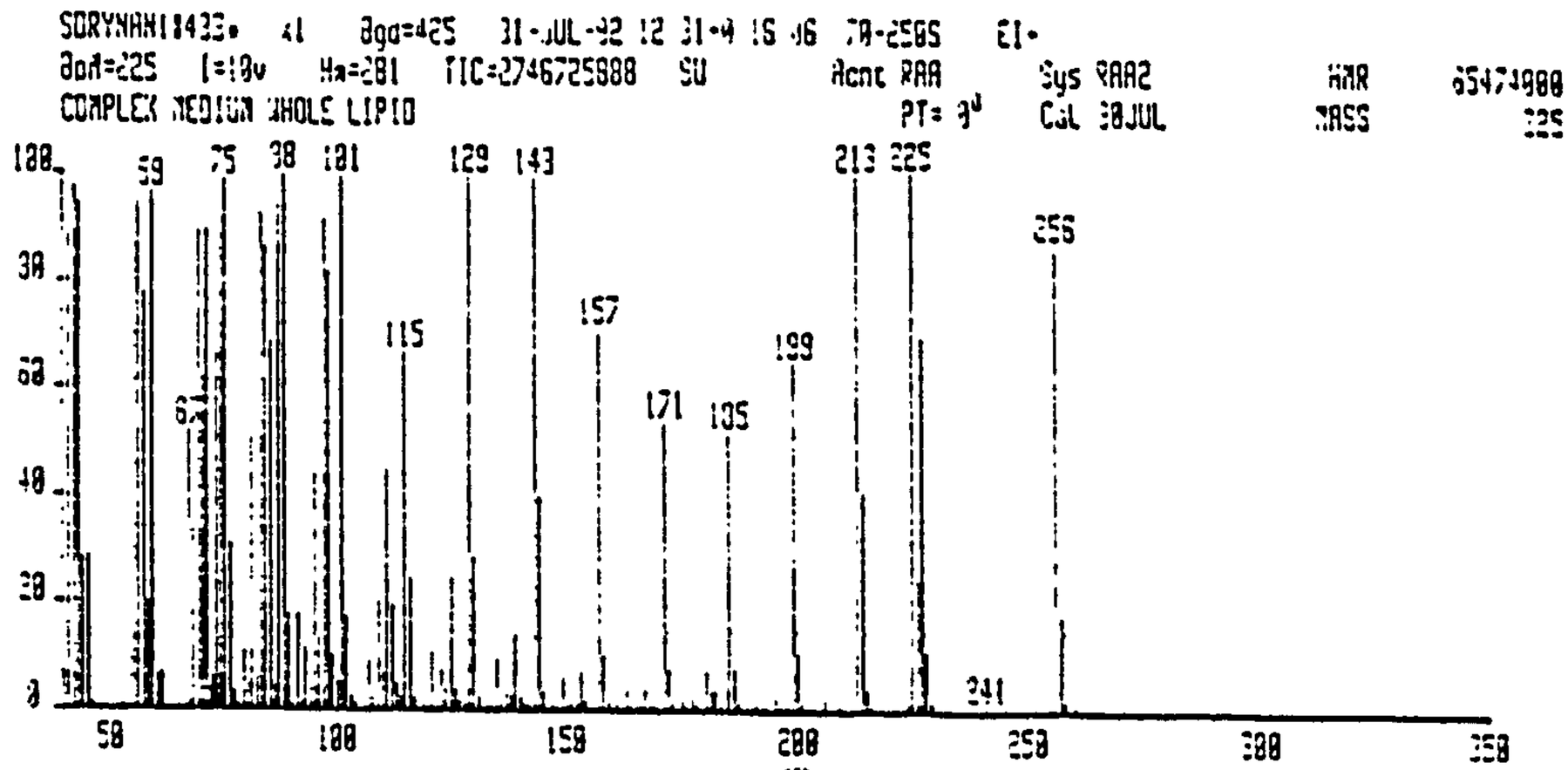
75
88
181
129
143
157
171
185
199
213
227
241
255

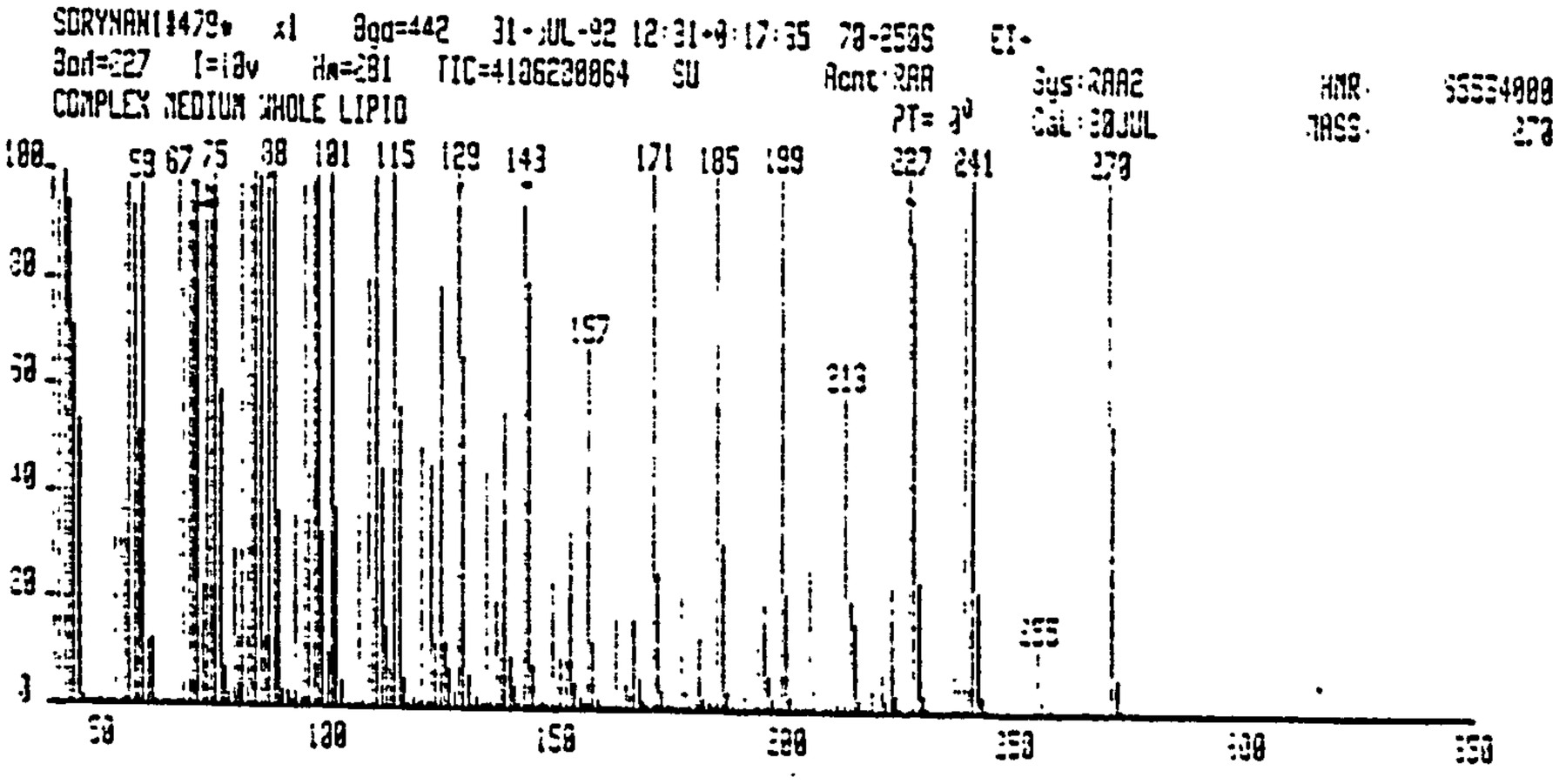
(g)


59
67
75
88
181
115
129
143
157
171
185
199
213
227
241
255

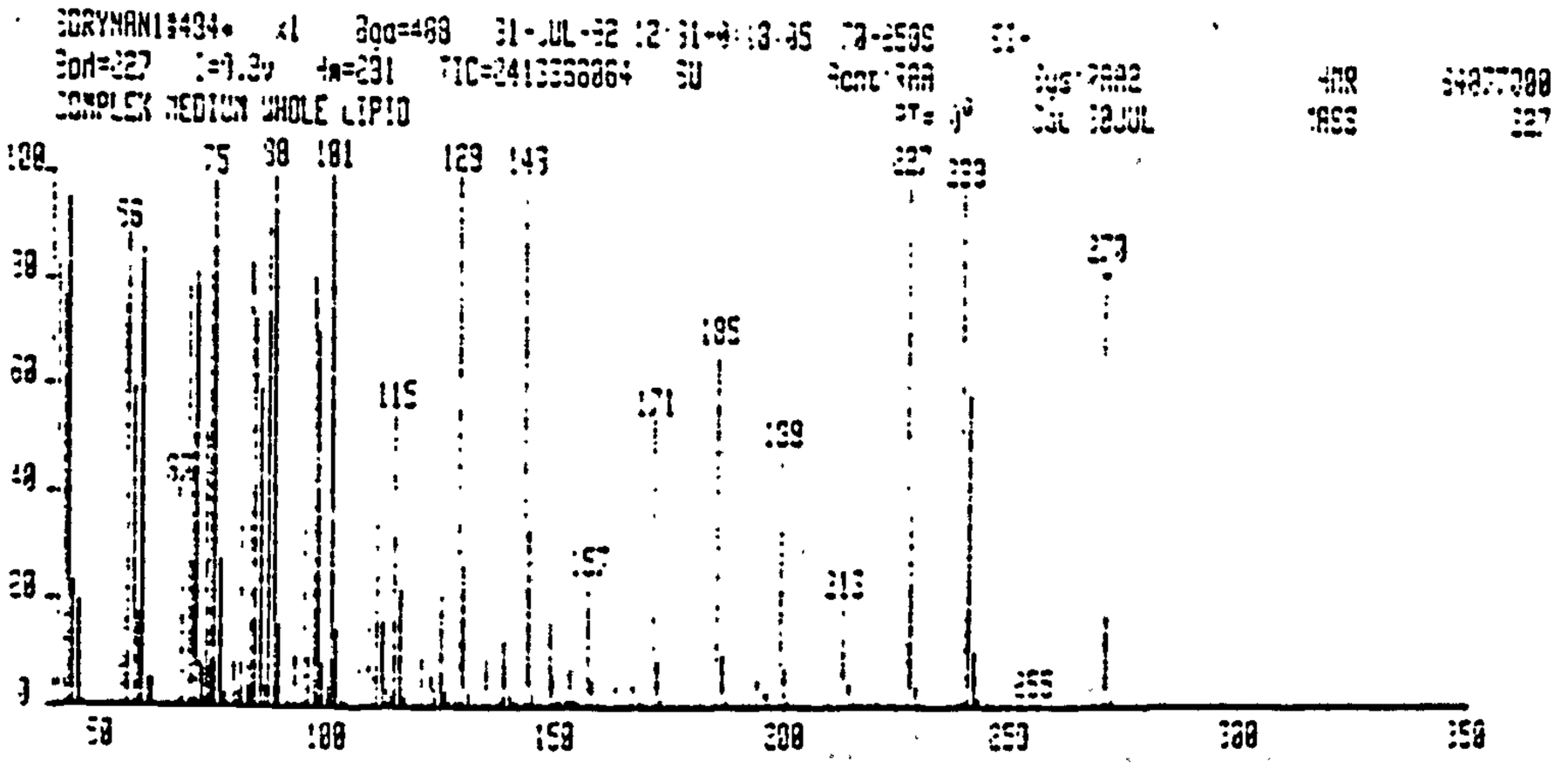
(h)


SORYNAN18433. 41 Bgd=425 31-JUL-92 12:31:49:16 78-2585 EI+
Bon=225 I=18v Hm=281 TIC=2746725888 SU Acnt RAA Sys RAA2 HMR 65474988
COMPLEX MEDIUM WHOLE LIPID PT= 3V Cal 30JUL MASS 225
100 59 75 38 181 129 143 213 225 256 115 157 199 171 185 241
50 100 150 200 250 300 350

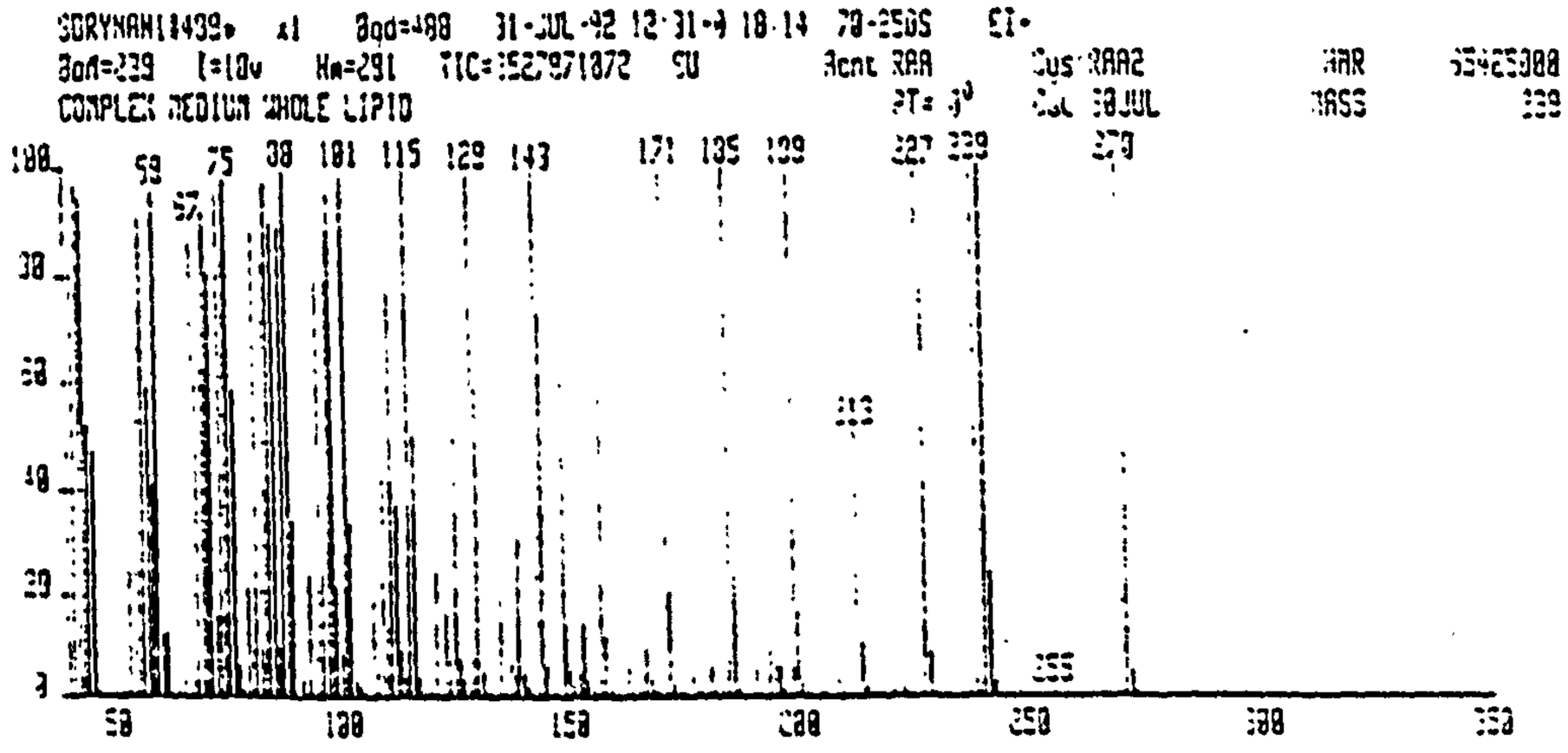
(i)


SORYNAN18479. 41 Bgd=442 31-JUL-92 12:31:49:17:35 78-2585 EI+
Bon=227 I=18v Hm=281 TIC=4186230864 SU Acnt RAA Sys RAA2 HMR 55554988
COMPLEX MEDIUM WHOLE LIPID PT= 3V Cal 30JUL MASS 278
100 59 67 75 38 181 115 129 143 171 185 199 227 241 278 157 213 256
50 100 150 200 250 300 350

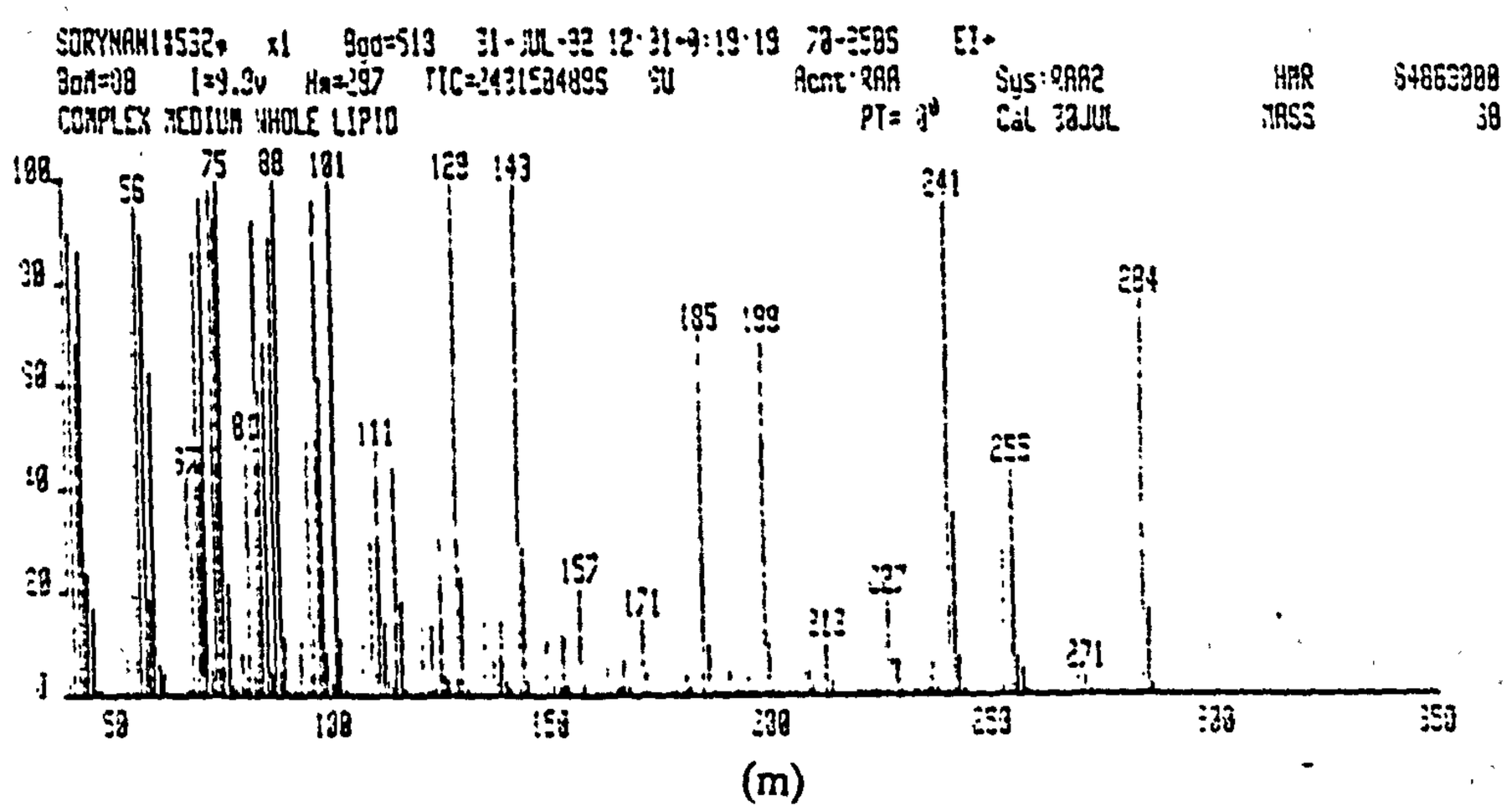
(j)


SORYNAN18434. 41 Bgd=468 31-JUL-92 12:31:49:18:45 78-2585 EI+
Bon=227 I=18v Hm=281 TIC=2413358864 SU Acnt RAA Sys RAA2 HMR 54877988
COMPLEX MEDIUM WHOLE LIPID PT= 3V Cal 30JUL MASS 227
100 75 38 181 129 143 227 233 59 115 171 185 199 213 253 157 255
50 100 150 200 250 300 350

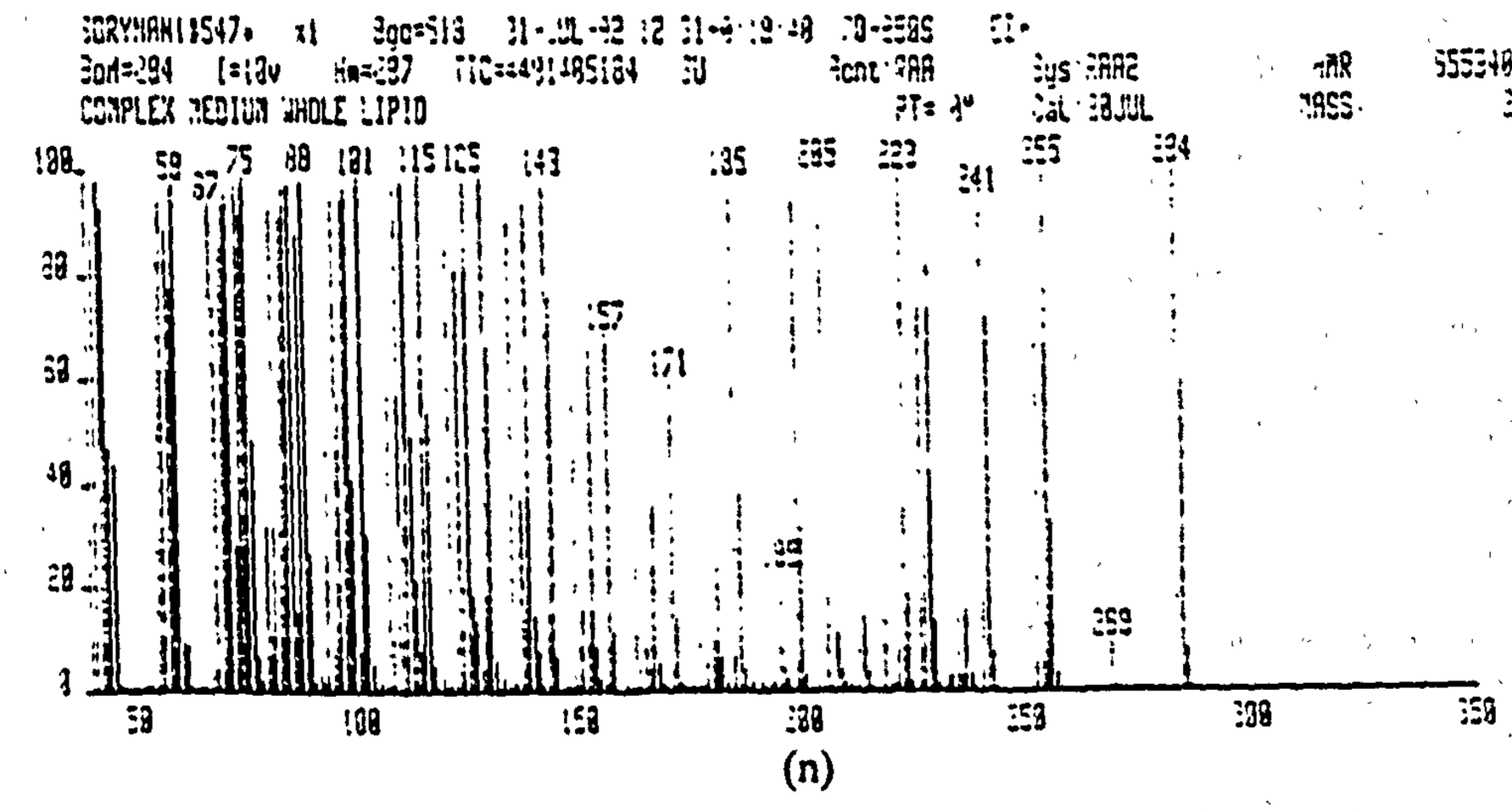
(k)


SORYNAN18439▸ x1 Bqd=488 31-JUL-92 12:31:9 18:14 78-2505 EI▸
Bon=239 I=10v Hm=291 TIC=152797872 SU Acnt RAA Sys:RAA2 HAR 55462388
COMPLEX MEDIUM WHOLE LIPID PT= 3° Cal 30JUL MASS 339
188 59 75 38 181 115 129 143 171 135 199 227 239 279
313
355
50 100 150 200 250 300 350

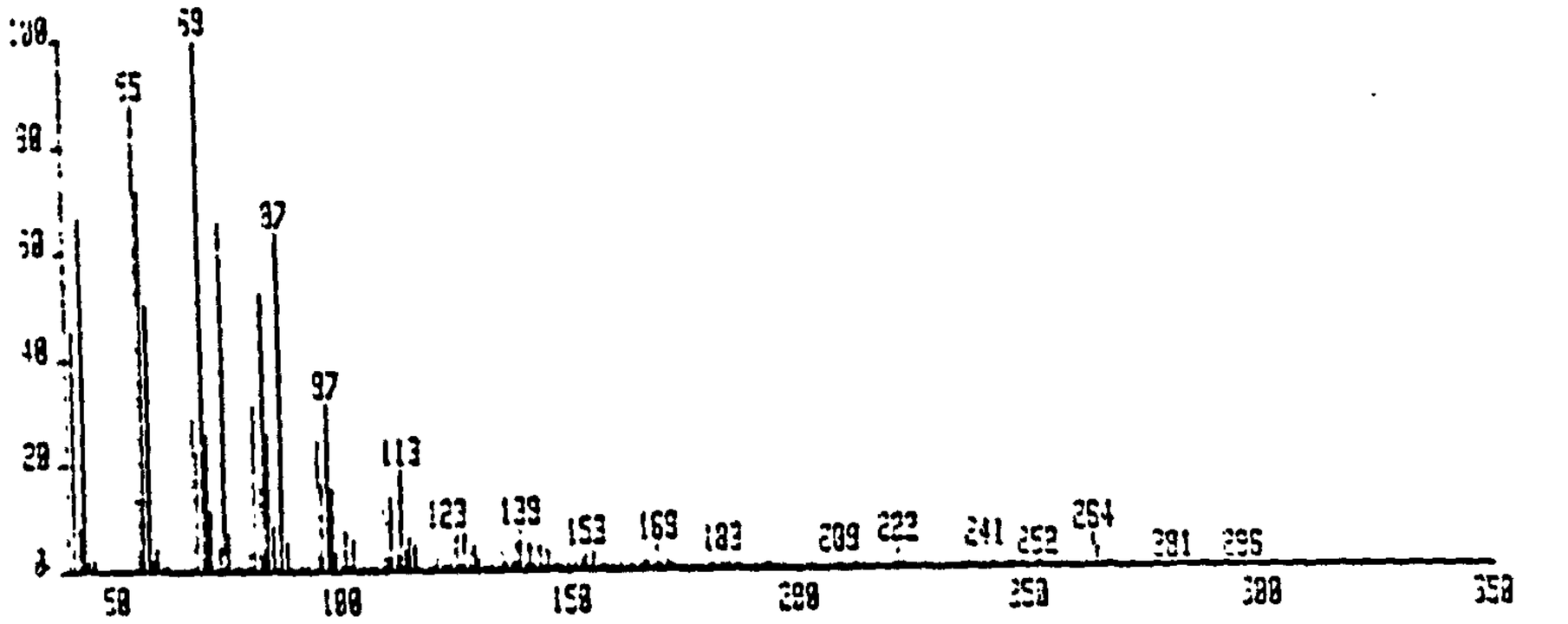
(l)


SORYNAN18532▸ x1 Bqd=513 31-JUL-92 12:31:9:19:19 78-2505 EI▸
Bon=98 I=9.3v Hm=297 TIC=24315848QS SU Acnt:RAA Sys:RAA2 HAR 54863008
COMPLEX MEDIUM WHOLE LIPID PT= 3° Cal 30JUL MASS 38
188 56 75 88 101 129 143 241
284
185 199
111
255
83
157
171
213
227
271
50 100 150 200 250 300 350

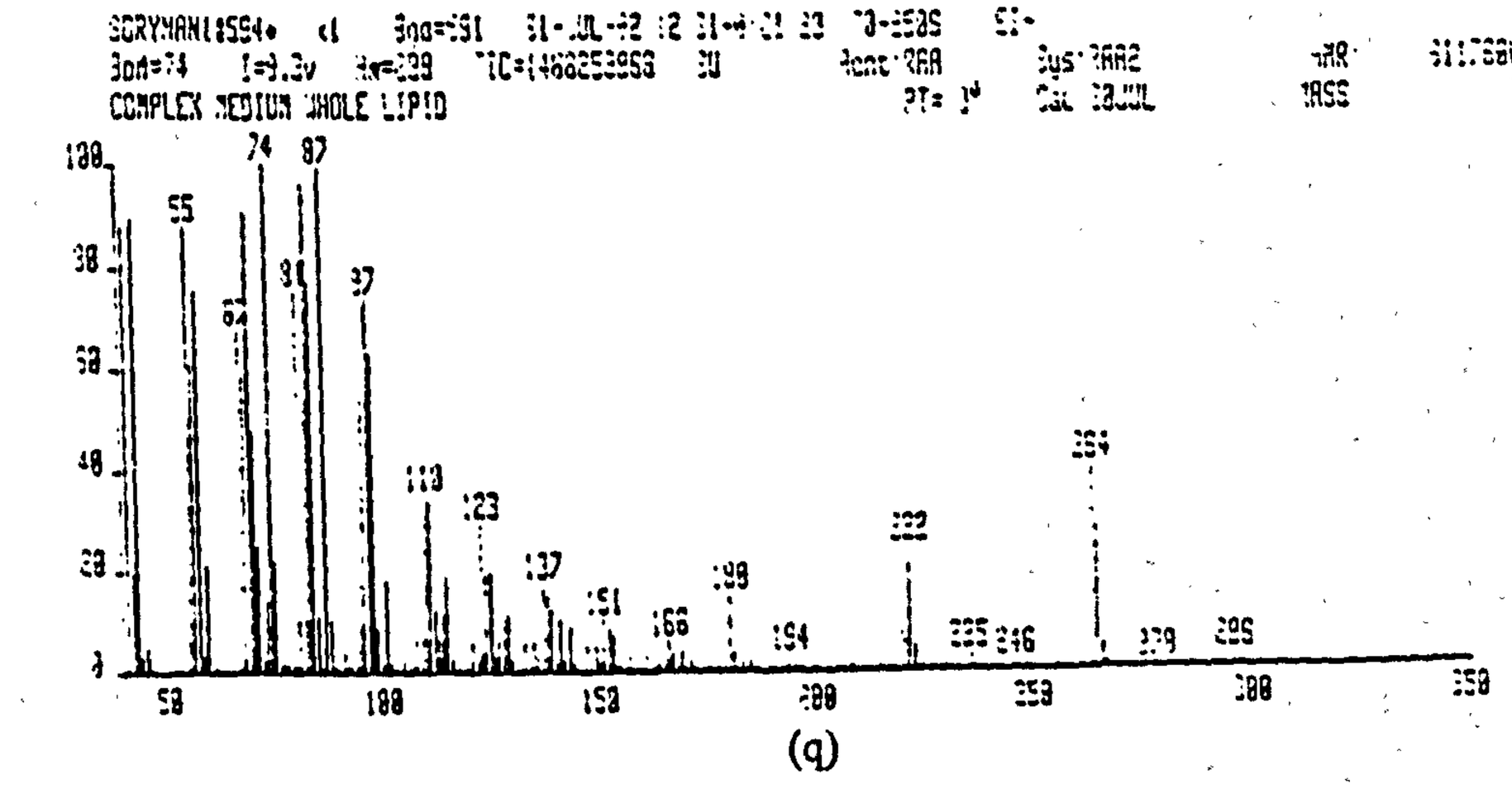
(m)


SORYNAN18547▸ x1 Bqd=513 31-JUL-92 12:31:9:19:49 78-2505 EI▸
Bon=294 I=10v Hm=287 TIC=44911485184 SU Acnt:RAA Sys:RAA2 HAR 55534000
COMPLEX MEDIUM WHOLE LIPID PT= 4° Cal 30JUL MASS 294
188 59 75 88 101 115 129 143 185 199 223 241 255 284
157
171
213
269
50 100 150 200 250 300 350

(n)


SDRYMAN18591• x1 Bgo=581 31-JUL-92 12:31:0:21:14 78-2585 EI•
BpM=143 I=9.3v Hm=388 TIC=1708352288 SU Acnt:RAA Sys:RAA2 HMR: 64656888
COMPLEX MEDIUM WHOLE LIPID PT= d⁰ Cal:30JUL MASS: 143
100
80
60
40
20
0
143
255
75
87
57
97
129
139
299
111
135
157
171
213
227
241
263
283
50
100
150
200
250
300
350

(p)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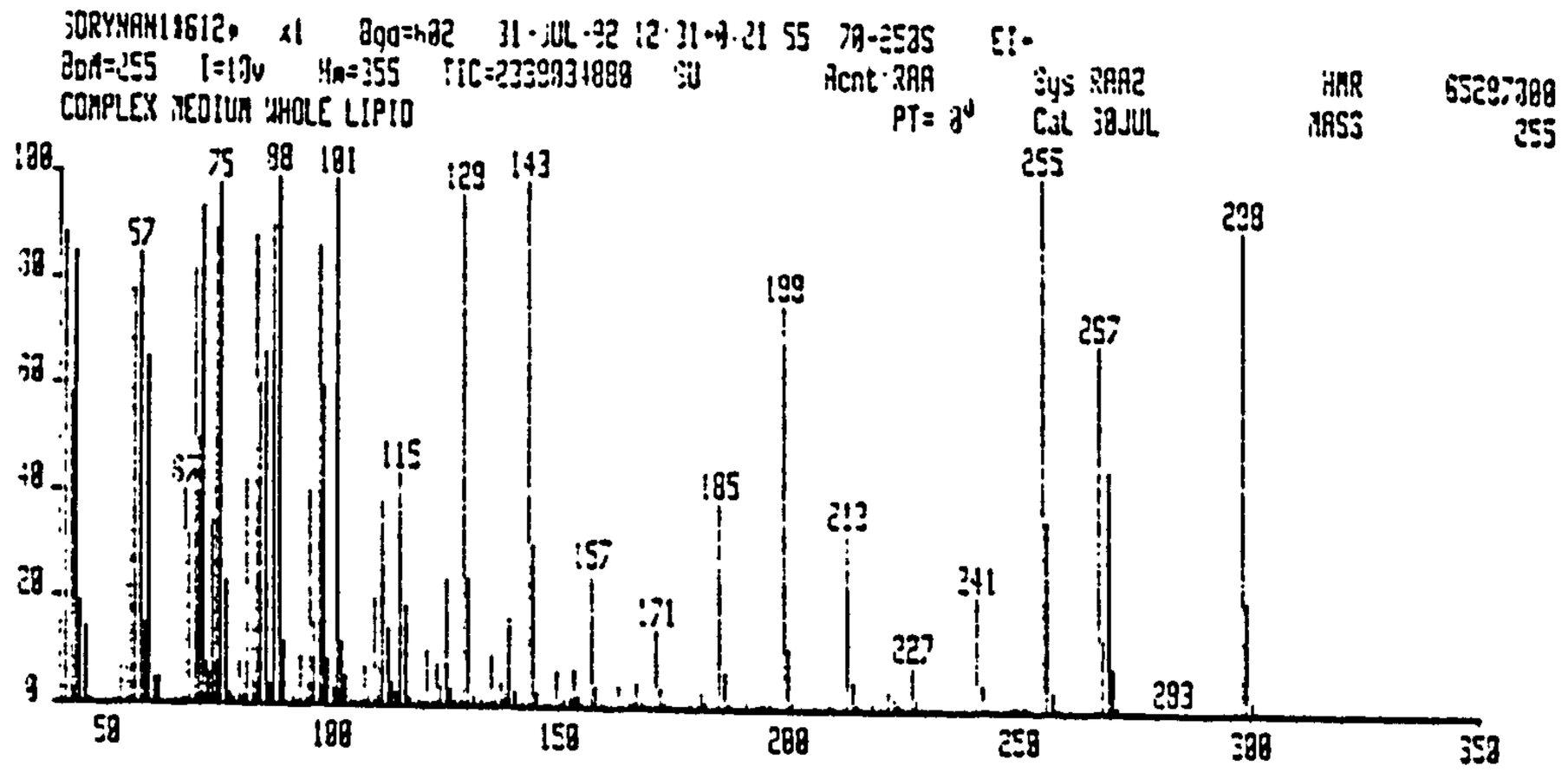
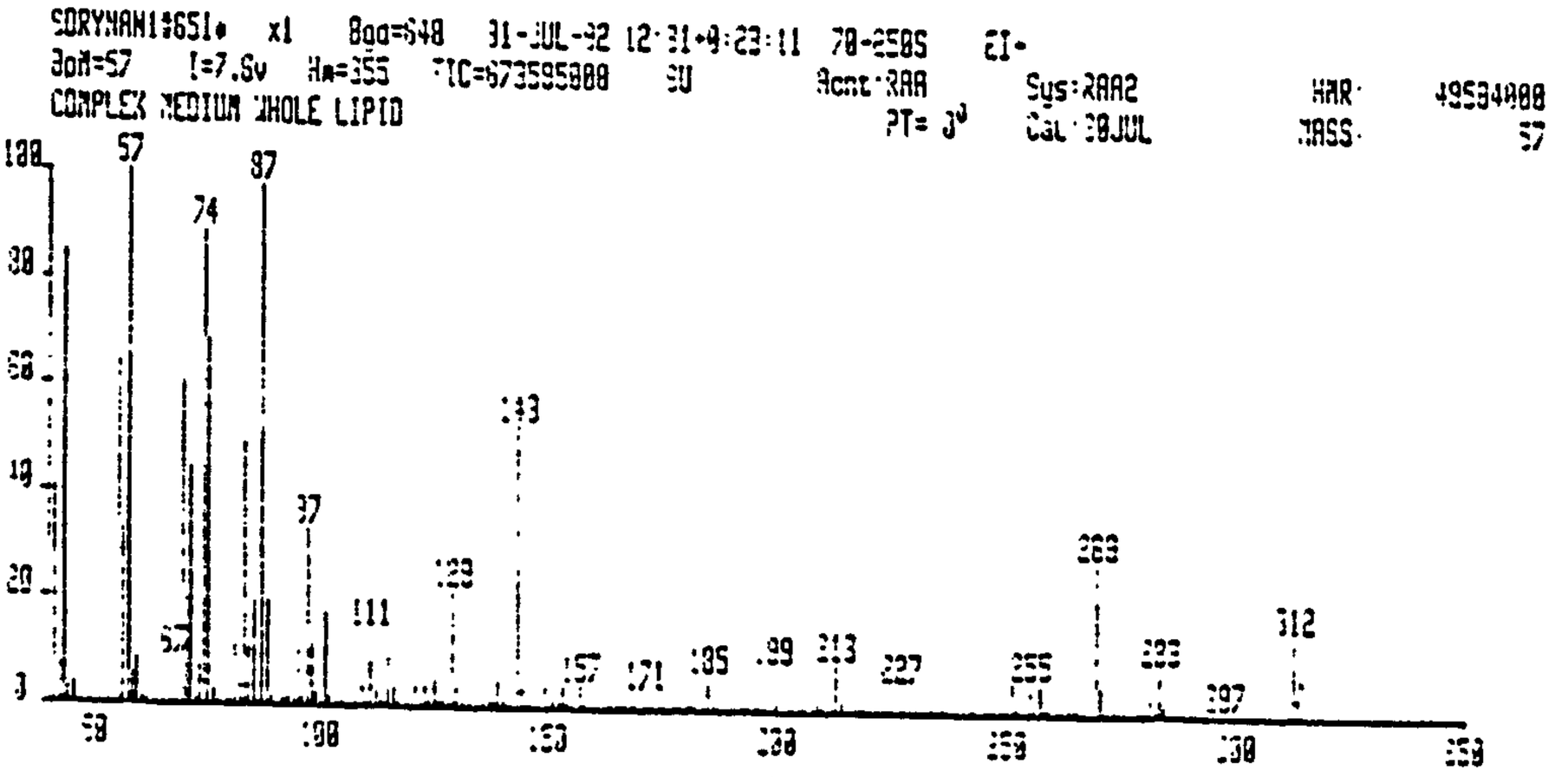
BpM=74 I=9.3v Hm=388 TIC=1468253853 SU Acnt:RAA Sys:RAA2 HMR: 64656888
COMPLEX MEDIUM WHOLE LIPID PT= d⁰ Cal:30JUL MASS: 74
100
80
60
40
20
0
74
87
55
97
57
113
123
137
151
166
194
222
246
264
279
295
50
100
150
200
250
300
350

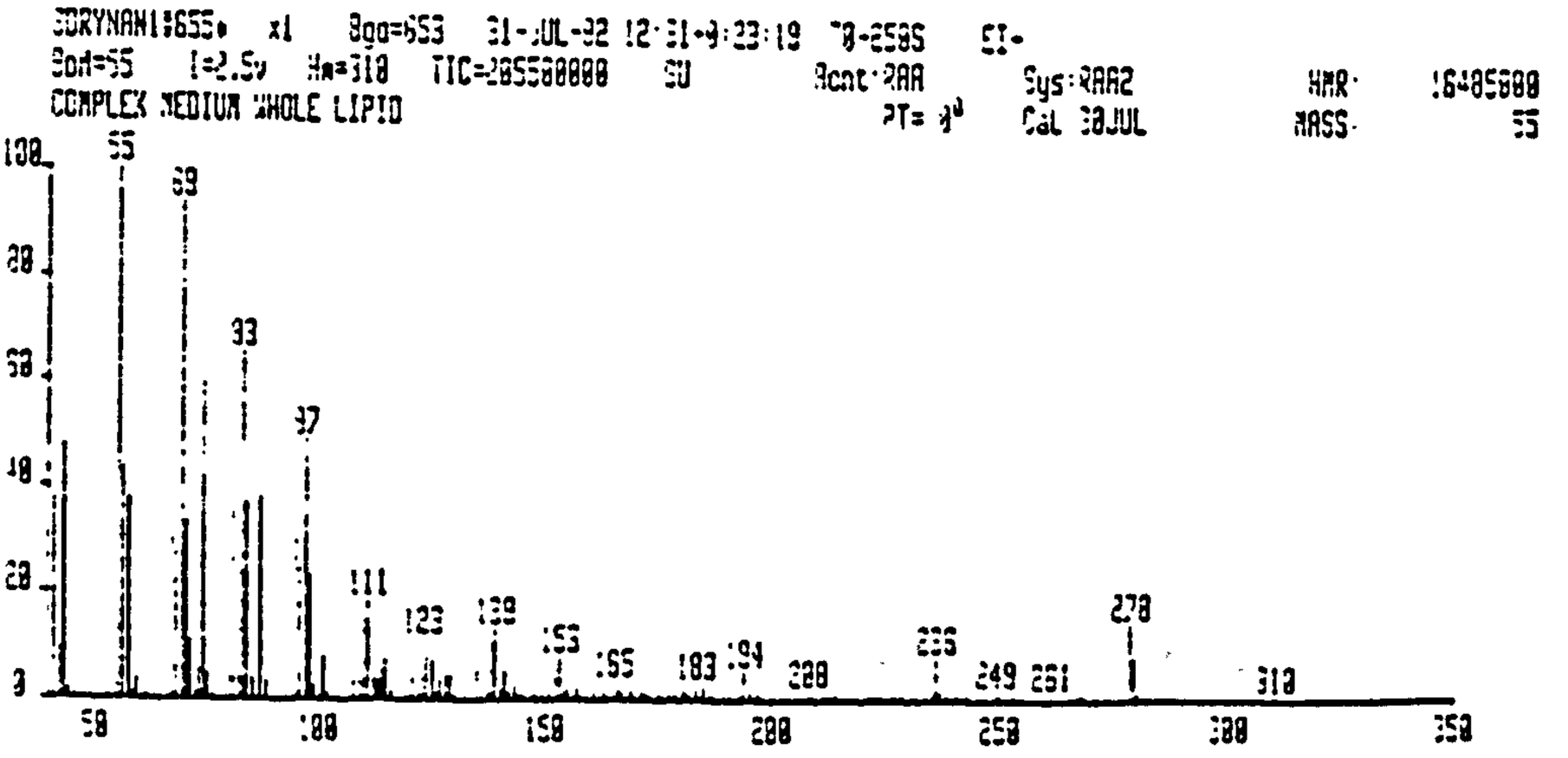
(q)


SORYNAN1%612• x1 Bqo=h82 31-JUL-92 12:31:4:21 55 78-2585 EI-
BoM=255 I=10v Hm=355 TIC=233983488 SU Acnt:RAA Sys RAA2 HAR 65287888
COMPLEX MEDIUM WHOLE LIPID PT= 8⁴ Cal 30JUL MASS 255
100 75 98 101 129 143 255 298 199 257 57 115 185 213 157 171 241 227 293
50 100 150 200 250 300 350

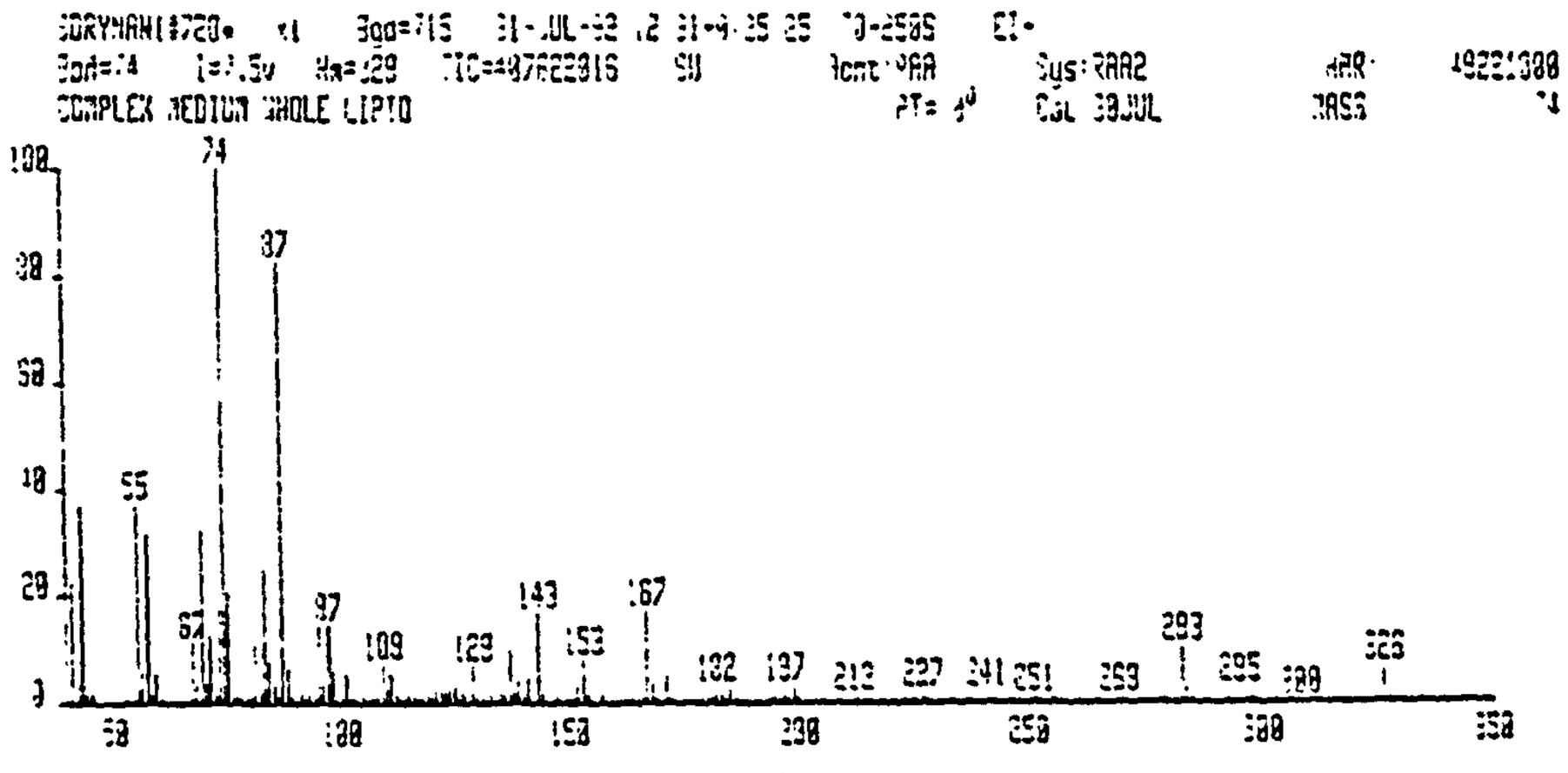
(r)


SORYNAN1%651• x1 Bqo=648 31-JUL-92 12:31:4:23:11 78-2585 EI-
BoM=57 I=7.5v Hm=355 TIC=673595888 SU Acnt:RAA Sys:RAA2 HAR: 49534888
COMPLEX MEDIUM WHOLE LIPID PT= J⁴ Cal:30JUL MASS: 57
100 57 87 74 43 97 111 123 99 213 227 255 263 283 312 297 157 171 185
50 100 150 200 250 300 350

(s)


SORYNAN1%655• x1 Bqo=653 31-JUL-92 12:31:4:23:19 78-2585 EI-
BoM=55 I=2.5v Hm=318 TIC=2855888888 SU Acnt:RAA Sys:RAA2 HAR: 16485888
COMPLEX MEDIUM WHOLE LIPID PT= 4⁴ Cal 30JUL MASS: 55
100 55 69 83 97 111 123 139 153 165 183 194 208 235 249 261 278 318
50 100 150 200 250 300 350

(t)


SDRYMAN(#720• x1 Bgd=715 31-JUL-82 12 31:4:35 25 10-2585 EI•
Bgd=74 I=3.5v Mx=629 TIC=4876223816 SU Acnt:MAA Sys:RAA2 MAR: 19221388
SCMPLEX MEDIUM WHOLE LIPID PT= 4⁰ Cal 30JUL MASS #1
100
80
60
40
20
0
74
87
55
67
97
109
123
143
153
167
182
197
213
227
241
251
263
283
295
309
323
50
100
150
200
250
300
350

(u)

Table 5.5 SUMMARY OF THE FATTY ACIDS PRESENT IN THE WHOLE LIPID EXTRACTED FROM *S.RIMOSUS* CULTURED IN YEME

S.rimosus was cultured in YEME (Methods 2.3.2 (c) and 2.5). Whole lipid fatty acid methyl esters from the biomass were prepared and run on GC-MS (Methods 2.23-2.30). This table provides a summary of the fatty acid identified (for mass spectra see Fig. 5.7), by comparison with known standards (see Table 5.4) and their identifying mass spectra (Methods 2.30.1). The peak number for each fatty acid refers to the GC traces (see Fig. 5.6). Where the symbol '>' is used it means that one peak is greater in height than another and where the symbol '=' is used it means that the peaks are of equal height. The symbols used to define types of fatty acids are: i - iso fatty acid, a - anteiso fatty acid, 2-OH - fatty acid containing a methyl group on carbon 2, 3-OH as before except the hydroxyl group is on carbon 3, ^{9,11,12} denote the position of the double bonds and Δ denotes a fatty acid containing a cyclopropane ring. 10C refers to a fatty acid of 10 carbon chain length, which could not be further characterised.

Peak no.		Fatty acid	M+	Characteristic features						
161	Minor	10 C	183	74						
233	Minor	12:0	214	74	M-31	M-43	M-57			
275	Minor	13:0	228	74	M-31	M-43				
280	Minor	a13:0	228	74	M-29	M-43	M-61			
307	Minor	x	x	no 74						
343	Major	14:0	242	74	M-31	M-43	M-57			
346	Minor	x	x	no 74						
366	Minor	14:0	242	74	M-31	M-43	M-57			
410	Major	methyl 15:0	256	74	M-29	M-43	M-57			
416	Major	a15:0	256	74	M-29	M-43	M-55	M-56	M-57	M-65
420	Major	i15:0	256	74	M-29	M-43	M-57	M-61	M-79	
433	Major	15:0	256	74	M-31	M-43	M-57			
479	Major	i16:0	270	74	M-29	M-43	M-57	M-65		
486	Major	x	x	no 74						
494	Major	16:0	270	74	M-31	M-43	M-57			
499	Major	16:0	270	74	M-31	M-43	M-57			
515	Major	x	x	no 74						
532	Major	methyl 17:0	284	74	M-29	M-43	M-57			
547	Major	a17:0	284	74	M-29	M-43	M-57	M-61	M-79	
578	Major	17:1	296	69>74	55	M-32				
591	Major	methyl 18:0	298	74	M-29	M-43	M-57			
594	Major	18:1	296	74	M-32	M-74	M-116			
600	Minor	x	x	no 74						
612	Major	18:0	298	74	M-31	M-43	M-57			
619	Minor	x	x	no 74						
645	Minor	x	x	no 74						
651	Minor	19:0	312	74	M-29	M-43	M-57			
655	Minor	19:0Δ	310	69>74	M-32					
666	Minor	x	312	74	M-31	M-43	M-57			
697	Minor	x	x	no 74						
711	Minor	x	x	no 74						
720	Minor	20:0	326	74	M-31	M-43	M-57			

Fig. 5.8 GC TRACE OF THE WHOLE LIPID FATTY ACID METHYL ESTERS EXTRACTED FROM *S.RIMOSUS* GROWN ON MODIFIED HMM MEDIUM

S. rimosus was grown according to Methods 2.5 in modified HMM [Methods 2.3.1 (b)]. The whole lipid fatty acid extracted from the biomass (see Methods 2.23-2.27 for preparation) was run on GC-MS (Methods 2.30). This is the GC trace from which the individual peaks were taken for further analysis by MS. Each individual peak (corresponds to a fatty acid) was assigned a peak number automatically by the machine.

DRYNAN2 81-1168 31-JUL-92 14 46 70-2505 (EI+) Sys RAA2

Chromatogram Identifiers BB LTIC

Text MINIMUM MEDIUM WHOLE LIPID

IHP

B 3863762944

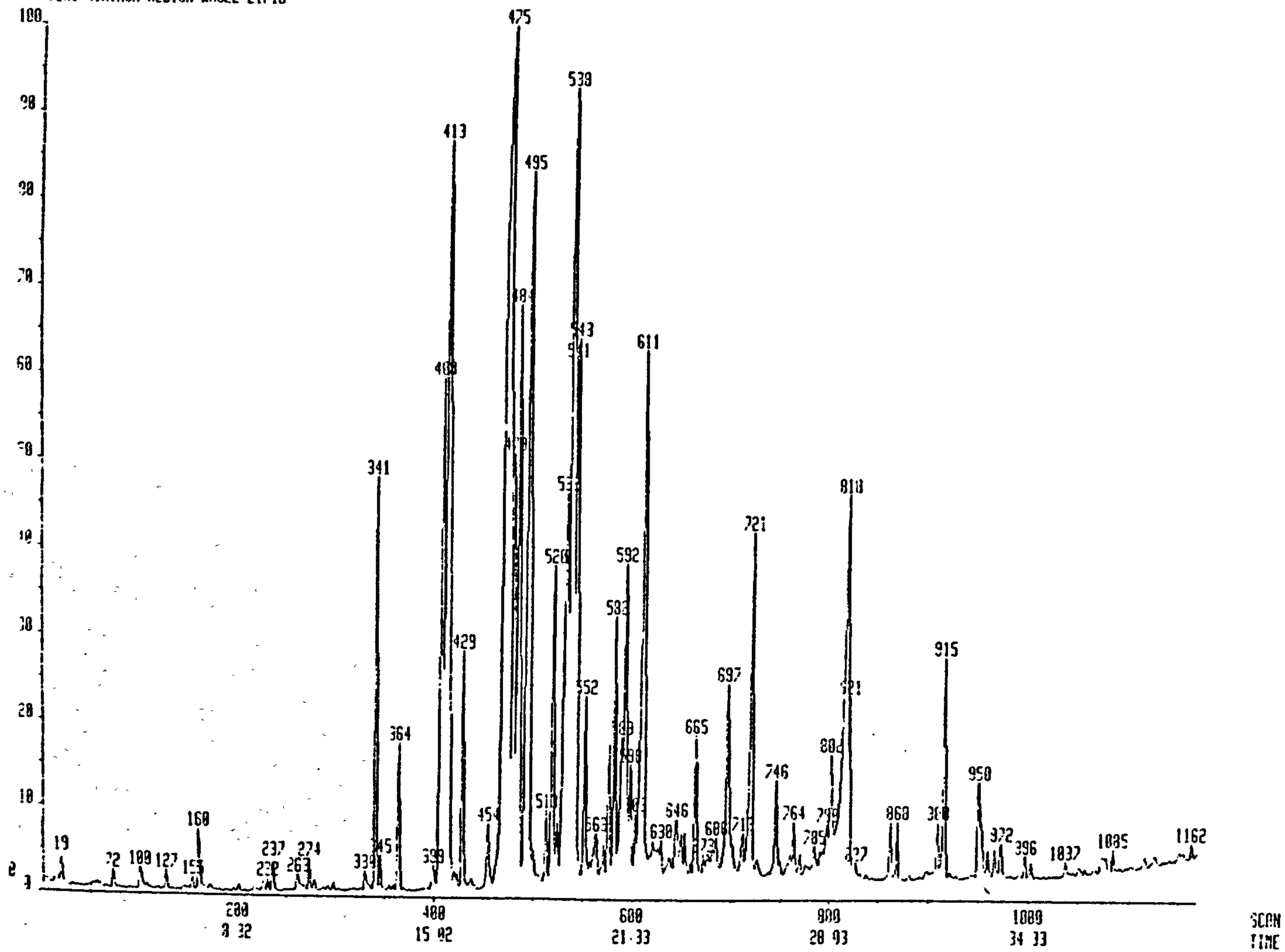


Fig. 5.9 INDIVIDUAL MASS SPECTRA FOR THE WHOLE LIPID FATTY ACID METHYL ESTERS FROM *S. RIMOSUS* CULTURED IN MODIFIED HMM

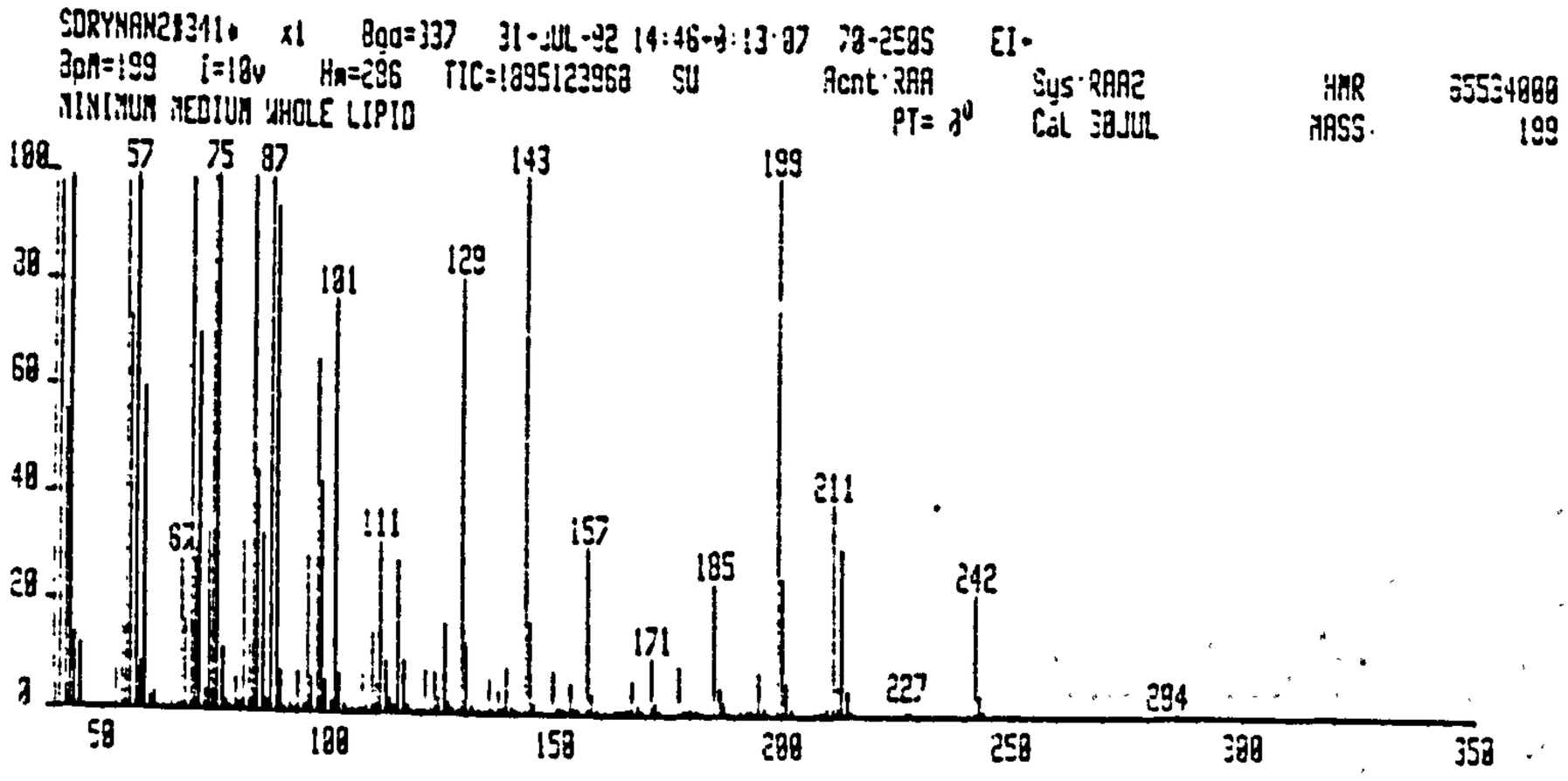
S.rimosus was cultured in modified HMM (Methods 2.3.1 (b) and 2.5). Whole lipid fatty acid methyl esters from the biomass were prepared and run on GC-MS (Methods 2.23-2.30). Individual peaks from the GC trace were further analysed by MS (Methods 2.30). The peak numbers correspond to the GC trace (see Fig. 5.8). The mass spectra for each peak are shown as follows:

Peak no.

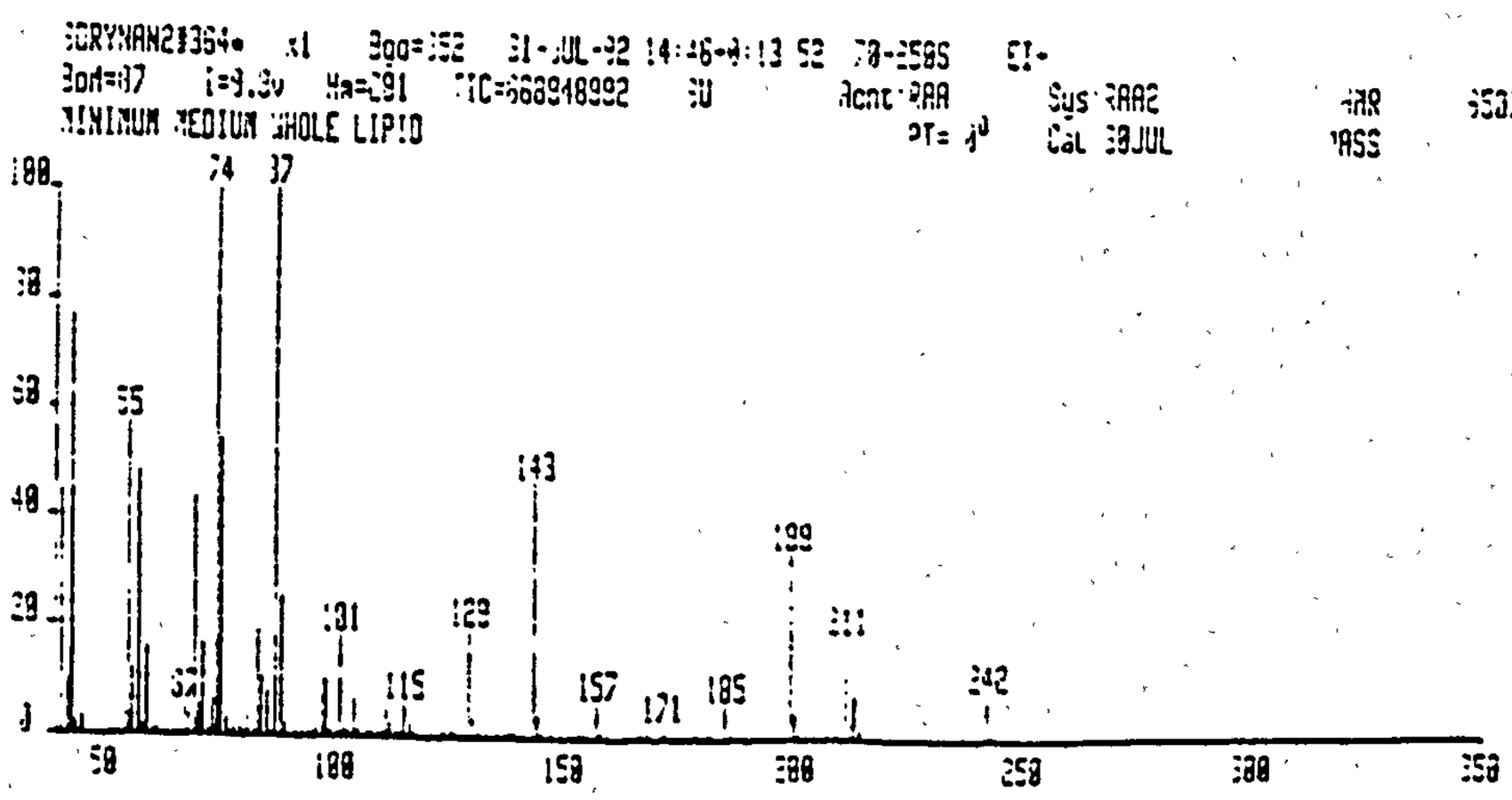
(a) 160
(b) 341
(c) 364
(d) 408
(e) 413
(f) 429
(g) 475
(h) 479
(i) 484
(j) 495
(k) 513
(l) 520
(m) 532
(n) 538
(o) 541
(p) 543
(q) 552
(r) 592
(s) 598
(t) 611
(u) 915
(v) 950


SDRYNAN21168• x1 Bgd=158 31-JUL-92 14:46-0 07 13 78-2585 EI•
BpM=74 I=5.9v Hm=291 TIC=298534816 SU Acnt RAA Sys:RAA2 HMR 38444888
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal:38JUL MASS 74
74
87
55
67
98
111
129
143
155
287
281
100
80
60
40
20
0
50
100
150
200
250
300
350

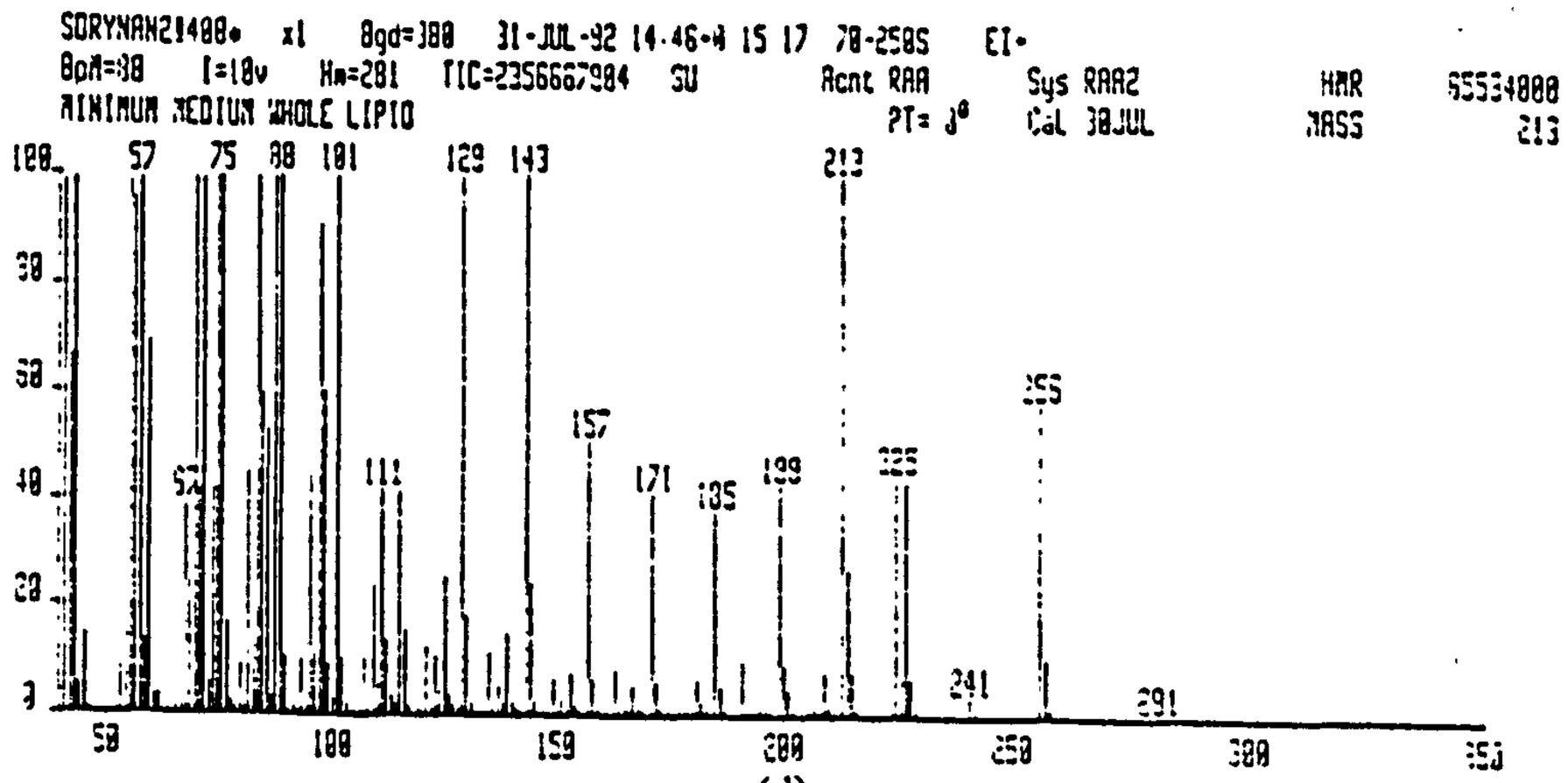
(a)


SDRYNAN21341• x1 Bgd=337 31-JUL-92 14:46-0 13 07 78-2585 EI•
BpM=199 I=18v Hm=296 TIC=1895123968 SU Acnt RAA Sys RAA2 HMR 35534888
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal 38JUL MASS 199
57
75
87
143
199
129
101
67
111
157
185
211
242
171
227
294
100
80
60
40
20
0
50
100
150
200
250
300
350

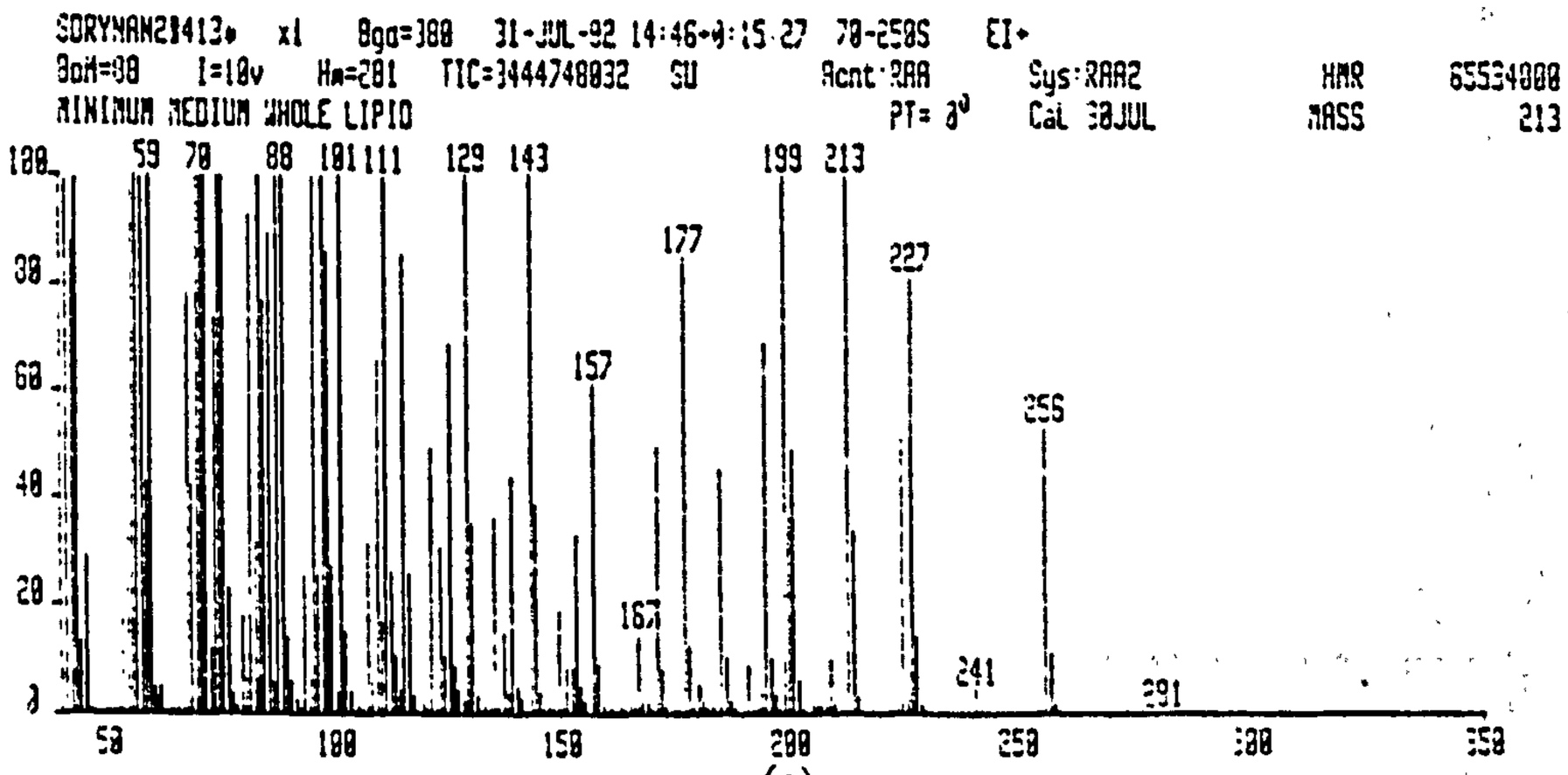
(b)


SDRYNAN21354• x1 Bgd=352 31-JUL-92 14:46-0 13 52 78-2585 EI•
BpM=87 I=9.3v Hm=291 TIC=868948992 SU Acnt RAA Sys RAA2 HMR 35374988
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal 38JUL MASS 87
74
87
55
143
199
131
129
111
157
185
211
242
171
115
100
80
60
40
20
0
50
100
150
200
250
300
350

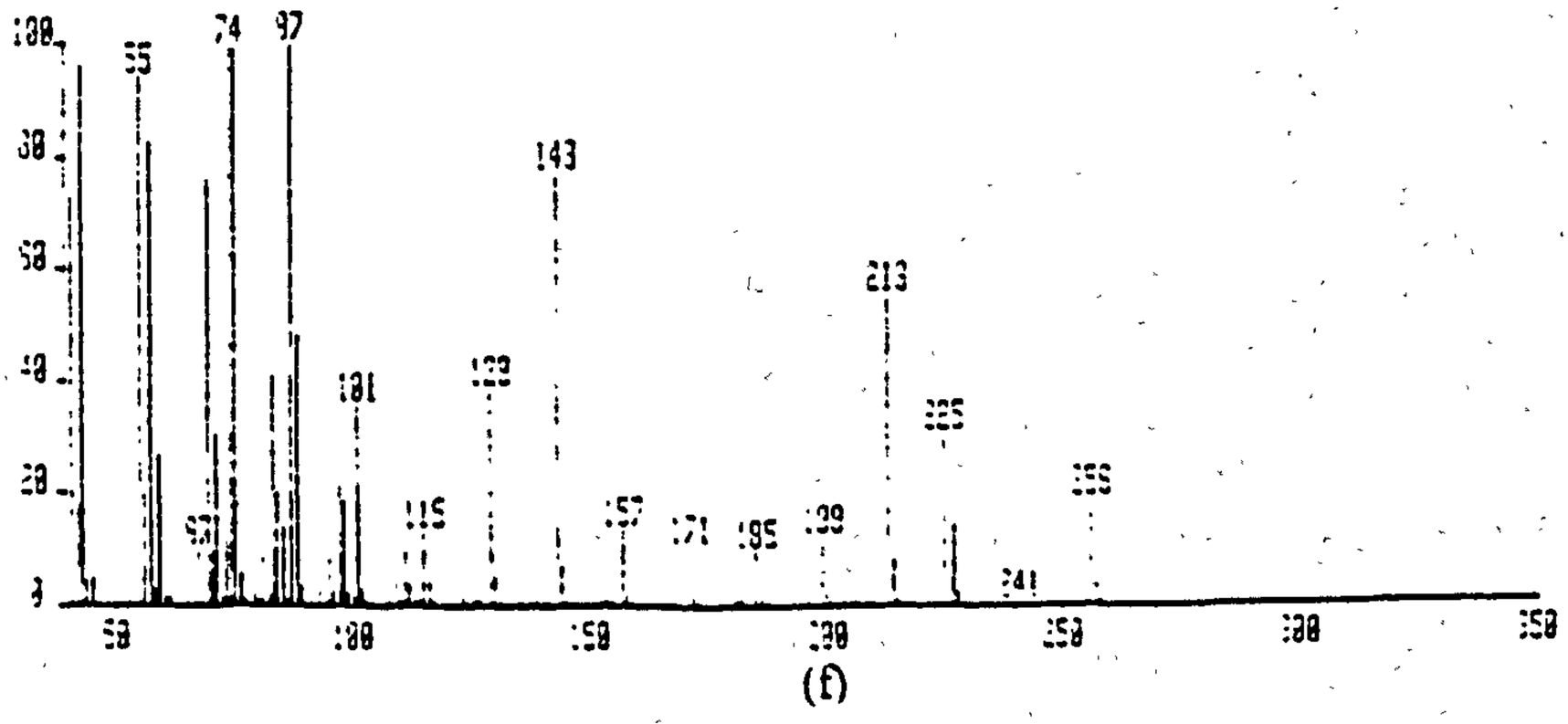
(c)


SORYNAN21408• x1 Bgd=388 31-JUL-92 14:46:4 15 17 78-2585 EI•
BpM=88 I=10v Hm=281 TIC=2356667984 SU Acnt RAA Sys RAA2 HMR 65534888
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal 30JUL MASS 213
100 57 75 88 101 129 143 213 255 241 291
57 111 157 171 185 199 227
50 100 150 200 250 300 350

(d)


SORYNAN21413• x1 Bgd=388 31-JUL-92 14:46:4:15:27 78-2585 EI•
BpM=88 I=10v Hm=281 TIC=3444748032 SU Acnt RAA Sys RAA2 HMR 65534888
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal 30JUL MASS 213
100 59 70 88 101 111 129 143 199 213 177 227
157 255 167 241 291
50 100 150 200 250 300 350

(e)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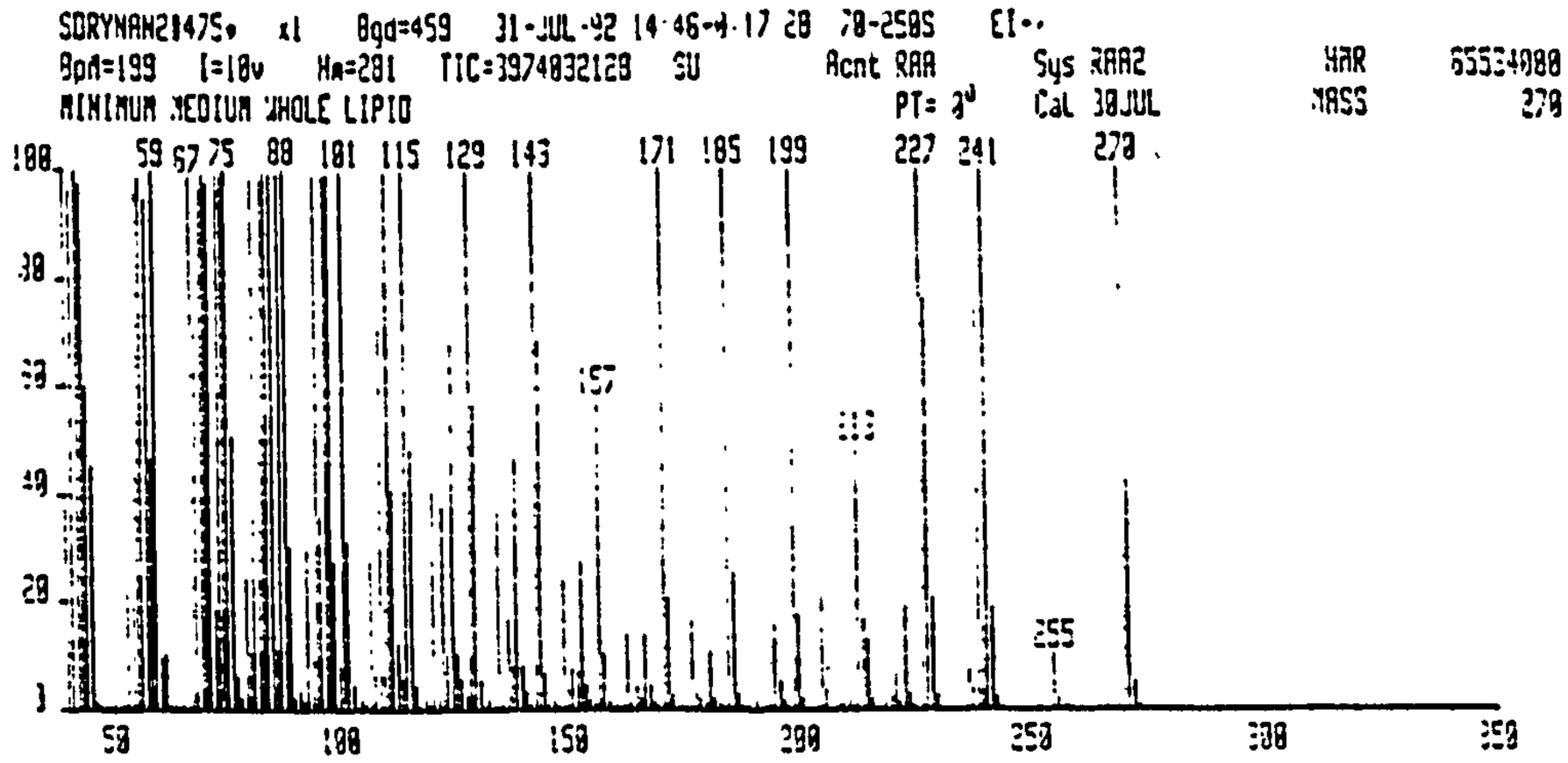
BpM=87 I=9.3v Hm=281 TIC=1848233888 SU Acnt RAA Sys RAA2 HMR 65991888
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal 28JUL MASS 37
100 74 97 55 143 213 181 129 225 115 157 171 185 199 255 241
50 100 150 200 250 300 350

(f)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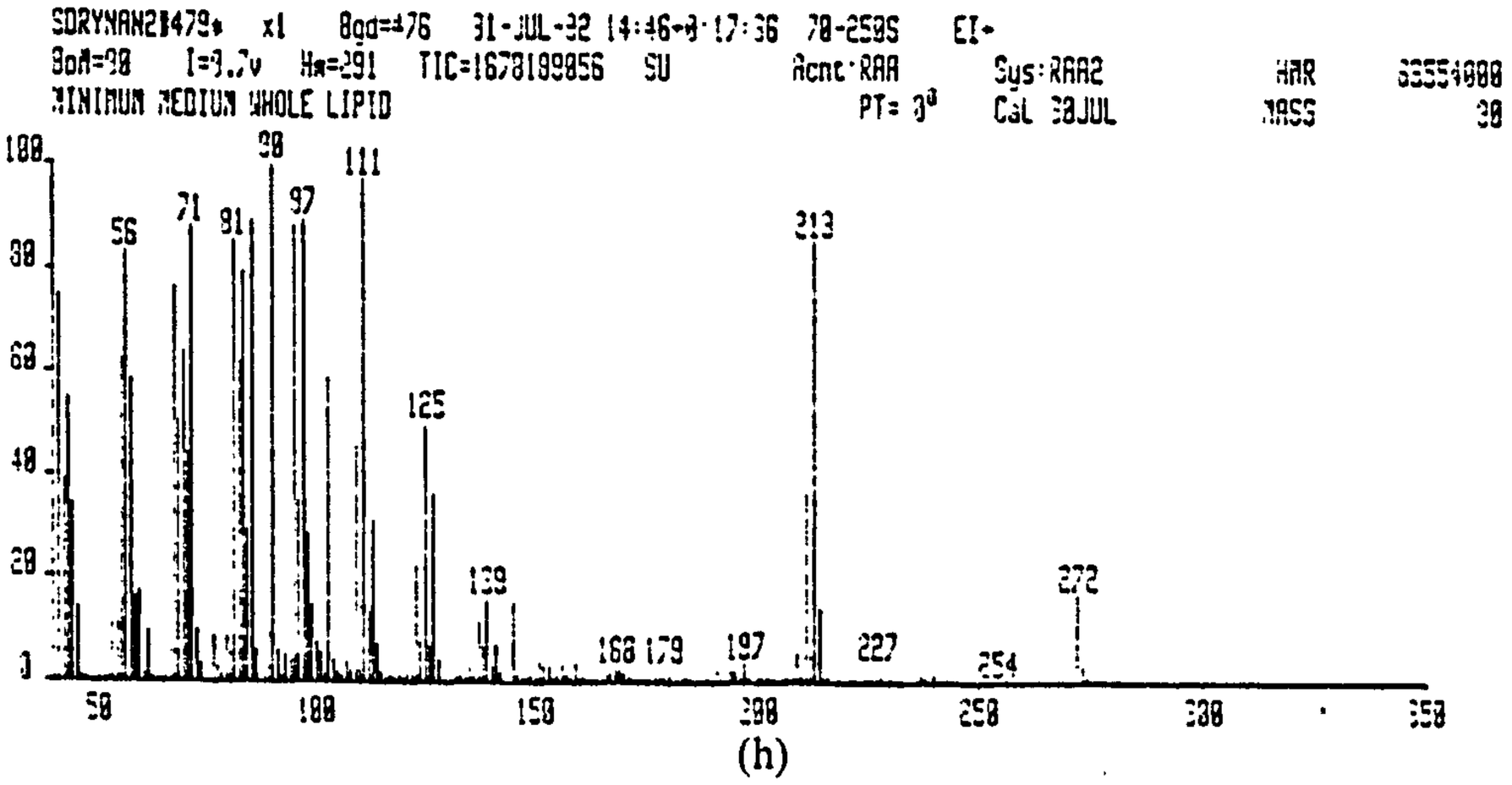
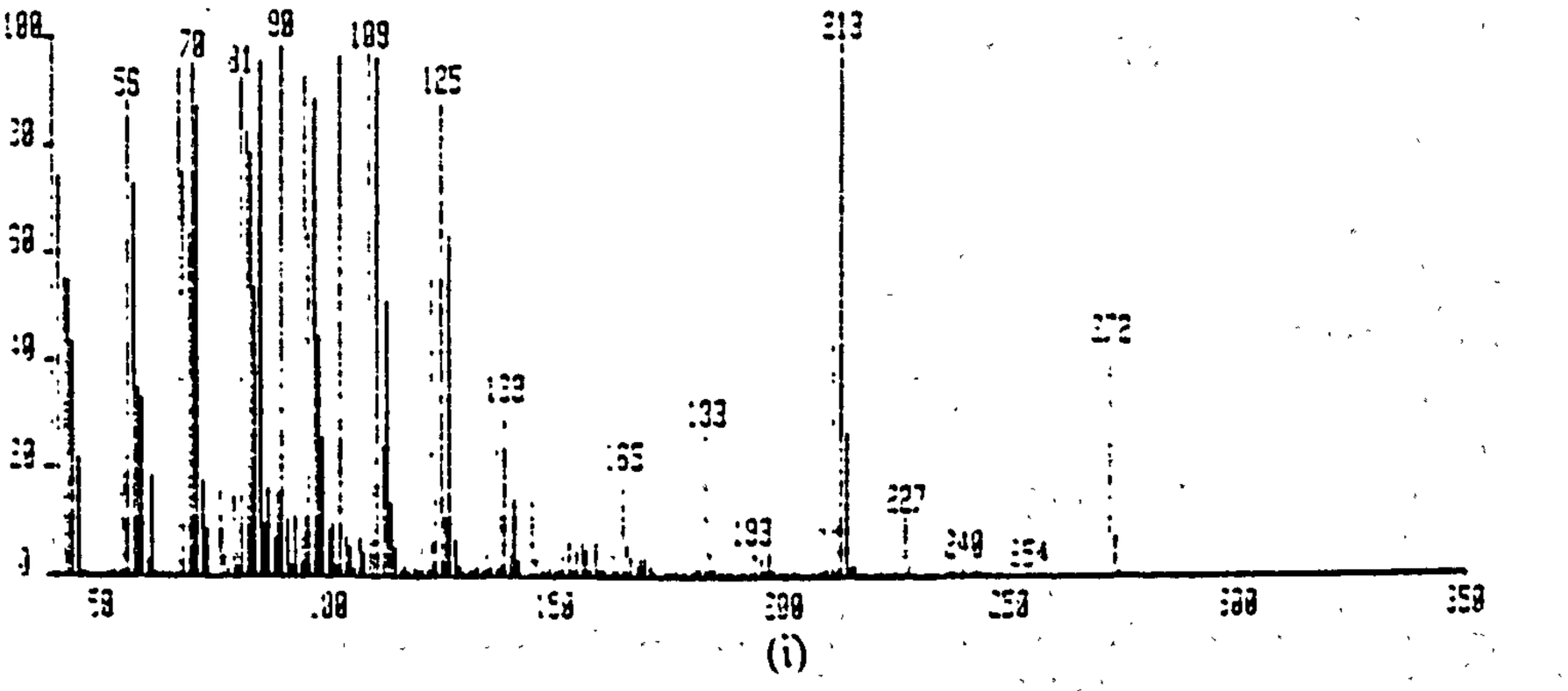
BpM=199 I=10v Hm=281 TIC=3974032128 SU Acnt RAA Sys RAA2 HAR 65534088
MINIMUM MEDIUM WHOLE LIPID PT= 3⁰ Cal 30JUL MASS 270
100 59 67 75 88 101 115 129 143 157 171 185 199 227 241 255 270
50 100 150 200 250 300 350

(g)


SORYNAN21479• x1 Bgd=476 31-JUL-92 14:46-4:17:36 78-2585 EI•
BpM=98 I=9.7v Hm=291 TIC=1678199856 SU Acnt:RAA Sys:RAA2 HAR 65534088
MINIMUM MEDIUM WHOLE LIPID PT= 3⁰ Cal 30JUL MASS 98
100 98 111 56 71 81 97 213 125 139 168 179 197 227 254 272
50 100 150 200 250 300 350

(h)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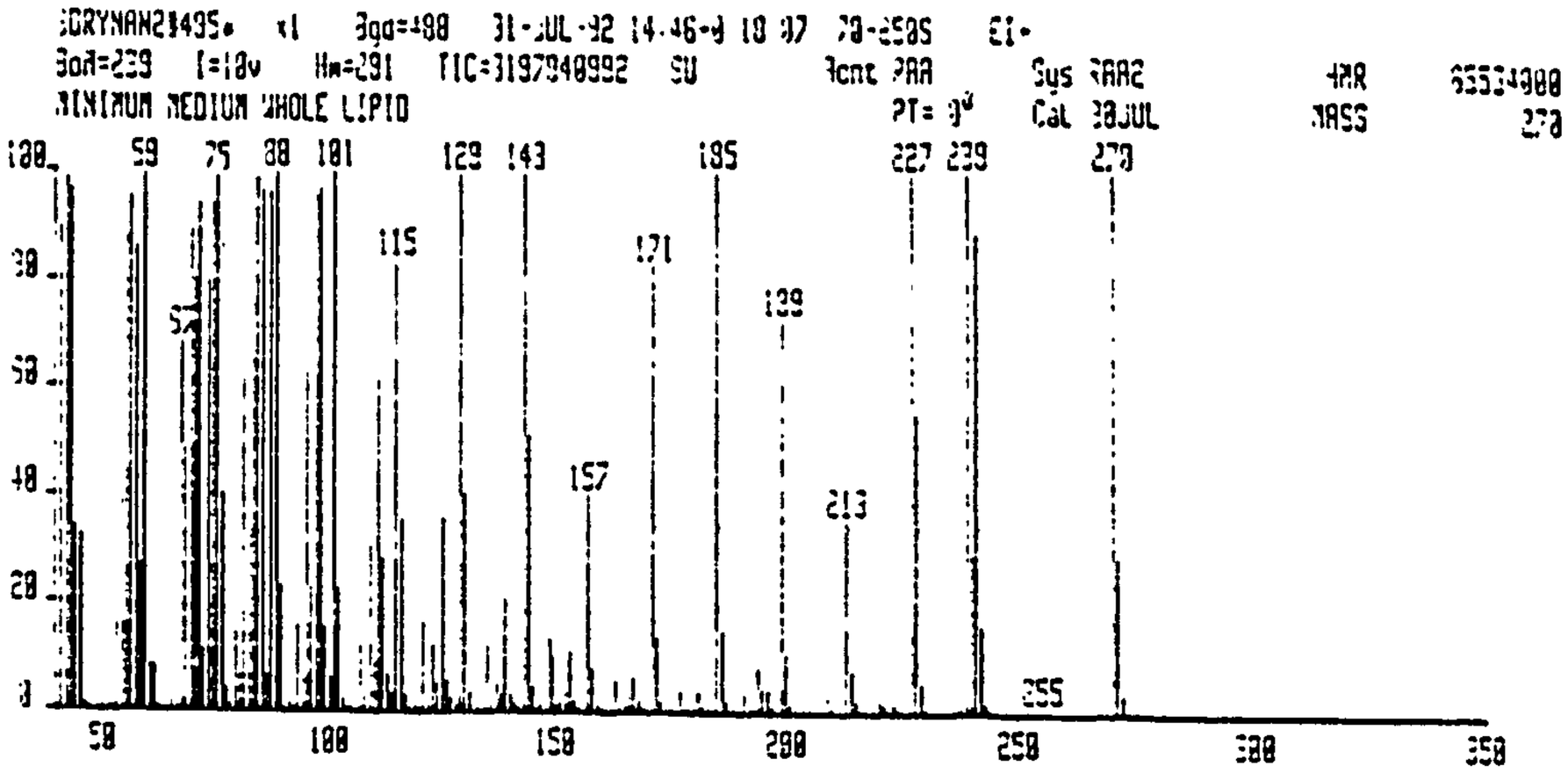
BpM=32 I=9.9v Hm=281 TIC=2458818888 SU Acnt:RAA Sys:RAA2 HAR 65813300
MINIMUM MEDIUM WHOLE LIPID PT= 3⁰ Cal 30JUL MASS 213
100 98 109 55 70 81 125 213 133 155 193 227 240 254 272
50 100 150 200 250 300 350

(i)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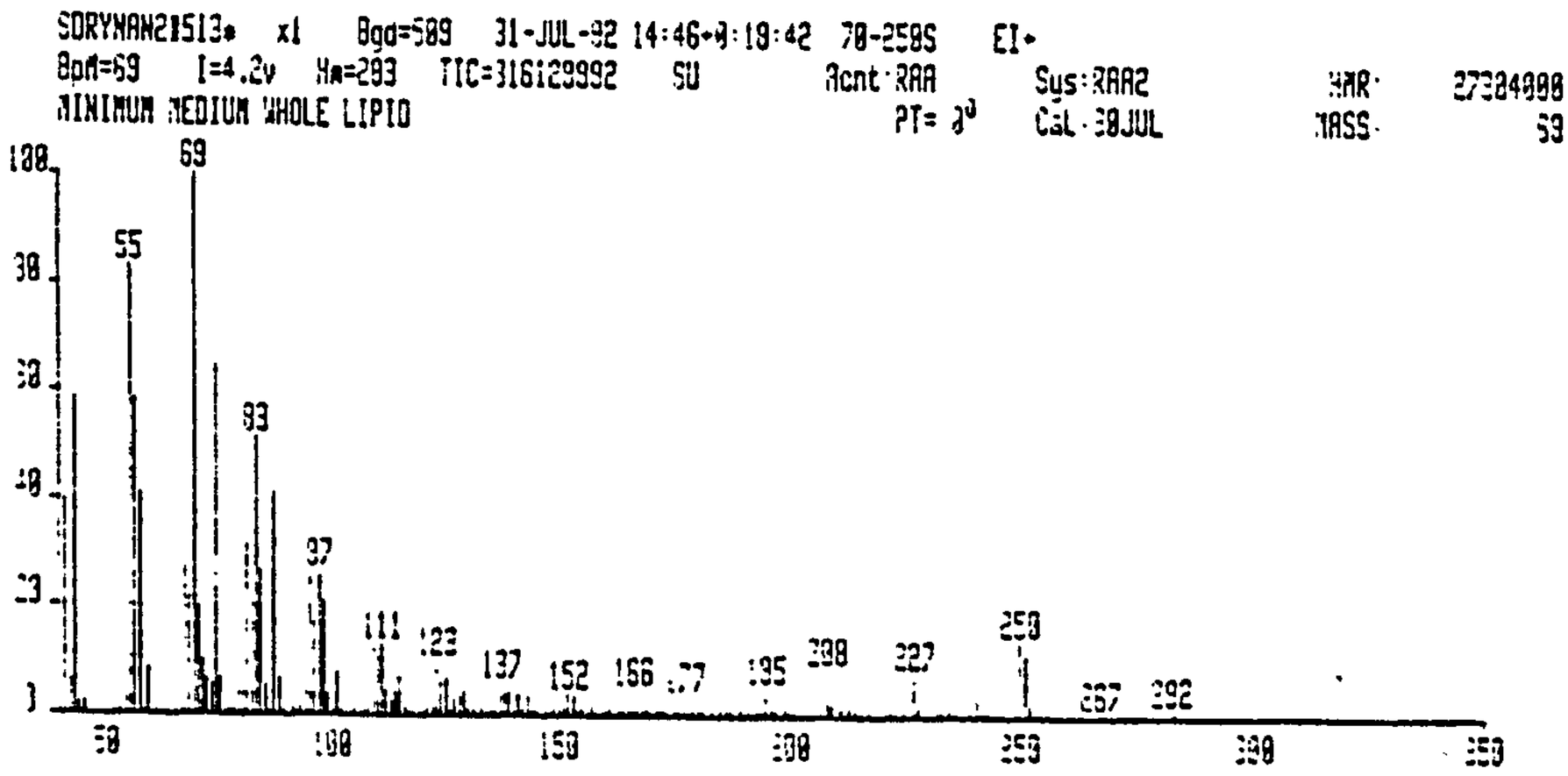
Bpk=239 I=18v Hm=291 TIC=3197940992 SU Acnt RAA Sys RAA2 MAR 65534980
MINIMUM MEDIUM WHOLE LIPID PT= 9° Cal 30JUL MASS 278
59 75 88 101 129 143 185 227 239 279
115 171 199 157 213 255
50 100 150 200 250 300 350

(j)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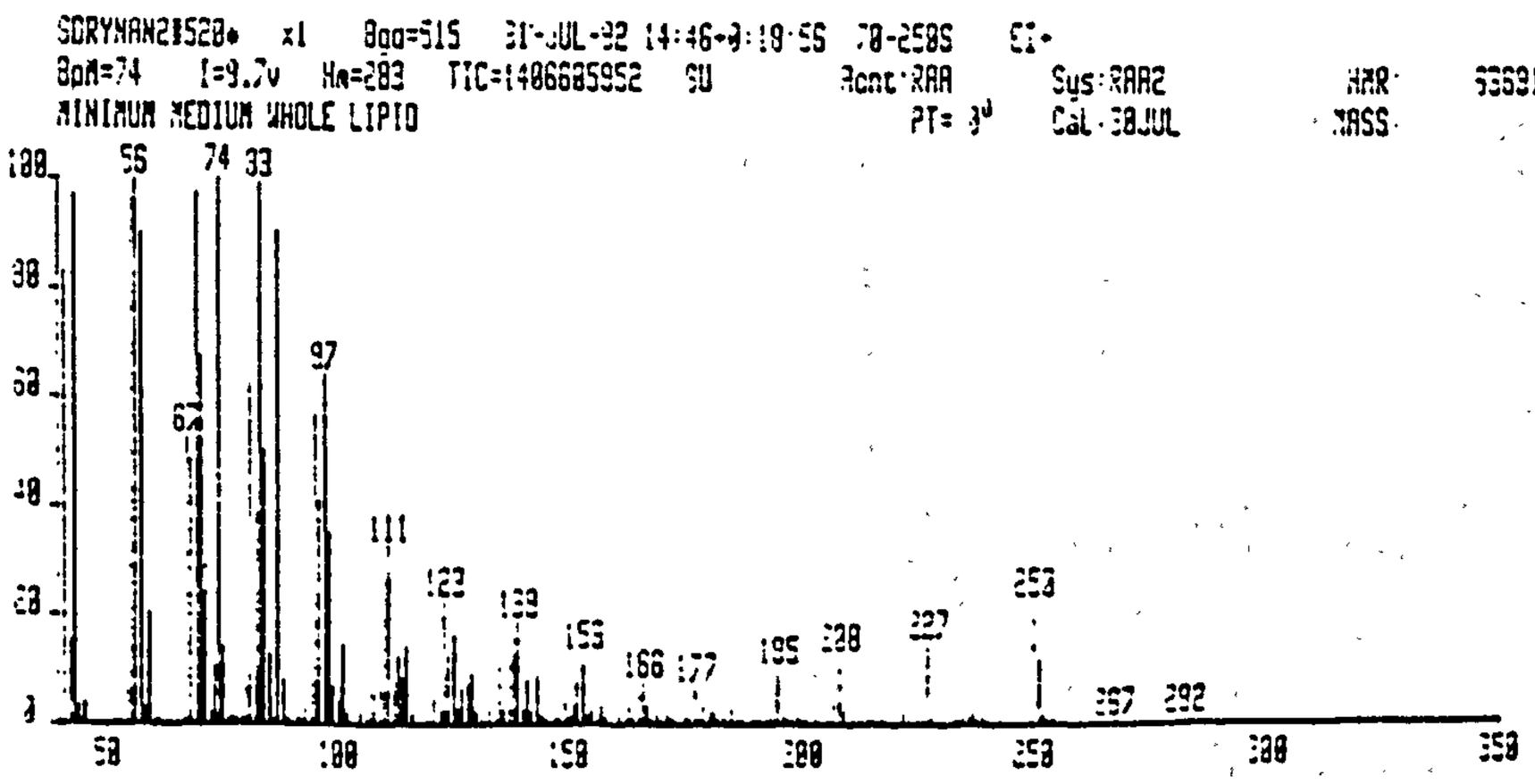
50 100 150 200 250 300 350

(k)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Bpk=74 I=9.7v Hm=283 TIC=1486685952 SU Acnt RAA Sys RAA2 MAR 53591000
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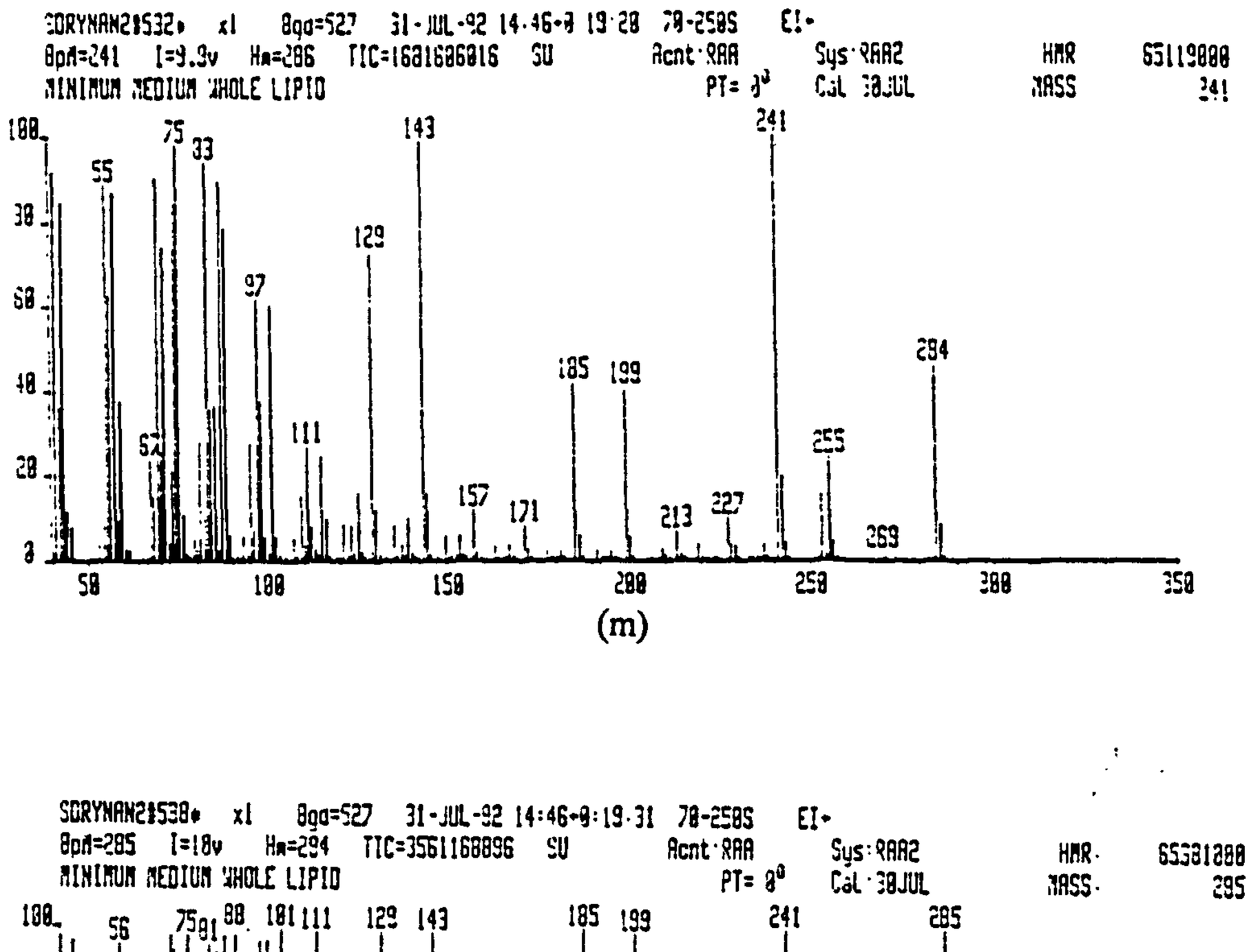
74 55 83 97 111 123 139 153 166 177 195 208 227 253 267 292
50 100 150 200 250 300 350

(l)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BpM=241 I=9.3v Hm=286 TIC=1681686816 SU Acnt:RAA Sys:RAA2 HMR 65119888
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal 30JUL MASS 241
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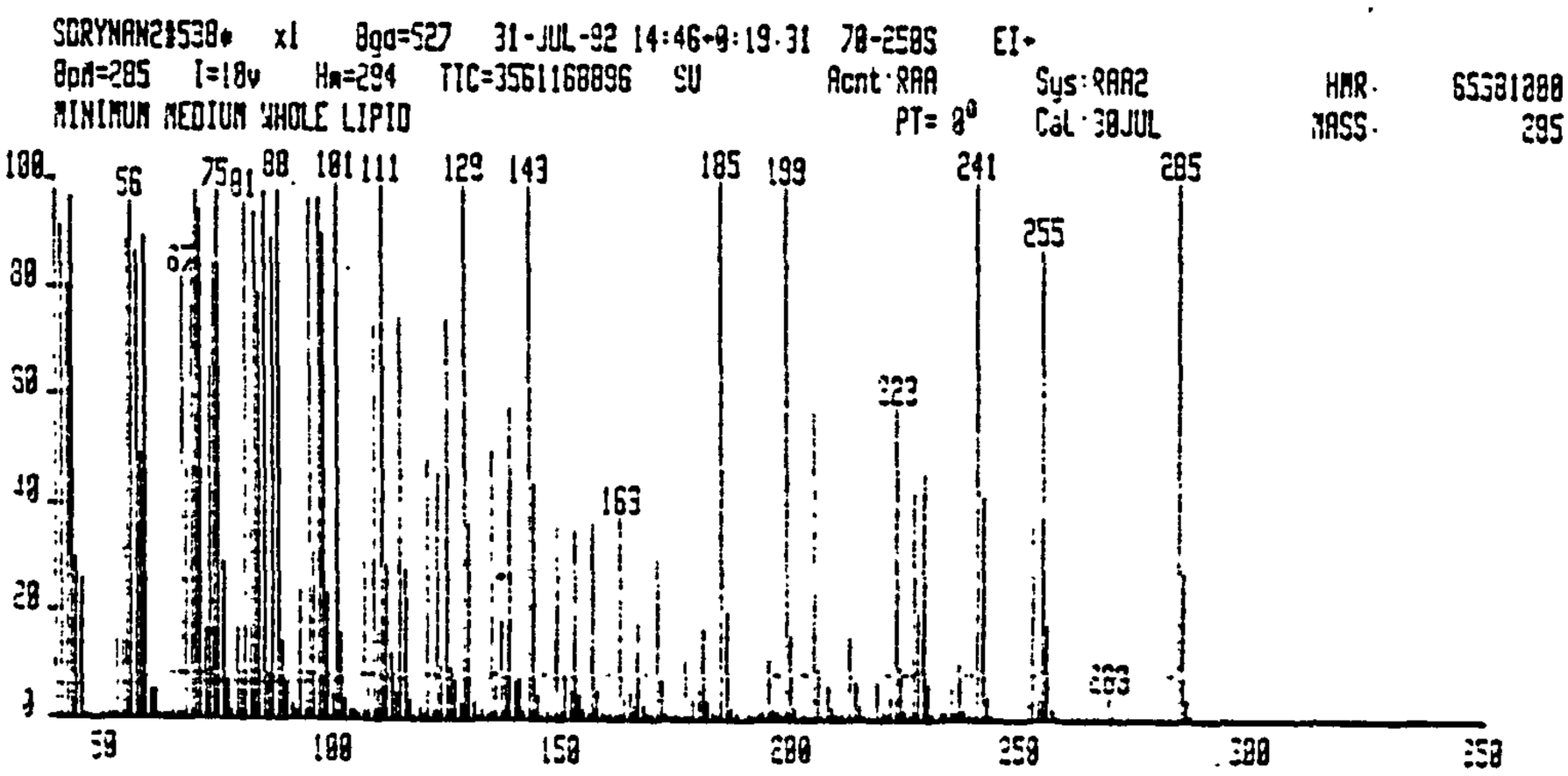
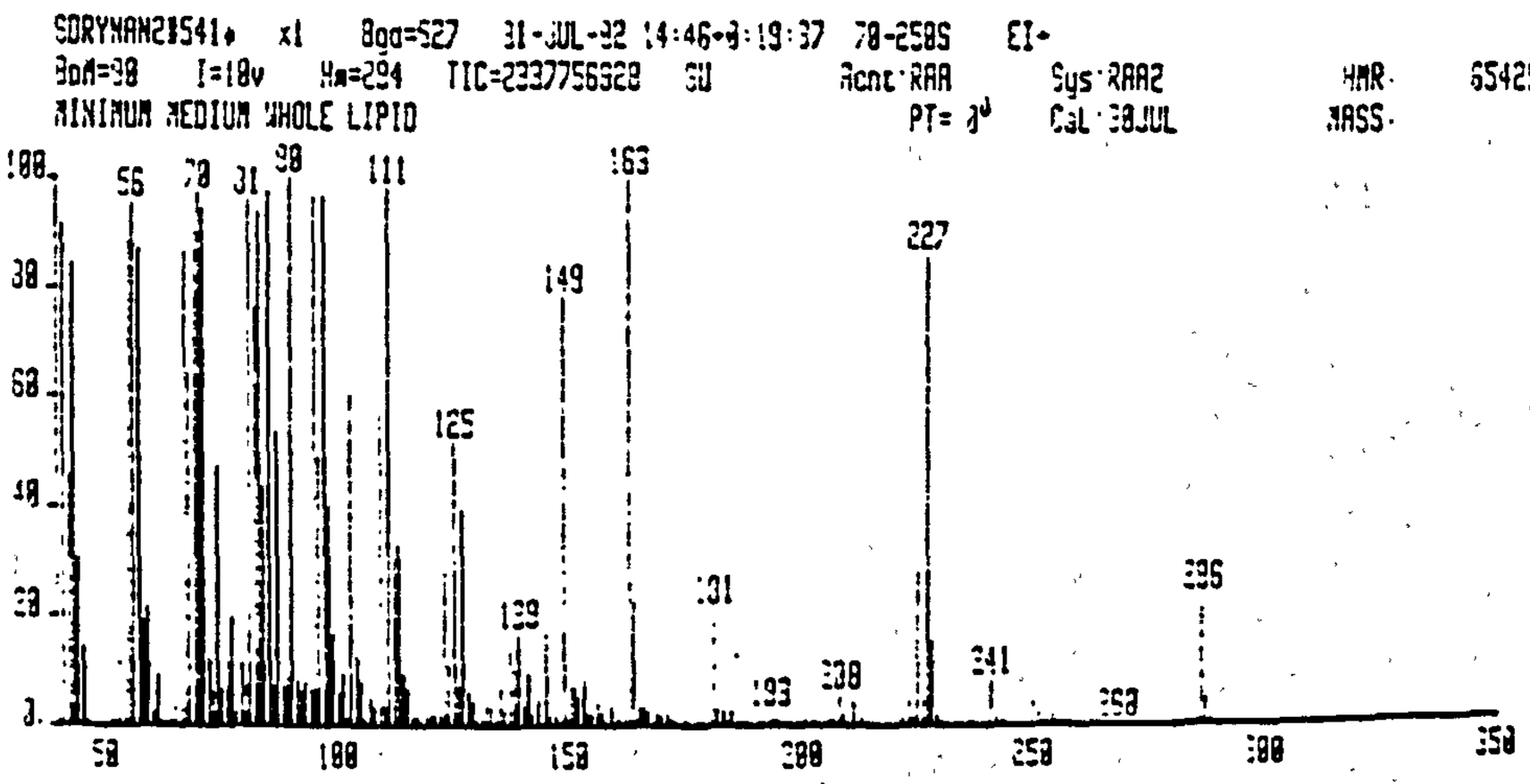
50 100 150 200 250 300 350

(m)


SDRYNAN2#538◆ x1 Bgd=527 31-JUL-92 14:46:9:19:31 78-2585 EI◆
BpM=285 I=18v Hm=294 TIC=3561168896 SU Acnt:RAA Sys:RAA2 HMR: 65381888
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal:30JUL MASS: 295
100 56 75 91 88 181 111 129 143 185 199 241 285 255 223 163 283
50 100 150 200 250 300 350

(n)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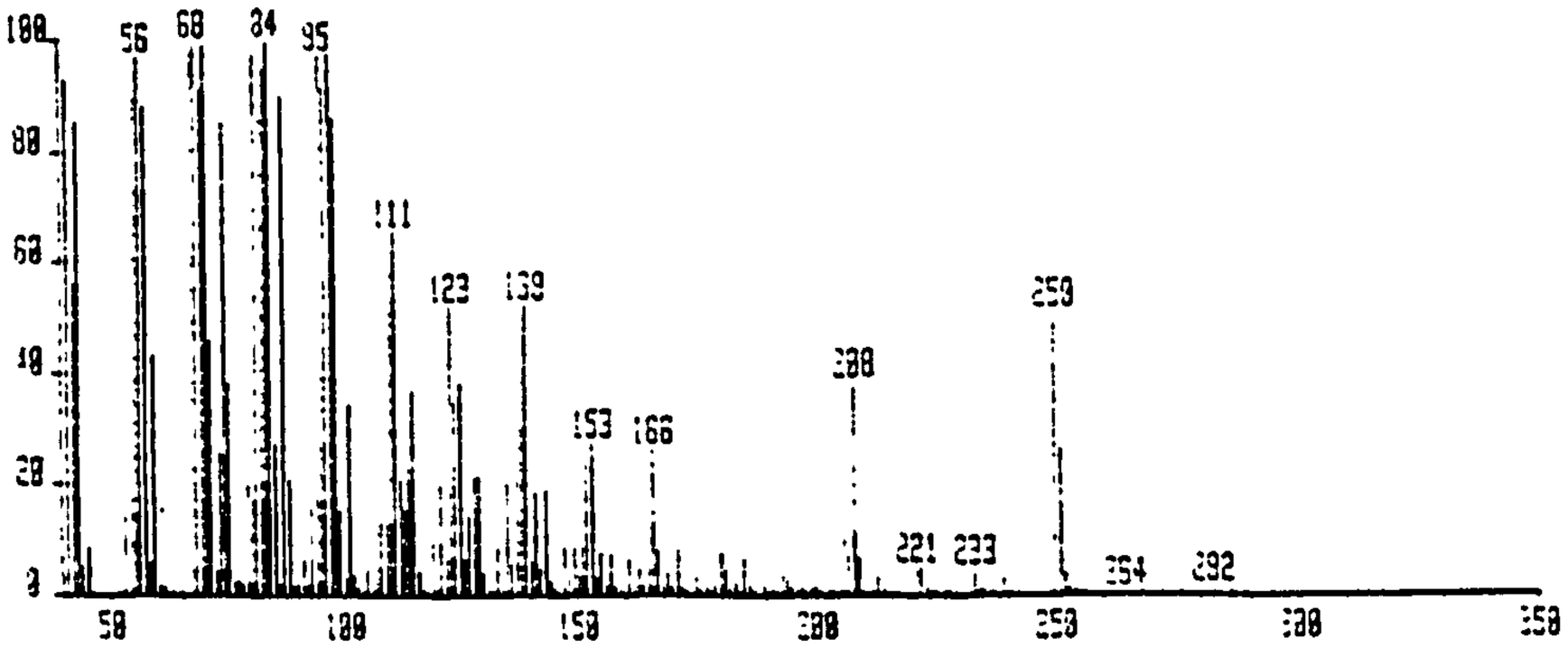
BpM=98 I=18v Hm=294 TIC=2337756928 SU Acnt:RAA Sys:RAA2 HMR: 65429888
MINIMUM MEDIUM WHOLE LIPID PT= 0° Cal:30JUL MASS: 98
100 56 78 31 98 111 163 227 149 125 139 131 193 208 241 258 286
50 100 150 200 250 300 350

(o)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BoM=84 I=3.8v Hm=355 TIC=2375486976 SU Acnt:RAA Sys:RAA2 HMR 54361088
MINIMUM MEDIUM WHOLE LIPID PT= d⁰ Cal 30JUL MASS 94
100
80
60
40
20
0
56
68
84
95
111
123
139
153
166
208
221
233
254
282
50
100
150
200
250
300
350

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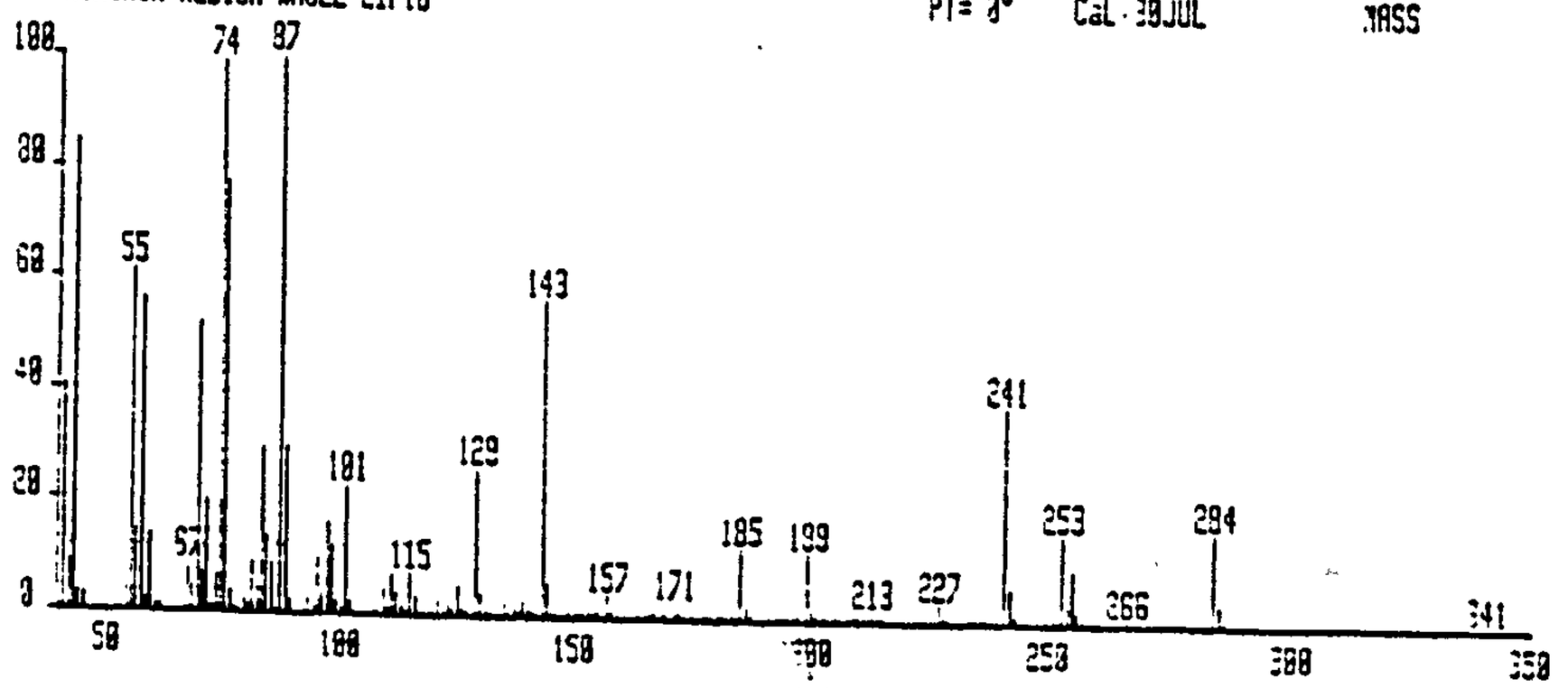
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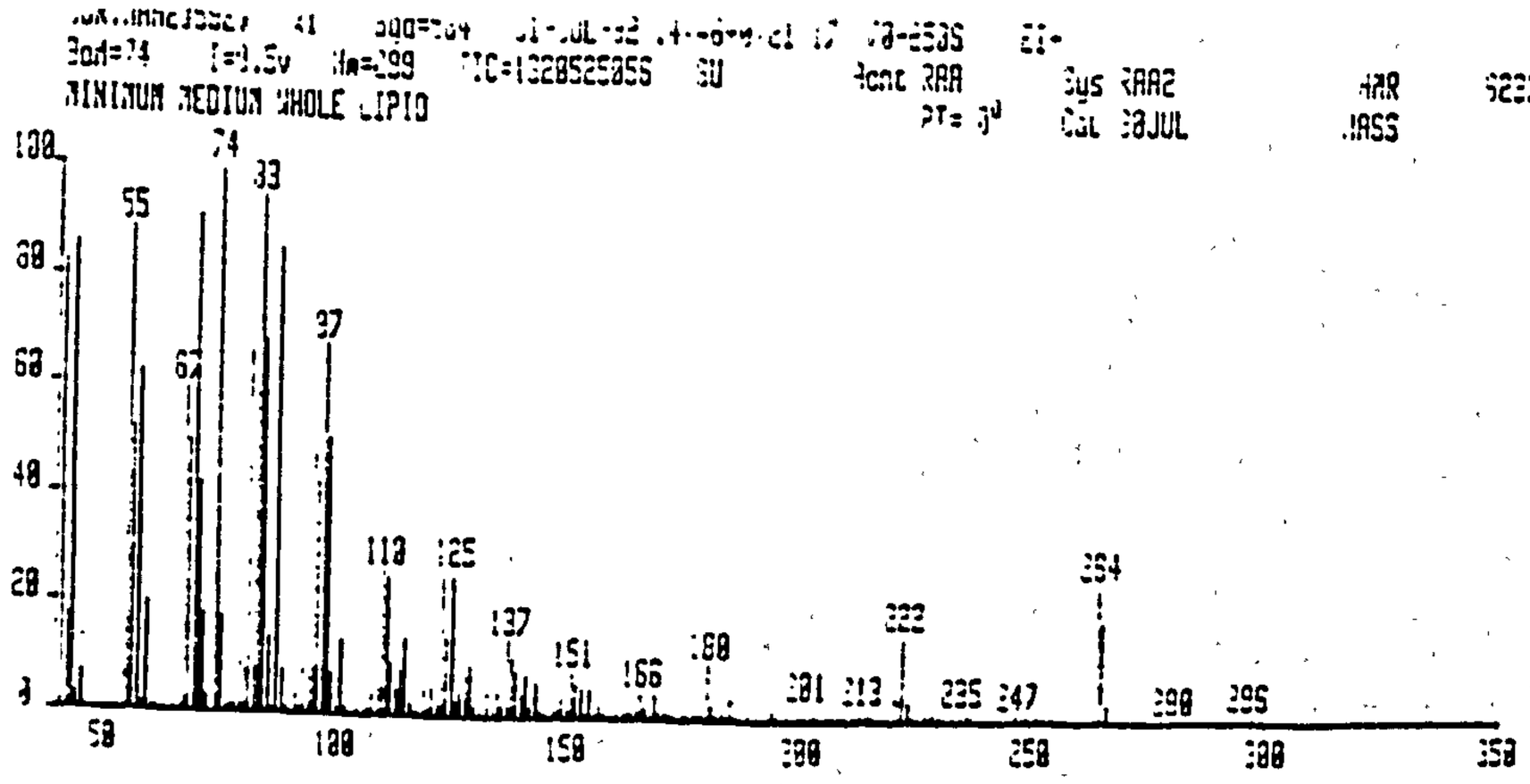
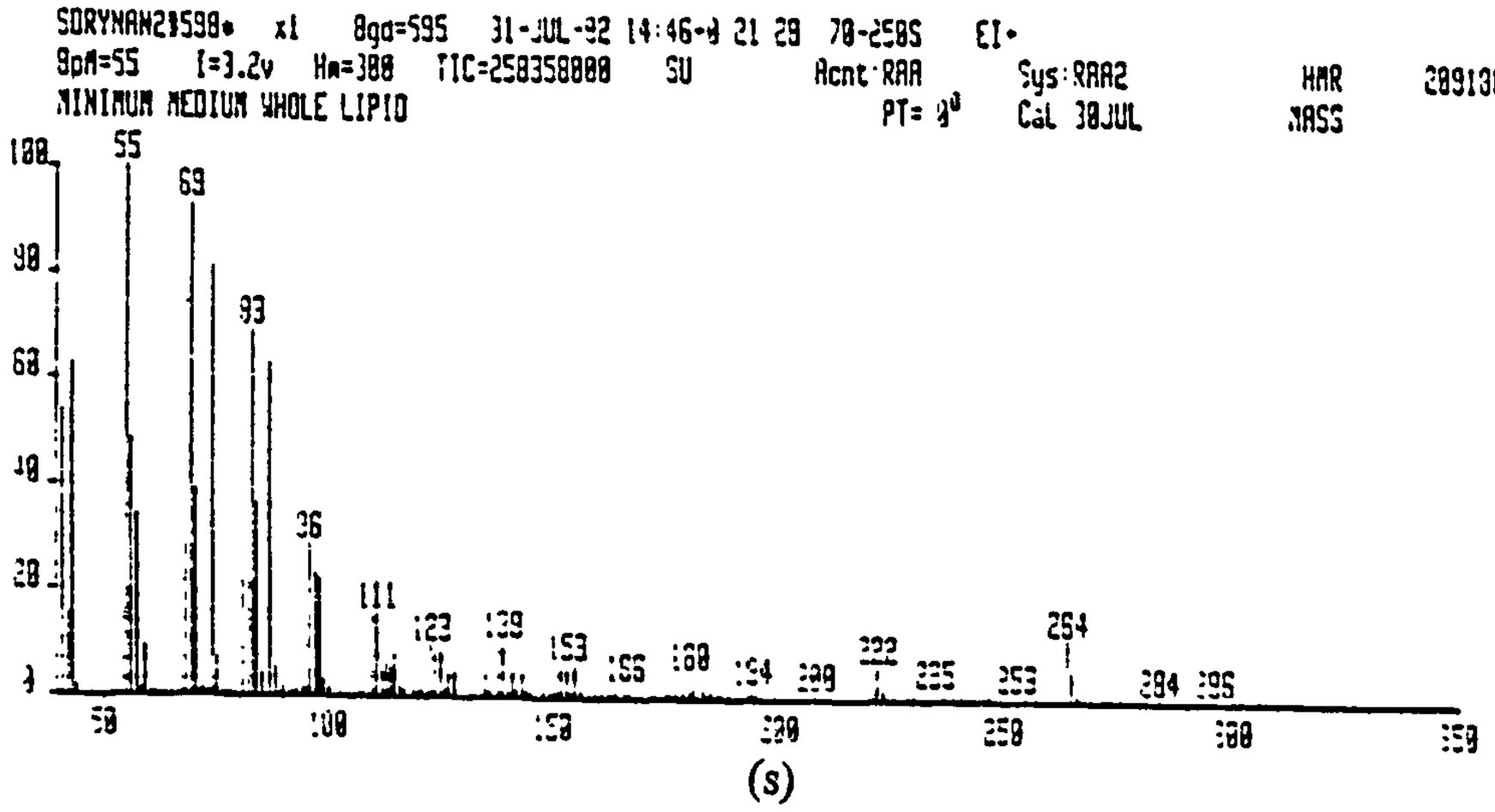
BoM=87 I=3.5v Hm=355 TIC=756838816 SU Acnt:RAA Sys:RAA2 HMR: 63847888
MINIMUM MEDIUM WHOLE LIPID PT= d⁰ Cal 30JUL MASS 87
100
80
60
40
20
0
55
74
87
97
101
115
129
143
157
171
185
199
213
227
241
253
266
294
341
50
100
150
200
250
300
350

(q)


100
80
60
40
20
0
55
74
83
97
110
125
137
151
166
180
208
222
235
247
264
290
295
50
100
150
200
250
300
350

(r)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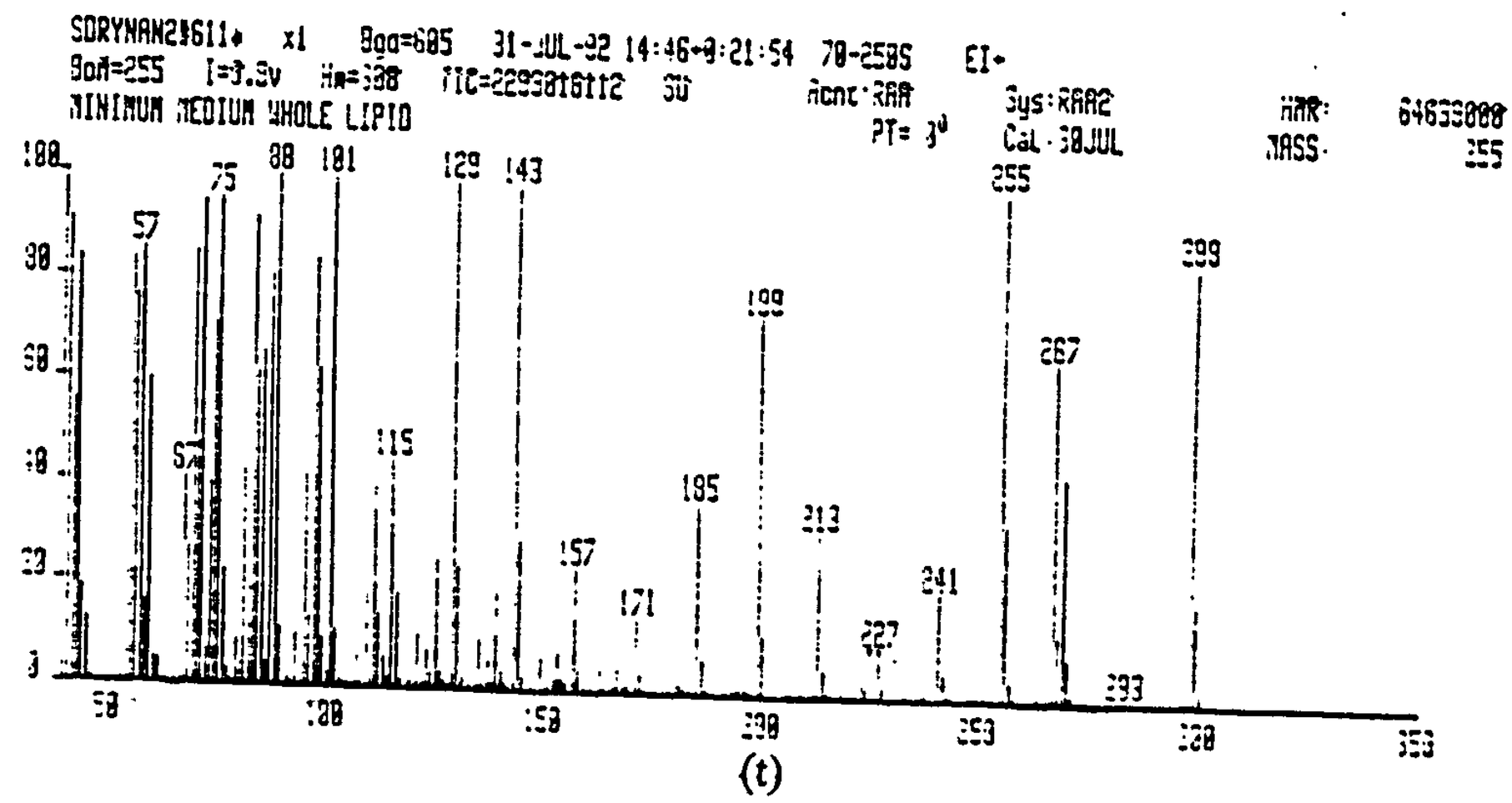
MINIMUM MEDIUM WHOLE LIPID PT= 9⁰ Cal 30JUL MASS 55
55
69
93
36
111
123
139
153
155
168
194
209
222
235
253
264
284
295
(s)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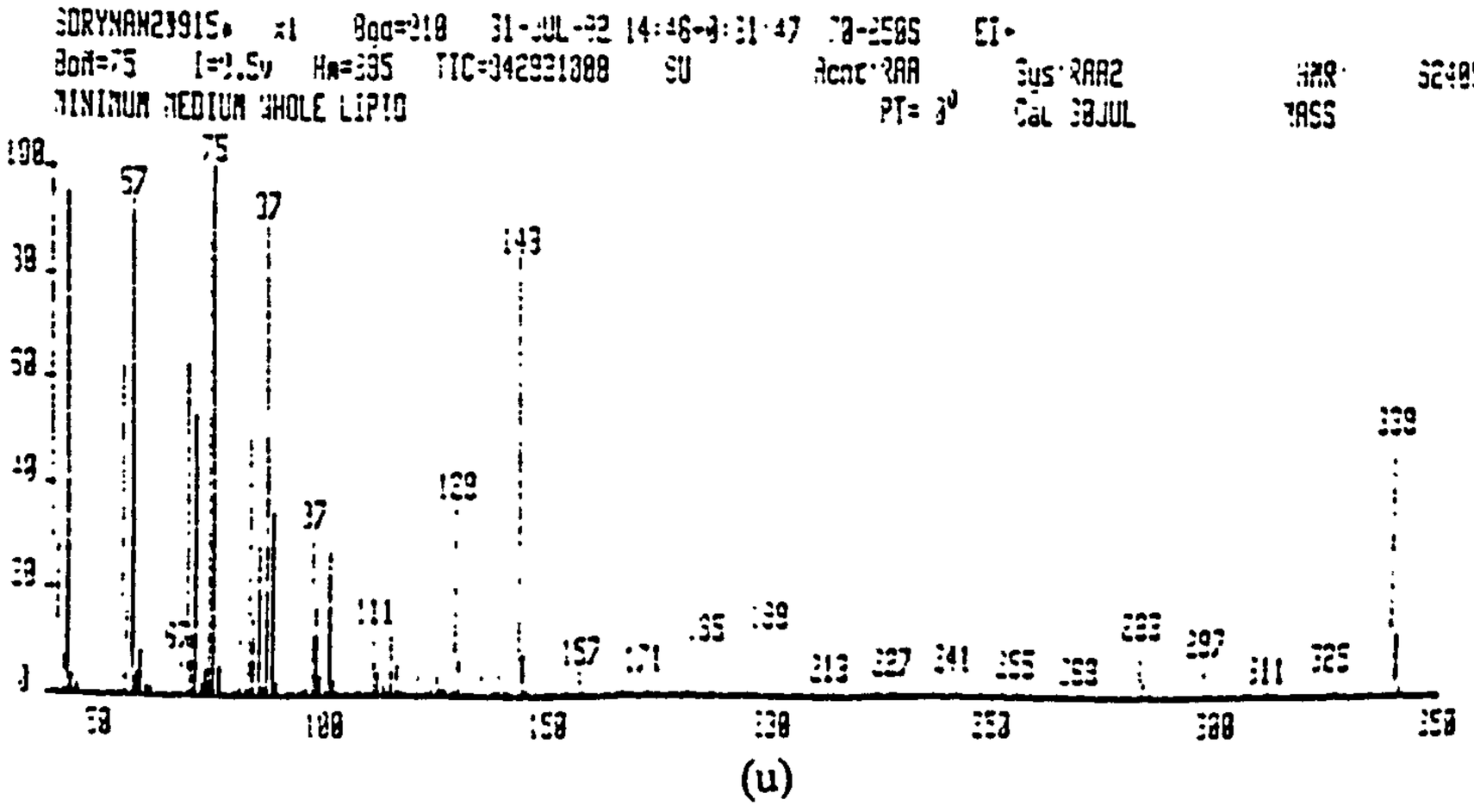
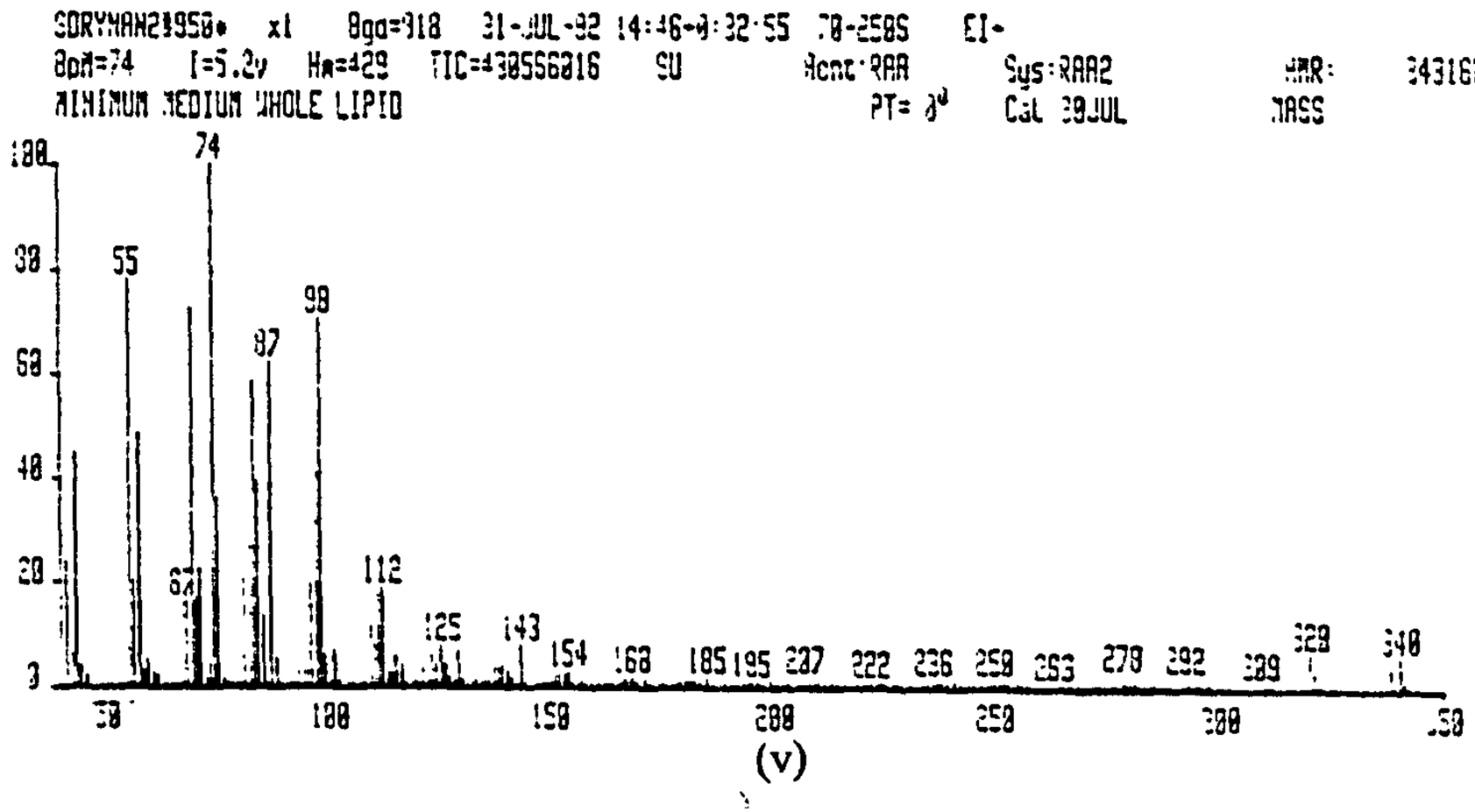
BpM=255 I=3.3v Hm=388 TIC=2293816112 SU Acnt:RAA Sys:RAA2 HMR: 64633888
MINIMUM MEDIUM WHOLE LIPID PT= 9⁰ Cal 30JUL MASS 255
75
88
101
129
143
255
299
57
189
267
51
115
185
213
157
171
227
241
283
(t)


SORYNAN2#915* x1 Bgd=218 31-JUL-92 14:46-9:31:47 70-2585 EI*
BpM=75 I=2.5v Hm=335 TIC=342831088 SU Acnt:RAA Sys:RAA2 HMR: 52485888
MINIMUM MEDIUM WHOLE LIPID PT= 9⁰ Cal 30JUL MASS 75
75
57
37
143
339
111
139
157
171
185
213
227
241
255
283
297
311
325
(u)


SORYMAN21958* x1 Bgd=318 31-JUL-92 14:46-9:32:55 78-2585 EI-
BpM=74 I=5.2v Hm=429 TIC=430556816 SU Acnt:RAA Sys:RAA2 AMR: 34316800
MINIMUM MEDIUM WHOLE LIPID PT= 0 Cal 29JUL MASS 74
100
80
60
40
20
0
74
55
98
87
62
112
125
143
154
168
185
195
207
222
236
250
263
278
292
309
328
340
50
100
150
200
250
300
350

(v)

Table 5.6 SUMMARY OF THE FATTY ACIDS PRESENT IN THE WHOLE LIPID EXTRACTED FROM *S.RIMOSUS* CULTURED IN MODIFIED HMM

S.rimosus was cultured in modified HMM (Methods 2.3.1 (b) and 2.5). Whole lipid fatty acid methyl esters from the biomass were prepared and run on GC-MS (Methods 2.23-2.30). This table provides a summary of the fatty acid identified (for mass spectra see Fig. 5.9), by comparison with known standards (see Table 5.4) and their identifying peaks in the mass spectra (Methods 2.30.1). The peak number for each fatty acid refers to the GC traces (see Fig. 5.8).). Where the symbol '>' is used it means that one peak is greater in height than another and where the symbol '=' is used it means that the peaks are of equal height. The symbols used to define types of fatty acids are: i - iso fatty acid, a - anteiso fatty acid, 2-OH - fatty acid containing a methyl group on carbon 2, 3-OH as before except the hydroxyl group is on carbon 3, ^{9,11,12} denote the position of the double bonds and Δ denotes a fatty acid containing a cyclopropane ring.

Peak no.		Fatty acid	M+	Characteristic peaks					
160	Minor	10 C	202	74	M-59				
341	Major	14:0	242	74	M-31	M-43	M-57		
364	Minor	14:0	242	74	M-31	M-43	M-57		
408	Major	i15:0	256	74	M-29=M-31	M-43			
413	Major	a15:0	256	74	M-29	M-43	M-57	M-61	M-79
429	Minor	15:0	256	74	M-31	M-43	M-57		
454	Minor	x	x	no 74					
475	Major	i16:0	270	74	M-29>M-31		M-43	M-57	
479	Major	2-OH 15:0	272	90	103	M-59			
484	Major	2-OH 15:0	272	90	103	M-32	M-59		
495	Major	16:0	270	74	M-31	M-43	M-57		
513	Minor	17:1	282	69>74	55	M-32	M-74	M-116	
520	Minor	17:1	282	74	M-32	M-74	M-116		
532	Major	i17:0	284	74	M-29	M-43	M-57		
538	Major	a17:0	285*	74	M-29	M-43	M-61	M-79	
541	Major	2-OH 16:0	286	74	90	M-59			
543	Major	17:1	282	74	M-32	M-74	M-116		
552	Minor	17:0	284	74	M-31	M-43	M-57		
582	Minor	x	x	no 74					
592	Major	18:1	296	74	55	M-32	M-74	M-116	
598	Minor	18:1	296	69>74	55	M-32	M-43	M-74	M-116
603	Minor	x	x	no 74					
611	Major	18:0	298	74	M-31	M-43	M-57		
915	Minor	methyl 21:0	339*	74	M-29	M-43	M-57		
950	Minor	21:0	340	74	M-31				

Further analysis of the individual spectra from the whole lipid extracted from the YEME grown biomass revealed the presence of methyl branched-chain fatty acids; methyl 15:0 (peak identification number 410), methyl 17:0 (peak identification number 532) and methyl 18:0 (peak identification number 591) but from the spectra it was difficult to determine which carbon the branches occurred on.

5.5 THE FATTY ACID PROFILE FROM *S. RIMOSUS* GROWN UNDER NITROGEN LIMITING CONDITIONS

5.5.1 DEVELOPMENT OF NITROGEN LIMITED GROWTH CONDITIONS

The key to lipid accumulation is to allow growth to occur under nitrogen limited conditions with the carbon source in excess (Ratledge, 1982). Therefore a number of different carbon/ nitrogen ratios in the growth media were investigated as potential nitrogen limiting conditions. *S. rimosus* was grown in 500 ml, aerated flasks (Methods 2.5), containing 200 ml of modified HMM with the following carbon and nitrogen concentrations:

(a) 10 mM glucose, 20 mM ammonium sulphate.

(b) 10 mM glucose, 2 mM ammonium sulphate.

(c) 20 mM glucose, 2 mM ammonium sulphate.

(d) 40 mM glucose, 2 mM ammonium sulphate.

5 ml samples were taken from each flask at various time intervals. Growth was followed by measuring optical density (Methods 2.6). Nitrogen concentrations were estimated by measuring ammonia concentration (Methods 2.20). Glucose (see Methods 2.19) was present at stationary phase in both the 20 mM and 40 mM glucose containing flasks. In all the flasks *S. rimosus* grew with a MGT of approximately 3 h. The growth and nitrogen concentration curves are shown in Fig. 5.10. Condition (d) was chosen as the condition best likely to promote nitrogen limited stationary phase.

5.5.2 GROWTH UNDER NITROGEN LIMITING CONDITIONS

S. rimosus was grown in a 10 l aerated flask, containing modified HMM, with 40 mM glucose and 2 mM ammonium sulphate. 1 ml samples were taken at frequent intervals for measurements of optical density, ammonia and glucose concentrations. Six 500 ml samples were taken; one during logarithmic growth, one at the end of logarithmic growth, and four during stationary phase. Fig. 5.11 follows growth and ammonia concentration. Arrows indicate where the 500 ml samples were taken [16 h 20 min (1), 26 h 35 min (2), 34 h 10 min (3), 55 h 10 min (4), 64 h 45 min (5) and 78 h 45 min (6)]. Lipid samples were extracted from the harvested biomass at each of these time points.

5.5.3 ANALYSIS OF THE LIPID CONTENT AND COMPOSITION

The concentration of lipid/ 100 mg dry weight biomass obtained at each time point are shown in Table 5.7. There does not appear to be any increase in lipid concentration, during growth and stationary phase, under nitrogen limiting conditions. Whole lipid, phospholipid containing and triglyceride spots from thin-layer chromatography plates were converted into fatty acid methyl esters and analysed by gas-liquid chromatography. Tables 5.8 (a), (b) and (c) give the percentages of the main fatty acids present, detected by GLC, in each of these fractions.

In the whole lipid i16:0 is the most predominant fatty acid present, although the percentage present does start to decline by the time sample was taken at 78 h and 45 min. Throughout the exponential growth phase and early stationary phase (sample numbers 1, 3 and 4) the proportions and concentrations of a15:0, 16:0, i17:0 and i15:0 remain relatively constant. Late on in stationary phase the concentrations of a15:0, i17:0 and i15:0 begin to decline but still remain present in the same 1:1 proportions. The concentration of 16:0 remains constant throughout exponential growth and stationary phase. The unidentified peak which appears at approximately 44 min increases in concentration with time. In the triglyceride fraction there are a large

number of unidentified peaks and the peak which occurs at approximately 44 min is always present at the highest concentration. The major peaks identified include i17:0; 20:0; 18:0; 19:0Δ; $18:1^{9}$ (double bond is at carbon 9) $18:1^{11}$ and $18:1^{9/12}$ Interestingly cyclopropane fatty acids 17:0Δ and 19:0Δ appear. 19:0Δ is a fairly major fatty acid by late stationary phase. Interestingly no 15 or 16 carbon chain length fatty acids occur as the major fatty acids in the triglyceride fraction.

In the phospholipid containing fraction i16:0 is the major fatty acid present until late stationary phase. In late stationary phase a change occurs which results in a major alteration in the fatty acids present in the phospholipid fraction. As with the triglyceride fraction the unidentified peak occurring at approximately 44 min is predominant especially so towards late stationary phase.

5.6 ANALYSIS OF LIPIDS IN *S. RIMOSUS* UNDER PHOSPHATE LIMITING CONDITIONS

5.6.1 ESTABLISHING PHOSPHATE LIMITING GROWTH CONDITIONS

Lipid accumulation by phosphate deprivation has been reported (Gill *et al.*, 1977)). Therefore a number of carbon/phosphate ratios in the growth media were investigated as potential phosphate limiting condition. *S. rimosus* was grown in 500 ml, aerated flasks (Methods 2. 5), containing 200 ml of modified HMM, 40 mM glucose and the following phosphate concentrations:

(a) 15 mM

(b) 1.5 mM

(c) 0.15 mM

(d) 0.07 mM

5 ml samples were taken from each flask at various time intervals. Growth was followed by measuring optical density (Methods 2.6). Phosphate concentrations were measured (Methods 2.21). Glucose (see Methods 2.19)was present throughout growth and

stationary phase. The growth curves are shown in Fig. 5.12. Phosphate concentrations at time zero and 71 h, glucose concentrations at 71 h and mean generation times are listed in Table 5.9. Condition (d) was chosen as the condition best likely to promote phosphate limited growth because growth occurred at a reasonable rate in media containing an excess of carbon and a limited supply of phosphate.

5.6.2 GROWTH OF *S. RIMOSUS* UNDER PHOSPHATE LIMITED CONDITIONS

S. rimosus was grown in a 10 l aerated flask, containing modified HMM, with 40 mM glucose, 0.07 mM phospate and 1.5 mM glucose. 1 ml samples were taken at frequent time intervals for measurements of optical density at 500 nm. Four 500 ml samples were taken; one during exponential growth, one during early stationary phase and two during late stationary phase. Fig. 5.13 follows the growth curve and arrows indicate when the 500 ml samples were taken. Lipids were extracted from the biomass harvested from each of these sample. The concentration of lipid/ 100 mg dry weight biomass are shown in Table 5.10. There does not appear to be any increase in lipid concentration, during growth and stationary phase, under phosphate limited conditions.

Fig. 5.10 DEVELOPMENT OF THE CONDITIONS WHICH WOULD ALLOW NITROGEN LIMITED GROWTH OF *S. RIMOSUS*

S. rimosus was grown in 500 ml aerated flasks (Methods 2.3.1(b)). The flasks contained 200 ml of modified HMM and one of the following:

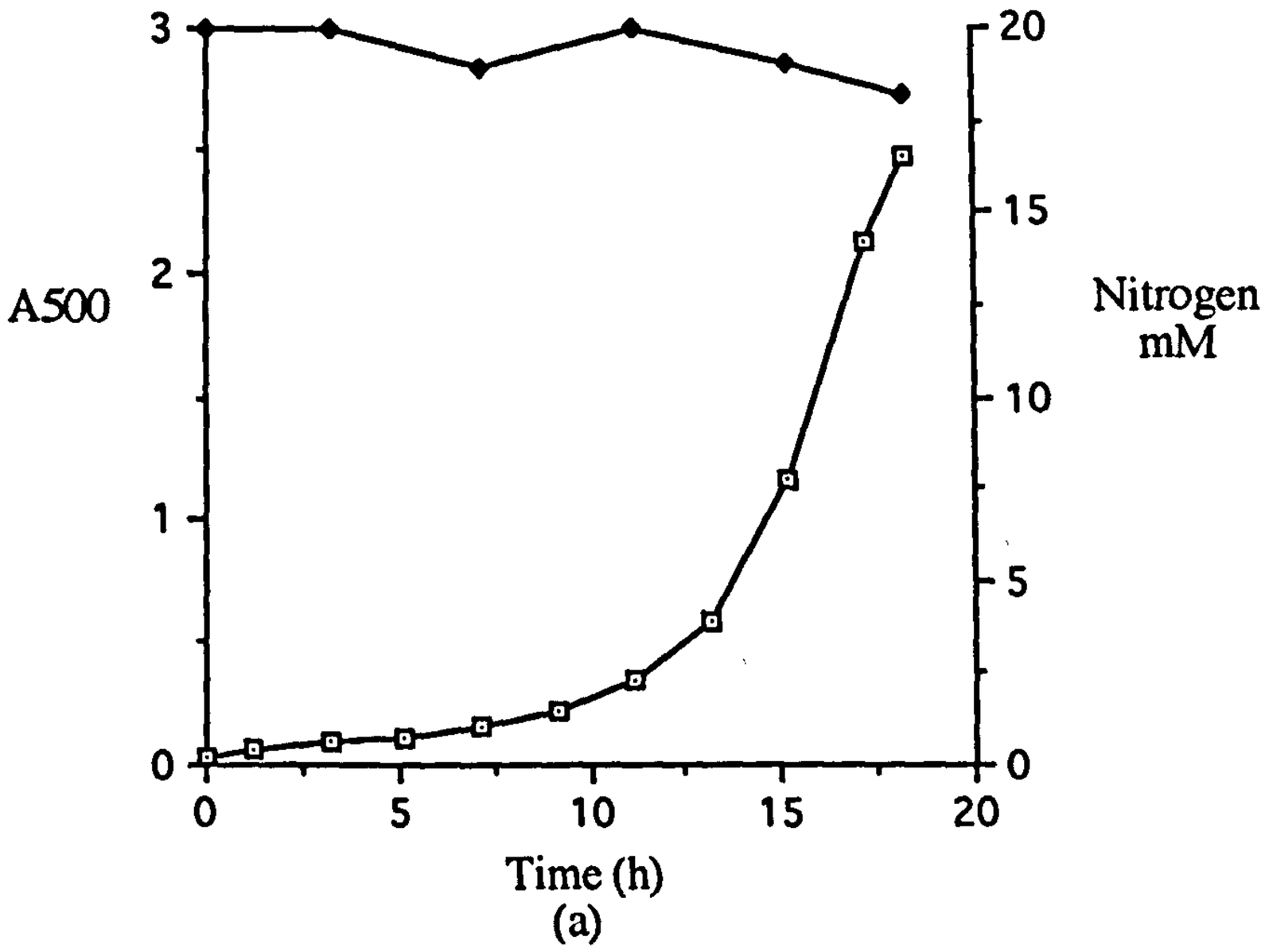
(a) 10 mM glucose, 20 mM ammonium sulphate

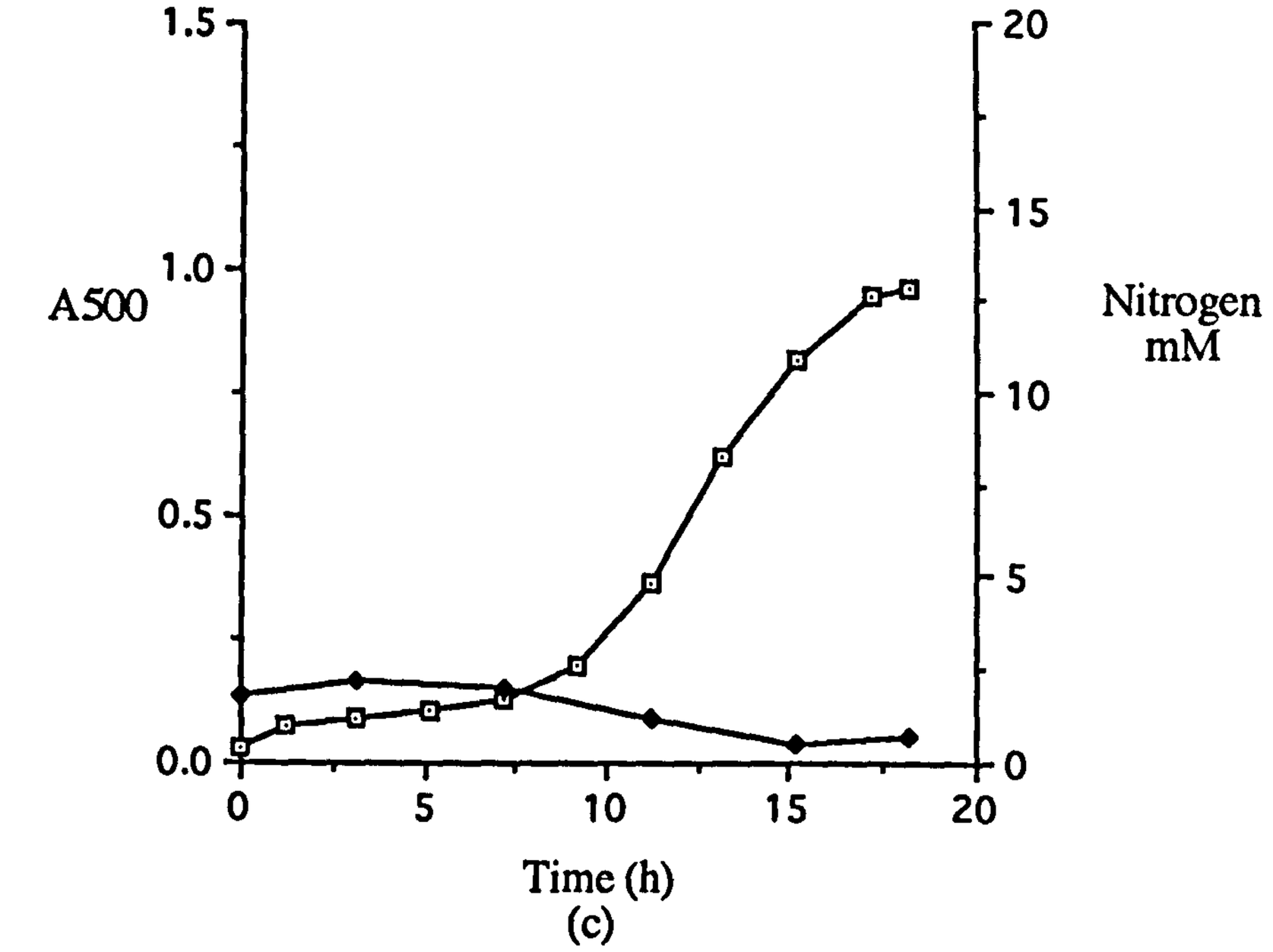
(b) 10 mM glucose, 2 mM ammonium sulphate

(c) 20 mM glucose, 2 mM ammonium sulphate

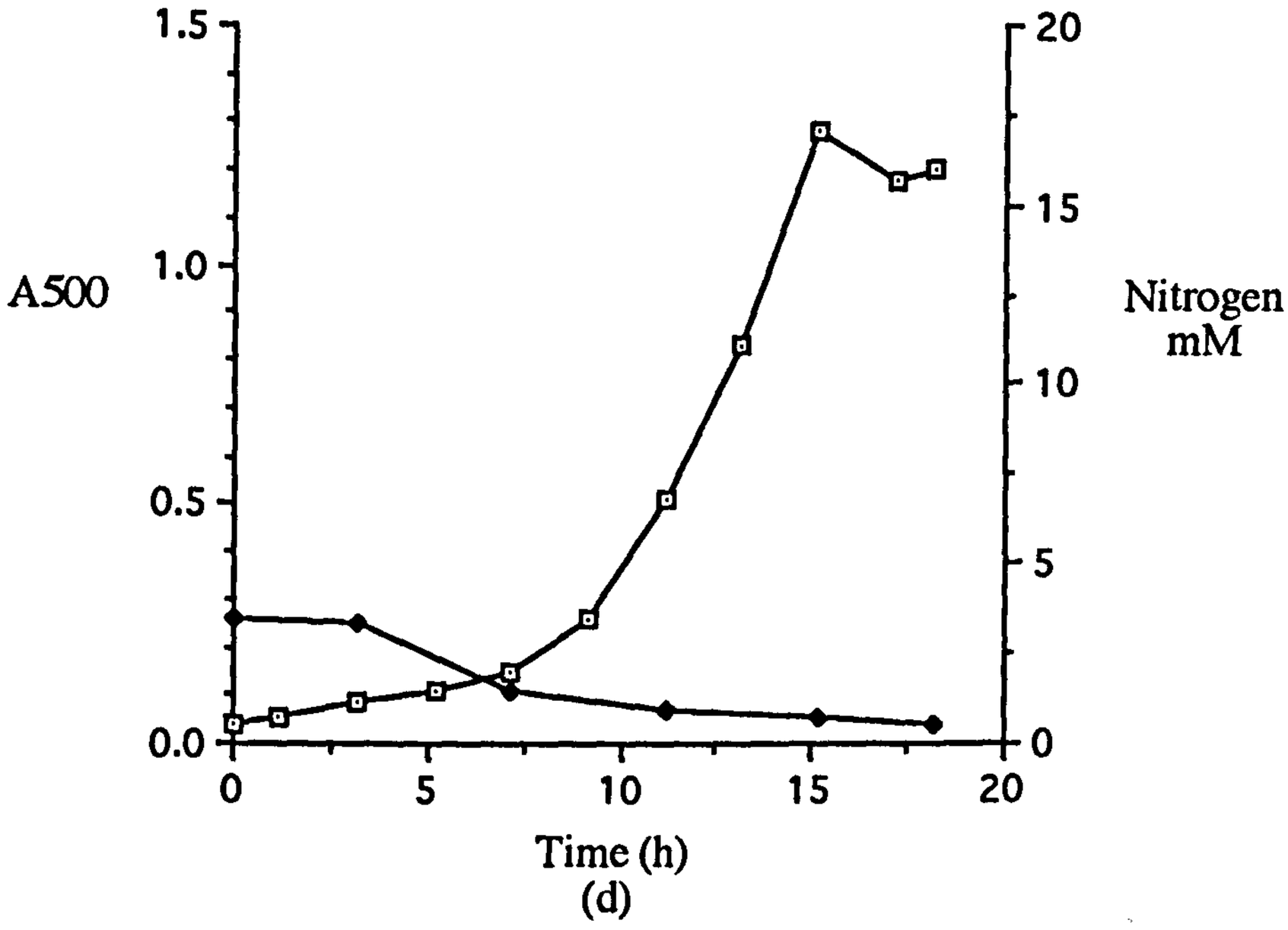
(d) 40 mM glucose, 2 mM ammonium sulphate

Growth was followed by measuring optical density at A500 (Methods 2.6) and nitrogen concentrations were estimated by measuring ammonia concentrations (Methods 2.20). The figures (a) - (d) give the growth curves (-o-) and nitrogen concentrations (-•-) corresponding to the above growth conditions ([a] - [d]).


A500
Nitrogen mM
Time (h)
(a)
A500
Nitrogen mM
Time (h)
(b)


1.5
1.0
0.5
0.0
A500
20
15
10
5
0
Nitrogen
mM
0
5
10
15
20
Time (h)

(c)


1.5
1.0
0.5
0.0
A500
20
15
10
5
0
Nitrogen
mM
0
5
10
15
20
Time (h)

(d)

Fig. 5.11 GROWTH OF *S. RIMOSUS* UNDER NITROGEN LIMITED CONDITIONS

S. rimosus was grown under the nitrogen limiting conditions developed in 5.4.1.

S. rimosus was grown in a 10 l flask (Methods 2.5), containing modified HMM, with 40 mM glucose and 2 mM ammonium sulphate present. Growth (□) was followed by measuring optical density at A500 (Methods 2.6) and nitrogen concentrations (mM) (-●) were estimated by measuring ammonia concentrations (Methods 2.20). Arrows indicate the time points where 500 ml samples were taken:

Sample no.	Time
1	16 h 20 min
2	26 h 35 min
3	34 h 10 min
4	55 h 10 min
5	64 h 45 min
6	78 h 45 min

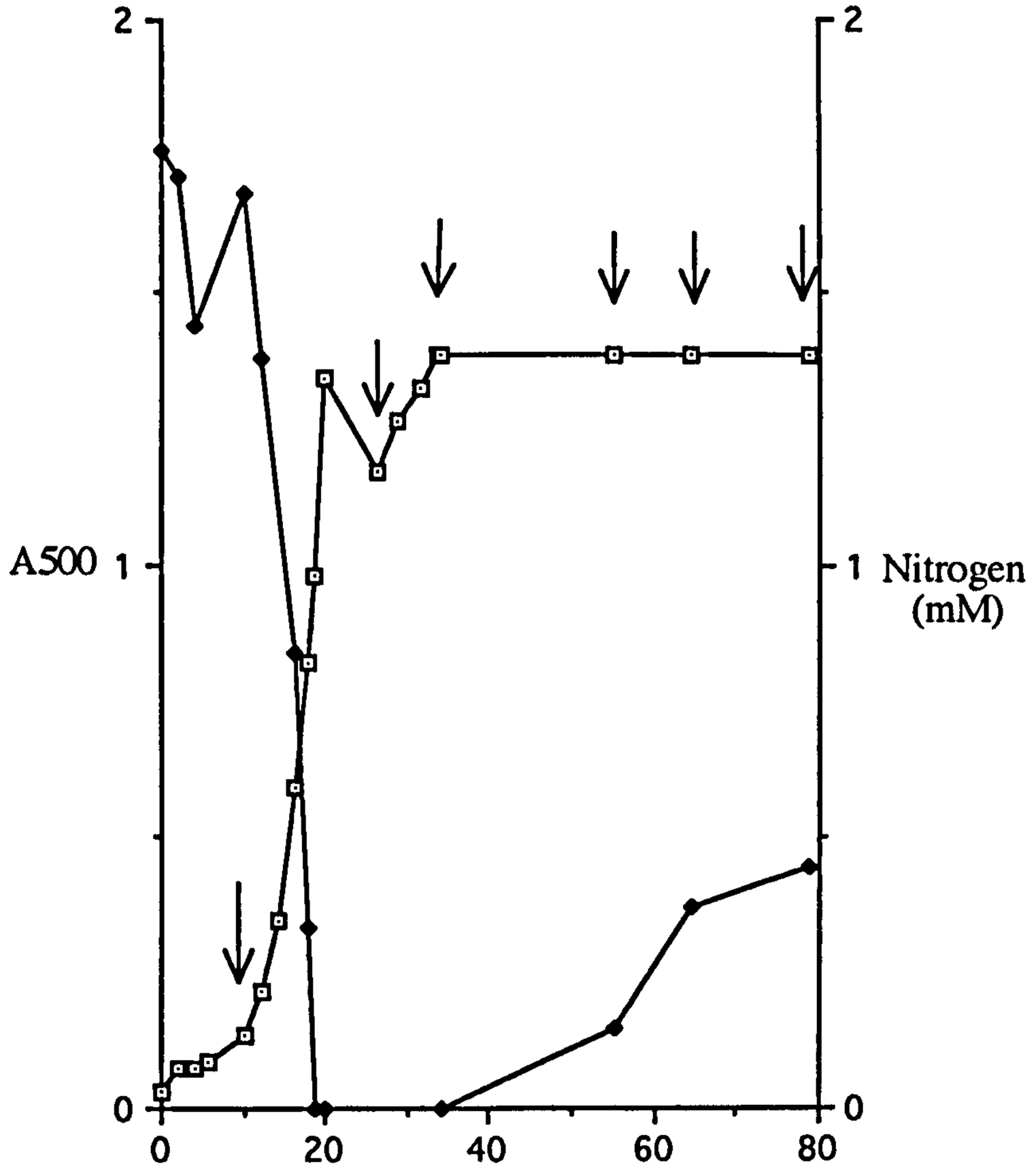

2
2
A500
1
1
Nitrogen
(mM)
0
0
0
20
40
60
80
Time (h)

Table 5.7 THE CONCENTRATIONS OF LIPID EXTRACTED FROM *S.RIMOSUS* GROWN UNDER NITROGEN LIMITED CONDITIONS

S.rimosus was cultured (Methods 2.5) under the nitrogen limited conditions developed in 5.5.1. Lipid was extracted from the biomass (Methods 2.23) and the concentrations (mg lipid/ 100 mg dry weight biomass) are listed in this table for each of the six samples taken throughout growth.

Sample no.	Growth phase	Time (h)	Lipid content mg lipid/100mg biomass[dry wgt.])
1	Exponential	16.3	7.9
2	Late Exponential	26.5	6.2
3	Early Stationary	34.2	3.3
4	Stationary	55.2	7.9
5	Stationary	66.8	8.0
6	Stationary	78.8	5.3

Table 5.8 THE PERCENTAGES OF THE MAIN FATTY ACIDS PRESENT IN THE LIPID FRACTIONS FROM *S. RIMOSUS* GROWN UNDER NITROGEN LIMITED CONDITIONS

S.rimosus was cultured (Methods 2.5) under the nitrogen limited conditions developed in 5.5.1. Six samples were taken throughout growth Lipid was extracted from the biomass and the whole lipid triglyceride and phospholipid fractions were converted into fatty acid methyl esters and run on GLC (Methods 2.23-2.26). The percentages of the main fatty acids found in each of these fractions are listed in the following tables (results are given as % by weight of weight of total fatty acid methyl esters):

(a) whole lipid

(b) phospholipid containing

(c) triglyceride

Sample times are listed below:

Sample no.	Time
1	16 h 20 min
2	26 h 35 min
3	34 h 10 min
4	55 h 10 min
5	64 h 45 min
6	78 h 45 min

Sample 1	%	Sample 3	%	Sample 4	%	Sample 5	%	Sample 6	%
i16:0	24.5	i16:0	25.8	i16:0	22.5	i16:0	19.2	i16:0	15.3
a15:0	8.8	a15:0	11.5	16:0	10.9	16:0	10.9	16:0	11.9
16:0	8.8	i15:0	10.0	a15:0	8.1	43.04	9.2	43.14	11.0
i17:0	8.7	16:0	9.7	i17:0	8.1	a15:0	6.6	18:0	5.9
18:0	8.5	i17:0	9.2	43.32	6.7	i17:0	6.5	i17:0	5.2
i15:0	5.0	18:1'	6.0	33.39	6.0	18:0	4.6	a15:0	5.0
26.29	5.0	22.34	5.7	i15:0	4.8	i15:0	4.1	18:1'	4.5
18:1'	4.2	26.18	5.1	18:0	4.7	18:1'	3.5	i15:0	4.1
16:1'	3.4	18:0	4.4	26.20	4.6	26.04	3.5	19:0	3.8
43.25	3.3	16:1'	3.3	18:1	4.0	17:0Δ	3.2	20:0	3.7

(a) whole lipid

Sample 1	%	Sample 2	%	Sample 3	%	Sample 4	%	Sample 5	%	Sample 6	%
i16:0	14.8	i16:0	27.5	i16:0	26.0	i16:0	25.7	43.24	21.7	18:2[9,12]	19.5
43.34	14.2	43.25	16.2	i17:0	9.2	42.23	9.4	i16:0	17.0	20.02	19.0
42.91	8.6	16:0	11.9	a15:0	8.6	i17:0	9.1	27.58	6.9	18:1[9]	14.1
32.96	8.2	i17:0	9.5	43.38	8.5	16:0	8.0	i17:0	6.6	16:0	11.9
16:0	7.3	a15:0	7.6	16:0	7.6	a15:0	7.5	18:1[9], 18:1[11]	5.7	14:0	11.3
27.6	6.5	27.59	5.8	26.24	5.8	26.14	5.8	a15:0	4.1	2-OH 12:0	9.8
i17:0	4.6	32.93	5.6	i15:0	5.4	i15:0	4.7	26.13	4.1	i16:0	7.9
18:1[9]	4.6	18:0	5.5	18:0	3.7	16:1[9]	3.7	18:0	3.7		
18:0	4.3	i15:0	5.2	16:1[9]	3.5	18:0	3.2	32.24	3.6		
i15:0	3.2	16:1[9]	4.4	18:1[9]	2.5	18:1[9]	3.2	16:1[9]	2.8		

(b) phospholipid

Sample 1	%	Sample 2	%	Sample 3	%	Sample 4	%	Sample 5	%	Sample 6	%
43.95	11.9	43.1	22.2	43.97	19.2	18:0	25.4	43.87	19.8	43.75	16.4
39.26	8.9	18:1, 18:1	8.4	18:0	9.3	18:1'	19.5	19:0Δ	11.2	i17:0	13.0
18:0	8.0	18:0	8.0	i17:0	9.2	43.3	19.3	20:0	6.4	35.86	11.1
39.04	7.7	19:0	7.2	19:0	8.2	$18:2^{9,12}$	11.2	43.45	6.0	19:0Δ	10.9
i17:0	6.3	35.15	5.9	36.67	7.4	32.12	9.9	19:0	5.9	35.56	10.8
20:0	5.7	i17:0	5.1	40.49	5.5	i17:0	7.5	32.41	5.8	36.39	8.5
42.19	5.4	17:0Δ	4.8	19:0Δ	5.2	27.63	7.3	35.95	5.7	20:0	8.0
19:0	4.8	12:0	4.6	33.45	4.6			i17:0	5.5	39.12	6.9
19:0Δ	4.4	32.7	4.2	42.45	4.1			40.03	5.4	12:0	4.3
35.8	4.4	19:0Δ	4.1	2-OH 14:0	3.8			26.9	4.8		

(c) triglyceride

Fig. 5.12 DEVELOPMENT OF THE CONDITIONS WHICH WOULD ALLOW PHOSPHATE LIMITED GROWTH OF *S. RIMOSUS*

S. rimosus was grown in 500 ml aerated flasks (Methods 2.3.1(b)). The flasks contained 200 ml of modified HMM and one of the following concentrations of phosphate:

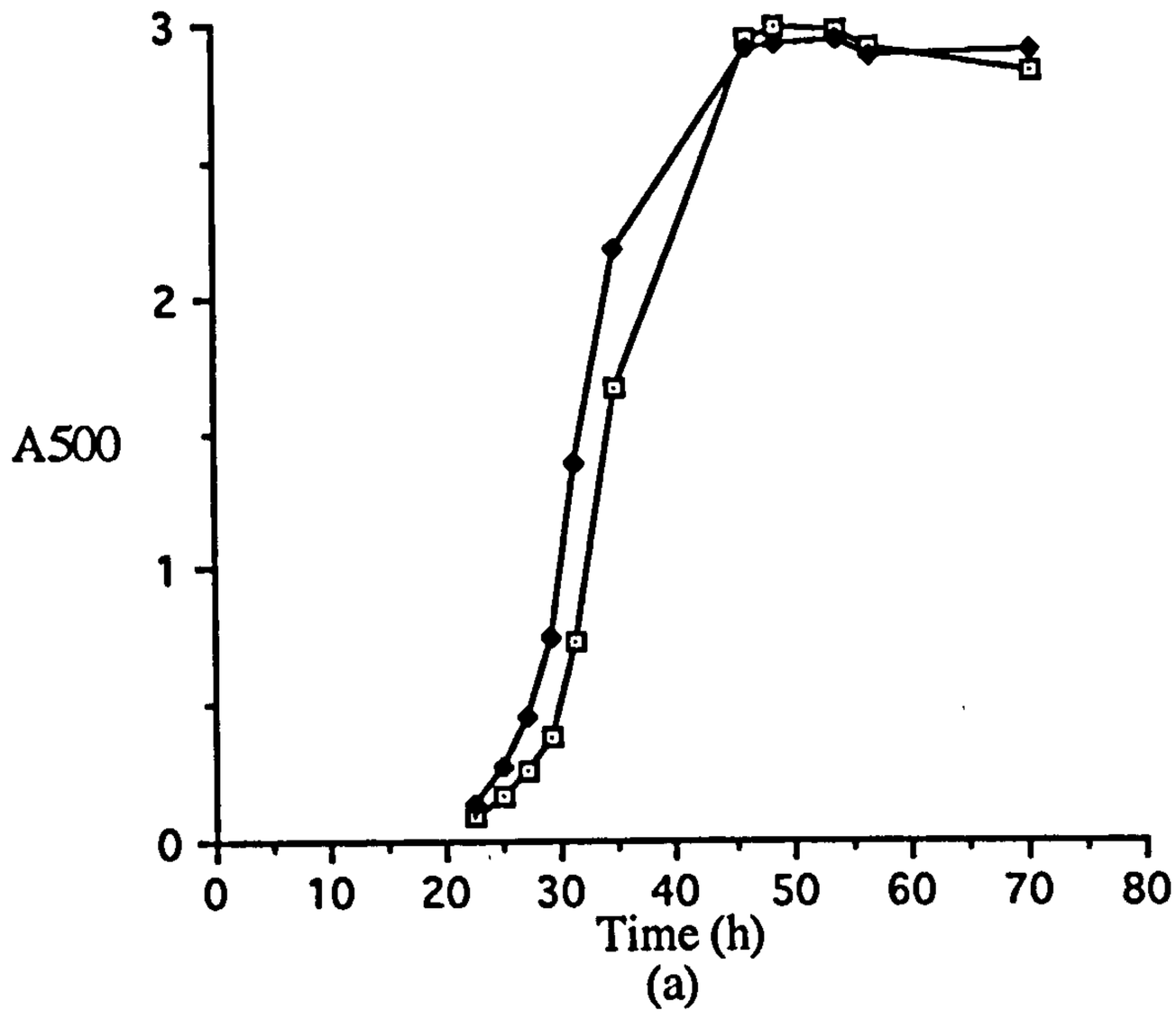
(a) 15 mM

(b) 1.5 mM

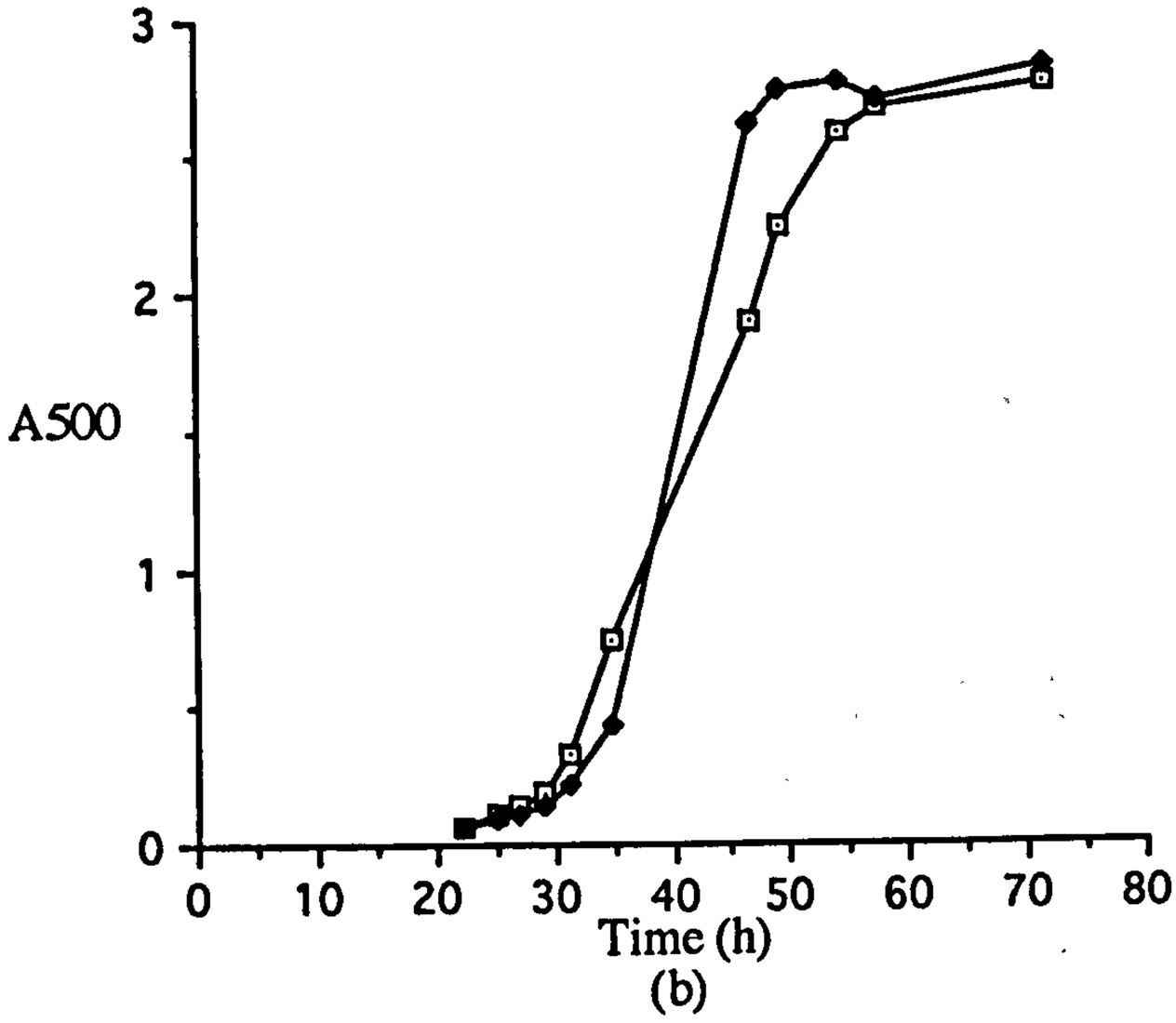
(c) 0.15 mM

(d) 0.07 mM

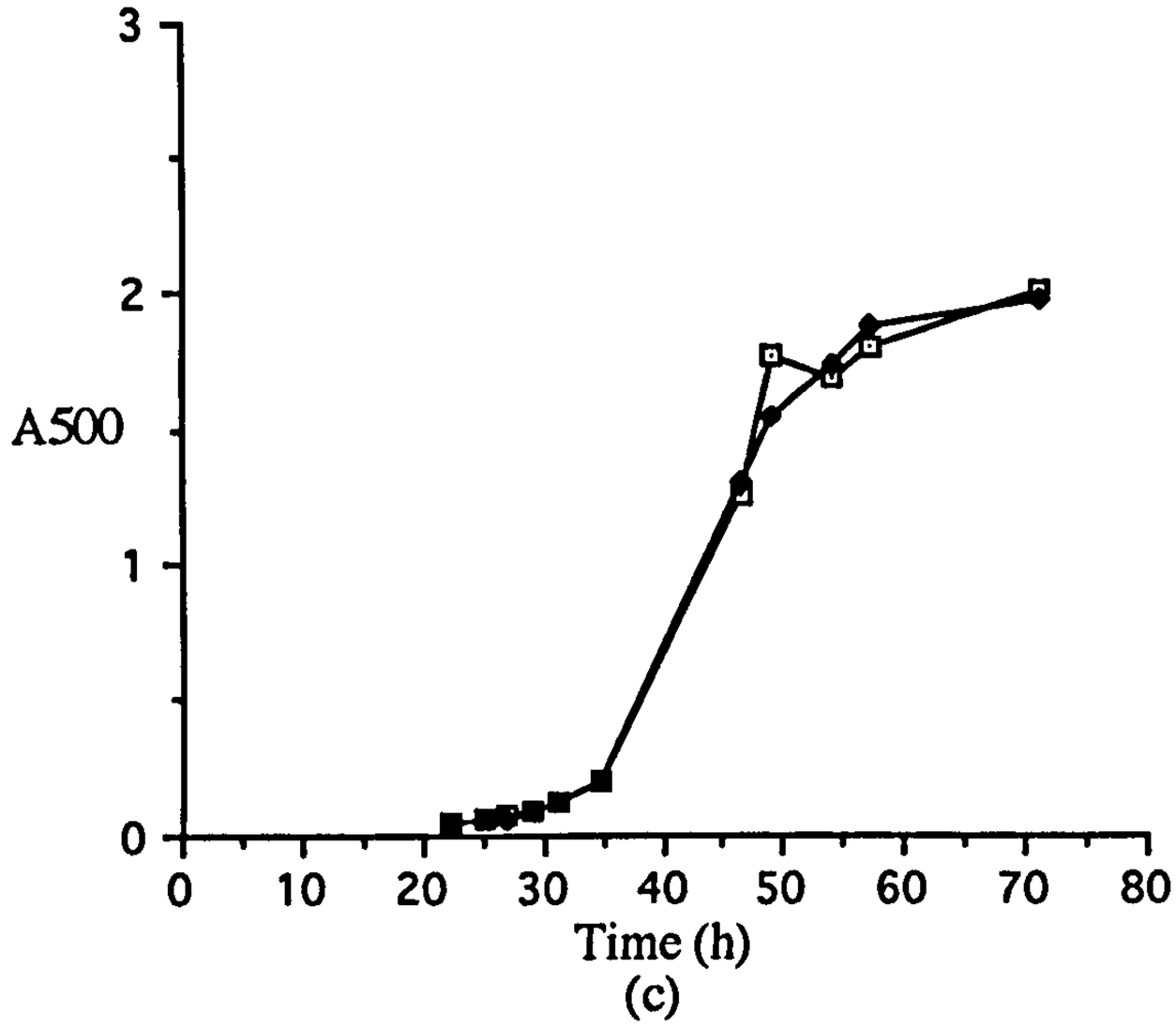
Growth was followed by measuring optical density at A500 (Methods 2.6). The figures (a) - (d) give the growth curves


A500
3
2
1
0
0
10
20
30
40
50
60
70
80
Time (h)

(a)


A500
3
2
1
0
0
10
20
30
40
50
60
70
80
Time (h)

(b)


3
2
1
0
A500
0
10
20
30
40
50
60
70
80
Time (h)

(c)

3
2
1
0
A500
0
10
20
30
40
50
60
70
80
Time (h)

(d)

Table 5.9 SUMMARY OF THE MEASUREMENTS TAKEN DURING THE DEVELOPMENT OF PHOSPHATE LIMITED GROWTH

S. rimosus was grown in 500 ml aerated flasks (Methods 2.3.1(b)). The flasks contained 200 ml of modified HMM and one of the following concentrations of phosphate:

(a) 15 mM

(b) 1.5 mM

(c) 0.15 mM

(d) 0.07 mM

Phospate concentrations were measured (Methods 2.21) at time zero and at 71 h. Glucose measurements (Methods 2.19) were taken at 71 h. Mean generation times (h) are also listed.

Condition	Flask	[Phosphate] mM		[Glucose] mM	MGT (h)
		O h	71 h	71 h	
a	1	19.2	17	0.91	3.2
	2	19.9	17.7	0.43	3.2
b	1	2.84	1.36	4.93	3.2
	2	2.8	1.22	4.11	32
c	1	0.31	0.047	30.6	4.8
	2	0.41	0.037	28.62	4.8
d	1	0.11	0.018	44.25	6.4
	2	0.11	0.02	45.15	6.4

Fig. 5.13 GROWTH OF *S. RIMOSUS* UNDER PHOSPHATE LIMITED CONDITIONS

S. rimosus was grown under the phosphate limiting conditions developed in 5.6.1. *S. rimosus* was grown in a 10 l flask (Methods 2.5), containing modified HMM, with 40 mM glucose and 20 mM ammonium sulphate present. Growth was followed by measuring optical density at 500 nm (Methods 2.6). Arrows indicate the time points where 500 ml samples were taken:

Sample no.	Time
1	20 h 30 min
2	44 h 30 min
3	70 h 30 min
4	96 h 30 min

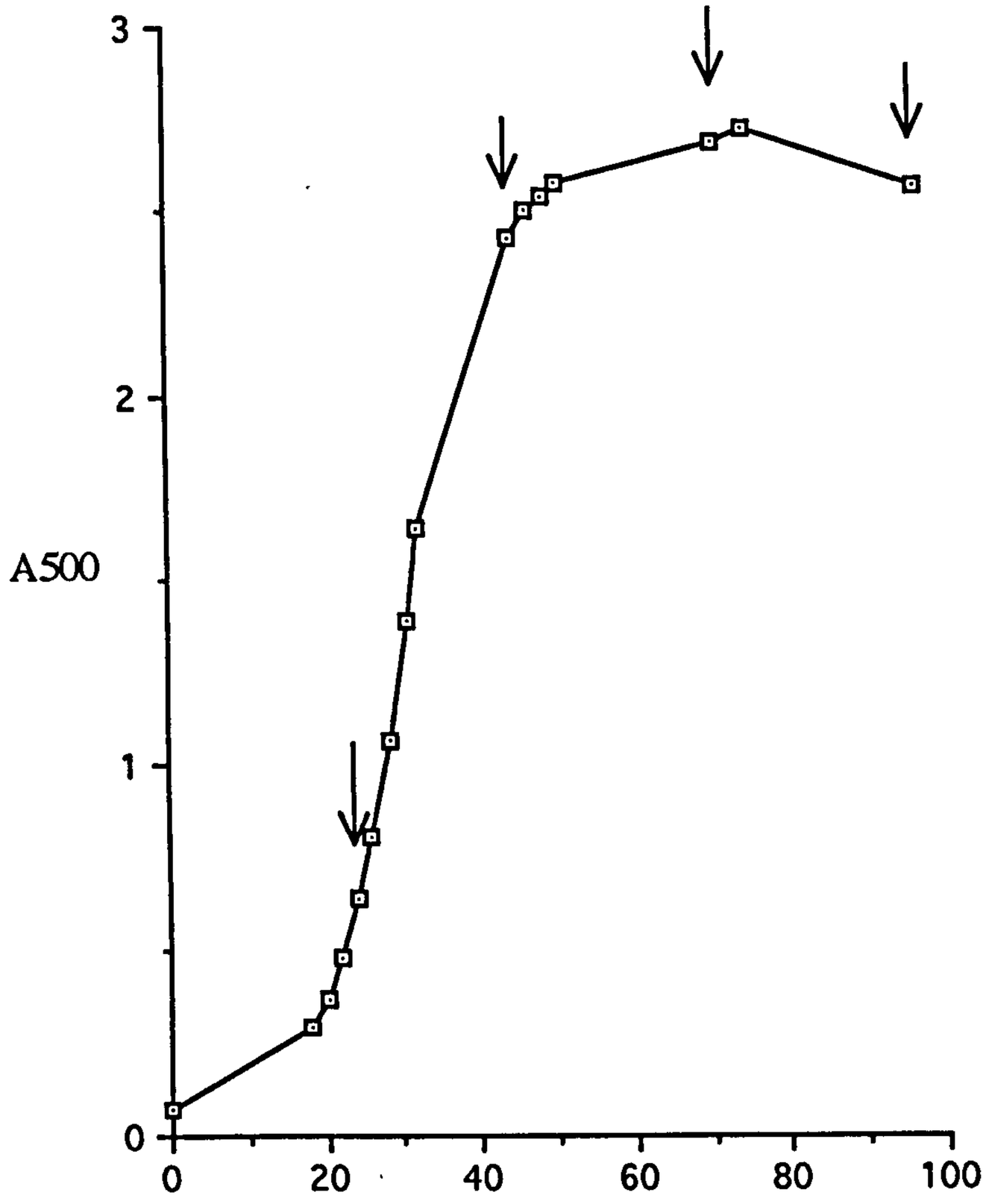

A500
3
2
1
0
0
20
40
60
80
100
Time (h)

Table 5.10 THE CONCENTRATIONS OF LIPID EXTRACTED FROM *S.RIMOSUS* GROWN UNDER PHOSPHATE LIMITED CONDITIONS

S.rimosus was cultured (Methods 2.5) under the phosphate limited conditions developed in 5.6.1. Lipid was extracted from the biomass (Methods 2.23) and the concentrations (mg lipid/ 100 mg dry weight biomass) are listed in this table for each of the four samples taken during growth.

Sample no.	Growth phase	Time(h)	Lipid content mg lipid/100mg biomass[dry wgt.])
1	Exponential	28.5	1.5
2	Early Stationary	44.5	2.7
3	Stationary	70.5	1.1
4	Stationary	96.5	0.7

5.7 DISCUSSION

5.7.1 A COMPARISON BETWEEN THE LIPID PROFILES OF *S. RIMOSUS*, *S.COELICOLOR* AND *S. CLAVULIGERUS*

All three species shared similar lipid profiles, characteristically containing a high percentage of branched-chain fatty acids. Of particular interest was the very high concentration (~ 40%) of branched-chain fatty acids present in the triglyceride lipid fraction extracted from *S. rimosus*. Triglyceride represents a possible lipid storage compound and could therefore provide a route for the diversion of important antibiotic precursors. *S. coelicolor* and *S. clavuligerus* also have branched-chain fatty acids present in their triglyceride fraction, but at a lower concentration than that of *S. rimosus*.

In general fatty acids do not exist as free carboxylic acids in organisms as major components (Gurr and James, 1985). Where free fatty acids are reported they are usually artefacts due to cell damage which allows lipases to act. Interestingly *S. clavuligerus* did not have any free fatty acids present whereas *S. coelicolor* and *S. rimosus* did. Perhaps this could be accounted for because of the different culture conditions.

When comparing the fatty acid constituents of the whole lipid fractions belonging to the three species a number of interesting differences were found. Of the fatty acids identified, *S. coelicolor* and *S. rimosus* contained approximately 50% odd numbered fatty acids in the whole lipid samples, while *S. clavuligerus* had a high proportion of even numbered fatty acids (~ 40%) present. *S. coelicolor* had saturated and unsaturated fatty acids present in approximately equal concentrations, while *S. clavuligerus* had a higher percentage of saturated fatty acids (2:1) and *S. rimosus* had an even higher percentage of saturated fatty acids present (5:1). The inclusion of monounsaturated fatty acids is normally how fluidity is achieved in membranes. A sinilar role is proposed for branched-chain fatty acids in the lipid membranes of branched-chain fatty acid

producers (Kaneda, 1991). This could provide an explanation for the low concentrations of unsaturated fatty acids found in *S. rimosus* because their presence is not necessary as a result of the high concentration of branched-chain fatty acids.

5.7.2 A COMPARISON BETWEEN THE LIPID PROFILES OBTAINED FROM *S. RIMOSUS* GROWN ON A MINIMAL MEDIUM AND A COMPLEX MEDIUM

The aim of this analyses was to determine whether the production of oxytetracycline by *S. rimosus* would alter the lipid profile of the biomass. Modified HMM is a chemically defined medium which allows the production of oxytetracycline by *S. rimosus*. YEME is a complex medium and as such it is therefore difficult to determine all the chemical constituents. Antibiotic production rarely occurs on complex media as the formation of secondary products is usually in response to reduced growth opportunities which can be caused as a result of limitation of an essential nutrient.

There were no significant differences between the whole lipid and phospholipid profiles from *S. rimosus* grown on the defined medium, modified HMM, and the complex medium, YEME, when analysis by GLC was carried out. The most abundant fatty acid was i16:0 and the other branched-chain fatty acids; i15:0, a15:0 and i17:0 together with the straight chain 16:0 were present in approximately the same high proportions. However, major differences were detected between the fatty acid compositions of the triglyceride fractions. The triglyceride fraction from the YEME grown, non-antibiotic producing, *S.rimosus* was made up predominantly of the branched-chain fatty acids; i16:0, i15:0 and a15:0. The triglyceride fraction of the modified HMM grown, oxytetracycline producing *S. rimosus* was quite different to the non-oxytetracycline biomass. The fatty acid composition of the modified HMM grown biomass consisted of fatty acids that were predominantly greater in length than 17 carbons and had i17:0 present as the only branched-chain fatty acid. It is very interesting to note that antibiotic

production does have an effect on the triglyceride fatty acid composition, especially the absence of branched-chain fatty acids from the triglyceride fraction.

A major fatty acid which remained unidentified and gave a peak corresponding to approximately 44 min, on the GLC traces, occurred in the biomass from both growth conditions. The standard Bacterial Acid Methyl Ester CP Mix did not contain standards with a fatty acid chain length long enough to allow the identification of this peak. However, an approximate estimate would be a compound containing 22 carbons. It would be unusual to find a fatty acid with 22 carbons in a *Streptomycete*. TLC analysis was carried out using samples of both modified HMM and YEME but no lipid components were detected. This would rule out unusual fatty acids being taken up from the growth media.

GC-MS analysis again demonstrated that the two biomass samples had essentially had the same whole lipid components. Major constituents , identified by GC-MS, were 15:0, i15:0, 16:0, i16:0 and a17:0. This differs slightly from the GLC analysis in both samples in that it showed i17:0 to be a major constituent while the GC-MS determines a17:0 to be a major constituent. This serves to highlight the difficulty in distinguishing between anteiso and iso branched chain fatty acids. GC-MS analysis did reveal some differences between the two biomass samples. Hydroxy fatty acids (2-OH 15:0 and 2-OH 16:0) were present in the modified HMM grown biomass but not in the YEME grown biomass. The YEME grown biomass had a number of methyl branched fatty acids, methyl 15:0, methyl 17:0 and methyl 18:0, which did not appear in the modified HMM biomass. Long-chain fatty acids were identified as 20:0, for the YEME grown biomass, and methyl 21:0 and 21:0 for the modified HMM grown biomass. A number of peaks were detected at the end of the GC traces, for both biomass samples, corresponding to longer chain compounds but none were identified as fatty acids. From this it could be assumed that the large peak, detected by GLC analysis, with a retention time of 44 min was in fact not a fatty acid.

Therefore, the use GLC and GC-MS has revealed that major difference between lipid extracted from oxytetracycline producing and non-producing *S.rimosus* lies in the triglyceride fractions. The triglyceride extracted from oxytetracycline producing *S.rimosus* contained fewer branched-chain fatty acids, particularly those containing 15 and 16 carbons, than *S.rimosus* grown under conditions which did not allow the production of antibiotic. This would suggest that in this case *S. rimosus* could be diverting the common precursors that are used in the biosynthesis of branched-chain 15 and 16 carbon fatty acids into oxytetracycline production.

5.7.3 ANALYSIS OF THE LIPID CONTENT AND COMPOSITION OF *S. RIMOSUS* GROWN UNDER CONDITIONS WHICH WERE KNOWN TO PROMOTE LIPID STORAGE

The conditions chosen were those most likely to produce nitrogen or phosphate limited growth. Taken into consideration was that a reasonable rate of growth had to be achieved in order for growth to be followed, by measuring optical density at A500. Both conditions allowed the production of antibiotic, which was determined by the appearance of a yellow colour signifying oxytetracycline production. Analysis of the lipid content revealed that neither condition showed any evidence of promoting lipid storage by *S. rimosus*. The lipid content of the nitrogen limited biomass did in general have a higher lipid content (up to 3-4 fold higher), than that of the phosphate limited biomass , but not at levels that would characterise an oleaginous organism or indeed any greater than growth on standard modified HMM.

Analysis of the fatty acid composition revealed that i16:0 was the most abundant fatty acid present in the whole lipid and phospholipid fraction. In the triglyceride fraction the longer chain fatty acids of greater than 17:0 appear to be the most predominant. In 5.7.2 it was noted that antibiotic production by *S. rimosus* affected the fatty acid composition of the triglyceride fraction. Now that the storage of lipids has been shown

not to occur it can be assumed that the common precursors for antibiotic synthesis and branched-chain fatty acid production are in this case being used to produce the polyketide antibiotic, oxytetracycline and not being stored as branched-chain fatty acid components of a stored triglyceride. Again the peak occurring at approximately 44 min was present as a major component but this has been shown, by GC-MS analysis, not to be a fatty acid.

Thus, attempts to promote lipid storage by *S. rimosus* were not successful. There are a number of possible explanations for this. *S. rimosus* might not have the necessary biochemical equipment (enzymes etc.) to allow lipid storage to take place. Alternatively the conditions chosen to promote nitrogen limited growth (carbon and nitrogen present in a 20 to 1 ratio) were not extreme enough to promote lipid storage. Growing *S. venezulae* in a medium with a high carbon to nitrogen ratio (15:1) did not result in an increase in lipid storage products (Ranade and Vining, 1993). Another possible explanation is that *S. rimosus* could store alternative energy reserves such as polysaccharides, the presence of which was not looked for in this study.

Perhaps it was naive to assume that either nitrogen or phosphate limitation would necessarily promote lipid storage instead of the production of polyketide antibiotics. The conditions of nutrient limitation which promote the production of storage lipids are the same conditions that promote antibiotic production in certain *Streptomycetes*. This is certainly the case for *S. coelicolor* whereby the production of the polyketide antibiotic, actinorhodin is elicited by nitrogen or phosphate limitation. I have found no available reports of the effects of nitrogen or phosphate depletion on the production of oxytetracycline by *S. rimosus*. With hindsight it would have been useful to compare the concentrations of oxytetracycline from cultures grown on ordinary minimal medium with those grown on a limited medium, in order to determine the effects of the nutrient limitations on oxytetracycline production.

An alternative way of tackling this investigation could have been to inhibit the production of antibiotic in the minimal medium and determined whether this altered the lipid content of the biomass. If antibiotic production was inhibited then perhaps this would have resulted in a greater branched-chain fatty acid content in the triglyceride fraction, or even promoted the storage of lipid as triglyceride. One such method of undertaking this would be to utilise the well known fact that inorganic phosphate at high concentrations will suppress the biosynthesis of secondary products, often under conditions which permit good cell growth (Martin, 1977; Weinberg, 1974 and Whitney, 1977). In fact, Gersch *et al.*, (1979) reported that inorganic phosphate inhibited the production of the biosynthesis of the macrolide antibiotic, turimycin, in strains of *Streptomyces hygroscopicus*.

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