STUDIES ON SOIL

AND

MICROBIAL LIPASES

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ABSTRACT

The relative sensitivities of four methods for the determination of lipase activity was found to be dependent upon the source of the enzyme, when comparisons were made using commercial porcine pancreatic lipase and two partially-purified bacterial lipases.

Lipase activity was dependent upon substrate surface area rather than substrate concentration *per se*. Long-chain insoluble fatty acids were inhibitory to lipase activity. A minimum concentration of fatty acid was required before inhibition of lipase activity occurred. This minimum inhibitory concentration was inversely related to the chain length of the fatty acid. Fatty acid inhibition of lipase activity could be partially reversed by the addition of Ca^{2+}.

A sensitive and precise assay for the determination of soil lipase activity was developed. The assay involves a 3h 0.1 M tetrasodium pyrophosphate (pH 7.5) extraction of soil, followed by a 10 min. assay on the extract using the fluorogenic substrate 4-methyl umbelliferone nonanoate (4 MUN). Basal lipase activities of thirteen New Zealand topsoils ranged from 28 to 597 nmol 4-methyl umbelliferone (4 MU) produced min^{-1} g^{-1} of oven-dried soil and were not strongly correlated with soil pH, clay content, cation-exchange capacity, or organic matter.

The addition of triacylglycerols to soil samples induced microbial production of lipase resulting in triacylglycerol degradation and associated oxygen consumption. With tallow applied to a Hamilton clay loam topsoil sample at the rate of 2.5 mg g^{-1} and incubated at 25°C, lipase levels increased from 160 nmol 4 MU min^{-1} g^{-1} to 490 nmol 4 MU min^{-1} g^{-1} after 8 days and the majority of the triacylglycerol was degraded within 14 days. The time for significant triacylglycerol degradation to occur was related to the level of lipase induction upon amendment and not upon the basal lipase

levels of the soil. The extent of lipase induction and, therefore, triacylglycerol degradation, was influenced by loading rate, type of triacylglycerol, soil type, temperature, and mineral nutrient additions. In synthetic mixtures of substrates the breakdown of triacylglycerol occurred after the microbial oxidation of alternative substrates.

The lipolytic microbiota of both unamended and triacylglycerol-amended soils were isolated. The bacterial population demonstrated the greatest increase upon amendment with triacylglycerols. A bacterial isolate obtained by enrichment culture, and designated *Pseudomonas* 017, was capable of producing high levels of extracellular inductive lipase when grown upon triacylglycerols, long-chain fatty acids and hydrocarbons. Growth upon these substrates was accompanied by the production of an emulsifying agent. The lipase of *Pseudomonas* 017 was partially purified and characterised.

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CONTENTS

CHAPTER 1 INTRODUCTION

1.1 The importance of lipases

The enzyme triacylglycerol acyl-hydrolase (EC 3.1.1.3, commonly termed lipase) cleaves the ester linkages of triacylglycerols or their analogs. Lipase is active against only those acyl-ester substrates that are insoluble (or in micellar form) in an aqueous environment (Brockerhoff and Jensen 1974), and therefore its activity may be readily distinguished from that of carboxylic-ester hydrolase (EC 3.1.1.1, commonly termed esterase).

The action of lipase represents a case of special interest to enzymologists, with a water-soluble enzyme acting upon a water-insoluble substrate. Studies on the kinetics of such heterogenous catalysis should prove of more value in understanding the principles of enzymatic reactions in natural biological systems (where most enzymatic activity occurs at interfaces) than classical studies in homogenous systems (Verger and de Haas 1976).

In the medical field, low serum lipase levels are implicated as the cause of congenital hyperlipemia (Type I hyperlipoproteinemia) (Fredrickson and Herbert 1978). The possibility exists that manipulation of serum lipase levels may be useful in controlling abnormal lipid accumulation. Elevated serum lipase levels provide a clinical indication of acute pancreatitis (Tietz *et al* 1959).

Microbial lipases are responsible for the development of rancidity in fat-containing foodstuffs (Alford *et al* 1963; Deeth and Fitz-gerald 1976). However, with certain products lipolytic action is necessary for flavour development (Lawrence 1967a; Seitz 1974; Deeth and Fitz-gerald 1976). Besides flavour development, microbial lipases have a variety of industrial uses being incorporated into

detergents, washing powders, pharmaceuticals, chewing gum and dentifrices (Seitz 1974). Several manufacturers are now marketing enzyme powders (containing lipase) which promote rapid and efficient breakdown of organic materials in wastewaters (*e.g.* Actizyme® marketed by Southern Cross Laboratories, Dural, New South Wales, Australia).

Triacylglycerols are the principal components of the lipid or "oil and grease" fraction of domestic sewage and various food-processing wastewaters (Hunter and Heukelekian 1965; Overcash and Pal 1979). During biological treatment of such wastes, sufficient lipolysis and subsequent oxidation, must occur if problems associated with triacylglycerol accumulation within the treatment plant are to be avoided, and suitable effluent quality with respect to organic levels is to be achieved (Banerji *et al* 1974).

1.2 Scope of the thesis

The prime objective of the research was to investigate the induction of lipase and the consequent degradation of triacylglycerol in soil. This was prompted by proposals by both the dairy industry (James, New Zealand Co-Operative Dairy Company, *pers comms*) and the meat processing industry (Cooper, Meat Research Institute of New Zealand, *pers comms*) to increasingly use land application as a method of treatment of wastewaters. As wastewaters from both of these industries contain triacylglycerol, concern was held that triacylglycerol accumulation may occur resulting in decreased rates of wastewater infiltration and oxygen diffusion.

In addition to studies on lipase in soil (Chapters 5 and 6) several other objectives arose once research had begun. Specifically, these were:

(1) To develop and compare methods of estimating lipase activity (Chapter 2);

(2) To further elucidate interface phenomena as it relates to lipase activity (Chapter 3);

(3) To study the production and properties of microbial lipases, particularly those produced in the presence of insoluble carbon and energy sources (Chapters 8, 9 and 10).

CHAPTER 2 THE MEASUREMENT OF LIPASE (TRIACYLGLYCEROL ACYL-HYDROLASE, EC 3.1.1.3) ACTIVITY

2.1 Assay methods : An overview

There are numerous published methods for the quantitation of lipase (triacylglycerol acyl-hydrolase EC 3.1.1.3) activity, each possessing its advantages and disadvantages. The method of choice becomes one that is best suited to the particular experimental system being investigated. Lipase assays can be broadly classified into those using the "natural" triacylglycerol substrates and those using "artificial" substrates.

(a) Lipase assays employing triacylglycerols as substrates

With lipase activity towards triacylglycerol substrates the primary reaction being investigated is: Triacylglycerol → Diacylglycerol + Fatty acid. The di- and monoacylglycerols may subsequently be attacked to release glycerol. The rate of the reaction can be measured by the rate of physical or chemical changes occurring in the assay system.

2.1.1 Turbidimetric determinations of lipase activity may be performed in semi-solid or liquid systems. Lawrence *et al* (1967a and b) have described an agar diffusion assay for lipase estimation which was "able to detect lower levels of lipase activity" than continuous potentiometric titration. The sample is placed in a well within a thin layer of buffered agar containing emulsified triacylglycerol. After incubation the zone of hydrolysis is measured, the square of its diameter being a measure of lipase activity. Zones of hydrolysis are seen as zones of clearing when short-chain ($<C_{12}$) triacylglycerols are used as substrates, due to the solubility of the released fatty acids and di- and monoacylglycerols. With long-chain ($>C_{12}$) triacylglycerols, zones of hydrolysis show up as increased opaque zones, presumably due to Ca-salt precipitation of the released fatty acids. Alternatively, the system may be flooded with Osmium tetroxide and translucent zones of hydrolysis appear against a dark background (Kuimova *et al* 1975).

In liquid systems the lipolytic hydrolysis of emulsified triacylglycerols leads to a decrease in the turbidity of the system (*e.g.* Borgstrom 1957; Rottem and Razin 1964; Shihabi and Bishop 1971). With short-chain triacylglycerols such a decrease results from the solubility of the hydrolysis products, whilst with long-chain triacylglycerols the effect is due to the lower extinction co-efficients of the insoluble products (Kok *et al* 1978). No values relating the sensitivity of this method to other methods could be found in the literature. However, a method where low enzyme activities would be determined by small differences between large absorbance values would appear to have an inherent lack of sensitivity. In addition, the possibility of substrate limitation exists as low levels of triacylglycerol (*e.g.* Kok *et al* 1978, 0.2 mg mL^{-1}) must be used to provide initial spectrophotometric absorbance readings that are meaningful (*i.e.* not above approximately 1.2). This possibility has not been discussed by users of the technique, although measurements presented elsewhere in this thesis (Section 3.2; Figs. 3.1, 3.2, and 3.3) suggest that substrate limitation is likely.

Both turbimetric procedures are suitable for assaying for lipase activity in microbial culture supernatants which may contain buffering agents, acidic by-products and other compounds which may interfere with other triacylglycerol-based lipase assays. Lawrence *et al* (1967a and b) successfully monitored lipase production by the agar diffusion assay in cultures of *Micrococcus* and *Pseudomonas* species. Aisaka and Terada (1979) used a liquid turbidimetric procedure to examine lipase production by the fungus *Rhizopus japonicus*. Both methods have in common the disadvantage of employing arbitrary units of lipase activity (diameter squared or Δ absorbance per unit of time) although Kok *et al* (1978) have described a procedure for calibration of the liquid turbidimetric method.

2.1.2 Stalagmometric determinations of lipase activity measure the rate of change of surface tension (usually by a Du Nuoy Tensiometer) in the assay mixture. The observed changes in surface tension upon incubation of lipase with triacylglycerols results from the surfactant and solubility properties of the hydrolysis products. When soluble hydrolysis products are formed the surface tension decreases whilst insoluble products lead to an increase (Lagocki *et al* 1970). The use of short-chain triacylglycerols releasing soluble hydrolysis products is preferred as surface tension changes are larger and the 'quality' of the interface is unaltered by accumulation of insoluble hydrolysis products (Dervichian 1971; Verger and de Haas 1976). The extent (as well as the direction) of surface tension change is dependent upon the substrate used (and hence the reaction products), the presence of surface-active amphipaths (*e.g.* contaminating proteins) and the degree of ionisation of the released fatty acids (Dawson 1969; Lagocki *et al* 1970; Brockerhoff and Jensen 1974). The technique is therefore a difficult one to perform and subsequently interpret and requires pure preparations of lipase. It can, however, provide valuable information on the kinetics of lipase action, lipase denaturation at the interface, and the role of colipase (Lagocki *et al* 1973; Verger and de Haas 1976; Verger *et al* 1977).

2.1.3 Conductimetric determinations of phospholipase activity have been described by Moores and Lawrence (1972). Increases in conductivity of a low ionic strength assay system are brought about by the production of fatty acid anions and protons. The method therefore requires that the released fatty acids be ionised and soluble. Moores and Lawrence (1972) ensured such conditions by performing the reaction in 20% v/v n-propanol or by using substrates releasing short-chain fatty acids. The method was described as "rapid and sensitive" and "readily adapted for routine large-scale determinations". The method has apparently not been used with triacylglycerol and lipase despite its potential.

Conductimetic methods are limited to relatively pure enzyme preparations (*i.e.* those not containing contaminating conducting materials) that are capable of activity in low ionic strength systems.

2.1.4 Analysis of released fatty acids by titration is the method most often used in lipase determinations. The titration may be performed directly upon the assay system, after addition of water-miscible organic solvents such as alcohol or acetone, or after extraction of the fatty acids into a water-immiscible organic solvent (Lawrence 1967b; Desnuelle 1972; Brockerhoff and Jensen 1974).

Direct titration procedures may be performed after a pre-set incubation period (Tietz *et al* 1959; Mates and Sudakevitz 1973) or continuously by maintenance of a predetermined pH using periodic manual additions of alkali or employing an automatic pH-stat (Benzonana and Desnuelle 1968; Vandermeers *et al* 1974).

Alkali titration of the released fatty acids after a pre-set incubation period requires that the assay system be unbuffered or only weakly buffered so that sample titration values are significantly higher than blank values. This essentially means that the pH of the assay system changes during the incubation period. This change in pH, coupled with lipase inactivation during the long incubation periods required to obtain sufficient sensitivity (Jonsson and Snygg 1974), results in sub-optimal rates being determined. Renshaw and San Clemente (1967) used a strong buffer (0.1M Tris-HCl, pH 8.3) and a short incubation period and titrated both blank and samples to a pH of 10.5. Such a method is likely to be quite insensitive as blank titration values will be high due to the presence of buffer.

Alkali titration may be performed potentiometrically or a suitable indicator may be used. When triacylglycerols releasing long-chain fatty acids are used as substrates the end-point of the titration should be at pH 9.0 or greater to ensure complete ionisation of the fatty acids (Shahani *et al* 1964; Mattson and Volpenheim 1966; Benzonana and

Desnuelle 1968). Titrations with an indicator should therefore use one with a sufficiently high pK_a such as thymol blue or thymolphthalien (pK_a's of 9.6 and 10.5 respectively). The problem does not arise with short-chain triacylglycerol substrates, such as tributyrylglycerol, which is completely ionised above pH 6.0 (Brockerhoff and Jensen 1974). At the end of the assay period miscible organics, such as alcohol or acetone are usually added to stop the reaction and/or to improve the solubility of released long-chain fatty acids (Tietz *et al* 1959; Mates and Sudakevitz 1973; Iwai *et al* 1975; Bashkatova and Severina 1978).

Some improvement in the sensitivity of titration procedures would appear to be possible by extraction and subsequent titration of released fatty acids within a water-immiscible organic phase (Cohen *et al* 1969; Lu and Liska 1969; Henderson 1971; Castberg *et al* 1975; Sugiura *et al* 1977). This method is dependent upon the released fatty acids partitioning into the organic phase and hence this organic phase must be chosen with care, particularly when water-soluble fatty acids are involved. Castberg *et al* (1975) obtained high extraction of water-soluble butyric acid into an organic phase of diethyl ether and light petroleum. The organic extraction method allows assay systems to be strongly buffered at optimal pH's and to contain buffering agents from the sample, and hence it has been successfully used with crude and purified microbial lipases (Lu and Liska 1969; Henderson 1971; Sugiura *et al* 1977).

Continuous titration of released fatty acids by addition of base provides initial reaction velocities essential for studies on lipase kinetics (Desnuelle and Savary 1963; Benzonana and Desnuelle 1965 and 1968; Brockman *et al* 1973; Vandermeers *et al* 1974). The method is dependent upon a non-buffering or weakly-buffering assay system and is therefore likely to be an insensitive method when the lipolytic activity of crude enzyme preparations such as microbial culture supernatants are being determined (Lawrence 1967b). When long-chain triacylglycerols

are used as substrates the procedure needs to be performed at pH ≥ 9.0 to ensure that all of the released fatty acid is ionised and hence titrable. The assay pH may be reduced if a high ionic strength system is used (Benzonana and Desnuelle 1968). If it is required to perform assays at lower pH's then correction for the degree of ionisation of the released fatty acid is necessary (Shahani *et al* 1964). Alternatively, short-chain triacylglycerols such as tributyrylglycerol may be used as substrates as the released fatty acids will be completely ionised down to pH 6.0 (Brockerhoff and Jensen 1974). It would appear that several workers have not realised this limitation and have constructed pH-activity curves with long-chain triacylglycerols (*e.g.* Renshaw and San Clemente 1967). As most lipases display maximal activity toward tributyrylglycerol as compared to other triacylglycerols and display pH optima between 7.0 to 8.0, the use of tributyrylglycerol as substrate provides the optimum sensitivity (Erlanson and Borgstrom 1970; Brockerhoff and Jensen 1974).

2.1.5 Analysis of released fatty acids by manometry is another short-duration lipase assay, but is rarely used (Lawrence 1967b). The method relies on the liberation of CO_2 from a bicarbonate buffered assay system by the released fatty acids. For the desired shift in the bicarbonate/CO_2 equilibrium to occur the fatty acids released must be ionised and preferably soluble. Thus, short-chain triacylglycerols are the preferred substrates (Drummond and Tager 1959a and b). The method would obviously be interfered with by any other reactions in the assay system releasing CO_2 (*e.g.* respiration) or indirectly by any reactions releasing acidic by-products which would upset the bicarbonate/CO_2 equilibrium. The method is, therefore, only likely to function successfully with purified enzyme preparations. In such situations the practical complications of the manometric method (gas purging, complications arising from pH and temperature changes) make the pH-stat method more desirable.

2.1.6 Analysis of C14-labelled fatty acids released from triacylglycerols by liquid scintillation counting is a very sensitive method of assaying for lipase activity (Brockerhoff and Jensen 1974). The separation of the labelled fatty acid from radioactive tri-, di- and mono-acylglycerols can be achieved by use of paper chromatography (Marsh and Fitzgerald 1972), anion exchange resins (Kelley 1968), Florisil columns (Chino and Gilbert 1965) or TEAE-cellulose columns (Zieve 1973). Liquid-liquid extractions of lipid and separations of labelled fatty acids usually involves two-step treatments of the assay mixture (Kaplan 1970; Schotz *et al* 1970). However, Belfrage and Vaughan (1969) have described a single-step method for separation of labelled fatty acids from the acylglycerols. Radioactively labelled triacylglycerol substrates have been successfully used for the determination of lipase activity in microbial cell cultures as well as after purification (Paznokas and Kaplan 1977; Proulx *et al* 1978).

2.1.7 Analysis of released fatty acids by gas-liquid chromatography is capable of providing qualitative information on the nature of fatty acids released by lipolytic action in mixed substrate systems, as well as quantitative information on activity (Alford and Pierce 1963; Alford *et al* 1964; Mencher and Alford 1967). The procedure described by Alford and co-workers involves ion-exchange or thin-layer chromatography for separation of fatty acids from the acylglycerols followed by methylation or silylation of the fatty acids prior to gas chromatographic analysis. As a method solely for quantitative determination of lipase activity the procedure described contains many steps which make it unfavourable for routine assays. However, modifications to the procedure could be made to improve speed and ease of analysis. For example, the single-step liquid extraction and separation of fatty acids described by Belfrage and Vaughan (1969) could be coupled to analysis of the unmodified fatty acids (*i.e.* no silylation or methylation step) using recently developed columns (*e.g.* the esterified carbowax's, FFAP and SP-1000).

Alternatively, both the acylglycerols and released fatty acids could be extracted into a general lipid solvent and subsequently separated during the chromatographic analysis using high-temperature columns such as the carborane silicones.

2.1.8 Analysis of released fatty acids by colourimetry provides a lipase assay with sensitivity (relative to titrimetric methods, see for example Mahadevan *et al* 1969) that is capable of functioning successfully with crude enzyme preparations such as microbial cell cultures (Breuil and Kushner 1975a). The method involves complexing of released fatty acids with copper or cobalt followed by subsequent extraction of the resultant soaps into an organic phase. The copper and cobalt in this phase is reacted with suitable chromogens (*e.g.* diethyldithiocarbamate, diphenylcarbazone) and the absorbance related to fatty acid level by way of a standard curve (Meinertz 1971). The colourimetric determination of fatty acids, described by Duncombe (1963) has been modified for use in assays of lipase activity in serum (Lippi *et al* 1972), and microbial cultures (Breuil and Kushner 1975a). Mahadevan *et al* (1969) have substituted diphenylcarbazone for diethyldithiocarbamate in Duncombe's method for measurement of pancreatic and lysosomal lipase activity as has Muller (1972) for serum lipase measurements. Such a modification increased the sensitivity by 4-6 fold.

The colourimetric assay must be performed with care so as not to contaminate the organic phase sample with unreacted copper from the aqueous phase. The method is not suitable for use with short-chain triacylglycerol substrates as the copper (or cobalt) salts of the released fatty acids do not partition effectively into the organic phase (Duncombe 1963; Meinertz 1971; this thesis, section 2.4). This limitation was apparently not realised by Breuil and Kushner (1975a and b) who used the method with tributyrylglycerol as a substrate.

2.1.9 Analysis of released fatty acids using bacterial bioluminescence has been reported by Ulitzur (1979). Myristic acid ($C_{14:0}$) released from lipolytic action upon trimyristylglycerol increased the luminescence of a mutant strain of the luminous bacterium *Beneckea harveyi*. The method was rapid and extremely sensitive, being capable of detecting the release of 10 pmol myristic acid per min.

2.1.10 An electron spin resonance method for serum lipase activity determination toward triacylglycerols has been described by Janzen and Burns (1977) although no information as to its sensitivity was given.

b) *Lipase assays employing "artificial" substrates*

The use of "artificial" substrates for determination of lipase activity has become increasingly popular due to their rapidity, sensitivity and precision. However, criticisms as to their validity as lipase substrates have been made on the basis of specificity (Nachlas and Seligman 1949; Barrowman and Borgstrom 1968; Barrowman 1969; Brockerhoff 1969; Melius and Doster 1970; Brockerhoff and Jensen 1974). The use of artifical esters with short acyl chains for lipase estimation is unwise as such substrates are available for attack by carboxylesterase (EC 3.1.1.1) if they possess sufficient solubility in the assay system.

2.1.11 Lipase assays using chromogenic substrates are the most common of the artifical substrate methods. Hydrolysis of p-nitrophenyl esters releases the yellow-coloured p-nitrophenol which can be assayed by measuring absorbance at 400 nm (Barrowman and Borgstrom 1968). Substrates for lipase assay should be insoluble (*e.g.* the esters of laurate and palmitate) but their absorbance due to turbidity interferes with the assay and must be removed by addition of a water-miscible organic solvent (*e.g.* acetone).

The liberation of β-napthol from its acyl-esters can be measured after diazotisation, protein precipitation and extraction into ethyl acetate (Nachlas and Seligman 1949). The purple pigment has an

absorbance maximum at 540 nm. Napthol esters have been successfully used for lipase assays in crude microbial cell cultures by Hobson and Summers (1966) and Breuil and co-workers (Breuil and Kushner 1975a; Breuil *et al* 1978). In both of these studies hydrolysis of the acetate ester was used as a measure of carboxylesterase activity whilst hydrolysis of longer-chained esters (laurate, palmitate) was regarded as lipase activity.

Brockerhoff *et al* (1970) have described a sensitive lipase assay using vinyl oleate as the substrate. The released unstable vinyl alcohol isomerizes to acetaldehyde to produce a dye which is analysed colourimetrically at 666 nm. The sensitivity of the method arises from both the relatively high rate at which vinyl oleate is hydrolysed by lipase (pancreatic) and the high molar absorptivity of the dye formed by acetaldehyde.

Varley (1969) has described a method for serum lipase determination using phenyl laurate as substrate. The released phenol was analysed using reaction with the Folin-Ciocalteau reagent and subsequent spectrophotometric measurement at 686 nm.

2.1.12 Lipase assays using fluorogenic substrates have been evaluated by Guilbault *et al* (1968). The fluorogenic substrates tested were esters of n-methyl indoxyl (excitation λ = 438 nm, emission λ = 500 nm), fluorescein (excitation λ = 490 nm, emission λ = 530 nm), and 4-methyl umbelliferone (excitation λ = 340 nm, emission λ = 450 nm). The medium-chain length (C_7-C_9) 4-methyl umbelliferone esters possessed the lowest detection limit and were suitably specific for lipase. Jacks and Kircher (1967) had earlier demonstrated the potential of 4-methyl umbelliferone esters as lipase substrates. The use of fluorescein dibutyrate as substrate (Kramer and Guilbault 1963; Guilbault and Kramer 1964) requires both ester linkages to be cleaved before the fluorochrome is released.

This requirement leads to low rates of measured activity and, hence, relatively high detection limits. Guilbault and Heiserman (1969) have determined that of six η-methylindoxyl esters used as substrates for pancreatic lipase, the myristate ester was the best, from the viewpoint of stability and specificity. The general use of fluorometric techniques in enzymology is described by Guilbault (1973).

The developmental work described above, for use of fluorogenic substrates, has been performed largely with animal and plant lipases. The method employing 4-methyl umbelliferone esters has subsequently been successfully employed for microbial lipases (Dooijewaard-Kloosterziel and Wouters 1976; Adoga and Mattey 1979; Roy 1980). Fluorometric assays of lipase activity are rapid, extremely sensitive (approximately 1000 x that of conventional titrimetric and potentiometric methods, *e.g.*, Guilbault *et al* 1968; Dooijewaard-Kloosterziel and Wouters 1976; Roy 1980) and capable of being used with crude samples such as microbial cell cultures. Of particular interest to the aims of this thesis are the publications of Pancholy and Lynd (1972 and 1973) which demonstrated the suitability of the 4-methyl umbelliferone butyrate substrate for the determination of lipase activity in crude extracts of soil.

2.2 Precision and detection limits

In deciding upon an analytical method the choice should be made with a knowledge of the precision and the detection limit of the methods available. The precision of a method (*i.e.* the scatter or dispersion of test results of subsamples) may be statistically measured by the use of variance, standard deviation (square root of the variance) or relative standard deviation (the standard deviation expressed as a percentage of the mean). Results suitable for statistical estimation of precision should be based on 5 or more determinations. When the precision of a method is being quoted it should be done for various levels of the substance, as the relative standard deviation is likely to increase as levels approach the detection limit. Also, determinations

of precision should include the variability in the blank values (Wilson 1961). Thus, if the results for the sample (A) and blank (B) have normal distributions, then the standard deviation of the corrected results is given by the expression

$$S_C = \sqrt{S_A^2 + S_B^2},$$

where S_C = the standard deviation of the "true" level (the true level, C, being A-B).

S_A = the standard deviation of the sample value

and S_B = the standard deviation of the blank value

When $S_A^2 >> S_B^2$ the effect of blank variability is negligible. When S_A^2 is approximately equal to S_B^2, however, the true standard deviation will be about 40% (*i.e.* a factor of $\sqrt{2}$) greater than the standard deviation calculated from the sample results only. The latter case will most frequently arise when sample values are close to blank values (*i.e.* approaching the detection limit).

The limit of detection of an analytical method can be determined statistically from a knowledge of the standard deviation of the blank values (Wilson 1961; Roos 1962). The detection limit (using the conventional level of significance of 95%) is that level where there is a 5% risk of concluding that the substance is absent when it is present, and a 5% risk of concluding that the substance is present when it is absent (Roos 1962). This can be shown to be equal to 2 x 1.645 S_C. If it is assumed that the standard deviation of the sample values at the detection limit are equal to the standard deviation of the blank values (*i.e.* $S_A = S_B$, and therefore $S_C = \sqrt{2}S_B$, then the detection limit is equal to 2 x 1.645 x $\sqrt{2}S_B$ or 4.65 S_B (Midgley 1979).

Previous studies on lipase determinations have often quoted a precision at one enzyme level only and/or with no account being taken of the variability in non-enzymic rates. The detection limit (or "sensitivity") of a method, when stated, is either stated intuitively (*e.g.* "extremely sensitive", "insensitive") or using a non-statistical basis (*e.g.* the level of enzyme giving twice the *rate* of the blank).

The following sections describe the procedures and give statistically based estimates of precision and detection limit, for several methods of estimating lipase activity. As enzymes from different sources display different substrate specificities, the precision and detection limit is defined for each method and each of the three enzymes. The three enzymes used were a commercially available porcine pancreatic lipase (Sigma Chem. Co. St Louis, USA), and bacterial lipases from *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017 (a soil isolate) partially purified and described in Chapter 10. As well as precision and detection limit other factors are discussed in assessing the methods (*e.g.* speed of analysis, limitations).

2.3 Continuous potentiometric titration assay of lipase activity

The standard procedure adopted for continuous potentiometric titration of released, fatty acids was as follows:

> A 15 mL aliquot of a 2% (v/v) emulsion of tributyrylglycerol (Sigma Chem. Co., St Louis, USA), in distilled water (created by sonication for 5 min, at maximum power in a Dawe Soniprobe) was placed in a water-jacketed reaction vessel and equilibrated to 30°C. The pH electrodes of a suitable meter (Radiometer 22 or expanded scale Pye Unicam Model 290) were placed in the vessel and the contents stirred. The system was adjusted to pH 7.5 ± 0.2, at which point an aliquot of the enzyme suspension (usually 1 mL) was added and a stop-clock initiated. The system was maintained at pH 7.5 ± 0.2 by periodic addition of small quantities (10-100 µL) of a

suitable concentration of standardised alkali (NaOH, 0.01 M or 0.1 M). The times at which the alkali additions were made were recorded and the subsequent butyric acid production rate determined. Results were expressed as µmol butyric acid released min^{-1} mL^{-1} (or mg^{-1} protein). Each assay was completed within a 10-minute period. Rates of non-enzymic hydrolysis were determined using boiled (5 min) enzyme samples.

Rates of non-enzymic and enzymic hydrolysis were linear for at least 30 min (Fig.2.1). At pH 7.5 the mean rate of non-enzymic hydrolysis calculated from 10 separate determinations was 0.05 µmol butyric acid min^{-1} with a standard deviation (S_B) of 0.009 µmol butyric acid min^{-1}. By definition, the detection limit is that level of enzyme releasing 0.042 µmol butyric acid min^{-1} (*i.e.* 4.65 x S_B). This corresponds to a detection limit of 0.129 µg Lowry protein for the *Pseudomonas* 017 lipase, 8.81 µg Lowry protein for the *Staphylococcus aureus* lipase and 0.035 µg Lowry protein for the commerical porcine pancreatic lipase.

The precision of the method was determined at a range of enzyme levels by variable dilution of the *Pseudomonas* 017 preparation. Each dilution was subsampled 10x for subsequent analysis. The data is presented in Table 2.1. When enzyme levels are near the detection limit the standard deviation of the blank values has a significant effect on the precision of the method. The activity obtained at levels near the detection limit may be between 50-150% of the "true" activity of the sample (using 95% confidence limits as $2S_C$). The minimum achievable relative standard deviation of the method obtained at enzyme levels well above the detection limit, is between 5.0-6.0%. The minimum 95% confidence limits are therefore 10-12% of the determined value.

The method described was capable of being modified to test the effect of pH and temperature on enzyme activity. Rates of non-enzymic hydrolysis were not affected by temperatures up to 50°C but were markedly increased by pH's above 7.5 (Fig.2.2). Base-catalysed hydrolysis

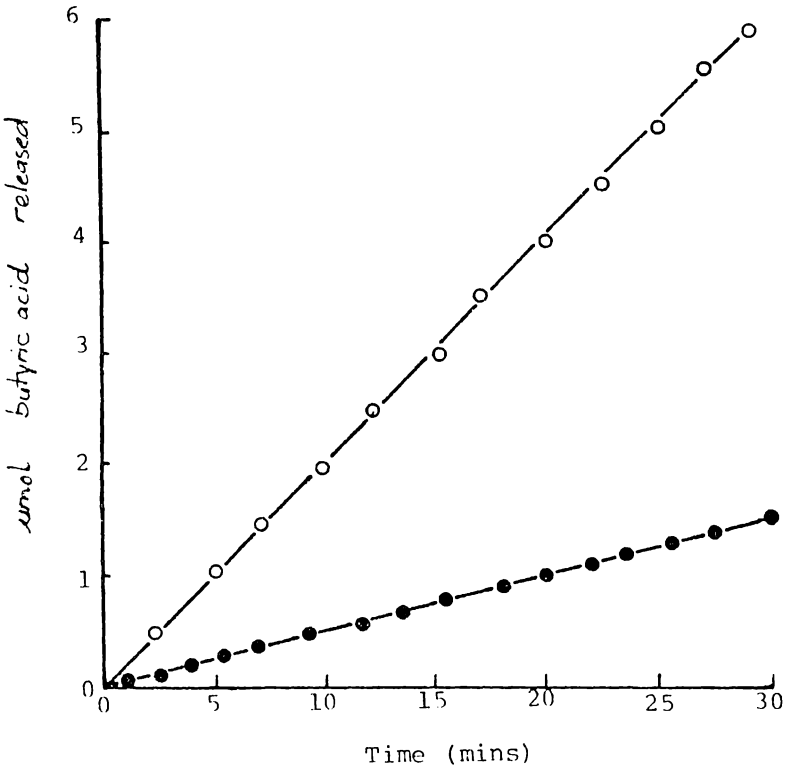


Fig. 2.1 Release of butyric acid with time in continuous potentiometric titration assay of a boiled enzyme control (-●-) and an enzyme system (-o-).
Enzyme source was purified *Pseudomonas* 017 lipase at a level of 0.45 μg Lowry protein.

TABLE 2.1 The precision of the continuous potentiometric titration method using partially purified *Pseudomonas* 017 lipase as the enzyme source. The detection limit is 0.129 µg Lowry protein.

Enzyme Level (µg Lowry protein)	Mean Activity (µmol butyric acid min^{-1}).	Standard Deviation* (µmol butyric acid min^{-1})			Relative Standard Deviation (% mean value)
		S_A	S_B	S_C	
0.15	0.054	0.010	0.009	0.035	25.0
0.20	0.073	0.013	0.009	0.0158	21.7
0.50	0.178	0.017	0.009	0.0192	10.8
1.00	0.358	0.023	0.009	0.0242	6.9
2.00	0.721	0.039	0.009	0.0400	5.6
5.00	1.790	0.105	0.009	0.1054	5.8

* S_A = Standard deviation of sample values (10 replicates)

S_B = Standard deviation of blank values (10 replicates)

S_C = Standard deviation of the "true" value, with the "true" value being the sample - blank.

S_C calculated as $\sqrt{S_A^2 + S_B^2}$

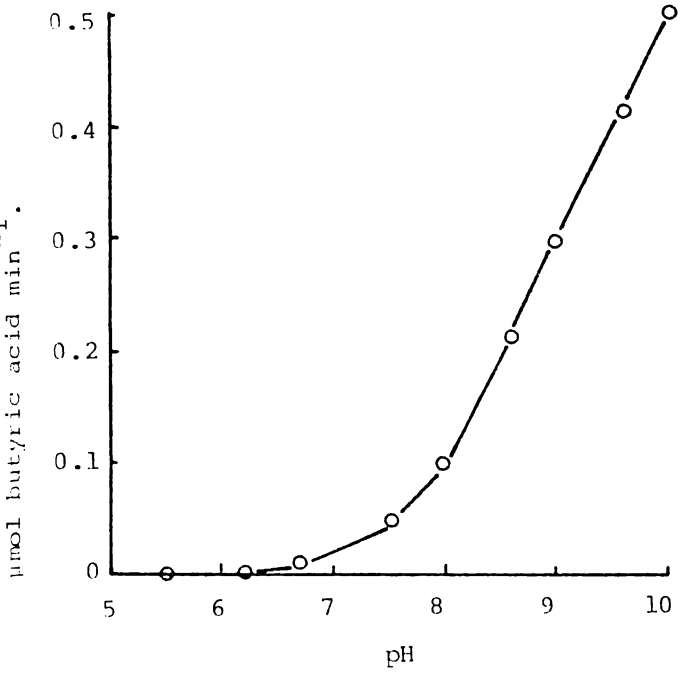


Fig. 2.2. The effect of pH on rates of non-enzymic hydrolysis of 15 mL of a 2% (v/v) tributyrylglycerol emulsion.

of tributyrylglycerol has been observed previously by Castberg *et al* (1975) and Erlanson and Borgstrom (1970). Thus, when pH-activity relationships were being determined blank rates were assayed for at each pH value. As the pK_a of butyric acid is at pH 4.81 (CRC Chemical and Physical Handbook) pH-activity relationships were investigated to a minimum pH of 5.81. Using the relationship,

$$\log \frac{[HA^-]}{[HA]} = pH - pK_a$$

it can be shown that at pH 5.81 91% of the butyric acid would be ionised.

The method was used in this thesis to test the substrate specificity of various enzyme preparations. However, this involved more than simply replacing tributyrylglycerol with the desired substrate. With substrates releasing fatty acids of acyl chain-length $\geq C_{10}$, two extra factors influenced the kinetics of the reaction.

i) The longer-chain fatty acids, when released, exerted a product inhibition effect with resultant non-linear kinetics and sub-optimal rates of hydrolysis. This effect was reversed by the addition of Ca^{2+} (as $CaCl_2$) to the assay mixture at a final concentration of 5 mM. A more detailed presentation and discussion of this phenomenon may be found in 3.3.

ii) The long-chain fatty acids, when released, may not be completely ionised and hence the titratable acidity (and therefore apparent activity) is less than the true value. This can be overcome by increasing the ionic strength of the system (Benzonana and Desnuelle 1968). Thus, the assay system was modified by addition of 0.1 M NaCl (final concentration) which lowers the pK of oleic acid ($C_{18:1}$) from 9.2 to 7.8 (Fig.2.3). The presence of both 0.1 M NaCl and 5 mM $CaCl_2$ lowers the pK_a of oleic acid to 6.3, which means that at the assay pH of 7.5 94% of the acid is ionised. Assay systems for substrates releasing long-chain fatty acids ($>C_{10}$) therefore contained 2% (v/v) of

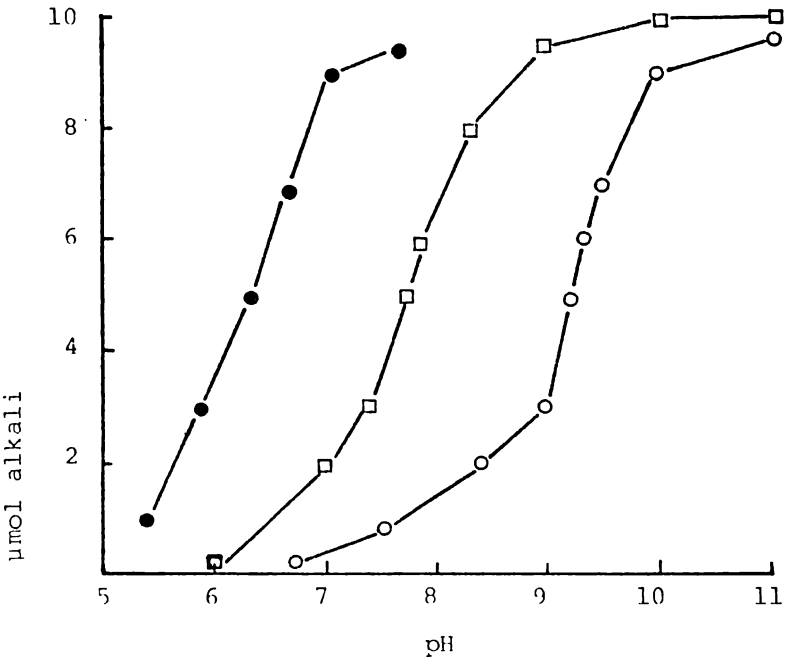


Fig. 2.3 Effect of NaCl and $CaCl_2$ on the titration of oleic acid. 10 µmol of oleic acid in 15 mL of 2% (v/v) olive oil in distilled water was titrated against 0.01 M NaOH. o-o, oleic acid; □ -□, oleic acid + 0.1 M NaCl; ● -●, oleic acid + 0.1 M NaOH + 5 mM $CaCl_2$.

emulsified substrate, 0.1 M NaCl, 5 mM $CaCl_2$ all in a total volume of 15 mL with the subsequent addition of the sample.

Investigation of the effect of pH on enzyme activity is theoretically possible with long-chain substrates providing corrections are made for the changes in degree of ionisation at each pH. In practice, it was found more satisfactory to use tributyrylglycerol or, if long-chain substrates were specifically of interest, to use the organic extraction-colourometric fatty acid analysis described in 2.4.

The continuous potentiometric titration method was unsuitable with enzyme preparations that contained appreciable levels of buffering capacity such as some bacteriological growth media. The effect of such buffering components in the growth media on the observed extracellular lipolytic activities of *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017 was investigated. The cell-free supernatant of these organisms was collected by centrifugation after 48h growth on trypticase soy broth (for *S. aureus*) or olive oil-mineral salts (for *Pseudomonas* 017) according to the procedures outlined in Section 10.2. Both of these media possess considerable buffering capacity. The trypticase soy broth is buffered by peptone and hydrogen phosphate constituents whilst the olive oil-mineral salts medium contains phosphate buffer at a final concentration of 70 mM.

Aliquots of the cell-free supernatants were analysed for activity towards tributyrylglycerol by the standard continuous potentiometric titration technique before and after dialysis against distilled water for 24h. In addition, the concentration of butyric acid at the end of the 10 min assay period was determined by gas chromatography using a Pye Unicam GCD.

Tributyrylglycerol was removed from samples by 5 min. end-on-end shaking with an equal volume of hexane and butyric acid assayed by direct injection of an aliquot (2-10 µL) of the aqueous phase. The glass column used was 1.8 m long x 4 mm internal diameter packed with

15% PEGA (polyethylene glycol adipate) on 100-120 mesh Diatomite C-AW (Pye Unicam, Cambridge, U.K). The column T was 130°C, FID detector T 158°C and injector T 135°C. The carrier gas was N_2 at a flow rate of 35 mL min^{-1}. Hydrogen and air flow rates were 40 and 160 mL min^{-1} respectively. Under the conditions employed butyric acid was eluted 3-4 min after injection. Preliminary experiments with a range of standards demonstrated that >90% of the butyric acid remained in the aqueous phase after hexane extraction of the tributyrylglycerol. The level of butyric acid in samples was determined by comparison to standards prepared in distilled water. The relative response of the detector to samples or standards was calculated by the following simple equation:

$$\text{Relative response (arbitrary units)} = \frac{\text{peak height x retention time x attenuation}}{\text{injection volume}}$$

The relative response of standards was linear ($r > 0.98$) over the range 5 nmol to 200 nmol of butyric acid injected.

The culture supernatants displayed little or no apparent lipase activity when assayed by the standard continuous potentiometric titration procedure (Table 2.2), despite the activity detected by gas-liquid chromatography being well above the apparent detection limit of the method. After dialysis, the activity of the *Pseudomonas* 017 supernatant detected by continuous potentiometric titration approached that observed by gas - liquid chromatography indicating dilution of the interfering buffering components. Activity with the *Staphylococcus aureus* supernatant was observable but still below that recorded by gas-liquid chromatography indicating the persistance of some non-dialysable buffering components. These results demonstrate the inapplicability of a continuous potentiometric titration technique for following patterns of lipase production in bacteriological growth media.

TABLE 2.2 Lipase activity of the cell-free supernatants of *Pseudomonas* 017 and *Staphylococcus aureus* NCIB 6571 as determined by continuous potentiometric titration before and after dialysis (24h, distilled water) and comparison to the activity as determined by gas-liquid chromotography.

METHOD	ACTIVITY (μmol butyric acid min^{-1}. mL^{-1}).	
	Pseudomonas 017	*Staph. aureus*
Continuous potentiometric titration		
i) before dialysis	2.2	0
ii) after dialysis	4.9	0.102
Gas liquid chromatography	5.0	0.132

2.4 Lipase activity assayed by colourimetric determination of released fatty acids

The standard colourimetric procedure adopted for assay of lipase activity by the rate of fatty acid release was as follows:

An emulsion of olive oil (high purity, Sigma Chem. Co., St Louis, USA) was prepared by sonicating a 3% v/v suspension using a Dawe Soniprobe at maximum power for 5 min. Into a 15 mL screw-cap test-tube (teflon-lined caps) was placed 1 mL of freshly-prepared (within 8h) emulsion and 2 mL of 0.1 M Tris-HCl buffer pH 7.5 (with 7.5 mM Ca^{2+} as $CaCl_2$). After 5 min equilibration at 30°C, an aliquot of sample (0.1-0.5 mL) was added and incubated for 30 min. The reaction was stopped by addition of 2 mL of 0.27 M $CU(NO_3)_2$. $3H_2O$ in 0.45 M triethanolamine buffer pH 7.8. Copper soaps of the fatty acids were extracted by addition of 10 mL chloroform (BDH, reagent grade) followed by vigorous end-on-end shaking for 2 min. Reaction tubes were subsequently centrifuged (2000 G for 4 min) to provide a distinct phase boundary. The upper blue aqueous phase was removed by suction, using a finely-drawn pasteur pipette connected to a water vacuum pumo, and discarded. A suitable aliquot (0.1-3.0 mL) of the chloroform layer was removed and diluted to a final volume of 3 mL with chloroform. To this was added 0.5 mL of 9 mM diethyldithiocarbamate (DDC) in n-butanol, mixed and allowed to stand for 10-60 min before measurement in a Beckman DU spectrophotometer at a wavelength of 440 rm with a 1 cm light path.

The reagent blank, used to zero the spectrophotometer contained buffer, copper reagent, chloroform and DDC. The substrat blank contained buffer, substrate, copper reagent, chloroform and DDC. This system provided a check on the purity of the olive oil substrate and its rate of hydrolysis. In addition, a sample blank containing buffer, sample, copper reagent, chloroform and DDC was run to determine the level of interference from fatty acids present in the sample.

Fatty acid standards were prepared using oleic acid ($C_{18:1}$) dissolved in chloroform to final concentrations of 5-100 µM.

10 mL aliquots of these standards were shaken vigorously with 2 mL of the copper reagent, centrifuged and 3 mL quantities of the chloroform phases were subsequently reacted with 0.5 mL of the DDC reagent. By use of this standard curve and suitable conversion factors lipase activity could be expressed as μmol oleic acid released min^{-1} mL^{-1} (or mg^{-1} protein).

With a 3 mL aliquot of the chloroform phase the substrate blank had a mean absorbance at 440 nm of 0.053 with a standard deviation of 0.021 (10 determinations). From the standard curve (Fig.2.4) this standard deviation corresponds to a fatty acid concentration of 2.4 μM in the 10 mL chloroform phase or a total amount of 0.024 μmol. By definition, the detection limit ($4.65 \times S_B$) is therefore that level of enzyme that releases 0.113 μmol oleic acid in the 30 min incubation period or 0.004 μmol oleic acid min^{-1}. This corresponds to a detection limit of 0.054 μg Lowry protein for the *Pseudomonas* 017 lipase, 1.67 μg Lowry protein for the *Staphylococcus aureus* lipase and 0.010 μg Lowry protein for the commercial porcine pancreatic lipase.

The precision of the method was determined at a range of enzyme levels by variable dilution of the *Pseudomonas* 017 preparation. Each dilution was subsampled 10x for subsequent analysis. The data presented in Table 2.3 demonstrates a minimum achievable relative standard deviation for the method of between 4-5%.

The method may be successfully used with incubation at different temperatures (5°C - 70°C) and different pH's (4.0-10.5) with no difference in substrate blank levels.

Whilst the method was routinely used with olive oil (principally trioleoylglycerol) it was capable of being used with other long-chain triacylglycerols down to trilaurylglycerol.

Fatty acid standards of chain length C_8 or less gave low colour responses (Table 2.4) presumably due to the low solubility of these fatty acids in the chloroform. This technique cannot, therefore, be used with short-chain triacylglycerol substrates.

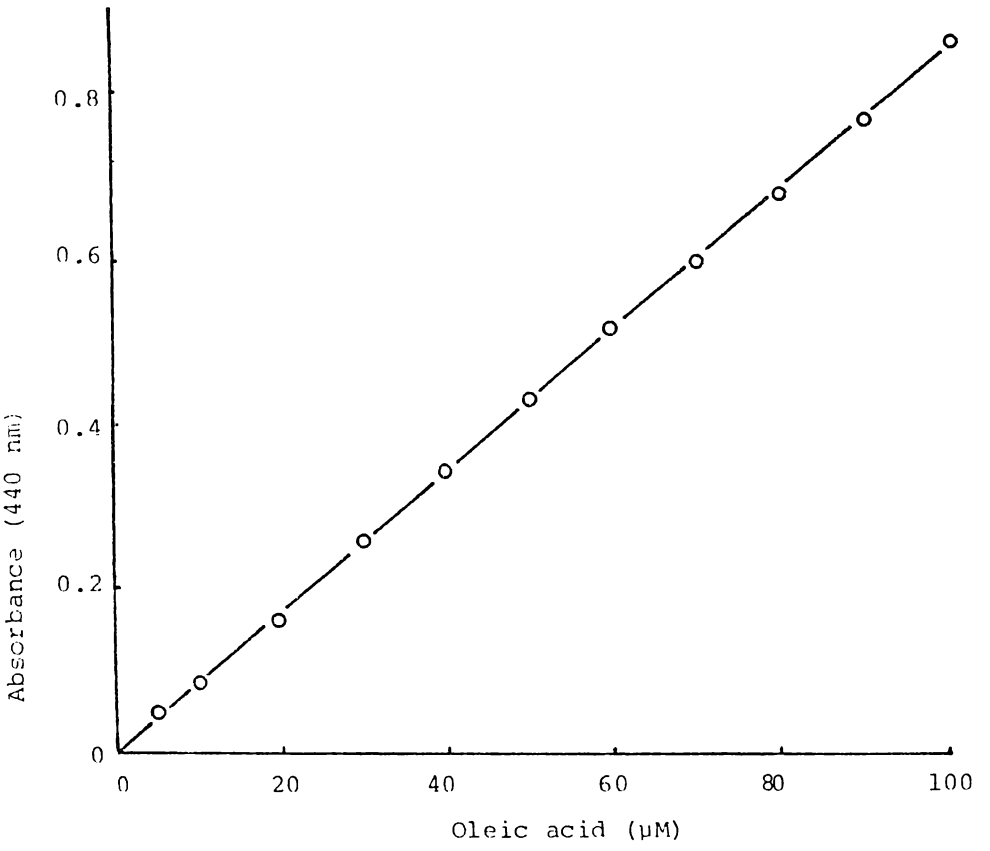


Fig. 2.4 Relationship between absorbance and concentration for solutions of oleic acid in chloroform as determined by the copper soap method.
Correlation coefficient, $r > 0.99$.
Equation : $y = 0.00846\ x + 0.0086$.

TABLE 2.3 The precision of the lipase assay using colourimetric determination of the rate of fatty acid release from an olive oil emulsion. The partially purified *Pseudomonas* 017 lipase was used as the enzyme source (detection limit 0.054 µg Lowry protein).

Enzyme Level (µg Lowry protein)	Mean Activity ($nmol$ fatty acid min^{-1})	Standard Deviation* (μmol fatty acid min^{-1})			Relative Standard Deviation (% mean value)
		S_A	S_B	S_C	
0.1	7	0.9	0.8	1.20	17.2
0.2	17	1.0	0.8	1.28	7.5
0.5	39	1.4	0.8	1.61	4.1
1.0	74	3.0	0.8	3.10	4.2
2.0	150	7.1	0.8	7.14	4.8
10.0	736	37.0	0.8	37.0	5.0

* S_A = Standard deviation of sample values (10 replicates)

S_B = Standard deviation of blank values (10 replicates)

S_C = Standard deviation of the "true" value, with the "true" value, being the sample-blank, calculated as:

$$\sqrt{S_A^2 + S_B^2}$$

TABLE 2.4 The colour response produced by various fatty acids when subjected to the copper soap-chloroform extraction technique. All fatty acids at 100 µM.

Fatty acid	Absorbance 440 nm
Acetic(2:0)	0
Butyric (4:0)	0.002
Caproic (6:0)	0.008
Caprylic (8:0)	0.196
Capric (10:0)	0.820
Lauric (12:0)	0.835
Myristic (14:0)	0.848
Palmitic (16:0)	0.864
Stearic (18:0)	0.865
Oleic (18:1)	0.855

The method was used successfully in this thesis with crude cell culture supernatants as has been done previously by Breuil and Kushner (1975a). However, the value of the method was diminshed when the sample contained high levels of fatty acid which provided a high interfering sample blank.

A problem sometimes encountered with the method was high substrate blanks. These high substrate blanks were occasionally associated with contaminated buffer but more often associated with high levels of fatty acid accumulating in the olive oil with time. For this reason, olive oil was purchased in small quantities and used within 6 months. Substrate blanks with an absorbance at 440 nm of >0.1 were regarded as unsatisfactory. No problems were experienced with the copper and DDC reagents which were routinely stored at 4°C and used within 1 month of preparation.

2.5 Assay of lipase activity using ρ-nitrophenyl laurate

The standard ρ-nitrophenyl laurate assay for lipase activity was:

> To 0.8 mL of 0.1 M Tris-HCl buffer pH 7.5 (with 7.5 mM Ca^{2+} as $CaCl_2$) was added 0.1 mL of 8 mM ρ-nitrophenyl laurate (Sigma Chem. Co., St Louis, USA) in 2-ethoxyethanol. The resultant emulsion was equilibrated at 30°C for 5 min. A 0.1 mL aliquot of sample was then added followed by incubation for 30 min. The final concentration of ρ-nitrophenyl laurate in the assay system was therefore 800 μM. After incubation, 3 mL of acetone was added, the whole was mixed and the absorbance measured at 400 nm in a Beckman DU spectrophotometer (1 cm path length) after 10 min. Non-enzymic rates of ρ-nitrophenyl laurate were determined in the same manner using boiled (5 min) enzyme. The reference cell of the spectrophotometer contained acetone and buffer (3:1). The increase in absorbance was related to a standard curve and converted to μmol ρ-nitrophenol released min^{-1} mL^{-1} (or mg^{-1} protein). The standard curve for ρ-nitrophenol was constructed using a matrix of 3 parts acetone to 1 part 0.1 M Tris-HCl

buffer pH 7.5 (with 7.5 mM Ca^{2+} as $CaCl_2$). The curve was linear over the range 0-1 mM (Fig.2.5).

The mean non-enzymic blank absorbance after 30 min incubation was 0.005 with a standard deviation of 0.002 (10 determinations). By definition, the detection limit is therefore that level of enzyme that produces an absorbance of 0.009 or a final p-nitrophenol concentration of 11.7 µM (from the standard curve) in the 4 mL of acetone-Tris. This converts to 46.8 nmol after 30 min or a rate of 1.56 nmol min^{-1}. This corresponds to a detection limit of 0.0368 µg Lowry protein for the *Pseudomonas* 017 lipase, 8.67 µg Lowry protein for the *Staphylococcus aureus* lipase, and 0.088 µg Lowry protein for the commercial porcine pancreatic lipase.

The precision of the method was determined at a range of enzyme levels by variable dilution of the *Pseudomonas* 017 preparation followed by subsequent repeated (10x) analysis of each dilution. The minimum achievable relative standard deviation of the method was between 4-5% (Table 2.5).

The method was capable of being used at temperatures up to 50°C with no change in mean non-enzymic blank values. When the method was used with incubation at different pH values it was necessary to correct for both the changes in non-enzymic blank values and the changes in the colour response of the released p-nitrophenol. The p-nitrophenyl laurate ester is increasingly unstable at pH's >8.0 resulting in higher rates of non-enzymic hydrolysis. The degree of colour produced by p-nitrophenol is also pH-dependent with highest absorbances occurring at pH's > 10. Consideration was initially given to adding alkali to the final acetone-Tris matrix to increase the colour response, as is done with other p-nitrophenyl ester enzyme assays (*e.g.* Tabatabai and Bremner 1970). However, the increased rate of non-enzymic hydrolysis coupled with its wider variability precluded such an approach.

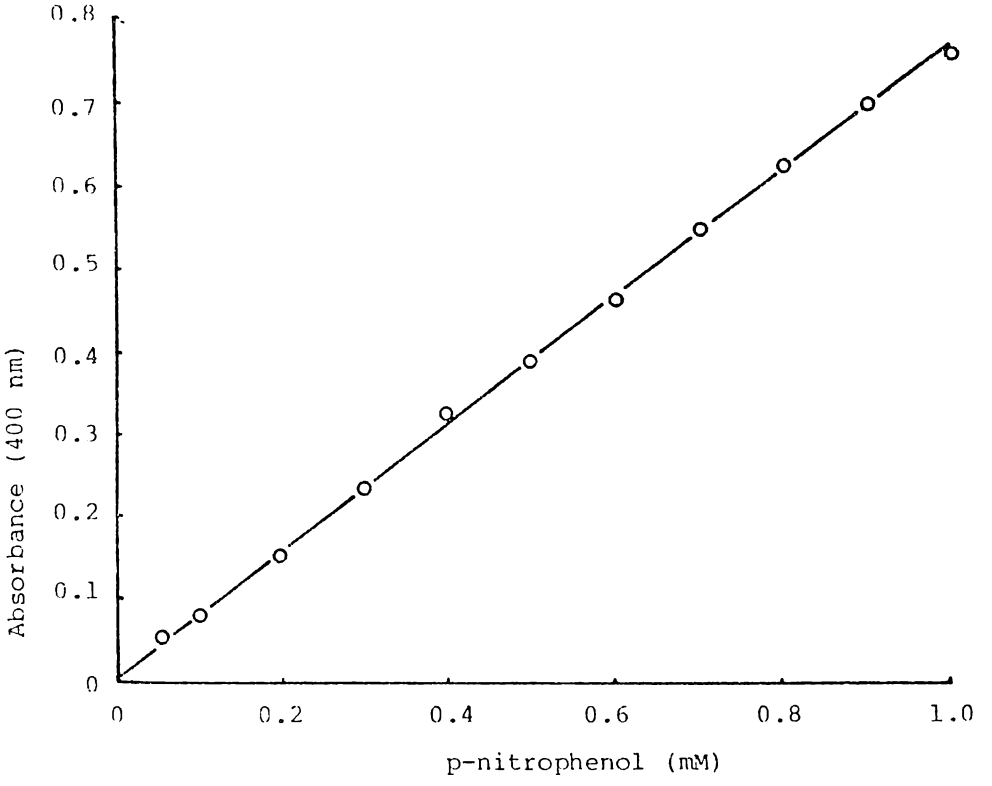


Fig. 2.5 Relationship between absorbance and concentration of p-nitrophenol in a 3:1 acetone-Tris (pH 7.5) matrix. Correlation coefficient, $r > 0.99$. Equation : $y = 0.746\ x + 0.0078$.

TABLE 2.5 The precision of the lipase assay using ρ-nitrophenyl laurate as the substrate. The partially purified *Pseudomonas* 017 lipase was used as the enzyme source (detection limit 0.0368 µg Lowry protein).

Enzyme Level (µg Lowry protein)	Mean Activity (µmol ρ-nitrophenol min^{-1}).	Standard Deviation* (µmol ρ-nitrophenol min^{-1})			Relative Standard Deviation (% mean value)
		S_A	S_B	S_C	
0.05	2.1	0.36	0.34	0.50	23.8
0.10	4.0	0.68	0.34	0.76	19.0
0.20	8.3	0.85	0.34	0.92	11.1
0.50	22.1	1.33	0.34	1.37	6.2
1.00	42.3	2.12	0.34	2.15	5.1
5.00	210.0	8.42	0.34	8.43	4.0

* S_A = Standard deviation of sample values (10 replicates)

S_B = Standard deviation of blank values (10 replicates)

S_C = Standard deviation of the "true" value, with the "true" value being the sample - blank, calculated as: $\sqrt{S_A^2 + S_B^2}$

To test substrate specificity, other esters of p-nitrophenol were used in place of the laurate, with the inclusion of suitable non-enzymic blanks. The p-nitrophenyl esters of acetate and caprylate were less stable with higher non-enzymic blank values, whilst the palmitate ester was more stable. Under the conditions of the assay p-nitrophenyl acetate was partially insoluble and hence can be regarded as a potential substrate for both lipase and esterase.

The p-nitrophenyl laurate substrate solution (8 mM in 2-ethoxyethanol) and the p-nitrophenol standards were stable for 2 weeks when stored at 4°C in the dark. Standards lost 50% of their colour when left at room temperature and exposed to the light for 24h.

2.6 Assay of lipase activity using the fluorogenic substrate 4-methyl umbelliferone nonanoate (4 MUN)

The standard fluorometric assay for lipase activity was:

To 3.0 mL of 0.1 M Tris-HCl buffer pH 7.5 was added 0.1 mL of sample (or suitable dilution) followed by equilibration at 30°C for 5 min. 0.1 mL of 10 mM 4-methyl umbelliferone nonanoate (Koch-Light Laboratories, Colnbrook, England) in 2-ethoxyethanol was added (giving a final concentration of 312 µM) and vortex mixed. The resultant emulsion was placed in a Farrand Mk I spectrofluorometer and zeroed with excitation at 340 nm and emission at 450 nm with 5 nm exit and entrance slits. The assay mixture was incubated at 30°C for 10 min and the increase in fluorescence determined. Rates of non-enzymic substrate hydrolysis were determined using the same procedure except that boiled (5 min) samples were used. The increase in fluorescence was related to a standard curve of 4-methyl umbelliferone (4 MU) and results expressed as µmol 4 MU released min^{-1} mL^{-1} (or mg^{-1} protein). The standard curve for 4MU was constructed with an identical matrix to assay mixtures using boiled samples. The procedure was thus: To 2.7 mL of 0.1 M Tris-HCl buffer pH 7.5 was added 0.1 mL of 10 mM 4 MUN and 0.1 mL of boiled enzyme. The spectrofluorometer was zeroed. To this matrix was added 0.3 mL of a standard 4 MU solution (prepared from a 10 mM stock standard dissolved

in 2-ethoxyethanol and diluted with 0.1 M Tris-HCl pH 7.5) and the fluorescence measured. The standard curve was linear over the final concentration range of 0 - 20 μM (Fig. 2.6).

The mean non-enzymic blank rate of hydrolysis was 0.03 nmol min^{-1} with a standard deviation of 0.01 nmol min^{-1} (10 determinations) By definition, the detection limit is that level of enzyme releasing 0.0465 nmol 4 MU min^{-1}. This corresponds to an enzyme level of 0.536 ng Lowry protein for the *Pseudomonas* 017 preparation, 25 ng Lowry protein for the *Staphylococcus aureus* preparation, and 7.8 ng for the pancreatic lipase.

The precision of the method, determined at various levels of *Pseudomonas* 017 lipase, had a minimum achievable relative standard deviation of 1-2% (Table 2.6).

Whilst the method was routinely used with 4 MUN as substrate, other fatty-acyl-esters of 4 MU were sometimes used to test substrate specificity. When this was done it was necessary to determine non-enzymic blank rates for each substrate as the shorter-chain acyl-esters (4 MU-acetate, 4 MU-butyrate, 4 MU-caproate) were particularly unstable. In addition, separate standards with the appropriate substrate present had to be run. The fluorescent response with the same standard concentration of 4 MU was found to vary, depending upon the substrate present.

The 4MUU substrate solution in 2-ethoxyethanol was stable for 1 month when stored at 4°C. Other fatty-acyl-esters of 4 MU possessed similar stability except for the more unstable 4 MU-acetate and 4 MU-butyrate which were prepared on the day required. The 10 mM stock standard of 4 MU prepared in 2-ethoxyethanol was stable for 6 months when stored at 4°C. Working standards prepared by dilution of this stock with 0.1 M Tris-HCl were stable for at least 1 week at 4°C (determined by ratio of fluorescent response to that given by 1 mg L^{-1} quinine sulphate in 0.05 M H_2SO_4).

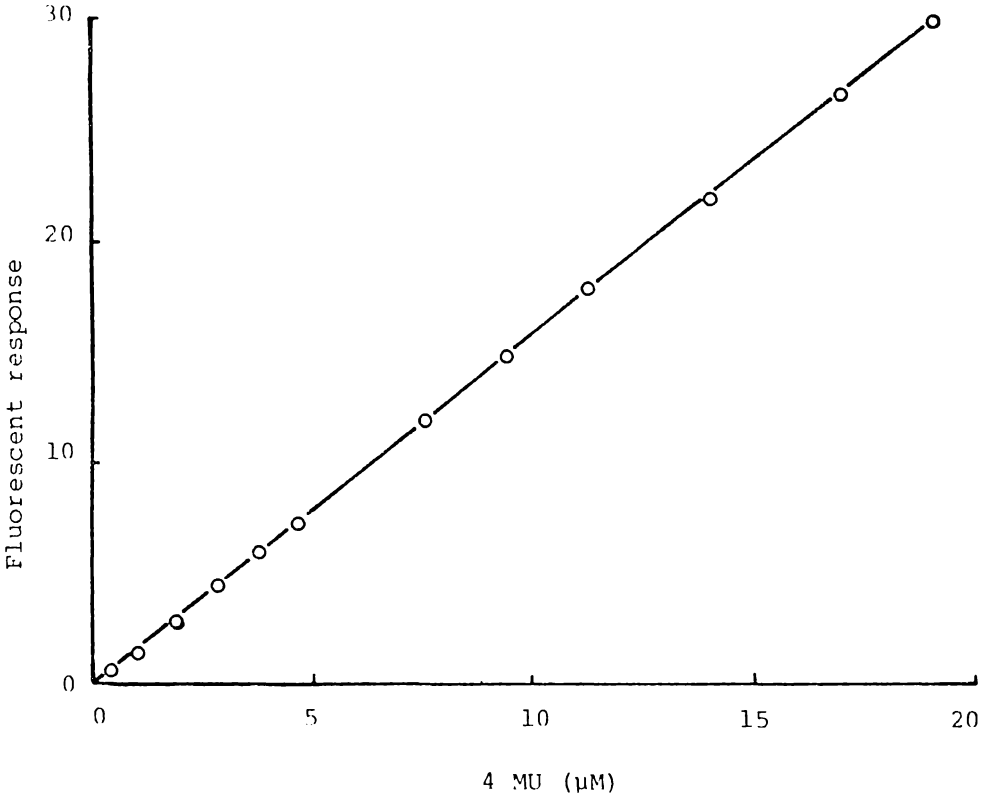


Fig. 2.6. Relationship between fluorescent response and 4-methyl umbelliferone (4 MU) concentration in a matrix of 0.1 M Tris buffer (pH 7.5) and 4-methyl umbelliferone non-anoate. $r > 0.99$.
Equation for the line, $y = 1.57x + 0.002$.

TABLE 2.6 The precision of the fluorometric lipase assay using 4-methyl umbelliferone nonanoate substrate. The partially purified *Pseudomonas* 017 lipase was used as the enzyme source (detection limit 0.536 ng Lowry protein).

Enzyme Level (ng Lowry protein)	Mean Activity (nmol 4MU. min^{-1}).	Standard Deviation (nmol 4MU . min^{-1}).			Relative Standard Deviation (% mean value)
		S_A	S_B	S_C	
1.0	0.08	0.01	0.01	0.014	17.5
2.0	0.17	0.013	0.01	0.016	9.7
5.0	0.44	0.015	0.01	0.018	4.1
10.0	0.87	0.017	0.01	0.020	2.3
20.0	1.73	0.021	0.01	0.023	1.3

The method described was capable of being used at different temperatures providing separate non-enzymic blank rates were determined at each temperature. Non-enzymic blank rates increased with temperatures above 30°C and became unacceptable at temperatures above 50°C.

When examining the relationship between pH and lipase activity with the fluorometric technique several factors had to be considered. The 4 MUN substrate became increasingly unstable at alkaline pH's (Fig. 2.7) and hence non-enzymic blanks had to be performed at each pH investigated. In addition, the fluorescent response of 4 MU and the optimum excitation wavelength varied with pH (emission maximum remained at 450 nm). Fink and Koehler (1970) have previously demonstrated this with the unsubstituted umbelliferone. Scans of excitation wavelength of 4 MU demonstrated that the maximum shifted from 340 nm at pH's <7.3 to 365 nm at pH's >7.5 (Fig. 2.8). At pH's below 7.3 the 365 nm excitation was observed as a shoulder on the main 340 nm peak and above pH 7.5 the situation was reversed. After examining the fluorescent response at each wavelength over the pH range 2.0 - 10.0 (Fig. 2.9), it was decided to adopt 340 nm as the excitation wavelength under all assay conditions. This was done because at the pH region of optimal activity for lipases (approximately pH 6-9) the fluorescent response at 365 nm was extremely pH-dependent.

The assay system did not routinely contain Ca^{2+}, a common co-factor for the expression of lipase activity. It was found that the addition of Ca^{2+} did not affect the rate of attack of 4 MUN by any of the three purified lipases providing the fluorescent response after the 10 min incubation period was in the linear range of 0-20 μM 4 MU (final concentration). The effect was probably due to the low enzyme levels required and the nature of the fatty acids being released (see Section 3.3 for a full discussion of this phenomenon). In systems where fatty acid levels in the sample were suspected to be high, assays were also run with Ca^{2+} (as $CaCl_2$) incorporated into the buffer to give a final concentration of 5 mM.

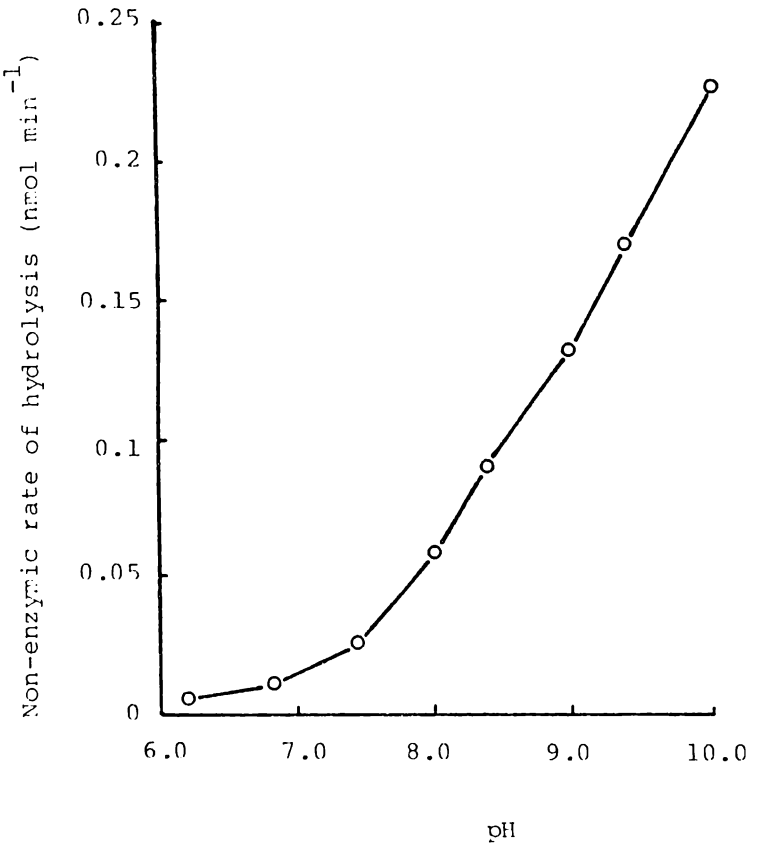


Fig. 2.7. The effect of pH on the non-enzymic rate of 4 MUN hydrolysis.

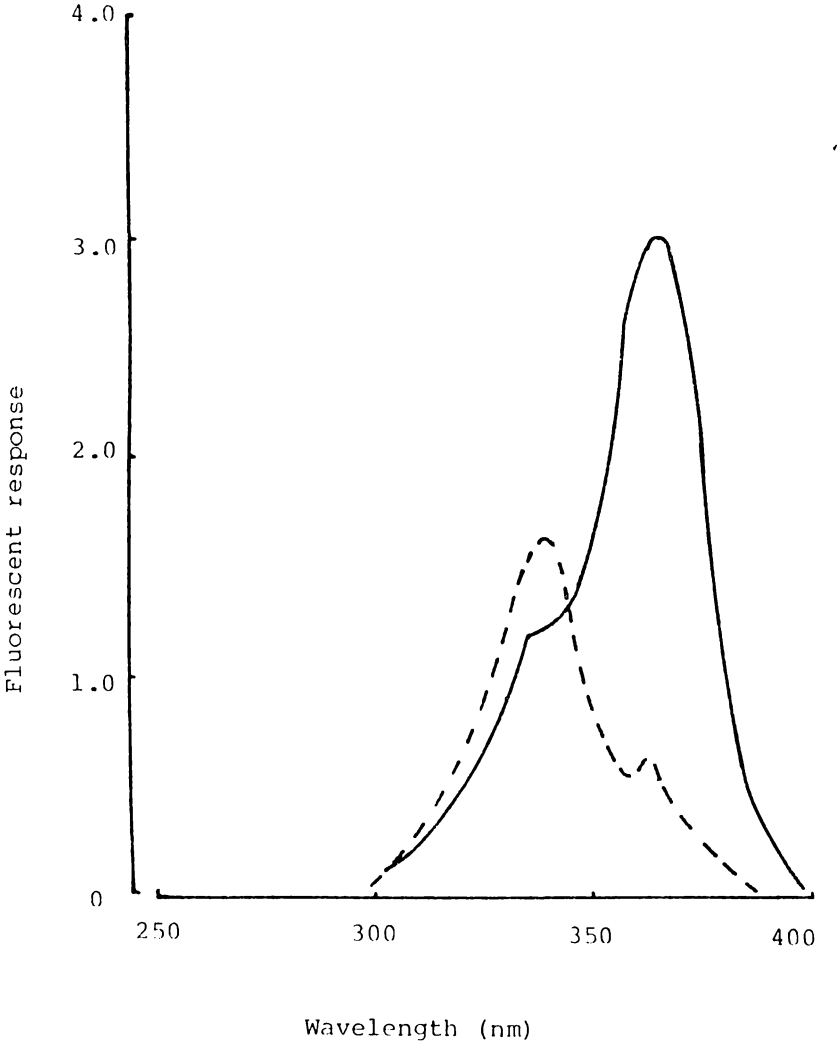


Fig. 2.8. Excitation spectrum of 4-methyl umbelliferone fluorescence at pH 7.0 (- - -) and pH 8.5 (——).

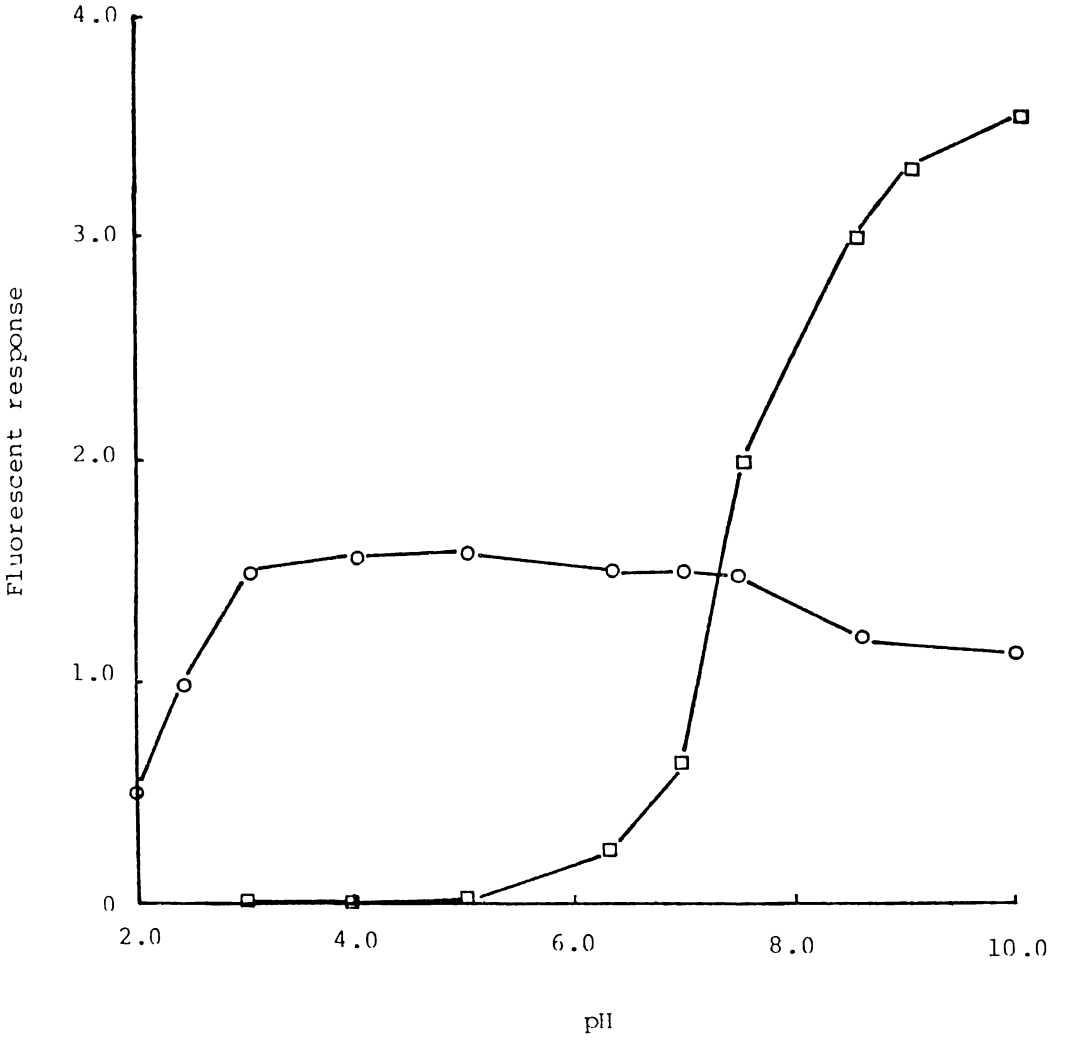


Fig. 2.9. Relationship between fluorescent response and pH for a 1 μM solution of 4-methyl umbelliferone at two excitation wavelengths. o-o, excitation λ = 340; □—□ , excitation λ = 365 nm.

2.7 Summary and discussion

Several assays for lipase activity were examined and detection limits and precision determined. These results are summarised for all four methods in Table 2.7. It becomes apparent from this table that the relative sensitivities of the methods varied depending upon the enzyme source. For example, the fluorometric method was 240 x more sensitive than the continuous potentiometric titration techniques for the *Psuedomonas* 017 lipase, 352 x for the *Staphylococcus aureus* lipase, and 4.5 x for the pancreatic lipase. These differences are simply a reflection of the relative substrate specificities of the 3 enzyme preparations. The pancreatic lipase has a high specificity for triacylglycerol substrates with relatively low rates of attack towards monoesters such as p-nitrophenyl laurate and 4 MUN. In contrast, the bacterial lipases display broader substrate specificities with considerable activity toward artificial esters as well as triacylglycerols.

The fluorometric method possessed the greatest sensitivity and highest precision. It was quick and simple to perform with no further manipulation being required after incubation (unlike the Cu-soap colourimetric fatty acid assay and p-nitrophenyl laurate assay). It was capable of being used with crude enzyme sources, providing suitable matrices for standards were prepared. The fluorometric method was therefore used for routine monitoring of lipase activity throughout this thesis. By definition, lipases are enzymes which attack triacylglycerols to release diacylglycerols plus fatty acids. There has therefore been some controversy in the literature as to the suitability of artificial esters for use in lipase assays, particularly from the viewpoint of enzyme specificity (Barrowman and Borgstrom 1968; Brockerhoff 1969; Melius and Doster 1970). However, Barrowman and Borgstrom (1968) have demonstrated that several artificial esters including p-nitrophenyl laurate, provided the necessary specificity. As the acyl chain-length of the artifical esters increased (and hence their insolubility) their

TABLE 2.7 The detection limits and minimum achievable precision of four methods for determining lipase activity.

METHOD	DETECTION LIMIT (µg Lowry Protein)			PRECISION
	SOURCE			(relative standard deviation)
	Pseudomonas	*S. aureus*	Pancreas	
Continuous potentiometric titration	0.129	8.81	0.035	5.6 %
Cu-soap, colourimetric fatty acid assay	0.054	1.67	0.010	4.1
p-nitrophenyl laurate	0.0368	8.67	0.088	4.0
Fluorometric (4 MUN)	0.000536	0.025	0.0078	1.3

specificity as substrates for lipase increased. However, 4-methyl umbelliferone caprate (C_{10}) was capable of being attacked by both pancreatic lipase and esterase. Therefore, in situations where the identity of the enzyme was in doubt preliminary tests with p-nitrophenyl laurate and/or triacylglycerol substrates were performed.

CHAPTER 3 STUDIES ON LIPASES AND SURFACE-RELATED PHENOMENA

3.1 Introduction

The kinetics of enzymatic catalysis in heterogenous systems has attracted considerable attention in recent years. Many enzymes function naturally in two-phase systems with either the enzyme and/or the substrate present at an interface (McLaren and Packer 1970; Verger and de Haas 1976). The commercial benefits derived from immobilisation of enzymes onto solid surfaces allowing continuous product harvesting has added impetus to studies of enzymatic action in heterogenous systems (Zaborsky 1973; Mosbach 1976). The action of hydrolytic enzymes on lipid substrates represents a system whereby a water-soluble enzyme acts upon a water-insoluble substrate. Such lipolytic enzymes include lipase (EC 3.1.1.3), cholesterol esterase (EC 3.1.1.13), the phospholipases (EC 3.1.1.4, 3.1.4.3, and 3.1.4.4) and lysophospholipase (EC 3.1.1.5)

As is common to the functioning of all enzymes, the essential prerequisite for lipolytic activity is the formation of an enzyme-substrate complex. As lipid substrates are insoluble, the crucial step for the performance of the catalytic reaction becomes the initial adsorption of the enzyme at the interface of the lipid and aqueous phases. Studies by Desnuelle and co-workers (Sarda *et al* 1957; Sarda and Desneulle 1958; Benzonana and Desnuelle 1965) demonstrated that rates of pancreatic lipase activity were dependent upon the interfacial area. They found that the adsorption of enzyme onto the substrate surface was an equilibrium phenomenon with increased adsorbed enzyme (and hence increased activity) occurring with increased surface area of substrate up to a saturation point.

The effect of certain substances on lipolytic activity has been related to modifications that such substances have (or may have) on the surface interaction between enzyme and substrate. Some of the more definitive studies are those of alcohol inhibition (Mattson *et al* 1970), effects of bile salts and colipase (Brown *et al* 1977; Lairon *et al* 1978) and inhibition by phenylcarboxylic acids (Rakhimov and Dzhanbaeva 1977) on lipase activity. The observation that product inhibition of lipolytic catalysis occurs when water-insoluble (but not water-soluble) fatty acids are being produced (Schonheyder and Volqvartz 1945; Shah and Wilson 1965) suggests that such inhibition may also be due to an effect on the properties of the interface (Benzonana and Desneulle 1968). The nature of fatty acid inhibition and its reversal by Ca^{2+} (and other divalent cations) remains poorly understood.

The purpose of the work presented here was to further investigate the role of substrate surface area on lipase activities and elucidate the nature of the effects observed with fatty acids and other surface-active substances.

3.2 Effect of substrate surface area on the activity of three purified lipases

Benzonana and Desnuelle (1965) demonstrated the significance of interfacial area in determining lipolysis rates using porcine pancreatic lipase and two olive oil emulsions of different particle size. Different particle size systems were created by centriguation of a sonicated emulsion and utilising either the larger particles in the upper 'cream' layer or the smaller particles beneath. This method was recognised by the authors as being rather crude, even though consistent results were obtained. The work reported here uses three lipase preparations and four systems with a different interfacial area per unit weight of substrate created by sonication and by adsorption of the substrate (olive oil) onto siliconised glass microspheres of defined

diameter. Unlike the studies of Benzonana and Desnuelle (1968), the assay system did not contain bile salts, which have been shown to alter the kinetics of lipid-lipase systems (Brown *et al* 1977; Lairon *et al* 1978; Sémériva and Desnuelle 1979).

The enzyme preparations used were porcine pancreatic lipase (Grade 1, Sigma Chem. Co., St Louis, USA), and two bacterial lipases obtained from *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017 (a soil isolate) and partially purified as described in 10.2.

Glass microspheres (Duke Scientific Co, Palo Alto, USA) with mean diameters (determined by microscopic observation) of 7.0 µm (standard deviation of 0.6 µm) and 40 µm (standard deviation of 2.0 µm) were siliconised and triacylglycerol coated in the following manner:

Microspheres were detergent rinsed (warm 2% Na_3PO_4), chromic acid soaked for 10h, rinsed to neutrality with distilled-deionised water and dried at 60°C. 1 g of microspheres were then rendered hydrophobic by 24h shaking incubation at 37°C with 10 mL of the silylation reagent 2% (w/v) dichlorodimethylsilane (Supelco Inc., Bellefonte, USA), in dry toluene. Subsequently the toluene was evaporated and 5 mL of a hexane solution of triacylglycerol added (high purity olive oil, Sigma Chem. Co., St Louis, USA) to give a final weight of added lipid of either 4 mg g^{-1} or 20 mg g^{-1} of microspheres. The hexane was evaporated at 60°C with orbital shaking at 200 rev min^{-1}.

An emulsion of olive oil (3% w/v) was prepared in 0.1 M Tris-HCl pH 7.5 (with 5 mM Ca^{2+} as $CaCl_2$) by sonication (2.4). This emulsion possessed a positively-skewed size distribution profile with a mean diameter of 1.11 µm (as determined by a Coulter electronic particle counter fitted with a 30 µm aperture and a multichannel analyser). As the emulsion was stable for at least 8h (number of particles mL^{-1} and size distribution remained the same), substrate was prepared daily, thereby avoiding the need for addition of 'stabilisers' or 'emulsifiers'.

The assays were performed in the following manner:

Into a 15 mL screw-cap test-tube (teflon-lined caps) was placed either various volumes of (0-1.0 mL) of sonicated olive oil emulsion or various weights (5-200 mg) of olive oil coated glass microspheres. All systems were adjusted to a total volume of 1.0 mL by addition of 0.1 M Tris-HCl pH 7.5 (with 5 mM Ca^{2+} as $CaCl_2$). After equilibration at 30°C, 10 µL of enzyme preparation was added, a stopclock initiated, and the system vigourously shaken. The reaction was stopped after 3 min incubation with the addition of 2 mL of the copper reagent used in 2.4. Subsequent determination of released fatty acids was performed by colourimetric analysis as described in 2.4. Enzyme levels (as Lowry protein) in the assays were 0.85 µg for commercial porcine pancreatic lipase, 400 µg for *Staphylococcus aureus* lipase, and 6 µg for *Pseudomonas* 017 lipase.

The amount of lipase not adsorped onto the substrate surface was determined by removing a 0.1 mL aliquot of the liquid phase of the glass microsphere systems, after the incubation period but before the addition of copper, and assaying flourometrically for lipase (2.6). The extent of lipase adsorption was determined by difference, using a control incubated without substrate.

Results obtained for the activity of the three lipases towards four different substrate systems are presented on a substrate weight per unit volume basis in Figs. 3.1 - 3.3 and on an interfacial area (IA) per unit volume basis in Figs. 3.4 - 3.6. Woolf plot transformations ($S.V^{-1}$ *vs* S or $IA.V^{-1}$ *vs* IA) revealed linear plots ($r > 0.95$) for all systems indicating apparent obedience to Michaelis-Menten kinetics. Gross changes in the apparent K_m's could be induced by altering the amount of substrate surface available per unit weight (Table 3.1), whilst the apparent v_{max}'s remained unaltered (within experimental error). However, the apparent K_m(IA) values were constant for each lipase.

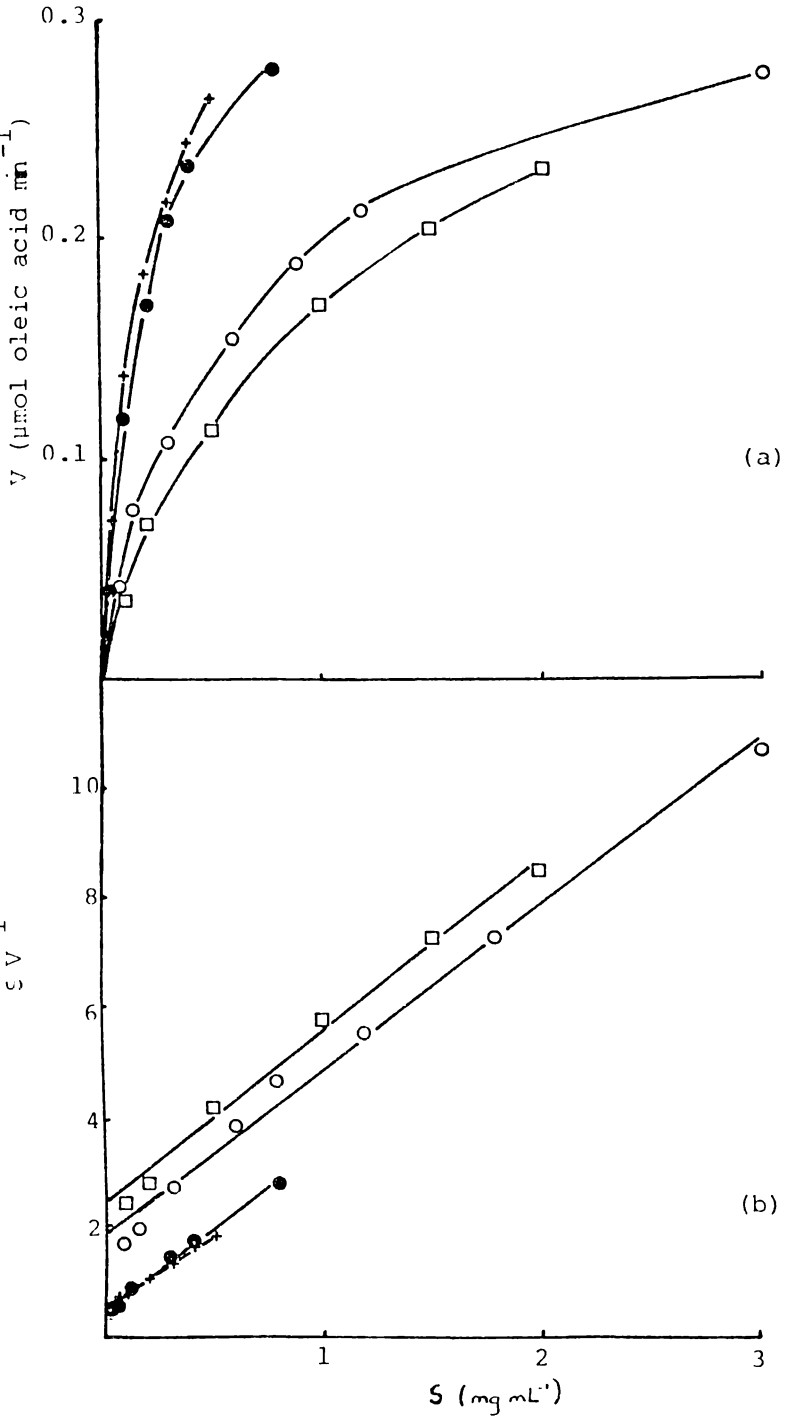


Fig. 3.1: (a) The effect of olive oil concentration(s) on the reaction velocity (v) of pancreatic lipase with systems of various surface area.

: (b) Woolf plot transformation of data.

o-o, sonicated emulsion (surface area = 38.6 cm^2 mg^{-1} substrate)

□ , 20 mg olive oil adsorbed onto 1 g of 40 µm diameter glass beads (surface area = 30.6 cm^2 mg^{-1} substrate).

● , 4 mg olive oil adsorbed onto 1 g of 40 µm diameter glass beads (surface area = 153 cm^2 mg^{-1} substrate).

+ , 20 mg olive oil adsorbed onto 1 g of 7 µm diameter glass beads (surface area = 174 cm^2 mg^{-1} substrate).

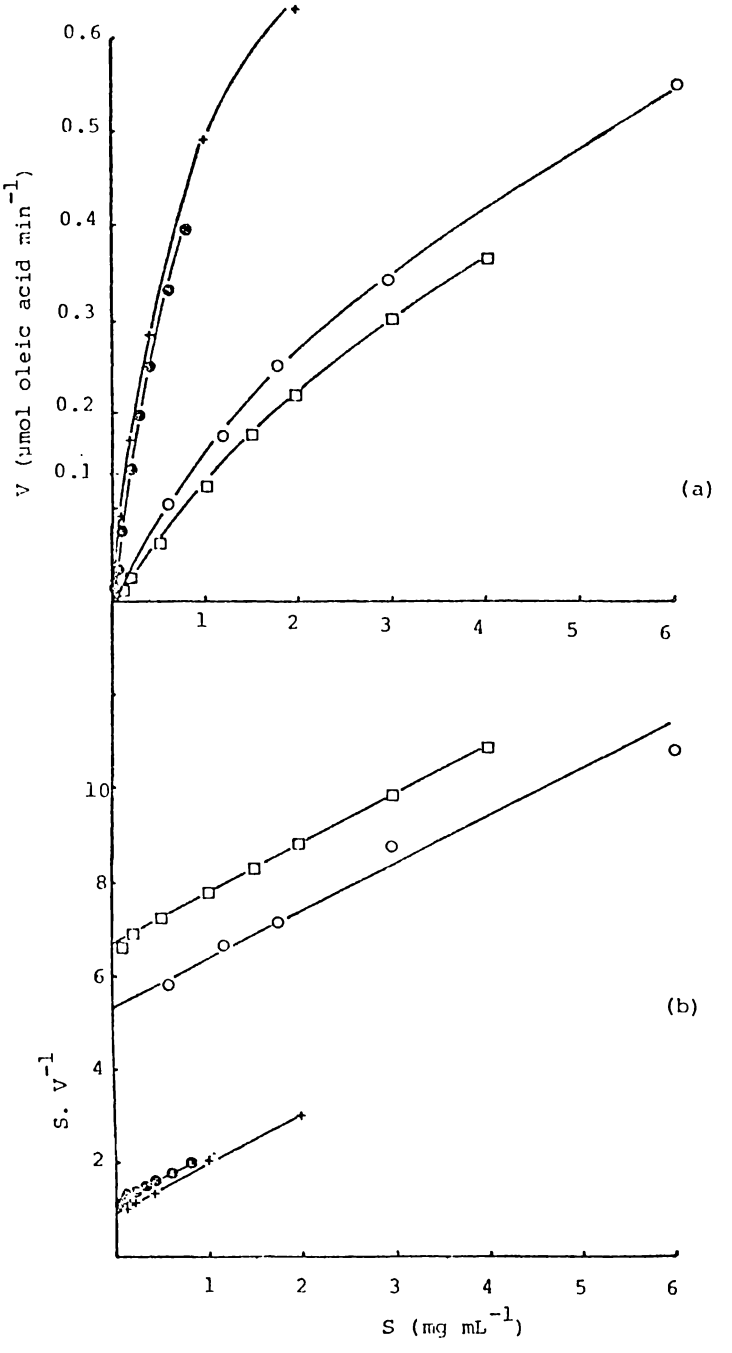


Fig. 3.2: (a) The effect of olive oil concentration(s) on the reaction velocity (v) of *Staphylococcus aureus* NCIB 6571 lipase with systems of various surface area.

: (b) Woolf plot transformation of data.

o-o, sonicated emulsion (surface area = 38.6 cm^2 mg^{-1} substrate)

□ , 20 mg olive oil adsorbed onto 1 g of 40 μm diameter glass beads (surface area = 30.6 cm^2 mg^{-1} substrate).

● , 4 mg olive oil adsorbed onto 1 g of 40 μm diameter glass beads (surface area = 153 cm^2 mg^{-1} substrate).

+ , 20 mg olive oil adsorbed onto 1 g of 7 μm diameter glass beads (surface area = 174 cm^2 mg^{-1} substrate).

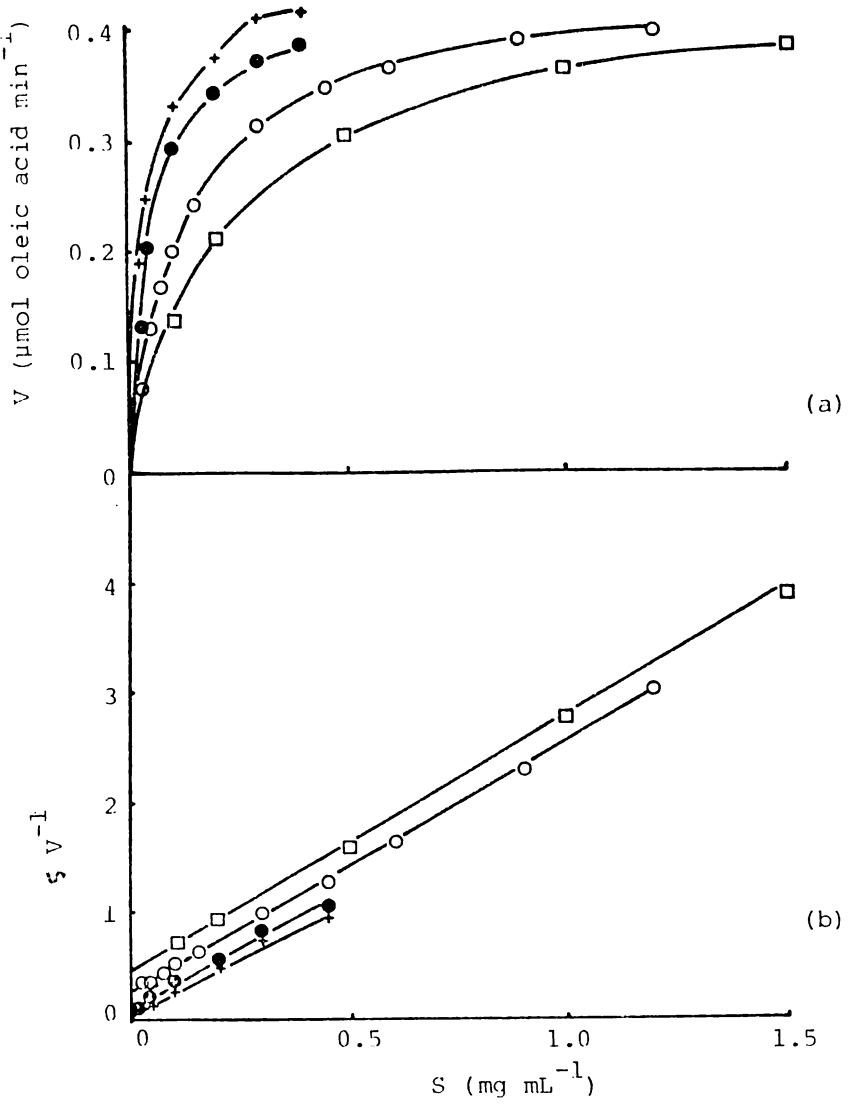


Fig. 3.3: (a) The effect of olive oil concentration(s) on the reaction velocity (v) of *Pseudomonas* 017 lipase with systems of various surface area.

: (b) Woolf plot transformation of data.

o-o, sonicated emulsion (surface area = 38.6 cm^2 mg^{-1} substrates).

□, 20 mg olive oil adsorbed onto 1 g of 40 µm diameter glass beads (surface area = 30.6 cm^2 mg^{-1} substrate).

●, 4 mg olive oil adsorbed onto 1 g of 40 µm diameter glass beads (surface area = 153 cm^2 mg^{-1} substrate).

+, 20 mg olive oil adsorbed onto 1 g of 7 µm diameter glass beads (surface area = 174 cm^2 mg^{-1} substrate).

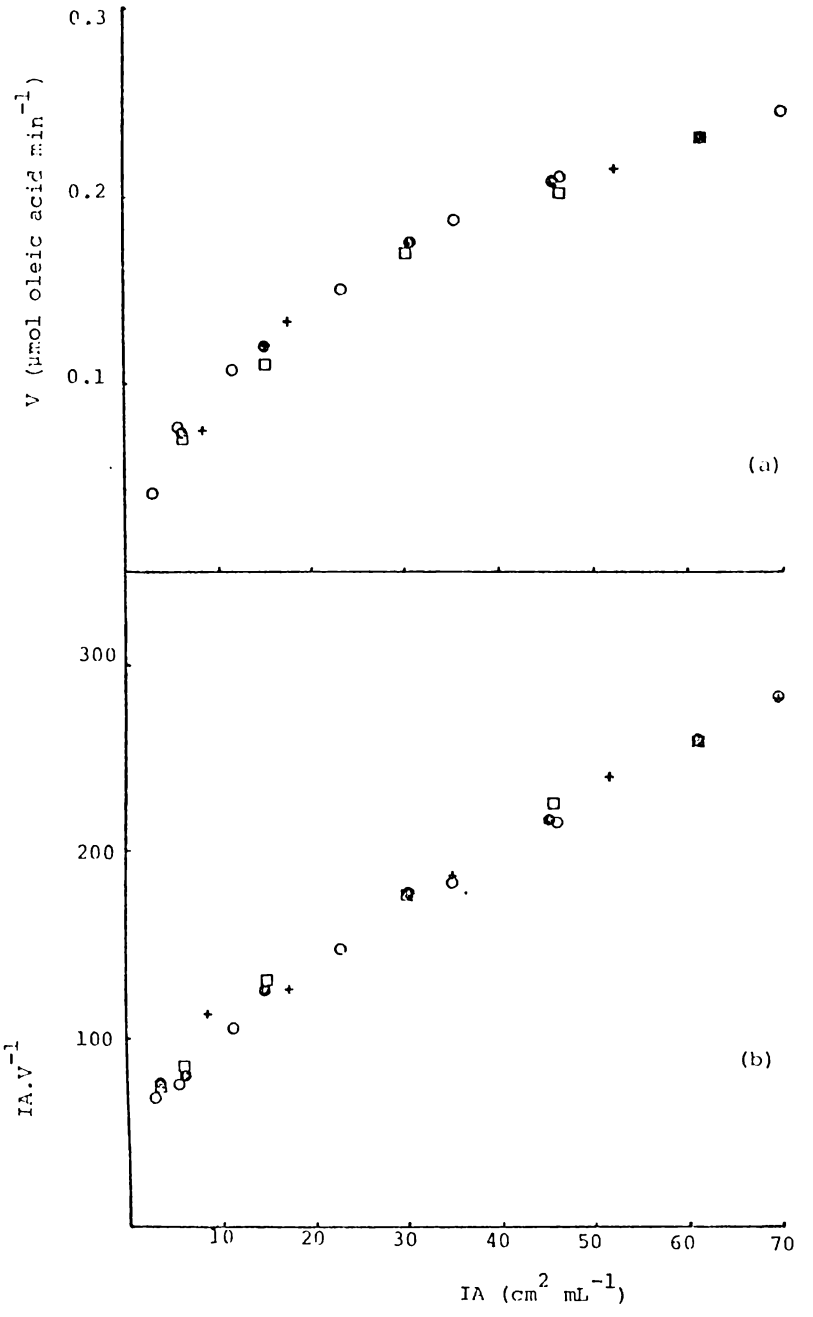


Fig. 3.4:: (a) The effect of interfacial area (IA) on the reaction velocity (V) of pancreatic lipase attack towards olive oil.

: (b) Woolf plot transformation of data.

o-o, sonicated emulsion (surface area = 38.6 cm^2 mg^{-1} substrates).

□ , 20 mg olive oil adsorbed onto 1 g of 40 µm diameter glass beads (surface area = 30.6 cm^2 mg^{-1} substrate).

● , 4 mg olive oil adsorbed onto 1 g of 40 µm diameter glass beads (surface area = 153 cm^2 mg^{-1} substrate).

+ , 20 mg olive oil adsorbed onto 1 g of 7 µm diameter glass beads (surface area = 174 cm^2 mg^{-1} substrate).

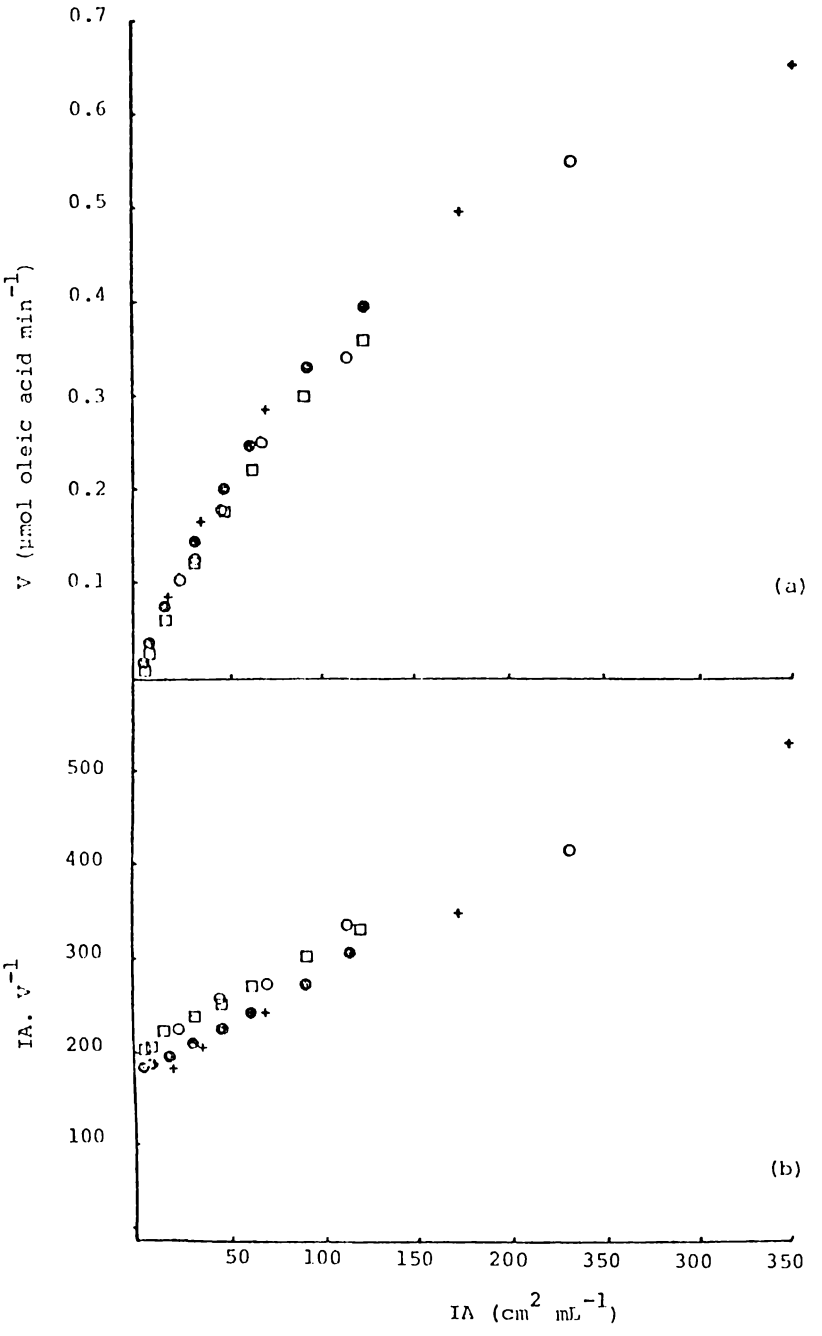


Fig. 3.5: (a) The effect of interfacial area (IA) on the reaction velocity (V) of *Staphylococcus aureus* NCIB 6571 lipase attack towards olive oil.

: (b) Woolf plot transformation of data.

o-o, sonicated emulsion (surface area = 38.6 cm^2 mg^{-1} substrates.

□, 20 mg olive oil adsorbed onto 1 g of 40 μm diameter glass beads (surface area = 30.6 cm^2 mg^{-1} substrate).

●, 4 mg olive oil adsorbed onto 1 g of 40 μm diameter glass beads (surface area = 153 cm^2 mg^{-1} substrate).

+, 20 mg olive oil adsorbed onto 1 g of 7 μm diameter glass beads (surface area = 174 cm^2 mg^{-1} substrate).

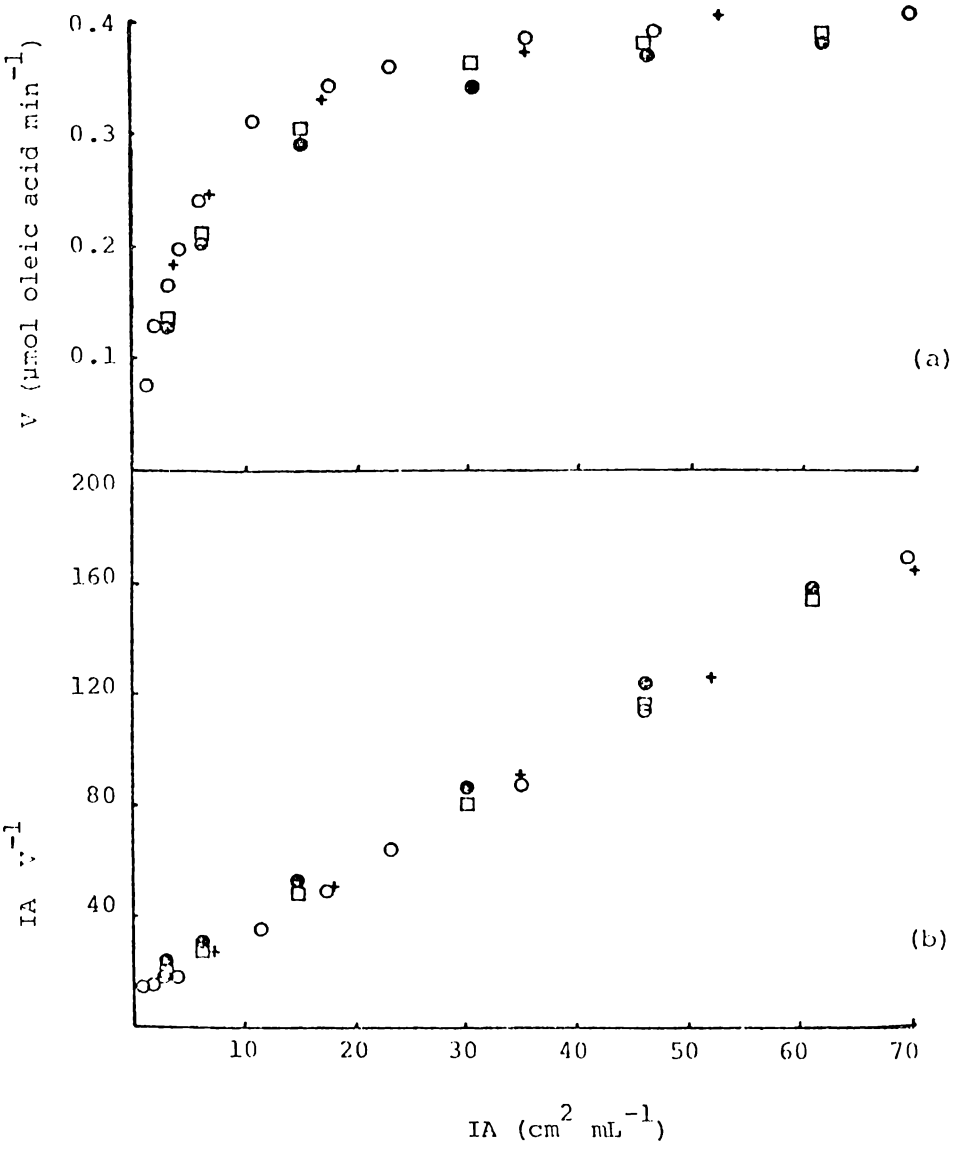


Fig. 3.6: (a) The effect of interfacial area (IA) on the reaction velocity (V) of *Pseudomonas* 017 lipase attack towards olive oil.

: (b) Woolf plot transformation of data.

o-o, sonicated emulsion (surface area = 38.6 cm^2 mg^{-1} substrates).

□ , 20 mg olive oil adsorbed onto 1 g of 40 µm diameter glass beads (surface area = 30.6 cm^2 mg^{-1} substrate).

● , 4 mg olive oil adsorbed onto 1 g of 40 µm diameter glass beads (surface area = 153 cm^2 mg^{-1} substrate).

+ , 20 mg olive oil adsorbed onto 1 g of 7 µm diameter glass beads (surface area = 174 cm^2 mg^{-1} substrate).

TABLE 3.1 Summary of kinetic data obtained for 3 lipases when acting upon olive oil systems containing different interfacial areas per unit of weight. Data obtained from Woolf plots.

Lipase source	Interfacial area per unit weight ($cm^2\ mg^{-1}$)	Woolf plot co-relation coeff. (r)	Apparent K_m $mg\ mL^{-1}$	Apparent v_{max} $\mu mol\ min^{-1}$	Apparent K_m (interfacial area) $cm^2\ mL^{-1}$
Pancreatic	30.6	0.98	0.76	0.31	22.9
	38.6	0.95	0.62	0.33	22.3
	153.3	0.99	0.17	0.34	25.8
	174.0	0.99	0.15	0.35	28.5
S. aureus	30.6	0.95	6.4	0.95	189.0
	38.6	0.99	5.2	0.97	181.0
	153.3	0.96	1.1	0.96	169.0
	174.0	0.99	0.9	0.96	162.0
Pseudomonas 017	30.6	0.99	0.21	0.44	6.3
	38.6	0.99	0.22	0.44	4.7
	153.3	0.97	0.05	0.43	7.1
	174.0	0.97	0.03	0.45	5.6

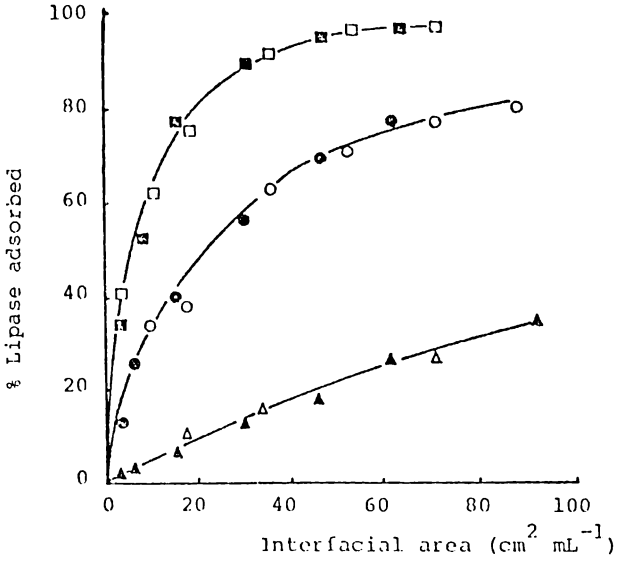
These results support the generally held concept that the kinetics of lipolysis can be explained by replacing the substrate concentration *per se* with an interfacial area term in the standard Michaelis-Menten equation (Benzonana *et al* 1964; Benzonana and Desnuelle 1965; Brockerhoff and Jensen 1974).

Assuming that the apparent K_m(IA) replaces K_m (substrate concentration at 0.5 v_{max}) then such values should be inversely related to the affinity of the lipolytic enzyme for the substrate surface. It would appear from Table 3.1 that the three lipases used possessed widely varying affinities for the substrate surface. As the system is heterogenous, with affinity for the substrate being synonymous with adsorption to the substrate, the relative levels of enzyme adsorption observed with the three lipases (Fig. 3.7a) was predictable. A strong correlation existed between lipase activity (% apparent v_{max}) and % lipase adsorbed (Fig. 3.7b).

The nature of the adsorption process of lipase to a lipid surface is presumably *via* a hydrophobic binding mechanism (Brockerhoff and Jensen 1974; Rakhimov and Dzhabaeva 1977), an assumption supported by the effect of substances which create or diminish surface charge at the interface (Brown *et al* 1977). It is therefore reasonable to suggest that the relative degrees of hydrophobicity of the three enzymes follows the inverse of their apparent K_m(IA)'s *i.e.*, *Pseudomonas* 017 lipase > pancreatic lipase > *Staphylococcus aureus* lipase.

3.3 Fatty acid inhibition of partially purified bacterial lipases

Despite the apparent acceptance that calcium ions should be added to lipase assay systems to overcome fatty acid inhibition (Brockerhoff and Jensen 1974), little definitive work on the nature of the inhibition could be found in the published literature. Fatty acid inhibition of lipase activity has been assumed to be a surface phenomenon whereby ionised water-insoluble fatty acids adsorb at the substrate-



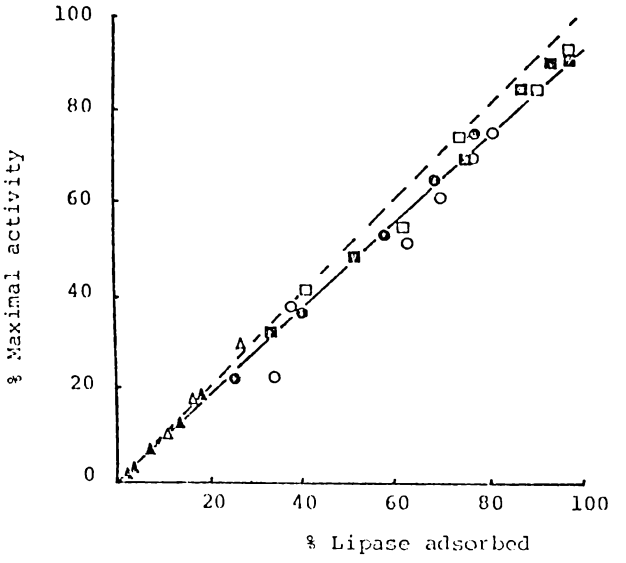


Fig. 3.7: (a) Adsorption of three lipase enzyme preparations as a function of available substrate interfacial area. Lipases of *Pseudomonas* 017, □—■; porcine pancreas, O-●; *Staphylococcus aureus*, Δ-▲. Open symbols represent data obtained from systems with 40 μm diameter glass beads. Closed symbols represent data obtained from systems with 7 μm diameter glass beads.

: (b) Relationship between adsorption and activity for the 3 lipase preparations. Correlation coefficient, r = 0.995, slope = 0.926. Dashed line represents ideal relationship (r = 1.0, slope = 1.0).

aqueous interface creating a negative surface charge thereby preventing formation of the lipid-lipase complex (Benzonana and Desnuelle 1968; Dawson 1968). However, the study of Smith and Alford (1966) with a *Pseudomonas* lipase demonstrated fatty acid inhibition that was time-dependent and not reversed (but neutralised) by Ca^{2+} addition. Apparently, the effect of fatty acid was to irreversibly denature the enzyme rather than to affect the surface interaction. Also, these workers found little or no inhibition occurred with a number of long-chain, water-insoluble saturated fatty acids. In this study, attempts were made to further elucidate the nature of fatty acid inhibition and the role of Ca^{2+}.

The differential effect that added Ca^{2+} ions (as $CaCl_2$, final concentration 5 mM) had on the observed catalytic activity of the two bacterial lipases towards various triacylglycerols is summarised in Table 3.2. The data presented was calculated from the total alkali consumed in 5 min of incubation in the potentiometric assay system (2.3). When soluble fatty acids were being released Ca^{2+} did not influence lipase activity whilst Ca^{2+} stimulation was observed with the release of insoluble fatty acids, a finding consistent with previous studies (Shah and Wilson 1965; Benzonana and Desnuelle 1968; Entressangles and Desnuelle 1968).

A closer examination of the pattern of alkali consumption during the assay period revealed linear rates when soluble fatty acids were being released with and without Ca^{2+} present. However, in systems where insoluble (or partially soluble) fatty acids were being released alkali consumption was linear in the presence of Ca^{2+} but hyperbolic in the absence of Ca^{2+} (Fig.3.8). With substrates containing long-chain fatty-acyl groups the time before divergence of the rate curves with and without Ca^{2+} was dependent upon the fatty acid being released and the level of enzyme in the assay system (Table 3.3). Apparently, a minimum level of fatty acid had to be reached before inhibition of

TABLE 3.2 Influence of calcium ions (5 mM) on the enzymatic activity of two purified bacterial lipases towards a variety of triacylglycerols.

SUBSTRATE	ENZYME ACTIVITY (% of activity without Ca^{2+})	
	Pseudomonas 017	*Staphylococcus aureus*
Tributyrylglycerol	100	100
Tricaprolyglycerol	100	100
Tricaprylylglycerol	100	100
Tricaprylglycerol	100	100
Trilaurylglycerol	165	118
Trimyristylglycerol	182	153
Tripalmintoylglycerol	265	154
Tristearoylglycerol	325	154
Trioleoylglycerol	580	182
Trilinolylglycerol	765	221

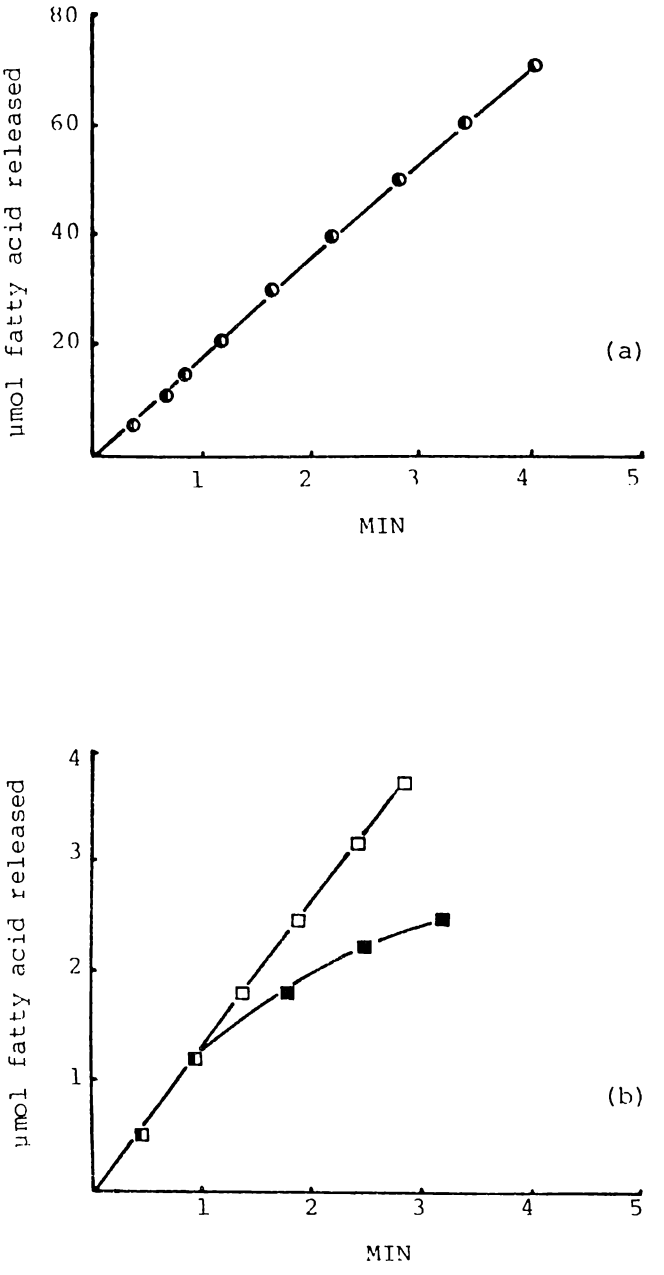


Fig. 3.8: Patterns of fatty acid release during the potentiometric assay of *Pseudomonas* 017 lipase activity toward the triacylglycerols, (a) tributyrylglycerol and (b), trimyristylglycerol. Open symbols with Ca^{2+} (5 mM), closed symbols without Ca^{2+}. Half-shaded symbols represent overlapping points.

lipase activity (and reversal by Ca^{2+}) became noticeable. This minimum inhibitory concentration (MIC) decreased with increasing fatty-acyl chain-length, indicating a relationship with relative aqueous solubilities. The further decreases in MIC observed when double-bonded fatty acids were being released may be a result of greater steric hindrance at the interface due to their 'kinked' structure.

The differential effect of Ca^{2+} on the enzymic hydrolysis of artificial lipase substrates is summarised in Table 3.4. All assays were performed according to the standard procedures of 2.5 and 2.6 except for the addition or deletion of Ca^{2+} and the use of shortened incubation periods. Comparison of results obtained for various lipase substrates (Tables 3.3 and 3.4) demonstrate that the effect of Ca^{2+} was dependent upon both the alcohol moiety of the esters and the fatty acid being released. The influence of the alcohol moiety is a reflection of the different levels of fatty acid released during the assay period. If each fatty acid has a minimum concentration before it inhibits the activity of a lipase on a specific substrate, then if the assay system was such that this MIC was not reached during the incubation period no effect of Ca^{2+} would be observed.

In order to gain further information on fatty acid inhibition (and, in particular, the "MIC concept") assay systems were amended with fatty acids. The use of substrates which apparently demonstrated an effect of released fatty acids on activity (*i.e.*, a Ca^{2+} influence) during the assay period were obviously unsuitable for these studies. Studies were performed primarily with 4-methyl umbelliferone non-anoate with some work using tributyrylglycerol.

Fatty acid amendments to assay systems were made as aqueous solutions of their neutralised sodium salts. The Na salts were prepared by dissolving the appropriate acids in ethanol and neutralising with ethanolic NaOH (1M). The precipitated salts were washed with ethanol and stored dry under vacuum over H_2SO_4, being prepared as

TABLE 3.4 Effect of Ca^{2+} (5 mM) on the activity of 2 bacterial lipases towards the esters of ρ-nitrophenol and 4-methyl umbelliferone and the level of fatty acid present at the end of the incubation period.

SUBSTRATE	LIPASE*			
	Staph. aureus		*Pseudomonas* 017	
	% of activity without Ca^{2+}	Fatty acid level (μM)	% of activity without Ca^{2+}	Fatty acid level (μM)
ρ-nitrophenol esters:				
acetate	100	35	100	140
caprylate	100	90	119	419
laurate	128	134	145	400
palmitate	134	70	156	256
4-methyl umbelliferone esters:				
	100	0.6	100	5.5
butyrate	100	0.6	100	5.5
nonanoate	100	3.6	100	10.5
laurate	100	2.0	100	8.4
palmitate	100	0.8	100	2.2
oleate	100	0.9	100	8.8

* Enzyme levels (as Lowry protein) were 10 μg per assay for *Staph. aureus* for both ρ-nitrophenyl and 4-methyl umbelliferone esters. Enzyme levels (as Lowry protein) were 4 μg and 0.4 μg per assay for *Pseudomonas* 017 for ρ-nitrophenol and 4-methyl umbelliferone esters respectively. Assay periods were 1 min for 4-MU ester assays and 10 min for ρ-nitrophenol assays.

aqueous solutions prior to use.

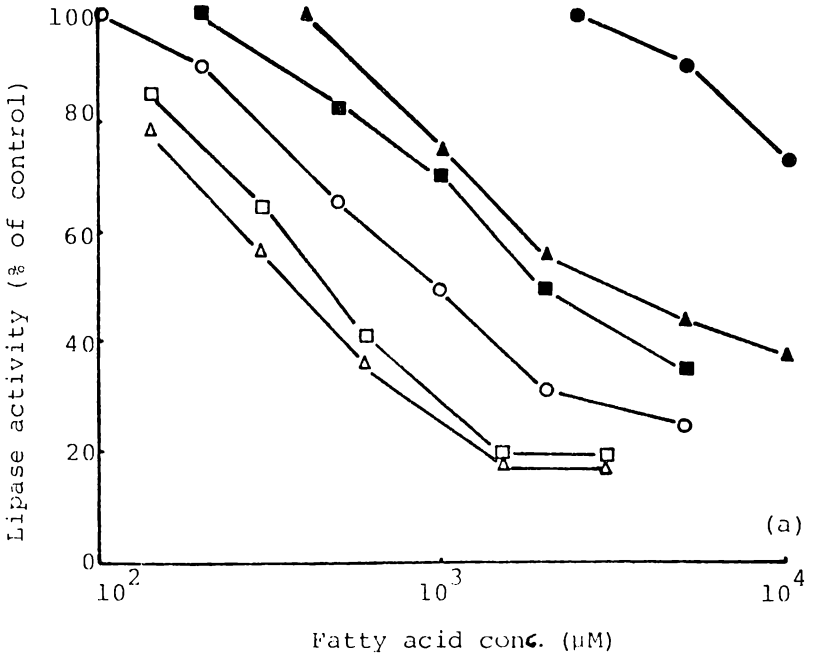
Both the staphylococcal and pseudomonad lipase preparations were inhibited in activity toward 4 MUN by insoluble or partially soluble fatty acids (Figs. 3.9 and 3.10). At any given concentration of the fatty acids the level of inhibition increased with increased insolubility (*i.e.*, increasing chain length), a finding consistent with the studies on triacylglycerol substrates (Table 3.3) and with those of Rakhimov and Dzhanbaeva (1977) with phenyl derivatives of a range of fatty acids. Plots of the logarithm of fatty acid concentration causing 50% inhibition against fatty acid chain-length (Log I_{50} *vs* n) gave straight lines for both lipases (Fig. 3.11a) as did a plot of the logarithm of fatty acid aqueous solubilities against chain length (Fig. 3.11b). It is apparent from Figs. 3.9 and 3.10 that a minimum inhibitory concentration (MIC) of fatty acid must be reached before lipase activities are depressed. This MIC was directly related to the aqueous solubilities of the fatty acids but did not coincide with saturation concentrations (Table 3.5). The previous observation (Table 3.4) that activity toward 4 methyl umbelliferone esters was unaffected by Ca^{2+} was due to the fact that the MIC's of the fatty acids were not reached during the assay period.

The MIC's were similar for both lipases despite the observation that at fatty acid concentrations above this MIC the staphylococcal lipase was inhibited to a greater extent (Fig. 3.11a). However, the apparent MIC's of fatty acids affecting lipase activity towards other substrates (traicylglycerols and ρ-nitrophenyl esters) were markedly different (Tables 3.3 and 3.4). In addition, the MIC of added oleate was higher in systems with tributyrylglycerol as substrate than in systems with 4 MUN (Fig 3.12). These results suggest that the inhibitory effect was an indirect one involving an interaction with the substrate rather than with the enzyme.

Fig. 3.9. Effect of various levels of fatty acids on the activity of the lipase of *Staphylococcus aureus*. Butyrate had no inhibitory effect up to 10^4 µM.

(a) Valeric (C_5), ●
Caproic (C_6), ▲
Caprylic (C_8), ■
Capric (C_{10}), ○
Lauric (C_{12}), □
Myristic (C_{14}), △

(b) Palmitic (C_{16}), ○
Stearic (C_{18}), ▲
Oleic ($C_{18:1}$), △
Linoleic ($C_{18:2}$), □


Lipase activity (% of control)
100
80
60
40
20
0
10^2
10^3
10^4
(a)
Fatty acid conc. (μM)

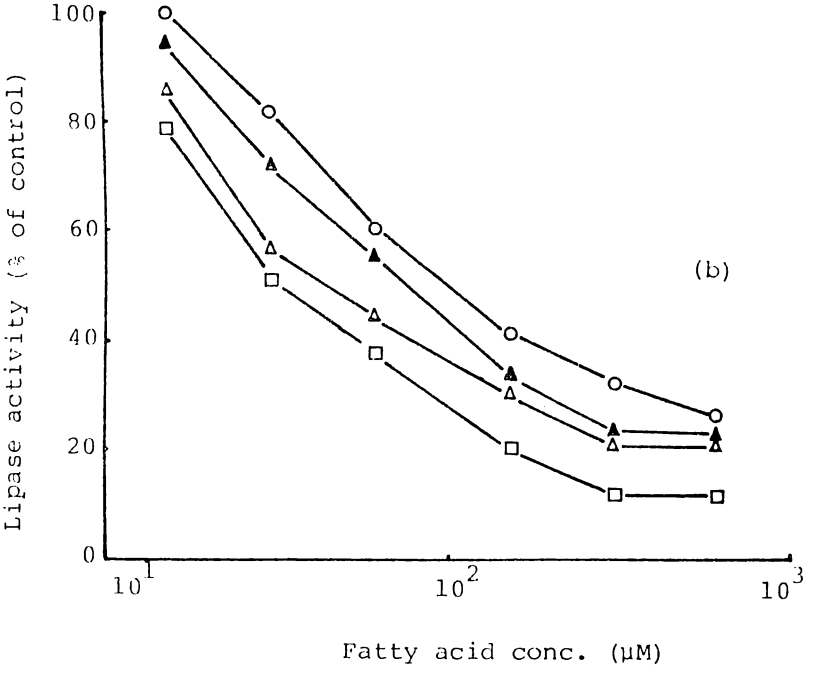
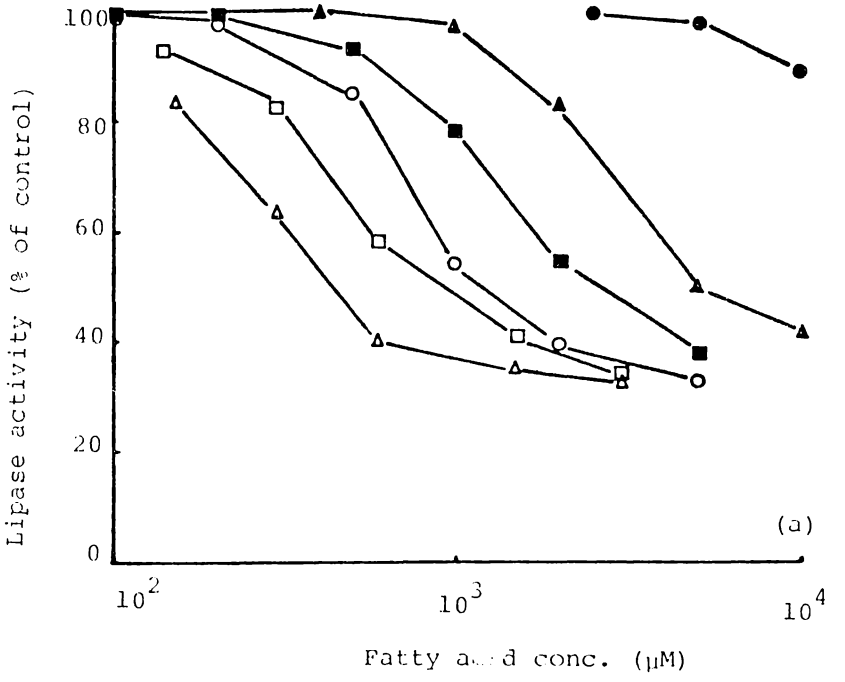
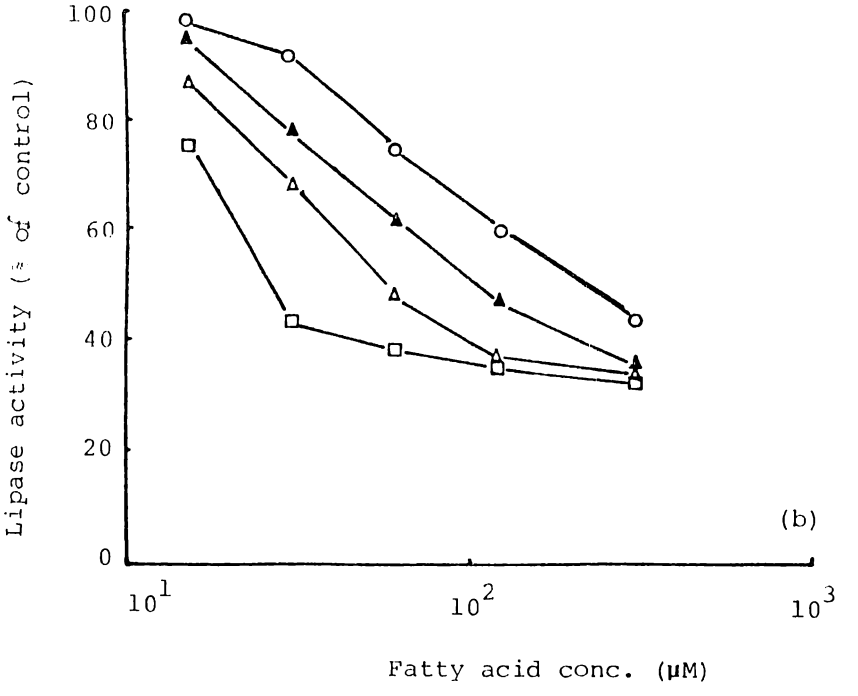

Lipase activity (% of control)
100
80
60
40
20
0
10^1
10^2
10^3
(b)
Fatty acid conc. (μM)

Fig. 3.10. Effect of various levels of fatty acids on the activity of the lipase of *Pseudomonas* 017. Butyrate had no inhibitory effect up to 10^4 μM

(a) Valeric (C_5), ●
Caproic (C_6), ▲
Caprylic (C_8), ■
Capric (C_{10}), o
Lauric (C_{12}), □
Myristic (C_{14}), Δ

(b) Palmitic (C_{16}), o
Stearic (C_{18}), ▲
Oleic ($C_{18:1}$), Δ
Linoleic ($_{18:2}$), □


Lipase activity (% of control)
100
80
60
40
20
0
(a)
10^2
10^3
10^4
Fatty acid conc. (μM)


Lipase activity (% of control)
100
80
60
40
20
0
(b)
10^1
10^2
10^3
Fatty acid conc. (μM)

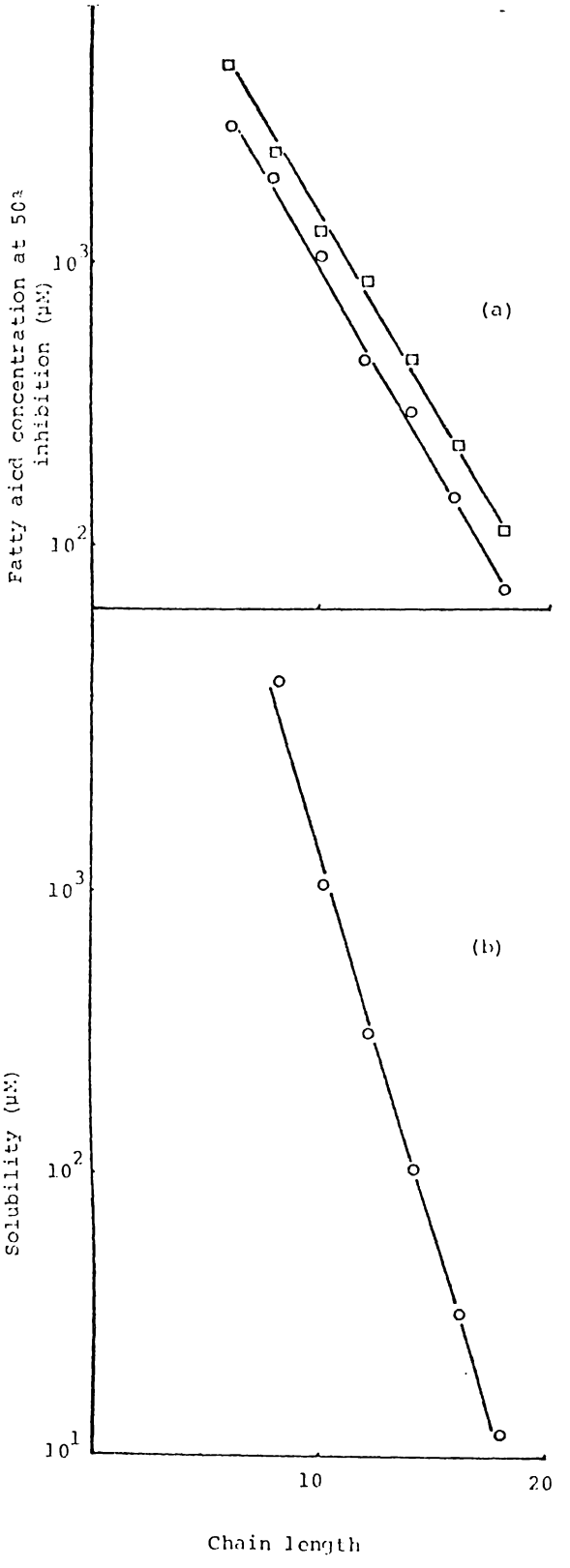


Fig. 3.11. Effect of fatty acyl chain length on:

(a) inhibition of lipases from *Staphylococcus aureus* (o-o) and *Pseudomonas* 017 (□-□).

(b) aqueous solubility (data from CRC Handbook of Biochemistry).

TABLE 3.5 Aqueous solubilities and minimum inhibitory concentrations of fatty acids in systems with 4 MUN as substrate and the lipases of *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017.

Fatty Acid	Aqueous Solubility (μM)	MIC (μM)	
		Staph.aureus	*Pseudomonas* 017
Caproate (6:0)	8.78×10^4	4.5×10^2	8.5×10^2
Caprylate (8:0)	5.48×10^3	2.3×10^2	3.8×10^2
Caprate (10:0)	1.05×10^3	1.4×10^2	1.7×10^2
Laurate (12:0)	3.14×10^2	80	80
Myristate (14:0)	1.05×10^2	65	65
Palmitate (16:0)	32.4	18	25
Stearate (18:0)	12	14	14
Oleate (18:1)	-	11	10
Linoleate (18:2)	-	9	9

* Enzyme levels (as Lowry protein) were 10 μg and 0.4 μg per assay for *Staph. aureus* and *Pseudomonas* 017 preparations respectively. Assay period was 1 min.

\- Data not available.

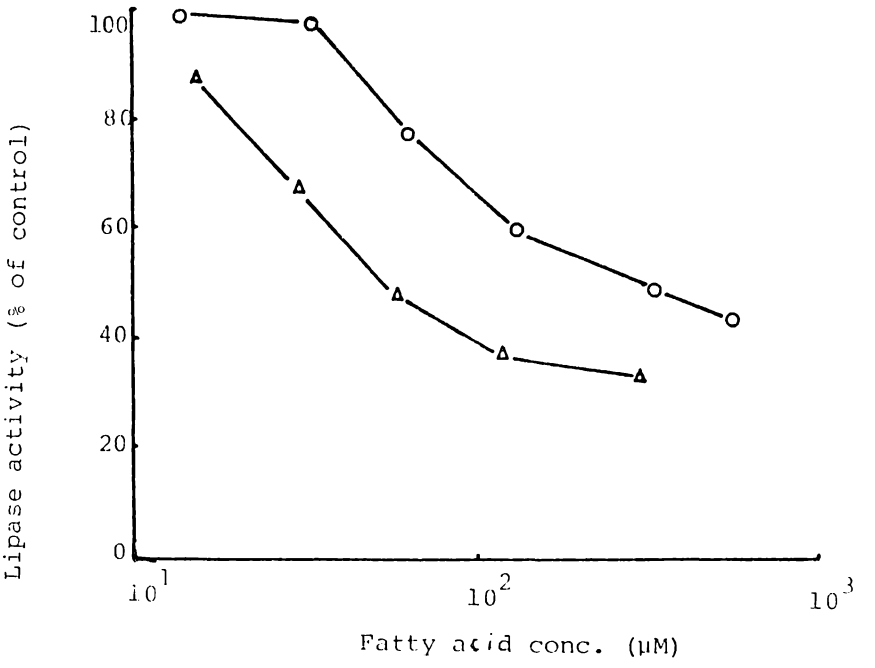


Fig. 3.12: Effect of various levels of oleate on the activity of *Pseudomonas* 017 lipase toward 4-MUN (Δ-Δ) and tributyrylglycerol (o-o).

Changes in substrate concentration induced a classical "competitive inhibition" response with Woolf plots ($S.V^{-1}$ *vs* S) of fatty acid inhibited systems (Figs. 3.13 and 3.14) demonstrating similar slopes (therefore similar apparent v_{max}'s) but different y-intercepts (therefore different apparent K_m's) (Table 3.6). Competitive inhibition, in the classical sense, results from competition between the inhibitor and the substrate for the active site of the enzyme. As the lipolytic reaction occurs at a substrate-aqueous interface the inhibition observed with fatty acids could be rationalised by a competition between inhibitor and enzyme for available substrate surface, an explanation offered by other workers (Benzonana and Desnuelle 1968; Rakhimov and Dzhanbaeva 1977). The concept of a minimum inhibitory concentration (MIC) and its dependence on fatty acid chain-length developed in the work reported here can be accommodated in such a theory by assuming that this MIC is the level where fatty acids are occupying enough of the surface to begin to limit the availability of substrate to the enzyme. The greater the insolubility of the fatty acid the more it would partition at the interface and therefore the more its inhibitory effect and the lower its MIC. However, such a "steric hindrance" theory has difficulty in explaining the reversals observed with Ca^{2+} ions. The Ca-fatty acid soaps formed are insoluble and would therefore still occupy the interface. An alternative explanation for fatty acid inhibition and reversal by Ca^{2+} ions may be postulated from consideration of surface charge phenomena. Adsorption of fatty acids at the interface would be with the uncharged alkyl groups at (or in) the lipid and the hydrophilic carboxyl end exposed to the aqueous medium (Dawson 1968). Thus, the previously uncharged lipid substrate surface would become negatively-charged and hence lipase adsorption and subsequent activity is diminished. This 'charge-repulsion' theory would accommodate the effects of Ca^{2+} (and other divalent cations) in the role of a neutraliser of this negative charge.

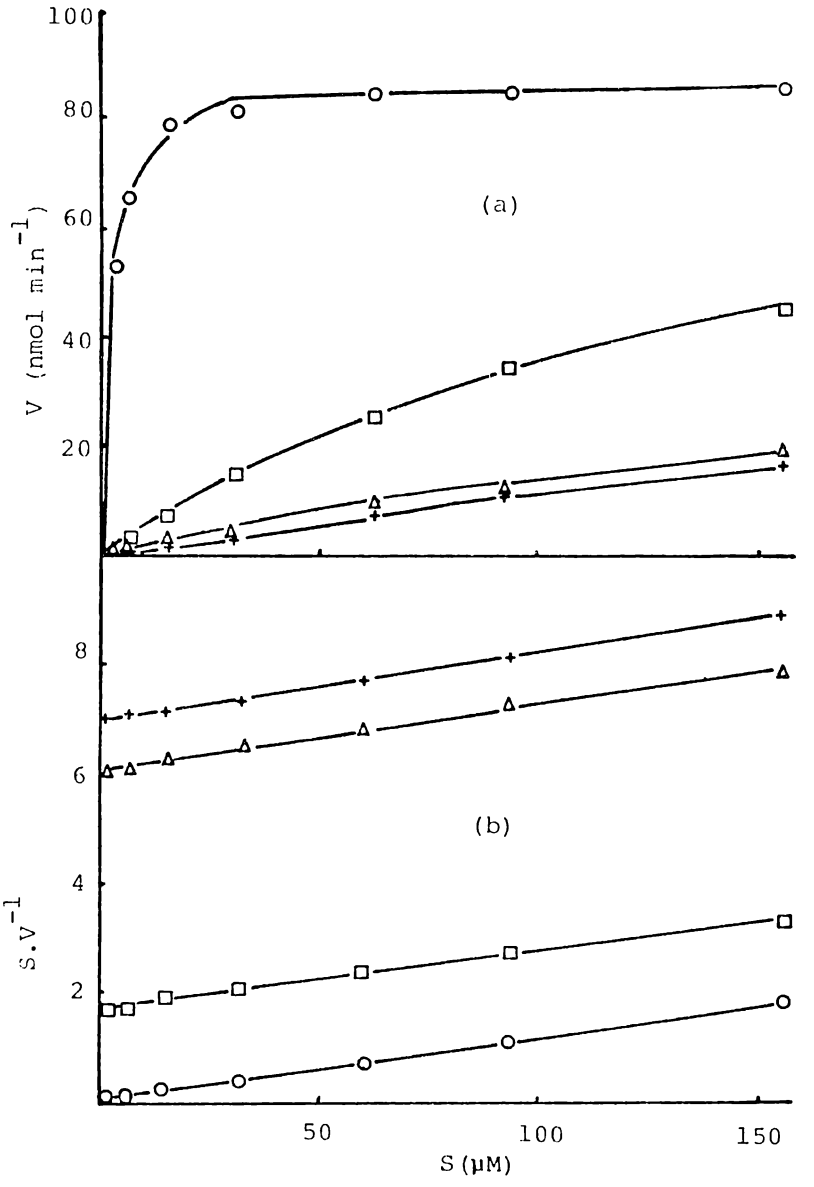


Fig. 3.13: (a) Inhibition of *Pseudomonas* 017 lipase activity towards 4 MUN substrate by Na-oleate. Na-oleate concentrations of zero (o-o), 30 μM (□-□), 150 μM (Δ-Δ) and 300 μM (+-+). Enzyme level in assay mixture was 1 μg protein.

(b) Woolf plot transformation of data.

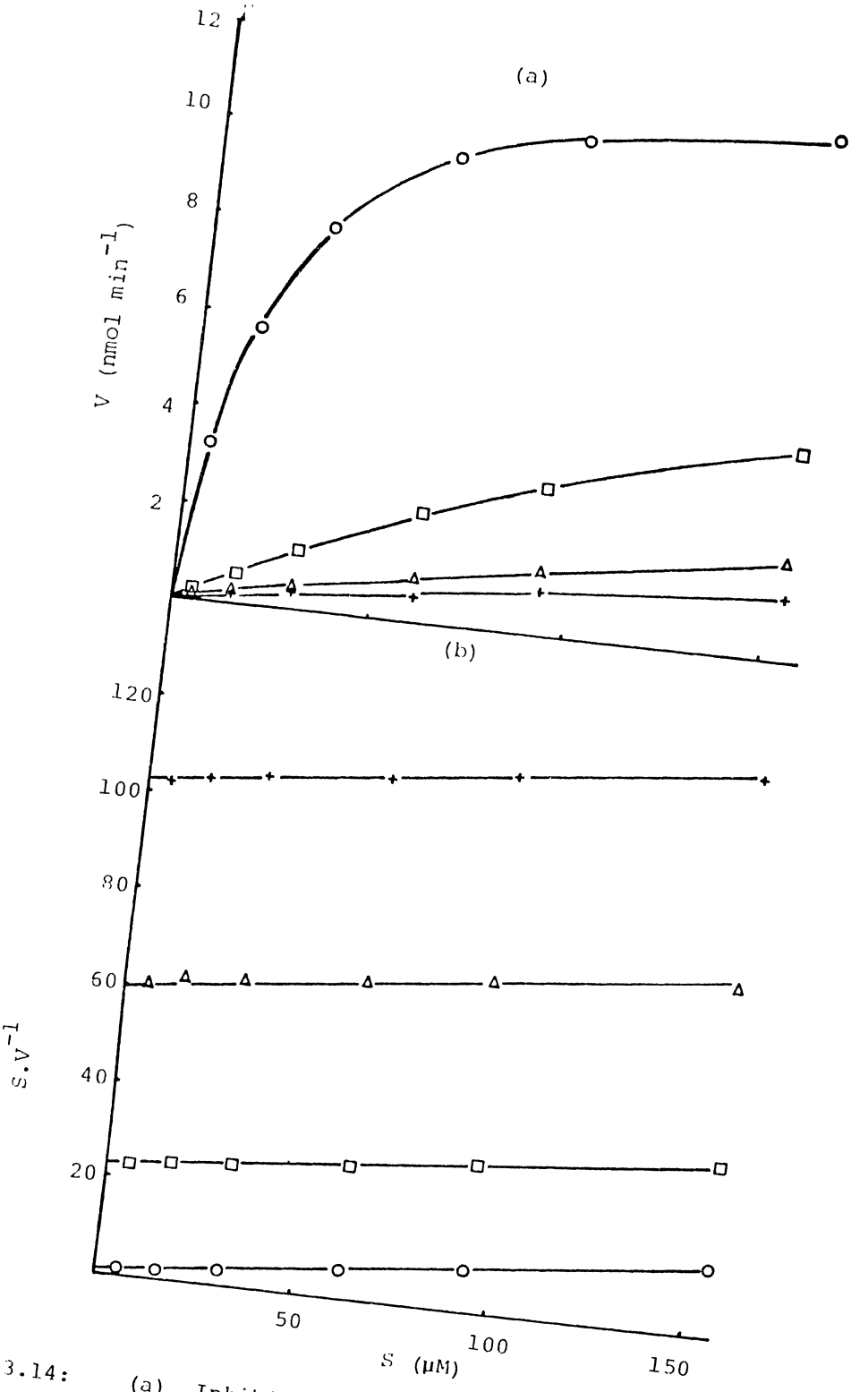


Fig. 3.14: (a) Inhibition of *Staphylococcus aureus* NCIB 6571 lipase activity towards 4 MUN substrate Na-oleate concentrations of zero (o-o), 30 μM(□-□), 150 μM(Δ-Δ), and 300 μM (+-+). Enzyme level in assay of 1 μg protein.

(b) Woolf plot transformation of data.

TABLE 3.6 Kinetic parameters calculated from Woolf plots for Na-oleate inhibition of the lipases of *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017.

Oleate level (μM)	*Staph. aureus* lipase*		*Pseudomonas* 017 lipase*	
	K_m(app) (μM)	ν_{max} (nmol min^{-1})	K_m(app) (μM)	ν_{max} (nmol min^{-1})
0	16	12	1.8	87.0
30	269	11.9	146	86.9
150	720	11.9	518	84.8
300	1180	11.5	585	83.3

* Enzyme level at 1 μg protein $assay^{-1}$.

TABLE 3.7 Inhibition of two bacterial lipases in hydrolysis of 4 MUN by increasing levels of Na-oleate and the reversal of inhibition by calcium. Enzyme levels (as Lowry protein) were 10 μg and 0.4 μg per assay for *Staph. aureus* and *Pseudomonas* 017 preprations respectively.

Na-oleate conc. μM	% lipase activity of control			
	Staph. aureus without Ca^{2+}	*Staph. aureus* with Ca^{2+}*	*Pseudomonas* 017 without Ca^{2+}	*Pseudomonas* 017 with Ca^{2+}*
0	100	100	100	100
30	56	89	67	89
60	45	87	49	86
150	30	72	35	76
300	21	69	33	62
600	20	60	n.d.**	n.d.**

* Ca^{2+} added at final concentration of 5 mM. Increases in Ca^{2+} up to 100 mM did not enhance the reversal effect.

** n.d. = not determined.

A similar surface charge approach was proven for the inhibitory effect of gum arabic (composed of sugar acids) on hydrolysis of tributyryl-glycerol and the reversal obtained by addition of Ca^{2+} ions (Brown *et al* 1977). However, this "charge-repulsion" theory has difficulty in explaining totally the effects of Ca^{2+} ions. In the present study, as increased levels of fatty acid were added to the assay system, the ability of Ca^{2+} to reverse the inhibition was diminished, even at elevated levels of Ca^{2+} (Table 3.7). It may be that fatty acid inhibition involves principally charge-repulsion at low concentrations but that at higher concentrations steric hindrance becomes important. A combination of both charge-repulsion and steric-hindrance mechanisms also allows for explanation of the greater inhibition observed with unsaturated fatty acids on the basis of their "kinked" alkyl chain (Gurr and James 1975) and therefore greater steric hindrance.

3.4 Effects of some surface-active agents on two partially purified bacterial lipases

The published literature contains examples of both stimulation and inhibition of lipase activity by a range of surface-active agents, variously described as emulsifiers, stabilisers, detergents, and surfactants (Brockerhoff and Jensen 1974; Sémériva and Desnuelle 1979). Stimulatory compounds may be added to substrate emulsions to stabilise them thereby preventing coagulation and subsequent decreases in available substrate surface area (Brockerhoff and Jensen 1974). Alternatively, they may act by reducing the extent of lipase denaturation sometimes associated with adsorption at the interface (Borgstrom and Erlanson 1973; Vandermeers *et al* 1974). Inhibition may be attributed to effects either directly upon the enzyme or indirectly by affecting the properties of the interface (Sémériva and Desnuelle 1979). The work reported here was performed to elucidate the effects of surface-active compounds on the activity of two bacterial lipases. It was

hoped that studies with an artificial substrate, 4 MUN, might clarify the mode of action of these surface-active compounds.

The effect of addition of several surface-active compounds to the potentiometric assay system (2.3) on the activity of the two bacterial lipases towards olive oil is summarised in Table 3.8. The effects of the same compounds when 4 MUN is the substrate is presented in Table 3.9. Compounds which demonstrated an effect influenced by time of contact with the enzyme may be assumed to act on the enzyme *per se*, whilst those compounds with no difference in effect with time act on the total reaction system (*e.g.*, by competitive inhibition or changing the properties of the substrate surface).

The time-independent stimulatory effect of Triton X-100 (alkylaryl polyether alcohol) observed with the two lipases at both concentrations with both substrates was apparently an effect of increasing substrate turnover rate, although the mechanism remains unknown. Similar stimulation of activity by Triton X-100 of an *Acinetobacter* lipase toward β-naphthyl laurate was observed by Breuil and Kushner (1975b). Stimulation by Triton X-100 at low concentrations followed by inhibition at high concentrations was observed for liver lipases (Kaplan and Teng 1971; Kariya and Kaplan 1971). Paznokas and Kaplan (1977) found that high concentrations of Triton X-100 (0.625-2.5%) inhibited the velocity at low substrate levels of a *Mycobacterium* lipase.

Sodium dodecyl sulphate (SDS, an anionic detergent) added to the reaction systems caused a time-dependent decrease in the activity of both lipases. The decrease in activity was similar with both substrates and subsequent dialysis (18h, against 5 L 5 mM Tris-HCl pH 7.5) did not regenerate activity. Both these results indicate that the enzymes were irreversibly denatured by SDS, a finding consistent with its effects on animal lipases (Borgstrom and Erlanson 1973). Paznokas and Kaplan (1977) were able to recover 50% of the activity after SDS electrophoresis of *Mycobacterium* lipase and demonstrated that

TABLE 3.8 Effect of surface-active compounds on the activity of partially purified lipases from *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017 towards olive oil as determined by continuous potentiometric titration.

COMPOUND (final conc).	% activity of control			
	Pseudomonas		*Staph. aureus*	
	Immediate	30 min. * preincub.	Immediate	30 min.* preincub.
Gum arabic (0.5%)	100	100	100	100
Sodium dodecyl sulphate (0.03%)	125	50	65	13
Triton x-100 (0.03%)	140	144	165	164
Triton X-100 (0.6%)	200	210	230	225
Tween 80 (0.03%)	100	145	100	100
Tween 80 (0.6%)	75	68	62	62
Na-taurocholate (0.1%)	68	68	130	133
Na-taurocholate (1%)	12	12	25	25

* 30 min. preincubation of compound with enzyme prior to addition of substrate.

TABLE 3.9 Effect of surface-active compounds on the activity of partially purified lipases from *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017 towards 4-methyl umbelliferone nonanoate.

COMPOUND (final conc)	% activity of control			
	Pseudomonas 017 lipase		*Staph. aureus* lipase	
	Immediate	Preincubation 30 min*	Immediate	Preincubation 30 min*
Gum arabic (0.5%)	100	100	100	100
Sodium decyl sulphate (0.03%)	172	54	65	12
Triton X-100 (0.03%)	158	162	161	160
Triton X-100 (0.6%)	252	250	225	228
Tween 80 (0.03%)	100	151	85	85
Tween 80 (0.6%)	81	80	65	62
Na-taurocholate (2 mM)	59	51	112	112
Na-taurocholate (20 mM)	9	8	35	32

* 30 min preincubation of compound with enzyme prior to addition of substrate.

the preparations activity was unaffected when assayed in the presence of 0.02% SDS. The *Pseudomonas* 017 lipase preparation was *stimulated* by the presence of SDS in the assay system providing the assay was performed immediately. It would appear that SDS may have a favourable effect on the substrate-enzyme interaction but an unfavourable effect on the stability of the enzyme.

The nonionic detergent, Tween 80, inhibited the activity of both enzymes when present at 0.6%. Variation in 4 MUN substrate level revealed that such inhibition was "kinetically competitive"(Figs.3.15 and 3.16). Similar results were obtained by Wouters (1967) for *Geotrichum* lipase and olive oil substrate. Work presented in Chapter 10 demonstrated that neither enzyme was capable of action upon Tween 80 which suggests that the inhibition was a competition with the enzyme for the substrate surface. At 0.03% Tween 80,a stimulatory effect on *Pseudomonas* 017 lipase was observed after 30 min preincubation. The effect was similar in magnitude with assay by both substrates and hence appeared to be either an influence on the enzyme *per se* or on an inhibitor present in the enzyme preparation.

The effect of the anionic bile salt Na-taurocholate was to inhibit the activity of *Pseudomonas* 017 lipase at both concentrations (0.1% and 1%) whilst the activity of *Staph. aureus* NCIB 6571 lipase was stimulated at 0.1% but inhibited at 1%. Studies by other workers have shown similarly variable results on the effects of Na-taurocholate on lipases. Bacterial lipases may be stimulated (Chander *et al* 1979; Severina and Bashkatova 1979) or inhibited (Finkelstein *et al* 1970; Breuil and Kushner 1975b; Severina and Bashkatova 1979) by Na-taurocholate. It is thought that stimulation by bile salts arises from their protection of the enzyme from denaturation upon adsorption to the substrate (Borgstrom and Erlanson 1973; Vandermeers *et al* 1974). The inhibitory effect of bile salts has been variously ascribed to

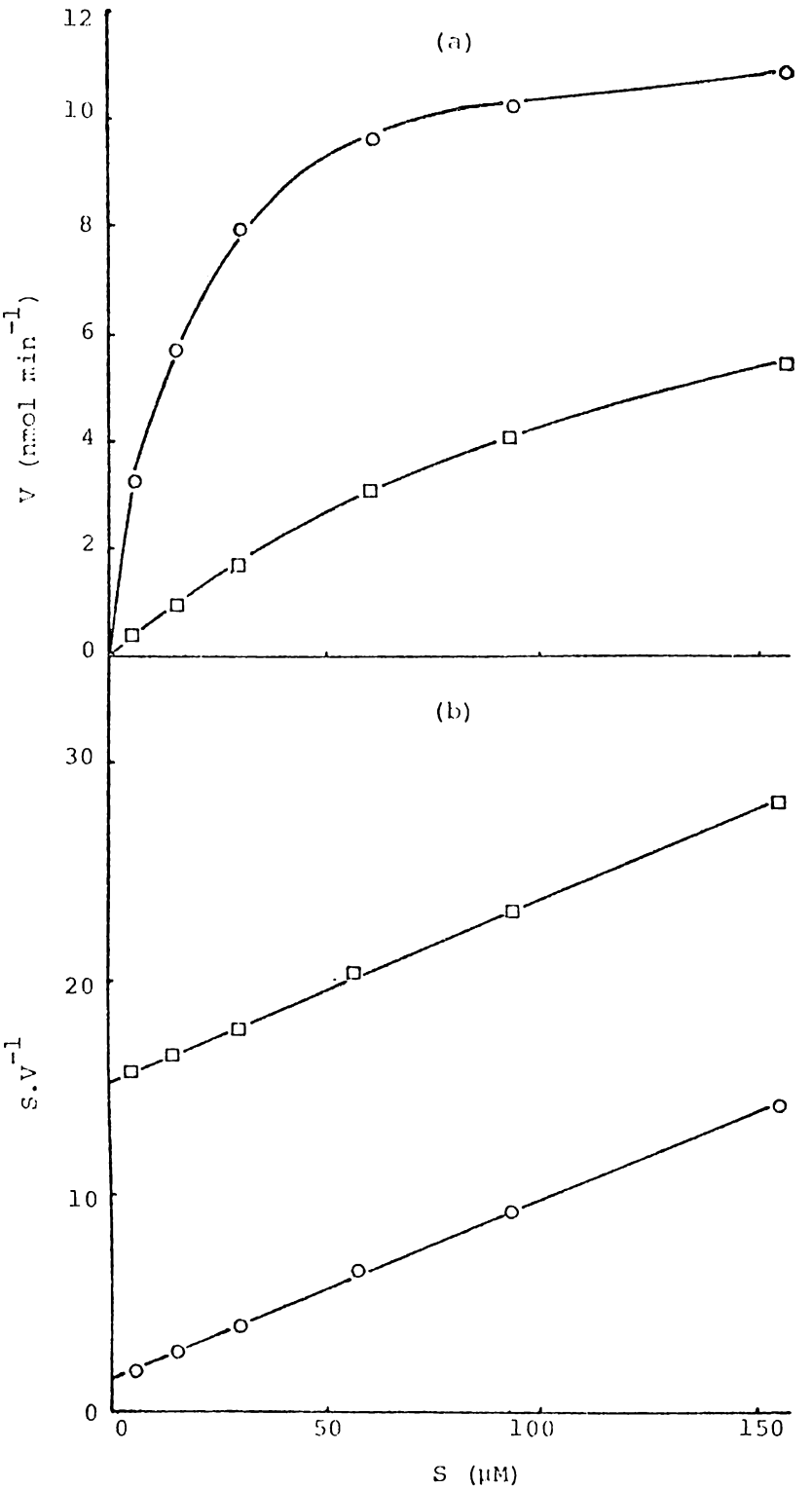


Fig. 3.15: (a) Competitive inhibition of 4 MUN hydrolysis by the lipase of *Staphylococcus aureus* NCIB 6571 by 0.6% Tween 80. Enzyme level in assay of 1 μg protein. o-o, control; □-□, 0.6% Tween 80.
(b) Woolf plot transformation of data.

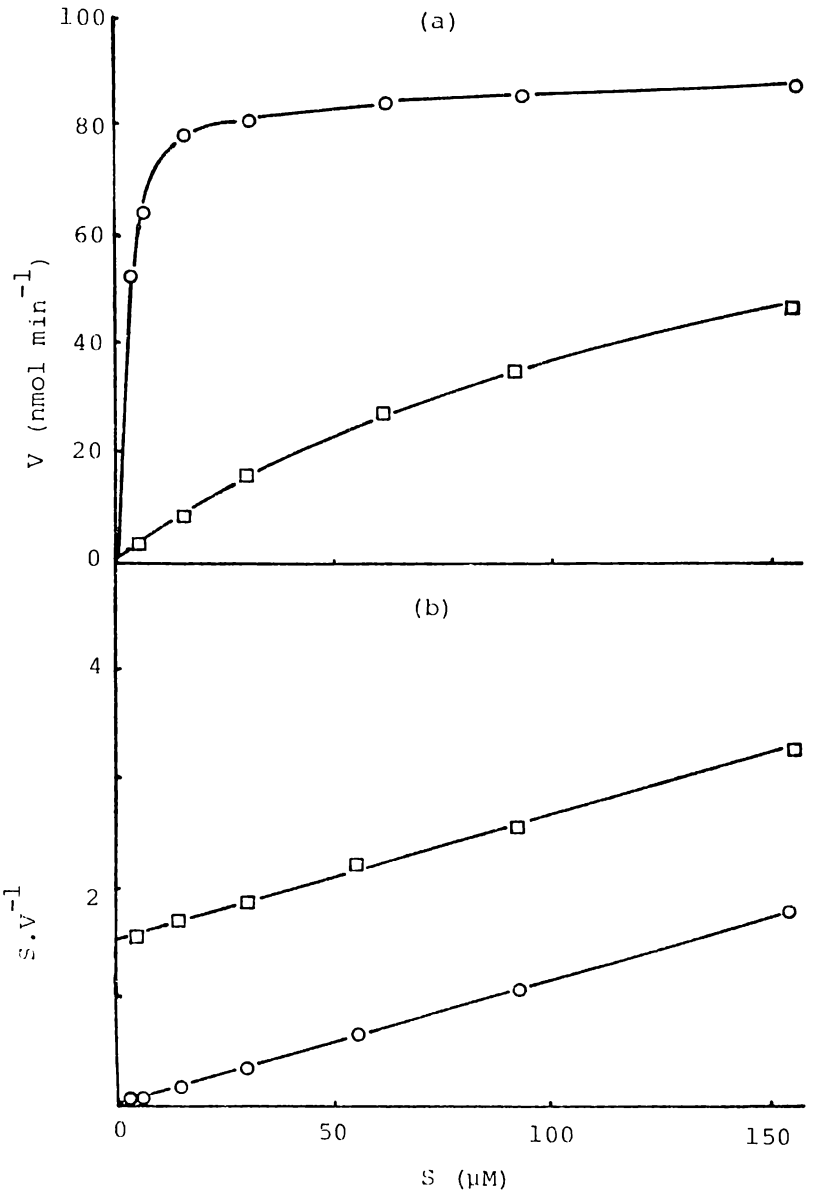


Fig. 3.16: (a) Competitive inhibition of 4 MUN hydrolysis by the lipase of *Pseudomonas* 017 by 0.6% Tween 80. Enzyme level in assay of 1 µg protein.

(b) Woolf plot transformation of data.

o-o , Control; □-□ , 0.6% Tween 80.

"charge-repulsion" and "steric-hindrance" at the interface and direct binding with the enzyme itself (Borgstrom and Erlanson 1973; Brown *et al* 1977). The situation is at present unclear (Sémériva and Desnuelle 1979).

The results with gum arabic (Tables 3.8 and 3.9) suggest that this compound has no effect on the activities of the two lipases. However, variation in the 4 MUN substrate concentration demonstrated a "kinetically competitive" inhibition probably resulting from "charge-repulsion" at the substrate surface (Wouter 1967; Brown *et al* 1977).

The results presented demonstrate that no generalisations as to the effects of surface-active compounds on lipase activity can be made. The effect of a surface-active compound is dependent upon its concentration, the origin of the enzyme, whether preincubation occurred and the substrate used. Inhibiting effects may be due to either enzyme denaturation or effects associated with the substrate-aqueous interface.

3.5 Summary

Studies in this chapter have helped to further the understanding of lipase action and surface-related phenomena. The importance of available substrate surface area to lipase adsorption and subsequent activity was demonstrated. The nature of fatty acid inhibition was somewhat clarified and the concept of a minimum inhibitory concentration developed. The influence of surface-active compounds on lipase activity was investigated. However, results did not allow any general theories on the nature of their influence to be developed.

CHAPTER 4 ENZYMES IN SOIL : AN OVERVIEW

4.1 Origin of soil enzymes

Despite the lack of conclusive evidence, it is accepted that enzymes in soil originate from animal, plant, and microbial sources (Ladd 1978). Furthermore, it is often tacitly assumed that, of these sources, microorganisms contribute the largest portion of the accumulated soil enzymic activity (Kiss *et al* 1975).

Numerous studies have shown that enzyme activity in soils may be influenced by the prevailing vegetation (Ross 1966; Khan 1970; Neal 1973; Pancholy and Rice 1973a and b; Kiss *et al* 1978; Duxbury and Tate 1981). These results, however, cannot be used to support the hypothesis that plants act as a significant source of soil enzymic activity. The effects may be indirect, whereby the changes in the type and quantity of plant-derived organic material added to the soil induces a differential response by the microorganisms within it (Kiss *et al* 1978; Ladd 1978). More acceptable (though still indirect) evidence for the existence of plant-derived enzymes in soil, comes from studies demonstrating the high enzyme activities associated with plant tissue (herbage and roots) and root exudates (Estermann and McLaren 1961; Hall and Davie 1971; Neal 1973; Ross 1975; Ross 1976; Speir and Ross 1976). These studies indicate that plants have the *potential* to make a considerable contribution to the soils enzymic capabilities. Whether such a potential is actually realised has not yet been conclusively proven and presumably depends upon the stability of plant-derived enzymes in the soil environment.

The contribution that animal-derived enzymes may make to the total soil enzymic pool has received scant attention. The excreta of earthworms contains significant levels of invertase (Kiss 1957) and phosphatase (De Jorge and Sawaya 1967). Presumably, other enzymes present in the digestive tract of animals, living within and upon the soil, are

excreted and therefore potentially available to contribute to the soil's enzymic pool. As with plant-derived enzymes, the realisation of this potential presumably depends upon the stability of the enzymes in the soil environment.

The significance of enzymes of microbial origin to the accumulated soil enzyme activity is not doubted (Kiss *et al* 1975; Skujins 1976, Skujins 1978; Speir and Ross 1978) even if not conclusively proven (Ladd 1978). The tacit assumption that microorganisms supply most of the soil enzymic activity arises from a knowledge of their growth and metabolic characteristics (Speir and Ross 1978). Microorganisms in soil are numerous, are generically (and hence, enzymically) diverse, and possess the capacity to inductively produce enzymes in response to the addition of substrates.

4.2 Range of enzymes in soil

Enzymic activity from four of the six major divisions of enzymes have been detected in soils. Oxidoreductases and hydrolases have received the most attention. There have been several investigations of transferase and lyase activities, but no reported studies on isomerase and ligase activities in soil. Reviews detailing the range of enzymic activities detected in soil include those of Skujins (1967), Kuprevich and Shcherbakova (1971), Kiss *et al* (1975), Skujins (1976) and Ladd (1978). The activities of approximately fifty different enzymes have been detected in soil (Ladd 1978). Considering the diversity in the soil microbial population, and the contribution that plants and animals may make, it seems likely that the range of enzymic activities detected in soil will increase in the future.

4.3 Stability and state of soil enzymes

The presence of enzymes in geologically preserved soils and sediments (Skujins and McLaren 1968; Radulescu and Kiss 1971; Nannipieri *et al* 1973) suggests that native soil enzymes are extremely stable. The stability of soil enzymes (relative to enzymes from other sources) is further demonstrated by their resistance to physical, chemical and enzymic treatments. Enzymic activity may remain in soils subjected to prolonged incubation at high temperatures (Conrad 1940; Halstead 1964; Chalvignac 1971; Zantua and Bremner 1977). Soil enzymes may be less affected by inhibitors than enzymes from other sources (Bremner and Douglas 1971, Kiss *et al* 1975; Lethbridge and Burns 1976) and may be stable to harsh chemical conditions used to extract them (Getzin and Rosefield, 1968). Several enzymes present in soil have been shown to be resistant to attack by added proteases (Burns *et al* 1972a and b; Satyanarayana and Getzin 1973).

A considerable proportion of the soil enzymic activity resides outside living cells (Kiss *et al* 1975; Skujins 1976; Burns 1978; Skujins 1978). These enzymes may be truly extracellular (*i.e.* released from live cells) or be intracellular enzymes, released into the soil upon cell lysis. To avoid confusion in terminology, Skujins (1976 and 1978) used the word *abiontic* to describe all enzymic activity existing outside living cells. The accumulation of abiontic enzymes in soils suggests that a mechanism (or mechanisms) exists for their stabilisation as it may be anticipated that such enzymes would be rapidly consumed as a proteinaceous substrate by soil microorganisms (Burns 1978).

The ability of certain purified components of soil to stabilise enzymes *in vitro* has been well demonstrated. Some workers have considered the formation of clay-enzyme complexes (and, more generally, clay-protein complexes) and the stability thereby conferred upon the enzyme (McLaren 1954; Estermann *et al* 1959; Aomine and Kobayashi 1964; Sørenson 1969; Harter and Stotzky 1973). However, clay-enzyme or other clay-protein

complexes do not necessarily exhibit increased stability (Burns *et al* 1972a, and b; McLaren and Barshad 1976; Morgan and Corke 1976). Numerous *in vitro* studies have examined model humus-enzyme systems from the viewpoints of mechanisms of formation, kinetics and stability (Ladd and Butler 1975; McLaren 1975). These model studies have revealed that enzymes (and, more generally, proteins) may be bound to humus by a variety of bonds (ionic, hydrogen, covalent, hydrophobic) and that such binding usually imparts an increased resistance to denaturation, although this increased stability is frequently accompanied by a decreased activity.

The capacity of isolated soil components to bind, and thereby stabilise, enzymes *in vitro* presents only indirect evidence for the operation of such mechanisms *in vitro*. More direct evidence is provided by the extraction and separation of enzymically-active complexes from the general soil milieu. Several workers have extracted humus-enzyme complexes from soil and discovered that such complexes are extremely stable (Burns *et al* 1972a and b; McLaren *et al* 1975; Nannipieri *et al* 1975; Cacco and Maggioni 1976; Ceccanti *et al* 1978). Whereas humus-enzyme complexes have been isolated from soil, humus-free clays with enzymic activity have not (McLaren 1975 and 1978). The observation that enzymic activity may be highest in the clay-size fraction of soils (Haig 1955; Burns *et al* 1972b) may merely reflect the relative distribution of humic materials (and hence humic-enzyme complexes) which have been shown to bind preferentially to clays (Mortland 1970).

4.4 Applied aspects of soil enzymology

Enzymes in soil are the fundamental catalytic agents responsible for the decomposition and mineralisation of organic material. Determination of the activity of enzymes in soil may therefore be of practical significance from a number of viewpoints.

In the agronomic context, work in the 1950's (particularly by Soviet and East-European workers) suggested that measurement of enzymic activity may provide a "biological index" of soils, from which an assessment of soil fertility could be made (Kiss *et al* 1975; Skujins 1978). Since that time, the increasing number of exceptions to this approach has led to the viewpoint that the complexity of the soil system and the factors influencing its enzymic activity, does not allow the overall fertility of a soil to be judged by an enzyme measurement (Howard 1972; Burns 1978; Skujins 1978). Nevertheless, measurement of specific enzymic activities in soil may provide information of value regarding nutrient cycling and hence the availability of these nutrients to plants. In this context, the release of inorganic nitrogen and phosphorus, by the activities of ureases and phosphatases respectively, are of most significance, as the availability of these nutrients often limits plant growth. With the increasing use of urea as a nitrogen fertilizer, problems associated with soil urease activity have been experienced (Bremner and Mulvaney 1978). The rise in pH and liberation of ammonia may damage seedlings, lead to nitrite accumulation and toxicity, and result in gaseous loss of applied fertilizer as ammonia. Soil urease, and compounds capable of inhibiting it, have therefore been well studied (*e.g.* Bundy and Bremner 1973).

Amendments to soil, associated with normal agricultural practices or with the land-application of wastes, may affect specific enzymic activities in soil in three basic ways:

(i) The amendment may contain substances that are inhibiting to the soil enzyme. Examples include the inhibitory effects of pesticides on various soil enzymes (reviews by Kiss *et al* 1975 and Cervelli *et al* 1978) and high phosphate fertilisation on phosphatases (Speir and Ross 1978). The inhibitory effect may be transient or long-term, its duration being dependent upon the persistence of the inhibitory substance.

(ii) The amendment may stimulate the production, by soil micro-organisms, of the enzyme(s) responsible for its degradation. This has been demonstrated for a large number of substances added to soil including various polysaccharides (reviewed by Kiss *et al* 1978), organic esters of sulphur (Houghton and Rose 1976), lipids (Pancholy and Lynd 1972) carbohydrates (Ladd and Paul 1973), pesticides (reviewed by Cervelli *et al* 1978) and others. It would seem possible, therefore, that the time taken for the induction of such degradative enzymes could be used as a tool for monitoring the breakdown of additives in soil (Nannipieri *et al* 1978 and 1979). However, not all substances added to soil are degraded by enzymes induced by their presence. Some substances may be primarily degraded by the stabilised accumulated enzymic activity present in the soil *before* the addition of its substrate. This appears to be the case with urease (Zantua and Bremner 1976) and possibly for a large number of other enzymes (Kiss *et al* 1975).

(iii) The amendment may stimulate general microbial activity and thereby lead to an increase in the levels of enzymes not directly responsible for its degradation (*e.g.* Ladd and Paul 1973; Zantua and Bremner 1976; Nannipieri *et al* 1979). This overall increase in enzymic activity may be expected to increase soil fertility due to an increased mineralisation of nutrients. However, the reverse may occur when amendments of organic substances high in C and low in P and N are added to soil. Such amendments may lead to the increased activity of enzymes associated with mineralisation (*e.g.* urease, phosphatase) merely providing nutrients for immobilisation within the expanding population of soil microorganisms thereby denying such nutrients to plants (*e.g.* Plice 1948; Ladd and Paul 1973; Smith 1974).

CHAPTER 5 LIPASES IN SOIL : EXTRACTION, MEASUREMENT, AND PROPERTIES

5.1 Measurement of enzymic activity in soil

An axiom of classical enzymology is that enzymes should be assayed under optimal conditions. The result obtained represents the maximum potential catalytic activity of the sample. Generally, this axiom has been followed in soil enzymology, with assays being performed with excess substrate, at optimum temperature, and with a buffer capable of maintaining optimal pH throughout the incubation period (Burns 1978). Several workers have pointed out that an assessment of the fate of a substrate when added to soil is best evaluated by performing enzyme assays at soil pH's (Zantua and Bremner 1975a; Roberge 1978). Presumably, their argument could be extended to include the requirements that assays be performed at soil temperatures and soil moisture levels. The method of choice depends upon the aims of the investigator. Measurement of maximum potential catalytic activity allows for comparative assessments of total accumulated enzyme levels between soils and, temporally and spatially, within soils. These measurements, therefore, allow for an assessment of the factors controlling the level of accumulated enzymes in soil and the extent to which microbially-produced enzyme induction occurs upon amendment of soil with an enzyme substrate. Measurement of enzymic activity under conditions existing in the field assesses the rate of substrate degradation likely to occur in that environment. This type of measurement is of direct, applied significance when evaluating the persistence of fertilizers, pesticides and various constituents of wastewaters when added to soils. The fate of the additive is of prime interest in such studies, rather than the enzyme itself.

In assessing the enzymic potential of a soil sample several problems exist that do not arise in assays performed with more pure systems.

(i) To measure the enzyme level present in the sample it is essential that no induction of further enzyme production occurs during the period of the assay. This has usually been accomplished by the addition to the assay mixture of toluene which, supposedly, acts to prevent microbial proliferation without influencing the activity of enzymes (Skujins 1967; Kiss *et al* 1975; Skujins 1976; Skujins 1978). However, these reviews demonstrate that toluene may not always achieve this objective. For this reason, assays of enzymic activity involving short-term incubation (<2h) with the substrate have been recommended as an alternative means of minimising microbial proliferation during the assay period (Burns 1978).

(ii) The product or substrate, whichever is used to assess enzymic activity, must be separated quantitatively from the soil particulate matter. For example, the sorptive powers of soil constituents have proved a problem with phosphatase assays based on the measurement of the release of inorganic-p from organic-p (Skujins 1967; Speir and Ross 1978) and β-napthol from β-napthol phosphate (Ramírez-Martínez and McLaren 1966).

(iii) The buffer system used in the assay should not solubilise soil substances which would subsequently interfere with the estimation of product or substrate (Rýsary and Macura 1972; Tabatabai and Singh 1976; Lethbridge *et al* 1978).

(iv) Ideally, freshly-collected and field-moist soils should be used for enzyme assays. When this is not practical, storage of soils should be under conditions favourable to the preservation of activity of the enzyme of interest (Skujins 1967; Speir and Ross 1975; Zantua and Bremner 1975b; The air-drying of soils prior to enzyme assays arose from the established use of this practice in other areas

of soil research. However, the air-drying of soils may have drastic effects on enzymic activity (Ross 1965; Tabatabai and Bremner 1970; Speir and Ross 1975b) and the continuation of the practice has been questioned (Roberge 1978).

(v) Products of enzymic action in soil may themselves act as substrates for other enzymes present in the soil, thereby giving an underestimate of the activity (Ross 1974).

Most assays of soil enzymes involve direct incubation of the soil with substrate (Roberge 1978). However, some assays involve determination of enzymic activity in soil extracts *e.g.* polyphenol oxidase (Mayaudon *et al* 1973a and b), peroxidase (Bartha and Bordeleau 1969), lipase (Pancholy and Lynd 1972) and uricase (Martin-Smith 1963). In studies related to the state of enzymes in soil and in attempted purification, activities have been determined in soil extracts of enzymes which are usually measured in the soil itself (Burns *et al* 1972a and b; Ladd 1972; McLaren *et al* 1975; Hayano and Katami 1977; Nannipieri *et al* 1980). These studies have usually demonstrated that a considerable proportion of the enzymic activity is not extractable. However, several studies have demonstrated that certain enzymic activities in the extracts of some soils may be equal to or greater than those observed with soil incubations (Ladd 1972; Nannipieri *et al* 1980). Increased activities upon extraction may indicate the removal of diffusion barriers between the substrate and the enzyme. Such an effect is more likely to be observed when the substrate involved is of high molecular weight (Ladd and Butler 1975; Nannipieri *et al* 1980). It may be argued, therefore, that the total level of enzymes in the soil is best assessed by measurement of activity in extracts, providing an efficient extractant is available. Furthermore, assaying enzymic activity in extracts avoids some of the problems associated with enzyme assays in a heterogenous environment (McLaren 1960; McLaren and Packer 1970; Cervelli *et al* 1973; Irving and Cosgrove 1976).

5.2 Measurement of lipase levels in soil*

5.2.1 Previous work : Lipase activity in soil has been measured by incubation of soil with emulsified tributyrylglycerol (Pokorná 1964; Mourey 1979). However, such methods require long-term incubations to obtain detectable concentrations of produced fatty acids, may be interfered with by other acids (Pancholy 1971) and are relatively insensitive (Pancholy and Lynd 1972). Furthermore, it seems possible that a proportion of the released fatty acid may be metabolised (Adelson *et al* 1957; Krause and Lange 1965) and/or bound to soil constituents (Mortland 1970) and therefore not measured. Another problem likely to occur is that of diffusion barriers. This problem would be exacerbated in lipase assays as the substrate is insoluble, existing as an emulsion with particle sizes in the micrometre range (3.2).

Mathur *et al* (1980) measured acetic acid production of soil, incubated with triacetylglycerol for 18h, and mistakenly termed this lipase activity. As lipases do not appreciably attack soluble substrates (Desneulle 1972), the determined activity must be regarded as primarily that of carboxylesterase (EC 3.1.1.1).

Pancholy (1971) and Pancholy and Lynd (1972 and 1973) have measured lipase activities in phosphate buffer extracts of soils using the fluorogenic substrate 4-methyl umbelliferone butyrate. The procedure was rapid, precise and sensitive. However, the efficiency of extraction of lipase was not determined, and hence the measured activities must be viewed with caution. Hayano and Katami (1977) demonstrated that phosphate buffer extracted only 0.3% of the β-glucosidase activity of a soil.

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* Some of the contents of this section have been published (Appendix III)

5.2.2 Standard assay for soil lipase activity : Previous assays for soil lipase would appear to have a number of shortcomings (5.2.1). An alternative assay method was therefore developed. The assay involves the use of the general soil organic matter extractant tetra-sodium pyrophosphate, used previously for enzyme extractions (Nannipieri *et al* 1974 and 1980), and subsequent incubation with the fluorogenic substrate 4-methyl umbelliferone nonanoate (4 MUN). Results leading to the adoption of this standard procedure are detailed in following sections. The standard assay was:

> To 1 g of soil (stored field-moist at 4°C) in a 50 mL Erlenmeyer flask was added 10 mL of 0.1 M tetra-sodium pyrophosphate buffer, pH 7.5. Flasks were incubated at 30°C with orbital shaking at 120 rev min^{-1} for 3 h, and the slurry centrifuged at 4000 G for 15 min. A 0.1 mL aliquot of the dark-brown supernatant was subsequently used as an enzyme source in the standard fluorometric lipase assay using 4-methyl umbelliferone nonanoate (4 MUN) as substrate (2.6). Incubation with substrate (final concentration 312 μM) was therefore of 10 minutes duration, at a temperature of 30°C, and with 0.1M Tris-HCl buffer pH 7.5. Rates of non-enzymic substrate hydrolysis were determined using boiled (5 min) soil extracts. Standard curves to relate fluorescent response to 4-methyl umbelliferone (4 MU) concentration (0-20 μM), were constructed using the same matrix as samples, again with boiled soil extract. The use of an identical matrix was essential as pyrophosphate extracts caused considerable quenching of fluorescence of 4 MU standards. Enzyme activity was expressed as nmol 4 MU produced min^{-1} g^{-1} of oven-dried soil.

5.2.3 Standard assay versus a direct incubation technique : As described in 5.1, efficient extraction of enzymes from soil is rarely achieved. For an extracted enzyme assay to be preferred over that involving direct incubation with soil as a measure of total enzyme level, then the activity of the extract must be equal to or greater

than that observed with the soil. The standard soil lipase assay of 5.2.2. was compared to the following assay involving direct incubation of the substrate, 4 MUN, with the soil. The direct incubation assay was:

> To 0.1 g of soil in a 50 mL Erlenmeyer flask was added 4.5 mL of 0.1 M Tris-HCl buffer pH 7.5 Flasks were equilibrated at 20°C by shaking for 10 min at 120 rev min^{-1}. in an orbital incubator. After equilibration, 0.5 mL of 10 mM 4 MUN in 2-ethoxyethanol was added (final concentration 1 mM) followed by shaking for a further 10 min. Flasks were then cooled by immersion in an ice bath before the contents were poured into pre-cooled centrifuge tubes, and centrifuged at 4000 G for 10 min at 2°C. Supernatants were analysed fluorometrically for 4 MU concentration using standards prepared in the same matrix. Controls were run in which substrate was added to the soil suspension immediately before flasks were transferred to the ice bath. Corrections were also made for the extent of 4 MU adsorption onto each soil by adding a concentration range of 4 MU solutions in 0.1 M Tris-HCl pH 7.5 to 0.1 g soil samples, incubating for 10 min, and measuring the fluorescence of the centrifuged supernatant. This fluorescence was compared to that of 4 MU solutions prepared in 0.1 M Tris-HCl pH 7.5 soil extracts. Enzyme activity was expressed as nmol 4 MU produced min^{-1} g^{-1} of oven dried soil.

With four New Zealand topsoils (0-15 cm depth, described in 5.3.1) the activity in 0.1 M pyrophosphate buffer extracts was equal to, or greater than, that determined when the substrate was incubated directly with the soil (Table 5.1). The higher activities observed in extracts may reflect the removal of diffusion barriers between the substrate and the enzyme that may limit activity in soil incubations (Ladd 1972; Ladd and Butler 1975; McLaren 1978). This high apparent extraction efficiency (>100%) can be compared to the efficiency of pyrophosphate in extracting soil phosphatase (30.7 - 65.6%), soil urease (5.4 - 8.6%), soil protease as casein hydrolysis (56.9 - 111%) and soil protease as benzoyl arginine amide hydrolysis

TABLE 5.1 Lipase activities of four New Zealand topsoils as determined by the pyrophosphate extraction method and the direct soil incubation method. Results are expressed as a mean ± two standard deviations of 10 separate assays. Amended soils were loaded with tallow at 2.5 mg g^{-1} for 8 days at 25°C prior to analyses.

S O I L	Treatment	Lipase activity (nmol 4 MU min^{-1} g^{-1})	
		Direct Incubation	Pyrophosphate
Hamilton clay loam	U	125 ± 19	161 ± 10
	A	419 ± 75	500 ± 22
Ruatangata clay	U	92 ± 18	95 ± 7
	A	298 ± 44	348 ± 18
Horotiu silt loam	U	162 ± 18	194 ± 9
	A	228 ± 30	292 ± 16
Waitete sand	U	363 ± 65	597 ± 25
	A	586 ± 84	840 ± 42

U = Unamended, A = Amended

(14.7 - 67.8%) (Nannipieri *et al* 1980). This indicates that with direct incubations, diffusion problems are more acute with lipase assays, presumably because of the large size of the substrate. In this respect the results of Nannipieri *et al* (1980), where higher apparent extraction efficiencies for protease were obtained when the high molecular weight substrate, casein, was used for assay in comparison to that obtained with the smaller benzoyl arginine amide substrate, further indicates the interplay between substrate size and apparent extraction efficiency.

The pyrophosphate extraction method was more precise than the direct soil incubation method (95% confidence limits between 4.2 - 7.4% and 11.1 - 23.4% respectively). The higher degree of variability with the direct soil incubation method arose principally from variations in lipase activity occurring after cooling in ice following incubation. Attempts to terminate the enzyme activity more abruptly by adding acid or alkali were unsuccessful, either due to quenching of the fluorescence of 4 MU or to non-enzymic hydrolysis of the substrate.

An additional complication with the direct soil incubation technique was the necessity to correct for adsorption to soil colloids of the 4 MU produced (Fig. 5.1). A similar problem was experienced by Ramírez-Martínez and McLaren (1966) in the fluorometric direct incubation assay of phosphatase, with released β-napthol (from β-napthol phosphate) being adsorbed to soil. Adsorption curves for 4 MU were different for each soil and sometimes non-linear. Hence, correction for adsorption needed to be evaluated for each soil and over the concentration range where assay systems were likely to fall. This difficulty, coupled with the lower precision and sometimes lower measured activities made the direct incubation method less desirable when compared to the standard pyrophosphate extraction technique.

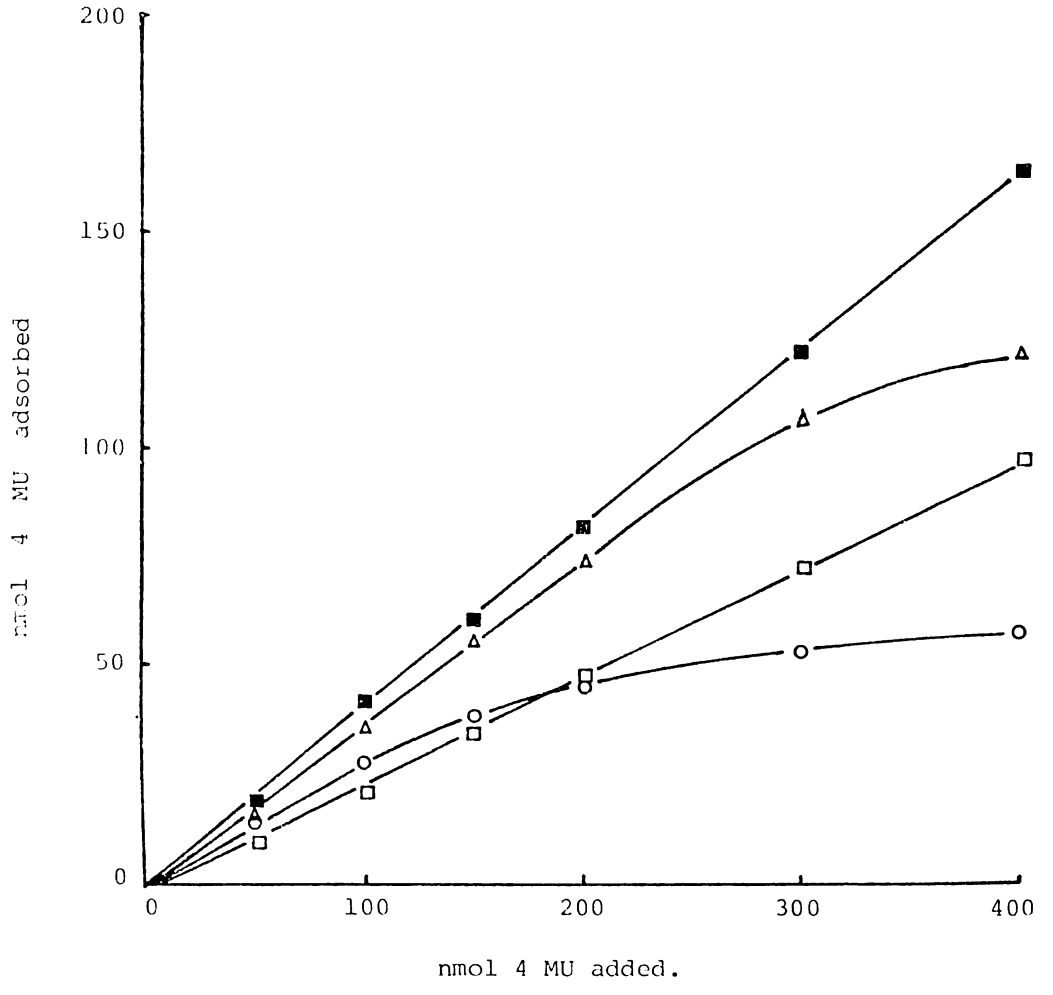


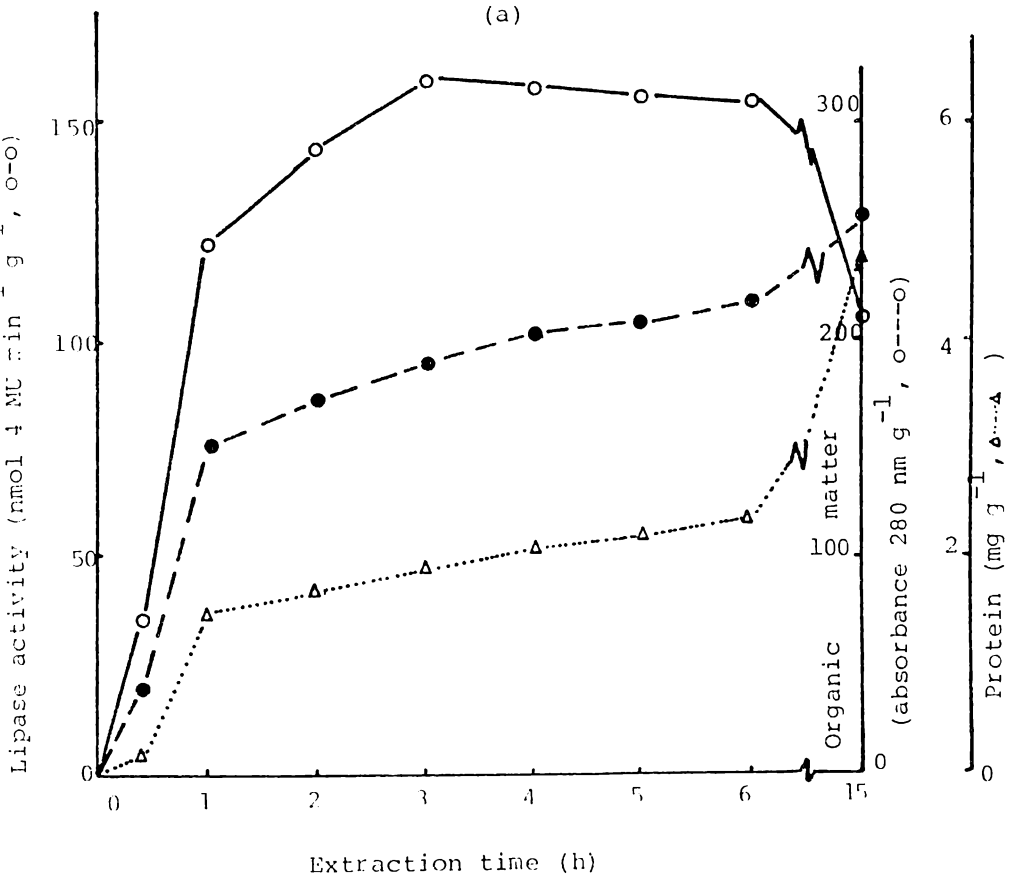
Fig. 5.1 Adsorption of 4-methyl umbelliferone to 0.1 g of four soils. o-o, Hamilton clay loam; □ , Ruatangata clay; Δ , Horotiu silt loam; ■ , Waitete sand.

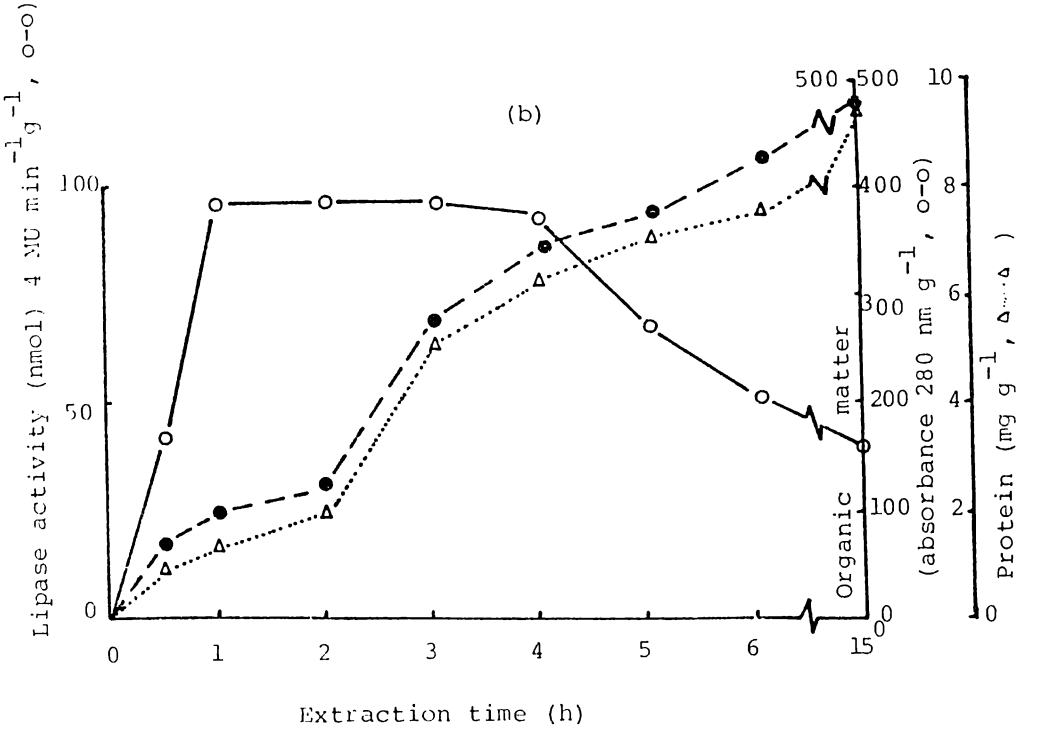
5.2.4 Extraction of lipase activity from soil : Measurement of lipase activity in pyrophosphate extracts, as a function of extraction time demonstrated maximal recovery of activity within 3 h (Fig. 5.2). With the Hamilton clay loam and Ruatangata clay, reduction in measured lipase activity was observed if the extraction extended over 3 hours. However, the activity in 3 h pyrophosphate extracts was relatively stable to incubation at 20°C (Table 5.8). These results suggest that, upon continued extraction, there was a subsequent release of either proteolytic enzymes capable of degrading lipase and/or inhibitory substances. The stability of lipase upon continued extraction with the two soils derived from volcanic ash may reflect the stability of organic matter in general in such soils (Wada and Inoue 1967; Wada and Harward 1974), or the absence of proteolytic activity in such soils (Aomine and Kobayashi 1964).

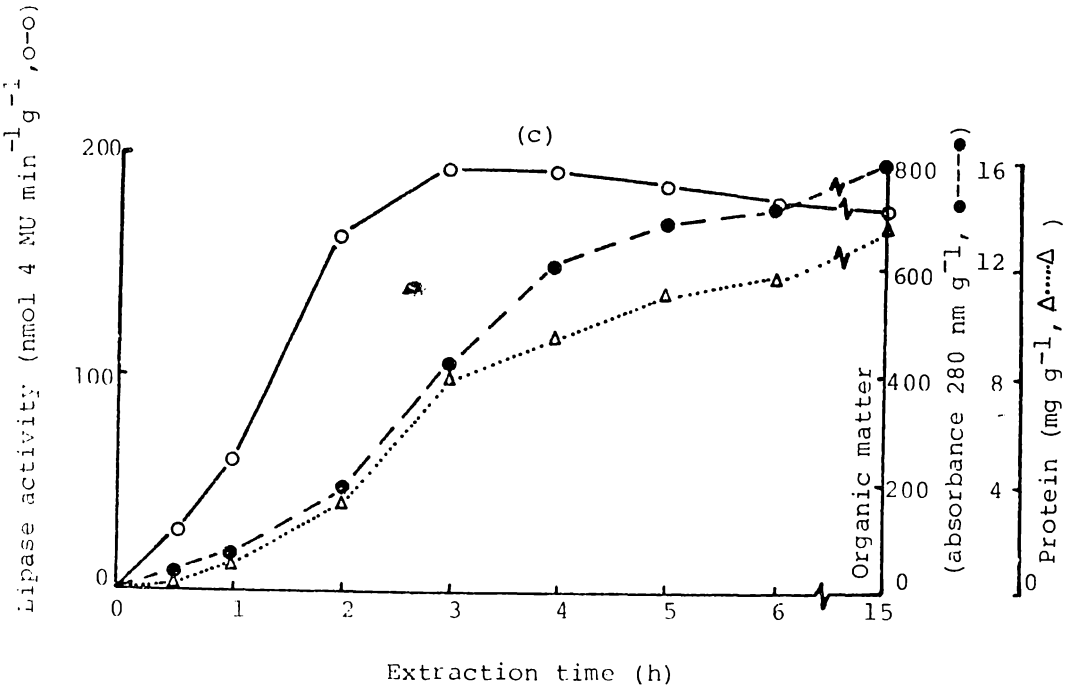
Measurement of Lowry protein (Appendix I) and organic material in the extracts obtained at various times revealed that considerably more protein and organic material was recovered after 3 h extraction (Fig. 5.2). Apparently the lipase was extracted prior to the bulk of soil proteins and organic material. Similar results with pyrophosphate extraction of phosphatase and protease from soil have been reported (Nannipieri *et al* 1980), whilst opposing results have been found for soil urease (Nannipieri *et al* 1974; Nannipieri *et al* 1980).

When soils were subjected to a range of extractants, highest lipase activities were obtained in extracts of 0.1 M tetra-sodium pyrophosphate (Tables 5.2, 5.3, 5.4, 5.5). Little further activity could be obtained by a second extraction of the soils with pyrophosphate. However, when the soil residue of other extractants used was re-extracted with pyrophosphate the combined activities of both extractants approached that present in a single pyrophosphate extraction. Exceptions were soils initially extracted with either 10 mM EDTA or 0.1 M NaOH, which apparently denatured the lipase. The extraction technique

Fig. 5.2. Effect of extraction time on levels of tetra-sodium pyrophosphate (0.1 M, pH 7.5) extractable lipase, organic material (absorbance 280 nm), and protein (Lowry) for four New Zealand topsoils. (a) Hamilton clay loam; (b) Ruatangata clay; (c) Horotiu silt loam; (d) Waitete sand


(a)
Lipase activity (nmol 4 MU min^{-1} g^{-1}, o-o)
Organic matter (absorbance 280 nm g^{-1}, o---o)
Protein (mg g^{-1}, Δ···Δ)
Extraction time (h)


(b)
Lipase activity (nmol) 4 MU min^{-1}g^{-1}, o-o)
Organic matter (absorbance 280 nm g^{-1}, o-o)
Protein (mg g^{-1}, Δ···Δ)
Extraction time (h)


(c)
Lipase activity (nmol 4 MU min^{-1} g^{-1}, o-o)
Organic matter (absorbance 280 nm g^{-1}, •---•)
Protein (mg g^{-1}, Δ···Δ)
Extraction time (h)

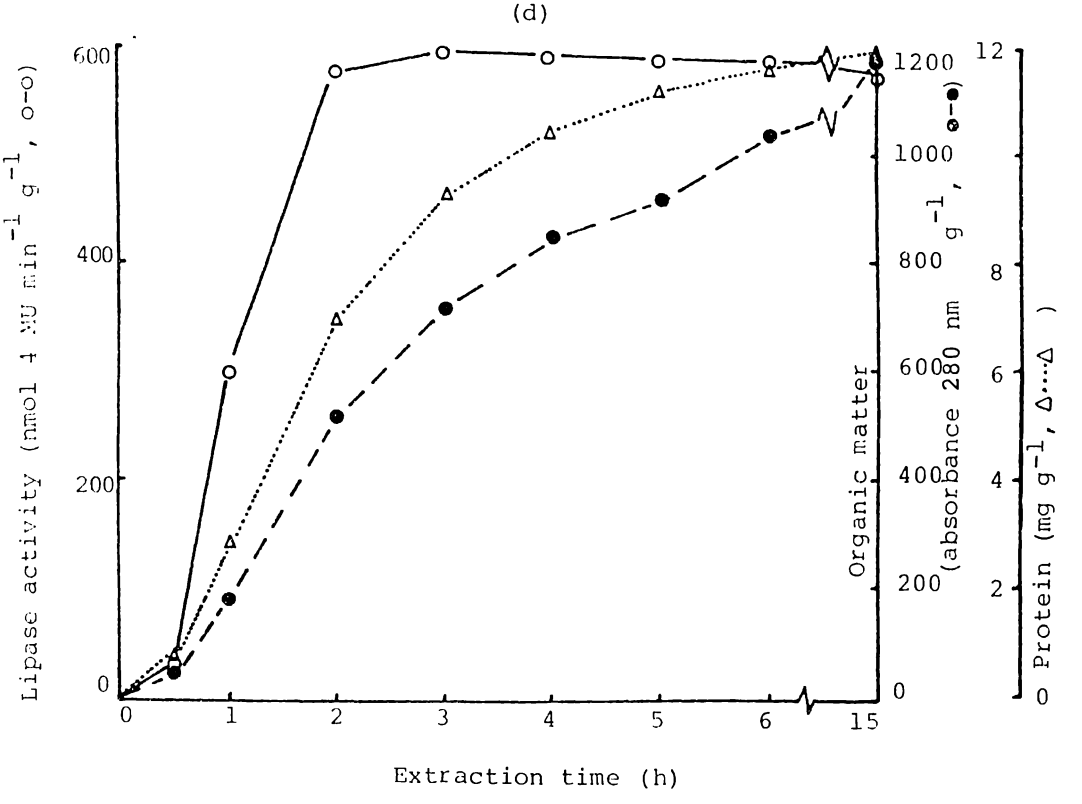

(d)
Lipase activity (nmol 4 MU min^{-1} g^{-1}, o-o)
Organic matter (absorbance 280 nm g^{-1}, •-•)
Protein (mg g^{-1}, Δ···Δ)
Extraction time (h)

TABLE 5.2 Comparison of the efficiency of extraction of lipase from Hamilton clay loam by 0.1 M tetra-sodium pyrophosphate and a range of other extractants all used at soil to extractant ratios of 10:1 for 3h.

Extractant	Lipase Activity (nmol 4 MU produced $min^{-1} g^{-1}$	
	In Extract	In Pyrophosphate Extract of Residue
Distilled water	16	142
Tris-HCl 0.1 M (pH 7.5)	38	120
Phosphate buffer 0.1 M (pH 7.5)	50	110
Phosphate buffer 0.1 M (pH 7.5)*	23	141
Phosphate buffer 0.1 M + 2 M urea	104	45
Phosphate buffer 0.1 M + 1 M KCl	54	100
Phosphate buffer 0.1 M + 1 M KCl + 10 mM EDTA	6	5
Acetate buffer 0.5 M (pH 5.8)	14	140
Tetra-Na pyrophosphate 0.1 M (pH 7.5)	160	5
Tetra-Na pyrophosphate 0.1 M + 10% ethanol	156	6
Tetra-Na pryophosphate 0.1 M + 2 M urea	155	8
Tetra-Na pyrophosphate 0.1 M + 10% toluene	155	7
NaOH 0.1 M	4	4

* Extracted according to the method of Pancholy and Lynd (1972) using a soil-to-extractant ratio of 2:1 for 30 min.

TABLE 5.3 Comparison of the efficiency of extraction of lipase from Waitete sand by 0.1 M tetra-sodium pyrophosphate and a range of other extractants all used at soil-to-extractant ratios of 10:1 for 3h.

EXTRACTANT	Lipase Activity (nmol 4 MU produced min^{-1} g^{-1})	
	In Extract	In Pyrophosphate extract of residue
Distilled water	12	582
Tris-HCl 0.1 M (pH 7.5)	46	541
Phosphate buffer 0.1 M (pH 7.5)	75	510
Phosphate buffer 0.1 M (pH 7.5)	36	555
Phosphate buffer 0.1 M + 2 M urea	180	402
Phosphate buffer 0.1 M + 1 M KCl	75	511
Phosphate buffer 0.1 M + 1 M KCl + 10 mM EDTA	12	18
Acetate buffer 0.5 M (pH 5.8)	24	571
Tetra-Na pyrophosphate 0.1 M (pH 7.5)	591	8
Tetra-Na-pyrophosphate 0.1 M + 10% ethanol	580	6
Tetra-Na-pyrophosphate 0.1 M + 2 M urea	575	12
Tetra-Na-pyrophosphate 0.1 M + 10% toluene	598	4
NaOH 0.1 M	10	14

* Extracted according to the method of Pancholy and Lynd (1972) using a soil to extractant ratio of 2:1 for 30 min.

TABLE 5.4 Comparison of the efficiency of extraction of lipase from Ruatangata clay by 0.1 M tetra-sodium pyrophosphate and a range of other extractants all used at soil-to-extractant ratios of 10:1 for 3h.

EXTRACTANT	Lipase activity (nmol 4 MU produced min^{-1} g^{-1})	
	In Extract	In pyrophosphate extract of residue
Distilled water	11	82
Tris-HCl 0.1 M (pH 7.5)	36	59
Phosphate buffer 0.1 M (pH 7.5)	41	52
Phosphate buffer 0.1 M (pH 7.5)*	20	74
Phosphate buffer + 2 M urea	70	21
Phosphate buffer + 1 M KCl	48	45
Phosphate buffer + 1 M KCl + 10 mM EDTA	2	2
Acetate buffer 0.5 M (pH 5.8)	18	71
Tetra-Na pyrophosphate 0.1 M (pH (pH 7.5)	95	3
Tetra-Na pyrophosphate 0.1 M + 10% ethanol	88	6
Tetra-Na pyrophosphate 0.1 M + 2 M urea	89	5
Tetra-Na pyrophosphate 0.1 M + 10% toluene	95	2
NaOH 0.1 M	1	1

* Extracted according to the method of Pancholy and Lynd (1972) using a soil-to-extractant ratio of 2:1 for 30 min.

TABLE 5.5 Comparison of the efficiency of extraction of lipase from Horotiu silt loam by 0.1 M tetra-sodium pyrophosphate and a range of other extractants all used at soil-to-extractant ratios of 10:1 for 3h.

E X T R A C T A N T	Lipase activity (nmol 4 MU produced $min^{-1}g^{-1}$)	
	In Extract	In pyrophosphate extract of residue
Distilled water	8	174
Tris-HCl 0.1 M (pH 7.5)	35	161
Phosphate buffer 0.1 M (pH 7.5)	38	142
Phosphate buffer 0.1 M (pH 7.5)*	25	162
Phosphate buffer + 2 M urea	85	105
Phosphate buffer + 1 M KCl	38	137
Phosphate buffer + 1 M KCl + 10 mM EDTA	7	12
Acetate buffer 0.5 M (pH 5.8)	11	178
Tetra-Na pyrophosphate 0.1 M (pH 7.5)	190	7
Tetra-Na pyrophosphate 0.1 M + 10% ethanol	190	5
Tetra-Na pyrophosphate 0.1 M + 2 urea	186	6
Tetra-Na pyrophosphate 0.1 M + 10% toluene	195	5
NaOH 0.1 M	12	12

* Extracted according to the method of Pancholy and Lynd (1972) using a soil-to-extractant ratio of 2:1 for 30 min.

of Pancholy (1971) and Pancholy and Lynd (1972, 1973), extracted between 6 - 21% of the activity detected in pyrophosphate extracts. In contrast to the enzyme activity measured in this study carboxylesterase activity was not extractable with pyrophosphate (Getzin and Rosefield 1968), but was extractable in strong alkali (Getzin and Rosefield 1968) and with extractants containing EDTA (Cacco and Maggioni 1976). This indicates that the activity being measured in this study was specifically lipase and not carboxylesterase.

No significant differences in activity were observed with the addition of toluene to the pyrophosphate extractant. This result was perhaps predictable, as little microbial lipase production would be expected to occur in a relatively short (3 h) extraction period with no substrate present. Pokorna (1964) observed a 15% decrease in lipase activity in the presence of toluene. However, his assay involved a long-term (72 h) direct incubation of soil with tributyryl-glycerol which may allow sufficient time for significant lipase induction to occur. Pyrophosphate may be regarded as a relatively mild extractant which does not disrupt cells present in soil (Nannipieri *et al* 1974). The measured activity thus represents accumulated abiontic soil enzyme.

Determination of the levels of Lowry protein and organic material in various extracts from Hamilton clay loam enabled an evaluation of the specificity of the extractants to be made (Table 5.6). On a protein basis, changes in specific activity were not large. The specific activity, on an organic material basis, was highest in the least efficient extractants indicating that a portion of the lipase may exist in a relatively labile state, with the rest being tightly bound and only extractable with an efficient organic matter extractant such as pyrophosphate. The increased lipase levels observed when urea was added to phosphate buffer reflected the ability of urea to extract more organic matter from soil.

TABLE 5.6 Specific activities of various extractants for soil lipase. The soil used was Hamilton clay loam.

EXTRACTANT	Specific Activity*	
	Protein Basis	Organics Basis
Distilled water	88	78
Tris-HCl 0.1 M (pH 7.5)	95	75
Phosphate buffer 0.1 M (pH 7.5)	102	51
Phosphate buffer + 2 M urea	100	18
Tetra-Na pyrophosphate 0.1 M (pH 7.5)	85	0.9

* Protein basis = specific activity expressed as nmol 4 MU min^{-1} mg^{-1} protein.

Organics basis = specific activity expressed as nmol 4 MU min^{-1} unit Abs 280 nm^{-1}.

The effect of extractant-to-soil ratio on the efficiency of extraction was determined for the four soils using pryophosphate as extractant (Fig. 5.3). The amount of activity recovered obeyed a Langmuir curve, with greater than 90% of the theoretical maximum activity (as determined from regression analysis) being extracted at solution-to-soil ratios of 10:1. At higher dilutions efficient extraction was compromised by excessive dilution.

5.2.5 Choice of substrate : A range of fatty-acyl-esters of 4 MU (prepared at 10 mM in 2-ethoxyethanol, the acetate ester prepared in ethanol) were tested for their suitability as substrates for extracted soil lipase determination. The acetate, butyrate, octanoate and laurate esters were obtained from ICN Laboratories, Cleveland, Ohio, USA; the heptanoate, nonanoate, palmitate and oleate esters were obtained from Koch-Light Laboratores, Colnbrook, England. Variations in the concentration of substrate (S) in the assay system were made in order to derive the Michaelis-Menten kinetic values K_m (substrate conc. at 0.5 v_{max}) and v_{max} (maximum velocity of enzyme activity). Whilst this exercise may be regarded as fundamentally invalid due to the insolubility of lipase substrates, it was a useful approach from the practical viewpoint, as it allowed for the choosing of the most desirable substrate and the level needed to avoid substrate limitation during an assay. All data obeyed Michaelis-Menten kinetics with the transformation $S.V^{-1}$ vs S (Woolf plot) possessing high linear correlation coefficients ($r>0.95$). From these linear regressions the apparent K_m (y intercept.slope^{-1}) and v_{max} (slope^{-1}) were calculated for each substrate for each of the four soils (Table 5.7). Maximal activities were observed towards the nonanoate (C_9) ester of 4 MU for all four soils. The substrate specificity was similar for the lipase extracted from all four soils and resembles that previously reported for pancreatic and plant lipases (Jacks and Kircher 1967) and for bacterial lipases (10.3.1). Maximal substrate affinity (minimal apparent K_m)

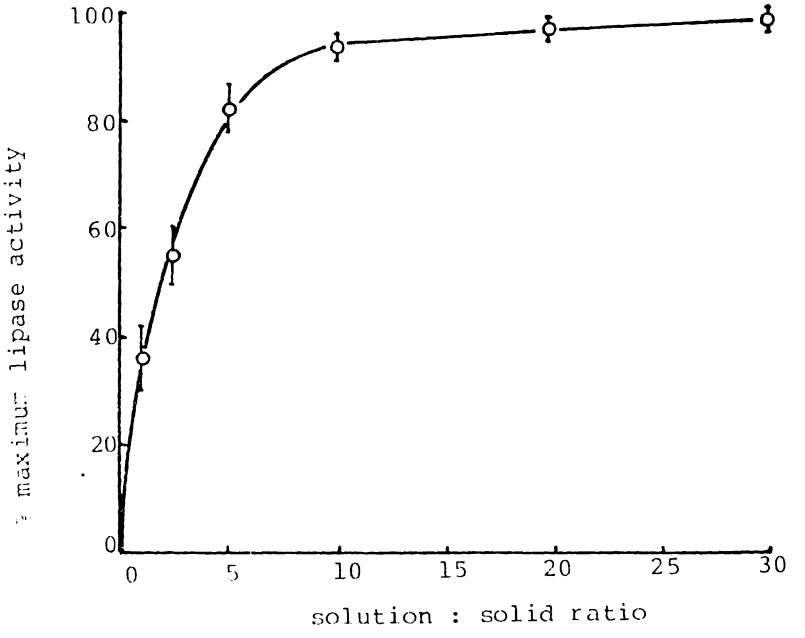


Fig. 5.3 Effect of pyrophosphate extractant to soil ratio on the recovery of lipase activity from four New Zealand soil samples. Error bars represent the spread of results for all four soils.

TABLE 5.7 Kinetic constants for the hydrolysis of fatty-acyl-esters of 4 MU by lipase extracted from four New Zealand topsoils. K_m values are uM. ν_{max} values are nmol 4 MU min^{-1} g^{-1}.

SUBSTRATE (ester of 4 MU)	SOIL							
	Hamilton clay loam		Ruatangata clay		Horotiu silt loam		Waitete sand	
	K_m	ν_{max}	K_m	ν_{max}	K_m	ν_{max}	K_m	ν_{max}
Acetate (C_2)	35	30	30	15	30	36	32	82
Butyrate (C_4)	17	36	15	20	12	47	16	112
Heptanoate (C_7)	12	74	10	43	9	90	12	260
Octanoate (C_8)	12	142	10	85	9	170	12	509
Nonanoate (C_9)	13	162	13	95	9	195	10	590
Laurate (C_{12})	19	120	20	78	28	142	28	432
Palmitate (C_{16})	38	32	35	22	45	45	55	115
Oleate ($C_{18:1}$)	18	47	15	31	45	61	45	181

was observed with the medium chain-length esters (C_7-C_9) for all four soils. Substrate affinities were remarkably similar between soils except for the longer-chain esters (C_{12}, C_{16}, $C_{18:1}$). The higher apparent K_m's observed for these substrates by the extracts from the two soils developed on volcanic ash (Horotiu silt loam and Waitete sand) indicates that steric hindrance problems were greater in these extracts. The apparent K_m's observed for hydrolysis of 4-methyl umbilliferone butyrate (12 - 17 µM) were slightly lower than that found by Pancholy and Lynd (1972) for soil lipase extracted by phosphate buffer (33 µM). The apparent K_m values observed for the 4 MU esters are similar to those previously reported for plant and animal lipases (Jacks and Kircher 1967; Guilbault *et al* 1968) and determined elsewhere in this thesis for bacterial lipases (10.3.2).

The substrate concentration chosen for routine assay with 4 MUN (312 µM) was well above the determined apparent K_m's for all four soils. The lack of substrate limitation was further demonstrated by the extent of 4 MUN hydrolysis by soil extracts being linear with time for at least the first 15 min, even with the high activity of the Waiteti sand extract (Fig. 5.4). Incubations for 10 min were used routinely.

5.2.6 Effect of temperature, air-drying and storage : Maximal rates of hydrolysis of 4 MUN by pyrophosphate extracts from the four soils occurred between 30 - 40°C, with relatively little change in activity up to 50°C (Fig. 5.5). As the 4 MUN substrate becomes increasingly unstable at higher temperatures (2.6), 30°C was selected for routine assay.

The lipase activity in pyrophosphate extracts was extremely stable and extracts could be conveniently stored prior to analysis. With the soil extracts tested, no activity was lost when stored at -20°C or 4°C for up to 5 days (Table 5.8). At 20°C, the most rapid

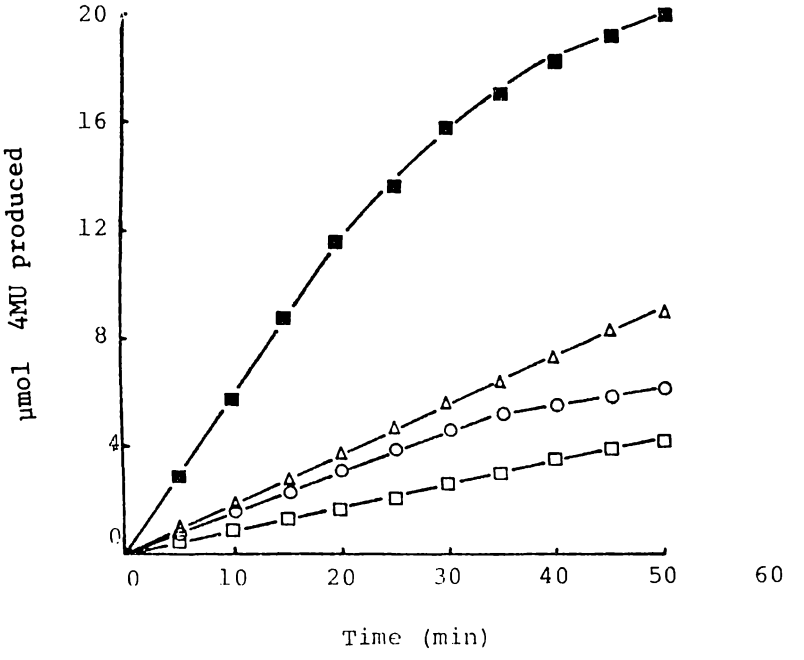


Fig. 5.4. Hydrolysis of 4 MUN with time by pyrophosphate extract of four soil types: o-o, Hamilton clay loam; □-□, Ruatangata clay; Δ-Δ, Horotiu silt loam; ■-■, Waitete sand.

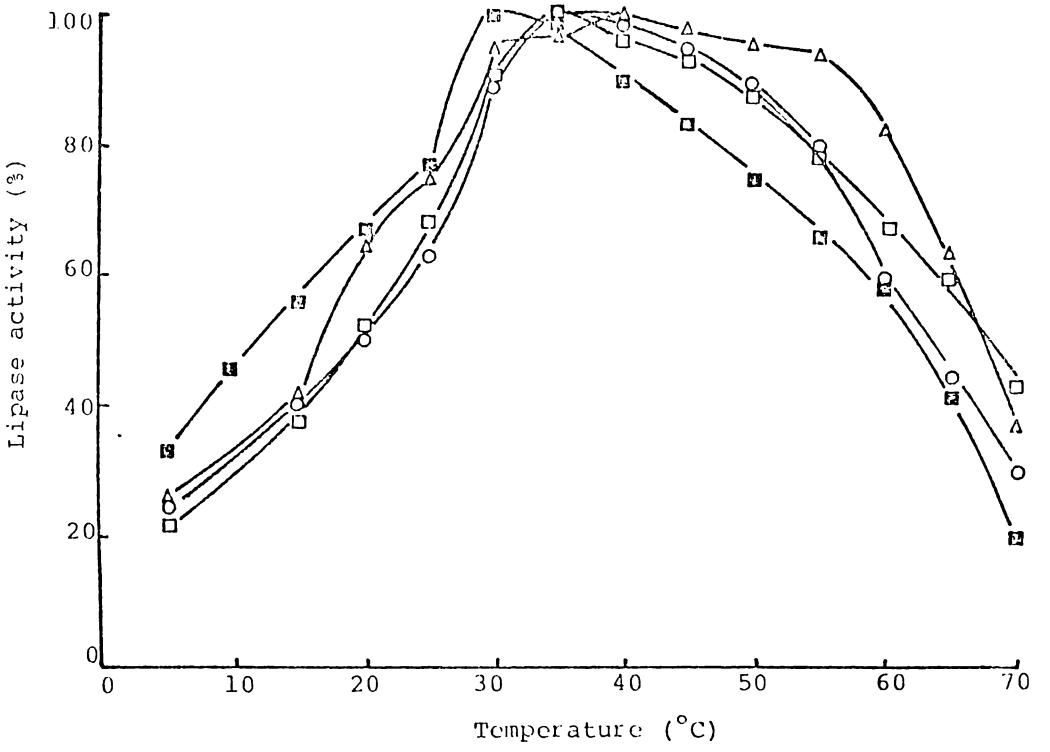


Fig. 5.5 Effect of temperature on lipase activity of pyrophosphate extracts of four soil types. o-o, Hamilton clay loam; □-□, Ruatangata clay; Δ-Δ, Horotiu silt loam; ■-■, Waitete sand.

TABLE 5.8 Effect of storage at different temperatures on relative activity of lipase extracted from soils by 0.1 M tetra-sodium pyrophosphate.

S O I L	Temperature of Storage					
	-20°C		4°C		20°C	
	5 days	10 days	5 days	10 days	5 days	10 days
	(% activity)					
Hamilton clay loam	100	95	100	84	88	73
Ruatangata clay	100	100	100	88	90	77
Horotiu silt loam	100	100	100	96	95	90
Waitete sand	100	100	100	100	100	97

loss of activity was observed in extracts from the Hamilton clay loam and Ruatangata clay.

The temperature stability of the pyrophosphate extracts was determined by pre-incubation for 1h at a range of temperatures prior to analysis (Fig. 5.6). All soil extracts were stable up to 50°C. Above this temperature the Hamilton clay loam and Ruatangata clay extracts were less stable (T at 50% inactivation = 57°C) than those from Horotiu silt loam and Waitete sand (T at 50% inactivation = 75°C). The temperature stability of the lipase in the soil (as opposed to the soil extracts) was determined by 1h pre-incubation of the soils at various temperatures prior to extraction and analysis. The lipase present in the soils proved more temperature stable than that in the extracts with no loss of activity occurring at the highest temperature used (90°C). Lipase activity of extracts or soils were totally destroyed by autoclaving at 121°C for 15min. The thermal stability of *in situ* soil lipase was considerably greater than that observed for lipases from microorganisms isolated from the soil (10.3.4).

No detectable difference in extractable lipase activity was apparent between soil when collected fresh or after storage at field-moisture at 4°C for up to 2 years before extraction and assay. Air-drying of soils before extraction and assay decreased measured lipase activity by 32% in Hamilton clay loam, 44% in Ruatangata clay, 14% in Horotiu silt loam, and 11% in Waitete sand. Similar decreases in lipase activity upon air-drying were observed by Pokorná (1964).

5.2.7 Effect of pH : The lipase activity of pyrophosphate extracts was determined in a range of buffers between pH 2.0 - 10.0 (Fig. 5.7) Equivalent lipase activities were obtained at the same pH in regions where the buffer systems overlapped, indicating that the buffers themselves did not have differing effects on enzyme activity. Lipase

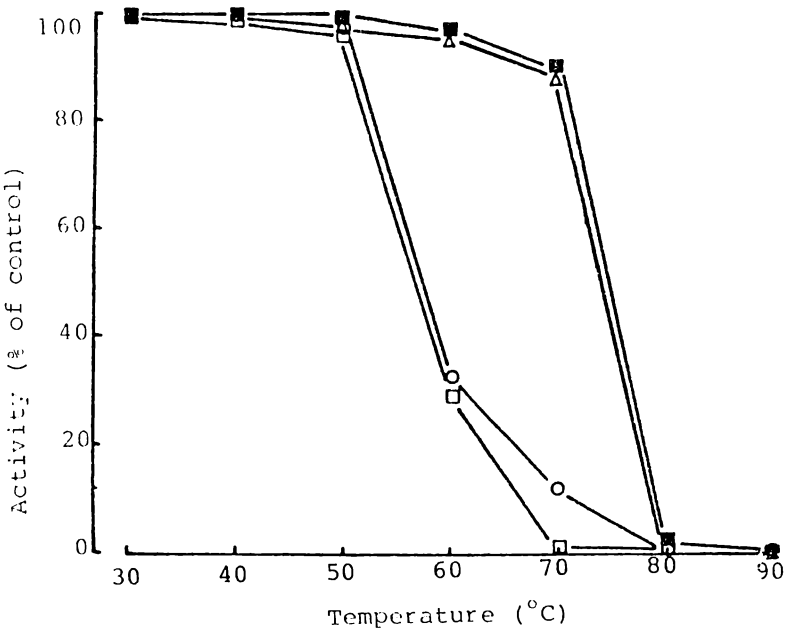


Fig. 5.6. Effect of pre-incubation temperature on the lipase activity of pyrophosphate extracts from various soils. Pre-incubation time was 1h. Soils were Hamilton clay loam (o-o), Ruatangata clay (□-□), Horotiu silt loam (Δ-Δ), and Waitete sand (■-■).

activity characteristically displayed two pH optima; one at pH 6.5 - 7.0, the other at pH 8.0 - 8.5. The proportion of activity present in each peak varied, depending upon the soil. The choice of pH for routine assay is therefore somewhat arbitrary and may be dependent upon soil types. A pH of 7.5 was chosen as it avoided the problems of increased non-enzymic substrate hydrolysis at alkaline pH, and depression of fluorescence of 4 MU standards (prepared in a matrix of buffer, extract and 4 MUN) at acid pH.

The dual peaks observed in pH/activity studies may reflect separate contributions from the bacterial and fungal components of the soil microbiota. Pure culture studies on soil isolates revealed that bacterial lipases characteristically display an alkaline pH optimum whilst fungal lipases characteristically have optima which are acidic (8.3.3). This appears to be a general phenomenon (Lawrence 1967b); The properties of each soil would be expected to regulate the relative contributions of bacteria and fungi to the soil lipase pool, and hence explain the differences in observed pH profiles. More definitive proof of this hypothesis has been provided by Hopkins (1982) who re-inoculated sterilised soils with bacterial and/or fungal lipolytic isolates, and observed the development, upon incubation, of a single alkaline lipase peak (bacterial inoculum), a single acidic lipase peak (fungal inoculum), and dual peaks (both bacteria and fungi).

When Hamilton clay loam was amended with triacylglycerol (as per 6.2.1) the relative proportions of the two peaks in the pH profile changed (Fig. 5.8). The alkaline peak became of greater relative significance, possibly indicating the dominant role of bacteria in the breakdown of added triacylglycerol. The large increase in the bacterial population capable of utilising triacylglycerol as a sole carbon and energy source (relative to that of fungi and actinomycetes) upon amendment of soil with triacylglycerol (8.2.3) further corroborates this suggestion.

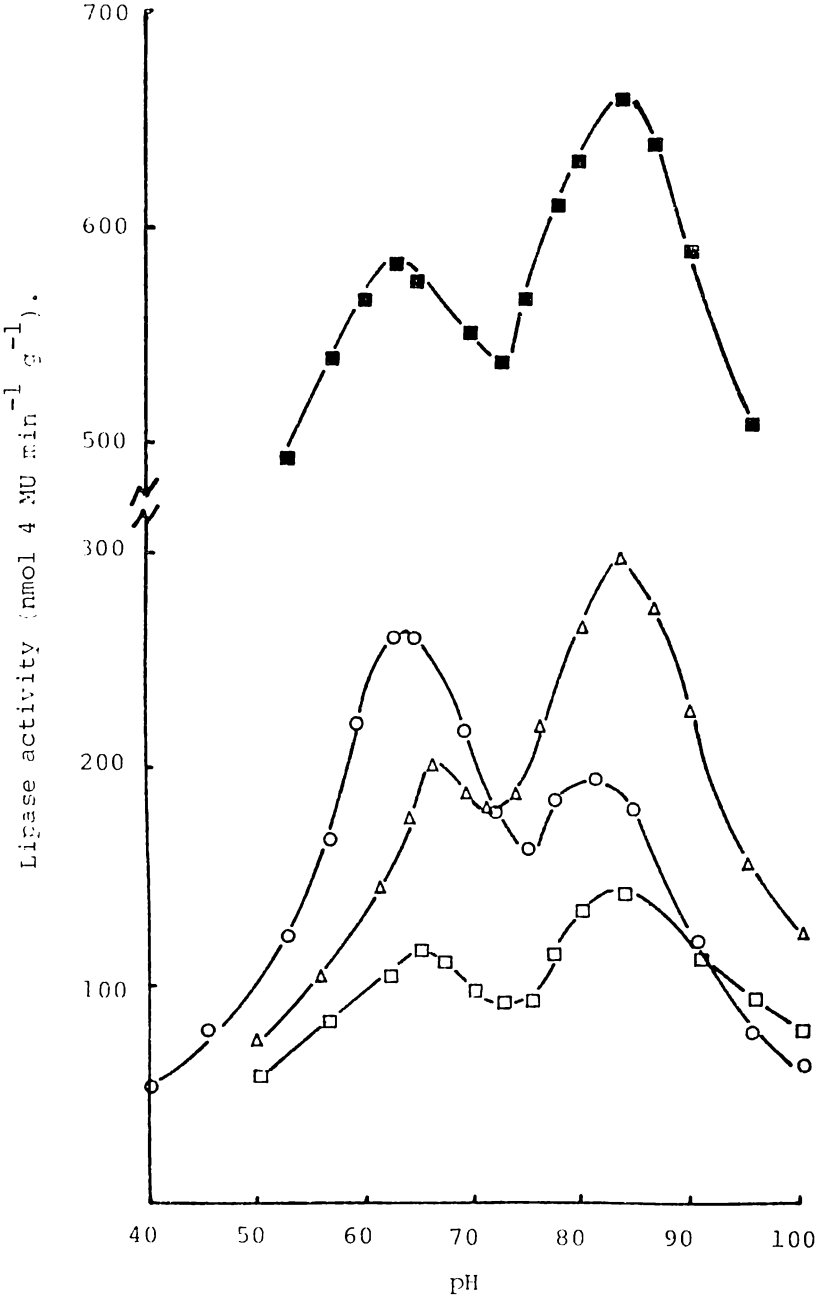


Fig. 5.7. Effect of pH on the lipase activity of soil extracts Hamilton clay loam (o-o); Ruatangata clay (□-□), Horotiu silt loam (Δ-Δ), Waitete sand (■-■).

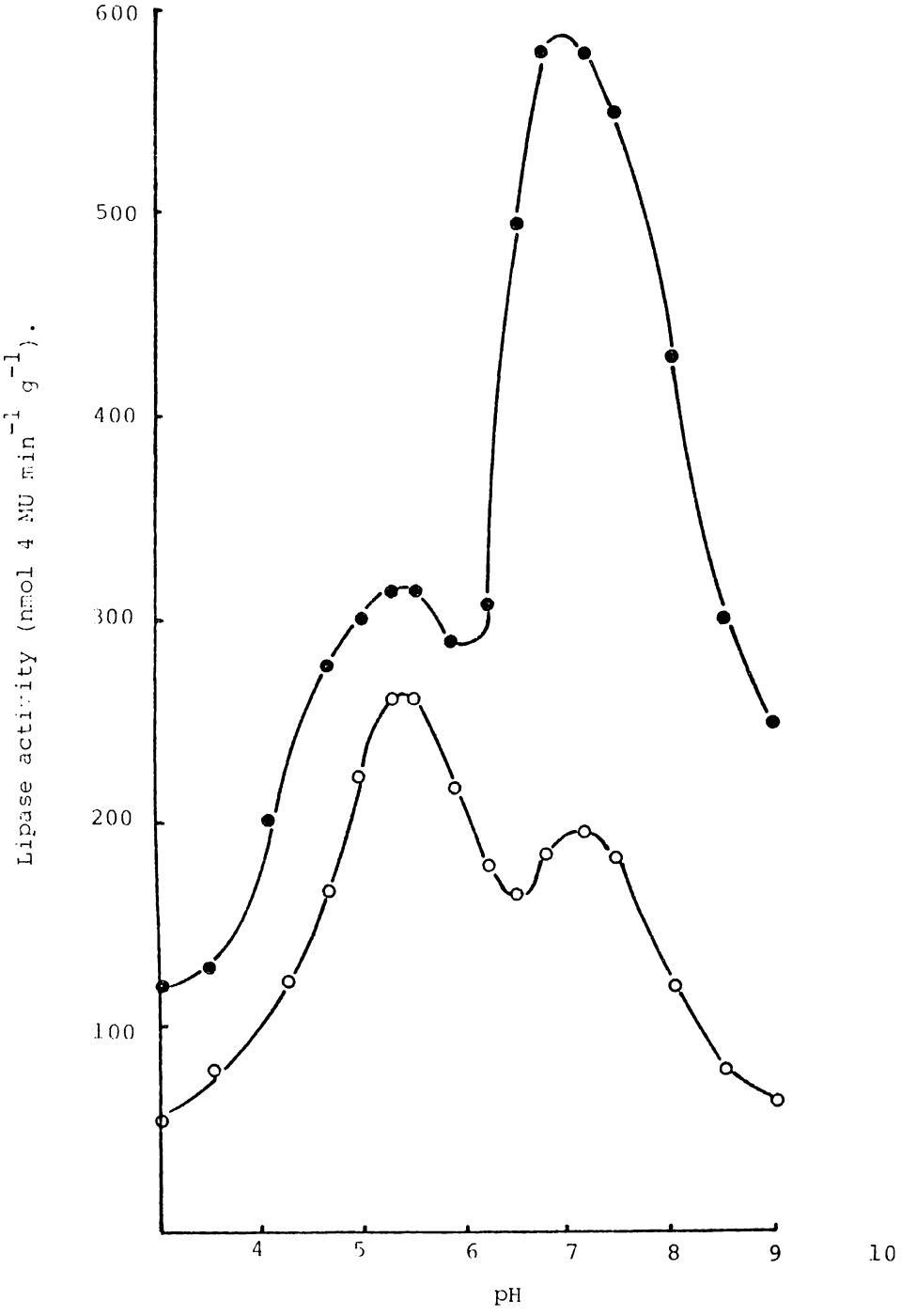


Fig. 5.8. Effect of pH on the lipase activity of soil extracts from an unamended (o-o) and amended (●-●) Hamilton clay loam soil. Amendment: Tallow at 2.5 mg g^{-1}; incubated for 8 days at 25°C.

5.2.8 Effect of calcium : Calcium is a common cofactor added to lipase mixtures (Desnuelle 1972). However, the addition of calcium to the standard fluorometric assay developed in this thesis did not stimulate activity of pancreatic or bacterial lipases towards 4 MUN (3.3). Addition of Ca^{2+} (final concentration 5 mM; as $CaCl_2$) to the fluorometric assay system had either no effect on extracted soil lipase activity (Hamilton clay loam and Ruatangata clay) or caused a decrease (Horotiu silt loam and Waitete sand) (Table 5.9). In assay systems from the latter two soils a brown precipitate formed upon addition of Ca^{2+} to the buffer-plus-extract system. As Ca^{2+} is capable of precipitating organic material (Tabatabai and Bremner 1969; Ladd 1972) it may be that co-precipitation of lipase and humic material occurred with a subsequent decrease in activity, possibly associated with increased steric hindrance.

5.3 Lipase activities of some New Zealand soils

5.3.1 Soils used : Surface soil samples (0-15 mm) collected from a range of New Zealand Soil Bureau reference sites were seived (<2mm), before storing at field moisture in sealed plastic bags at 4°C until analysed. Some properties of the collected soils were determined using the methods described by New Zealand Soil Bureau (1968) and are presented in Table 5.10. In addition to surface samples, depth profile samples were collected for Waitete sand, Tirau silt loam and Hamilton clay loam.

5.3.2 Lipase activities and soil types : The lipase activity of 13 New Zealand topsoils was determined by the procedure described in 5.2.2. In addition, the amount of extractable organic material and extractable protein (Appendix I) was measured. Results (Table 5.11) demonstrated a wide range of soil lipase levels (28 - 597 nmol 4 MU min^{-1} g^{-1}), extractable organic material (190 - 1260 absorbance units at 280 nm g^{-1}), and extractable protein (1.70 - 10.91 mg g^{-1}). Even

TABLE 5.9 Effect of Ca^{2+} on the activity of lipase extracted from four New Zealand topsoils.

S O I L	Lipase activity (nmol 4 MU $min^{-1}g^{-1}$)	
	No Ca^{2+}	Ca^{2+} (5 mM)
Hamilton clay loam	161	165
Ruatangata clay	92	94
Horotiu silt loam	195	135
Waitete sand	595	410

TABLE 5.10 Properties of thirteen soil samples (0-15 cm depth) collected from New Zealand Soil Bureau reference sites.

S O I L	Classification	pH	Organic C (%)	Clay (%)	CEC
Hamilton clay loam	brown granular loam	5.3	3.2	28	18.7
Horotiu silt loam	yellow-brown loam	5.8	9.8	24	31.6
Kaawa hill soil	yellow-brown earth	5.6	5.9	29	27.2
Naike clay	brown granular loam	5.6	6.4	50	24.2
Netherton clay loam	gley	5.5	5.6	30	21.5
Ruatangata clay	brown loam	5.3	5.3	58	34.7
Rukuhia peat	organic	5.0	60.5	<1	62.1
Taupo sandy silt	yellow-brown pumice	5.8	7.1	12	26.2
Te Kowhai silt loam	yellow-brown loam	5.9	11.2	21	36.0
Tirau silt loam	yellow-brown loam	5.5	7.5	22	32.2
Waimete North clay	red-brown loam	5.6	3.9	41	32.0
Waitete sand	yellow-brown loam	5.7	8.5	10	24.6
Wharekohe silt	podzol	5.1	5.5	10	21.1

TABLE 5.11 Levels of 0.1 M pyrophosphate (pH 7.5) extractable lipase, organic material and protein in some New Sealand topsoils.

S O I L	Lipase (nmol min min^{-1} g^{-1})	Organic material (Abs 280 nm g^{-1})	Protein ($mg\ g^{-1}$)
Hamilton clay loam	161	190	1.90
Horotiu silt loam	194	412	8.20
Kaawa hill soil	181	293	3.09
Naike clay	307	190	1.70
Netherton clay loam	210	580	7.90
Ruatangata clay	95	288	5.15
Rukuhia peat	28	1260	10.91
Taupo sandy silt	82	220	3.23
Ke Kowhai sandy loam	265	158	3.08
Tirau silt loam	344	205	3.75
Waimate North clay	278	295	3.11
Waitete sand	597	720	9.38
Wharekohe silt	220	236	4.64

disregarding the organic soil (Rukuhia peat), there was no correlation between lipase and organic material or between lipase and protein in the extracts (Table 5.12). Of the soil properties measured (Table 5.11), none gave strong correlations with lipase activities, the best correlation obtained being that with organic C (r = 0.465). Some previous work has demonstrated strong correlations between soil enzymic activities and organic C in a range of topsoils (*e.g.* Tabatabai and Bremner 1970; Cooper 1972; Dalal 1975; Zantua *et al* 1977) whilst other studies have revealed only weak relationships (Ladd 1972; Pancholy and Rice 1973a; Speir 1977).

5.3.3 Lipase activities in soil profiles : The lipase levels through a depth profile were determined for Waitete sand, Tirau silt loam and Hamilton clay loam (Table 5.13). Within each soil there was a strong correlation (r>0.90) between lipase activity and both extractable organic material and soil organic C. Similar results have been reported for other soil enzymes (Myers and McGarity 1968; Gould *et al* 1973; Tabatabai 1977; Speir and Ross 1978; Juma and Tabatabai 1978) and probably reflects the trends in relative biological activity.

5.3.4 Lipase activities and season : The seasonal variation in lipase activity was studied by collection and analysis of monthly surface samples (0-15 cm) from the Hamilton clay loam reference site over the period March 1978 - March 1979 (Fig. 5.9). Activities were relatively constant throughout the year, except for a transient rise in October - November. These higher spring-early summer values may reflect a general increase in microbial activity associated with warmer soil temperatures (Ramírez-Martínez and McLaren 1966; Khaziev 1969). The relatively stable level of lipase indicates that the opposing processes of synthesis and denaturation were equal. In 5.4, the concept of a constant stabilised level of lipase in soil is presented. Enzyme produced in excess of this stabilised level is unprotected and therefore has only a short lifetime.

TABLE 5.12 Relationship between 0.1 M pyrophosphate extractable soil lipase levels and other soil properties. Correlations derived from linear regression analysis of 12 mineral soils using data of Table 5.10 and 5.11.

Soil property	Correlation coefficient (r)*
Extractable organic material	-0.069
Extractable protein	0.097
pH	0.173
Organic C	0.465
Clay content	-0.317
Cation-exchange capacity	-0.096

* Linear regressions of soil property against lipase activity.

TABLE 5.13 Effect of soil depth on lipase levels of three New Zealand soils.

DEPTH (cm)	Lipase activity (nmol 4 MU min^{-1} g^{-1})		
	Hamilton clay loam	Tirau silt loam	Waitete sand
0-5	202	485	755
5-10	165	380	570
10-15	151	312	495
15-20	88	268	450
20-25	60	161	403
25-30	47	90	228
30-40	22	41	148

Correlations

Hamilton clay loam : lipase vs extractable organics, r = 0.91
lipase vs organic C, r = 0.97

Tirau silt loam: lipase vs extractable organics, r = 0.94
lipase vs organic C, r = 0.96

Waitete sand: lipase vs extractable organics, r = 0.94
lipase vs organic C, r = 0.94

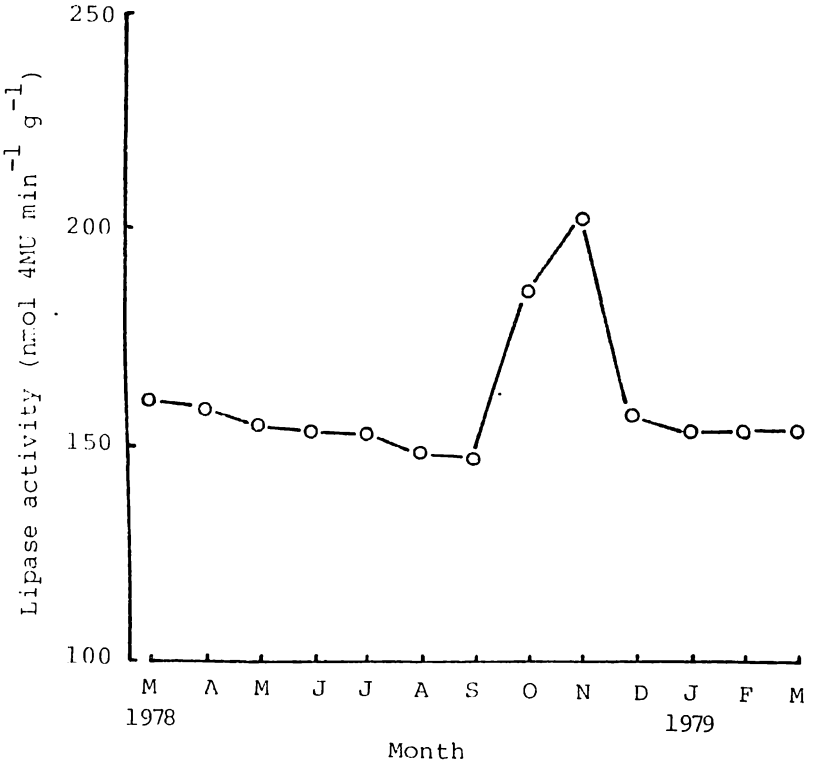


Fig. 5.9. Lipase activity in Hamilton clay loam soil at different times of the year.

5.4 The state of lipase in soil

A conceptual representation of the state of accumulated abiontic enzymes in soil has been discussed by Kiss *et al* (1975), Ladd and Butler (1975), Skujins (1976) and Burns (1978). The lipase present in soil appears to exist as abiontically accumulated enzyme (5.2.3), with activities in cell-free extracts of soils being as high or higher than that observed with the soil as a whole.

The level of an enzyme in soil is the net result of the opposing processes of synthesis and denaturation (Kiss *et al* 1978). The observation that lipase activities in soil remain relatively constant throughout the year (5.3.4) indicates that these processes are equal. When triacylglycerol-amended soils are incubated the lipase levels are transiently induced but subsequently return to pre-amendment levels (6.3.1). These studies suggest that the soil has a finite capacity for stabilisation of lipases and that lipases produced in excess of this capacity are unprotected and subsequently denatured by proteolytic activity. A similar approach has been used by other workers to explain the long-term constancy of an enzyme level in a particular soil both seasonally and in response to various amendments (Ladd and Paul 1973; Zantua and Bremner 1976 and 1977; Bremner and Mulvaney 1978).

As discussed in 4.3, evidence suggests that accumulated abiontic enzymes in soil may exist bound to humic material and hence display increased stability. It may seem logical, therefore, to assume that soils with high levels of organic material will possess the greatest capacity for binding and thereby stabilising enzymes. The results presented in 5.3.2 demonstrated only a weak relationship ($r = 0.465$) between lipase levels and organic matter contents for 12 New Zealand topsoils. Other evidence, however, suggests that a stable humus-lipase complex may exist. Specifically:

(i) Within each soil a strong relationship existed between lipase level and organic matter content through a depth profile (5.3.3).

(ii) The highest extraction of lipase from soils was obtained with an extractant that also extracted the most organic material (5.2.4).

(iii) Attempts at purification of soil lipase from other non-active organic material was unsuccessful (Table 5.14). Similar results were found for soil urease, also postulated to exist as a humus-enzyme complex (Ceccanti *et al* 1978).

The poor correlation obtained between lipase levels and organic matter contents for a range of soils may indicate that lipases are stabilised in soil by specific components of the humic material. The level of these specific components may regulate the capacity of the soil for stabilisation of lipase, rather than the total organic content. As lipases have an affinity for hydrophobic surfaces (Brockerhoff and Jensen 1974), it may be that hydrophobic regions of the humic material act as stabilisation sites. The increased lipase activity obtained with phosphate buffer extracts upon the inclusion of urea (5.2.4) indicates that hydrophobically-bound components may be involved in stabilisation of soil lipase. The non-uniform distribution of lipase in the humic fraction that such a postulate implies was demonstrated by the variation in specific activity (on an organic matter basis) that was observed with variations in extraction time or extractant (5.2.4). Similar non-uniform distribution of soil enzymic activity has been demonstrated by Cacco and Maggioni (1976) for esterase and by Nannipieri *et al* (1974) for urease.

An insight into the binding mechanisms whereby abiontic lipases may exist in soil can be gained by examining the relative efficiencies of the extractants used (5.2.4). Enzymes extracted from soil by phosphate buffer may be taken to represent enzymes bound by

TABLE 5.14 Procedures attempted for purification of soil lipase from pyrophosphate extracts.

METHOD *	BEHAVIOUR
$(NH_4)_2SO_4$ (60% saturation)	Co-precipitation of organics and lipase.
Protamine sulphate (1 mg mL^{-1})	Co-precipitation of organics and lipase.
Alcohol (50% v/v)	Both organics and lipase not precipitated.
Acetone (50% v/v)	Some precipitate formed, loss in activity.
Ultrafiltration	>80% activity lost.

* Methods used were as described for bacterial lipases in 10.2.2 with 0.1 M pyrophosphate pH 7.5 extracts of soil as the starting material.

relatively weak ionic bonds to soil constituents (Skujins 1976; Hayano 1977). When an enzyme can *only* be extracted from soil concomitantly with the extraction of humic material, this is presumptive evidence for the enzyme being more strongly bound to the humic material (Ladd and Butler 1975; Skujins 1976; Burns 1978). In all four soils examined (5.2.4) the greatest proportion of lipase (56 - 87%) could only be extracted concomitantly with the humic material (by pyrophosphate) and therefore would seem to exist in a strongly-bound form.

When Hamilton clay loam was amended with triacylglycerol and incubated for several weeks (6.3.1) there was a change in the distribution of the soil lipase (Table 5.15). Large fluctuations occurred in the phosphate-buffer extractable lipase levels whilst little change was observed in the lipase extractable only by pyrophosphate. This suggests that lipase produced in response to substrate addition did not become strongly-bound to the humic material and hence was susceptible to proteolytic attack. Apparently, no further "stabilisation sites" existed for the binding of the newly-produced enzyme.

The studies of 5.2 demonstrated the higher stability of lipases in soils derived from volcanic ash (Horotiu silt loam and Waitete sand). Lipases from these soils were more resistant to denaturation during extraction, air-drying and exposure to high temperatures. As a higher proportion of the lipase activity in these soils was strongly bound (extractable only with pyrophosphate) it may be suggested that such binding has conferred the observed stability.

5.5 Summary

A precise and rapid method for the assay of soil lipases was developed using the fluorogenic substrate 4-methyl umbelliferone nonanoate (4 MUN). The method utilises a 3h 0.1 M tetra-sodium pyrophosphate (pH 7.5) extraction, followed by a 10 min assay. Optimum activity in extracts was observed between 30 - 40°C. The lipases

TABLE 5.15 Extraction of lipase activity from unamended and amended Hamilton clay loam soil. Amended was with tallow at a loading rate of 2.5 mg g^{-1} with incubation at 25°C.

SOIL TREATMENT	Lipase activity (nmol 4 MU min^{-1} g^{-1})	
	Phosphate buffer extractable	Pyrophosphate extract of residue*
Unamended	50	110
Amended: 8 days incubation	368	125
Amended: 30 days incubation	58	120

* The soil residue of samples initially extracted with phosphate buffer (0.1 M, pH 7.5) was re-extracted with pyrophosphate.

were thermostable and extracts could be conveniently stored at 40°C or -20°C for 5 days without loss of activity. Extracted lipases characteristically displayed two pH optima; one each side of neutrality. These dual peaks in the pH profile are tentatively ascribed to the separate contributions of fungi (acidic peak) and bacteria (alkaline peak).

Lipase activities of 13 New Zealand topsoils ranged from 28 to 597 nmol 4 MU min^{-1} g^{-1} and were not strongly correlated with pH, clay, cation-exchange capacity or organic matter.

Attempts to separate a soil lipase fraction from non-active organic material were unsuccessful. The possible state of lipase in soil was discussed and the existence of a humus-lipase complex proposed.

CHAPTER 6 TRIACYLGLYCEROL DEGRADATION IN SOILS

6.1 Introduction

6.1.1 Principles of land application of wastewaters : Increasing public pressure, accompanied by increasingly severe legal restrictions on the preservation of surface water quality, has brought about the need for a higher level of waste treatment before discharge. Secondary and tertiary treatment of wastes, necessary to meet stricter effluent standards, involve high capital and maintenance costs. This has provided an economic incentive for the consideration of land treatment of wastes as an alternative (Overcash and Pal 1979). In addition, the application of waste to land at suitable loading rates may result in beneficial effects on soil fertility by increasing nutrient levels, rates of nutrient cycling and providing water (Bouwer and Chaney 1974).

The two main design criteria for land treatment of wastewaters are minimum total cost (and usually, therefore, minimum land area) and minimum impact on the environment (Bouwer and Chaney 1974). Excessive waste applications to soil may result in undesirable effects including malodours (Mosier *et al* 1977), soil clogging (Rice 1974), contamination of surface waters and/or groundwaters (Norstadt *et al* 1977), and toxic effects on vegetation and/or microbially-mediated nutrient cycling (Jones *et al* 1970; Chaney and Giordano 1977; Overcash and Pal 1979). Thus, to meet the design criteria, it is necessary to assess the assimilative capacity of the receiving soil for each of the constituents of the waste. The least easily assimilated constituents determines the maximum acceptable loading rate and, therefore, the land area required (Overcash and Pal 1979). Assessment of

the assimilative capacity of a soil for a biodegradable organic constituent usually involves addition of the constituent to a soil and determination of its rate of breakdown by the respiratory response of the soil microbiota (*e.g.* Dibble and Bartha 1979).

6.1.2 Biodegradation of lipids in soil : Previous work : Studies on lipids in soil have concentrated on their extraction and composition (see reviews by Howard and Hamer 1960; Stevenson 1966; Morrison 1969; Braids and Miller 1975; Fridland 1976). The lipid fraction of soil contains hydrocarbons, alcohols, organic acids and their esters, sterols, terpenes, pigments and heterocyclics (Morrison 1969; Braids and Miller 1975). Lipids may constitute between 1 - 5% of the soil organic matter in mineral soils and up to 30% in organic soils (Stevenson 1966; Fridland 1976; Chae and Lowe 1980).

Little work has been performed on the fate of lipids in soil, either those occurring naturally or as added wastes (Braids and Miller, 1975).

The lipid that has received the most attention from the viewpoint of degradation in soil is crude oil and its distillates (*e.g.* Jobson *et al* 1974; Grudin and Syratt 1975; Lehtomaki and Niemela 1975; Jensen 1975; Raymond *et al* 1976; Dibble and Bartha 1979). In contrast, triacylglycerol breakdown in soil has received scant attention (Pancholy and Lynd 1972; Smith 1974). Triacylglycerols (the primary storage fat of plants and animals) are a common component in vegetable, fish and meat processing and domestic wastewaters (Heukelekian and Mueller 1958; Hunter and Heukelekian 1965; Loehr and de Navarra 1969; Kramer 1971; Banerji *et al* 1974; Smith 1974; Green *et al* 1976; Lordi and Lue-Hing 1976; Barker and Worgan 1981; Barnett *et al* 1981; Cooper 1981; Hrudey 1981). Lipids in wastewaters have been shown to cause problems in conventional treatment

systems (Heukelekian and Mueller 1958; Simpson 1970; Kramer 1971; Novak and Kraus 1973; Banerji *et al* 1974; Given *et al* 1974; Lordi and Lue-Hing 1976). The potential therefore exists for such problems to manifest themselves in land treatment systems, particularly if loadings are at such a rate as to exceed the lipid-degrading capabilities of the soil and thereby lead to lipid accumulation. The observation that lipids may be degraded less rapidly than the other major organic constituents of wastes (Loehr and Roth 1968; Novak and Kraus 1973) suggests that the assimilative capacity of the soil for lipids may regulate the loading rates, and hence the land area required, for the disposal of some wastewaters to land. The research presented in this chapter was performed in order to assess the assimilative capacity of soils for lipids and determine factors affecting the rate of lipid degradation.

6.2 Basic procedures for lipid degradation studies in soil

6.2.1 Amendment of triacylglycerol to soils : A 10% (w/v) emulsion of the desired lipid in distilled water was prepared by sonication for 5 min with a Dawe Soniprobe at maximum power. Lipids that were solid at room temperature were melted by heating to 60°C in a water bath prior to sonication. A suitable quantity of the lipid emulsion (or a dilution) was added to thinly-spread soil samples (usually 200 g) in the bottom of a plastic aquarium as a fine mist *via* a hand-operated spray unit. The soil was continually mixed during the application to ensure an even distribution of lipid. Both lipid-loaded soils and untreated control soils were brought to 60% of their water-holding capacity by addition of distilled water as a fine mist. Soils were then placed in plastic pots, weighed, covered, and incubated in the dark at the appropriate temperature. Moisture contents were maintained throughout the incubation by periodic addition of distilled water.

Loading rates ranged from 1 - 10 mg of lipid g^{-1} of soil (0.1 - 1.0% w/v). With the usual conversion factors to the plough layer of field soil, this converts to approximate areal loading rates of 2 - 20 tonnes ha^{-1} (Dibble and Bartha 1979; Overcash and Pal 1979).

6.2.2 Measurement of residual triacylglycerol levels in soil : Determination of the time taken for the degradation of triacylglycerol when applied to soils, was a fundamental requirement of the investigation. To effectively meet this requirement, a reliable technique for efficient extraction and subsequent quantitative determination of the triacylglycerol had to be developed.

Previous work on lipids in soil has involved investigations into the naturally-occurring lipid component (Braids and Miller 1975). These naturally-occurring lipids may be of a diverse nature, with widely varying solubilities in a range of organic extractants, and may exist in strongly-bound organic matrices, or on clay minerals (Goring and Bartholomew 1949; Stevenson 1966; Braids and Miller 1975). For this reason, the lipid extraction techniques giving the highest extraction efficiencies from soils may involve the use of soil pretreatment (usually with HCl-HF), heat, long extraction periods, and successive extractions with different organic mixtures (Hance and Anderson 1963; Morrison and Bick 1967; Wang *et al* 1969; Kowalenko and McKercher 1970). Such exhaustive extraction techniques are time-consuming and unsuitable when large numbers of samples are to be extracted. An alternative, simple and rapid extraction procedure was employed which was capable of effeciently extracting the lipid of interest (applied triacylglycerol) from soil samples. The procedure of Kowalenko and McKercher (1970) was modified with a single 3h 10:1 (extractant:soil ratio) hexane-acetone (1:1 v/v) extraction, followed by subsequent colourimetric determination of triacylglycerol (Fletcher 1968). The standard procedure was:

To 1.0 g of soil in a 15 mL borosilicate test-tube (teflon-lined screw-cap) was added 10 mL of hexane-acetone (1:1 v/v). The tubes were subsequently placed horizontally on an orbital incubator and shaken at 200 rev min^{-1} for 3h. After centrifugation at 2000 G for 4 min a suitable aliquot (0.1-1.0 mL) was withdrawn, and placed into a fresh screw-cap test-tube. With no caps, the tubes were placed in a 60°C water bath and evaporated to dryness (10-15 min). 2 mL of freshly-redistilled (within 1 week) "Aristar" grade isopropanol was added and tubes capped and shaken for 1 min. Subsequent saponification was performed by addition of 0.5 mL of 1M KOH and incubating for 5 min at 60°C. After cooling to room temperature, the released glycerol was oxidized to formaldehyde by addition of 0.5 mL of Na m-periodate (0.25% in 2 M acetic acid) and subsequent incubation for 10 min. The formaldehyde was converted to diacetyldihydrolutidine by addition of 3 mL of a colour reagent (prepared at least a day beforehand by mixing 80 mL redistilled isopropanol, 40 mL 2M ammonium acetate, and 0.3 mL acetylacetone) followed by incubation for 30 min at 60°C. The resultant yellow colour was measured within 20 min at 410 nm on a Beckman DU spectrophotometer with isopropanol in the reference cell. A standard curve was constructed (Fig. 6.1) with trioleoylglycerol (Sigma Chemical Co, St Louis, USA) over the range 0 - 240 µg by using 0 - 2 mL of a standard 120 $mg.L^{-1}$ solution in hexane-acetone (1:1 v/v) Blank absorbances were determined by extraction and analysis of a control soil that had not received lipid amendment.

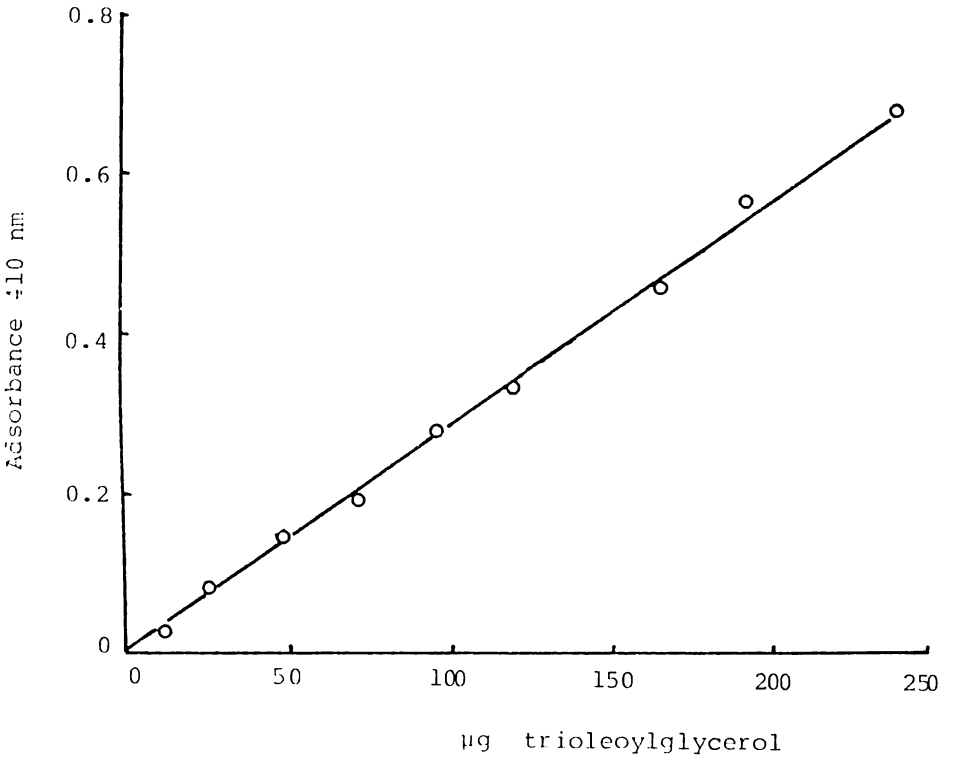


Fig. 6.1 Relationship between absorbance and trioleoylglycerol level. r = 0.998; equation for the line is: y = 0.00262x + 0.00452

Results obtained with applied lipids were corrected for the different amount of glycerol released per unit weight of lipid in comparison to that of the trioleoylglycerol standard. This was merely the ratio of molecular weights (the "average molecular weight" of mixed triacylglycerols being obtained from Appendix II) with the data derived from the trioleoylglycerol standard curve being multiplied by 0.34 for tributyrylglycerol, 0.99 for olive oil, 0.94 for tallow, and 0.88 for anhydrous milk fat to arrive at µg triacylglycerol.

Results were expressed as µg triacylglycerol g^{-1} of soil.

The technique described provided >95% recovery of triacylglycerols when sprayed onto soil as an aqueous emulsion (Table 6.1). Repeat extraction of the soil residue yielded no further triacylglycerol. The minimum extraction time which provided optimum recovery was 3 h (Fig. 6.2).

Blank absorbances varied, depending upon the soil being extracted. However, the standard deviation of the blank absorbances (S_B) was relatively constant between soils at 0.015. The detection limit of the method may therefore be defined as that triacylglycerol level giving an absorbance at 410 nm of 0.070 (4.65 x S_B) above the blank (2.2). From the standard curve this corresponds to a trioleoylglycerol level of 25 µg. With a maximum of 2 mL of a 10 mL extract from 1 g of soil being used in the assay, this detection limit converts to 125 µg trioleoylglycerol g^{-1} of soil. For other triacylglycerols this is a detection limit of 43 µg tributyrylglycerol g^{-1} of soil, and 110 µg anhydrous milk fat g^{-1} of soil. Analysis of 10 subsamples of an olive oil amended soil (loading rate 2.5 mg g^{-1}) yielded a relative standard deviation of 7%. The amount of triacylglycerol

TABLE 6.1 Extraction of various triacylglycerols from Hamilton clay loam at three different loading rates.

Triacylglycerol	Extraction efficiency (%)*		
	Loading rates (mg g^{-1} of soil)		
	0.25	2.5	10.0
Trioleoylglycerol	100	96	96
Tributyrylglycerol	100	100	100
Trilaurylglycerol	98	95	95
Tripalmitoylglycerol	95	96	95
Olive oil	101	97	95
Tallow	99	100	95
Anhydrous milk fat	98	95	97

* Extraction efficiency was determined by comparing the triacylglycerol content of soil extracts with that in the emulsion used.

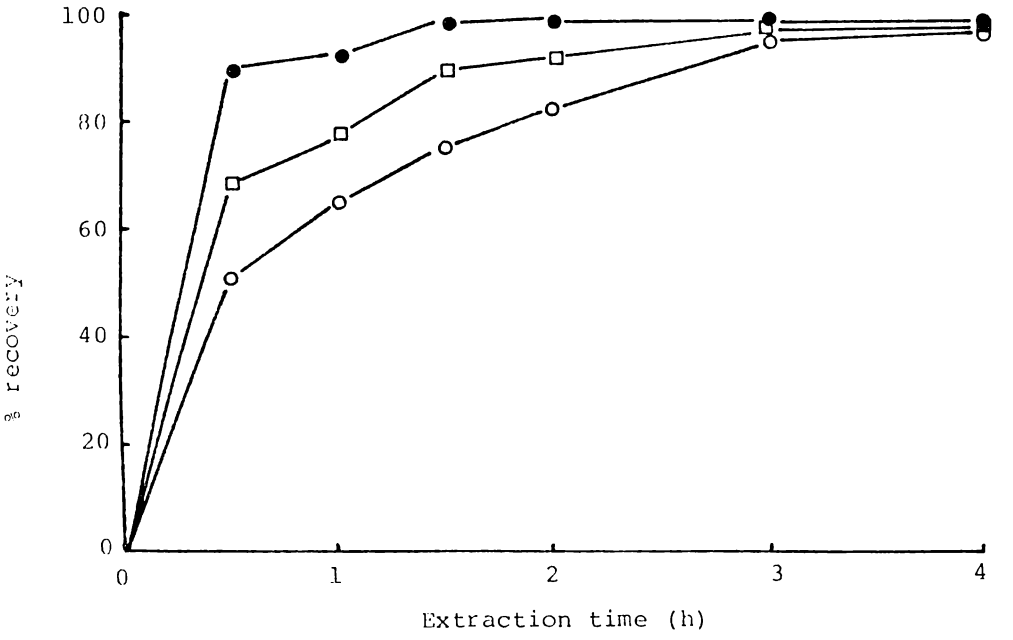


Fig. 6.2. The effect of extraction time on the recovery of olive oil from Hamilton clay loam soil at 3 different loading rates. Loading rates of 0.25 mg g^{-1} of soil (●-●), 2.5 mg g^{-1} of soil (□-□), and 10.0 mg g^{-1} of soil (o-o).

degraded was calculated as the difference between the level applied and the level remaining.

The technique described will measure, in addition to triacylglycerol, any hexane-acetone extractable glycerol or alkali susceptible glycerol ester (including monoacylglycerol and diacylglycerol). With triacylglycerol-amended soils the possibility exists that these lipolytic breakdown products and intermediates may accumulate in the soil. As free glycerol would be an amenable substrate for microbial utilisation its accumulation would not be expected. Nevertheless, levels of free glycerol in the hexane-acetone extracts were occasionally determined using the above described technique with the exclusion of the alkaline saponification step. The presence of tri-, di- and monoacylglycerols and fatty acids in the extracts was qualitatively examined using thin-layer chromatographic separation. The TLC system was essentially that described by Skipski and Barclay (1969; p 549) and used previously by Clément *et al* (1962) and Mencher and Alford (1967) for investigations into lipolytic hydrolysis products. The procedure was:

> Aliquots (10 - 100 µL) of the hexane-acetone soil extract were spotted 4 cm from the bottom of a pre-coated silica-gel (0.20 mm thick), aluminium-backed plate (Merck, Darmstadt) and developed using ascending chromatography with petroleum ether: ethyl ether: acetic acid (90:30:1) until the solvent front had migrated 12 - 15 cm from the origin (approximately 30 min). Plates were subsequently sprayed with 20% (v/v) NH_4HSO_4 and charred at 180°C for 1 h to detect lipids. On each chromatogram 10 µL of a standard mixture containing trioleoylglycerol, 1,2-dioleoylglycerol, monooleoylglycerol, and oleic acid (all (Sigma Grade I) all at 1 $g.L^{-1}$ (in hexane-acetone) was run.

When run separately each lipid standard produced a single spot (indicating purity) and migrated at a different rate to the others (indicating discrimination). Approximate R_f's obtained were 0.60 for triacylglycerols, 0.32 for fatty acids, 0.12 for diacylglycerols and 0 for monoacylglycerols.

The TLC technique described was capable of detecting 0.5 µg of lipid on the chromatogram. With 100 µL of the 10 mL extract being applied, this represents a detection limit of 50 µg lipid g^{-1} of soil.

6.2.3 Measurement of lipase induction : The level of lipase in amended soils was monitored using the standard pyrophosphate extraction - fluorometric assay technique described in 5.2.2. In samples where lipase activities exceeded 1000 nmol 4 MU min^{-1} g^{-1}, measurements were made using 0.1 mL of a 10-fold diluted (in 0.1 M Tris-HCl, pH 7.5) pyrophosphate extract to ensure linear reaction kinetics during the 10 min assay period.

In some studies (as a comparision) the lipase activities in the amended soils were measured using the pyrophosphate extracts as the enzyme source for the standard p-nitrophenyl laurate (2.5) and olive oil emulsion (2.4) assays.

6.2.4 Measurement of respiratory response : Immediately after loading soils with lipid, 2 g subsamples were placed in Warburg flasks for manometric measurement of oxygen uptake, at the appropriate temperature, using 0.2 mL of 20% (w/v) KOH (on filter paper wicks) in the centre wells as a CO_2 absorbent. Oxygen uptake rates were measured periodically throughout the incubation period, control soil rates subtracted and the difference expressed as µmol O_2 consumed h^{-1} g^{-1} of soil. Cumulative respiration curves were subsequently derived by integration of this rate data.

6.3 Biodegradation of lipids applied to soil

6.3.1 Basic response pattern : The responses of a Hamilton clay loam surface soil sample when amended with 2.5 mg of tallow g^{-1} of soil and incubated at 25°C are shown in Fig. 6.3. The composition of the tallow is detailed in Appendix II.

A large proportion of the applied lipid was degraded within 14 days. The maximum rate of lipid degradation (0.36 mg g^{-1} day^{-1}) occurred 8-10 days after application to the soil. No significant changes occurred in the lipid content of an autoclaved (121°C for 2h), amended soil during 30 days of incubation. Thin-layer chromatography of hexane-acetone soil extracts (6.2.2) revealed no accumulation (either transiently or permanently) of the lipolytic breakdown products *i.e.* diacylglycerol, monoacylglycerol or fatty acids (Table 6.2). Fatty acids initially present in the applied tallow were apparently rapidly degraded being non-detectable after 3 days incubation.

The lipase activity of tallow-amended soil increased 3-fold over its initial level. Unamended control soils showed only a small increase in activity to 200 nmol 4 MU min^{-1} g^{-1} after 5 days incubation and returned to initial activities after 10 days. After reaching a peak of activity at 7 days lipase levels gradually declined to reach pre-amendment levels after 30 days. The induced lipase apparently acted as a proteolytic substrate for microbes and was therefore relatively unstable in contrast to the accumulated lipase already present in the soil (see 5.4 for a more detailed discussion). This induction of microbial lipase production in response to lipid amendment to soil has previously been demonstrated by Pancholy and Lynd (1972).

Addition of toluene (20% v/w) to tallow-amended soil prevented any increase in lipase activity, presumably by preventing microbial proliferation. A toluene-treated soil did not appreciably degrade applied lipid, with 85% of the lipid remaining after 20 days,

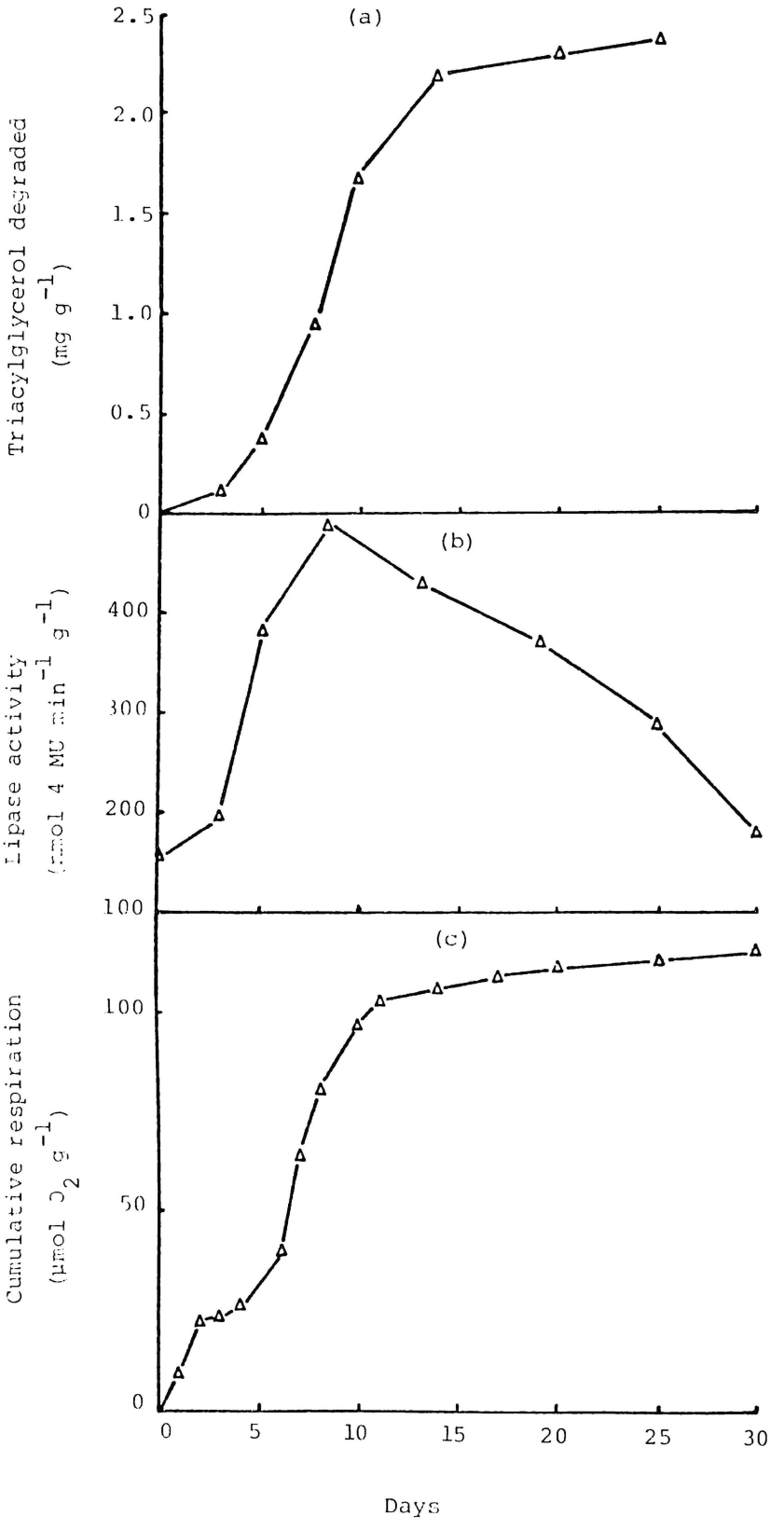


Fig. 6.3 Effect of tallow addition at a loading rate of 2.5 mg g^{-1} of soil on triacylglycerol degradation (a), lipase levels (b), and respiration (c). Incubation temperature of 25°C Soil: Hamilton clay loam.

TABLE 6.2 The presence (+) or absence (-) of various lipid fractions in hexane-acetone (1:1 v/v) extracts of a Hamilton clay loam soil sample amended with tallow (loading rate of 2.5 mg g^{-1} of soil) and incubated at 25°C. Presence or absence based on a detection limit of 50 µg lipid g^{-1} of soil.

Days after Amendment	Lipid			
	Triacyl-glycerol	Diacyl-glycerol	Monoacyl-glycerol	Fatty Acid
0	+	-	-	+
1	+	-	-	+
3	+	-	-	-
5	+	-	-	-
10	+	-	-	-
20	-	-	-	-

despite still possessing the initial lipase activity of 160 nmol 4 MU min^{-1} g^{-1}. These results demonstrate that the majority of the degradation of applied lipid occurred *via* induced lipase rather than by the accumulated lipase already present at the time of application. The failure of previously accumulated lipase to attack applied lipid probably reflects the difficulty of obtaining substrate-enzyme contact in a system involving an insoluble, large-sized substrate and a bound, and therefore immobilised, enzyme (5.4) all in the presence of further spatial barriers imparted by soil particles.

Respiratory activity increased markedly in response to tallow amendment. Unlike the lipase activity and lipid degradation patterns observed, some respiratory response was observed during the first 2-3 days. This initial response probably represents degradation of the fatty acid impurities present in the tallow prior to triacylglycerol breakdown. This is supported by the rapid disappearance of fatty acids from TLC plates of hexane-acetone extracts (Table 6.2).

The amount of oxygen required to completely oxidize 2.5 mg of tallow to CO_2 and H_2O can be estimated at 220 µmol O_2 (using data of Russel 1980). Approximately 50% of this calculated maximum oxygen demand was actually exerted (Fig. 6.3c) suggesting that a considerable proportion of the applied lipid was transformed to new microbial biomass rather than used for respiration.

6.3.2 Effect of loading rate : Tallow was applied to Hamilton clay loam soil at loading rates of 1.0, 2.5, 5.0 and 10.0 mg g^{-1} of soil (approximately equivalent to 2.0, 5.0, 10.0, and 20 tonnes ha^{-1}). and incubated at 25°C. Lipid degradation, lipase induction, and respiratory response were monitored (Fig. 6.4).

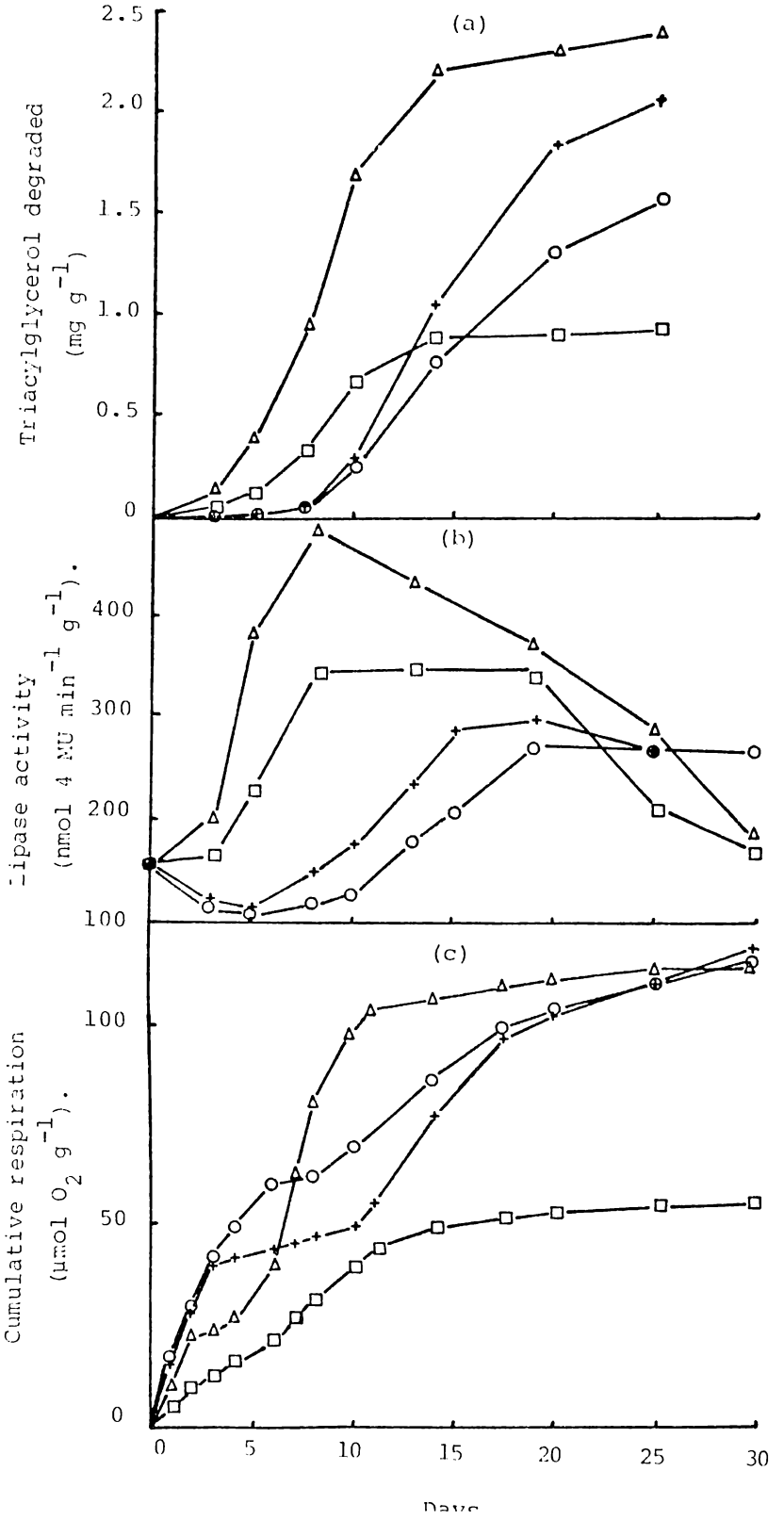


Fig. 6.4 The effect of tallow loading rate on triacylglycerol degradation (a), lipase induction (b), and respiration (c). Incubation temperature of 25°C.

Soil : Hamilton clay loam. Loading rates as mg tallow g^{-1} of soil were 1.0 (□-□), 2.5 (Δ-Δ), 5.0 (+-+), and 10.0 (o-o).

After 25 days incubation all of the lipid added at loading rates of 1.0 and 2.5 mg g^{-1} of soil was degraded. At loading rates of 5 and 10 mg g^{-1} of soil 45% and 16% of the applied lipid had been degraded in 25 days. On a mass basis the greatest degradation occurred at a loading rate of 2.5 mg g^{-1} of soil. Maximal rates of lipid degradation were 0.16 mg g^{-1} day^{-1} for the 1.0 mg g^{-1} loading, 0.36 mg g^{-1} day^{-1} for the 2.5 mg g^{-1} loading, 0.19 mg g^{-1} day^{-1} for the 5.0 mg g^{-1} loading, and 0.12 mg g^{-1} day^{-1} for the 10 mg g^{-1} loading. The two highest loading rates displayed the longest lag period before measurable lipid degradation occurred.

The extent to which lipase activity was induced in tallow-amended soil depended upon the loading rate and reflected the extent of observed lipid degradation. Highest lipase activity occurred in soil amended with tallow at a loading rate of 2.5 mg g^{-1} of soil. The lower extent of induction of lipase (and hence lower maximal lipid degradation rates) observed with the 1.0 mg g^{-1} loading rate probably reflects the smaller population of microbes in contact with substrate and hence able to inductively respond. The lower lipase induction observed at the two highest loading rates suggests an inhibitory effect of the applied lipid on the microbial population. This inhibitory effect resulted in increased lag periods before significant lipid degradation, lower maximal degradation rates, and less total lipid degraded than at the 2.5 mg g^{-1} loading rate. At the two highest loading rates, lipase activities were lower than the unamended soils for the first 5-10 days.

The initial respiratory response was not associated with triacylglycerol degradation. The extent of this initial response was dependent upon the loading rate and probably represents metabolism of fatty acid impurities prior to triacylglycerol degradation. Subsequent respiratory activity reflected the triacylglycerol degradation patterns with an apparent inhibition at high loading rates.

The inhibition observed in this study with high triacylglycerol loadings was not found by Pancholy (1971) or Smith (1974). The results of Pancholy (1971) exhibited increased lipase production up to the maximum loading rate attempted of 50 mg g^{-1} of soil. Smith (1974) demonstrated increased respiratory activity up to the maximum loading rate attempted of 50 mg g^{-1} of soil. Both of these workers added triacylglycerol to the soil in a non-emulsified state. In contrast, the triacylglycerol was added in this study as an emulsion. Emulsified triacylglycerol would possess a greater surface area and therefore would have been more effective in coating soil crumbs and inhibiting oxygen diffusion. When soil crumbs taken from a soil amended with tallow at a rate of 10 mg g^{-1} were examined under low-power magnification (x10) a coating of lipid could be seen. As wastewaters applied to land have usually undergone some pretreatment involving removing of floatables (Bouwer and Chaney 1974; Overcash and Pal 1979) the majority of the lipid reaching the soil is in an emulsified form. In this respect, the results presented in this study better represent the conditions likely to be encountered in the actual land treatment systems.

6.3.3 Effect of soil type : The amendment of five New Zealand topsoils (see Table 5.10 for properties) with tallow at a loading rate of 2.5 mg g^{-1} of soil resulted in differing patterns of lipid degradation and lipase induction, (Fig. 6.5). The most rapid degradation of tallow occurred in those soils in which the most rapid and greatest lipase induction above the basal soil activity was observed (Table 6.3). The lack of any relationship between the basal lipase activities of the various soils and the observed degradation rates of applied lipid is further evidence for the insignificant role that pre-accumulated soil lipase plays in the subsequent degradation of applied lipid (see 6.3.1 for possible reasons for this).

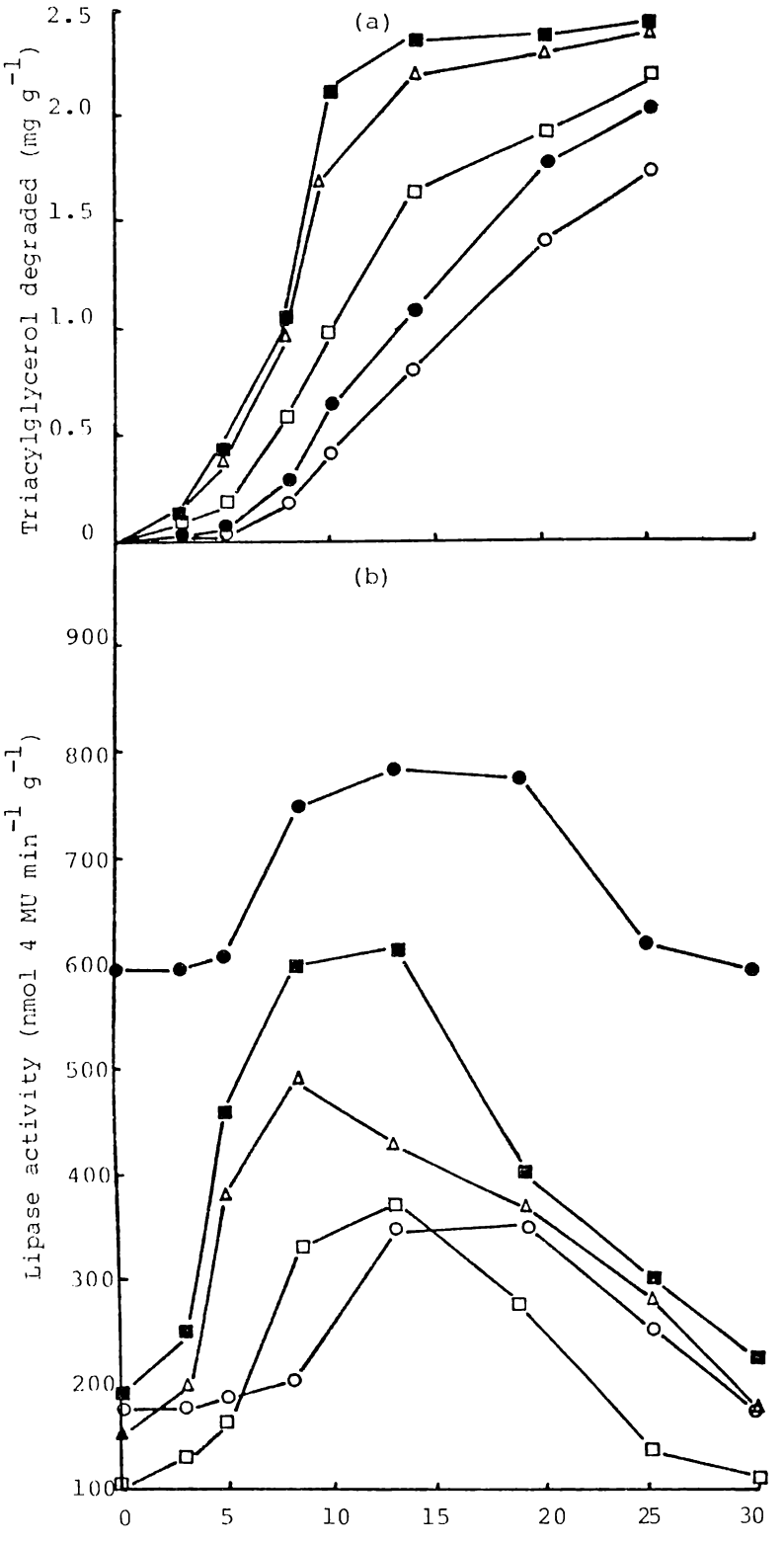


Fig. 6.5. The effect of soil type on triacylglycerol degradation (a), and lipase induction (b). Soils used were Hamilton clay loam (Δ-Δ), Kaawa hill soil (o-o), Ruatangata clay (□-□), Horotiu silt loam (■-■) and Waitete sand (●-●). Incubation temperature of 25°C. Tallow loading rate of 2-5 mg g^{-1} of soil

TABLE 6.3 The responses of five soils to amendment with 2.5 mg g^{-1} of tallow and subsequent incubation at 25 C.

S O I L	Lipase activities (nmol 4 MU min^{-1} g^{-1})			
	Initial	Inductive lipase	Lag period (days)	Lipid (½ life)
Hamilton clay loam	160	330	<3	8.5
Horotiu silt loam	195	420	<3	8.2
Ruatangata clay	100	270	<3	11.6
Waitete sand	595	180	5	15.5
Kaawa hill soil	181	170	8	18.5

The observed differences between soils in the extent of lipase induction and in lag periods reflects the relative abilities of the resident microbiota to take advantage of an externally applied energy source. Long lag periods may result from a small and/or predominantly dormant lipolytic microbial population. Low maximal levels of lipase induction may result from high lipase instability and/or limitations imposed on the growth of lipolytic microorganisms, possibly by the lack of available mineral nutrients (see 6.3.7).

6.3.4 Effect of triacylglycerol type : The addition of various triacylglycerols to Hamilton clay loam each produced their own characteristic lipase induction and lipid degradation pattern (Fig. 6.6). Tallow was the most rapidly degraded (half-life of 8.5 days), followed by anhydrous milk fat (half-life of 12 days), tributyrylglycerol (half-life of 15.5 days) and olive oil (half-life of 18 days). The patterns of lipase induction reflected the lipid degradation patterns. Both olive oil and tributyrylglycerol amended soils possessed long lag periods before lipase induction in comparison to tallow and anhydrous milk fat.

The four lipids used differed markedly in their composition (Appendix II). The tallow and anhydrous milk fat samples possessed some non-esterified fatty acid as well as the triacylglycerols. Rapid metabolism of this free fatty acid may result in a subsequent high degree of lipase inducement and rate of lipid degradation (6.4.2) The relative degrees of lipase inducement resemble those for pure cultures of lipolytic microorganisms isolated from soil (9.5). The rate at which an applied lipid will degrade in soil is dependent upon both its ability to induce lipase production and its amenability to lipase action. In this respect, tributyrylglycerol was a poor inducer of lipase production (9.5) but it was an amenable substrate for the lipases of a soil microorganism, (10.3.1). This resulted in

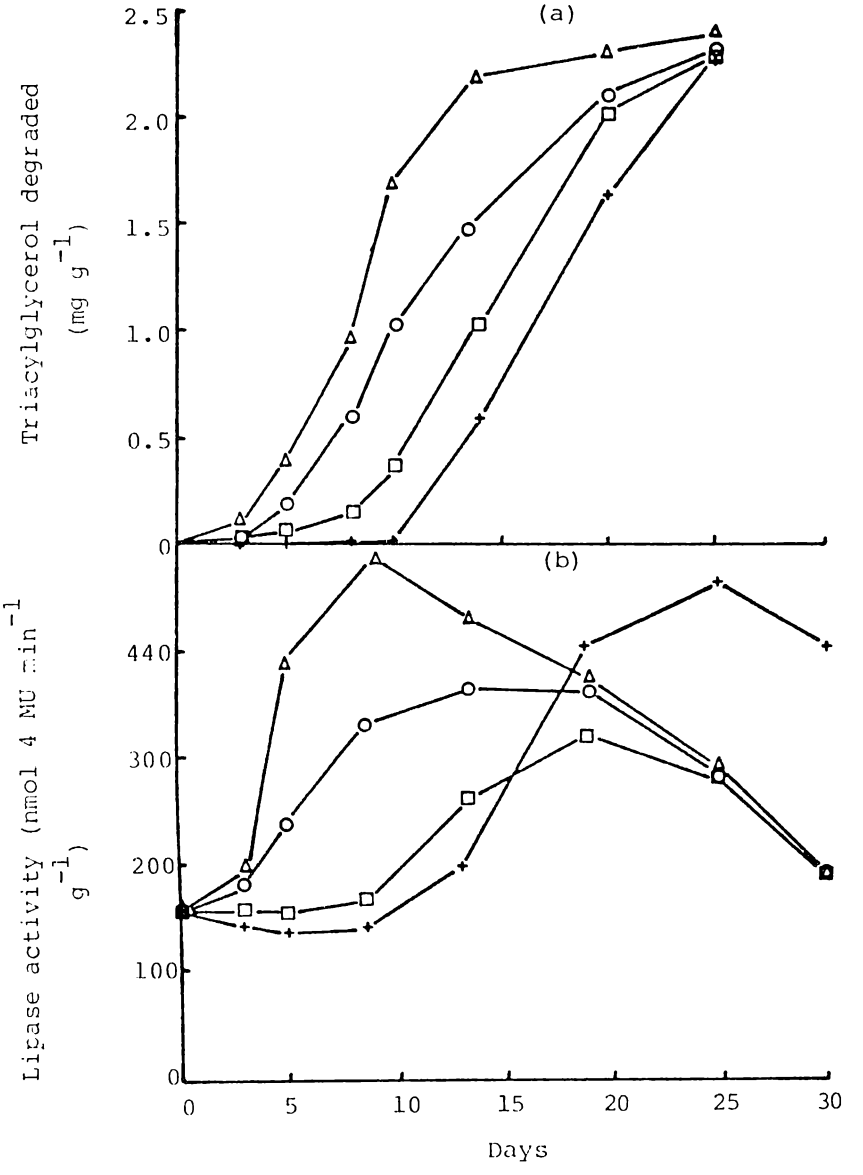


Fig 6.6. The effect of triacylglycerol type on triacylglycerol degradation (a), and lipase induction (b). Triacylglycerols used were tallow (Δ-Δ), tributyrylglycerol (□-□), anhydrous milk fat (o-o), and olive oil (+-+) all at loading rates of 2.5 mg g^{-1}. Incubation temperature of 25°C.

Soil: Hamilton clay loam.

the maximal rate of tributyrylglycerol degradation (0.19 mg g^{-1} day^{-1}) being similar to that for anhydrous milk fat (0.21 mg g^{-1} day^{-1}) which was a better lipase inducer (9.5) but poorer substrate (10.3.1).

6.3.5 Effect of temperature : The induction of lipase and the degradation of lipid in soil was markedly temperature dependent (Fig 6.7) Most rapid lipase induction and lipid degradation occurred at the highest temperature tested (25°C). A longer lag period existed at 17°C but maximal lipase levels obtained were higher than at 25°C. At 12°C, an even longer lag period was observed. Despite higher lipase levels occurring at 17°C maximal lipid degradation rates were observed at 25°C (maximal rate at 17°C of 0.15 mg g^{-1} day^{-1} and at 25°C of 0.36 mg g^{-1} day^{-1}). This probably reflects the effect of temperature on the activity of soil lipase (optimal at 30-40°C, 5.2.6).

6.3.6 Effect of reloading : The reloading of a tallow-amended soil at an equivalent loading rate 2.5 mg^{-1} g^{-1}) 15 days after the initial amendment, resulted in a further inducement of lipase and an immediate rapid degradation of lipid (Fig. 6.8). This indicated that the soil microorganisms were pre-adapted to metabolism of lipids by the first amendment. Over the 25-day period 90% of the lipid added in the two loadings (total addition of 5 mg g^{-1}) was degraded. In contrast, with a single loading of 5 mg g^{-1} only 40% of the added lipid was degraded over the same incubation period. When an amendment of 5 mg g^{-1} was made to a pre-induced soil the lipid was degraded more rapidly than with non-induced soil but still at a rate less than that observed with repeated 2.5 mg g^{-1} loadings. It would appear therefore, that at 25°C a loading rate of 2.5 mg g^{-1} (approximately 5 tonnes ha^{-1}) at 15-day intervals provides for the most rapid degradation of applied lipid.

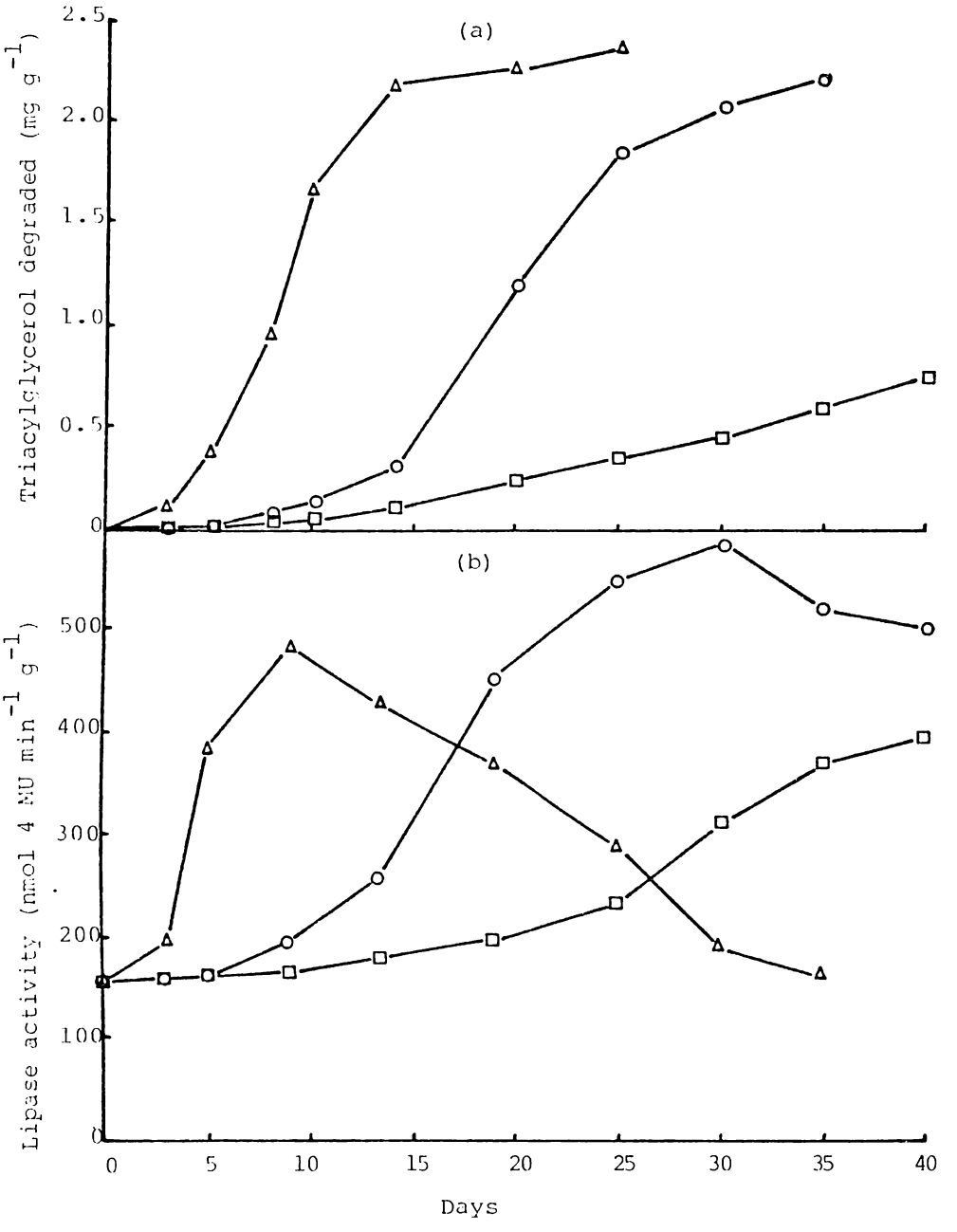


Fig. 6.7. The effect of temperature on triacylglycerol degradation (a) and lipase induction (b). Temperatures used were 12°C (□-□), 17°C (o-o), and 25°C (Δ-Δ). Loading rate of 2.5 mg tallow g^{-1} of soil.

Soil: Hamilton clay loam.

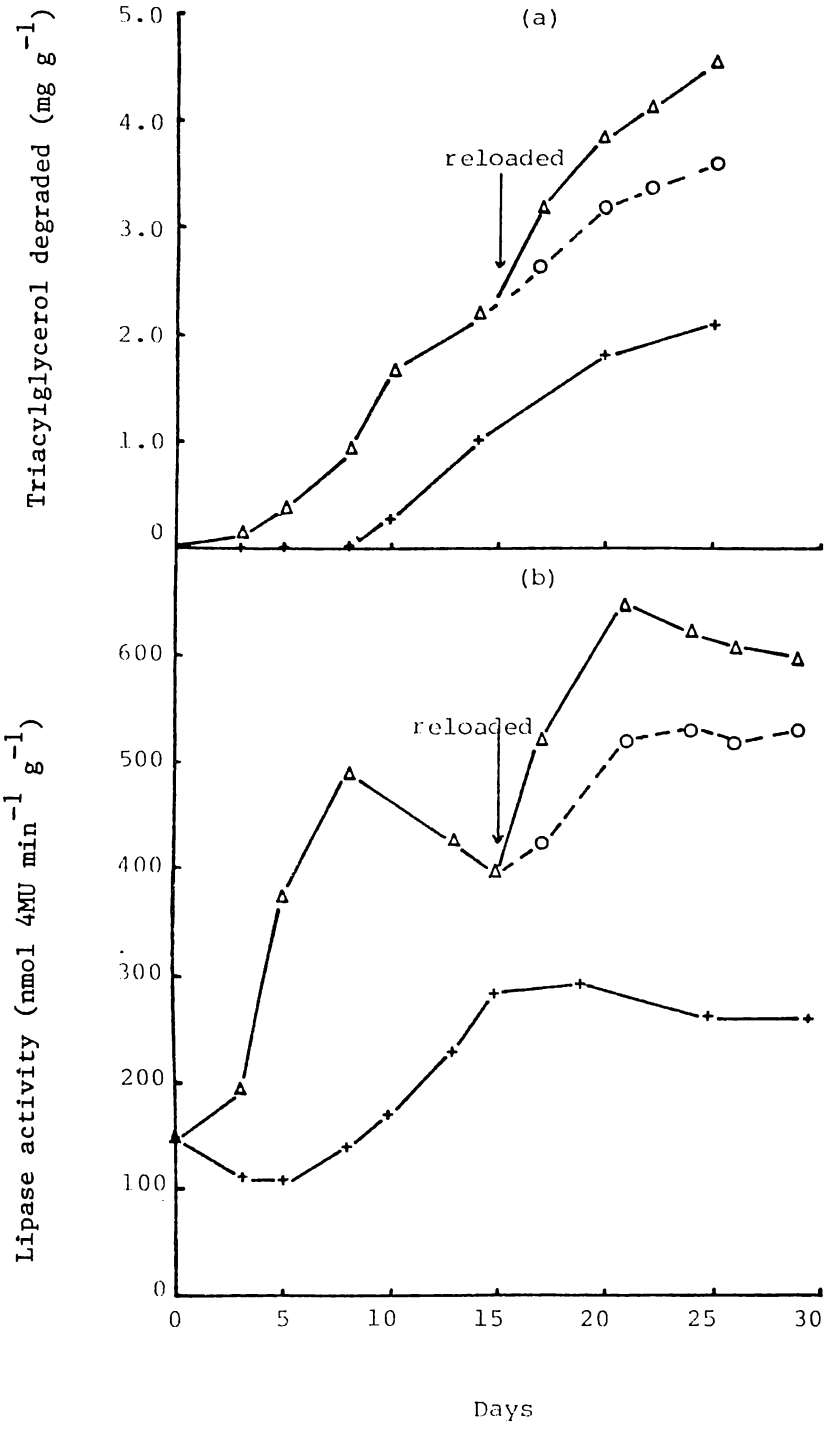


Fig. 6.8. The effect of reloading Hamilton clay loam soil with tallow on triacylglycerol degradation (a) and lipase induction (b). Δ-Δ, tallow loaded at 2.5 mg g^{-1} of soil at day 0 and day 15; o-o, tallow loaded at 2.5 mg g^{-1} of soil at day 0 and at 5.0 mg g^{-1} of soil at day 15; +-+, a single loading of tallow at 5.0 mg g^{-1} of soil at day 0.

6.3.7 Effect of fertilizer additions : Nitrogen (as NH_4NO_3) and phosphorus (as KH_2PO_4) were added to tallow-amended soil by dissolving the salts in the distilled water used for tallow emulsification. Concentrations used were 10.2 g NH_4NO_3 L^{-1} (3.6 g N L^{-1}) and 1.52 g KH_2PO_4 L^{-1} (0.35 g P L^{-1}). As the 10% (w/v) tallow emulsion contains approximately 70% by weight as carbon the carbon content was 70 g L^{-1}. The amendment therefore possessed an approximate C:N ratio of 20:1 and a C:P ratio of 200:1. At the tallow loading rate of 2.5 mg g^{-1} (5 tonnes ha^{-1}) the C added was 3.5 tonnes ha^{-1} and therefore the N and P were added at a rate equivalent to 175 kg ha^{-1} (or 87 µg N g^{-1}) and 17.5 kg ha^{-1} (7.7 µg P g^{-1}) respectively.

With the Hamilton clay loam soil, fertilizer addition had only a minor influence on lipase induction and lipid degradation rates (Fig. 6.9). With the Waitete sand, addition of fertilizer markedly increased the rate and extent of lipase induction and decreased the lipid half-life from 15.5 days to 9 days (Fig. 6.10). These results suggest that mineral limitations are probably soil-specific. Apparently, Hamilton clay loam soil already possessed sufficient available N and P (or possessed the ability to rapidly cycle N and P) to support increased microbial growth in response to addition of a carbon and energy source (in this case lipid), whereas Waitete sand did not.

6.4 Biodegradation of lipids in mixed substrates applied to soil

6.4.1 Principles : When a microbial population is provided with a range of energy-yielding substrates, those substrates are degraded sequentially (McCarty 1966; Alexander 1977). Low molecular weight soluble organics such as simple sugars and alcohols are utilised for microbial respiration and growth prior to high molecular weight, insoluble substances such as cellulose and lipid. The consequences of this sequential substrate utilisation for land treatment systems is

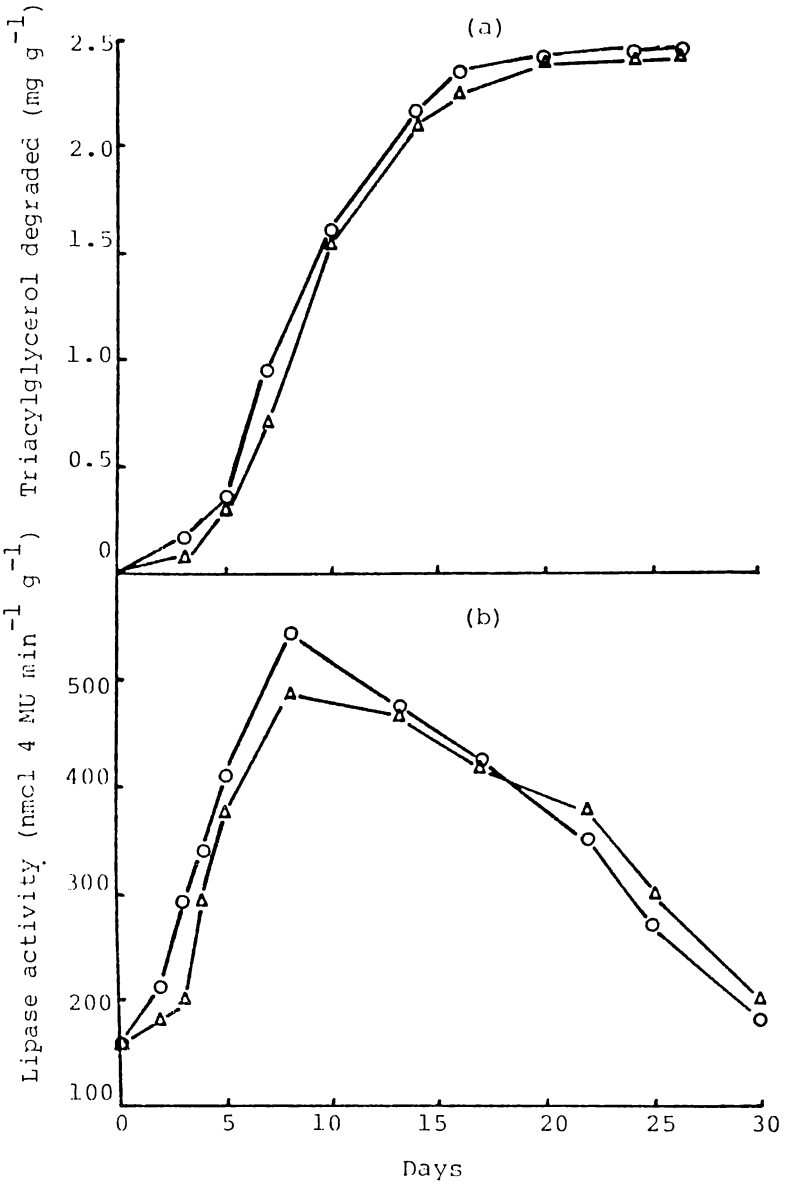


Fig. 6.9. The effect of fertiliser addition on triacylglycerol degradation (a) and lipase induction (b) in tallow-amended soil. Loading rate 2.5 mg g^{-1} of soil. Soil: Hamilton clay loam. Incubation temperature of 25°C. Δ-Δ, no fertiliser addition; o-o, fertiliser added at the rate of 87 g N.μg^{-1} (175 kg ha^{-1}) and 8.7 μg P. g^{-1} (17.5 kg ha^{-1}).

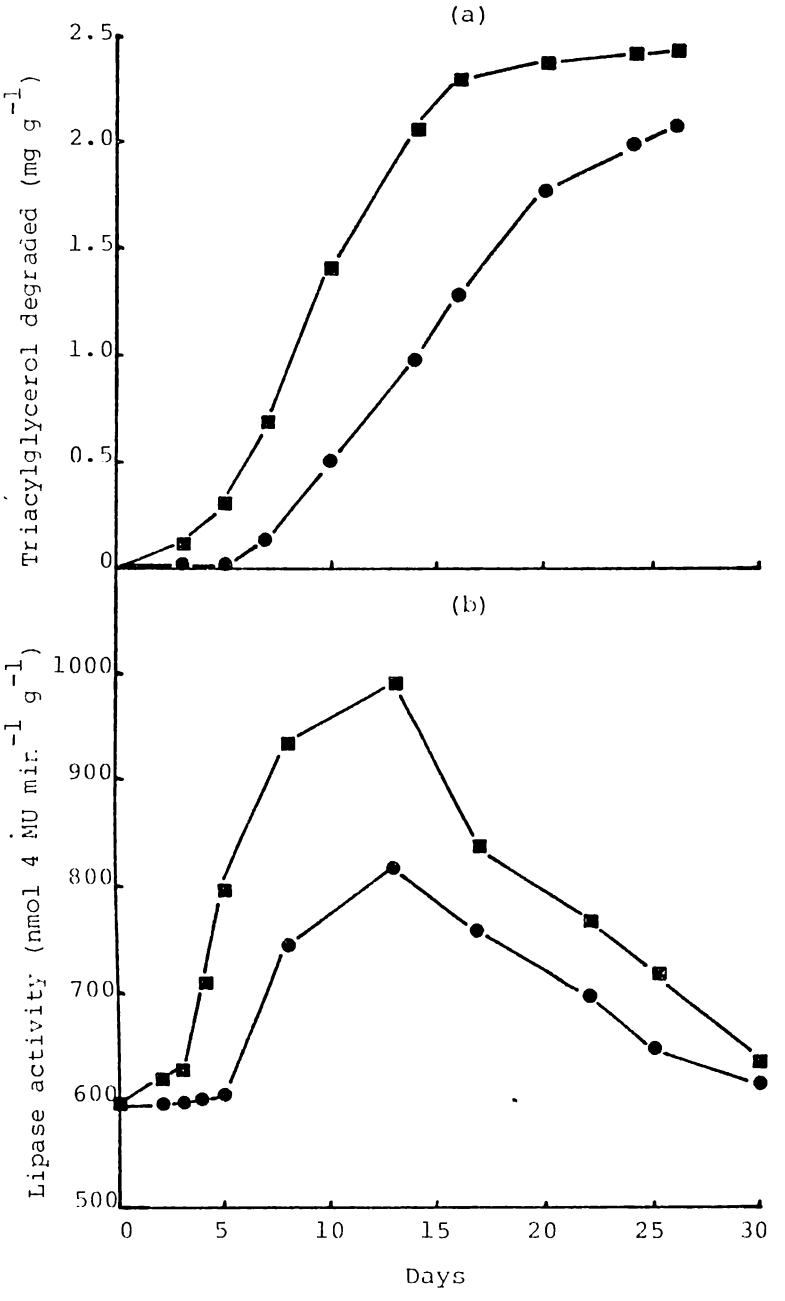


Fig. 6.10. The effect of fertiliser addition on triacylglycerol addition (a) and lipase induction (b) in tallow-amended soil. Loading rate 2.5 mg g^{-1} of soil.
Soil: Waitete sand.
Incubation temperature of 25°C. ●-●, no fertiliser addition; ■-■, fertiliser added at the rate of 87 µg N g^{-1} (17.5 kg ha^{-1}) and 8.7 µg P g^{-1} (17.5 kg ha^{-1})

that interval times between wastewater loadings should be based upon the time required for degradation of the least readily assimilable organic if accumulation of this substance in the soil-plant system is likely to create problems (Overcash and Pal 1979; 6.1.1). Furthermore, the breakdown of these less amenable substrates should be assessed in the waste as a whole as the presence of preferential substrates may be expected to delay their degradation. It was for this reason that triacylglycerol degradation in mixed substrate systems was determined.

6.4.2 Degradation of triacylglycerols in synthetic mixtures when applied to soil : Synthetic mixtures of substrates (glucose-olive oil, bovine serum albumin-olive oil, and oleic acid-olive oil) were prepared by adding the desired quantities to distilled water before emulsifying (6.2.1). This mixture was then added to Hamilton clay loam soil to give loading rates of 1 mg g^{-1} for the non-triacylglycerol component and 2.5 mg g^{-1} for the triacylglycerol. Olive oil (high purity; Sigma Chem. Co., St Louis, USA), was used as it contained low levels of contaminating fatty acid (Appendix II).

With all three mixtures a two-step respiratory response was observed (Fig. 6.11). The initial response was not associated with triacylglycerol degradation or marked lipase induction (Fig. 6.12) and must therefore represent microbial utilisation of the non-triacylglycerol substrate. The secondary respiratory response coincides with degradation of the triacylglycerol. The short intermediate plateau was probably a result of the exhaustion of the amenable substrate and the time required for induction of enzymes necessary for triacylglycerol metabolism. Despite the presence of a preferentially-utilised substrate, the extent of lipase induction and the degradation of triacylglycerol was greater in mixtures than when olive oil was added by itself. It would appear that the presence of alternative, rapidly-utilised substrates increased the microbial activity of the

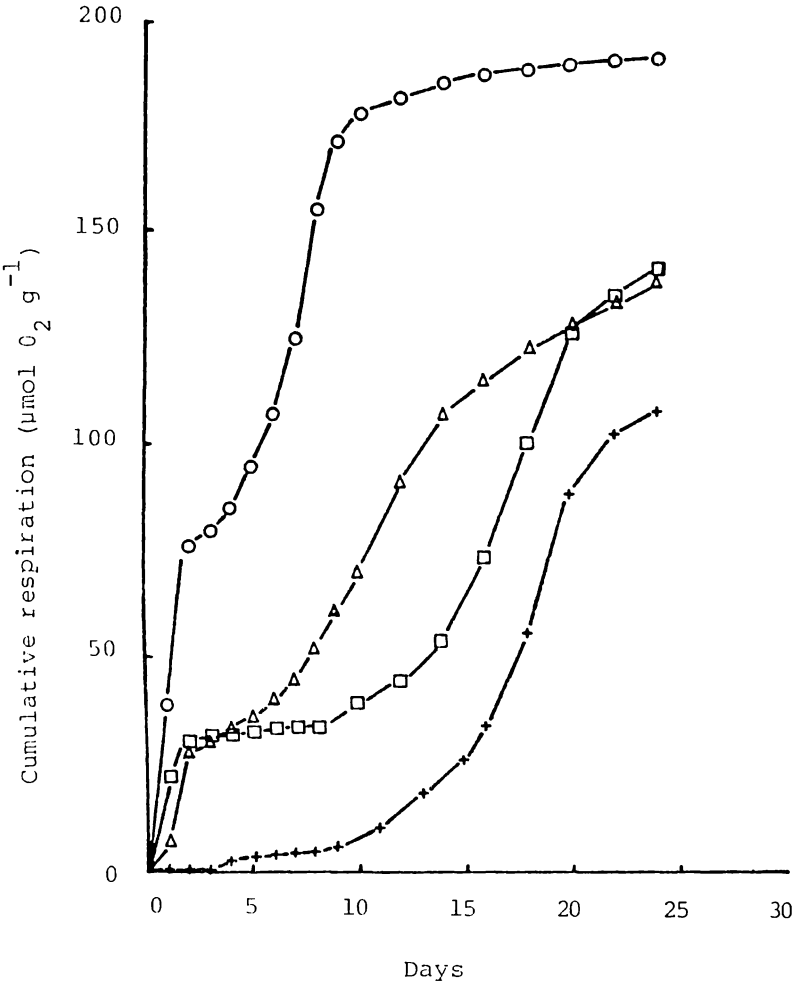


Fig. 6.11. Cumulative respiratory response of Hamilton clay loam soil when amended with various substrates and incubated at 25°C. o-o, 1 mg oleic acid + 2.5 mg olive oil per g of soil; Δ-Δ, 1 mg bovine serum albumin + 2.5 mg olive oil per g of soil; □-□, 1 mg glucose + 2.5 mg olive oil per g of soil; +-+, 2.5 mg olive oil per g of soil.

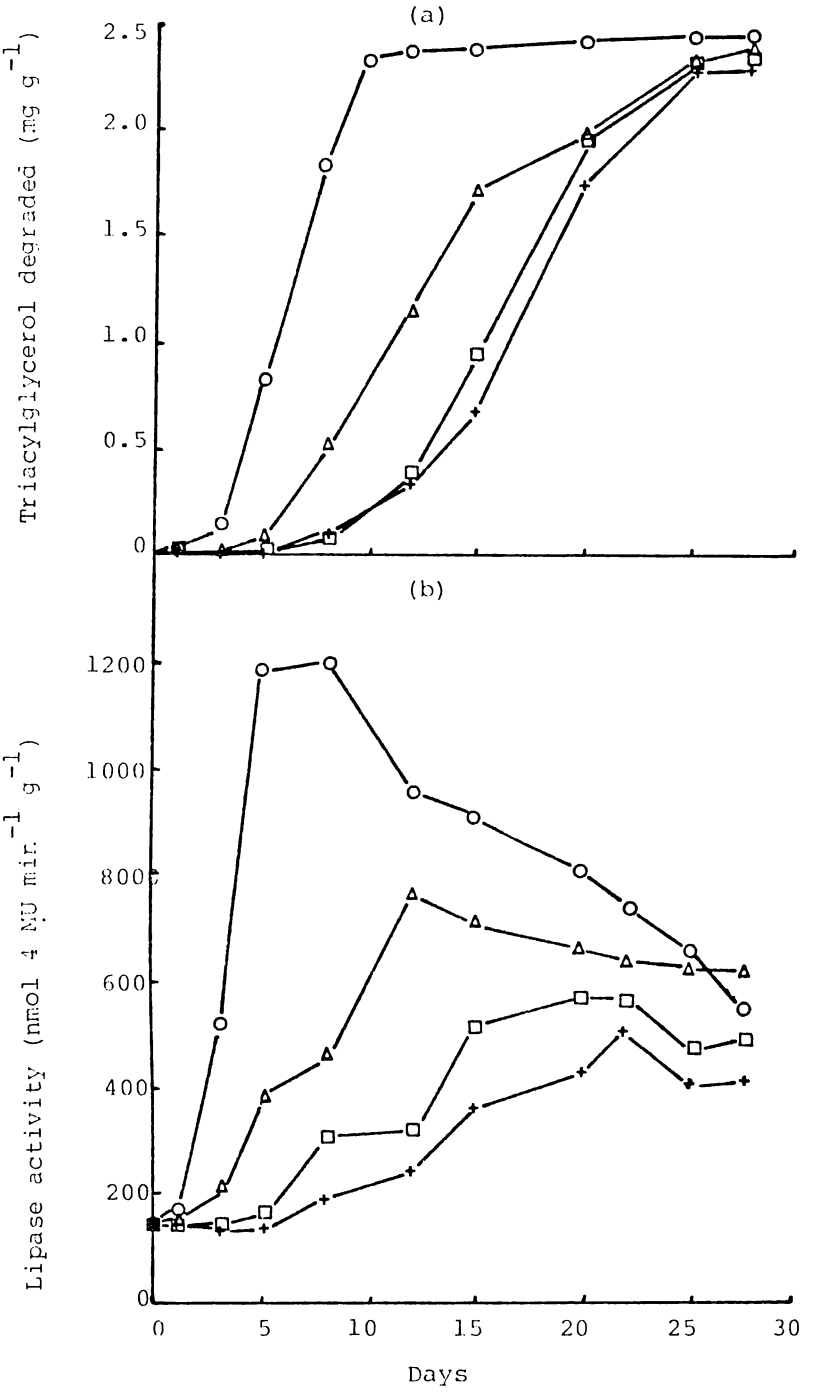


Fig. 6.12. The degradation of triacylglycerol (a) and the induction of lipase (b) in Hamilton clay loam amended with various substrates and incubated at 25 C.

o-o, 1 mg oleic acid + 2.5 mg olive oil per g of soil;

Δ-Δ, 1 mg bovine serum albumin + 2.5 mg olive oil per g of soil;

□-□, 1 mg glucose + 2.5 mg olive oil per g of soil;

+-+, 2.5 mg olive oil per g of soil.

soils such that a larger population of active microbes were present when the switch to triacylglycerol degradation occurred.

The most rapid triacylglycerol degradation and highest level of lipase induction occurred in soil amended with a fatty acid-triacylglycerol mixture. When fatty acid (oleate) was added to soil, as a sole source of carbon and energy, rapid oxidation occurred with the majority of the respiratory response completed within 2 days (Fig. 6.13). After 3 days no fatty acid could be detected by thin-layer chromatography. There was a subsequent increase in the lipase level of the soil, reaching a maximum after 8 days. Previous studies (Tsujisaka *et al* 1973; Sugiura *et al* 1975), demonstrated that lipase production by microorganisms in pure culture may be stimulated by the addition of fatty acid to the medium. Studies presented in this thesis (9.5) demonstrate that an isolate obtained from soil is capable of high lipase production when grown on fatty acids as a sole source of carbon and energy. A similar induction of lipase production by fatty acids apparently occurred within the soil microbial population.

As with oleate-olive oil mixtures, combinations of fatty acid and other triacylglycerols produced a high level of lipase and a rapid degradation of the triacylglycerol (Fig. 6.14). The level of lipase reached in fatty acid-triacylglycerol mixtures was considerably higher than that observed with either substrate alone. The previously observed differences in triacylglycerol degradation and lipase inducement with various triacylglycerol amendments (6.3.4) were negated when all systems contained fatty acid. This effect probably explains the results observed in 6.3.4, where those triacylglycerols possessing fatty acid impurities (tallow and anhydrous milk fat) were degraded faster than the pure triacylglycerols (olive oil and tributyrylglycerol).

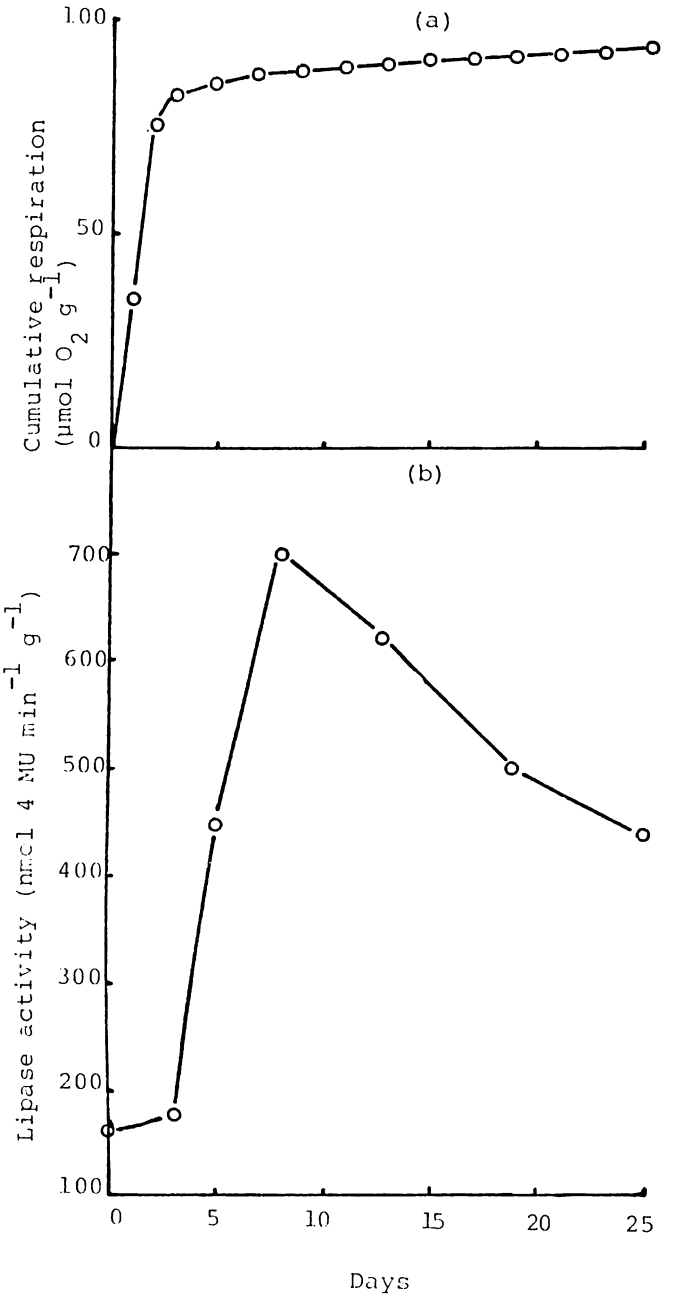


Fig. 6.13. Cumulative respiration (a) and lipase levels (b) of Hamilton clay loam soil amended with oleate at a loading rate of 1 mg g^{-1} of soil.
Incubation temperature 25°C.

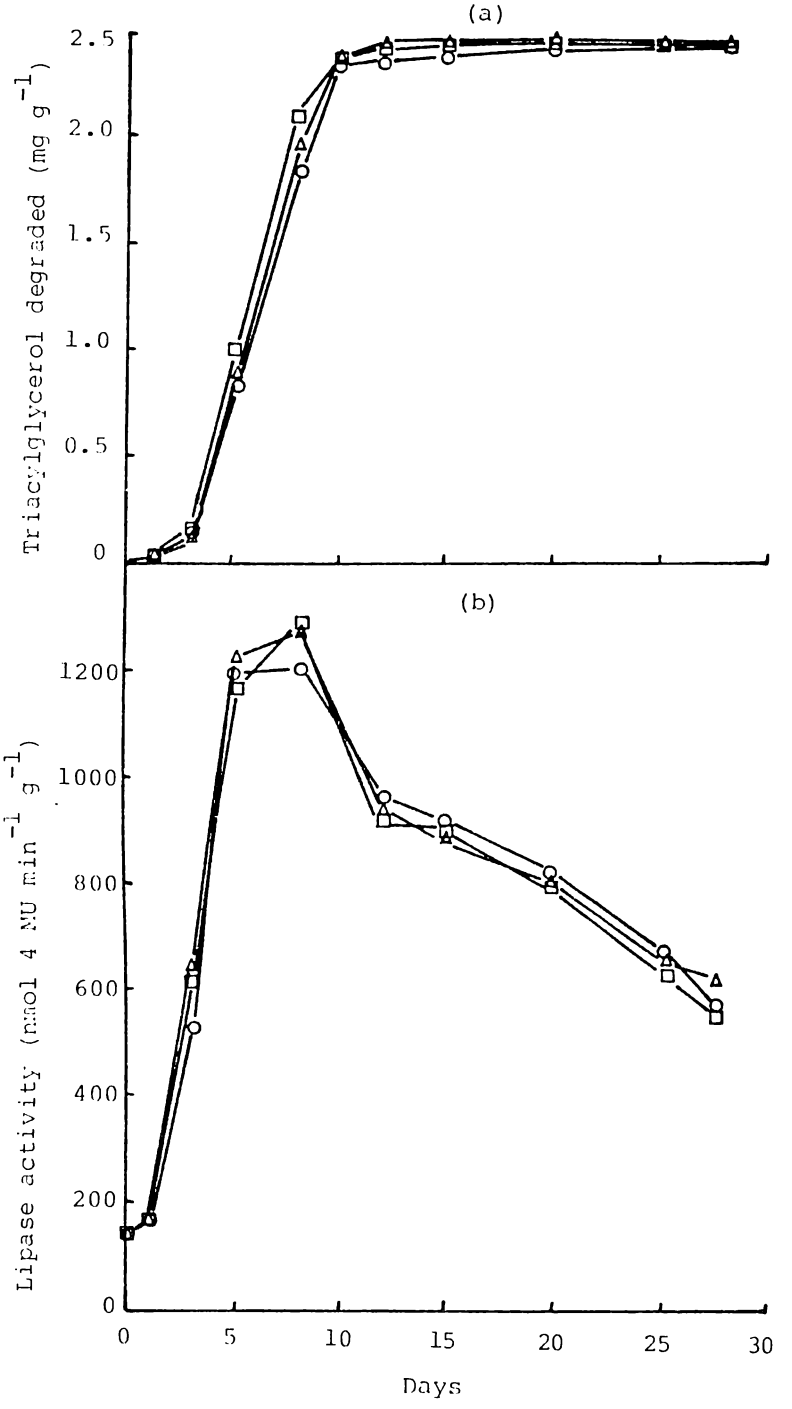


Fig. 6.14. The degradation of triacylglycerol (a) and the level of lipase (b) when various fatty acid-triacylglycerol mixtures are applied to Hamilton clay loam soil.
Incubation temperature of 25°C.
o-o, 1 mg oleate + 2.5 mg olive oil;
Δ-Δ, 1 mg oleate + 2.5 mg tallow;
□-□, 1 mg oleate + 2.5 mg anhydrous milk fat.

The relationship observed, between increased lipase levels and triacylglycerol degradation when both pure triacylglycerol and mixed substrates were amended to soil indicates that the activity observed by the 4 MUN assay truly represented levels of triacylglycerol-hydrolysing (lipase) activity. This was further confirmed by performing the standard p-nitrophenyl laurate and olive oil emulsium assays (2.5 and 2.4) using pyrophosphate extracts from unamended and amended soils as the enzyme source (Table 6.4). Furthermore, this table demonstrates that the activity observed in fatty acid amended soils can be attributed to triacylglycerol-hydrolysing activity.

6.5 Biodegradation of milk applied to soil*

6.5.1 Introduction : In March 1979, industrial action at six dairy processing factories in the Waikato region resulted in large-scale dumping of raw wholemilk into nearby waterways. The biochemical oxygen demand (BOD) of the water increased dramatically, with a consequent fall in dissolved oxygen resulting in fish kills and undesirable odours (Tocker 1979).

The Dairy Wastes Advisory Committee has since investigated alternative means of disposal to be used should such a situation arise again (Leather 1980). The committee proposed land disposal of milk at an application rate of 60 m^3 ha^{-1} at 14-day intervals. They also stated that "further research is required in this area before any heavier applications could be attempted" (Leather 1980). In comparison to conventional wastewaters, milk has a high BOD (102, 500 g m^{-3}; Nemerow 1963) the main components being carbohydrates (principally lactose), protein (principally casein), and lipid (principally triacylglycerols). Of these three components, it is probable that the triacylglycerols would be the most recalcitrant (6.4.2).

*Some of the contents of this section have been published (Appendix III)

TABLE 6.4 Lipase activities of pyrophosphate extracts of unamended and amended Hamilton clay loam soils using three different substrates.

Enzyme Source	Lipase activity (nmol product min^{-1} g^{-1})		
	4 MUN	p-nitrophenyl laurate	Olive Oil
Unamended soil	160	250	280
Amended: tallow (2.5 mg g^{-1}); incubated 8 days at 25°C	490	830	920
Amended: oleate (1 mg g^{-1}); incubated 8 days at 25°C	700	1610	1420
Amended: Oleate (1 mg g^{-1}) + tallow (2.5 mg g^{-1}); incubated 8 days at 25°C	1260	2600	2560

The work presented here was conducted under laboratory-controlled conditions to provide some basic information on the degradation patterns of milk when applied to soils, with particular emphasis on the fate of the lipid component.

In all experiments raw wholemilk was obtained from the Waikato Milk Treatment Station, Riverlea Road, Hamilton. This milk possessed a low bacterial count (<4 x 10^3 mL^{-1}; determined as per 8.2.2) and a triacylglycerol content of 50 g L^{-1} (determined as per 6.2.2).

6.5.2 Effect of milk loading rate : Raw wholemilk was applied to Hamilton clay loam topsoil samples at rates of 0, 20, 50 and 100 mg g^{-1} of soil (or 0, 40, 100 and 200 m^3 ha^{-1}). The loading rate of the lipid component was therefore 0, 2, 5 and 10 tonnes ha^{-1} respectively. Lipid degradation, lipase induction, and respiratory response were followed during incubation at 25°C.

At all loading rates there was a rapid respiratory response which was mainly completed within 6 days (Fig. 6.15). The maximum attainable respiratory rate and total oxygen consumed was related to the milk loading rate (Table 6.5). All of the applied bichemically oxidisable organic matter was consumed by the soil microorganisms at all loading rates.

Significant induction of lipase, and hence degradation of lipids present in the milk, did not begin to occur until 4 days after the initial application of milk (Fig 6.16). Lipase levels reached a peak 8-10 days after initial application of milk and subsequently fell rapidly. The maximum lipase level reached was related to the loading rate. The higher lipase levels associated with higher loading rates resulted in maximal rates of lipid degradation. The maximal rates of lipid degradation were 0.28, 0.75 and 1.42 mg g^{-1} day^{-1} for loading rates of 20, 50 and 100 mg g^{-1} respectively.

TABLE 6.5 Maximum respiration rates and the total oxygen consumed by Hamilton clay loam soil samples incubated with raw wholemilk at 25°C at 3 different loading rates.

Loading rate ($mg\ g^{-1}$)	BOD applied ($\mu mol\ g^{-1}$)	BOD exerted ($\mu mol\ g^{-1}$)	Maximum resp. rate ($\mu mol\ g^{-1}\ day^{-1}$)
20	60	55	13
50	150	140	35
100	300	275	85

6.5.3 Effect of reloading : When a Hamilton clay loam soil sample initially loaded with 50 mg milk g^{-1} (or 100 m^3 ha^{-1}) was reloaded after 15 days at the same rate, the response of the microorganisms was rapid (Figs 6.15 and 6.16). Respiratory response was nearly complete within 3 days (*c.f.* 6 days for the initial application). A second peak of lipase activity was reached 4 days after reloading with milk (*c.f.* 8-10 days for the initial application) and the triacylglycerol component was degraded within this time.

6.5.4 Effect of soil type : The amendment of 4 different soils with milk resulted in patterns of oxygen consumption and triacylglycerol degradation that were not markedly different (Fig. 6.17). This was despite the basal lipase activities of the 4 soils ranging from 100 to 595 nmol 4 MU min^{-1} g^{-1}. The maximum extent to which lipase activities increased over these basal levels was similar for all soils (range 460-560 nmol 4 MU min^{-1} g^{-1}). This suggests that degradation of the triacylglycerol component of milk was achieved by the induced lipase rather than the accumulated lipase present in the soil before application. This was also observed when triacylglycerol alone was added to soil (6.3.3).

6.5.5 Effect of temperature : Incubation temperatures lower than 25°C resulted in longer lag periods before significant oxygen consumption occurred. (Fig. 6.18). At 17°C, after this increased lag period, respiration rates were similar to those at 25°C. At 12°C, longer lag periods were accompanied by lower respiration rates. To exert the majority of the oxygen demand required 5 days at 25°C, 8 days at 17°C, and 15 days at 12°C.

Incubation temperature influenced the time required for significant lipase induction and triacylglycerol degradation (Fig. 6.18). The triacylglycerol half-life was 5.5 days at 25°C, 9.5 days at 17°C, and 18 days at 12°C.

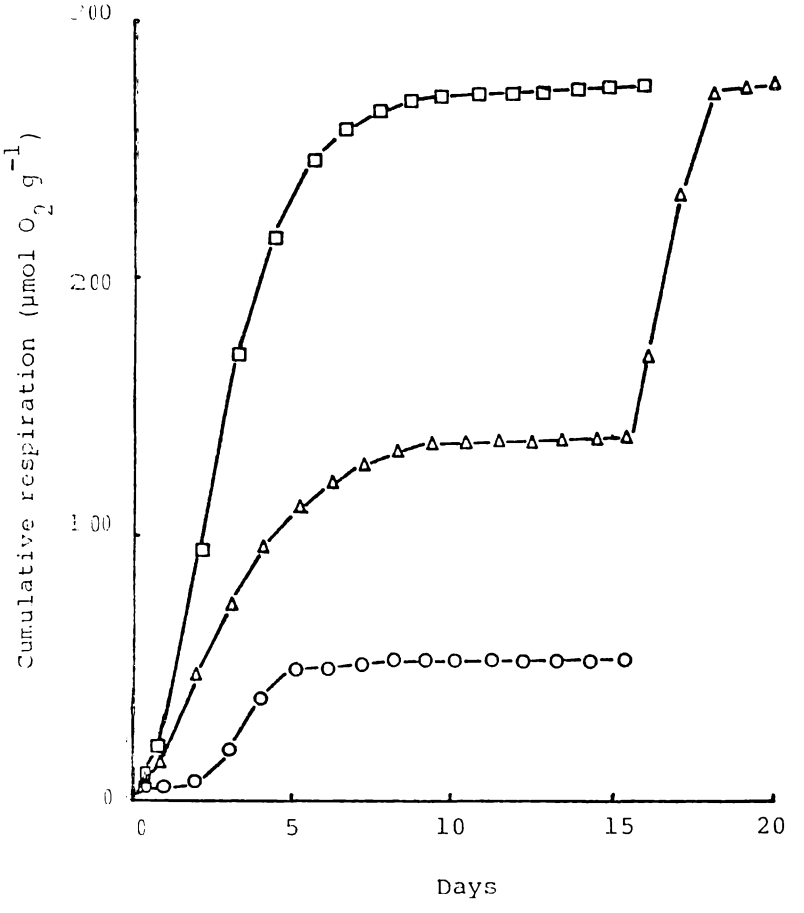


Fig. 6.15. Patterns of oxygen consumption when raw wholemilk was applied to Hamilton clay loam soil. Loading rates of 20 mg g^{-1} (40 m^3 ha^{-1}), o-o; 50 mg g^{-1} (100 m^3 ha^{-1}), Δ-Δ; 100 mg g^{-1} (200 m^3 ha^{-1}), □-□. Incubation temperature of 25°C.
The soil initially loaded at a rate of 50 mg g^{-1} was reloaded after 15 days with another 50 mg g^{-1}.

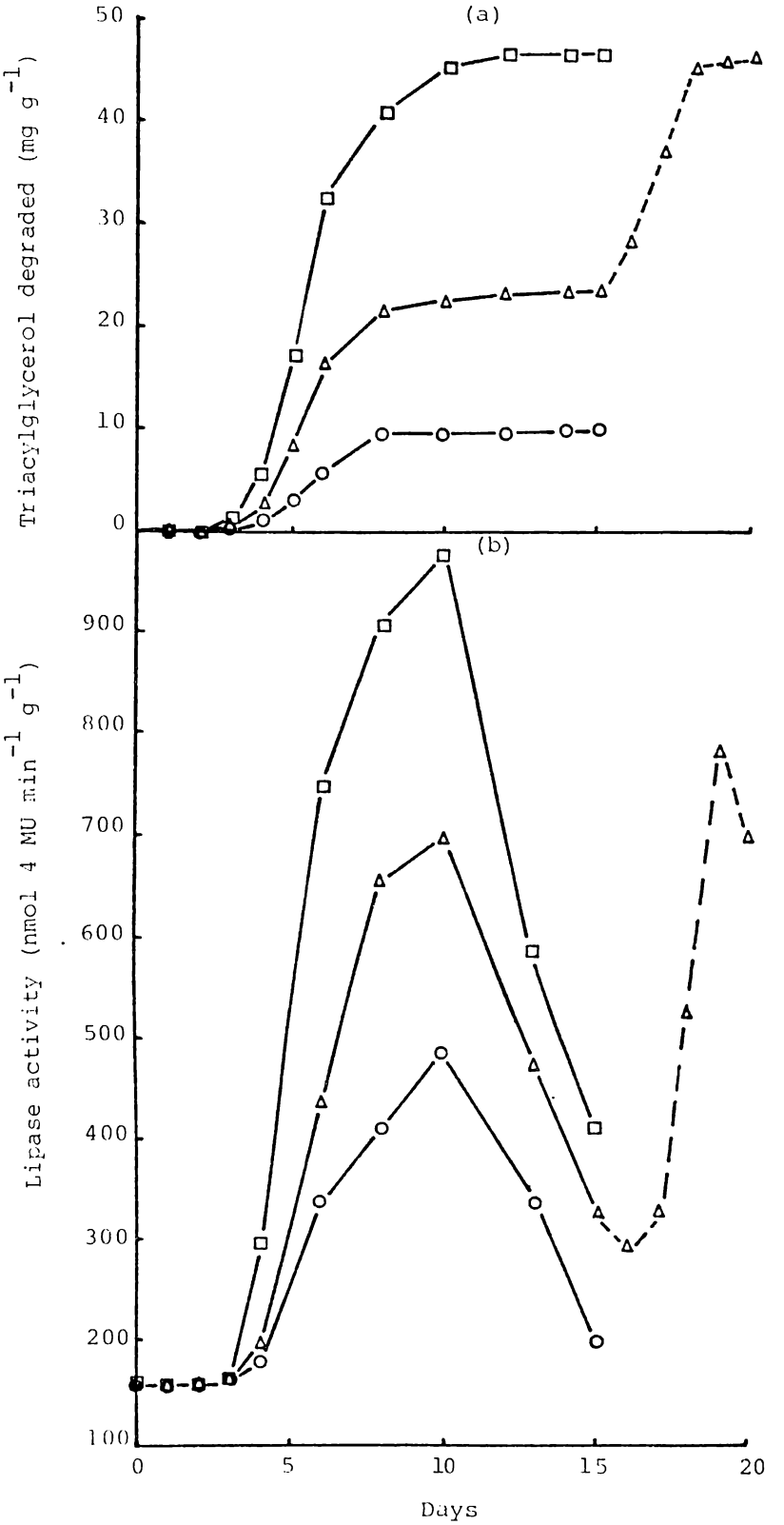


Fig. 6.16. Degradation of lipid (a) and induction of lipase (b) when wholemilk was added to Hamilton clay loam soil. Loading rates were: 20 mg g^{-1}, o-o
50 mg g^{-1}, Δ-Δ, reloaded with 50 mg g^{-1} after 15 days
and 100 mg g^{-1}, □-□.
Incubation temperature of 25°C

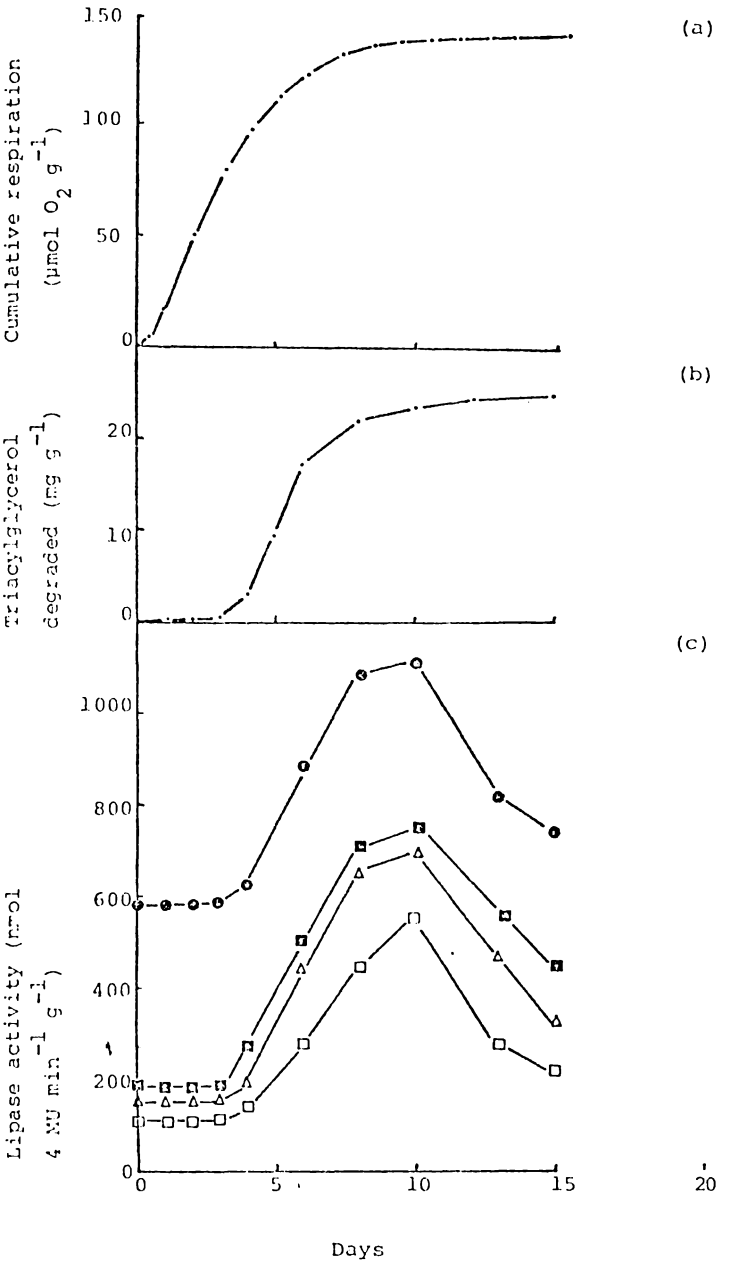


Fig. 6.17. The effect of soil type on the patterns of oxygen consumption (a), triacylglycerol degradation (b), and lipase activities (c) of soils amended with raw wholemilk.
Soils used were: Hamilton clay loam, Δ-Δ,
Ruatangata clay, □-□,
Horotiu silt loam, ■-■,
Waitete sand, ●-●.
Incubation temperature of 25°C. Loading rate of 50 mg g^{-1}.
All four soils demonstrated the one curve shown for (a) and (b).

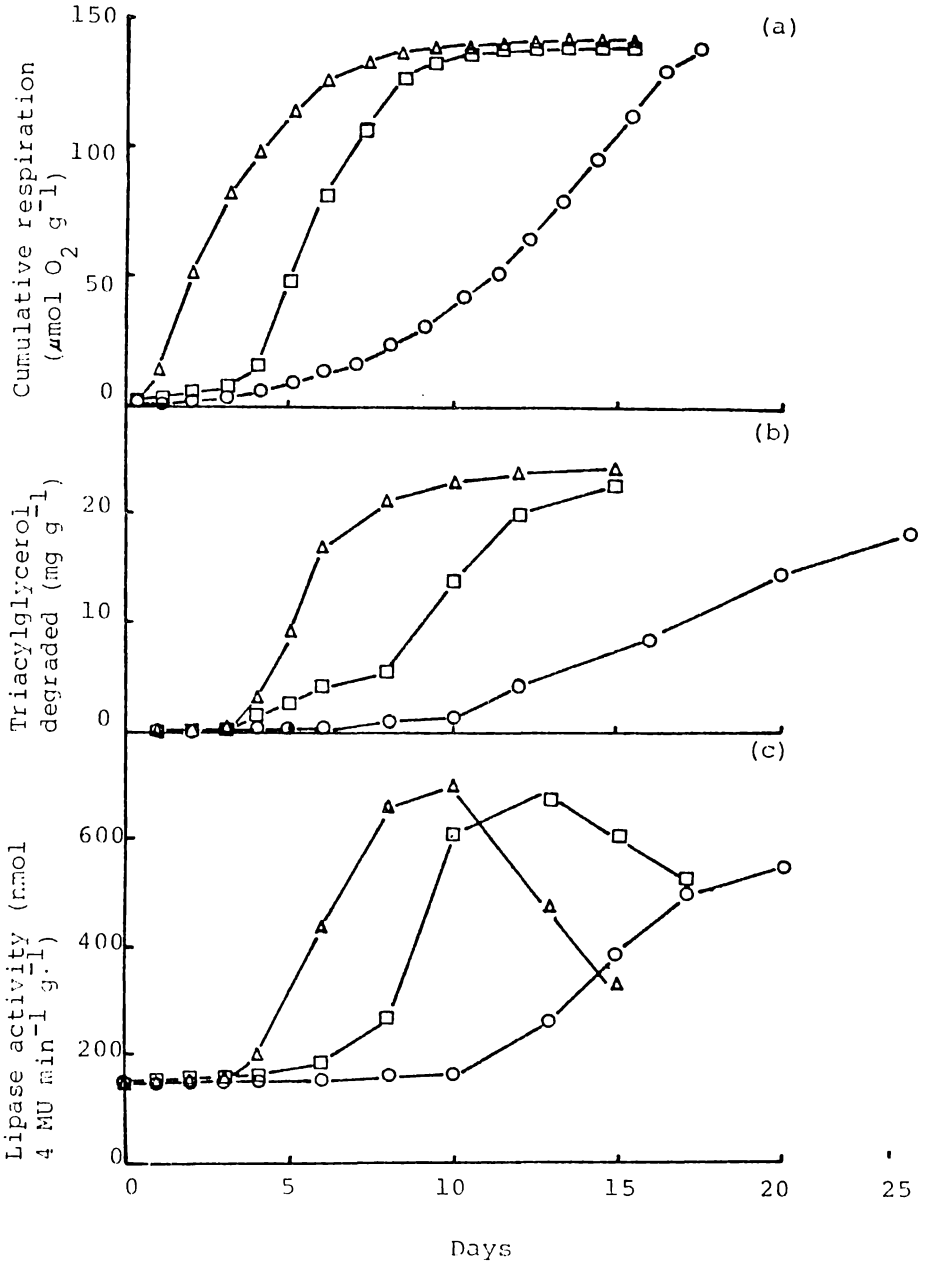


Fig. 6.18. The effect of temperature on patterns of oxygen consumption (a), triacylglycerol degradation (b), lipase activities (c) of Hamilton clay loam soil amended with raw whole-milk at a loading rate of 50 mg g^{-1}.
o-o, 12°C; □-□, 17°C; Δ-Δ, 25°C.

6.5.6 Effect of milk loading on microbial populations : Soil incubated with milk at the highest application rate (100 mg g^{-1} or 200 m^2 ha^{-1}) became festooned with a white mycelial growth 2-3 days after application (Plate 6.1). Soils at all application rates attempted developed a distinct 'musty' odour. Microscopic observation of the mycelial growth revealed both fungal and actinomycete filaments. Plate counts demonstrated that all groups had increased considerably in number (Table 6.6), consistent with the observed respiratory response. Tentative identification (as per 8.3.1), of randomly-selected colonies revealed bacteria of the genera *Arthrobacter Bacillus, Pseudomonas,* and *Lactobacillus* (presumably the *Lactobacillus spp* were derived from the original milk sample). Dominant actinomycetes were *Streptomycetes* and *Micromonospora.* Common fungal isolates were *Penicillium* and *Aspergillus.*

6.5.7 Possible implications for emergency milk disposal onto land: The above experiments were carried out under laboratory-controlled conditions and hence any extrapolations to the field must be treated with caution. Nevertheless, the data does reveal some interesting points which may influence recommendations on milk loading rates and interval times. The main design criteria for land treatment of unwanted milk shou should be the same as for conventional wastewaters *i.e.,* the use of the maximum loading rate (and, therefore, minimum land area) that is allowed for by the constraint of minimum impact on the environment (6.1.1).

Despite milk possessing a high biochemical oxygen demand the soil microbiota appeared capable of rapidly degrading even the highest application rate attempted (200 m^3 ha^{-1}). Whilst triacylglycerol was apparently the last of the major oxidisable organics to be degraded times for triacylglycerol degradation at both 17°C (12 days) and 25°C (6 days) were still rapid enough to suggest that in a land treatment programme, long-term accumulation would not be a problem.

Plate 6.1 Appearance of unamended control and wholemilk amended Hamilton clay loam soil sample after imcubation for 3 days at 25°C.
Wholemilk amendment was at the rate of 100 mg g^{-1} of soil.

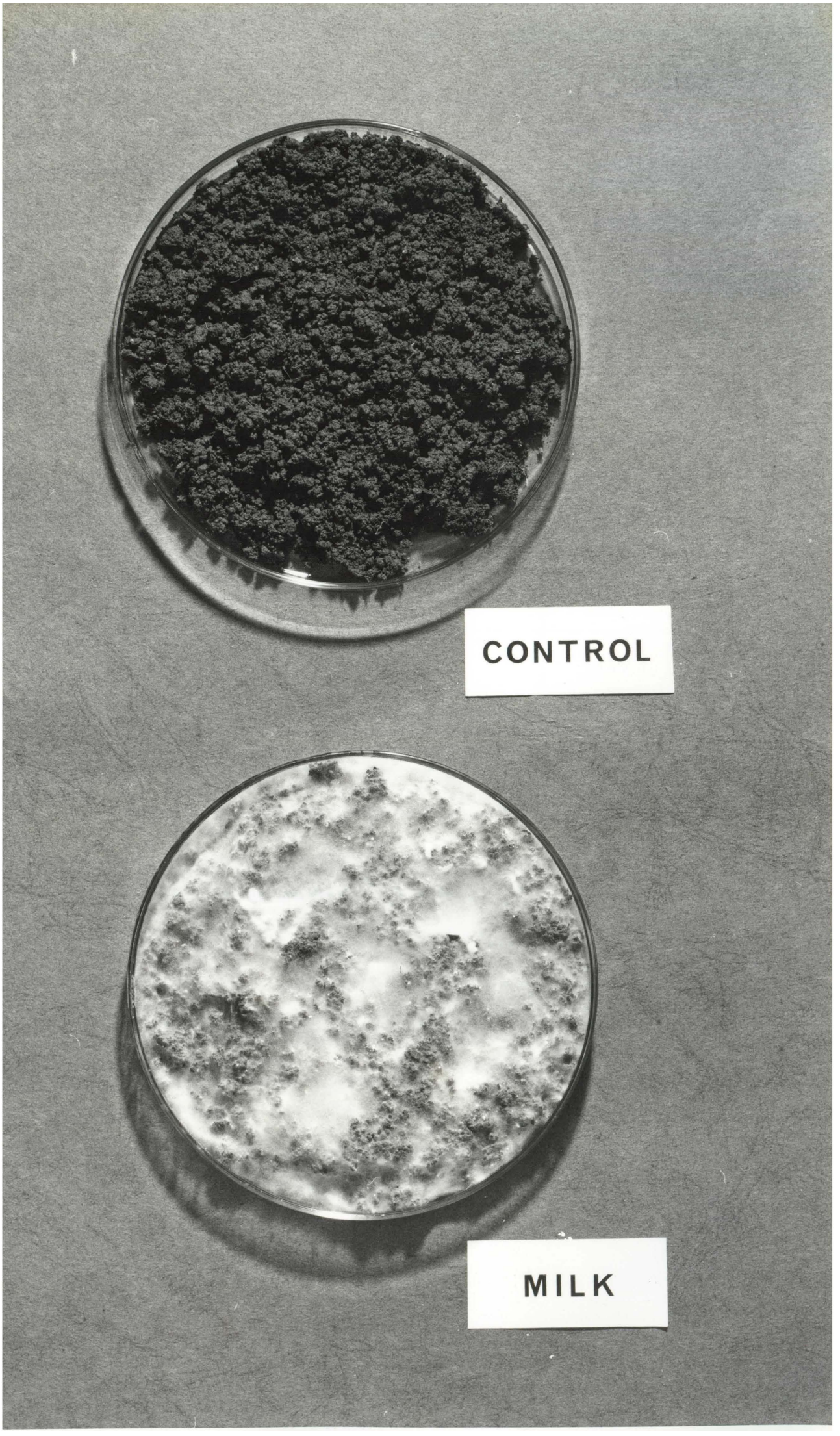

CONTROL
MILK

TABLE 6.6 Effect of wholemilk addition to soil on microbial numbers* after 10 days (colony-forming units x 10^{-6} g^{-1})

	Control soil	Milk-loaded soil +
Bacteria	4.0	410
Fungi	1.3	200
Actinomycetes	0.5	120

* Counts determined on dilution plates using media and methods described in 8.2.2.

+ Loading rate of 50 mg g^{-1} of soil.

However, at 12°C, the slow breakdown of triacylglycerol may restrict milk applications at this temperature to a 'one-off' event.

From the viewpoint of maximal organic degradation, it may be suggested that milk loading rates up to 200 m^3 ha^{-1}, with an initial interval time of 12 days and subsequently of 6 days, could be successfully assimilated by the soil if soil temperatures were higher than about 15°C. For Hamilton, this occurs from November-April (New Zealand Metereological Service 1978). Based on a large-sized dairy factory collecting 400 m^3 of wholemilk day^{-1} (Barnett *et al* 1981), these recommendations would require 24 ha of land (2 ha of land per day over the first 12 days). Alternatively, it may be more feasible for individual farmers to land apply milk *via* normal spray irrigation equipment used for dairy shed effluent or with spray booms on tractors.

It may well be that milk loading rates are not limited by the rate of degradation of the oxidisable organics. Other factors that may be limiting would presumably include pasture 'burn', waterlogging of soil, malodours, and run-off into watercourses (Leather 1980).

6.6. Implications : Triacylglycerols and land treatment

Typical lipid (principally triacylglycerols) concentrations in a variety of wastewaters are given in Table 6.7. The wastewaters of major interest in New Zealand are domestic sewage and those arising from slaughterhouses and dairy factories.

Land application of secondary-treated domestic sewage is practised by approximately 20 small towns (Cameron *pers comm*, MWD, Wellington). This secondary-treated effluent is weak in organics (biochemical oxygen demand of 20-60 mg L^{-1}) and may be expected to contain little triacylglycerol (Heukelekian and Hunter 1956). The factor limiting the loading rates of such waste is usually the capacity of the soil to receive the volumes of water without waterlogging, excessive run-off, or groundwater contamination.

TABLE 6.7 Lipid contents of some wastewaters

Waste	Lipid content ($mg\ L^{-1}$)	Reference
Domestic sewage	30-150	Loehr & De Navarra 1969
Slaughterhouses	100-900	Cooper *et al* 1979
Dairy factories	0-5000	Nemerow 1963
Fish processing	300-18000	Paessler 1956
Palm oil mills	1250-23800	Chin & Wong 1981
Potato processing	10	Smith 1974

There are 38 export slaughterhouses currently operating in New Zealand, each producing a pollution load equivalent to a city of 60-100,000 population (Cooper *et al* 1979). In 1978, 3 slaughterhouses employed land treatment of post-primary effluent and 3 more used land treatment of secondary-treated effluent (Cooper *et al* 1979). By 1980, this had increased to a total of 8 slaughterhouses using land treatment, with 5 using secondary treated effluent. As with domestic sewage, application rates of secondary-treated slaughterhouse effluent to land are limited by the capacity of the soil to receive the volume of liquid. With higher strength post-primary effluent, the organic loading may become significant. The main components of oxidisable organic material in post-primary effluent from slaughterhouses are protein and lipid (Russell 1980). Recommended overall land application rates for slaughterhouse effluents are approximately 600 mm yr^{-1} or 6000 m^3 ha^{-1} yr^{-1} (Cooper *pers comm;* Meat Research Institute of New Zealand, Hamilton). With a mean lipid (mainly triacylglycerol) level of 500 mg L^{-1} (or 500 g m^3) (Cooper 1981) in primary effluent, this represents a lipid loading rate of 3 tonnes ha^{-1} yr^{-1}. This would typically be applied under a regime of weekly or two-weekly intervals over a 30 week killing season. Individual lipid loadings would therefore be 0.1-0.2 tonnes ha^{-1}. Based upon the work presented for lipid breakdown in soil (6.3) this lipid loading should be easily assimilated by the soil system. Furthermore, the presence of protein in the wastewater should aid in this degradation (6.4.2).

Land application of dairy factory effluents is a common practice in New Zealand, with 45 of 84 plants operating in the 1979-80 season using spray irrigation (Barnett *et al* 1981). Dairy factory effluents are usually applied to land without any pretreatment. The organic strength and composition of the effluent is controlled by the type of product being manufactured (Parkin and Marshall 1976; Barnett *et al* 1981). In terms of biochemical oxygen

demand (BOD_5) and lipid content, wastewaters derived from butter and casein manufacture are the strongest with BOD_5's of 30-60,000 mg L^{-1} and lipid levels of 5,000 mg L^{-1} (Nemerow 1963; Barnett *et al* 1981). Typical spray irrigation programmes involve 10-60 mm (100-600 m^3 ha^{-1}) of waste application with a 2 week interval time (Parkin and Marshall 1976; McAuliffe *et al* 1979). Assuming the extreme condition of a dairy factory waste possessing a lipid level of 5,000 mg L^{-1} (or 5,000 g m^3) being applied at the rate of 600 m^3 ha^{-1} this represents a lipid loading rate of 3 tonnes ha^{-1} every 2 weeks. This is a far higher loading than commonly occurs with slaughterhouse effluent. Based upon the results obtained with lipid-alone systems (3.3) it may be anticipated that lipid accumulation would occur in soils receiving this loading of effluent, particularly when soil temperatures fall below 15°C. However, as the organic fraction of dairy factory effluents contains carbohydrates and proteins as well as lipid and may be regarded as a dilution of wholemilk (Barnett *et al* 1981), the results of 6.4 and 6.5 are probably more applicable. These sections revealed that lipids in a mixed organic system were degraded more rapidly by the soil microbiota than when lipid was applied alone. The results of 6.5 suggest that dairy factory effluent applied at a rate to give a lipid loading of 3 tonnes ha^{-1} could be adequately assimilated by the soil system within 2 weeks providing soil temperatures were above 15°C.

6.7 Summary

The factors affecting the breakdown of triacylglycerols were investigated using patterns of respiration, triacylglycerol degradation, and lipase activity. Triacylglycerol degradation was influenced by loading rate, type of triacylglycerol, soil type, temperature, and mineral nutrient additions. Triacylglycerol amendment at a loading rate of $\geq$5.0 mg g^{-1} of soil resulted in an inhibition of subsequent

degradation. In synthetic mixtures of substrates the breakdown of triacylglycerol occurred after the microbial oxidation of alternative substrates. Despite this, the time taken for triacylglycerol degradation in such mixtures was more rapid than when triacylglycerol was added by itself. The degradation of milk when applied to soil was studied to provide some information on the possibilities of land treatment of milk under emergency situations. The implications of the study, with respect to land treatment of wastewaters common in New Zealand, were discussed.

CHAPTER 7 MICROBIAL LIPASES ; PRODUCTION AND PROPERTIES - AN OVERVIEW

7.1 Lipase production by microorganisms

The production of lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3) is a property common to a wide variety of microorganisms (Lawrence 1967a; Brockerhoff and Jensen 1974). All of the major divisions of microorganisms ('true' bacteria, actinomycetes, molds and yeasts), possess members capable of producing lipase. The majority of microbial lipases studied are extracellular, being secreted into the culture medium (Brockerhoff and Jensen 1974). The role of these cell-free lipases is to hydrolyse exogenous triacylglycerol to yield products more amenable to cellular uptake and subsequent metabolism (Lawrence 1967a).

There has been controversy as to the existence of truly intracellular lipases and their physiological role (Lawrence 1967a; O'Leary 1967; Brockerhoff and Jensen 1974). In those microorganisms that secrete an extracellular lipase the presence of activity associated with the cells may merely reflect inefficiency in the process of excreting newly-formed enzyme (Finkelstein *et al* 1970; Tsujisaka *et al* 1973; Aisaka and Terada 1979). With other microorganisms, where solely cell-bound lipases are produced, a specific intracellular metabolic function may be obvious. For example, Paznokas and Kaplan (1977) demonstrated the presence of maximal levels of intracellular lipase in *Mycobacterium phlei* during the late stationary phase of growth when the pool of cellular triacylglycerol shows rapid turnover and acts as a source of endogenous energy. In other cases the absence of triacylglycerol in cellular constituents (O'Leary 1967) makes the production of an intracellular lipase puzzling (Brockerhoff and Jensen 1974). However, it should be pointed out that the distinction between intracellular lipases and lipases bound to the external cell surface has not been made in these studies (*e.g.* Rottem and Razin 1964; Oterholm *et al* 1970).

Presumably, lipase bound to the cell surface could perform the same function as cell-free lipase in metabolising exogenous triacylglycerol, providing that adsorption between cell and substrate occurred.

Microbial lipases may be produced constitutively (*i.e.* in the absence of an identifiable inducer) or inductively. Constitutive lipase production is usually greatest in media containing peptone and may be delayed or diminished when growing upon carbohydrates (Lawrence *et al* 1967a; Mates and Sudakevitz 1973; Chander *et al* 1977). The addition of triacylglycerol to the growth medium of constitutive lipase producers may either have no effect on measured lipase levels (Lawrence *et al* 1967a; Pablo *et al* 1974) or cause a decrease (Eitenmiller *et al* 1970; Jonsson and Syngg 1974). This decrease may represent inhibition of the lipase activity by released fatty acids rather than an influence on lipase synthesis (Smith and Alford 1966). In contrast, inductive producers of lipase show a higher level of lipase activity in media in which triacylglycerol has been incorporated (Goodman 1950; Ota *et al* 1968; Yoshida *et al* 1968; Tsujisaka *et al* 1973; Akhtar *et al* 1980). Induction of lipase production may occur with substances other than triacylglycerols including phospholipids (Ota *et al* 1968; Aisaka and Terada 1979), bile salts (Ota *et al* 1968; Sugiura *et al* 1975), artifical esters such as the tweens (Wouters 1967; Ota *et al* 1968), fatty acids (Ota *et al* 1968; Yoshida *et al* 1968; Tsujisaka *et al* 1973, Sugiura *et al* 1975), and hydrocarbons (Breuil *et al* 1978). All of these inducers are either water-insoluble and/or possess hydrophobic alkyl chains, suggesting that such properties play an important role in lipase induction.

In addition to the medium composition, other conditions of cultivation influence lipase production. Lipase commonly does not reach maximal levels until the stationary phase of growth (Troller and Bozeman 1970; Fulton *et al* 1974; Jonsson and Snygg 1974; Paznokas and Kaplan 1977). The influences of incubation pH (Mates and Sudakevitz 1973; Jonsson and Snygg 1974; Akhtar *et al* 1980), temperature

(Lawrence *et al* 1967a; Mates and Sudakevitz 1973; Jonsson and Snygg 1974; and aeration (Alford *et al* 1963; Lawrence *et al* 1967a; Vovk *et al* 1974) on lipase levels attained in culture are species - specific. As the level of enzyme measured is the net difference between synthesis and denaturation, optimal conditions for the accumulation of lipase in the culture may not necessarily be those of maximal synthesis (Jonsson and Snygg 1974).

7.2 Properties of microbial lipases

7.2.1 Stability : The majority of microbial lipases are relatively stable enzymes, as are lipases in general (Desnuelle 1972). Both crude and purified microbial lipase preparations have been conveniently stored under refrigerated or frozen conditions with little loss in activity (Renshaw and San Clemente 1967; Troller and Bozeman 1970; Pablo *et al* 1974; Bashtakova and Séverina 1978). However, some studies have demonstrated microbial instability under cold conditions particularly with purified preparations (Lawrence *et al* 1967a Mencher and Alford 1967). Significant thermal inactivation of microbial lipases does not usually occur until temperatures of at least 50°C are reached (Renshaw and San Clemente 1967; Yamaguchi *et al* 1973; Pablo *et al* 1974; Watanabe *et al* 1977; Séverina and Bashtakova 1979) Some microbial lipases, however, have proved quite thermolabile (Mencher and Alford 1967; Ingham *et al* 1981).

Microbial lipases may posses a broad (Fulton *et al* 1974; Pablo *et al* 1974; Sugiura *et al* 1977; Chander *et al* 1979) or a narrow (Mencher and Alford 1967; Ingham *et al* 1981) tolerance to a range of pH's. The pH range of maximum stability may not necessarily coincide with that of maximal activity (Mencher and Alford 1967; Renshaw and San Clemente 1967).

The resistance of microbial lipases to denaturation facilitates their purification. Microbial lipases have been initially concentrated and purified using ammonium sulphate precipitation (Lawrence *et al* 1967a; Lu and Liska 1969; Breuil and Kushner 1975b; Sugiura *et al* 1977; Chander *et al* 1979), ethanol precipitation (Renshaw and San Clemente 1967; Vadehra and Harmon 1967; Pablo *et al* 1974), ultrafiltration (Mencher and Alford 1967; Pablo *et ai* 1974; Ingham *et al* 1981) and acetone precipitation (Ota and Yamada 1966) usually with high yields. Subsequent manipulations such as gel-chromatography, ion-exchange chromatography, electrophoresis and lypholisation have been performed with varying degrees of success but usually with acceptable yields and purification factors (Brockerhoff and Jensen 1974).

7.2.2 Molecular weight and structure : Reported molecular weights for microbial lipases demonstrate a wide range (<10 000 - >200 000) (Brockerhoff and Jensen 1974). Individual preparations subjected to gel chromatography or gel electrophoresis may show both high and low molecular weight fractions with activity (Lawrence *et al* 1967a; Mencher and Alford 1967; Breuil and Kushner 1975b; Ingham *et al* 1981). In some studies, these fractions have been shown to be interconvertible (Mencher and Alford 1967; Sémériva *et al* 1969; Ingham *et al* 1981), indicating that they represent the same enzyme with the high molecular weight form being either an aggregate of the enzyme or an association of the enzyme with material not necessary for its catalytic function. Studies on purified microbial lipases have demonstrated the presence of non-proteinaceous material principally carbohydrates (Brockerhoff and Jensen 1974). The presence of carbohydrates may assist in the passage of lipases through the microbial cell membrane (Sémériva *et al* 1969) or in the orientation of lipases at the substrate-water interface (Brockerhoff 1973). Several studies have indicated that some microbial lipases

may be bound to lipid material (Chorvath and Fried 1970; Henderson and Hodgkiss 1973; Tsujisaka *et al* 1973; Breuil and Kushner 1975b; The role of this lipid material may well be to increase the hydrophobicity of the enzyme thereby increasing its affinity for the substrate. Alternatively, the presence of lipid material in lipase preparations may merely represent non-specific binding to hydrophobic regions of the protein. Amino acid analyses of several microbial lipases have shown a high proportion of hydrophobic residues in some cases (Tomizuka *et al* 1966; Chiba *et al* 1973; Liu *et al* 1973) but not always (Isobe and Sugiura 1977). It has been suggested that the tertiary structure of lipases is such that the enzyme possesses both hydrophobic and hydrophilic regions which allows for attraction to the substrate surface whilst maintaining water solubility (Brockerhoff 1973; Isobe and Sugiura 1977). Such a tertiary structure would result in an orientation at the substrate-water interface similar to that achieved by detergent molecules, with the hydrophobic region in or upon the lipid and the hydrophilic region in the aqueous phase (Brockerhoff and Jensen 1974; Verger and de Haas 1976). Thus, this tertiary structure would not only allow enzyme-substrate attraction but would control the orientation of the enzyme, presumably with the active site within the hydrophobic region (Brockerhoff and Jensen 1974).

7.2.3 Substrate specificity : The numerous studies on microbial lipases and their substrate specificity have demonstrated, in general, similarities to plant and animal lipases(Desnuelle 1972; Brockerhoff and Jensen 1974). Studies with triacylglycerols commonly show highest activities towards tributyrylglycerol, low activities towards esters with long-chain saturated fatty acids (*e.g.* tripalmitoylglycerol, tristearolylglycerol), and an enhanced activity when the esters contain unsaturated fatty acids (*e.g.* trioleoylglycerol) (Shah and Wilson 1965;

Eitenmiller *et al* 1970; Hassing 1971; Fulton *et al* 1974; Pablo *et al* 1974). However, some microbial lipases may demonstrate maximal activities towards medium or long-chain-length esters (Vadehra and Harmon 1965; Iwai *et al* 1975; Sugiura *et al* 1977), or, in the case of *Geotrichum candidum*, a maximal activity toward trioleoylglycerol (Alford and Pierce 1961; Wouters 1967).

The positional and fatty-acyl linkage specificities of microbial lipases are species-specific. Most microbial lipases appear to preferentially attack the outer (*i.e.* 1- or 3-) ester linkages of triacylglycerols in a manner similar to that of pancreatic lipase (Alford *et al* 1964; Pablo *et al* 1974; Okumura *et al* 1976). However, some microbial lipases show no positional specificity attacking all three ester linkages at similar rates (Alford *et al* 1963; Alford *et al* 1964; Hassing 1971; Pablo *et al* 1974; Ingham *et al* 1981). The lipase from *Geotrichum candidum* preferentially attacks the oleic acid ester linkage regardless of its position (Alford *et al* 1964; Marks *et al* 1968; Okumura *et al* 1976).

In addition to activity towards triacylglycerols, microbial lipases may demonstrate activity towards di- and mono-acylglycerols (Mencher and Alford 1967; Hassing 1971), methyl esters (Yamaguchi *et al* 1973; Iwai *et al* 1975; Proulx *et al* 1978), and various other monoesters including the "Tweens" and "Spans" (Iwai *et al* 1964; Yamaguchi *et al* 1973) and artificial substrates (Breuil and Kushner 1975a; Dooijewaard-Kloosterziel and Wouters 1976; Paznokas and Kaplan 1977; Roy 1980). The range of substrates hydrolysed by a lipase is species-specific (Lawrence 1967b).

Some microbial lipases defy the definition of lipases as enzymes that attack only insoluble (or micellar) substrates. Homogenous microbial enzyme preparations have demonstrated hydrolytic

activity towards both soluble and insoluble esters (Iwai *et al* 1964; Lawrence *et al* 1967b; Fulton *et al* 1974; Iwai *et al* 1975) thereby exhibiting both esterase (carboxylic-ester hydrolase, EC 3.1.1.1) and lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3) activity. Several of these studies have indicated that the same catalytic site was probably involved (Lawrence *et al* 1967b; Fulton *et al* 1974).

7.2.4 Optimal conditions for activity : The optimal conditions for the expression of lipase activity vary with the source of the enzyme (Lawrence 1967b; Brockerhoff and Jensen 1974). In general, fungal lipases possess acidic pH-optima whilst bacterial lipases possess neutral or alkaline pH-optima. However, an acidic bacterial lipase (Alford *et al* 1963) and several alkaline fungal lipases have been reported (Oi *et al* 1967; Marks *et al* 1968; Nagaoka and Yamada 1973). The pH-activity profiles are usually broad (*e.g.* Alford *et al* 1963; Renshaw and San Clemente 1967; Marks *et al* 1968; Roy 1980).

Microbial lipases characteristically display activity over a broad range of temperature (Alford *et al* 1963; Lawrence 1967b; Troller and Bozeman 1970). The optimal temperature for the expression of microbial lipase activity is usually in the range 30°- 40°C (Lawrence 1967a; Brockerhoff and Jensen 1974) although optima at lower (Fukumoto *et al* 1963; Knoche and Horner 1970) and higher (Sugiura *et al* 1977; Watanabe *et al* 1977) temperatures have been reported.

The addition of cofactors, such as calcium ions (and other divalent cations) and bile salts, commonly used to increase the activity of plant and animal lipases (Desnuelle 1972) may increase, decrease or have no effect on, microbial lipases (Lawrence 1967b; Brockerhoff and Jensen 1974). These effects are related to the conditions of assay as well as the enzyme source (3.4) thereby making comparisons of published literature somewhat tenuous.

CHAPTER 8 LIPOLYTIC MICROORGANISMS FROM SOIL

8.1 Introduction

The wide range of microorganisms found in soil possess a diversity of metabolic capabilities (Gray and Williams 1971; Alexander 1977). This metabolic diversity has resulted in the soil being a common source from which microorganisms capable of performing a particular process (usually of commercial benefit) have been isolated. As microbial lipases have numerous industrial uses (Seitz 1974), and previous studies have shown that lipolytic microorganisms may be readily isolated from soil (Breuil and Gounot 1972; Yamaguchi *et al* 1973; Mourey and Kilbertus 1976; Watanabe *et al* 1977) it seemed that an investigation of the soil microbiota might result in the isolation of potentially valuable lipolytic microorganisms.

The work presented in subsequent sections describes the enumeration, isolation and characterisation of soil microorganisms that produce lipase constitutively and/or inductively. Particular emphasis was placed on the isolation of microorganisms producing a high level of stable, extracellular lipase with a high specific activity in the cell-free supernatant after growth in a simple medium. These factors are of importance when considering the potential of the microorganisms for producing lipase for subsequent purification and industrial application.

8.2 Isolation of lipolytic microorganisms from soils

8.2.1 Introduction

Lipolytic microorganisms have previously been isolated from soil using three fundamentally different methods:

i) Emulsified tributyrylglycerol may be incorporated into agar media and lipolytic colonies that develop from the streaking of soil samples distinguished by a surrounding clear zone of hydrolysis (Aaronson 1970). The technique has been used to quantify the proportion of colony-forming units (CFU's) that are lipolytic by using the dilution plate technique. The total number of CFU's and the proportion thereof that are lipolytic can be evaluated on a single plate. (Breuil and Gounot 1972; Mourey and Kilbertus 1976). As there is a plentiful supply of alternative carbon and energy sources in the agar medium the method will primarily detect those organisms capable of constitutive lipase production.

ii) Soil samples may be streaked or dilution plated (for enumeration) onto an agar medium containing a triacylglycerol as the sole carbon and energy source (Aaronson 1970; Watanabe *et al* 1977). With this method the colonies that develop represent those organisms present in the sample that are capable of producing sufficient lipase on a triacylglycerol substrate to support growth..

iii) A soil sample may be introduced into a liquid medium containing triacylglycerol as the sole source of carbon and energy. After growth, a subsample is transferred to fresh medium, the process being repeated several times. Finally, the last of these enrichment cultures is streaked onto its equivalent agar medium and the colonies isolated (Yamaguchi *et al* 1973; Watanabe *et al* 1977). This enrichment technique selects for those microorganisms capable of rapid growth on triacylglycerol under the conditions of cultivation. As growth on this substrate is dependent upon lipase production, the method effectively selects for those microorganisms capable of rapid and substantial inductive production of lipase.

In the context of this study, method (i), when performed on an unamended soil sample, probably isolates those organisms responsible for the accumulated lipase described in 5.3. Method (ii), when performed on a triacylglycerol-amended soil, probably isolates those organisms responsible for the degradation of triacylglycerol described in 6.3. Method (iii) probably isolates those organisms present in soil most likely to be of commercial value. All three methods were used in this study.

8.2.2 Enumeration and isolation of lipolytic microorganisms from soil using tributyrylglycerol-amended media : Evaluation of the total colony-forming units (CFU's) and the proportion that were lipolytic was performed for the different microbial groups ("true" bacteria, actinomycetes, fungi) using the dilution spread plate technique onto selective media that contained emulsified tributyrylglycerol. The media and incubation conditions used were:

(i) Trypticase soy agar (BBL, Maryland, USA); at 25°C for 5 days for bacteria;

(ii) Czapek-Dox agar (BBL, Maryland, USA) adjusted to pH 5.0 and amended with the antibacterial antibiotics polymixin β-sulphate (5 mg L^{-1}) and sodium benzylpenicillin (1 mg L^{-1}); at 25°C for 5 days for fungi;

(iii) Starch-casein agar (Aaronson 1970) with the antifungal antibiotics actidione (50 mg L^{-1}) and nystatin (50 mg L^{-1}) (Davies and Williams 1970); at 25°C for 10 days for actinomycetes.

Antibiotics (all Sigma Chem. Co., St Louis, USA) were prepared as concentrated stock solutions (aqueous for polymixin β-sulphate sodium benzylpenicillin, and actidione; in dimethyl sulphoxide for nystatin), filter-sterilised (0.22 µm Sartorius), and added to autoclaved and cooled media prior to the pouring of plates.

Prior to autoclaving, all media received an addition of 10% (v/v) tributyrylglycerol emulsion (formed by sonication for 5 min in a Dawe Soniprobe at maximum power) to yield a final concentration of 0.5% (v/v).

The total CFU's and proportion that were lipolytic (as indicated by surrounding clear zones of hydrolysis) were determined for five unamended New Zealand topsoils (Table 8.1). The range in the proportion of lipolytic bacteria (21-55%) is similar to that obtained by Mourey and Gilbertus (25-61%). The results presented in Table 8.1 indicate that both fungi and actinomycetes in soil contain a considerable proportion of lipolytics and may therefore make an important contribution to the accumulated lipase observed in soils. The high proportion of lipolytic actinomycetes (55-86%) is similar to the findings of Ko and Hora (1970) with phospholipase-producing soil actinomycetes.

When the same tributyrylglycerol-amended media were used with soil previously treated with triacylglycerol (tallow at 2.5 mg. g^{-1} of soil for 8 days at 25°C as per 6.2.1) all three groups demonstrated an increase in countable numbers, with the change in the bacterial population being the greatest (Table 8.2). The proportion of lipolytics increased within all groups, as may be expected.

Randomly selected colonies were streaked onto fresh agar and individual colonies subsequently picked off and maintained on agar slants of the same composition as that used for isolation.

8.2.3 Enumeration and isolation of lipolytic microorganisms from soil using olive oil-mineral salts media: The microorganisms present in the soil that were capable of growth on triacylglycerol as a sole source of carbon and energy were enumerated and subsequently isolated using the dilution spread plate procedure with olive oil-mineral salts media of the following composition : (L^{-1}) :

TABLE 8.1 Total number of colony-forming units and proportion that were lipolytic for various groups of microorganisms in five New Zealand topsoils.

S O I L	Total No. g^{-1} ($x10^{-5}$) (% lipolytic)		
	Bacteria	Actinomycetes	Fungi
Hamilton Clay Loam	4.0 (32)	0.50 (64)	1.30 (57)
Horotiu Silt Loam	1.8 (55)	0.35 (82)	0.72 (50)
Taupo Sand	0.43 (21)	0.11 (75)	0.32 (45)
Wharekohe Sand	0.72 (55)	0.14 (86)	0.55 (60)
Ruatangata Clay	2.1 (30)	0.61 (55)	0.45 (38)

TABLE 8.2 Total number of colony-forming units and proportion that were lipolytic for various groups of microorganisms in unamended and triacylglycerol amended* Hamilton clay loam.

S O I L	Total No. g^{-1} ($x10^{-5}$) (% lipolytic)		
	Bacteria	Actinomycetes	Fungi
Unamended	4.0 (32)	0.5 (64)	1.3 (57)
Amended	165.0 (95)	0.9 (80)	12.0 (88)

* Amended with tallow at a loading rate of 2.5 mg. g^{-1} of soil followed by incubation at 25°C for 8 days.

NH_4NO_3, 2.0 g; KH_2PO_4, 4.0 g; Na_2HPO_4, 6.0 g; $MgSO_4.7H_2O$, 0.2 g; $FeSO_4.7H_2O$, 0.01 g; $CaCl_2$, 0.05 g; Olive oil (Sigma Chem. Co, St Louis, USA), 8.0 g. The pH was adjusted (pH 7.0-7.2 for bacteria, pH 8.0-8.2 for actinomycetes, and pH 5.0 for fungi), the whole sonicated (5 min Dawe Soniprobe maximum power), agar added (15 g L^{-1}), and autoclaved (121°C for 15 min). After cooling to 45°C, antibacterial antibiotics (polymixin β-sulphate, 5 mg. L^{-1}; sodium benzylpenicillin, 1 mg L^{-1}) were added to the fungal media, antifungal antibiotics (actidione, 50 mg L^{-1}; nystatin, 50 mg. L^{-1}) to the bacterial media and both antifungal and antibacterial antibiotics to the actinomycete media. Incubation of plates was for 5 days at 25°C for bacteria and fungi and for 10 days at 25°C for actinomycetes. Plates were examined using illumination from beneath, with a sheet of thin red cellophane between the light source and the plate which facilitated the detection of white, cream or translucent colonies against the cloudy background of the medium.

All three microbial groups possessed members capable of growth upon triacylglycerol as a sole carbon source (Table 8.3). Numbers increased markedly upon triacylglycerol amendment in soil indicating an induction in the soil of those organisms capable of using tributyrylglycerol as a sole source of carbon for growth and energy. The largest increase was that observed for the bacterial population (250x) suggesting that bacteria were the primary agents responsible for the degradation of triacylglycerol added to soil. A comparison of Tables 8.2 and 8.3 (obtained from the same experimental run) shows that the proportion of the total countable population capable of growth on triacylglycerol as a sole source of carbon and energy increases from 9% to 53% for bacteria, 6% to 61% for actinomycetes, and 9% to 54% for fungi as a result of triacylglycerol amendment of soil.

TABLE 8.3 Number of colony-forming units present in unamended and triacylglycerol amended* Hamilton clay loam soil that developed upon olive oil-mineral salts agar.

S O I L	No. g^{-1}		
	Bacteria	Actinomycetes	Fungi
Unamended	3.5×10^4	3.0×10^3	1.2×10^4
Amended	8.8×10^6	5.5×10^4	6.5×10^5

* Amended with tallow at a loading rate of 2.5 mg. g^{-1} of soil and incubated for 8 days at 25°C

Randomly selected colonies were streaked onto fresh agar and individual colonies subsequently picked off and maintained on slants of trypticase soy agar for bacteria, Czapek-Dox agar for fungi, and starch-casein agar for actinomycetes.

8.2.4 Isolation of lipolytic microorganisms from soil using enrichment culture: An olive oil-mineral salts liquid medium (composition as per 8.2.3, at pH 7.0-7.2) dispensed as 100 mL aliquots in 250 mL Erlenmeyer flasks was used as the enrichment medium. A sample (0.1 g) of either unamended or amended Hamilton clay loan top-soil was used as the inoculum. After incubation for 24-36h at 25°C and 120 rev min^{-1}, 1 mL was transferred to fresh medium. This procedure was repeated 7x, with a sample of the last enrichment flask being streaked onto an agar medium of the same composition. Colonies that developed (all bacterial) were subsequently maintained on trypticase soy agar slants.

8.3 Characterisation of lipolytic soil isolates

8.3.1 Identification: Bacterial isolates (both "true" bacteria and actinomycetes) were identified to generic level by examining cell morphology, gram reaction, spore formation and position, motility, oxidative-fermentative reactions, catalase reaction and API 20 B profile (Analytab Inc., Plainview, USA). Additional tests were sometimes required to classify an organism into one of two similar genera. All tests outside those of the API 20 B test strip were performed according to the procedures of Cowan and Steel (1965) and classifications made according to the 8th edition of Bergey (Buchanan and Gibbons 1974). Fungi were identified by morphological features (Ainsworth and Sussman 1973).

The identity of the bacterial isolates obtained from unamended and triacylglycerol-amended soils using three different isolation techniques (8.2) are given in Table 8.4. Lipolytic bacteria isolated on tributyrylglycerol - amended media with unamended soil were principally *Bacillus spp.* whilst with amended soils *Pseudomonas spp.* dominate. In contrast, Breuil and Gounot (1972) found that *Pseudomonas spp.* dominated the lipolytic bacterial flora of unamended Canadian soils, although their technique was specifically designed to isolate psychrophiles. Both *Pseudomonas spp.* and *Acinetobacter spp.* dominated the bacterial flora obtained on olive oil-mineral salts agar in both unamended and triacylglycerol amended soils. Since the bacterial population of the soil shows the greatest increase in numbers on this agar after amendment (8.2.3), it would seem likely that these *Pseudomonas spp.* and *Acinetobacter spp.* are the principal organisms responsible for the degradation of triacylglycerol added to soil.

The actinomycete flora demonstrated little apparent change in composition, although it was noted that no *Nocardia spp.* could be isolated on olive oil-mineral salts agar whilst *Micromonospora spp.* were the most frequently isolated actinomycete on this media. The possibility existed that a significant proportion of the actinomycetes were not capable of growing on this media which possessed antibacterial antibiotics to increase its selectivity (Davies and Williams 1970).

The lipolytic fungal flora was dominated by *Penicillium spp.* in unamended soils whilst in amended soil *Penicillium spp.* and *Fusarium spp.* were dominant isolates. The increase in *Fusarium spp.* upon the addition of triacylglycerol to soil is similar to that observed by Krause and Lange (1965) for a range of water-soluble substances added to soil.

8.3.2 Lipase production and distribution: A range of soil isolates were selected from those characterised in 3.1 and the production and location of lipase investigated. For comparison, two stock cultures of lipolytic bacteria (*Staphylococcus aureus* NCIB 6571 and *Pseudomonas*

TABLE 8.4 Classification of randomly-selected lipolytic isolates obtained from unamended and triacylglycerol Hamilton clay loam.

Medium and soil*		Bacteria					
		Total	*Pseudomonas*	*Acinetobacter*	*Bacillus*	*Chromobacterium*	Others
1	U	40	3	7	22	1	7
1	A	40	20	12	4	3	1
2	U	30	12	14	1	2	1
2	A	30	14	10	3	3	1
3	U	4	2	2	0	0	0
3	A	4	4	0	0	0	0
		Fungi					
		Total	*Penicillium*	*Aspergillus*	*Cladosporium*	*Fusarium*	Yeasts
1	U	20	13	4	0	1	2
1	A	20	8	1	2	8	1
2	U	20	17	0	1	2	0
2	A	20	8	0	1	11	0
		Actinomycetes					
		Total	*Streptomycetes*	*Micromonospora*	*Norcardia*		
1	U	10	5	2	3		
1	A	10	4	5	1		
2	U	10	4	6	0		
2	A	10	2	8	0		

* 1 = tributyrylglycerol-amended media,
2 = olive oil-mineral salts agar
3 = enrichment cultures,
U = unamended soil,
A = amended soil.

fluorescens NCIB 9046) were used.

Constitutive lipase production was assessed in trypticase soy broth (BBL, Maryland, USA) for "true" bacteria, peptone-yeast extracts broth (composition : 10 g L^{-1} trypticase peptone, 1 g L^{-1} yeast extract, pH 6.5) for fungi, and actinomyces broth (BBL, Maryland, USA) for actinomycetes. All of these media possessed peptone which has been shown to encourage constitutive lipase production (7,1). Lipase production was also assessed in the olive oil-mineral salts media of 8.2.3 Loopfuls of the isolates were taken from agar slants and inoculated into 50 mL of media in 250 mL Erlenmeyer flasks. Incubation was static at 25°C. Growth and lipase production were evaluated after 2 days for bacteria, after 5 days for fungi, and after 10 days for actinomycetes. Growth was assessed visually (presence or absence) whilst lipase activity was determined using the standard olive oil hydrolysis assay described in 2.1.8. The location of the lipase activity was assessed using the scheme described in Fig.8.1. The activity of Fraction I represents extracellular lipase. The activity of fraction II most probably represents lipase bound to the external cell surface since it is unlikely that the large-sized olive oil substrate particles would have penetrated the cell and been acted upon by intracellular lipases (Finkelstein *et al* 1970). Intracellular lipase is represented by the activity associated with Fraction III. Fraction IV contained principally disrupted cell walls and therefore probably represents the same cell surface bound activity as Fraction II.

The media used to test for constitutive lipase production allowed for growth of all isolates within the time periods prescribed. (Table 8.5). In contrast, 5 of the 14 isolates examined were unable to grow in the olive oil-mineral salts media. All isolates were capable of some constitutive lipase production, although the measured activities and distributions of these lipases showed considerable differences between organisms. As expected, those organisms capable

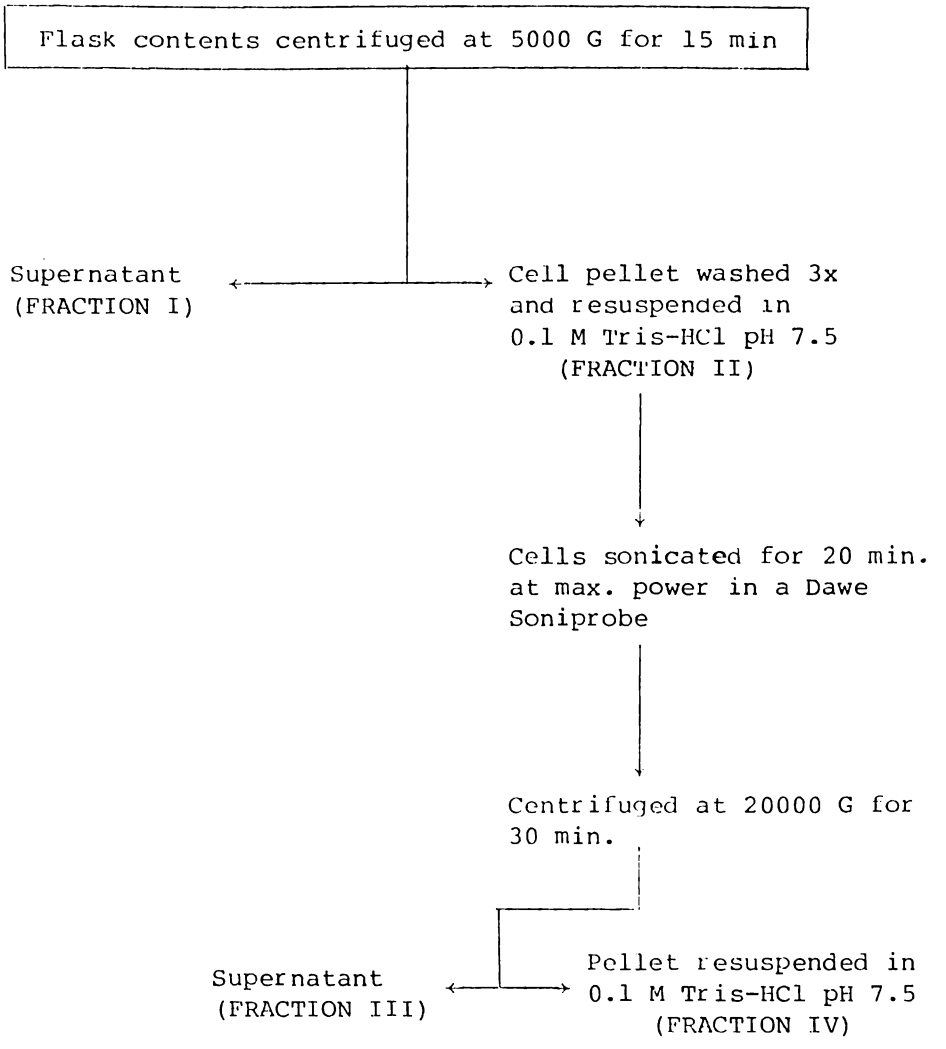


FIG. 8.1 Procedure used to determine location of Lipase activity.

TABLE 8.5 The production and distribution of lipase by soil isolates and two stock bacterial cultures.

ORGANISM	MEDIUM*	GROWTH	LIPASE ACTIVITY** (nmol min^{-1} mL^{-1})			
			I	II	III	IV
Stock cultures:						
S. aureus NCIB 6571	TSB	+	35	0	0	0
	OO	-	-	-	-	-
P. fluorescens NCIB 9046	TSB	+	7	0	0	0
	OO	+	25	0	0	0
Bacterial isolates:						
Pseudomonas 017	TSB	+	22	0	0	0
	OO	+	594	0	0	0
Acinetobacter A30	TSB	+	45	17	0	15
	OO	+	38	65	0	72
Bacillus licheniformis T-C	TSB	+	9	0	0	0
	OO	+	44	8	0	8
Bacillus cereus SUB	TSB	+	0	13	8	16
	OO	-	-	-	-	-
Bacillus sp L-C	TSB	+	0	17	9	19
	OO	-	-	-	-	-
Pseudomonas PTD	TSB	+	7	0	0	0
	OO	+	16	0	0	0
Pseudomonas fluorescens X-P	TSB	+	17	0	0	0
	OO	+	42	0	0	0
Fungal isolates:						
Penicillium FA	PYE	+	159	10	0	12
	OO	+	19	25	4	18
Penicillium HO	PYE	+	13	0	0	0
	OO	+	8	24	4	24
Fusarium T5	PYE	+	97	13	4	12
	OO	+	17	54	2	60
Aspergillus A4	PYE	-	49	0	0	0
	OO	-	-	-	-	-
Cryptococcus Y2	PYE	+	17	2	28	0
	OO	-	-	-	-	-
Actinomycetes isolates						
Streptomycete RB	AB	+	2	28	0	28
	OO	-	-	-	-	-
Micromonospora BB	AB	+	2	0	18	0
	OO	+	34	0	0	0

* TSB = trypticase soy broth, PYE = Peptone-yeast extract media, AB = actinomyces broth, OO = olive oil-mineral salts media.

** Lipase activity as determined by fatty acid release from olive oil

of growth on the olive oil-mineral salts medium produced lipase, with the activities and distributions again differing between organisms.

The bacterial isolates *Bacillus* SUB and *Bacillus* L-C produced a constitutive lipase which was primarily associated with the cell surface (fractions II and IV) although some intracellular activity was also observed (fraction III). The lipase activity of the *Acinetobacter* isolate A30 was found both extracellularly (fraction I) and cell-surface bound (fraction II and IV). Interestingly, the majority of the constitutively-produced lipase of the *Acinetobacter* was extracellular whilst the lipase produced on olive oil-mineral salts was cell-surface bound. All other bacterial isolated produced extracellular lipase.

The *Streptomycete* isolate RB produced a lipase which was primarily cell-surface bound (fractions II and IV). The *Micromonospora* isolate BB produced an intracellular constitutive lipase (fraction III) but an extracellular lipase when growing on triacylglycerol (fraction I).

The fungal isolates *Penicillium* FA, *Penicillium* HO and *Fusarium* T5 produced primarily an extracellular constitutive lipase (fraction I) but possessed both extracellular (fraction I) and cell-surface bound (fractions II and IV) activity when growing upon olive oil. *Aspergillus* A4 produced an extracellular constitutive lipase (fraction I) whilst the constitutive lipase activity of the yeast *Cryptococcus* Y2 was both extracellular (fraction I) and intracellular (fraction III).

Several of the soil isolates produced higher lipase levels than those of the stock cultures, particularly the bacterial isolates *Pseudomonas* 017. The inductive extracellular lipase activity of this isolate was 24x that of the stock culture of *Pseudomonas* NCIB 9046.

TABLE 8.6 Optimal pH's for the expression of lipase activity of crude extracts of various soil isolate cultures. Assays were performed using 4 MUN as substrate.*

C U L T U R E		Optimal pH
Bacteria:	*Pseudomonas* 017	7.5 - 8.0
	Acinetobacter A30	8.0 - 8.5
	B. Licheniformis T-C	8.0 - 8.5
	Bacillus Sp L-C	7.5 - 8.0
	Pseudomonas fluorescens X-P	8.5 - 9.0
Actinomycetes:	*Streptomycete* RB	8.5 - 9.0
	Micromonospora BB-extracellular	8.5 - 9.0
	Micromonospora BB-intracellular	7.0 - 7.5
Fungi:	*Penicillium* FA	6.0 - 6.5
	Penicillium HO	6.0 - 6.5
	Fusarium TS	5.0 - 5.5

* Buffers used were citric acid - Na citrate (pH 4.0-6.2) potassium phosphate (pH 5.2-8.0), and Tris-HCl (pH 7.0-10.0) all at 0.1 M.

The constitutive lipase production of fungal isolates FA and T5 were also high, but production when growing on triacylglycerol was low.

8.3.3 Influence of pH on activities of crude lipase preparations: Studies on soil lipases have demonstrated the presence of dual pH peaks, one each side of neutrality (5.2.7). The alkaline pH peak preferentially increased upon triacylglycerol amendment (5.2.7) and bacterial numbers increased more dramatically than fungi (8.2.2 and 8.2.3). This evidence suggests that the alkaline pH peak represented bacterial lipase whilst the acidic pH peak represented fungal lipase. This hypothesis was further corroborated by investigating the influence of pH on the lipase activity of various soil isolates. Activity was assessed using the crude preparations of 8.3.2 and the standard fluorometric assay of 2.6 with modifications to the pH of the assay mixture by the use of different buffers. Similar results were obtained between buffer systems at points of pH-overlap indicating no effect of buffer composition on enzyme activity.

All bacterial preparations demonstrated maximal activity toward 4 MUN at an alkaline pH (range of optimal pH's was 7.5-9.0) whilst maximal activity in all fungal preparations was at an acidic pH (range of optimal pH's was 5.0-6.5) (Table 8.6). The cell-bound lipase of *Streptomycete* RB possessed optimal activity at pH 8.5-9.0. as did the extracellular lipase of *Micromonospora* BB. However, it would appear that the constitutive intracellular lipase of *Micromonospora* BB is a different enzyme since it possessed an optimal activity at pH 7.0-7.5.

8.4 Summary

The lipolytic microbia of both unamended and triacylglycerol-amended soils were isolated using three different techniques. Subsequent identification of these isolates revealed that changes in species dominance occurred with triacylglycerol amendment and with the use of different isolation media. The bacterial population demonstrates the greatest increase upon triacylglycerol amendment particularly those bacteria capable of growth on triacylglycerol as a sole source of carbon and energy. The production and location of lipase by the microbial isolates was investigated on two media that tested for constitutive and inductive lipase. Several bacterial and fungal isolates produced considerably more lipase than stock bacterial cultures, particularly the bacterial isolate *Pseudomonas* 017. This organism produced high levels of extracellular inductive lipase.

CHAPTER 9 GROWTH AND LIPASE PRODUCTION OF TWO SOIL BACTERIA

9.1 Introduction

The initial screening of lipolytic soil microorganisms (8.3.2) revealed one isolate, *Pseudomonas* 017, that was capable of inductively producing a high level of extracellular lipase. The prime objective of the work presented in this chapter was to investigate the factors influencing the growth and lipase production of *Pseudomonas* 017, with a view to assessing its potential for commercial exploitation.

Studies were also performed with another soil isolate, *Acinetobacter* A30, for comparative purposes. This organism produced an extracellular lipase when grown upon complex media but, unlike *Pseudomonas* 017, primarily a cell-bound lipase when grown upon triacylglycerol (8.3.2).

9.2 Characteristics of the organisms

The detailed characteristics of the two soil isolates are as follows:

Acinetobacter A30 - Creamy, mucoid colonies on trypticase soy agar, Gram negative. Cells pleomorphic being short, plump rods (1.5 - 2.0 x 1.0 - 1.5 μm) in logarithmic phase becoming coccoid (1.0 - 1.5 μm diameter) in stationary phase. Non-motile, often in pairs. Optimal growth temperature 30°C, range 4 - 42°C. Cytochrome oxidase negative, catalase positive. Strictly aerobic. With API 20B strips, negative results were observed for gelatin hydrolysis, nitrate reductase, β-galactosidase (ONPG hydrolysis), urease, tryptophanase, H_2S production, acetoin production, citrate utilisation, and acid metabolism of saccharose, mannitol, starch and sorbitol. Weak acid production occurred with L-arabinose, fructose, glucose, maltose, rhamnose, galactose, mannose and glycerol. The organism was resistant to 1U of

penicillin, but not to 5U.

This *Acinetobacter* sp may be separated from the morphologically similar *Moraxella* by its negative oxidase test, its moderate resistance to penicillin, and its ability to utilise carbohydrates (Lautrop 1974). Whilst only one species is formally recognised in the genus *Acinetobacter* (*Acinetobacter calcoaceticus*) this isolate appears to fall within the subgroup "Lwoffi" which comes closest to deserving status as a separate species (Lautrop 1974) since it was unable to utilise citrate, was susceptible to 5U of penicillin, gave a weak acid reaction with glucose, and was unable to hydrolyse gelatin. Breuil *et al* (1975) have isolated a "Lwoffi" *Acinetobacter* from river sediment which was capable of producing lipase (Breuil and Kushner 1975a and b; Breuil *et al* 1978).

Pseudomonas 017 - White, glossy colonies on trypticase soy agar, turning fawn-brown upon prolonged incubation. Gram negative. Cells single, straight or slightly curved rods (0.5 - 0.8 x 1.5 - 2.0 μm). Motile, with 3 - 8 polar flagella. No diffusible pigment formed on Kings media A or B. Optimal growth temperature 25°C, range 4 - 40°C. Cytochrome oxidase positive, catalase positive. Strictly aerobic. With API 20B strips, negative results were observed for gelatin hydrolysis, nitrate reductase, β-galactosidase (ONPG hydrolysis), urease, tryptophanase, H_2S production, acetoin production and acid metabolism of saccharose, L-arabinose, mannitol, maltose, starch, rhamnose, mannose and sorbitol. Positive results were observed for citrate utilisation and acid metabolism of fructose, glucose, galactose, and glycerol. No growth at pH 4.5 on glucose or ethanol. No growth on DL-β-hydroxybutyrate.

This *Pseudomonas* sp may be separated from *Acetobacter* spp by its polar flagellation, lack of growth at pH 4.5, and inability to utilise ethanol. It is distinguished from *Gluconobacter* spp by its inability to grow at pH 4.5 and to utilise ethanol (De Ley and Frateur 1974). *Pseudomonas* 017 may be placed in Group I of the classification scheme of Doudoroff and Palleroni (1974) for the genus *Pseudomonas*

since it is capable of growth in a mineral medium with the addition of a single carbon source. Since it does not grow on DL-β-hydroxybutyrate it can be placed in Section I of the classification scheme. Whilst *Pseudomonas* 017 does not produce a fluorescent pigment, other characteristics (including its multitrichous flagellation) excludes it from the non-fluorescent sub-group. *Pseudomonas* 017 differs from *P. aeruginosa* due to its multitrichous flagella, inability to denitrify, to grow at 41°C, and to hydrolyse gelatin. The isolate cannot be classified with *P. fluorescens* since it does not produce a diffusible fluorescent pigment or hydrolyse gelatin. *Pseudomonas* 017 may be tentatively identified as a non-fluorescent strain of *Pseudomonas putida*. According to Stanier *et al* (1966) *Pseudomonas putida* is nutritionally diverse and is a frequent isolate obtained from enrichment culture, with soil as the inoculum and mineral salts plus a single carbon source as the medium. *Pseudomonas* 017 was isolated under such conditions.

9.3 Maintenance and preculture of isolates

Both isolates were maintained on trypticase soy agar (BBL Maryland, USA) slants at 4°C with once-monthly transfers. A standard preculture procedure was followed with both organisms prior to growth and lipase production experiments. This procedure was as follows:

To 100 mL of trypticase soy broth (BBL Maryland, USA) in a 250 mL Erlenmeyer flask was added a loopful of inoculum from the maintenance slant. After incubation at 25°C and 120 rev min^{-1} on an orbital shaker for 14 - 20h, the flask contents were centrifuged (5000 G for 20 min), washed 3x with saline (NaCl 8.5 g L^{-1}), and finally resuspended in saline to the same volume. This washed cell suspension was used as the inoculum for growth and lipase production experiments. Both the *Pseudomonas* 017 and the *Acinetobacter* A30 cultures harvested at this time were in mid- to late-exponential phase. Viable cell counts using spread-plate dilution onto trypticase soy agar revealed that the washed cell suspensions obtained from repeated runs contained 1.1 - 4.2 x 10^8 cells

mL^{-1} and 2.5 - 7.0 x 10^8 cells mL^{-1} for the *Pseudomonas* 017 and *Acinetobacter* A30 respectively. In studies with complex media *Staphylococcus aureus* NCIB 6571 was used for comparative purposes. This organism was maintained and precultured in the same way as the soil isolates except that the incubation temperature was 37°C. At the time of harvest of this *Staphylococcus aureus* preculture cells were in mid-exponential phase at a viable cell concentration of 3.0 - 8.2 x 10^8 mL^{-1}.

9.4 Growth and lipase production in complex media

9.4.1 Effect of incubation time : Growth and lipase production by *Acinetobacter* A30, *Pseudomonas* 017 and *Staphylococcus aureus* NCIB 6571 was followed after inoculation of 1 mL aliquots of washed cell suspension (9.3) into 100 mL of trypticase soy broth in 250 mL Erlenmeyer flasks. Incubation was with orbital shaking at 120 rev min^{-1} and at temperatures of 25°C (for *Acinetobacter* A30 and *Pseudomonas* 017) and 37°C (for *Staphylococcus aureus*). Samples were periodically removed and growth assessed by turbidity (absorbance at 660 nm) and lipase activity assayed by both the fluorometric procedure of 2.6 and the olive oil hydrolysis procedure of 2.4.

Lipase activity as assessed by either method, was closely related to the extent of growth for all three organisms (Fig. 9.1). No further increase in lipase activity was observed upon cultures entering stationary phase. Prolonged incubation led to a decrease in lipase activity with the *Staphylococcus* and *Acinetobacter* cultures but not with the *Pseudomonas* 017 culture.

Previous work on *Acinetobacter* isolates has also demonstrated a close relationship between growth and lipase production in a complex medium (Breuil and Kushner 1975a). The results obtained with *Staphylococcus aureus* NCIB 6571 are at variance with previous reports on staphylococcal lipase production where maximal activities were obtained after cultures entered the stationary phase of growth

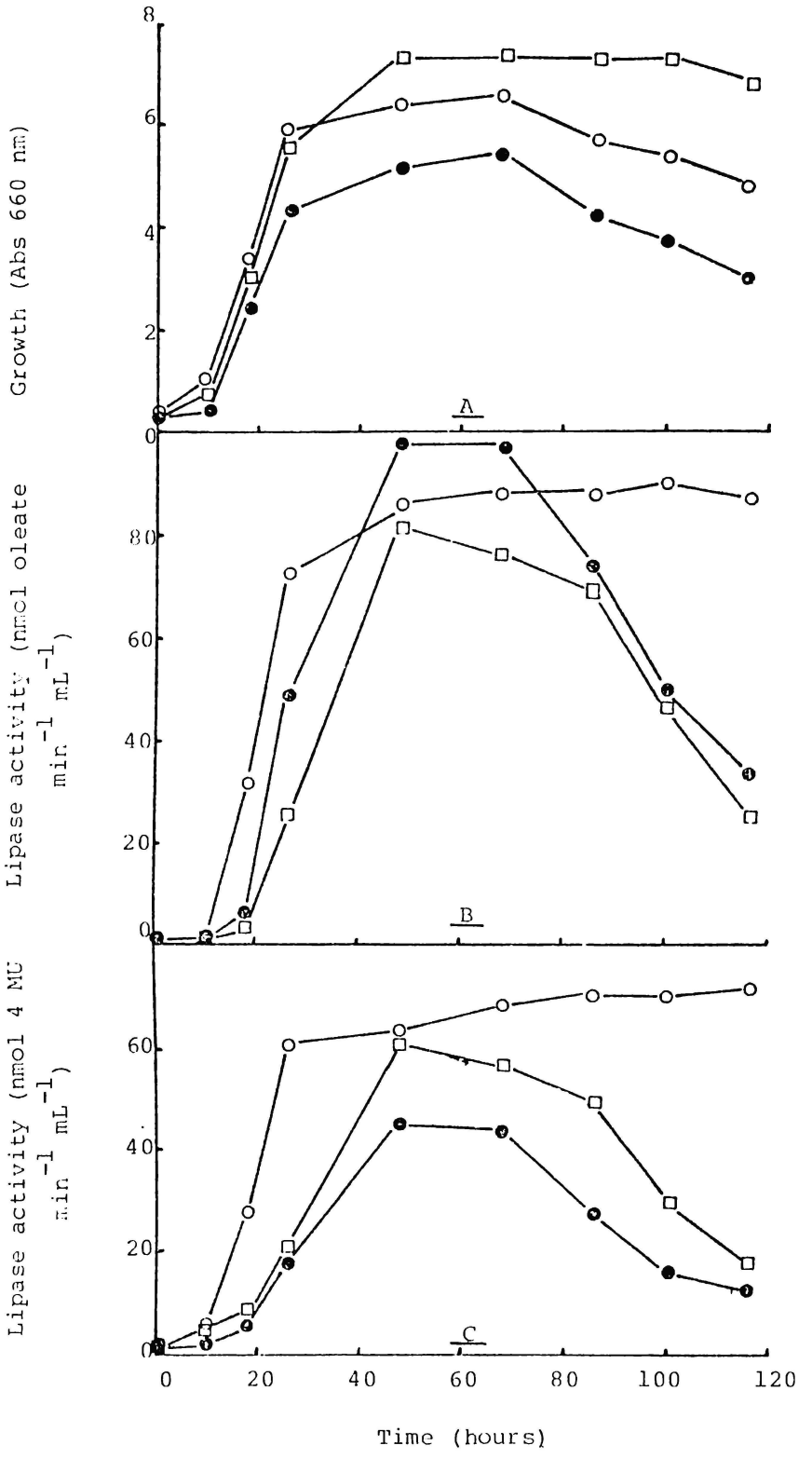


Fig. 9.1. Growth and lipase production of *Staphylococcus aureus* NCIB 6571 (●-●), *Acinetobacter* A30 (□-□), and *Pseudomonas* 017 (o-o), when grown on trypticase soy broth. Growth (A) was assessed by turbidity, and lipase activity by olive oil hydrolysis (B) and 4-methyl umbelliferone nonanoate hydrolysis (C).

(Troller and Bozeman 1970; Jonsson and Syngg 1974; Pablo *et al* 1974). However, Jurgens *et al* (1981) did find a close relationship between growth and lipase activity. Lawrence *et al* (1967a and b) demonstrated a close relationship between growth and lipase production for a pseudomonad growing in complex medium.

The decreased lipase activity observed upon prolonged incubation of the staphylococcal culture may have been due to proteolytic attack since this organism was proteolytic (demonstrated activity toward gelatin in API 20B tests). Decreased activity in the *Acinetobacter* A30 cannot be a result of proteolysis since the organism was gelatin negative (9.2) and therefore indicates that the enzyme was unstable to the incubation conditions (Jonsson and Snygg 1974) unlike the *Pseudomonas* 017 lipase.

9.4.2 Effect of medium composition : Aliquots (1 mL) of washed cell suspensions (9.3) were inoculated into 100 mL of various media in 250 mL Erlenmeyer flasks. Flasks were subsequently incubated with orbital shaking at 120 rev min^{-1} at 25°C for *Acinetobacter* A30 and *Pseudomonas* 017 and 37°C for *Staphylococcus aureus* NCIB 6571. Growth (Absorbance 660 nm) and lipase activity (fluorometric method of 2.6) were assessed after 48h.

The effect of medium composition on growth and lipase production was similar for all three organisms (Table 9.1). The higher lipase levels obtained with trypticase soy broth and brain heart infusion broth as compared to nutrient broth and the positive effect peptone has on lipase production have previously been demonstrated for bacteria (Mates and Sudakevitz 1973; Jonsson and Snygg 1974).

The influence that addition of two triacylglycerols and their free fatty acids have on growth and lipase production in trypticase soy broth is detailed in Table 9.2. Triacylglycerols did not affect the growth of any of the organisms. The presence of oleate in the medium prevented any growth of the gram positive

TABLE 9.1 Growth and lipase production of *Staphylococcus aureus* NCIB 6571, *Acinetobacter* A30, and *Pseudomonas* 017 after 48h incubation.

ORGANISM	MEDIUM*	GROWTH (Abs 660 nm)	LIPASE ACTIVITY (nmol 4MU min^{-1} mL^{-1})
Staph. aureus	TSB	5.22	45
	BHI	6.85	52
	NB	4.21	0
	NB + P	4.22	18
Acinetobacter A30	TSB	7.31	61
	BHI	7.52	58
	NB	4.33	2
	NB + P	4.44	22
Pseudomonas 017	TSB	6.40	64
	BHI	7.12	63
	NB	5.10	2
	NB + P	4.95	18

* TSB = trypticase soy broth; BHI = brain heart infusion; NB = nutrient broth; NB + P = nutrient broth + 1% peptone.

TABLE 9.2 Growth and accumulated lipase levels of three bacteria incubated for 48h in trypticase soy broth with various amendments.

ORGANISM	MEDIUM*	GROWTH† (N_0 mL_9^{-1} x 10^{-9})	LIPASE ACTIVITY (nmol 4MU min^{-1} mL^{-1})
Staph. aureus	TSB	3.8	45
	TSB + B	3.9	44
	TSB + TB	4.0	45
	TSB + O	0	0
	TSB + OO	3.8	21
Acinetobacter A30	TSB	10.2	61
	TSB + B	10.5	60
	TSB + TB	10.3	60
	TSB + O	10.4	13
	TSB + OO	10.1	31
Pseudomonas 017	TSB	7.2	64
	TSB + B	7.1	63
	TSB + TB	7.4	63
	TSB + O	7.3	12
	TSB + OO	7.2	22

* TSB = trypticase soy broth; + B = 0.1% butyrate; + TB = 0.1% tributyrylglycerol; O = 0.1% oleate; OO = 0.1% olive oil.

† Growth was assessed by viable counts using the spread plate dilution procedure onto trypticase soy agar. Plates were incubated for 3 days at 37°C for *Staph. aureus* and at 25°C for *Acinetobacter* A30 and *Pseudomonas* 017.

Staphylococcus aureus without influencing the growth of the gram negatives, *Acinetobacter* A30 and *Pseudomonas* 017. The selective inhibition of growth of gram positives by long-chain fatty acids is a well-documented phenomenon (Galbraith *et al* 1971; Sheu and Freese 1973). The inclusion of either tributyrylglycerol or butyrate into the culture medium did not affect lipase levels after 48h. In contrast, both olive oil (principally trioleoylglycerol) and oleate led to decreased lipase levels after 48h in all cultures which demonstrated growth. Decreased lipase levels upon incorporation of long-chain triacylglycerols into complex media have previously been reported for a range of microorganisms (Smith and Alford 1966; Eitenmiller *et al* 1970; Jonsson and Snygg 1974). The decrease may represent inhibition of the lipase activity by the released insoluble fatty acids (Smith and Alford 1966; 3.3) rather than an influence on lipase synthesis. The lack of effect of butyrate or its esterified triacylglycerol can therefore be explained by its lack of inhibition of lipase activity (3.3).

The decreased lipase activity of *Pseudomonas* 017 cultured in trypticase soy broth upon the inclusion of oleate or olive oil is puzzling. When *Pseudomonas* 017 was cultured in simple media with mixed substrates, the exhaustion of the non-lipid component subsequently led to high lipase levels being induced by the lipid component (9.5). The absence of such a phenomenon when trypticase soy broth was used indicates complete suppression of inductive lipase synthesis. An explanation for this suppression may be that the high level of growth in the trypticase soy broth made conditions unfavourable for subsequent lipase synthesis (*e.g.* the pH of the culture fluid rose to 8.7 - 9.0)

9.5 Growth and lipase production in simple media

The growth and lipase production of *Acinetobacter* A30 and *Pseudomonas* 017 was assessed in simple media containing mineral salts and a sole carbon and energy source (at 8 g L^{-1}). The mineral salts was the same as that used by Breuil *et al* (1978), with the following composition L^{-1} : NH_4NO_3, 2 g; KH_2PO_4, 4 g; Na_2HPO_4, 6 g; $MgSO_4.7H_2O$, 0.2 g; $FeSO_4.7H_2O$, 0.01 g; $CaCl_2$, 0.05 g; initial pH 7.2 ± 0.2. Aliquots (1 mL) of bacterial precultures (9.3) were inoculated into 100 mL of the desired media in 250 mL Erlenmeyer flasks and incubated at 25°C with orbital shaking at 120 rev min^{-1}.

Initial screening of a range of substrates demonstrated that both organisms produced higher levels of lipase when grown upon hexadecane or olive oil than when grown upon sugars or sugar derivatives (Table 9.3). The *Pseudomonas* 017 isolate also produced high levels of lipase when grown upon long-chain fatty acids, unlike *Acinetobacter* A30. The induction of microbial enzyme production in response to the presence of its substrate is a common phenomenon and has previously been reported for the lipase-triacylglycerol situation (Tsujisaka *et al* 1973; Akhtar *et al* 1980). The physiological role of lipase induction under such conditions is obvious, since the utilisation of the triacylglycerol as a source of carbon and energy is dependent upon its initial hydrolysis. Induction of microbial lipase production when growth occurs upon hydrocarbons (Breuil *et al* 1978) or fatty acids (Ota *et al* 1968; Sugiura *et al* 1975), has previously been reported. The role of lipases in the growth and metabolism of organisms utilising these substrates remains obscure. Breuil *et al* (1978) found that all their bacterial isolates that were capable of growth upon hydrocarbons were lipase-positive suggesting a causal relationship. The possibility exists that the presence of long alkyl-chain compounds stimulates lipid metabolism in general, rather than lipase production in particular (9.7).

TABLE 9.3 Viable count and lipase levels of cultures of *Acinetobacter* A30 and *Pseudomonas* 017 after incubation in single substrate-mineral salts media for 48h at 25°C.

ORGANISM	SUBSTRATE*	VIABLE COUNT† (No mL^{-1} x 10^{-9})	LIPASE ACTIVITY nmol min^{-1} mL^{-1}	
			A^{sp}	B^{te}
Pseudomonas 017	glucose	7,2	16	19
	glycerol	5.8	32	40
	hexadecane	2.4	182	215
	olive oil	3.4	732	922
	butyrate	1.1	0	0
	stearate	6.2	162	592
	oleate	6.4	196	863
Acinetobacter A30	pyruvate	5.3	44	35
	glycerol	0	0	0
	hexadecane	1.5	118	88
	olive oil	3.5	151	112
	butyrate	0	0	0
	stearate	3.8	0	0
	oleate	4.1	0	0

* A = activity toward olive oil as per 2.4;

B = activity toward 4-methyl umbelliferone nonanoate as per 2.6.

† Viable count determined by spread plate dilution procedure using trypticase soy agar. Plates were incubated for 3 days at 25°C.

Patterns of growth (as monitored by oxygen consumption) and lipase production were examined in more detail with three substrates. Oxygen consumption was monitored by setting up a parallel range of media (total volume 2 mL) in Warburg respirometric flasks to that placed in the main incubation flasks. The Warburg flasks were inoculated with the same preculture at the same relative inoculum size (*i.e.*, 1%), 0.2 mL of 20% (w/v) KOH added to filter paper wicks in the centre well, connected to manometers, and incubated at 25°C and 120 rev min^{-1}. Oxygen consumption rates were periodically determined and cumulative respiration calculated by integration of this rate data. Preliminary experiments demonstrated that growth (as viable count) and lipase production within these Warburg flasks were the same as that which occurred in the main incubation flask. Fatty acid levels were monitored in the main incubation flask using the colourimetric analysis of 2.4.

The patterns of oxygen consumption, culture fatty acid level and lipase level are presented for *Acinetobacter* A30 and *Pseudomonas* 017 in Figs. 9.2 and 9.3 respectively. Both organisms demonstrated the shortest lag period when grown upon a soluble substrate and the longest lag period when grown upon hexadecane. The amount of added carbon and the proportion that was respired is presented in Table 9.4. The amount of respired carbon was calculated from the reported respiratory quotients (ratio of CO_2 produced to O_2 consumed on a molar basis) of the substrates (Dennis and Irvine 1981). The proportions of added carbon respired with glucose or pyruvate as substrate is similar to that reported by others (Pirt 1975). In comparison, both organisms respired proportionally less added carbon with either olive oil or hexadecane as substrates. Similar results for microbial growth on a range of hydrocarbons have been observed previously (Pirt 1975; Payne and Weibe 1978). Studies by Kennedy and co-workers (Kennedy

TABLE 9.4 Total carbon added and carbon respired by *Acinetobacter* A30 and *Pseudomonas* 017 growing on different substrates.

ORGANISM	SUBSTRATE	CARBON ADDED (μmol mL^{-1})	CARBON* RESPIRED (μmol mL^{-1})	RESPIRED %
Acinetobacter A30	pyruvate	273	108	40
	olive oil	515	108	21
	hexadecane	567	122	21
Pseudomonas 017	glucose	266	120	45
	olive oil	515	104	20
	hexadecane	567	130	23

* Carbon respired calculated assuming a respiratory quotient for glucose and pyruvate of 1.0 and a respiratory quotient for olive oil and hexadecane of 0.72 (Dennis and Irvine 1981).

and Finnerty 1975; Kennedy *et al* 1975) have demonstrated that a hydrocarbon-oxidising *Acinetobacter* sp. was capable of pooling unmetabolised hydrocarbon within intracytoplasmic membranes. The occurrence of such a phenomenon in the present study would explain the low proportions of respired carbon observed with insoluble substrates (Payne and Wiebe 1978).

The action of the microbial lipases on olive oil produced fatty acids which initially accumulated in the culture fluids of both organisms (Figs. 9.2 and 9.3). These fatty acids were subsequently metabolised. Apparently, during the early stages of growth upon olive oil, the rate at which fatty acids were being produced by lipolytic activity exceeded the rate at which they were being metabolised.

The production of fatty acids when growing upon hexadecane has previously been reported by Makula *et al* (1975) for an *Acinetobacter* sp. Finnerty (1977) has suggested that the role of fatty acids in hexadecane metabolism may be to act as an emulsifier of the hexadecane, thereby increasing the available substrate surface area and consequently increasing the growth rate of the organism. However, the studies of 9.6 indicate that the emulsification of insoluble growth substrates by *Pseudomonas* 017 was brought about by an emulsifier with properties not consistent with that of fatty acids *per se* (non-dialysable, unstable in the presence of organic solvents, unstable to ultrafiltration). The possibility exists that fatty acids were combined with other compounds to form more effective emulsifying agents (Zajic and Panchal 1976).

The lipase levels obtained in the cultures of both organisms were highest with olive oil as substrate (Fig. 9.2 and 9.3) as previously observed in Table 9.3. The patterns of lipase production upon olive oil preceeded oxygen consumption patterns with both organisms. This simply reflects the necessity of initial lipase action upon the

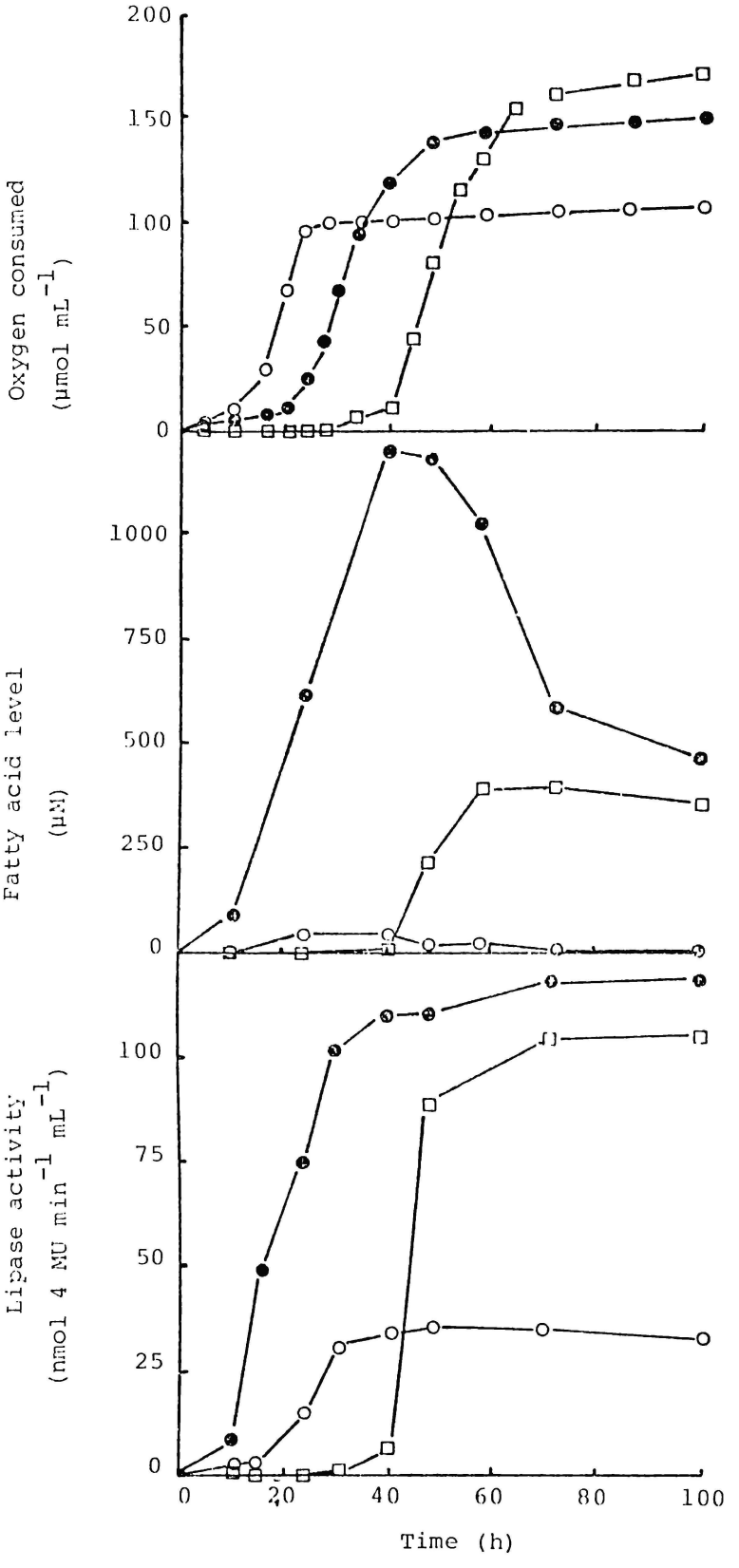


Fig. 9.2. The patterns of oxygen consumption, culture fatty acid level, and lipase level for *Acinetobacter* A30 growing on three different substrates all at 8 g L^{-1} -o-, pyruvate; ●-●, olive oil; □-□, hexadecane.

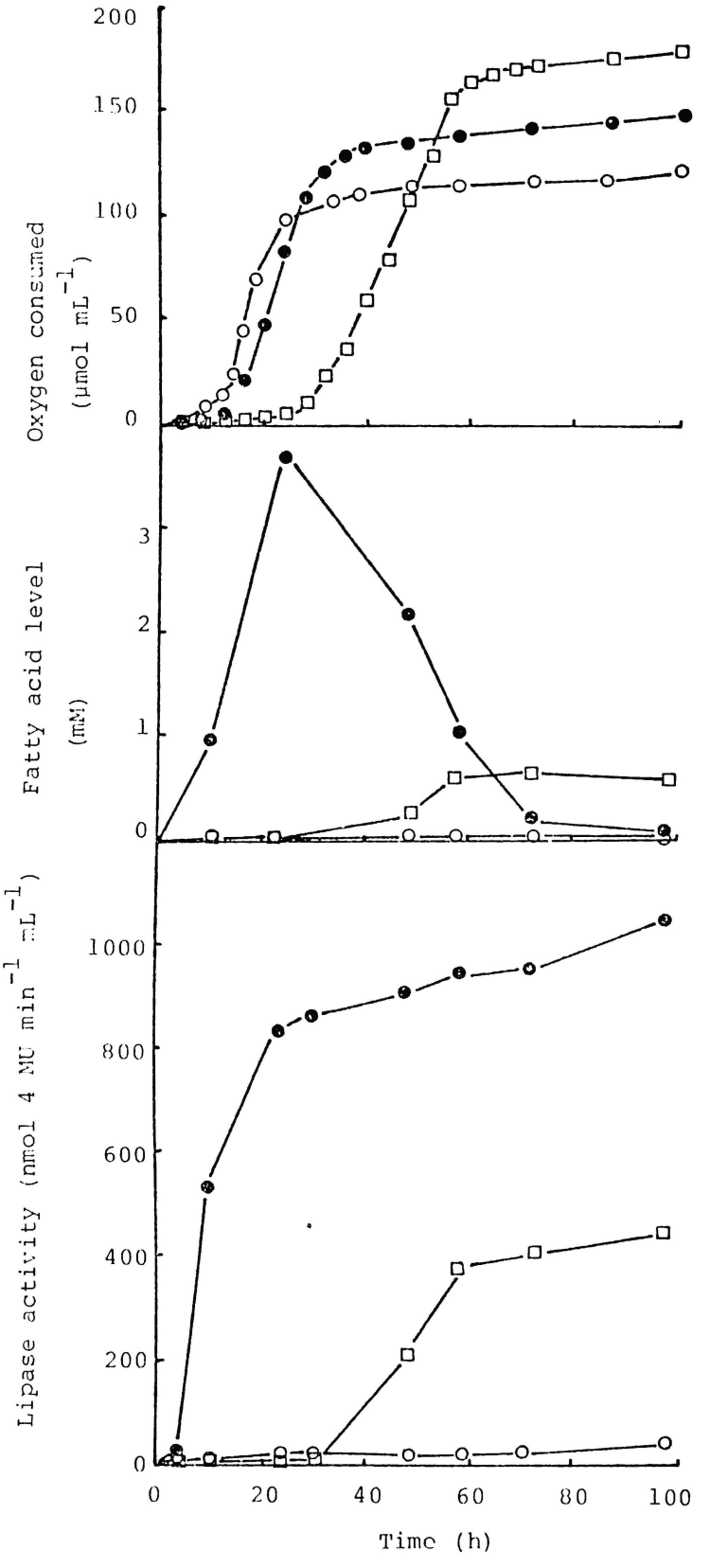


Fig. 9.3. The patterns of oxygen consumption, culture fatty acid level, and lipase level for *Pseudomonas* 017 growing on three different substrates all at 8 g L^{-1}. o-o, glucose; ●-●, olive oil; □-□, hexadecane.

triacylglycerol before oxidation can occur. With hexadecane as substrate, lipase production was closely associated with growth. This suggests that lipase production was a consequence, rather than a cause, of growth upon hexadecane.

The patterns of growth and lipase production of *Pseudomonas* 017 when cultured with a variety of triacylglycerols was examined (Fig. 9.4). Tributyrylglycerol was not able to support rapid growth of *Pseudomonas* 017, nor was it capable of inducing high levels of lipase, despite being an amenable substrate for lipolytic activity (10.3.1). Growth was most rapid, and lipase production highest, when tallow was supplied to the organism. The possibilities of using this byproduct lipid in the commercial production of lipase is discussed in 10.4.

Growth and lipase production of *Pseudomonas* 017 in fatty acid -mineral salts media was dependent upon the acyl chain-length (Table 9.5). The inability of *Pseudomonas* 017 to grow upon medium chain-length fatty acids (C_6 - C_{10}) may be related to the inefficiency of its extracellular emulsifier to act upon these substances (9.6.5). The high levels of lipase production observed with long chain-length fatty acids are similar to that previously reported for a yeast (Sugiura *et al* 1975).

The long alkyl-chain hydrocarbons (C_{16}, C_{18}) were capable of supporting considerable growth of *Pseudomonas* 017 along with the accumulation of high lipase levels (Table 9.6). As with fatty acids, the inability to grow upon short chain-length hydrocarbons may be related to the inefficiency of the emulsifier of *Pseudomonas* 017 to act upon these substances (9.6.5), or the lack of a suitable uptake mechanism (9.7).

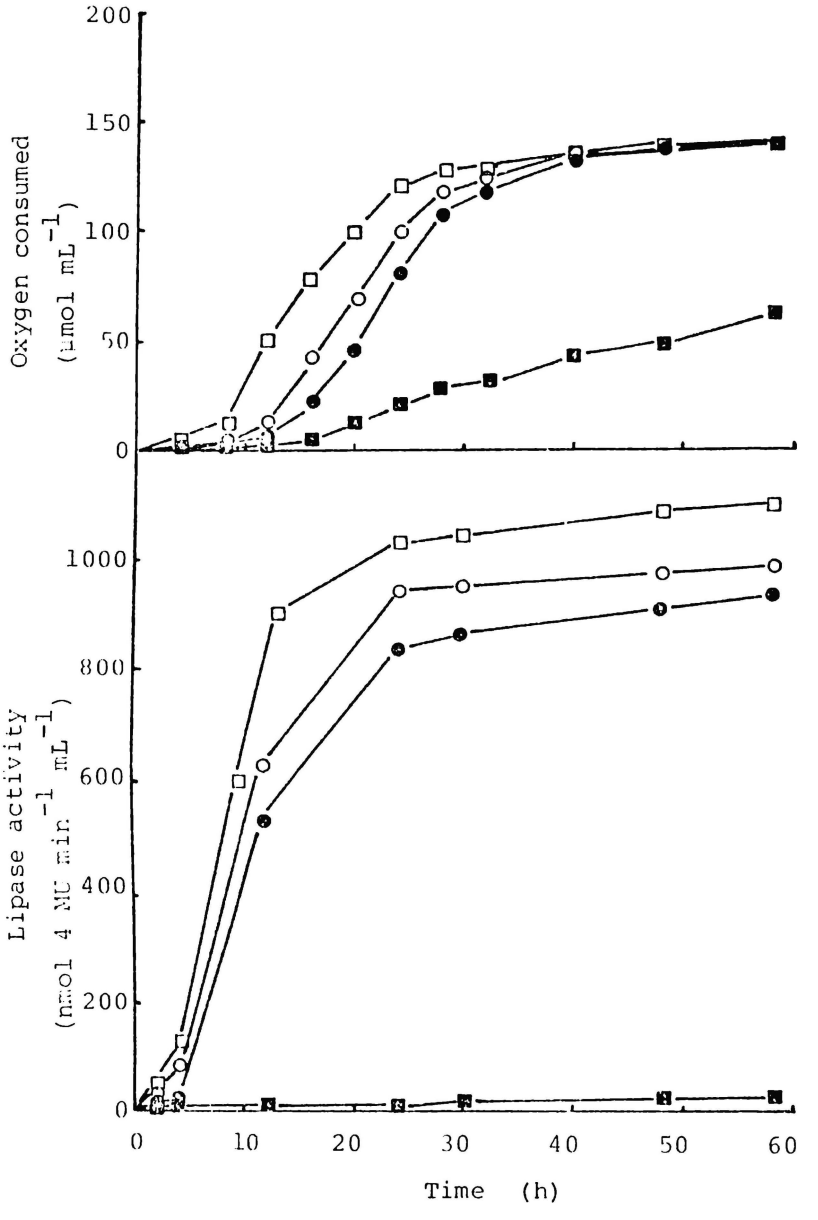


Fig. 9.4. Oxygen consumption and lipase levels for *Pseudomonas* 017 growing on different triacylglycerols all at 8 g L^{-1}. ■-■, tributyrylglycerol; ●-●, olive oil; o-o, anhydrous milk fat; □-□, tallow.

TABLE 9.5 Viable count and lipase levels of cultures of *Pseudomonas* 017 after incubation in fatty acid-mineral salts media for 48h at 25°C.

FATTY ACID	VIABLE COUNT (No. mL^{-1} x 10^{-9})	LIPASE ACTIVITY (nmol 4-MU min^{-1} mL^{-1})
Acetate (C_2)	1.3	10
Butyrate (C_4)	1.1	10
Caproate (C_6)	0	0
Octanoate (C_8)	0	0
Decanoate (C_{10})	0	0
Laurate (C_{12})	1.2	705
Myristate (C_{14})	3.4	458
Palmitate (C_{16})	4.1	355
Stearate (C_{18})	6.2	590
Oleate ($C_{18:1}$)	6.4	842
Linolenate ($C_{18:3}$)	6.3	208

TABLE 9.6 Viable count and lipase levels of cultures of *Pseudomonas* 017 after incubation in aliphatic hydrocarbon-mineral salts media for 48h at 25°C.

HYDROCARBON	VIABLE COUNT (No mL^{-1} x 10^{-9})	LIPASE ACTIVITY (nmol 4 MU min^{-1} mL^{-1})
Hexane (C_6)	0	0
Octane (C_8)	0	0
Decane (C_{10})	0.2	17
Hexadecane (C_{16})	2.4	206
Octadecane (C_{18})	1.9	168

Several cross-inoculation experiments were performed with *Pseudomonas* 017 cultures. After inoculation of precultures (9.3) and growth in one particular medium for 48h, cells were harvested, washed 3x in saline (8.5 g NaCl L^{-1}) and resuspended to their original volume. Aliquots (1 mL) were subsequently used to inoculate flasks of three different media, one of which was the same composition as the previous growth medium. The results presented in Fig. 9.5 demonstrate that prior growth on any one of the substrates oleate, olive oil or hexadecane facilitated subsequent growth and lipase production on all three. The organism was induced or primed for growth upon insoluble substrates in general rather than being selectively induced towards the initial growth substrate only.

When *Pseudomonas* 017 was grown on a mixed glucose-olive oil medium a diauxic pattern of oxygen consumption was observed (Fig. 9.6). The initial period of oxygen consumption represents preferential utilisation of the glucose, with the olive oil remaining as a distinct phase on the top of the culture fluid. The secondary phase of oxygen consumption was accompanied by a rapid increase in lipase level and emulsification of the olive oil. This secondary growth therefore represents utilisation of the olive oil. When incubation flasks contained bovine serum albumin in addition to olive oil, no difference in growth or lipase production from that observed in systems with only olive oil was noted. As *Pseudomonas* 017 was not proteolytic (9.2) it would seem that the presence of non-utilisable sources of carbon and energy have no effect on lipase production.

The lipase level reached by *Pseudomonas* 017, when grown upon olive oil, was dependent upon the initial level of triacylglycerol below a concentration of 2.0 g L^{-1} (Table 9.7). Above this concentration, no further increases in lipase level were observed.

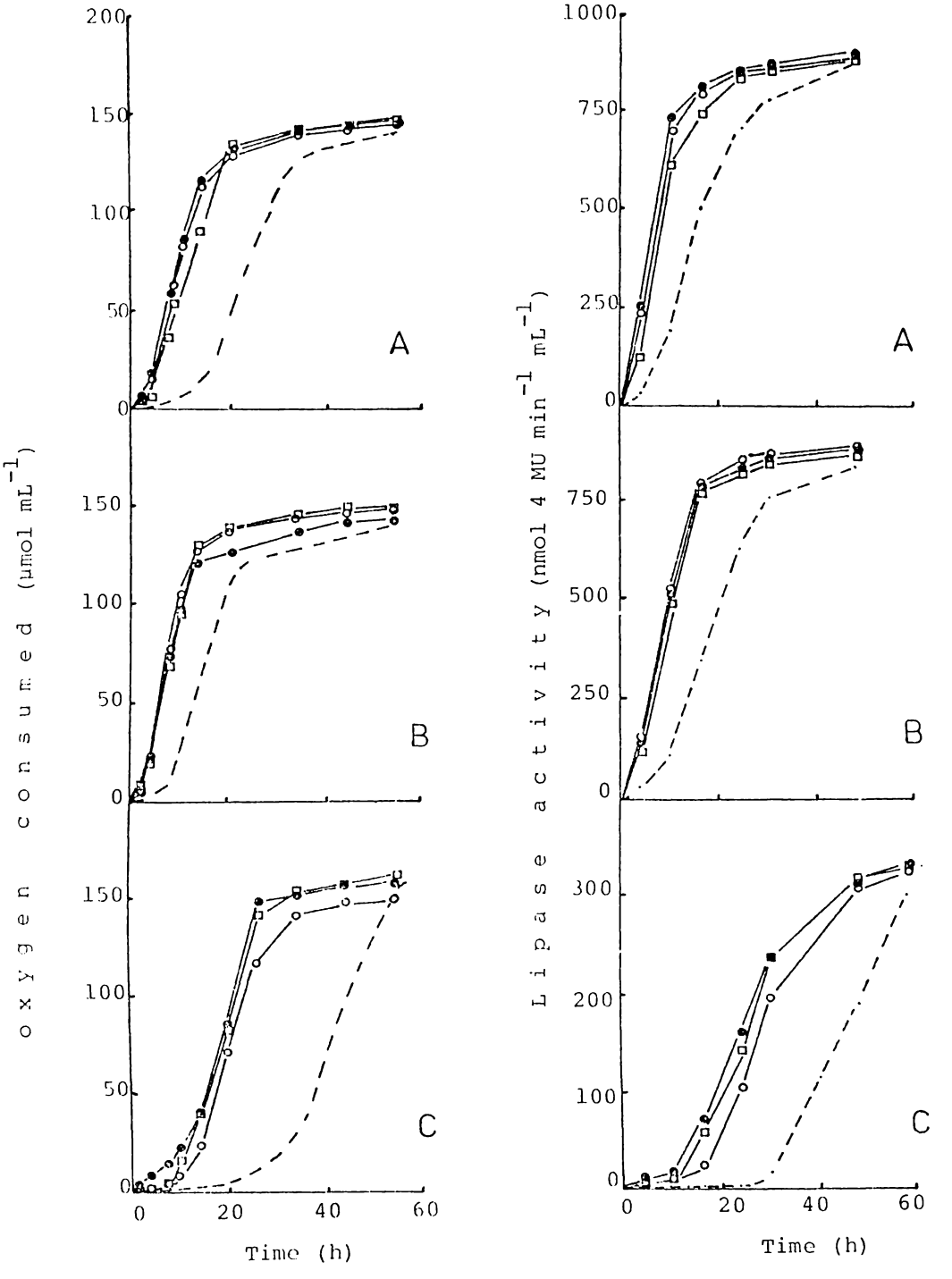


Fig. 9.5. Oxygen consumption and lipase levels for *Pseudomonas* 017 growing on olive oil (A), oleate (B), and hexadecane (C) after initial 48h growth in olive oil (o-o), oleate (●-●), or hexadecane (□-□). Dashed line represents results obtained after initial preculture in trypticase soy broth.

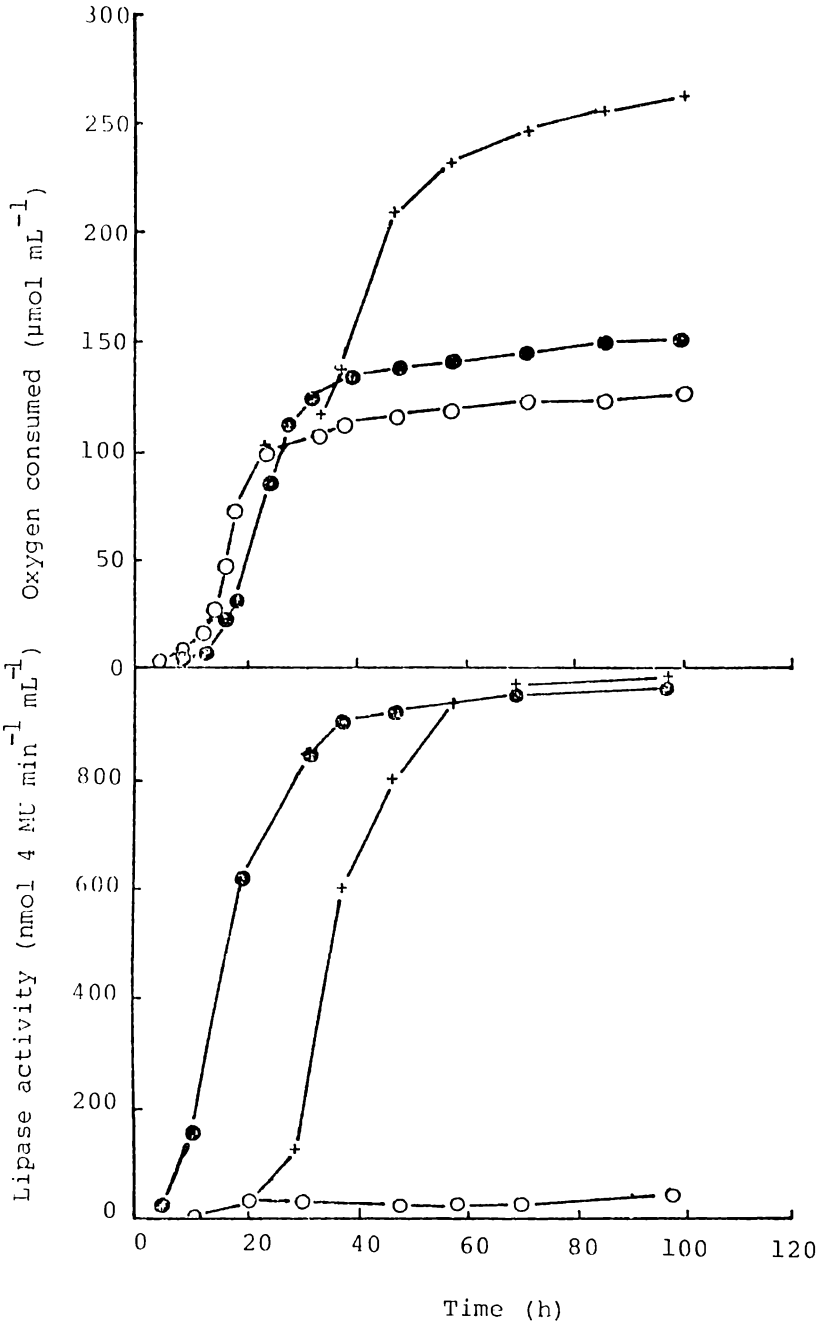


Fig. 9.6. Oxygen consumption and lipase levels for *Pseudomonas* 017 growing on glucose (o-o), olive oil (●-●), and glucose + olive oil (+-+). All substrates at 8 g L^{-1}.

TABLE 9.7 Lipase levels of *Pseudomonas* 017 when cultured in olive oil-mineral salts media with different concentrations of olive oil for 48h at 25°C.

Olive oil concentration ($g\ L^{-1}$)	Lipase level (nmol 4 MU min^{-1} mL^{-1})
0.1	35
0.2	70
0.5	185
1.0	402
2.0	912
4.0	895
8.0	910

9.6 Studies on the emulsifier of *Pseudomonas* 017

9.6.1 Introduction : The production of emulsifying agents by microorganisms is a phenomenon closely associated with rapid growth upon insoluble carbon and energy sources (Hisatsuka *et al* 1971; Erickson and Nakahara 1975; Zajic *et al* 1977; Floodgate 1978). Emulsification of insoluble substrates exposes a greater surface area for enzyme hydrolysis, thereby leading to more rapid rates of decomposition. Ecologically, this emulsification-decomposition relationship may be of great importance in determining the persistence, and hence environmental impact, of insoluble lipid substrates (Rosenberg *et al* 1975; Gutnick and Rosenberg 1977).

The production of extracellular emulsifying agents has been reported for a wide range of microorganisms (Zajic and Panchal 1976). In some studies emulsifier levels were highest when grown upon soluble substrates rather than insoluble substrates (Itoh and Suzuki 1972; Floodgate 1978; Rosenberg *et al* 1979a), suggesting non-specific or constitutive production. However, other studies have shown highest emulsifier levels in response to the presence of an insoluble substrate (Iguchi *et al* 1969; Zajic *et al* 1977a), suggesting inductive production.

Several workers have determined the chemical composition of microbially-produced emulsifiers (Iguchi *et al* 1969; Suzuki *et al* 1969; Hisatsuka *et al* 1971; Zajic *et al* 1977b; Zuckerburg *et al* 1979). These studies have demonstrated that microbial emulsifiers possess hydrophilic and hydrophobic constituents which are both necessary for effective emulsification (Zajic and Panchal 1976). Synthetic emulsifiers are used commercially in dispersion of oil slicks, in increasing efficiency of oil recovery, and in a variety of foodstuffs, (Zajic and Panchal 1976). It has been suggested that microbial emulsifiers may be more acceptable environmentally, since they are readily

biodegradable (Zajic *et al* 1977a).

The production of emulsifying agents by microorganisms has usually been assessed qualitatively by visual examination of the fate of added insoluble compounds (*e.g.*, Iguchi *et al* 1969; Floodgate 1978). However, Rosenberg *et al* (1979a) have described a simple quantitative assay of extracellular emulsifying activity based upon the spectrophotometric determination of the increase in turbidity brought about by emulsification.

The work described in subsequent sections details the effects of emulsification of insoluble substrates on bacterial growth, levels of emulsifier production, and preliminary attempts to characterise and purify the emulsifier produced by the soil isolate *Pseudomonas* 017.

9.6.2 Standard emulsifier assay : The standard quantitative assay for emulsifier level was based upon that described by Rosenberg *et al* (1979a) and was as follows:

> To 7.5 mL of cell-free supernatant (obtained by centrifugation at 6000 G for 20 min) in a 50 mL Erlenmeyer flask was added 0.1 mL of hexadecane. The flask was subsequently shaken at 200 rev min^{-1} for 1h at 30°C in an orbital shaker at which time the turbidity of the flask contents was measured as absorbance at 450 nm using a Bausch and Lomb Spectronic 20 Spectrophotometer. Where necessary, dilutions were made using cell-free supernatants. The reference tube used to zero the spectrophotometer contained cell-free supernatant alone. Controls to test for the degree of self-emulsification of hexadecane were performed using the mineral salts of the media employed for culture growth (9.5) in place of the cell-free supernatant. In this case, the reference tube used to zero the spectrophotometer contained mineral salts. Emulsifying activity was determined as the difference between the turbidity of incubated samples and the control and was conveniently expressed as milliunits mL^{-1} of sample where 1 unit represents an increase of 1.0 absorbance units at 450 nm during the 1h incubation period. The increase in absorbance was linearly related to the level of emulsifier added (Fig. 9.7).

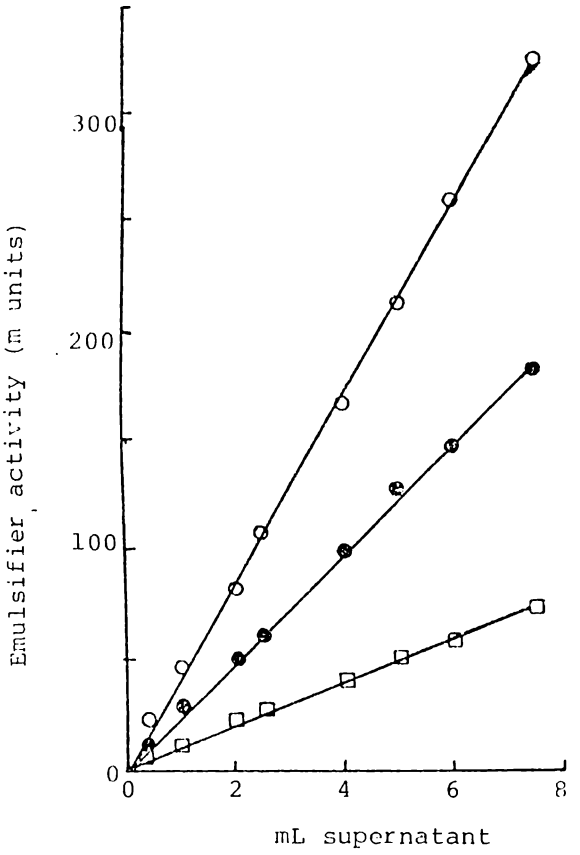


Fig. 9.7 Relationship between emulsifier activity and quantity of supernatant used in the assay. Supernatants were obtained from olive oil grown *Pseudomonas* 017 (o-o), *Bacillus licheniformis* T-C (●-●), and *Acinetobacter* A30 (□-□).

9.6.3 Emulsifier production : Several soil isolates capable of growth on olive oil as a sole carbon source (8.3), precultured (9.3), then inoculated into an olive oil-mineral salts medium (9.5) and incubated at 25°C with orbital shaking at 120 rev min^{-1}. After 48h cultures were centrifuged (6000 G for 20 min) and emulsifier activity determined in the cell-free supernatant. Whilst growth occurred with all cultures (as witnessed by a cell pellet forming upon centrifugation) the visual appearance of flask contents differed markedly. Cultures of *Acinetobacter* A30, *Pseudomonas* PTD, and *Pseudomonas* X-P were translucent whilst cultures of *Pseudomonas* 017 and *Bacillus licheniformis* T-C were opaque blue-white indicating a high degree of emulsification. Plate 9.1 demonstrates the visual appearance of *Pseudomonas* 017 after growth upon an insoluble substrate. The emulsifying activity of cell-free supernatants demonstrated a comparatively high level of extracellular emulsifier production by *Pseudomonas* 017 and *Bacillus licheniformis* T-C (Table 9.8).

Extracellular emulsifying activity in cultures of *Pseudomonas* 017 was closely associated with growth (Fig. 9.8) a finding similar to that reported for an *Arthrobacter* sp (Rosenberg *et al* 1979a). Emulsifier production by *Pseudomonas* 017 was apparently an inductive process, with levels being highest when growing upon insoluble substrates (Table 9.9). In dual-substrate systems emulsifier production was delayed until growth upon the insoluble substrate occurred (Fig. 9.8).

9.6.4 The influence of emulsification on growth : When precultures (9.3) of soil isolates were inoculated into olive oil-mineral salts media that had been emulsified by sonication (Dawe Soniprobe, maximum power for 5 min), reductions in lag periods occurred over those observed in non-sonicated media (Fig. 9.9). The extent of the lag period reduction upon sonication was greatest with those organisms that did not produce their own emulsifier (*Pseudomonas* X-P and *Pseudomonas* PTD).

Plate 9.1 Flasks of tallow-mineral salts media uninoculated (*left*) and after 12 hours growth of *Pseudomonas* 017 (*right*).

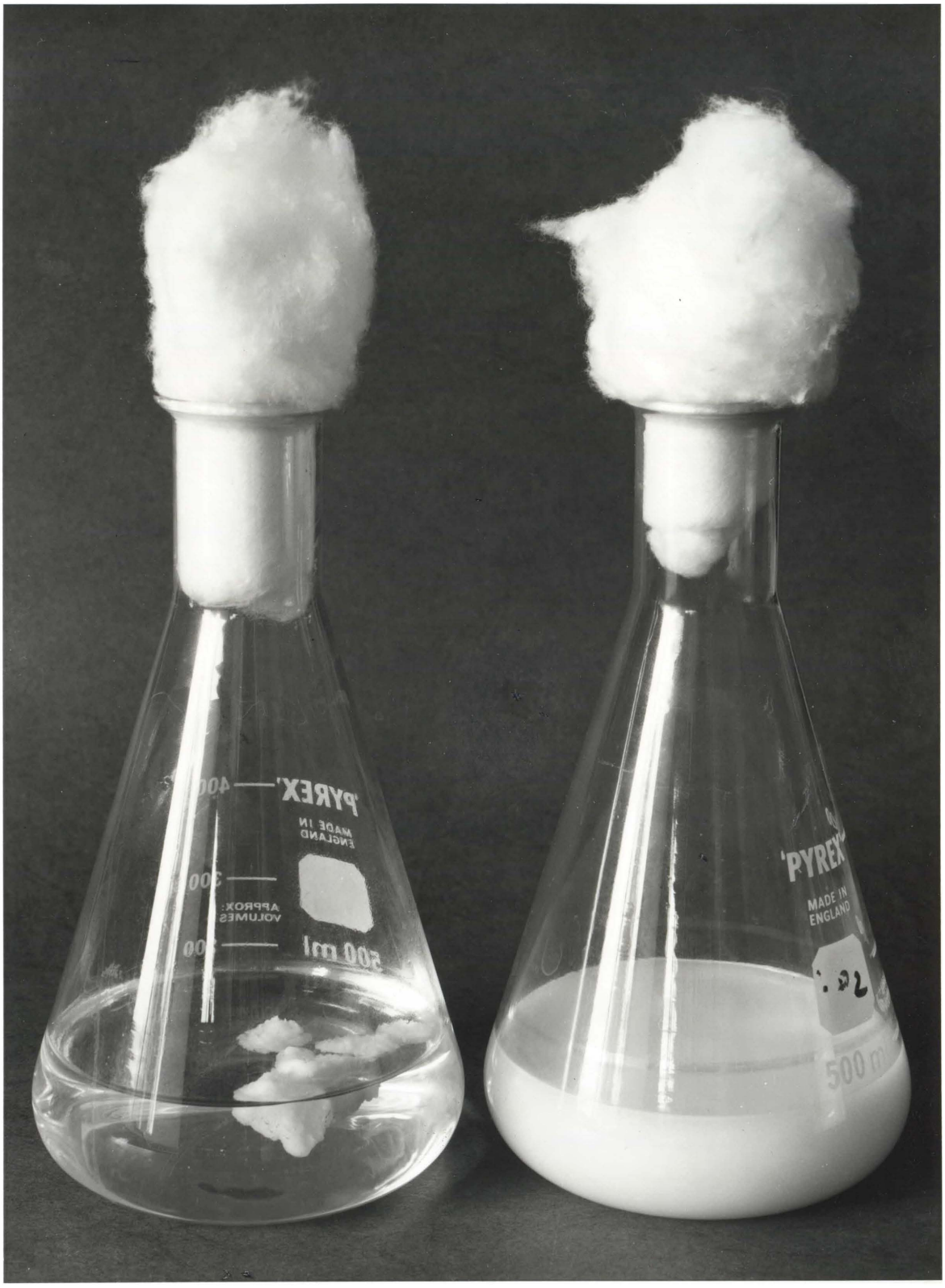

'PYREX'
MADE IN ENGLAND
500 ml
'PYREX'
MADE IN ENGLAND

TABLE 9.8 Extracellular emulsifying activity of some soil isolates after 48h growth in olive oil-mineral salts media.

Isolate	Emulsifier act. (munits mL^{-1})
Acinetobacter A30	71
Pseudomonas PTD	0
Pseudomonas X-P	13
Pseudomonas 017	323
Bacillus licheniformis T-C	183

TABLE 9.9 Extracellular emulsifying actvity of *Pseudomonas* 017 after 48h growth on various substrates.

GROWTH SUBSTRATE	EMULSIFIER ACT. (munits mL^{-1})
Glucose	13
Glycerol	13
Hexadecane	160
Olive oil	323
Tallow	315
Tributyrylglycerol	27

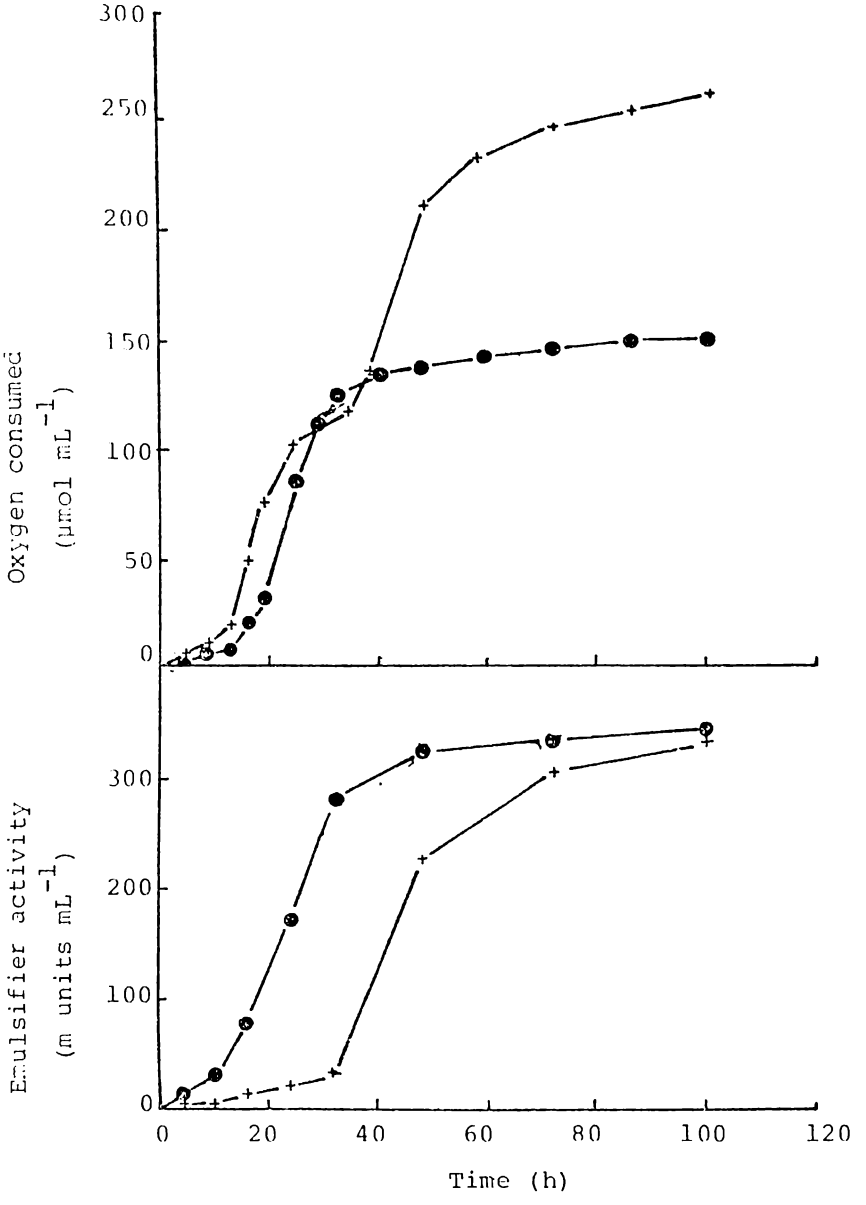


Fig. 9.8. Oxygen consumptin and emulsifier levels for *Pseudomonas* 017 growing on olive oil (●-●) or glucose + olive oil (+-+).

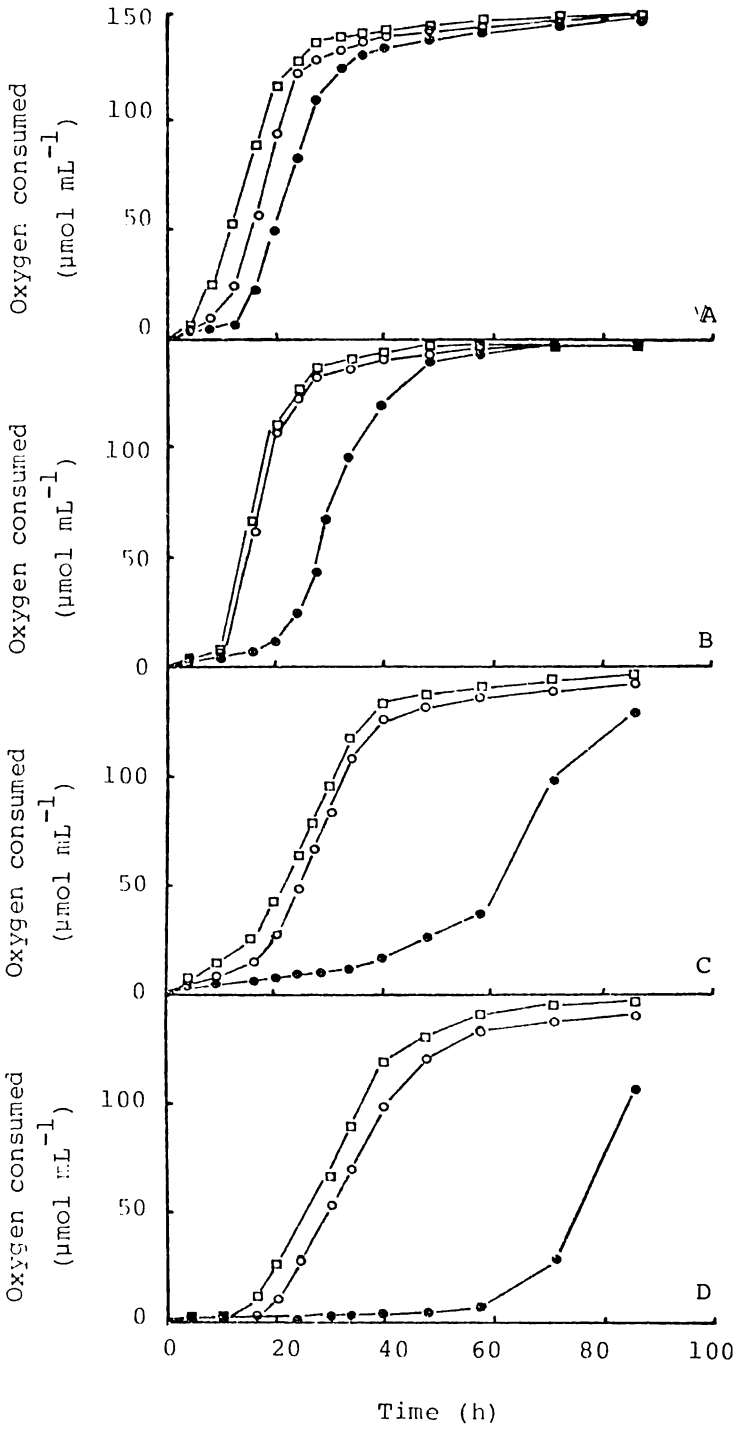


Fig. 9.9. Patterns of oxygen consumption for four bacterial cultures growing on olive oil media that were non-sonicated (●-●), sonicated (□-□), or prepared in spent *Pseudomonas* 017 media (o-o). A = *Pseudomonas* 017; B = *Acinetobacter* A30; C = *Pseudomonas* X-P, D = *Pseudomonas* PTD.

The lag period could also be reduced by inoculating precultures into media containing olive oil and autoclaved, spent media from a *Pseudomonas* 017 culture previously grown on olive oil (Fig. 9.9). Autoclaving the spent media served to destroy lipase activity whilst maintaining some emulsifying activity (9.6.5). The spent media rapidly (within 2h) emulsified the olive oil and thereby led to rapid growth.

The results obtained demonstrate the importance of the interfacial area of the insoluble substrate in regulating the rate of its microbial degradation. Whilst the enzyme responsible for the initial degradation of olive oil may be extracellular (*i.e.*, lipase), the induction of lipase production is likely to be dependent on direct substrate-cell contact. The increased interfacial area of systems that have been emulsified (either by sonication or by the emulsifying factor of *Pseudomonas* 017) would allow for greater substrate-cell contact and hence more rapid induction of hydrolytic enzymes. Microscopic observations of cultures during the early phases of growth revealed strong adsorption between cells and substrate.

9.6.5 Some properties of the emulsifier produced by *Pseudomonas* 017:

The extracellular emulsifier of *Pseudomonas* 017 was capable of emulsifying a range of insoluble substances, although the degree of emulsification varied (Table 9.10). Emulsifying activity towards short-chain insoluble substances (hexane, octane, octanoate, decanoate, tributyrylglycerol) was low or non-existent with maximal activity occurring towards long-chain insoluble liquids (hexadecane, olive oil). The emulsifier was capable of some activity towards substances that were solid at the assay temperature of 30°C (octadecane, tallow, anhydrous milk fat) unlike the emulsifier from an *Arthrobacter* sp. (Rosenberg *et al* 1979b). The addition of either octanoate or decanoate to an assay system containing hexadecane or olive oil

TABLE 9.10 Activity of *Pseudomonas* 017 emulsifier towards a range of insoluble substances.

SUBSTANCE	Emulsification (munits mL^{-1})
Octanoate	0
Decanoate	0
Laurate	40
Palmitate	71
Oleate	101
Tributyrylglycerol	5
Olive oil	308
Tallow	115
Anhydrous milk fat	157
Hexane	8
Octane	9
Decane	36
Hexadecane	323
Toluene	5
1-methyl naphthalene	9
Kerosene	168
Paraffin oil	121
Vacuum oil	146
Hexadecane + octanoate	0
Hexadecane + decanoate	0
Olive oil + octanoate	0
Olive oil + decanoate	0

completely prevented emulsification. Presumably, these fatty acids partitioned at the interface with the hydrophilic carboxyl groups directed towards the aqueous phase. The emulsifier would, therefore, have been presented with a fatty acid surface which it was unable to emulsify.

The activity of the emulsifier was markedly altered by changes in the physical state of the insoluble substances brought about by variation in the assay temperature (Fig. 9.10). Large increases in emulsifying activity occurred when the assay was performed just above the melting points of both hexadecane (18.2°C) and tallow (45°C, Merck Index). The emulsifier was stable to 1h exposure to temperatures up to 90°C and retained 73% of its activity upon autoclaving at 121°C for 15 min.

The activity of the emulsifier from *Pseudomonas* 017 was markedly decreased when the assay was performed at pH's below 4.0 (Fig. 9.11). Activity was similar over the pH range 4.0 - 9.5. The emulsifier was stable to 1h exposure to pH's in the range pH 1.0 - 11.0. Hence, the decreased emulsification observed below pH 4.0 was an effect on activity *per se* rather than on stability. Rosenberg *et al* (1979a) attributed a similar decrease in the activity of an emulsifier from an *Arthrobacter* sp below pH 4.0 to the protonation of a carboxyl group.

9.6.6 Attempted purification of the emulsifier of *Pseudomonas* 017

The initial step in the purification of extracellular microbial emulsifiers has involved extraction of the cell-free supernatant culture fluid with an organic solvent into which the emulsifier partitioned (Hisatsuka *et al* 1971; Rosenberg *et al* 1979a). However, when the cell-free supernatant fluid of *Pseudomonas* 017 was shaken with a variety of organic liquids (toluene, chloroform, heptane) the emulsifying activity could not be recovered from either the organic or

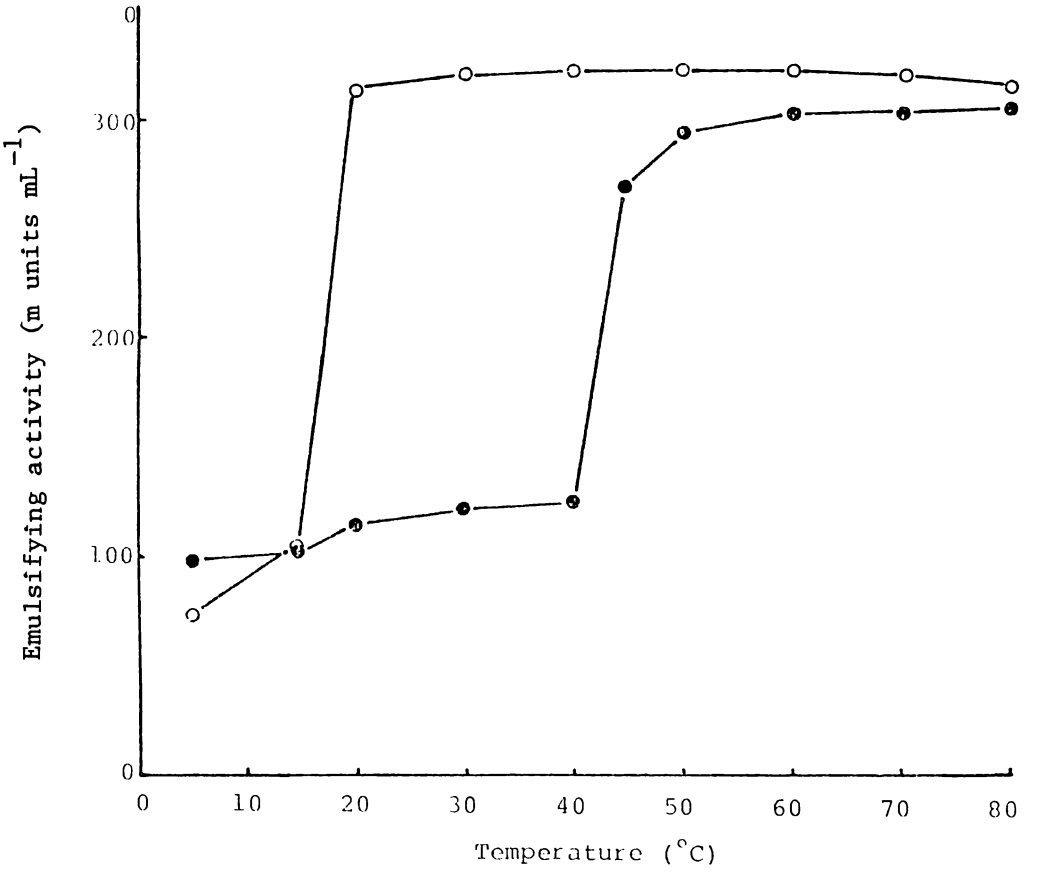


Fig. 9.10. Effect of assay temperature on the emulsifying activity of *Pseudomonas* 017 supernatant towards hexadecane (o-o) and tallow (●-●).

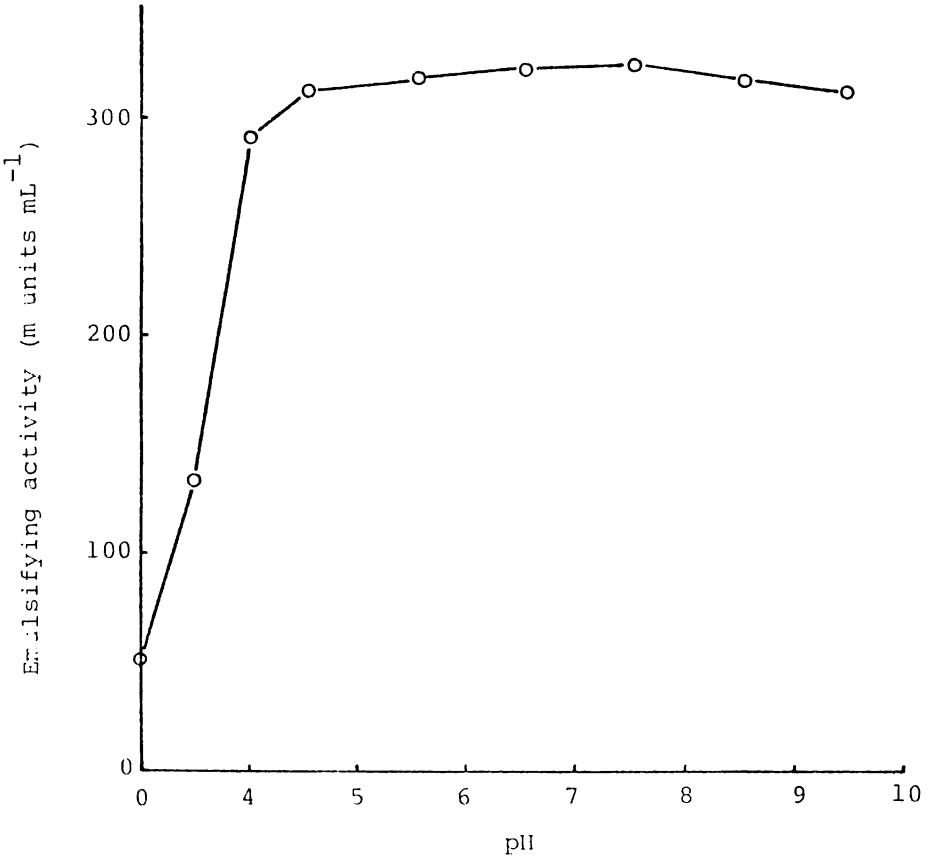


Fig. 9.11. Effect of assay pH on the emulsifying activity of *Pseudomonas* 017 supernatant towards hexadecane.

aqueous phases. Obviously, further work is required if the emulsifier is to be purified sufficiently to allow chemical characterisation. The purification may best include an initial precipitation step (ammonium sulphate? ethanol? acetone?) since organic extraction was a failure. The following additional observations should be borne in mind when purification is attempted:

i) the emulsifier was non-dialysable;

ii) the emulsifier was unstable to rotary evaporation but stable to lyophilisation;

iii) the emulsifying activity was lost upon ultrafiltration.

9.7 Growth and metabolism of *Pseudomonas* 017 : A discussion

The diverse metabolic capabilities of pseudomonads is a well-documented phenomenon, and many of the catabolic pathways are totally or partially delineated (Stanier *et al* 1966; Ornston 1971). The initial stages in the metabolism of long-chain hydrocarbons by pseudomonads occurs *via* an inducible alkane hydroxylation, alcohol dehydrogenation, and aldehyde dehydrogenation sequence to yield the corresponding fatty acid (Thijsse and van der Linden 1963; Benson and Shapiro 1975; Neider and Chapiro 1975; Grund *et al* 1975). Evidence has been presented which suggests that this initial sequence of events occurs external to the cell membrane (van Eyk and Bartels 1970; Benson and Shapiro 1976). Subsequent long-chain fatty acid uptake occurs *via* an inducible group translocation mechanism (Toscano and Hartline 1973). Breakdown of the fatty acids occurs *via* β-oxidation (Heringa *et al* 1961; Ornston 1971).

The observation that *Pseudomonas* 017 was induced for growth towards a fatty acid, hydrocarbon, and triacylglycerol by prior growth on any one of these three substrates (Fig. 9.5) indicates a common mechanism for metabolism. Both the hydrocarbon and triacylglycerol

are presumably externally converted to fatty acids. Hexadecane would be converted to its corresponding fatty acid, palmitate, by the mechanism described in the preceding paragraph. The accumulation of fatty acid in cultures of *Pseudomonas* 017 grown on hexadecane indicates the occurrence of this mechanism. Triacylglycerols yield fatty acids *via* the extracellular lipase. Thus, on all three substrates, growth of the organism is dependent upon the uptake of long-chain fatty acids. Since such uptake occurs *via* an inducible translocation mechanism (Toscano and Hartline 1973), prior growth on any one of the substrates provides a ready-primed inoculum for subsequent growth on the other substrates. However, whilst such an explanation would explain the situation of initial growth on hexadecane or triacylglycerol followed by growth on oleate, it does not adequately explain the ability of initial growth on oleate facilitating subsequent growth on hexadecane and triacylglycerol. The requirement for an induced fatty acid uptake mechanism would obviously be met by initial growth on oleate. In addition, however, facilitated growth on hexadecane and triacylglycerol requires the prior induction of the enzymes required to convert these substrates to fatty acids. Induction of lipase production by oleate was demonstrated for *Pseudomonas* 017 (Table 9.5 and Fig. 9.5). Apparently, induction of the alkane-oxidising enzyme system required to convert hexadecane to palmitic acid must also have occurred with oleate as the growth substrate. These results suggest that the genetic information coding for long-chain fatty acid uptake is co-transcribed with that coding for both alkane-oxidation and lipase production. Previous studies with other *Pseudomonas putida* strains have demonstrated that the genetic material coding for enzymes of alkane oxidation reside in extrachromosomal DNA or plasmids (Grund *et al* 1975; Benson and Shapiro 1976; Palchaudhuri and Chakrabarty 1976). It seems likely, therefore, that *Pseudomonas* 017 contains its

genetic material responsible for alkane oxidation and lipase production in such extrachromosomal DNA.

The inability of *Pseudomonas* 017 to grow upon medium-chain hydrocarbons and fatty acids (C_6 - C_{10}) whilst growth was observed with short-chain (C_2, C_4) and long-chain ($>C_{12}$) substrates is puzzling. Growth upon fatty acids by pseudomonads occurs *via* β- or ω- oxidation with acetate (C_2) units being transferred to coenzyme A (Ornston 1971). Since *Pseudomonas* 017 was capable of growth upon long-chain ($>C_{12}$) fatty acids the organism presumably possesses the necessary enzyme complement for these reactions. The inability to grow on C_6 - C_{10}) substrates, therefore, indicates an inability to transport them across the cell membrane, rather than an inability to metabolise them within the cell. Previous studies have suggested separate uptake mechanisms for short-chain fatty acids (C_2 - C_6) and medium- to long-chain fatty acids ($>C_8$) for pseudomonads (Toscano and Hartline 1973). However, with *Pseudomonas* 017 it appears that C_6 - C_{10} fatty acids were not able to be efficiently taken up by either the short-chain (C_2 - C_4) or long-chain ($>C_{12}$) transport mechanisms present.

The work presented in this chapter has provided an initial insight into the metabolism of insoluble substances by *Pseudomonas* 017 Extension of this work would provide a fruitful area for further research. The apparent ability of insoluble substrates to co-induce fatty acid uptake mechanisms, alkane oxidation, and lipase production deserves further attention, particularly at the genetic level. Such studies may reveal information of industrial value. Already, *Pseudomonas putida* strains have been genetically engineered *via* plasmid transfer to yield a "superbug" capable of degrading a wide range of hydrocarbon fractions found in oil (Hopwood 1981).

9.8 Summary

The growth, lipase production, and emulsifier production of *Pseudomonas* 017 has been investigated. Lipase production was low when *Pseudomonas* 017 was grown in complex media. Growth upon a variety of insoluble substances including triacylglycerols, long-chain fatty acids and hydrocarbons induced the production of high levels of lipase. Prior growth on any one of these substances facilitated growth on the others, suggesting a co-induction of degradative enzymes. Rapid growth of *Pseudomonas* 017 on insoluble substances was associated with the production of an extracellular emulsifying agent.

CHAPTER 10 PARTIAL PURIFICATION AND CHARACTERISATION OF LIPASES FROM *STAPHYLOCOCCUS AUREUS* NCIB 6571 AND *PSEUDOMONAS* 017 (A SOIL ISOLATE).

10.1 Introduction

The soil isolate, *Pseudomonas* 017, was capable of producing a high level of extracellular inductive lipase in simple media containing byproduct lipid (tallow) after a relatively short incubation period (Chapter 9). The organism, therefore, appeared to have the potential for commercial production of lipase. The work presented in this chapter represents a preliminary investigation into methods for subsequent purification of the enzyme and a study on the properties of the partially purified lipase obtained. For comparative purposes, the lipase of *Staphylococcus aureus* NCIB 6571 was also purified and characterised.

There have been numerous reports on the purification of lipases from pseudomonads (*e.g.*, Mencher and Alford 1967; Lu and Liska 1969; Finkelstein *et al* 1970; Sugiura *et al* 1977; Severina and Bashkatova 1979), and staphylococci (*e.g.*, O'Leary and Weld 1964; Renshaw and San Clemente 1967; Vadehra and Harmon 1967; Troller and Bozeman 1970; Jürgens *et al* 1981). In order to optimise the yield and obtain maximal purification of the lipases from the two organisms studied, initial work involved an investigation into a variety of methods for preliminary concentration of the enzyme from cell-free supernatants.

10.2 Purification

10.2.1 Preparation of the cell-free supernatant fluid: Organisms were precultured for 18h in 100 mL of trypticase soy broth (BBL,

Maryland, USA) in 250 mL Erlenmeyer flasks with orbital shaking at 120 rev min^{-1} and temperatures of 37°C for *Staphylococcus aureus* NCIB 6571 and 25°C for *Pseudomonas* 017. Precultures were centrifuged (9000G for 20 min) and washed 3 x with sterile saline (8.5 g L^{-1} NaCl). After the final suspension in saline, aliquots (2 x 20 mL) were inoculated into either 2 x 750 mL trypticase soy broth for *Staphylococcus aureus* or 2 x 750 mL olive oil-mineral salts media (8.2.3., modified by lowering olive oil concentration to 2 g L^{-1}) for *Pseudomonas* 017, in 2 L Erlenmeyer flasks. Flasks were incubated with orbital shaking at 120 rev min^{-1} for 2 days at 37°C for *Staphylococcus aureus* and for 4 days at 25°C for *Pseudomonas* 017. Cultures were subsequently centrifuged (9000 G for 20 min) and the supernatant vacuum-filtered (0.22 µm Sartorius).

The cultivation conditions described were designed to produce the maximal levels of lipase activity (Chapter 9). The growth medium for *Pseudomonas* 017 contained 2 g olive oil L^{-1} as this level gave near maximal lipase production (9.5) and a clear supernatant after 4 days incubation. Lower substrate concentrations resulted in lowered lipase levels (9.5) whilst higher levels failed to provide a sufficiently clear supernatant to facilitate subsequent purification.

The level of lipase in the cell-free supernatant of *Pseudomonas* 017 was 15x that in the *Staphylococcus aureus* supernatant based upon olive oil hydrolysis or 36x based upon 4 MUN hydrolysis (Table 10.1). The specific activity of *Pseudomonas* 017 supernatant was 600x that of the *Staphylococcus aureus* supernatant based upon olive oil hydrolysis and 1500x using 4 MUN hydrolysis. The high specific activity of *Pseudomonas* 017 supernatant reflects the low level of extracellular proteins with no proteinaceous material being present in the medium (*c.f.* peptone constituents of staphylococcal medium) and apparently a low level of extracellular proteins originating from the cell.

TABLE 10.1 Lipase and protein levels in the cell-free supernatant liquid of a 2-day-old culture of *Staphylococcus aureus* NCIB 6571 growing on trypticase soy broth and a 4-day-old *Pseudomonas* 017 growing on olive oil-mineral salts medium.

Culture	Substrate	Activity (μmol min^{-1} mL^{-1})	Protein (mg mL^{-1})	Specific act. (μmol min^{-1} mg^{-1})
Staph. aureus	Olive oil	0.066	8.25	0.008
	4 MUN	0.033	8.25	0.004
Pseudomonas 017	Olive oil	0.98	0.2	4.9
	4 MUN	1.20	0.2	6.0

10.2.2 Evaluation of initial purification procedures : The cell-free supernatants obtained in 10.2.1 were subjected to four different initial purification procedures,

(i) Ammonium sulphate precipitation was performed by step-wise addition of solid $(NH_4)_2SO_4$ to stirred cell-free supernatant (1500 mL) at 4°C until a 60% saturation was reached, followed by 4h standing and subsequent centrifugation (13000 G for 30 min at 4°C). The precipitate was resuspended in distilled water and dialysed against 10 L of 0.01 M Tris-HCl pH 7.5 for 20h at 4°C.

(ii) Ultrafiltration of cell-free supernatant (1500 mL) was performed at 4°C utilising a ChemLab apparatus equipped with an Amicon PM-10 filter (nominal molecular weight cut-off of 10 000) and with positive N_2 pressure of 345 kPa.

(iii) Alcohol precipitation was performed by drop-wise addition of pre-cooled (-20°C) 95% ethanol to stirred and pre-cooled (-4°C) cell-free supernatant (1500 mL) until a 50% (v/v) mixture was obtained. Stirring was continued for 1h followed by 16h standing at 4°C and subsequent centrifugation at -10°C for 30 min at 13 000 G. The precipitate was resuspended in distilled water and dialysed against 10 L of 0.01 M Tris-HCl pH 7.5 for 20 h at 4°C.

(iv) Acetone precipitation was performed by drop-wise addition of pre-cooled (-20°C) acetone to stirred and pre-cooled (-4°C) cell-free supernatant (1500 mL) until a 30% (v/v) mixture was obtained. Stirring was continued for 1h followed by 16h standing at 4°C and subsequent centrifugation at -10 C°for 30 min at 13 000 G. The precipitate was resuspended in distilled water and dialysed against 10 L of 0.01 M Tris-HCl pH 7.5 for 20h at 4°C.

The results presented in Table 10.2 demonstrate that the purification and yields obtained with the four initial procedures were markedly different between the two culture supernatants. With the staphylococcal lipase, high purification factors were achieved by alcohol and acetone precipitations whilst the greatest purification for the pseudomonad lipase was obtained with $(NH_4)_2SO_4$ precipitation. The yields of staphylococcal lipase were higher than those achieved with the pseudomonad lipase, indicating either more effective concentration and/or a greater resistance to denaturation. As little of the initial total activity of the cell-free supernatant of *Pseudomonas* 017 was in the supernatant of precipitations (Table 10.3) it is apparent that considerable denaturation occurred, particularly with alcohol and acetone. With ultrafiltration, less than 2% of the total activity of the cell-free supernatant of *Pseudomonas* 017 was present in the filtrate and 22% in the retentate. However, soaking of the ultrafilter in 20 mL of 2 M urea for 16h, followed by dialysis of this solution against 10 L of 0.01 M Tris-HCl pH 7.5 for 20h at 4°C, recovered 45% of the initial total activity. It proved impossible to concentrate cell-free supernatants of *Pseudomonas* 017 by more than 10x using ultrafiltration due to a rapid decrease in flow rates. The upper side of the ultrafilters became 'greasy' to touch indicating the formation of a lipid coating. It would seem that the lipase may adsorb to this lipid material and be dislodged only upon disruption of hydrophobic bonds by addition of urea.

10.2.3 Gel chromatography : The alcohol-precipitated preparation of *Staphylococcus aureus* lipase and the $(NH_4)_2SO_4$-precipitated preparation of *Pseudomonas* 017 lipase obtained in 10.2.2 were each applied to a Sephadex G-100 column (Pharmacia Co., Uppsala, Sweden) 70cm in length and 2.5cm in internal diameter, and eluted with 0.1 M Tris-HCl pH 7.5 at a flow rate of 0.5 mL min^{-1}. The column effluent was passed through a Shimadzu UV-200 spectrophotometer set at 280 nm and 5 mL portions were collected by an LKB fraction collector. Fractions were

TABLE 10.2 Comparison of various methods for the initial purification of lipases from cell-free supernatants of *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017.

Treatment	*Staph. aureus*		*Pseudomonas* 017	
	Purification factor*	Yield %	Purification factor*	Yield %
$(NH_4)_2SO_4$ precipitation	20	84	4.8	72
Ultrafiltration	7	42	0.8	22
Alcohol precipitation	60	90	3.5	46
Acetone precipitation	51	84	3.3	30

* Fold- increase in specific activity over that in the cell-free supernatant

TABLE 10.3 The proportion of the initial total activity of *Pseudomonas* 017 lipase recovered in various dialysed fractions.

Treatment	% total activity recovered in:	
	Precipitate	Supernatant
$(NH_4)_2SO_4$	72	10
Ultrafiltration	Retentate = 22	filtrate = 2
Alcohol	46	19
Acetone	30	12

assayed for lipase activity by the standard fluorometric assay of 2.6.

The column was initially calibrated using Blue Dextran (MW 2×10^6) for void volume determination and 5 proteins (bovine albumin, MW 67 000; ovalbumin MW 45 000; α-chymotrypsinogen A, MW 25 000; ribonuclease A MW 13 700; cytochrome c, MW 12 400; (all Sigma Chem. Co., St Louis, USA) for molecullar weight calibration (Fig. 10.1).

The staphylococcal lipase was eluted as a single symmetrical peak in the void volume (Fig. 10.2) indicating an enzyme with a molecular weight >100 000. Previous studies on the lipases of various *Staphylococcus aureus* strains have also reported high molecular weights. (Renshaw and San Clemente 1967; Vadehra and Harmon 1967; Tirunarayanan and Lundbeck 1968; Troller and Bozeman 1970; although Jürgens *et al* (1981) reported purification of staphylococcal lipases with molecular weights of 43 000 and 44 000. The fractions from 55 - 80 mL were dialysed for 10h against 10 L of distilled water at 4°C and subsequently lyophilised. The preparation obtained demonstrated a 5-fold increase in specific activity over the alcohol-precipitated material and a purification of 300x over the original cell-free supernatant (Table 10.4). This lyophilised preparation was stored at -20°C and used as the enzyme source in characterisation studies (10.3), in an examination into the precision and detection limit of various assays for lipase activity (2.3 - 2.6), and in studies on lipases and surface-related phenomena (Chapter 3).

The lipase from *Pseudomonas* 017 displayed a broad elution profile when chromatographed on Sephadex G-100 (Fig. 10.3). The major peak of activity was eluted in the void volume (MW >100 000) and was followed by some activity in all fractions up to an elution volume of 130 mL. A second minor peak of activity occurred at an elution volume of 120 mL (MW ∿ 30 000). Previous studies have demonstrated both high (>200 000) molecular weight (Lawrence *et al* 1967a; Finkelstein *et al* 1970; Severina and Bashkatova 1979) and low (32 000) molecular

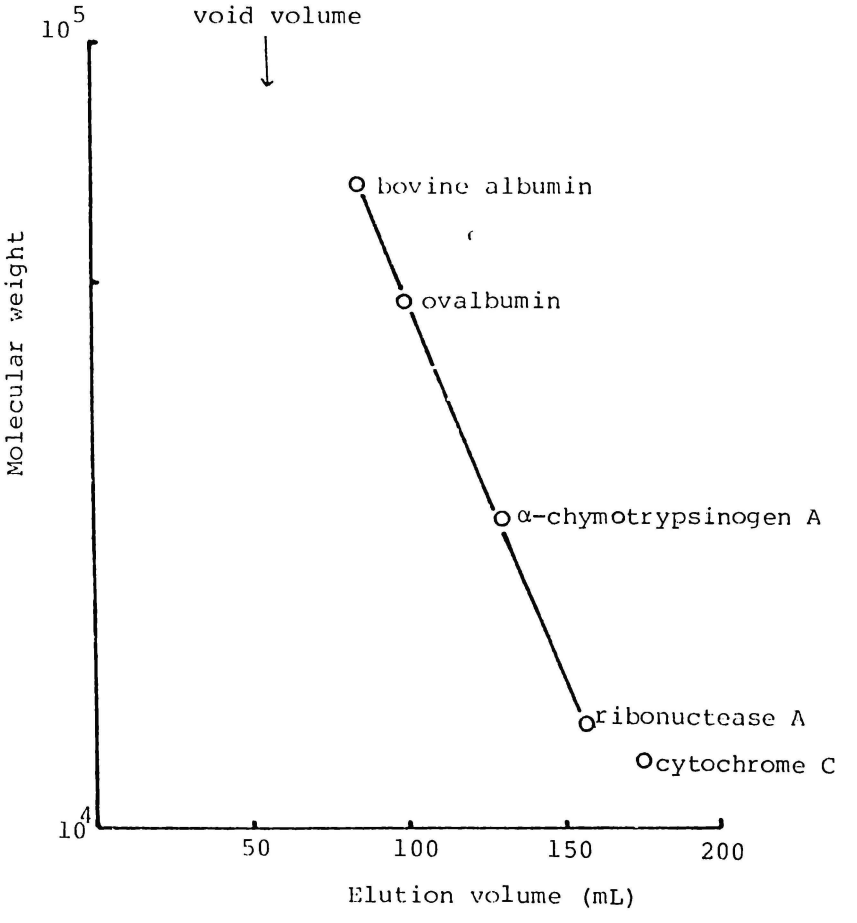


Fig. 10.1. Molecular weight calibration of Sephadex G-100 column (70cm x 2.5cm).

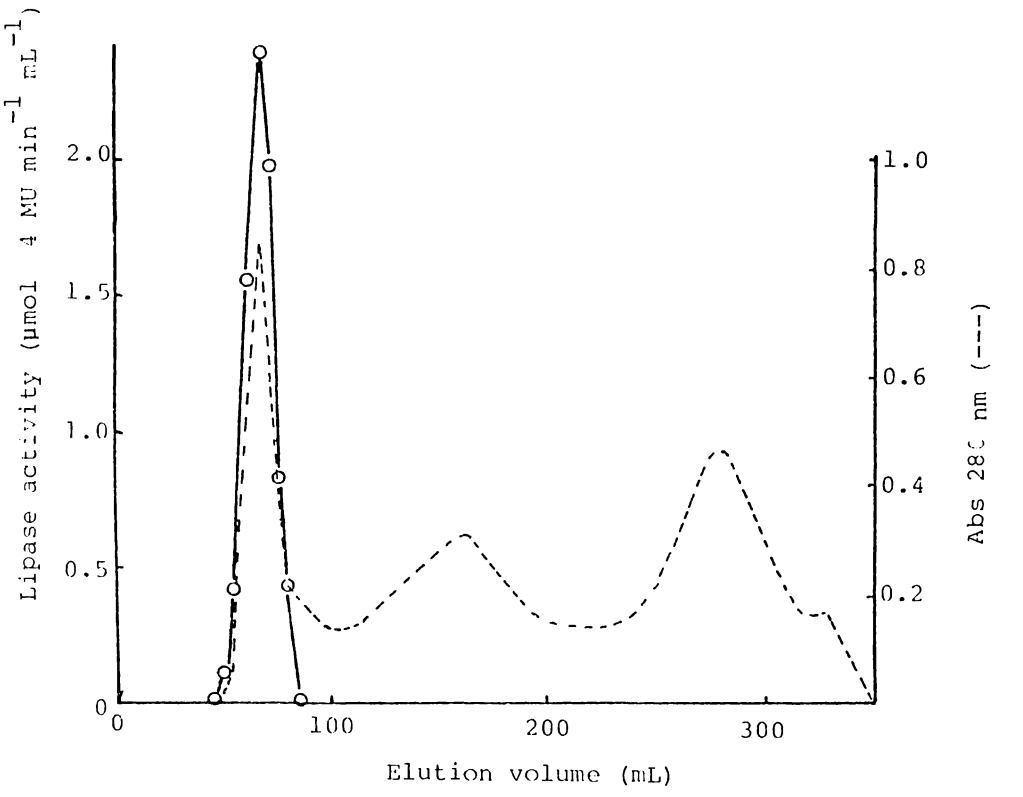


Fig. 10.2. Elution pattern of alcohol-precipitated *Staphylococcus aureus* lipase from Sephadex G-100 using 0.1 M Tris-Hcl pH 7.5 as eluant. 12.5 mg of protein was loaded onto the column.

TABLE 10.4 Summary of the purification of lipase from *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017.

S T E P	Specific activity (μmol 4 MU min^{-1} mg^{-1})	Purification factor	Yield (%)
	Staphylococcus aureus		
Cell-free supernatant	0.004	1	100
Alcohol precipitation	0.24	60	91
Gel chromatography	1.20	300	80
	Pseudomonas 017		
Cell-free supernatant	6.0	1	100
$(NH_4)_2SO_4$ precipitation	28.8	4.8	72
Gel chromatography	86.7	14.4	54

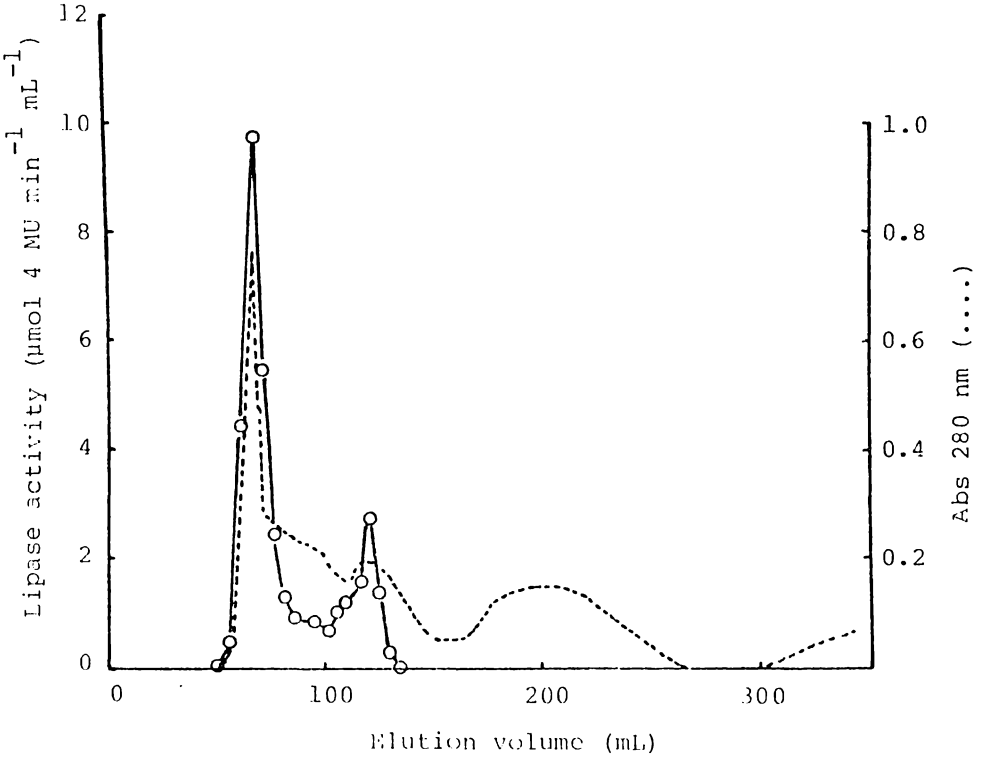


Fig. 10.3. Elution pattern of $(NH_4)_2SO_4$ precipitated *Pseudomonas* 017 lipase from Sephadex G-100 using 0.1 M Tris-HCl pH 7.5 as eluant. 7.5 mg of protein was loaded onto the column.

weight (Sugiura *et al* 1977) lipases from pseudomonads. The apparent presence of two forms of *Pseudomonas* 017 lipase has previously been reported for other pseudomonad lipases (Mencher and Alford 1967; Lu and Liska 1969) and for pancreatic lipase (Verger *et al* 1969). As lipases have been shown to be associated with non-proteinaceous material (Brockerhoff and Jensen 1974), it seems likely that the high molecular weight fraction of *Pseudomonas* 017 lipase represents such an associated form whilst the low molecular weight (30 000) fraction represents free lipase. The broad elution profile may indicate that such an association is relatively non-specific, with the free enzyme binding to a wide size range of material. As *Pseudomonas* 017 was grown upon a triacylglycerol substrate and the enzyme possesses a strong affinity for hydrophobic surfaces (3.2), this associated material may be lipid.

The elution volumes from 55 - 80 mL were dialysed for 10h against 10 L of distilled water at 4°C and subsequently lyophilised. This *Pseudomonas* 017 lipase preparation demonstrated a 3-fold increase in specific activity over the preparation applied to the column and 14.4x over the original cell-free supernatant (Table 10.4). This lyophilised preparation was stored at -20°C and used as the enzyme source in characterisation studies (10.3), in an examination into the precision and detection limit of various assays for lipase activity (2.3 - 2.6) and in studies on lipases and surface-related phenomena (Chapter 3).

10.2.4 Gel electrophoresis : The preparations obtained in 10.2.3 were subjected to polyacrylamide gel electrophoresis according to the procedures of Weber and Osborn (1969). Electrophoresis was performed in glass tubes (70mm x 6mm) with a current of 8 mA per tube, a gel concentration of 10% (w/v), and 0.1 M Tris-HCl pH 7.5 as buffer. All samples and standards were run until the bromothymol blue tracking dye was within 5mm of the bottom of the gel (approx 5h). Standards used

were bovine albumin (MW 67 000), ovalbumin (MW 45 000), pepsin (34 200) α-chymotrypsinogen (MW 25 000), and lysozyme (MW 14 300). Gels were either stained for protein (Weber and Osborn 1969) or for lipase activity (gels soaked in 4 MUN emulsion, 312 μM, for 5 min followed by observation for blue fluorescent 4 MU under UV light excitation at 354 nm).

Both the staphylococcal and pseudomonad lipase preparations obtained in 10.2.3 (both void volume fractions on Sephadex G-100) lacked mobility on polyacrylamide gels, with protein and activity remaining at or near the gel surface. This confirms their apparent high molecular weight as observed by gel chromatography (10.2.3). Attempts to improve mobility by 16h pre-incubation of preparations with 1% (w/v) sodium dodecyl sulphate and/or incorporation of sodium dodecyl sulphate (1% w/v) into the gel were unsuccessful, with protein remaining at the top of the gel and enzyme activity being lost, presumably due to denaturation (3.4).

10.3 Properties

10.3.1 Substrate specificity : The substrate specificities of the two bacterial lipase preparations were examined using the methods described in 2.3 (for acylglycerols, Tweens, ethyl butyrate and natural triacylglycerols), 2.5 (for ρ-nitrophenyl esters) and 2.6 (for 4-methyl umbelliferone esters).

Both the staphylococcal and pseudomonad preparations displayed no observable activity towards soluble esters (triacetylglycerol at 10 g L^{-1}, Tween 80, Tween 20, ethyl butyrate and 4 MU acetate) but considerable activity towards insoluble esters (Table 10.5) and can therefore be regarded as true lipases (Desnuelle 1972). Under the conditions employed, ρ-nitrophenyl acetate was partially insoluble (a turbid emulsion formed) and hence was susceptible to hydrolysis by lipase.

TABLE 10.5 Rates of hydrolysis of various substrates by lipase preparations of *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017.

SUBSTRATE	Rate of hydrolysis (μmol min^{-1} mg^{-1} protein)	
	Staphylococcus lipase	*Pseudomonas* 017 lipase
Triacetylglycerol (10 g L^{-1})	0	0
Triacetylglycerol (150 g L^{-1})	0.24	38
Tributyrylglycerol	4.80	360
Tricaprolylglycerol	2.25	90
Tricaprylylglycerol	2.05	200
Tricaprylglycerol	1.85	120
Trilaurylglycerol	0.59	76
Trimyristylglycerol	0.78	24
Tripalmitoylglycerol	0.16	16
Tristearoylglycerol	0.14	12
Trioleoylglycerol	2.41	74
Trilinolylglycerol	2.85	90
Dioleoylglycerol (1, 3)	0.65	98
Dioleoylglycerol (1, 2)	0.65	76
Tween 80	0	0
Tween 20	0	0
Ethyl butyrate	0	0
Olive oil	2.40	66
Coconut oil	n.d.*	116
Milk fat	n.d.*	140
Tallow	n.d.*	104
p-nitrophenyl acetate	0.05	1.3
p-nitrophenyl caprylate	0.12	10.5
p-nitrophenyl laurate	0.18	10.2
p-nitrophenyl palmitate	0.09	6.5
p-nitrophenyl sulphate	0	0
p-nitrophenyl phosphate	0	0
4-methyl umbelliferone acetate	0	0
4-methyl umbelliferone butyrate	0.18	45.2
4-methyl umbelliferone caproate	0.41	100.3
4-methyl umbelliferone caprylate	1.08	92.4
4-methyl umbelliferone nonanoate	1.20	86.7
4-methyl umbelliferone laurate	0.67	69.0
4-methyl umbelliferone palmitate	0.27	18.0
4-methyl umbelliferone stearate	0.15	7.3
4-methyl umbelliferone oleate	0.32	73.0
4-methyl umbelliferone elaidate	0.28	63.2
4-methyl umbelliferone sulphate	0	0
4-methyl umbelliferone phosphate	0	0

* n.d. = not determined.

Neither preparation demonstrated activity towards sulphate or phosphate esters.

Tributyrylglycerol was hydrolysed most rapidly by both lipase preparations. All insoluble triacylglycerols were hydrolysed, although rates were low with solid substrates (tripalmitoylglycerol and tristearoylglycerol). Unsaturated esters were hydrolysed more rapidly than their saturated equivalents, an apparently general phenomenon with lipases (Brockerhoff and Jensen 1974). The *Pseudomonas* 017 lipase displayed an unexpectedly low activity toward tricaprolyglycerol (6-carbon fatty-acyl chain). Both lipases displayed activity towards the dioleoylglycerols, with the *Pseudomonas* 017 lipase hydrolysing these substrates at similar or greater rates than the equivalent triacylglycerol.

The rates at which the natural esters were hydrolysed reflects their triacylglycerol composition (Appendix II). Tallow, milk fat and coconut oil all contain significant quantities of the more rapidly attacked short-chain acyl-esters, unlike olive oil which is principally trioleoylglycerol.

The insoluble fatty-acyl esters of p-nitrophenol and 4-methyl umbelliferone were hydrolysed by both bacterial lipases. The rates of hydrolysis relative to that observed with trioleoylglycerol are presented in Table 10.6, along with the relative rates with highly-purified porcine pancreatic lipase (Sigma Chem. Co., St Louis, USA). The low relative rates of hydrolysis of both p-nitrophenyl and 4-methyl umbelliferone esters observed with the pancreatic lipase are similar to results reported previously (Desnuelle and Savary 1963; Brockerhoff 1969; Melius and Doster 1970). In contrast, the bacterial lipases were capable of comparatively high relative rates of hydrolysis, particularly towards 4-methyl umbelliferone esters. As soluble substrates (4 MU-acetate, ethyl butyrate, Tweens, triacetylglycerol) were not hydrolysed, it must be assumed that both bacterial preparations

TABLE 10.6 Rates of hydrolysis of various substrates relative to that observed with trioleoylglycerol using lipases from *Staphylococcus aureus* NCIB 6571, *Pseudomonas* 017 and porcine pancreas (Sigma Chem. Co., St Louis, USA).

SUBSTRATE	Relative hydrolysis rate		
	Lipase		
	S. aureus	*Pseudomonas* 017	Pancreatic
Trioleoylglycerol	100	100	100
p-nitrophenyl acetate	2.1	1.8	0.1
p-nitrophenyl caprylate	5.0	14.2	1.4
p-nitrophenyl laurate	7.5	13.8	4.4
p-nitrophenyl palmitate	3.7	8.8	3.2
4-methyl umbelliferone acetate	0	0	0
4-methyl umbelliferone butyrate	7.5	61.1	0.1
4-methyl umbelliferone caproate	17.0	135.5	0.2
4-methyl umbelliferone caprylate	44.8	124.9	0.3
4-methyl umbelliferone nonanoate	49.8	117.2	1.5
4-methyl umbelliferone laurate	27.8	93.2	1.7
4-methyl umbelliferone palmitate	11.2	24.3	0.8
4-methyl umbelliferone stearate	6.2	9.9	0.6
4-methyl umbelliferone oleate	13.3	98.6	2.1
4-methyl umbelliferone elaidate	11.6	85.4	2.1

contained no esterase but rather, lipase with a markedly different substrate specificity from that observed with pancreatic lipase.

10.3.2 Effect of substrate concentration : The catalytic activity of *Pseudomonas* 017 lipase was influenced by substrate concentration in one of two fundamentally distinct ways, depending upon the substrate used (Figs. 10.4 and 10.5). Trioleoylglycerol, 4-methyl umbelliferone nonanoate and oleate and ρ-nitrophenyl laurate, all displayed apparent Michaelis-Menten kinetics, obeying the linear Woolf transformation ($S.V^{-1}$ *vs* S, $r > 0.98$) with apparent K_m's of 0.22 mg L^{-1}, 1.8 μM and 20 μM, and 0.09 mM respectively. Triacetylglycerol and ρ-nitrophenyl acetate displayed sigmoidal kinetics, with no or little activity at low substrate concentrations. Activity towards triacetylglycerol increased markedly at substrate concentrations beyond the solubility limit of 71.7 mg mL^{-1} (Merck Index), a finding that distinguishes lipases from esterases (Sarda and Desnuelle 1958; Desnuelle 1972). Similarly, marked increases in the hydrolysis of ρ-nitrophenyl acetate occurred beyond a concentration of 0.1 mM, presumably its solubility limit. The esters whose kinetics of hydrolysis obeyed the Michaelis-Menten equation must possess extremely low aqueous solubilities relative to the substrate levels at which measurable activities were observed.

10.3.3 Effect of pH : The effect of hydrogen-ion concentration on the stability of the bacterial lipases was determined by incubating the preparations for 30 min at 30°C in distilled water adjusted to a range of pH's (3 to 11) by addition of HCl or NaOH . The residual activity was measured at pH 7.5 using the continuous potentiometric titration assay with tributyrylglycerol (2.3). The staphylococcal lipase was stable over the pH-range 6 to 10 and was particularly labile at acidic pH's (Fig. 10.6). The pseudomonad lipase was stable over the complete pH-range of 3 to 11, (Fig. 10.6).

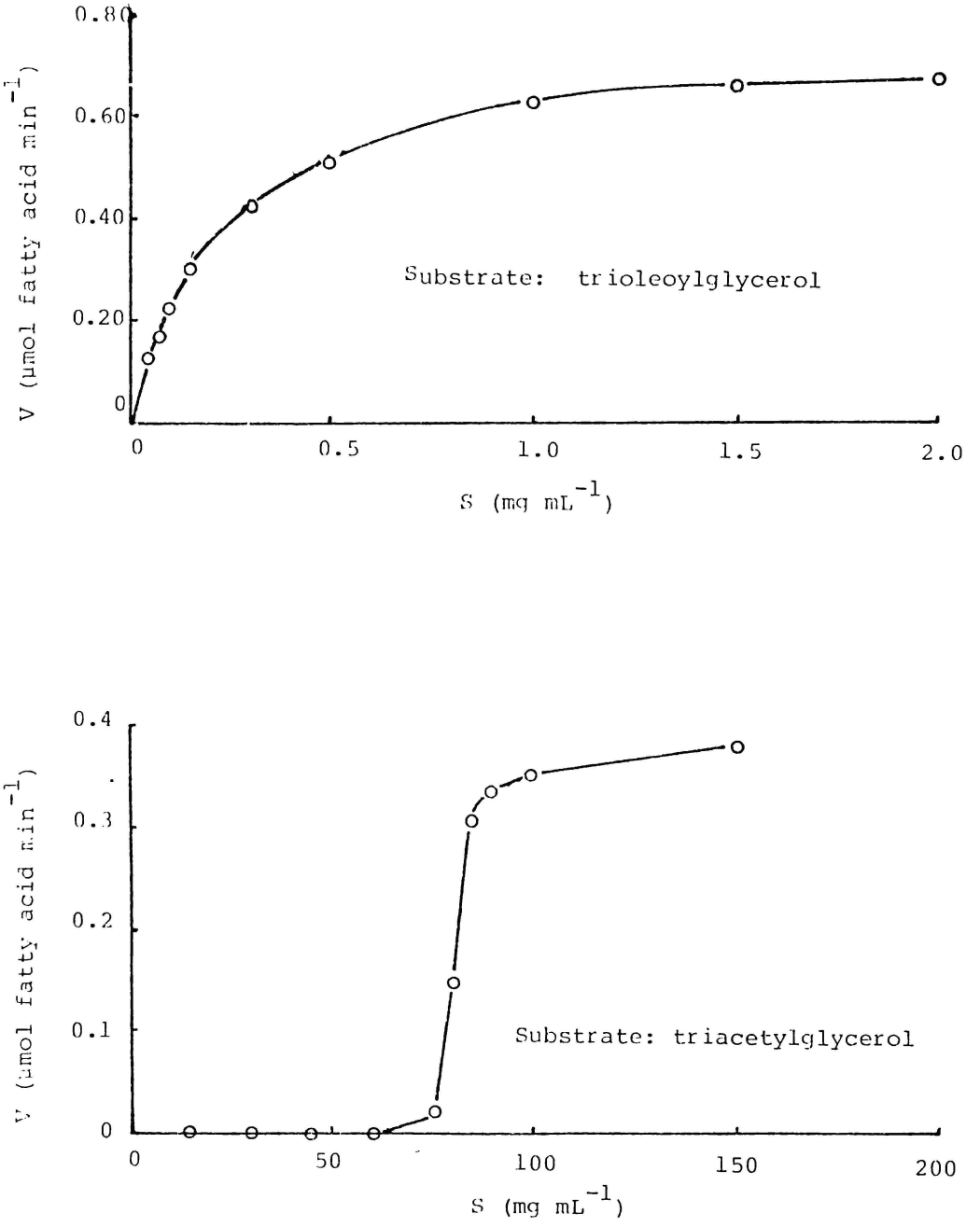


Fig. 10.4. The effect of substrate concentration on the activity of *Pseudomonas* 017 lipase towards trioleoylglycerol and triacetylglycerol. Enzyme level = 10 µg Lowry protein.

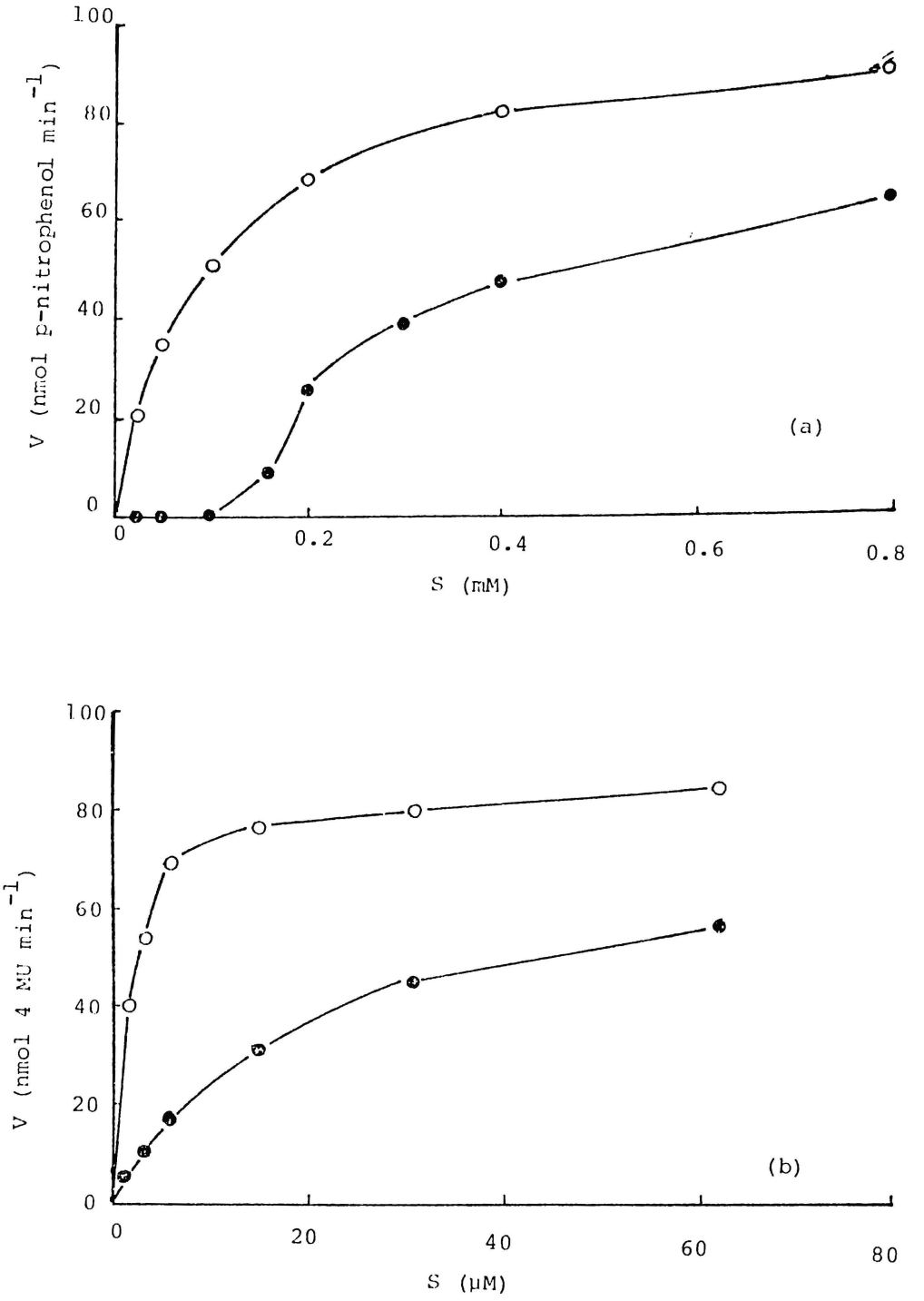


Fig. 10.5. The effect of substrate concentration on the activity of *Pseudomonas* 017 lipase towards various esters. Enzyme levels = 10 μg Lowry protein.
(a) o-o, p-nitrophenyl laurate; ●-●, p-nitrophenyl acetate
(b) O-O, 4-methyl umbelliferone nonanoate;
●-●, 4-methyl umbelliferone oleate.

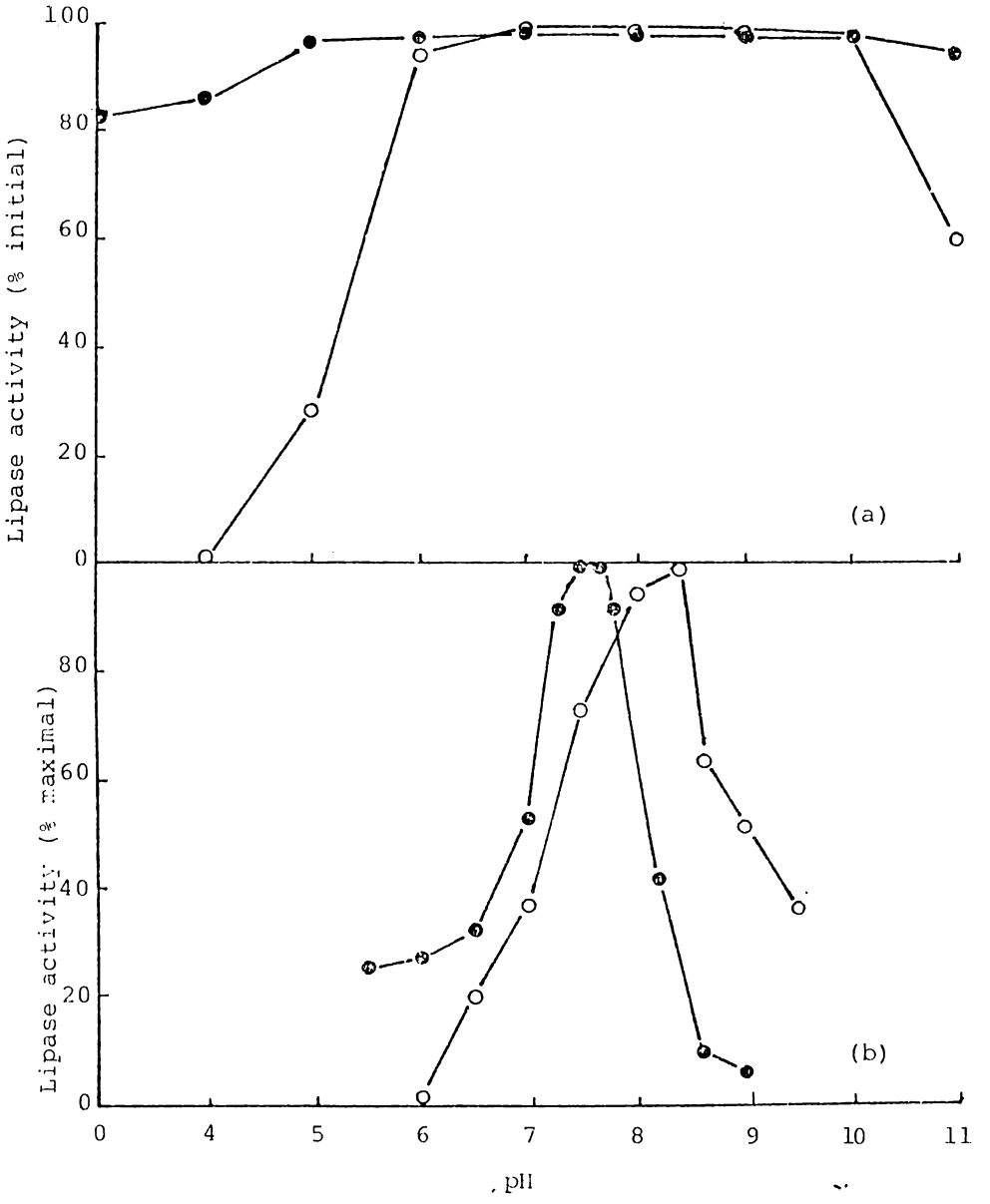


Fig. 10.6. Effect of pH on stability (a) and activity (b) of *Staphylococcus aureus* lipase (o-o) and *Pseudomonas* 017 lipase (●-●).

The activity of both bacterial lipases towards tributyryl-glycerol was markedly influenced by pH (Fig. 10.6). The staphylococcal lipase demonstrated an optimum at pH 8.2, the same as that found by Renshaw and San Clemente (1967). The pseudomonad lipase possessed optimal activity at pH 7.5 - 7.7. The same pH profiles were obtained when 4-methyl umbelliferone nonanoate was used as substrate.

10.3.4 Effect of temperature : The thermal stability of the enzyme preparations was determined by incubation in distilled water at the desired temperatures (25°C - 80°C) for 30 min followed by immediately plunging into an ice bath. The residual activity was subsequently determined using the continuous potentiometric titration assay with tributyrylclycerol as substrate (2.3). Both the staphylococcal and pseudomonad lipases were stable to 30 min exposure to temperatures up to 50°C (Fig. 10.7). Above 50°C, the staphylococcal lipase was more thermolabile than the pseudomonad lipase.

The activity of the staphylococcal lipase was maximal at 37°C with a rapid decrease in activity at higher temperatures. In contrast the lipase from *Pseudomonas* 017 displayed a broad temperature profile, with maximal activity occurring at 50°C. The observed temperature optimum for the staphylococcal lipase is similar to that previously reported for staphylococcal lipases (Renshaw and San Clemente 1967) and within the 30 - 40°C range where microbial lipases are generally most active (Lawrence 1967b; Brockerhoff and Jensen 1974). The high temperature optimum for *Pseudomonas* 017 is unusual for microbial lipases although Sugiura *et al* (1977) reported a similar optimum for the lipase from *Pseudomonas fluorescens*.

Both lipases were stable to storage at -20°C for at least 6 months. The staphylococcal lipase lost 10% of its activity when stored at 4°C for 7 days, whilst the lipase from *Pseudomonas* 017 was stable under these conditions. At 20°C both lipases lost 15% of their activity in 2 days.

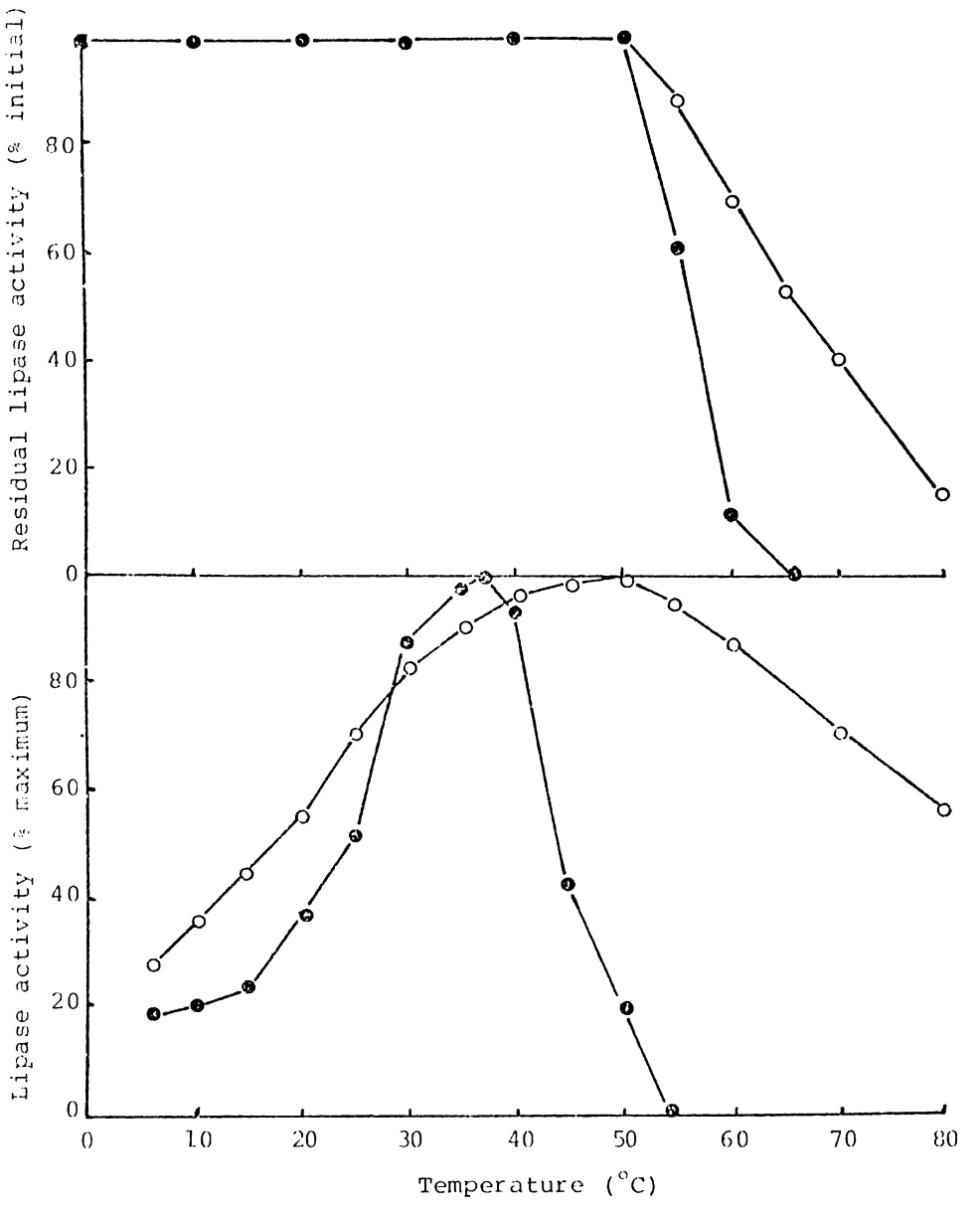


Fig. 10.7. The effect of temperature on stability (a) and activity (b) of *Staphylococcus aureus* lipase (●-●) and *Pseudomonas* 017 lipase (o-o).

10.3.5 Effect of enzyme inhibitors : A range of various enzyme inhibitors was incubated with the bacterial lipase preparations for 30 mins at 30 °C and residual activity analysed using continuous potentiometric titration with tributyrylglycerol as substrate (2.3) (Table 10.7). The lack of inhibition of lipase activity with both preparations by ethylenediaminetetra-acetic acid (EDTA) demonstrates the absence of metal ions at the catalytic site. A role for sulphydryl groups near or at the catalytic site for the *Pseudomonas* 017 lipase is suggested by the increased activity observed upon reduction with dithiothreitol and the decreased activity upon treatment with H_2O_2, iodoacetic acid and p-chloromercuribenzoic acid. Verger *et al* (1971) and Dooijewaard-Kloosterziel and Wouters (1976) came to similar conclusions for a pancreatic and fungal lipase respectively. Apparently, sulphydryl groups were not important to the catalytic activity of the lipase from *Staphylococcus aureus*. Phenylmethylsulphonyl fluoride (PMSF) did not influence the activity of either lipase preparation, suggesting the absence of serine at the catalytic site. PMSF has been used to differentiate between esterases (inhibited) and lipases (not inhibited) (Knoche and Horner 1970).

10.4 Industrial potential of *Pseudomonas* 017 lipase

The industrial potential of *Pseudomonas* 017 lipase is difficult to evaluate as information on the lipases currently produced commercially is scarce (Seitz 1974). Intuitively, the following observations suggest that *Pseudomonas* 017 lipase may be of commercial value.

(i) Lipase production is higher and more rapid than other microorganisms (8.3.2), including fungi from which commercial lipases are commonly produced (Seitz 1974);

(ii) Lipase production is high during the logarithmic phase of growth (9.5), suggesting that continuous cultivation and

TABLE 10.7 The effect of various enzyme inhibitors on the activity of two bacterial lipases.

EFFECTOR	Lipase activity (%)		
	Conc. (mM)	*S. aureus*	*Pseudomonas* 017
None	-	100	100
EDTA	10	98	100
	100	98	100
Dithiothreitol	1	100	140
	10	98	165
H_2O_2	0.1	94	25
	1	80	0
Iodoacetic acid	1	85	50
	10	70	35
p-chloromercuri-benzoic acid	1	97	65
phenylmethyl-sulphonyl fluoride	10	95	45
	0.1	100	100
	1	100	98

enzyme harvesting may be feasible;

(iii) High levels of lipase (9.5) with high specific activities (10.2) can be produced on readily-available primary processing byproducts (tallow and anhydrous milk fat);

(iv) The lipase is stable to storage, temperatures up to 50°C, and extremes of pH (10.3). Activity occurs over a broad temperature range and is maximal at or near pH's encountered in most environments (10.3). The lipase of *Pseudomonas* 017 displays considerable activity towards solid substrates (10.3).

A simple procedure for the purification of the lipase of *Pseudomonas* 017 needs to be developed if a high-purity enzyme preparation is desired. The use of hydrophobic interaction chromatography whereby lipases can be adsorbed onto and subsequently desorbed from hydrophobic materials (Isobe and Sugiura 1977b; Jürgens 1981) deserves investigation.

10.5 Summary

The extracellular lipases from *Staphylococcus aureus* NCIB 6571 and *Pseudomonas* 017 (a soil isolate) were purified 300-fold and 14.4-fold respectively over that in the cell-free supernatants. Elution profiles upon Sephadex G-100 chromatography indicated that both lipases were of a high molecular weight (>100 000), although the preparation from *Pseudomonas* 017 demonstrated activity in fractions corresponding to a molecular weight of 30 000.

Both lipase preparations displayed maximal activity towards tributyrylglycerol. Activity was observed towards all insoluble esters tested (including esters of p-nitrophenol and 4-methyl umbelliferone) but not towards soluble esters. The lipase of *Staphylococcus aureus* was unstable at pH's below 6, whilst the lipase of *Pseudomonas*

017 was stable over the pH range 3 - 11. Optimal activity occurred at pH 8.2 and 37°C for the staphylococcal lipase and at pH 7.5 - 7.7 and 50°C for the pseudomonad lipase. Both lipases were stable at temperatures up to 50°C. Neither enzyme possessed metals or serine residues at the catalytic site. Sulphydryl groups were necessary for the catalytic activity of the lipase from *Pseudomonas* 017.

The potential for commercial production and industrial application of *Pseudomonas* 017 lipase was discussed.

CHAPTER 11 CONCLUSIONS

The prime objective of this research at its inception was to determine the rate at which triacylglycerol was degraded when applied to soil (Chapter 1). The initial step in this degradation is the hydrolysis of the glycerol-ester linkages by lipase. The work described in Chapter 2 provides statistical estimates of the precisions and detection limits of four methods for analysing for lipase activity. The choice of a method for a particular experimental system can be based on the sensitivity required and/or the ease of the assay for routine use. In this context, the fluorometric assay, using 4-methyl umbelliferone nonanoate as substrate, proved most suitable in the present study. However, in systems where esterases may be present, questions as to its specificity have been raised. The assay described by Brockerhoff *et al* (1970), using vinyl oleate as the substrate, warrants further investigation since it was apparently both sensitive and specific for lipase.

The kinetics of lipolysis and the influence of various surface-active agents was investigated in Chapter 3. The activity of lipases was found to be dependent upon the substrate surface area rather than the concentration *per se*. Lipase action is dependent upon adsorption of the enzyme to the insoluble substrate surface. Factors that affect this adsorption therefore influence the observed activity of the enzyme. The inhibitory effect that fatty acids have on lipase activity could be explained by a combination of two phenomena:

1) Fatty acids, due to their combination of hydrophobic and hydrophilic components, partition at the substrate-aqueous interface thereby reducing the substrate effectively available to the enzyme.

2) The carboxyl group of the fatty acids imparts a charge to the surface, thereby repelling lipase. This charge repulsion could be overcome by neutralisation with Ca^{2+}.

The sensitivity of the fluorometric lipase assay allows for low enzyme levels to be used and thus, fatty acid levels do not reach inhibitory concentrations.

The fluorometric technique developed and defined in Chapter 2 was used to measure soil lipase activities (Chapter 5). The assay involves initial extraction of lipase from the soil with sodium tetrapyrophosphate followed by the fluorometric determination of lipase activity in the extract. This technique proved sufficiently sensitive and repeatable for measuring both basal soil lipase levels and lipase induction in soils after application of triacylglycerols (Chapter 6).

Studying patterns of oxygen consumption, triacylglycerol degradation, and lipase induction in triacylglycerol-amended soil samples, provided basic information on the factors influencing the ability of the soil microbiota to degrade triacylglycerols (Chapter 6). These studies showed that the basal soil lipase activity plays no role in the degradation of added triacylglycerol, but that the induction of lipases by growing lipolytic microorganisms is the determining feature of triacylglycerol removal. The induced lipase exists only transiently with lipase activities, returning to preamendment levels within 30 days. This suggests that each soil is capable of offering protection and hence long-term stability to a certain basal level of lipase (*via* a humus-lipase complex?) with lipase produced in excess of this level being unprotected and therefore unstable.

The studies of Chapter 6 suggest that triacylglycerol accumulation problems were unlikely to occur with wastewater application to land at commonly-used loading rates. The work needs to be extended to field-trials before more definitive statements can be made.

An examination of the microbiota in triacylglycerol-amended soils indicated that bacteria were the principal components responsible for the degradation of triacylglycerol (Chapter 8). One isolate, *Pseudomonas* 017, produced extraordinarily high levels of extracellular lipase when grown on triacylglycerols, fatty acids, or hydrocarbons (Chapter 9). The lipase could be produced by growth upon tallow (a byproduct of slaughterhouses), a relatively cheap source of substrate. This finding, coupled with the high specific activity of the crude supernatant, the high stability, and diverse substrate range, suggests that the enzyme may be suitable for commercial production and subsequent use in a variety of products (e.g., washing powders, dentifrices, sewage digestion activators). The production of lipase by *Pseudomonas* 017 under conditions of continuous cultivation would seem the next step in evaluating its potential for commercial purposes.

This thesis has examined lipases at the subcellular level, the cellular level, and the system level. The findings have furthered the knowledge at each of these levels and, hopefully, has provided a stimulation for further research into the areas not answered.

APPENDIX I

Protein Estimation

Protein levels were determined using a modification of the method of Lowry *et al* (1951) as follows:

To 50 mL of 2% (w/v) Na_2CO_3 in 0.1 M NaOH was added 0.5 mL of 2% (w/v) K-Na tartrate and 0.5 mL of 1% (w/v) $CuSO_4.5H_2O$ immediately before use. 5 mL of this mixture was added to 1 mL of sample (or dilution), mixed and left to stand for 10 min. After this time, 0.5 mL of Folin-Ciocalteau reagent (Sigma Chem. Co., St Louis, USA; diluted 1:2 with distilled water) was added, the solution mixed and left for 30 min. The absorbance at 750 nm was read using a Spectronic 20 and compared to a standard curve (0 - 500 $\mu g\ mL^{-1}$ bovine serum albumin).

APPENDIX II

Characterisation of triacylglycerols

i) Free fatty acid level : The level of contaminating non-fatty acid was determined using the colourimetric assay of 2.4. 300 mg of lipid was dissolved in 100 mL of chloroform and 10 mL removed for subsequent fatty acid determination. The levels of non-esterified fatty acid (as wt. %) were 0.05% for olive oil (high purity, Sigma product), 0.20% for anhydrous milk fat and 0.56% for tallow.

ii) Fatty acyl composition : The fatty acyl components of the triacylglycerols were determined by gas-liquid chromatography after methylation (Christie 1973) using the following procedure: To 50 mg of sample and 5 mg of heptadecanoic acid (internal standard) dissolved in 1 mL of dichloromethane was added 2 mL of methanolic (5% v/v H_2SO_4. The mixture was refluxed for 2h then 5% (w/v aqueous NaCl added. The methyl esters were extracted into hexane (2 x 5 mL), washed with 2% (w/v) aqueous $KHCO_3$ and dried over anhydrous Na_2SO_4. The solution was filtered, the hexane removed by rotary evaporation, and the methyl esters resuspended to 0.5 mL in hexane. Aliquots (5 µL) were injected into a Pye Unican GCD gas-liquid chromotograph fitted with a flame ionisation detector and a 1.8 m column of 10% EGSS-X on Gas Chrom P (mesh 100-120) (Applied Sciences, USA). Chromatography was performed with an N_2 flow of 45 mL min^{-1}, an H_2 flow of 50 mL min^{-1}, and an air flow of 25 mL min^{-1}. Column temperature was 180°C, injection temperature was 240°C and detector temperature was 250°C. The approximate elution time of the methyl heptadecanoate interval standard was 9.5 min.

Peaks were identified by their

retention times relative to that of methyl heptadecanoate and compared to that of methyl ester standards (Applied Sciences, USA). Compositions were expressed as weight % by calculation of relative quantities using peak height x retention time (Christie 1973).

The fatty-acyl compositions (wt. %) were as follows:

CHAIN LENGTH	TRIGLYCERIDE		
	OLIVE OIL	MILK FAT	TALLOW
4:0	0	6.0	1.0
6:0	0	1.5	0
8:0	0	2.0	0
10:0	0	2.0	0
12:0	0	5.8	2.2
14:0	0	17.0	3.3
16:0	8.5	23.1	25.0
16:1	0	1.3	0.2
18	1.7	13.0	23.1
18:1	85.3	26.8	45.9
18:2	4.4	0.5	0.2
20:0	0	1.0	1.4

APPENDIX III

Publications from the thesis

To date, two papers have been published from the material contained in this thesis:

i) Cooper, A.B., and Morgan, H.W., (1981). Improved fluorometric method to assay for 5 soil lipase activity. Soil biology and biochemistry 13 : 307-311. (Reprint in back pocket).

ii) Cooper, A.B., and Morgan, H.W., (1982). Biodegradation of milk when applied to soil. New Zealand journal of agricultural research 25 : 15-20 (Reprint not available)

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