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PURIFICATION AND CHARACTERIZATION OF LIPASE FROM *VICIA FABA MINOR*

by

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ABSTRACT

The purpose of this study was to ascertain whether a lipolytic enzyme, lipase, was present in faba bean (Vicia faba L var. minor). Lipase is believed to be one of the enzymes responsible for the initiation of the development of rancidity and off-flavour in processed faba bean.

Faba bean lipase was partially-purified from a faba bean acetone powder by ethanol fractionation. Purification was approximately 9-fold over the crude extract. Further purification was attempted by filtration on Sephadex G - 100 gel columns with a linear NaCl gradient, or by the incorporation of sodium deoxycholate which were responsible for 18-fold and 40-fold increases in lipase activity respectively. Comparison of the gel filtration profile of faba bean lipase was made against that of commercial hog pancreatic lipase.

The assay of the enzyme was based on a potentiometric titration of released fatty acid utilizing a pH-stat method.

The electrophoretic patterns of the faba bean lipase and commercial hog pancreas lipase were compared using polyacrylamide disc gel electrophoresis. There was one active lipase band detected in the Sephadex G - 100 purified faba bean lipase, with good correlation between protein bands and lipolytic bands. The isoelectric point of the enzyme was determined to be 4.8.

The molecular weight of faba bean lipase was estimated

by sodium dodecyl sulphate-gel electrophoresis to be 210,000 $\pm$ 20,000.

The course of the hydrolytic reaction was linear with respect to enzyme concentration. The enzyme exhibited greater activity towards short-chain triglyceride emulsions rather than long-chain triglyceride emulsions. The K_m for the reaction was determined to be 22.0 mM using tributyrin as the emulsified substrate.

The optimum pH of the enzyme was determined to be 8.5 while the lipase was stable over a pH range of 6.5 - 9.0 for a 10-minute period.

In solution the faba bean lipase was inactivated by exposure to 65°C for 2 minutes, indicating a comparatively heat-labile enzyme. The optimum temperature was determined to be 38°C.

The effects of various activators on the activity of the faba bean lipase were investigated. It was shown that NaCl was necessary for the lipolytic reaction to proceed to a zero-order rate with a maximum concentration of 0.7 M. The enzyme was influenced by the bile salts, sodium deoxycholate and sodium taurocholate, which resulted in maximal activation at a 12.0 mM concentration. The enzyme was not sensitive to high concentrations of either calcium or magnesium chlorides.

The faba bean lipase was inhibited by a high concentration of mercuric chloride (5.0 mM), with over 60% of the original activity being inhibited at this level. Faba bean

lipase was inhibited very slightly by a high concentration of p-chloromercuribenzoate. A 10.0 mM concentration resulted in a 20% reduction in lipase activity.

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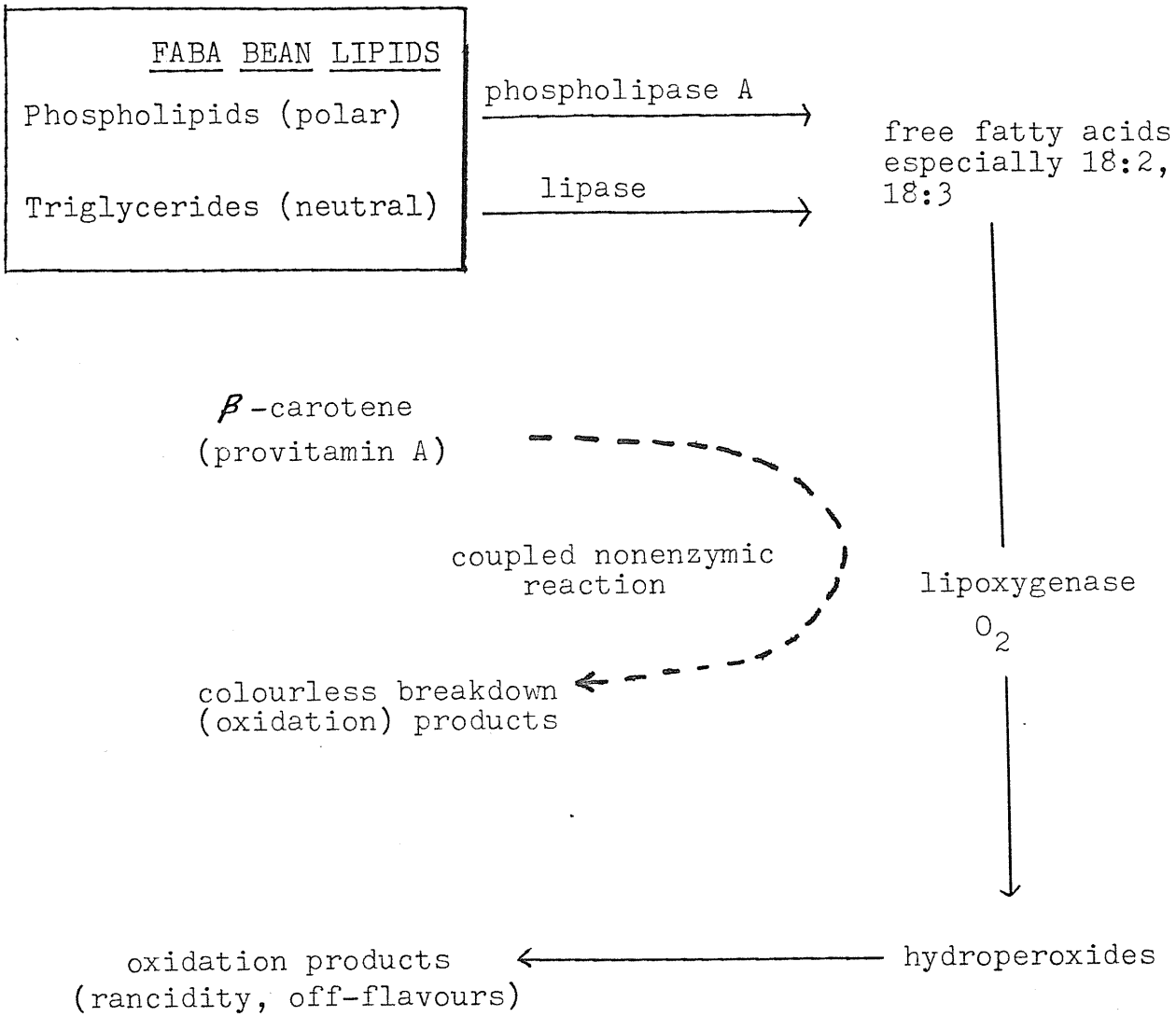
INTRODUCTION

The small faba bean, or horse bean, Vicia faba L var. minor, is being considered as a field crop for the Prairie region of Canada (Presber, 1972). At present the faba is being used to a considerable extent for livestock feed. Processed faba beans become rancid after mechanical disruption of the tissues, representing major storage problems and quality-acceptability problems, to livestock, and for possible use in human foods. The presence of increased free fatty acids during storage of faba beans as either a concentrate or flour has been detected (Hinchcliffe et al, 1974). This increase in free fatty acid content during storage, indicates the presence of esterases converting the stored triglycerides to free fatty acids. The rapid development of rancidity is due to the relatively high proportion of unsaturated fatty acids present in the lipid fraction, especially linoleic acid. This acid is particularly susceptible to oxidation, due to the presence of an active lipoxygenase system (Eskin and Henderson, 1976) which is highly specific towards unsaturated free fatty acids (Figure 1). Since the majority of fatty acids present are predominantly in the esterified form of either triglycerides or phospholipids, it was decided to investigate the mechanism of release of free fatty acid in the triglyceride fraction. Lipase (glycerol ester hydrolase, EC 3.1.1.3) is the enzyme responsible for the hydrolysis of acyl esters of fatty acids.

In plants, lipase investigations have been on olea-

FIGURE 1

Lipid oxidation pathway in the faba bean.



ginous seeds where lipase activity generally is manifest upon germination. Seed lipases have received relatively little attention, and have been almost exclusively concerned with lipases that exhibit exceptional characteristics for example, the acid lipase of castor bean. In the literature, studies on lipase are very entangled and confusing, indicating the difficulty in studying the enzyme, the major drawback being the limitations imposed by the water-insoluble substrate, lipase being active only at the lipid-water interface.

The object of this study was the detection of lipase in faba bean, and the purification and characterization of the enzyme. The determination of the effect of certain conditions (pH, temperature, activators and inhibitors) on faba bean lipase activity was undertaken. The determination of the molecular weight of the enzyme, along with comparison of lipolytic activity with commercial hog pancreatic lipase, was undertaken using polyacrylamide gel electrophoresis.

CHAPTER 2

REVIEW OF LITERATURE

The enzyme lipase belongs to the widely-distributed group of enzymes known as the esterases, which are involved in the splitting of ester linkages by the addition of water. Enzymes hydrolysing triglycerides have studied for well over a hundred years, but in the literature, studies on lipase are entangled with studies on esterases. The diversity of data seems to be partly due to the use of unpurified enzyme preparations and partly to the wide choice of substrates, assay conditions and methods used to determine lipase activity. The first workers in the field assumed that lipase hydrolysed only natural triglycerides, while more extensive investigations of specificity showed that lipases were very unspecific enzymes, and that there appeared to be considerable overlap with the equally unspecific esterases. The nature of either the fatty acid or the alcohol moiety has a secondary effect on the role of lipolysis. The real difference between ordinary esterases and lipases appears to reside in the physical nature of the substrates, with lipases being unable to attack substrate molecules fully dispersed in water, acting only at the water-lipid interface (Sarda and Desnuelle, 1958). The minimum degree of molecular aggregation of the substrate compatible with lipase action is still unknown.

Thus, lipases form a rather indefinite section of the esterase group of enzymes, but it is useful to distinguish lipases from other esterases by the definition recommended by the International Union of Biochemistry (1961), namely, that lipases hydrolyse emulsified esters of glycerol, whereas other water-soluble esterases hydrolyse water-soluble substrates.

All lipolytic enzymes are hydrolases and therefore, belong to class 3 within the classification recommended by the Enzyme Commission (Florkin and Stotz, 1965). Lipases are currently classified among the hydrolases and are ester hydrolases, enzyme group 3.1. No bonds other than carboxyl ester bonds have ever been found to be hydrolysed by lipases and they are therefore, defined as carboxyl ester hydrolases, and as lipase acts on esters of glycerol the enzyme is fully classified as glycerol ester hydrolase (EC 3.1.1.3).

Lipases were considered to be enzymes hydrolyzing glycerol esters according to the equation:

$$\text{A Triglyceride} + H_2O \rightarrow \text{Diglyceride} + \text{fatty acid ion}$$

Present information shows that the equation above is not quite correct, with triglyceride hydrolysis by most lipases now known to go beyond the diglyceride stage and to form substantial amounts of monoglycerides and sometimes of free glycerol (Entressangles and Desnuelle, 1968). Also, lipases have been recently shown to rapidly hydrolyse ester substrates other than glycerides (Seneriva and Dufour, 1972).

2.1 DISTRIBUTION OF LIPASES

Lipases are widely distributed in animals, plants and micro-organisms. Although lipases from many different sources have been described, only relatively few have been investigated in detail. A comprehensive list of lipases definitely or tentatively identified in animals, plants or micro-organisms will be found in the review of Wills (1965).

2.1.1 Animal Lipases

Mammalian lipases have received greatest attention in recent years. Three groups of enzymes may be distinguished in mammals: the lipases discharged into the digestive tracts by specialized organs, tissue lipases, and milk lipases.

Among the digestive lipases the enzyme synthesized by the pancreas is the best known and most often investigated, as well as being one of the earlier enzymes to be recognized (Claude Bernard, 1856). Despite its low level when compared to that of other pancreatic enzymes (1.2% of the total proteins in cattle pancreatic juice, 2.5% in pig and 3.4% in rat)(Marchis-Mouren, 1965), this lipase plays an essential role during the intraluminar digestion of dietary triglycerides. In addition, the existence of gastric and intestinal lipases, often disputed, now appears to be definitely proved. Lipases have been reported to be present in a number of tissues or organs of mammals such as heart, brain, muscle, adipose tissue and serum, where they are known as lipoprotein lipases. They have been identified in milk.

2.1.2 Plant Lipases

Few studies have been made so far on the distribution of lipases in whole plants except in seeds and fruits. Most of the effort in this area has been devoted to seed lipases. Seeds are generally rich in triacylglycerols, which serve as a compact source of energy for the newly-emerging plant. During germination of the seed, the triacylglycerol stores disappear. Since the fatty acids cannot be oxidised to provide energy until they are released from the triacylglycerols, lipolytic enzymes are probably rate-controlling during germination. Germination is usually rapid and lipolytic activity is relatively high at that time.

Crushing or storage generally activates dormant lipases in a seed, and the resulting accumulation of free fatty acids can cause an industrially-important oil to become unacceptable or to require additional processing to remove the acids. Nevertheless, investigators have neglected the lipases in the most important food oil-seeds, for example, soybean, cottonseed, corn, safflower, coconut, sesame, and other industrial seeds. Very few reports on the lipases of these seeds are available. Most attention has been paid to seed lipases that exhibit some unusual property, for example, the acid lipase of castor bean (Ory et al, 1960). Lipase activity has been reported in wheat (Sullivan and Howe, 1933), palm (Savary et al, 1957), barley and malt (Lowy, 1945), coconut (Sadasivan, 1951), peanuts (Sanders and Pattee, 1972), cotton (Olcott and Fontaine, 1941) wheat

germ (Singer and Holfstee, 1948; Stauffer and Glass, 1966), and oats (Martin and Peers, 1953).

2.1.3 Microbial Lipases

In the past, interest in microbial lipases resulted from investigation of food spoilage, especially of dairy products. The short-chain fatty acids are directly responsible for flavour defects, while the long-chain fatty acids could presumably be converted more readily to carbonyls and other volatile compounds such as free acids. In contrast, free fatty acids in some dairy products, notably cheese, contribute to desirable flavour, such as the action of lipase in Penicillium roqueforti contributing to the flavour of Roquefort cheese (Eitenmiller et al, 1970). The production of lipases may assist in the classification of micro-organisms, and the detection of those that are pathogenic (Lawrence et al 1967). Lipases are present in many strains of bacteria and fungi. Most of the lipases are intracellular, but some species, such as Stapylococcus, (Davis, 1954) secrete extracellular lipases.

2.2 SUBSTRATES

Lipases hydrolyse emulsified triglycerides, which are saturated or unsaturated fatty acid esters of glycerol. The reaction of lipolytic enzymes should apply to the rate constants of kinetic equations and it has been demonstrated that the hydrolysis of triglycerides by pancreatic lipase obeys the fundamental Michaelis-Menten equation (Sarda and Desnuelle, 1958). Nevertheless, this agreement is very

formal and does not explain the complexity of the nature of lipolytic reactions. Conventional enzyme kinetics has been developed for reactions in aqueous solutions, but the enzymic hydrolysis of lipids is different in one essential aspect: it is a heterogeneous reaction because the enzyme is water-soluble but its substrate is not. Therefore, the enzyme-substrate interaction must take place at the interface of the aggregated substrate and water. The difficulties of lipolytic reactions are therefore, inherent in the insolubility or the physical nature of the substrate. The major problem is the measurement of the concentration of an insoluble substrate, and as the enzyme forms an enzyme-substrate complex only at the oil-water interface, it is believed that the rate of lipolysis is dependent on the available surface area. As the substrate concentration is zero in the aqueous phase, at best only those molecules at the interface, on the surface of the oil droplets, are available to the enzyme. The oil-water interface increases as the concentration of the oil in water, or the saturation of the aqueous phase with emulsification increases. Thus, the available surface area is used as a measurement of the concentration of substrate as applied to the Michaelis-Menten equation. The concept of a surface-dependent interfacial reaction was demonstrated by studying the rate of hydrolysis of triacetin emulsified in gum arabic (Sarda and Desnuelle, 1958). The moderately water-soluble substrate triacetin is dissolved in water up to saturation point, whereupon the substrate is offered as an emulsion

with an increasing interfacial area. When the concentration of added triacetin is low, a true solution is produced and the rate of lipolysis is very slow, but increases sharply as the concentration of substrates increases above saturation to form a heterogeneous system.

Lipases hydrolyse natural triglycerides such as oils. Glycerol trioleate (triolein) is the most universal of substrates. It fulfills the definition of a lipase substrate by containing long-chain fatty acids only, and is liquid at the usual assay temperatures. A good surrogate for triolein is olive oil, which contains over 70% oleic ester and has the advantage of being cheap. Tributyrin is a convenient substrate, because it is easily dispersed in water by shaking or stirring without the addition of emulsifiers. Other fatty acid substrates that have been used for the reaction with lipase include tripropionin, trioctanoin, tripalmitin, tricaprylin and methyl oleate. Tributyrin and triolein have been most commonly employed as substrates, but while there appears to be differences between lipases, they all hydrolyse tributyrin at the fastest rate (Whitaker, 1972), and this is normally the substrate of choice. It must be realized that tributyrin does not meet the requirements for a substrate of lipase as defined, and although most lipases hydrolyse tributyrin, so do most of the esterase group. Therefore, if lipase activity is detected with tributyrin, it should be verified with triolein (olive oil). If the substrate is not emulsified or the emulsification is inadequate, the rate of shaking becomes an important factor

that affects the rate of hydrolysis. Emulsification of the triglyceride is normally desirable, and egg albumen and gelatin were first used as emulsifiers (Willstätter et al, 1923), but more recently a 10% gum arabic solution has been suggested for the emulsification of tributyrin or olive oil (Desnuelle et al, 1955).

Now that it has been clearly established that lipases hydrolyse only water-insoluble substrates in a heterogeneous system, any method proposing the use of water-soluble substrates for the measurement of lipase activity must be rejected. Examples of water-soluble substrates that have been used are the Tweens (Boissonnas, 1948), p-nitrophenyl acetate, and p-nitrophenyl butyrate (Gad, 1949).

2.3 SUBSTRATE SPECIFICITY

The specificity of an enzyme is normally defined by the chemical structure of its substrates. In the case of lipase, however, it is the physical form of the substrate that is used to determine the specificity of this enzyme. The substrate specificity of a lipase is defined by its positional specificity, that is the ability to hydrolyze only the primary or both primary and secondary ester bonds of a triglyceride: by its stereospecificity, that is the ability to hydrolyze only ester linkage 1 or ester linkage 3 of the triglyceride: by its preference for longer or shorter saturated or unsaturated fatty acids, and the sequence of splitting of fatty acids, from triglycerides and hydrolysis of mono- and diglycerides.

The question of which ester groups, primary or secondary, or both, are hydrolysed by lipase, was answered by Balls and Matlack (1938) in favour of the hydrolysis of primary esters of glycerol. Nevertheless, it was thought that the outer primary ester groups of triglycerides were the only ones attacked by lipase (Schonheyder and Volqvartz, 1952). Final proof for the hydrolysis at the primary esters at the 1 and 3 positions was presented by Mattson and Beck (1955) and Savary and Desnuelle (1956).

The two primary groups of glycerol esters are sterically distinct, although the molecule has a plane of symmetry, so that substitution with two different fatty acid groups will lead to optically-active derivatives (Hirshman, 1960). Since stereospecificity is a typical feature of enzymes, it might be expected that lipase makes a distinction between these positions, but the fact that the enzyme hydrolyzes both of the primary esters argues against this assumption. Strict proof that lipase is not stereospecific was supplied by Tattrie et al (1958).

The influence of fatty acid chain length on substrate specificity has been the subject of many studies on lipases. There is general agreement that short-chain fatty acids are released at the maximum rate. Among triglycerides, triacetin is a poor substrate, whereas tripropionin (Wills, 1961) and tributyrin (Entressangles et al, 1961) are hydrolysed faster than other triglycerides. Evidence has recently been presented that the total size of a triglyceride rather than the length of a particular fatty acid, regulates the rate of lipolysis (Sampugna et al, 1967). The effect of

fatty acid unsaturation on substrate activity is not so clear cut. It was noted by Brockerhoff (1965) that the polyunsaturated fatty acid esters of marine oils were hydrolysed at much slower rates by lipase than the saturated esters. Later work, however, failed to confirm these results and Brockenhoff (1970) showed that fats with a low degree of unsaturation were hydrolysed almost as readily as oleic esters. Savary and Desnuelle (1956) concluded that as a general rule, the existence of one or two double bonds in the fatty acid did not affect the rate of hydrolysis.

Since triglycerides contain three esterified fatty acids, it is clear that their hydrolysis must proceed in steps via diglyceride and monoglyceride to glycerol and free fatty acids. Starting with a triglyceride, the relative rates of hydrolysis are that diglycerides are rapidly formed, monoglycerides more slowly and glycerol even more slowly (Desnuelle et al, 1947). A course study of the hydrolysis of olive oil showed that the first product, diglyceride, gives rise to the monoglyceride, with glycerol appearing only when all the triglyceride and more than 50% of the ester bonds have been hydrolyzed (Constantin et al, 1960).

2.4 DETECTION AND ASSAY

The definition given above for lipases requires that the substrates employed for the quantitative evaluation of these enzymes are in an emulsified form. In principle, an esterolysis or lipolysis reaction can be followed either

through the disappearance of the substrate, the triglyceride, or by the rate of production of the resulting fatty acids or alcohol. Methods for the quantitation of lipolytic activity can be divided into three groups: (1) the measurement of physical changes in the reaction system, (2) assays of the liberated alcohols, and (3) assays of the liberated fatty acids (Brockerhoff and Jensen, 1974).

2.4.1 Measurement of Physical Changes in Triglyceride Disappearance

The first of these methods utilized stalagmometry, which measured the changes in surface tension brought about by the surface-active products of hydrolysis (Rona and Michaelis, 1911). With tributyrin as the substrate, the surface tension of the emulsion approaches that of water as the tributyrin is hydrolysed. Here the drop rate of the reaction mixture is counted and compared with that of a control, or the weight of each drop is determined (Lagerlof, 1962). Alternatively, the changes in surface tension can be measured directly with a Du Nuoy tensiometer and has been used to detect milk lipase activity (Dunkley and Smith, 1951). These methods do not give reliable quantitative estimates of lipase activity, because changes in surface tension are dependent on the amount and types of free fatty acids present, and on the ratio of soaps to free fatty acids. A rapid and convenient physical method is measuring the rate of clarification of an emulsion (Borgstrom, 1957). The continuous decrease in optical density of an olive oil emulsion can be followed with a photometer. This

procedure cannot be used below pH 8.0 because the emulsion collapses.

2.4.2 Assay of the Liberated Alcohols

The methods involving assay of the liberated alcohols are colorimetric or fluorometric and therefore, sensitive and fast, but suffer from the fact that most chromogenic alcohols are phenols, and their esters are, therefore, not proper substrates for lipases (Brockerhoff et al, 1970). Several methods have been described that employ special substrates that give coloured end products after hydrolysis. The p-nitrophenol liberated from hydrolysis of p-nitrophenyl butyrate is readily measured directly (Brockerhoff et al, 1970). α Naphthol is liberated from α naphthyl laurate, which combines with tetrazontized o-dianisidine to give a pigment, and has been used as a substrate in the assay of serum lipase (Gomori, 1957). Several esters of 2-naphthol 6-sulphonic acid have been used as substrates for milk lipase, the naphthol-sulphonic acid liberated being determined by Folin-Ciocalteu reagent (Forster et al, 1955).

2.4.3 Assay of Freed Fatty Acids

The vast majority of methods described for estimating lipase activity are based on the determination of liberated fatty acids released being measured directly by a titration (Dole and Meinertz, 1960). In a typical method the substrate, emulsified with gum arabic, is mixed with buffer and calcium ions, whereupon the lipase is added and

the mixture incubated for a definite period and titrated with base (Balls et al, 1937; Sigiura and Isobe, 1974). Alternatively, samples may be removed at definite intervals for titration (Peterson et al, 1943; Fiore and Nord, 1949). Other methods involve the extraction of the liberated fatty acids by chromatography (Marsh and Fitzgerald, 1972), or by the use of solvent extraction (Kaplan, 1970). These methods require long periods of incubation and as a result, do not provide accurate determination of initial velocities.

Finally, the method of choice utilizes a continuous potentiometric titration, where base is added as the pH decreases. This technique is most generally used in the assay of lipase, and is most conveniently carried out by a pH-stat coupled with an automatic burette and a recorder. Long periods of incubation are not required, with the pH being constantly maintained and the mixture being kept emulsified by constant stirring. The procedure widely used for routine experiments consists of continuous titration of the protons released at pH 9.0 and 37°C from an olive oil emulsion stabilized by gum arabic (Desnuelle et al, 1955). Addition of an optimal concentration of bile salts is necessary in the system in order to obtain linear kinetic curves, and calcium ions are required to shift the ionization range of long-chain fatty acids towards more favourable values (Benzonana and Desnuelle, 1968). This method has been used to measure the activity of pancreatic lipase (Marchis-Mouren et al, 1959), and a lipase from Staphylococcus

aureus (San Clemente and Vadehra, 1967). The pH-stat is valuable in investigations of lipase kinetics as initial velocities can be measured with a high degree of accuracy although the method is relatively insensitive and requires a pH of 8.0 - 9.0. At pH levels below 8.0, less and less fatty acid can be titrated with base (Mattson and Volphenhein 1966; Benzonana and Desnuelle, 1968).

As an alternative to the pH stat, the rate of acid production may be measured manometrically by determining the rate of liberation of CO_2 from a bicarbonate buffer and free fatty acid (Singer and Hofstee, 1948; Martin and Peers, 1953), and by colour change of acid - base indicators (Kason et al, 1972).

2.5 PURIFICATION

Lipase is perhaps one of the most difficult enzymes to isolate and purify. This is due mainly to the association of the enzyme to tightly-bound lipids, especially phospholipids, which are known to be insoluble in aqueous phase and require drastic conditions for separation which lead to extensive loss of enzymic activity.

The first lipase to be isolated was from the porcine pancreas, which led to some enrichment of the enzyme, but a pure enzyme was not obtained (Willstätter and Memmen, 1928). The initial stage of most preparations from pancreas involves dehydration and defatting by means of acetone, which results in a thorough delipidation of the tissue before extraction

of the lipases, and which does not affect the enzyme. The general method used in the purification of pancreatic lipase is the use of fractional precipitation of the dissolved acetone powder with electrolytes such as ammonium and magnesium sulphates. The outstanding contribution to methods of purification of pancreatic lipase has been made by Desnuelle and co-workers, whose procedure involved two precipitations with ammonium sulphate and two with acetone, followed by electrophoresis on starch gel of porcine lipase, resulting in a 36-fold purification (Sarda et al, 1957). Subsequently, the method was improved by the use of calcium phosphate gel followed by zone electrophoresis to yield a nearly pure enzyme (Marchis-Mouren et al, 1959). This method, however, yielded no more than a milligram of enzyme per run due to the fact that the enzyme was absorbed onto the gel system. An attempt was also made to further purify the proteins precipitated with magnesium sulphate by the use of dialysis, where lipase activity was concentrated about 20-25 times, with the enzyme being subjected to electrophoresis on plastic sponge, a procedure which gave a further 3.5-fold purification (Wills, 1958). Other methods for the purification of pancreatic lipase were attempted by Verger et al, (1969), where precipitates obtained by 0.32-0.52 ammonium sulphate saturation were treated with n-butanol and dialysed. Borgstrom (1955) subjected rat pancreatic juice to electrophoresis and obtained 40 to 50-fold purifications.

The lipase activity of porcine pancreatic extracts on

long-chain triglycerides in the presence of bile salts has been reported to be markedly depressed upon chromatography on DEAE - cellulose (Baskys et al, 1963). Addition of boiled pancreatic extract restored the activity. Morgan et al (1969) found that a heat-stable cofactor with a molecular weight of around 12,000 could be separated from rat pancreatic lipase. Available data suggest that it is normally bound to lipase in a 1:1 molar ratio (Mayie et al, 1971).

The purification of lipase from plant and microbial sources has proved to be more difficult than that from pancreatic sources. With a few exceptions, such as the purification of peanut alkaline lipase (Sanders and Pattee, 1972), the use of electrolytes in the fractional precipitation of lipase has been generally unsuccessful (Martins and Peers, 1953). The adjustment of the pH of a lipase extract to a pH range of 4.5 - 5.5, which is the known isoelectric point range of the enzyme, has resulted in a complete precipitation of the enzyme, but the denaturation of the enzyme makes other purification steps unsuccessful (Stauffer and Glass, 1966), or to render the enzyme almost inactive (Martin and Peers, 1953). The general method used in the purification of lipase from microbial sources has been the use of organic solvents to produce fractional precipitation, due to changes in dielectric constants in the medium (Nason, 1955). Modifications of the procedure of Fiore and Nord (1949) have been used for the fractionation of lipase from such sources as Leptospiral pomona (Patel et al 1964), Leptospiral bifexa (Chorvath and Fried, 1970; Chorvath and Benzonana,

1971), Chromobacterium viscosum (Sugiura and Isobe, 1974) and Staphylococcus aureus (Blobel et al, 1961; Shah and Wilson, 1963). Another method employed in the purification of lipase has been the use of differential centrifugation. This method has been used to isolate the enzyme from seed sources which are rich in oil, such as the extensively-researched acid lipase of castor bean (ory et al, 1962). A cofactor has also been isolated from castor bean, this being a heat-stable protein activator as in the case of pancreatic lipase (Ory et al, 1967). A lipase has been purified by differential centrifugation from the seed of Veronia anthelmintica with a thirty-fold purification (Olney et al, 1968).

2.6 GEL FILTRATION

Lipase has been purified to a fairly high degree by the application of gel filtration. The use of Sephadex gels which provides pores and cavities, created by the cross-linkages of dextran molecules, provides a good molecular sieve for the separation of proteins (Determan, 1968).

When aqueous extracts of porcine pancreatic lipase are chromatographed on Sephadex G-100, the lipase appears in the first protein fraction that emerges from the column (Gelotte, 1964; Downey and Andrews, 1965) with a very high apparent molecular weight. This preparation has been called

the "fast" lipase (Sarda et al, 1964). The elution of fast lipase on Sephadex G-200, or DEAE - cellulose ion exchange columns, resulted in a second more slowly migrating peak of lipase of an apparent low molecular weight, known as "slow" lipase (Gelotte, 1964; Berndt, et al 1968). Extracts of rat pancreas have been purified by Sephadex gel filtration to yield both fast and slow lipases (Morgan et al, 1968) while elution of Micrococcus extracts on Sephadex G-200 yielded two peaks, the first appearing directly after the void volume corresponding to the fast lipase, and the slow lipase migrating at a much slower rate (Lawrence et al, 1967).

Downey and Andrews (1965) found that the application of extracts of porcine lipase on Sephadex G-200 resulted in the yield of slow lipase being increased at the expense of fast lipase. It has been proposed that the fast lipase is a multimolecular aggregate of the slow monomolecular form (Gelotte, 1964; Sarda et al, 1964; Downey and Andrews, 1965). Chorvath and Fried (1970) showed that fast lipase can indeed be converted into slow lipase by treatment with deoxycholate. This is believed to be accomplished by the action of the bile salt resulting in the removal of some associated lipid, a concomitant reduction in molecular weight and a dissociation of lipase (Morgan et al, 1969). Also significant amounts of lipase might be adsorbed on the gel during filtration and the bile salt might promote the desorption of this bound enzyme (Desnuelle et al, 1963). The nature of the fast multimolecular enzyme complex is now believed to be an aggregate of lipase molecules and lipids, especially phos-

pholipids which form a tenacious association with the enzyme (Verger et al, 1969). Fast lipase is believed to be an artifact formed during the extraction of fresh tissue or powder with water (Schoor and Melius, 1969).

2.7 CRITERIA OF PURITY

The degree of purification of the enzyme with each purification step should be measured to determine when an enzyme solution is pure or if further application of purification techniques is necessary. Two of the most definitive determinations of purity are polyacrylamide gel electrophoresis and isoelectric focusing. Disc gel electrophoresis provides a technique whereby the isoenzyme patterns of the protein may be identified and studied, as well as the degree of purity of the enzyme isolated, while isoelectric focusing indicates the isoelectric point of the protein and the homogeneity of the enzyme.

Concentrated lipase preparations from Sephadex gel filtration columns have been shown to contain only a single esterase band when detected with α-naphthol esters on gel electrophoresis (Lawrence et al, 1967; Sugiura and Isobe, 1974). No isoenzymic patterns have been detected for lipase, indicating that activity is confined to one protein fraction (Lawrence et al, 1967).

Sugiura and Isobe (1974) obtained a homogeneous enzyme when an isoelectric focusing gradient was applied to a slow lipase preparation from Chromobacterium viscosum, while an isoelectric focusing profile obtained from a fast lipase

preparation of Corynebacterium acnes illustrated that the lipase activity did not completely coincide with the one peak, indicating the presence of other material (Hassing, 1971). The isoelectric point of the slow lipase of Chromobacterium viscosum was found to be pH 6.9 (Sugiura and Isobe, 1974), while that of the fast lipase Corynebacterium acnes was estimated to be pH 3.8 (Hassing, 1971). The isoelectric point of hog pancreatic lipase has been estimated to be pH 5.2 (Marchis-Mouren, 1959).

2.8 MOLECULAR WEIGHT OF LIPASE

The molecular weight determination of proteins by gel filtration has been achieved by the construction of calibration curves relating elution volume to molecular size. The molecular weight of "fast" lipase of hog pancreas was first estimated by gel filtration to be 300,000 (Gelotte, 1964; Ramachandran et al, 1970), while the slow form was estimated to be 38,000 (Sarda et al, 1964). Downey and Andrews (1965) experienced some problems arising in the application of gel filtration to the study of lipase systems, as several esterase fractions of varying molecular weights were fractioned. Extracts of rat adipose tissue gave molecular weights of 200,000, 75,000, 55,000 and 39,000 while that of hog pancreas was estimated to be 42,000. Activity of acetone-dried hog pancreatic lipase was confined in material of molecular weights of 10^6 and of 180,000, which were suggested to be aggregate forms of the low-molecular weight enzyme. It is now believed that the fast lipase, being an aggregate of slow

lipase, has a molecular weight in excess of 200,000, while the slow monomolecular lipase has a range of 55,000-25,000 (Vandermeers and Christee, 1968). The molecular weight of the pig pancreas lipase, determined by ultracentrifugation, is now revised to 48,000 (Verger et al, 1969), while those of some bacteria are relatively small molecules with values of 27,000 for Chromobacterium viscosum (Sugiura and Isobe, 1974) and 25,000 for Micrococcus (Lawrence et al, 1967), with 250,000 for the "fast" lipase form. Lipase isolated from Veronia anthelimintica had a molecular weight in excess of 200,000 (Olney, et al 1968), while a molecular weight of less than 50,000 was demonstrated for tobacco leaf lipase (Conners and Diffendall, 1968).

Molecular weight determination by electrophoresis on polyacrylamide gel in the presence of solium dodecyl sulphate has been used in more recent years because of the advantage of the high resolving power of this method over gel filtration. Disc gel electrophoresis has been used to determine molecular weights of rat pancreatic lipase, with a value of approximately 32,000 (Gidez, 1973), and of peanut alkaline lipase with a value of 55,000 (Sanders and Pattee, 1972).

2.9 CHEMICAL PROPERTIES OF LIPASE

2.9.1 Substrate Concentration

The Michaelis-Menten constant (Km), calculated using a Lineweaver-Burk plot, indicates the substrate concentration at which one-half of the enzyme reaction maximum velocity is developed. Using tributyrin as the general substrate, Km

values have been obtained for oat lipase of 6.0 mM (Martin and Peers, 1953), 0.26 mM for peanut alkaline lipase (Sanders and Pattee, 1972) and 0.031 mM for wheat germ lipase (Pancholy and Lynd, 1972), while a value of 47.6 mM has been obtained for Leptospiral lipase (Patel et al, 1964).

2.9.2 Effect of pH

The effect of pH on the rate of hydrolysis by lipase is shown not only on the enzyme itself, but also on the emulsified substrate. For most lipases the pH optimum ranges between 7.5 and 9.2. The optimum pH of pancreatic lipase is between 7.5-8.0, (Sarda et al, 1964). The normal pH range for lipase from plant and microbial sources lies between 8.0 and 9.0, with an optimum of pH 8.5 determined for peanut alkaline lipase (Sanders and Pattee, 1972) and for Leptospiral lipase (Chorvath and Benzonana, 1971).

2.9.3 Temperature

With a few exceptions, lipases are most active within the temperature range of 30° to 40°C, with an optimum temperature of 37°C. Several studies have been made of lipase stability at elevated temperatures. Purified fractions of the enzyme were virtually destroyed at 60°C for 25 minutes, while temperatures approaching 50°C drastically altered activity (Wills, 1960). Noticeable loss in enzyme activity is observed in 6-8 hours on standing at room temperature (Sanders and Pattee, 1972).

2.9.4 Action of Activators

Kinetic investigations on lipase action are advantageously performed in simple systems where no molecules

other than those of the substrate are present at the interface. However, lipase activity depends on various compounds which play an important part in the lipolysis of long-chain triglycerides.

(a) Sodium chloride activates the lipolysis of insoluble long-chain and short-chain triglycerides. In the complete absence of the salt, lipolysis does not proceed (Benzonana and Desnuelle, 1968), and it has been suggested that the sodium ions suppress the pH gradient at the interface and lower the apparent pK_a of the fatty acids, which can then be more quantitatively titrated (Erlanson and Borgstrom, 1970).

(b) Bile salts have a specific activating effect on lipase, but as they are surface-active agents, the increased rate of triglyceride hydrolysis produced is primary an emulsification effect resulting in an increase of the fat-water interfacial area, and does not directly affect the enzyme.

While bile salts cannot be regarded as true cofactors of lipase, they promote lipolysis and this is based on the theory that bile salts retard quite appreciably the inhibitory effects of fatty acids and soaps (Borgstrom, 1964). Benzonana and Desnuelle (1968) showed that deoxycholate does not augment the initial lipolysis rate, but improves the linearity of hydrolysis with time. High concentrations of bile salts display strong inhibitory effects, especially on pancreatic lipase, this inhibition being attributed to

substrate displacement and charge effects at the interface (Morgan et al, 1969). Another known effect of bile salts is to shift the optimum pH of pancreatic lipase from pH 8.0-9.0 to 6.0 (Borgstrom, 1954), but other workers found that activation could be observed in an alkaline medium but not in an acid medium (Wills, 1954).

(c) Calcium chloride - the activation of lipolysis by calcium chloride was demonstrated by Willstätter et al (1923) and has been confirmed on numerous occasions. The effect of calcium ions has been assumed to result from the removal of unionized fatty acids from the interface through the formation of insoluble calcium soaps (Benzonana and Desnuelle, 1968). Calcium ions do not affect the initial velocity of lipolysis, but in the absence of calcium the reaction is soon inhibited, while increasing concentrations lead to more linear reaction rates. Constantin et al (1960) found that in the absence of calcium, diglycerides were the main end-product of lipase hydrolysis of a triglyceride, but the addition of the salt causes the formation of monoglycerides and glycerol.

2.9.5 Inhibition of Lipase

In common with many hydrolytic enzymes, lipase is inhibited by heavy metals. Pancreatic lipase is strongly inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+} (Wills, 1960), while peanut alkaline lipase was completely inhibited by a concentration of 1.5×10^{-4}M $HgCl_2$ (Sanders and Pattee, 1972).

The use of specific sulphydryl reagents has been made to establish whether or not lipases are -SH enzymes. Lipase treated with a relatively high concentration of 1.0 mM p-chloromercuribenzoate resulted in only 40% inhibition of the enzyme (Wills, 1960). Experiments with -SH reagents led to the general opinion that lipases do not contain -SH groups as part of their active site.

CHAPTER 3

MATERIAL AND METHODS

MATERIALS

3.1 ENZYME SOURCE

Lipase was isolated from small faba beans (Vicia faba L var. minor cv. Ackerperle) which were obtained from the Department of Plant Science, University of Manitoba. The beans were harvested in 1975 and subsequently stored at room temperature. Commercial hog pancreatic lipase was purchased from the Sigma Chemical Co.

3.2 CHEMICALS

Tributyrin, olive oil U.S.P., gum arabic, sodium deoxycholate and α-naphthyl acetate were purchased from Fisher Scientific Co., New Jersey. Acrylamide, N,N-methylene bisacrylamide, N,N,N',N'- tetramethylethylenediamine (TEMED) and Riboflavin were obtained from Eastman Kodak Co., Organic Chemicals Division, Rochester, New York. Sephadex G-100, ribonuclease A, chymotrypsinogen A, pepsin, ovalbumin and aldolase were obtained from Pharmacia Fine Chemicals. Ammonium persulphate, bovine serum albumin and LKB Ampholine carrier ampholyte, pH 3.0-10.0, were obtained from British Drug Houses Chemicals, Poole, U.K., while sodium taurocholate was purchased from ICN - K & K Laboratories. Mercaptoethanol and Brilliant Blue G were purchased from the Sigma Chemical

Company and sodium dodecyl sulphate (SDS) was purchased from Matheson, Coleman and Bell. Mazola corn oil was purchased locally and other chemicals and reagents were of analytical grade.

METHODS

3.3 ASSAY PROCEDURE

The lipase assay was a modification of the titratable acidity procedure of San Clemente and Vadehra (1967). Lipase activity was determined by measuring the rate of hydrolysis of a triglyceride emulsion by potentiometric titration, the initial velocity of lipolysis being followed by continuous titration with 0.01 N NaOH at a constant pH (8.5) at 37°C by using a pH stat (Radiometer pH meter pHM 26C connected to a Radiometer titrator type TTT 1), coupled to a recorder (Honeywell Electronic 19), an electric interval timer (model 167 by Dimco-Gray Co.), and a Blue M. Electric Company Magni Whirl constant-temperature water-bath. The emulsified substrate (5% olive oil or corn oil, or 5.0-40.0mM tributyrin stabilized with 10% gum arabic solution by high-speed blending for 1-3 minutes) was introduced into the reaction flask with activators (5.0 mM $CaCl_2$,1.0 N NaCl and 10.60 mM Na deoxycholate or taurocholate) to a final volume of 15 millilitres. The assay mixture was continuously stirred and bubbled with CO_2 - free N_2. The pH was adjusted to pH 8.5 after which between 1.0 - 5.0 millilitres of enzyme solution were added and the pH readjusted to 8.6. When the pH meter

nulled at pH 8.5, zero time was recorded, and the rate of base addition was determined for 5 - 10 minutes. Controls were carried out to check nonenzymic hydrolysis of the substrate, which was found to be negligible below pH 10.0. Wherever possible, the final assay volume of emulsion was not more than three times the initial volume, to avoid complete dissolution of the emulsified substrate by excessive dilution. One unit of lipase activity is equal to one micromole of acid produced per minute at 37°C. This is calculated from the formula:

$$\text{Lipase Activity} = \frac{\text{ml NaOH/min} \times \text{N NaOH} \times 1000}{\text{mg. protein used}}$$

3.4 PROTEIN DETERMINATION

Protein was determined by the method of Lowry *et al*, (1951). Reagents were modified according to Miller (1959). Commercial Folin-Ciocalteu reagent was diluted ten times immediately before use. Crystalline bovine serum albumin served as the standard for the construction of the calibration curve. The absorbance of the blue solutions was determined on a Unicam SP 600 spectrophotometer at 680 nm.

3.5 PARTIAL PURIFICATION

3.5.1 Preparation of Acetone Powder

Whole faba beans were dehulled and ground to a powder in a Wiley Mill. The powder was extracted in 3-4 volumes of acetone by high-speed blending in a Waring blender, and filtered with suction through Whatman No. 4 filter paper. A light-coloured, low-density solid which decanted with the

acetone was collected, while considerable dark fibrous residue remained in the blender. The tan precipitate was washed with several successive portions of acetone, air-dried and broken up before being stored at 4°C.

3.5.2 Partial Purification

A crude extract was obtained by blending 10-50 g. of acetone powder with 10 volumes of 0.9% sodium chloride solution. The solution obtained was centrifuged at 10,000 r.p.m. for 20 minutes at 0°C and the supernatant collected and assayed for lipase activity, and stored at 4°C.

Many methods were tried in attempting to purify the crude extract, such as ammonium sulphate precipitation which did not precipitate the enzyme, while adjustment of the pH precipitated the enzyme, but was unsuitable as the enzyme was denatured and could not be reprecipitated. Ethanol precipitation proved to be successful with some limitations and some loss of enzyme activity. The crude extract was concentrated by the slow addition of precooled (-40°C) 95% absolute ethanol with constant stirring, the temperature of the mixture being continuously checked to ensure that the temperature did not rise above -15°C. The final ethanol concentration was first brought to 30% whereupon the mixture was immediately centrifuged at 8,000 r.p.m. for 20 minutes at 0°C, with the precipitate obtained being dissolved in a small volume of water, assayed for lipase activity, and discarded. The supernantant was then brought to a final ethanol concentration of 50% and then centrifuged at 8,000 r.p.m. for 20 minutes at 0°C. The precipitate obtained was dis-

solved in a small volume of water, assayed for lipase activity, and stored at 4°C. The ethanol - precipitated lipase was used for the kinetic studies.

3.5.3 Gel Filtration

Sephadex G-100 gel filtration was performed on the ethanol-precipitated fraction, using a 2.5 x 30 cm column previously equilibrated with 0.01 M sodium phosphate buffer, pH 7.0 at 4°C. Five-millilitre fractions were collected automatically using an LKB 7000 UltraRac fraction collector, and protein elution was monitored continuously by absorbance at 280 nm on a ISCO Model UA-4 absorbance monitor. Runs were carried out by applying to the column a linear gradient of 0.0 - 0.4 M NaCl solution, or by the incorporation of 10.60 mM sodium deoxycholate, in the eluting phosphate buffer (0.01 M pH 7.0). Each fraction was then assayed for lipase activity. Active fractions were pooled and stored.

The column was prepared by first allowing the gel to swell in 20 volumes of phosphate buffer at 90°C for three hours, after which the mixture was placed under vacuum for one hour to remove all entrapped air. The column was poured by allowing the gel bed to develop under minimum pressure (Determan, 1968).

3.6 DISC GEL ELECTROPHORESIS

Disc electrophoresis on polyacrylamide gel was performed essentially by the method of Ornstein and Davis (1964). For all experiments described, an anionic system was adopted with a 7.5% polyacrylamide gel and a running pH of 9.3.

3.6.1 Gel Preparation

Gel solutions and gels employed are described in the Polyanalyst Instruction Manual (Buchler Instruments Inc. Fort Lee, New Jersey), with the modification of the incorporation of 1.0% (final concentration) of soluble starch in the lower gel. The compositions of the gel and buffer solutions are given in Table 1. The stock solutions were stored individually in brown bottles at $4^{o}C$ for four weeks, while fresh solutions of ammonium persulphate and of riboflavin were prepared before each experiment. Gels were formed in tubes of 10 cm in length and an internal diameter of 6.0 mm, and were polymerized by the use of photopolymerization lamps.

3.6.2 Sample Applications

Samples were saturated with sucrose and layered directly on top of the medium-pore gel. Large volumes of samples were necessary (0.1-0.2 millilitres of extract) because of the low concentration of enzyme in the extracts. The sucrose solution was used to keep the samples in the tubes without mixing and floating in the upper buffer, by virtue of its higher density.

3.6.3 Electrophoretic Separation

Protein separation was carried out using the Savant disc electrophoresis apparatus with a Buchler power supply #31014 A. The proteins were first concentrated in the gel by the application of a current of 3 mA across each tube for the initial 30 minutes and then increased to a constant current of 5 mA per tube until the marker dye (bromophenol blue)had migrated near the end of the gels (approximately 50-60 minutes). Electrophoresis was conducted at $4^{o}C$ to

TABLE 1

ANIONIC GEL SYSTEM
RUNNING pH 9.3

GEL	VOLUME RATIOS	COMPONENTS/100 ML.		pH (25°C)
Lower Gel	1	Acrylamide	30 gm	
		Bisacrylamide	0.8 gm	
		Water to Volume		
	1	Tris	18.15 gm	9.1
		1N HCL	24 ml	(at 0°C pH 9.56)
		TEMED	0.24 ml	(or 0.4 ml at 0°C)
		Water to Volume		
	1	Ammonium Persulphate	0.28 gm	(or 0.8 gm at 0°C)
2		Water to Volume		
	1	4% Starch Solution		
Upper Gel	1	Acrylamide	10 gm	
		Bisacrylamide	0.8 gm	
		Water to Volume		
	1	Tris	2.23 gm	(or 2.16 gm at 0°C)
		1M H_3PO_4	12.8 ml	(at 0°C pH 6.86)
		TEMED	0.1 ml	6.7
		Water to Volume		
	1	Riboflavin	4 mgm	
		Water to Volume		
	1	Ammonium Persulphate	160 mgm	
		Water to Volume		

TABLE 1 (Cont'd)

	COMPONENTS/LITER		pH (25°C)
Upper Buffer	Tris	5.16 gm	8.91 (at 0°C pH 9.64)
	Glycine	3.48 gm	
Lower Buffer	Tris	14.5 gm	8.07 (at 0°C pH 8.84)
	1.0 N HCL	60 ml	
	Water to Volume		

maintain optimum enzyme stability.

3.6.4 Staining

(a) Detection of Protein Bands

The protein bands were developed by the immersion of the gels in 5% Amido Black in 7% acetic acid solution, and then destained electrophoretically using 7% acetic acid as the electrolyte, with a current of 7mA for 1½ hours. Gels were removed and the R_f values of the protein bands obtained using the dye marker as the electrophoretic front

(b) Detection of Lipase Activity Bands

After separation of proteins by electrophoresis, the gels were flooded with freshly-prepared buffered solutions containing 0.04% α-naphthyl acetate and 0.01% (w/v) Fast Blue B salt. The gels were incubated in a mixture of three parts of ethanol and two parts of 10% acetic acid for two hours at 37°C. Lipase activity was shown by the appearance of dark red bands (Marker and Hunter, 1959; Lawrence et al, 1967).

3.7 ISOELECTRIC FOCUSING

Polyacrylamide gel was used as the medium to carry out isoelectric focusing experiments in order to stabilize the pH gradient. The procedure required disc gel electrophoresis equipment similar to that used for protein separation (Maurer, 1971). The gel system used was that of Wrigley (1970) shown in Table 2, employing LKB Ampholine carrier ampholyte # 8141 (40% solution). The sample was applied directly into the gel by dissolving the sample into the work-

TABLE 2

GEL ISOELECTRIC FOCUSING SYSTEM

STOCK SOLUTION NO.	COMPONENTS/100 ML AQUEOUS SOLUTION	MIXING RATIO OF STOCK SOLUTIONS
1	Acrylamide 30 g. Bisacrylamide 0.8 g.	8.2 parts water 3.0 parts No. 1 0.8 parts No. 2 0.3 parts Ampholine
2	TEMED 1.0 g. Riboflavin 14.0 mg. Ampholine: LKB Ampholine Carrier Ampholytes, 40% Solution	

ELECTRODE SOLUTIONS

Upper Bath ⊕	Lower Bath ⊖
0.2% Sulphuric Acid	0.4% Ethanolamine

ing solution (Table 2), with the prescribed amount of water of the working solution being reduced correspondingly. High levels of samples were used due to the low concentration of lipase. The gels were photopolymerized and developed under running conditions of 3 mA constant current with the voltage increasing up to 350 volts for four hours at 4°C, using 0.2% sulphuric acid as the anode electrolyte and 0.4% ethanolamine at the cathode.

The pH range of the ampholyte was determined by segmenting the gels into 5 mm. lengths and suspending them in 2 ml. of distilled water for two hours. The pH was measured with microelectrodes using a Metrohm pH meter E 280 A. The sample gels were stained according to Grasslin et al (1970) by gentle agitating for 30-60 minutes in a solution of 0.2% Coomassie Brilliant Blue G250 in ethanol: water: glacial acetic acid (45:50:5 v/v) mixture and destained in a mixture of ethanol: water: glacial acetic acid (45:50:5 v/v).

3.8 MOLECULAR WEIGHT DETERMINATION

Sodium dodecyl sulphate-gel electrophoresis of proteins in the active peak of the Sephadex G-100 fractions was carried out to determine the molecular weight of the lipase enzyme from faba bean. The method described by Dunker and Rueckert (1969) was adopted. A calibration curve for molecular weight was obtained by the use of standard protein markers. The protein markers and enzyme samples were denatured by dissolving 2.0 mg./ml. of protein in 1.0% 2-mercaptoethanol, 4 M urea and 1.0% sodium dodecyl sulphate

in 0.1 M phosphate buffer pH 7.2, and incubated at 45°C for one hour. The stock solutions and gels were prepared according to the procedure of Shapiro et al (1967), and photopolymerised for 30 minutes. The protein markers and enzyme samples were then layered directly on top of the gels and developed under running conditions of 7-9 mA per tube for six hours.

The gels were soaked in 20% sulfosalicylic acid for 24 hours to leach out the sodium dodecyl sulphate, and then stained by immersion for 12 hours in 0.02% Coomassie Brilliant Blue saturated with trichloroacetic acid. The dye was removed and gels allowed to stand in 10% trichloroacetic acid, until greatest resolution of the stains was obtained. Reference values were obtained from each gel by the measurement of distance travelled by sample proteins and protein markers and by the electrophoretic front and the results obtained were shown in the form of a semilogarithmic plot.

3.9 DETERMINATION OF MICHAELIS CONSTANT

Tributyrin was used as the substrate for the determination of the Michaelis constant (Km) since the enzyme exhibited greatest activity with this substrate. The effect of enzyme concentration on the reaction was determined prior to the Michaelis-Menten studies resulting in a linear relationship up to concentration of 34 mg/ml, which was used in the estimation of the Km value.

The Km value was established by determination of the initial velocities of various substrate concentrations

(5.0 - 40.0 mM), all runs being carried out in triplicate. The initial velocities were then plotted against their respective substrate concentrations. A reciprocal plot of velocity against substrate concentration was used to determine the K_m (Lineweaver and Burk, 1934).

3.10 EFFECT OF pH

Saturated substrate mixtures (40.0 mM tributyrin) were assayed for rates of hydrolysis using a fixed protein concentration (34 mg/ml) for the determination of the optimum pH of faba bean lipase. The pH range was obtained by varying the pH of the assays directly in the reaction vessel with 0.01 N NaOH or acetic acid.

In the determination of the effect of pH on the stability of the enzyme, aliquots of enzyme preparation (24 mg/ml) were adjusted to the desired pH, and at various time intervals, 1.0 ml. aliquots were removed and assayed at pH 8.5 for residual activity. The pH was adjusted as above.

3.11 EFFECT OF TEMPERATURE

Faba bean lipase was incubated at various temperatures from 15-60°C for two hours and were assayed for activity to determine the optimum temperature of the enzyme.

The heat stability of the enzyme was determined by the exposure of aliquots of enzyme in the absence of substrate, to different temperatures. Aliquots were removed at one-minute intervals and directly assayed for residual activity at 37°C at pH 8.5.

3.12 INHIBITORS AND ACTIVATORS

A series of experiments was performed to determine the effects of various compounds on the rate of activity of faba bean lipase. Inhibitors investigated were mercuric chloride and p-chloromercuribenzoate. Sodium chloride, calcium chloride and the bile salts sodium deoxycholate and taurocholate as potential activators were also tested. Inhibition or activation was calculated after determination of the rate of hydrolysis of tributyrin (40.0 mM) in the presence and absence of the various substrates at different levels of concentration. All investigations were completed in triplicate and reactions were allowed to proceed with an enzyme concentration of 24.0 mg/ml at pH 8.5 and 37°C.

CHAPTER 4

RESULTS

ENZYME PURIFICATION

Lipase activity in faba beans was first detected in a crude extract using an emulsified olive oil substrate. Enzyme activity was very low in the crude extracts and long periods of incubation were necessary to detect rates of hydrolysis, so that the enzyme had to be partially-purified before further studies could be undertaken.

Heat treatment at 55°C for 10-20 minutes was unsuccessful, while pH 5.0 treatment was unsatisfactory as the enzyme was denatured and precipitated with a great amount of unwanted protein. Attempts at salt fractionation revealed that the enzyme was labile to precipitation by ammonium sulphate.

A nine-fold purification of faba bean lipase was obtained using ethanol precipitation (Table 3). Concentration by ethanol was carried out by precipitation of the enzyme in a 30-50% final ethanol concentration range. Extreme care had to be taken that the temperature of added ethanol did not exceed -15°C, as it was found that the enzyme was easily destroyed at higher precipitation temperatures. Enzyme yields of 75-80% could be obtained with this method. This enzyme preparation was used in the initial experiments to determine some of its properties.

TABLE 3

PURIFICATION OF FABA BEAN LIPASE

PROCEDURE	VOLUME (mls)	ACTIVITY (units/ml)	TOTAL ACT. (units/ml)	PROTEIN (mg/ml)	SPECIFIC ACT. (units/mg)	YIELD %	PURIFICATION FOLD
Crude	1000	2.0	2000	18.0	0.11	100	1
30-50% Ethanol ppt.	30	53	1590	56.0	0.946	79.0	8.6
Sephadex G-100	45	20	900	10.0	2.0	45.0	18.2
Sephadex G-100 with 10.60 mM Na deoxycholate	25	20	500	4.5	4.4	25.0	40.0

Further purification of faba bean lipase was undertaken. Greater purification was essential as such properties as the molecular weight and isoelectric point of the enzyme were to be determined, and the partially-pure enzyme preparation obtained from ethanol fractionation was not sufficiently pure for these purposes. At this stage, a comparison of the properties of faba bean lipase to be further investigated was made with those of commercial hog pancreatic lipase, which was obtained as a crude lyophilized powdered preparation having a minimum activity of 10 units per milligram (one enzyme unit is that amount of enzyme which catalyzes the release of one μ equivalent of fatty acid per minute at 25°C). (Worthington Enzyme Manual, 1972).

4.2 GEL FILTRATION

Gel filtration was carried out on a Sephadex G-100 column previously equilibrated with 0.01 M phosphate buffer (pH 7.0) containing either a 0.0-0.2 M sodium chloride gradient, or a 10.60 mM sodium deoxycholate solution which was added directly to the phosphate buffer. Protein was estimated by measurement of absorbance at 280 nm, while lipase activity was determined by continuous potentiometric titration of fatty acids. The elution of concentrated lipase preparations from both faba bean and hog pancreatic sources on Sephadex G-100 with a salt gradient resulted in a single peak of lipase activity. Lipase obtained from runs of ethanol-fractionated preparation through the column resulted in an

18-fold purification over the original crude extract, with a yield of 45% (Table 3).

A typical diagram from the elution of commercial hog pancreatic lipase is shown in Figure 2, showing the elution pattern of lipase expressed as activity units, compared with the fractionation of the total protein being removed from the column. It is shown that lipase is detected in the first protein band that is eluted from the column, with activity being detected in a wide range, containing some 80 mls of effluent with detectable activity. In comparison, the typical diagram from the elution of faba bean lipase (Figure 3) resulted in a single lipase activity peak corresponding to the first protein band recovered from the column, but in this case, the lipase peak is detected almost immediately after the elution run has started, lipase activity being detected in 50 mls of effluent from the column. A greater proportion of nonenzyme protein is removed in the elution of faba bean lipase than that of pig pancreatic lipase, this being due to the fact that the latter preparations are of much greater purity than the ethanol-fractionated faba bean lipase extracts. The detection of the two single lipase peaks in similar positions on the elution patterns of both lipase preparations would indicate the similarity of the molecular size and shape of both enzymes. No attempt was made to determine the sodium chloride content in each fraction. Therefore, the gradient illustrated on the chromatographic patterns in Figures 2 and 3 is theoretical.

FIGURE 2

GEL FILTRATION OF PORCINE PANCREATIC LIPASE ON SEPHADEX G-100
ELUTION AND EQUILIBRATION WITH 0.01 M PHOSPHATE BUFFER pH 7.0

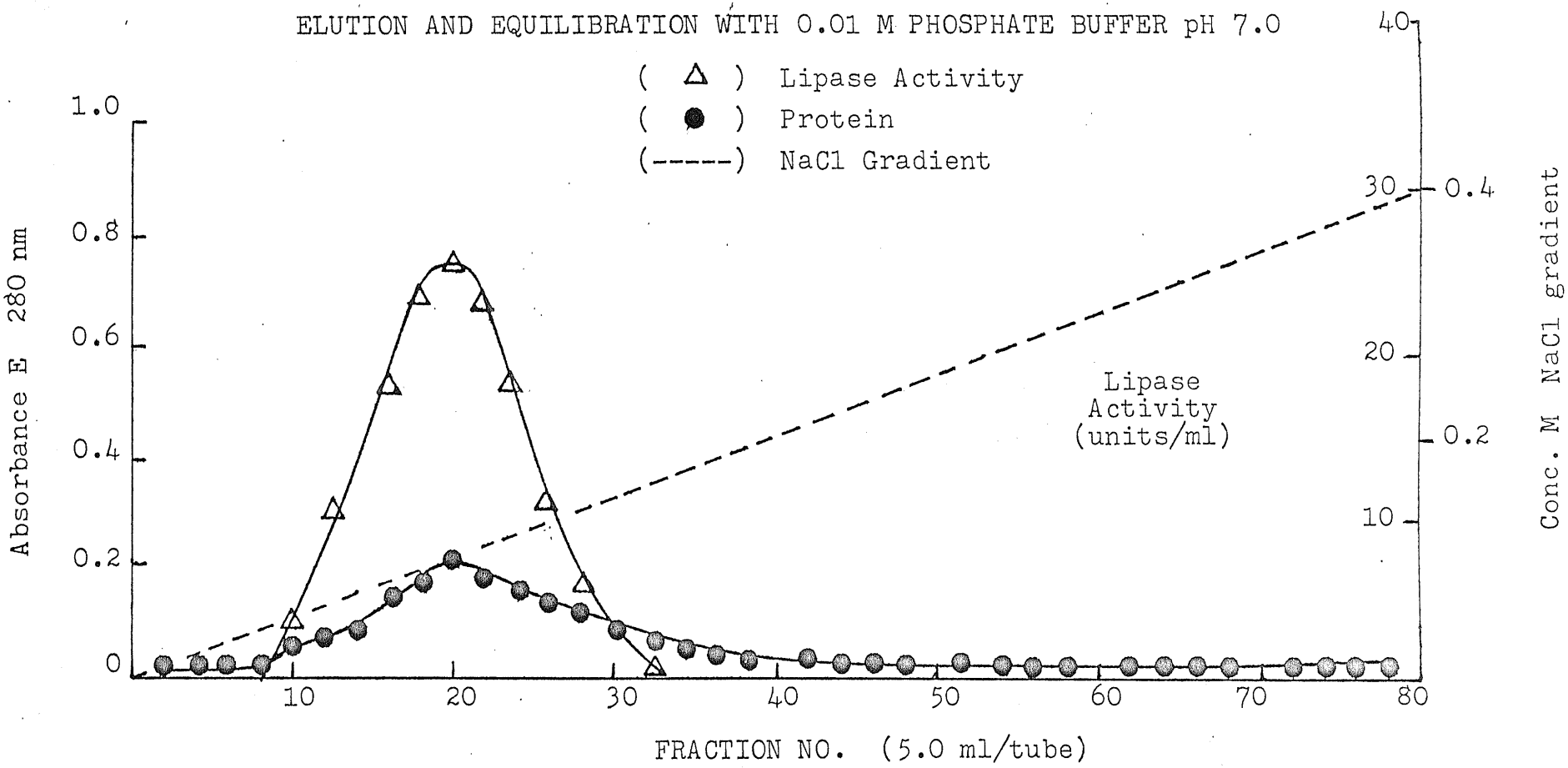
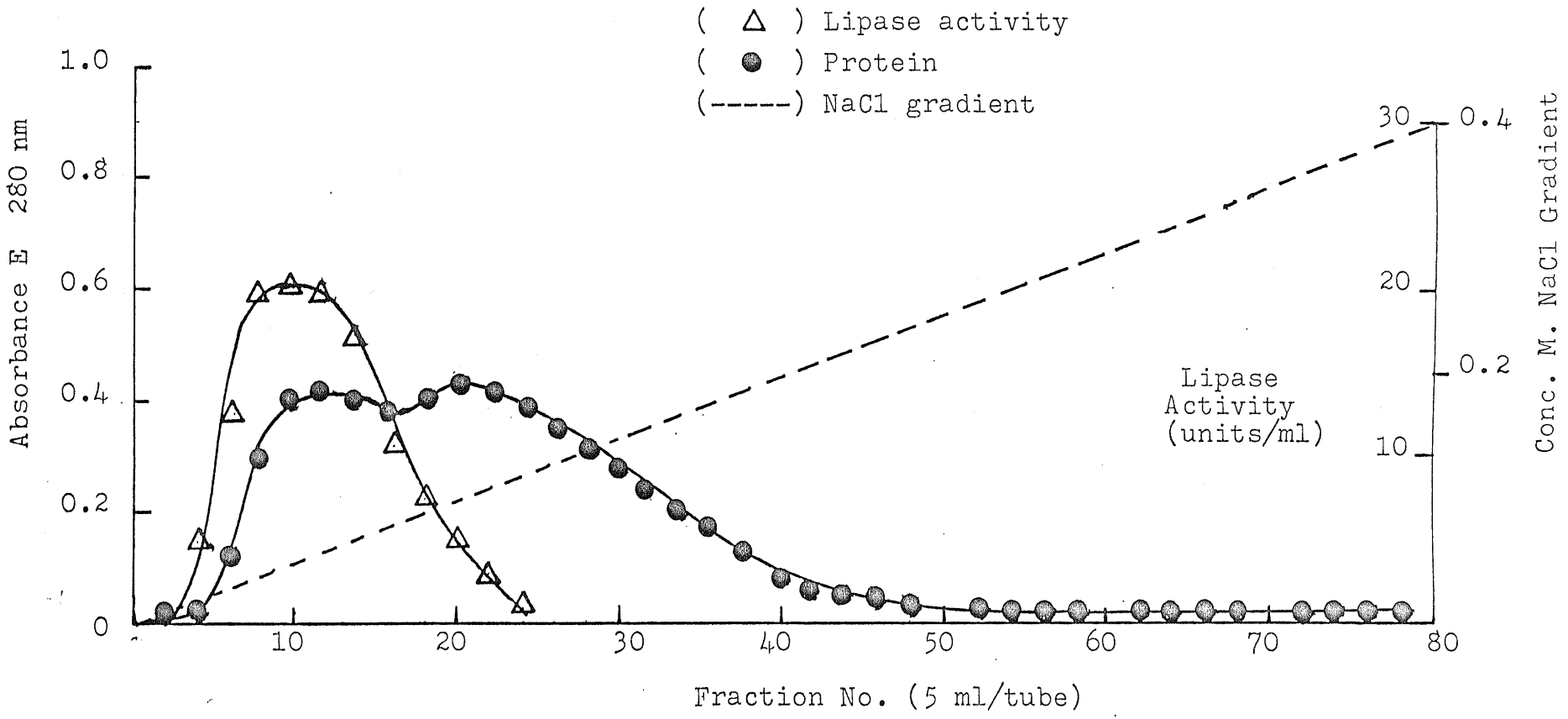


FIGURE 3 .

BEHAVIOR OF FABA BEAN LIPASE ON SEPHADEX G-100
ELUTION AND EQUILIBRATION WITH 0.01 M PHOSPHATE BUFFER pH 7.0



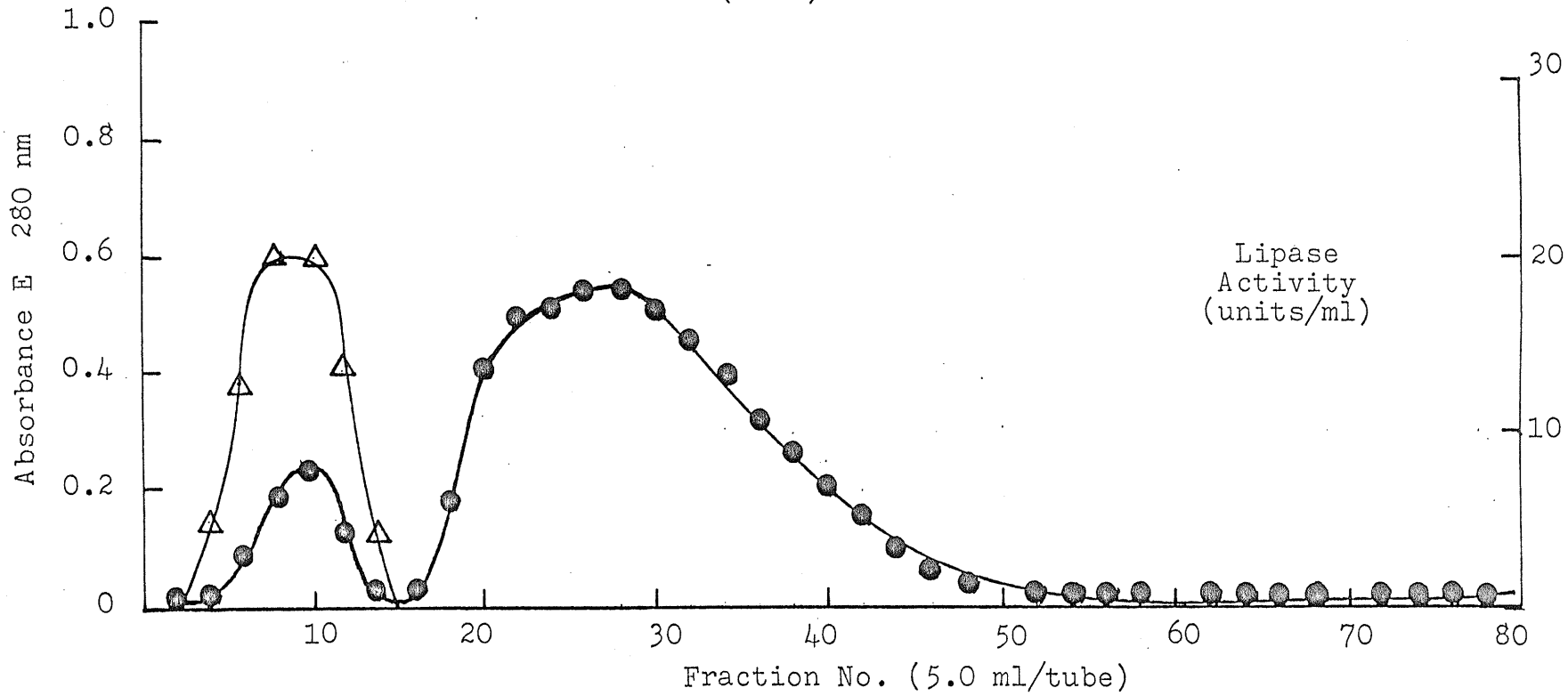
The presence of a single lipase peak detected in the first protein peak indicates that the lipase obtained by elution from Sephadex G-100 corresponds to the "fast lipase" described in the literature, and may be a high-molecular weight form of lipase. Gel filtration runs on faba bean lipase were undertaken in the presence of 10.60 mM sodium deoxycholate, hoping that the action of deoxycholate upon faba bean lipase would result in the removal of some associated phospholipid and inactive protein fractions. This would lead to the deaggregation of the multimolecular form of lipase, with the result that most of the lipase would appear further down the elution run as the low-molecular weight form known as "slow lipase". The result of this application of sodium deoxycholate to the elution of faba bean lipase from Sephadex G-100 is shown in a typical elution pattern (Figure 4). As with the other elution patterns, a single lipase activity peak is detected immediately after the start of the filtration run, corresponding to the "fast lipase" obtained in Figure 3. A "slow lipase" was not detected as had been expected, but some breakdown of the lipase band occurred in the presence of deoxycholate, with considerable inactive protein released behind the single active peak being shown in the profile, resulting in a sharper lipase peak with increased specific activity. Lipase obtained from deoxycholate-treated runs resulted in a 40-fold purification compared to the original crude extract and a yield of 25% (Table 3.)

FIGURE 4

GEL FILTRATION OF FABA BEAN LIPASE ON SEPHADEX G-100
ELUTION AND EQUILIBRATION WITH 0.01 M PHOSPHATE BUFFER pH 7.0
INCORPORATED WITH 10.60 mM Na DEOXYCHOLATE

(△) Lipase activity

(●) Protein



Runs with sodium deoxycholate on commercial pig pancreatic lipase were similar to those with a sodium chloride salt gradient, possibly due to the low protein content of the lipase preparation used.

4.3 EFFECT OF ENZYME CONCENTRATION

The correlation between the amount of enzyme and the reaction rate was investigated in order to determine protein concentration and the practicability of the titration technique for later studies on enzyme activity. The effect of enzyme concentration on the reaction rate is shown in Figure 5, with the initial velocity being strictly a linear function of the amount of enzyme present. A standard protein concentration of 34 mg/ml was used for later enzyme studies (except in the determination of the effect of pH on enzyme stability and the effect of various inhibitors on the enzyme, where a standard protein concentration of 24 mg/ml was used), this standard protein concentration being determined to be equal to one-half of the original enzyme preparation.

4.4 RATES OF SUBSTRATE HYDROLYSIS

The rates of hydrolysis by faba bean lipase of long and short-chain triglycerides are shown in Figure 6. Faba bean lipase demonstrated a slower rate of hydrolysis with long-chain triglycerides represented by olive oil U.S.P., and commercial Mazola corn oil, than that of short-chain triglycerides represented by tributyrin, and is in agreement with values reported for peanut alkaline lipase (Sanders and

FIGURE 5

EFFECT OF ENZYME CONCENTRATION ON
FABA BEAN LIPASE ACTIVITY

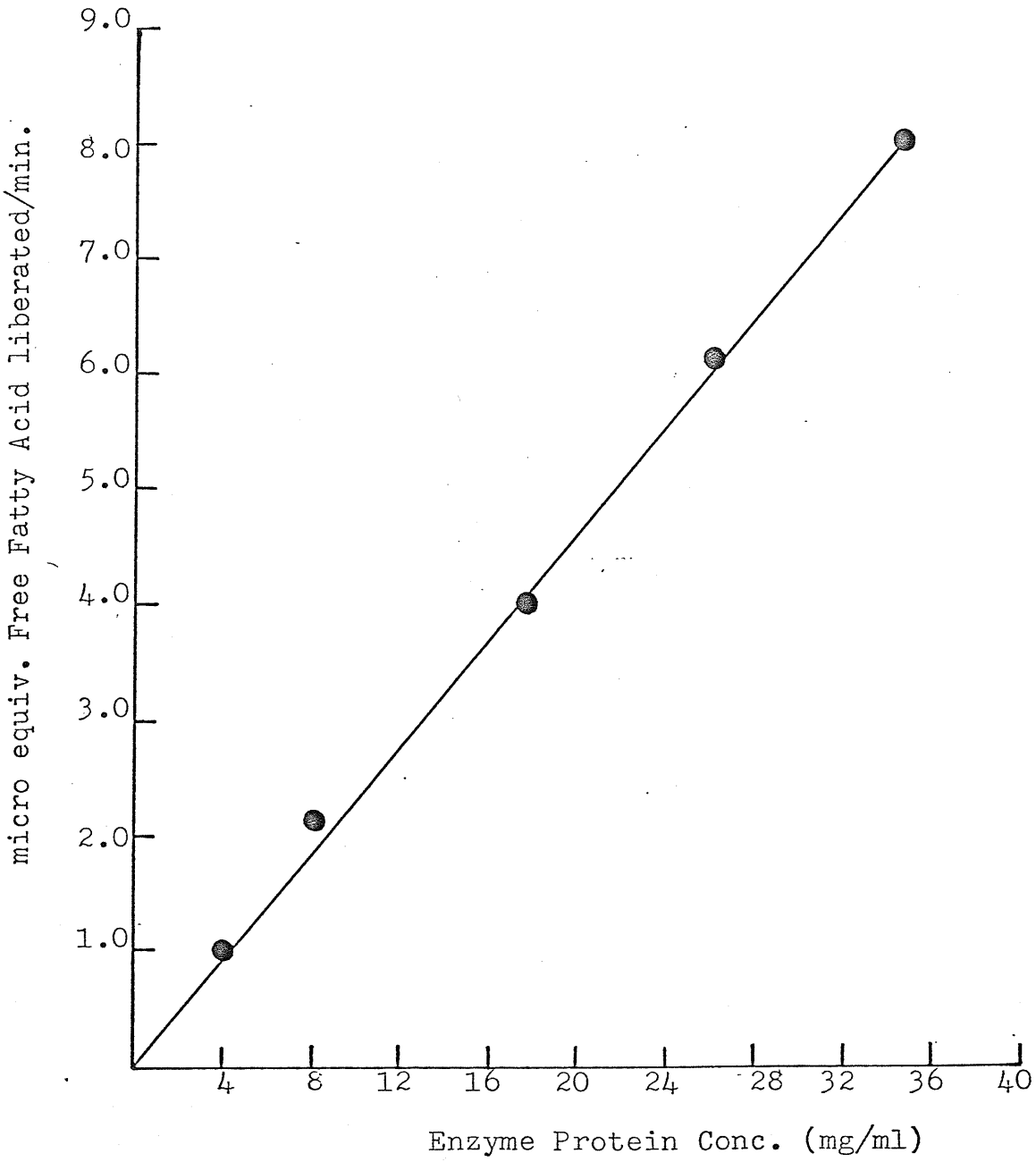
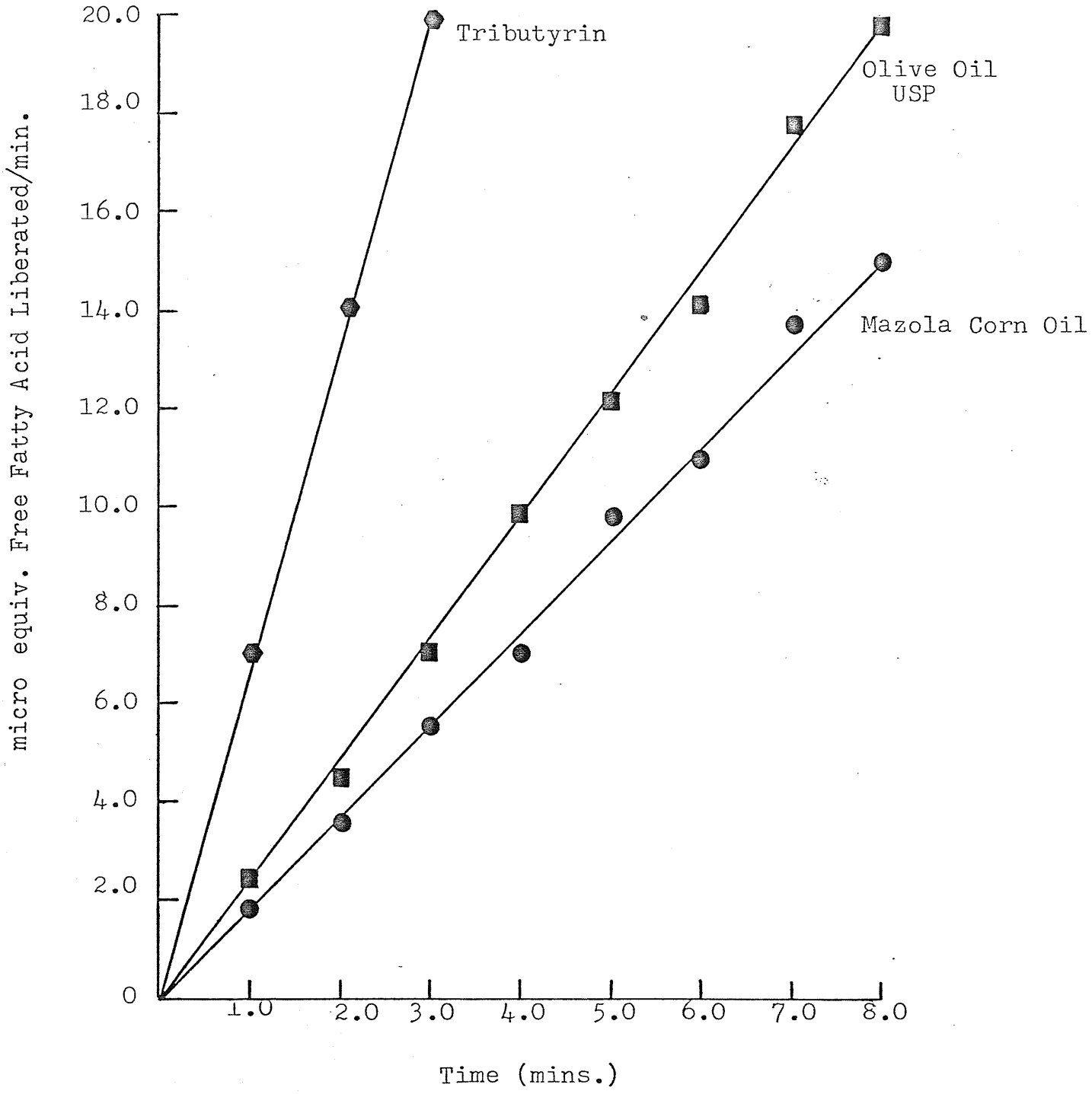


FIGURE 6

RATE OF HYDROLYSIS OF SHORT- AND LONG-CHAIN TRIGLYCERIDES BY FABA BEAN LIPASE



Pattee, 1972) and castor bean lipase (Ory et al, 1962). This indicates that tributyrin is the most suitable substrate for further studies on lipase. It must be noted that the lower rate of hydrolysis obtained for commercial corn oil may be in part due to the presence of a preservative, mono-isopropyl citrate.

4.5 SUBSTRATE CONCENTRATION AND CALCULATION OF MICHAELIS CONSTANT

The initial velocities in the presence of various tributyrin concentrations were ascertained and plotted against their respective substrate concentrations. The substrate tributyrin is expressed as a molar concentration, but as lipase-catalyzed hydrolysis occurs only in heterogeneous systems, substrate concentration is not strictly governed by the Michaelis rule relating the rate of the reaction to the molar concentration of the substrate in homogeneous systems. Here substrate concentration means the "emulsion" concentration, and as such is expressed as a molar term.

The effect of substrate concentration on lipase activity is shown as a typical rectangular hyperbole (Figure 7). Figure 7 shows the initial velocities plot of tributyrin in the presence of sodium deoxycholate and the addition of the bile salt is necessary in order to obtain a maximum linear kinetic curve, as well as the lower initial velocities in the absence of the bile salt.

Lineweaver-Burk plots gave straight lines (Figure 8) and from the intercept on the abscissa the apparent Michaelis

FIGURE 7

EFFECT OF SUBSTRATE CONCENTRATION ON FABA BEAN LIPASE

(■) In the Presence of Na Deoxycholate

(●) In the Absence of Na Deoxycholate

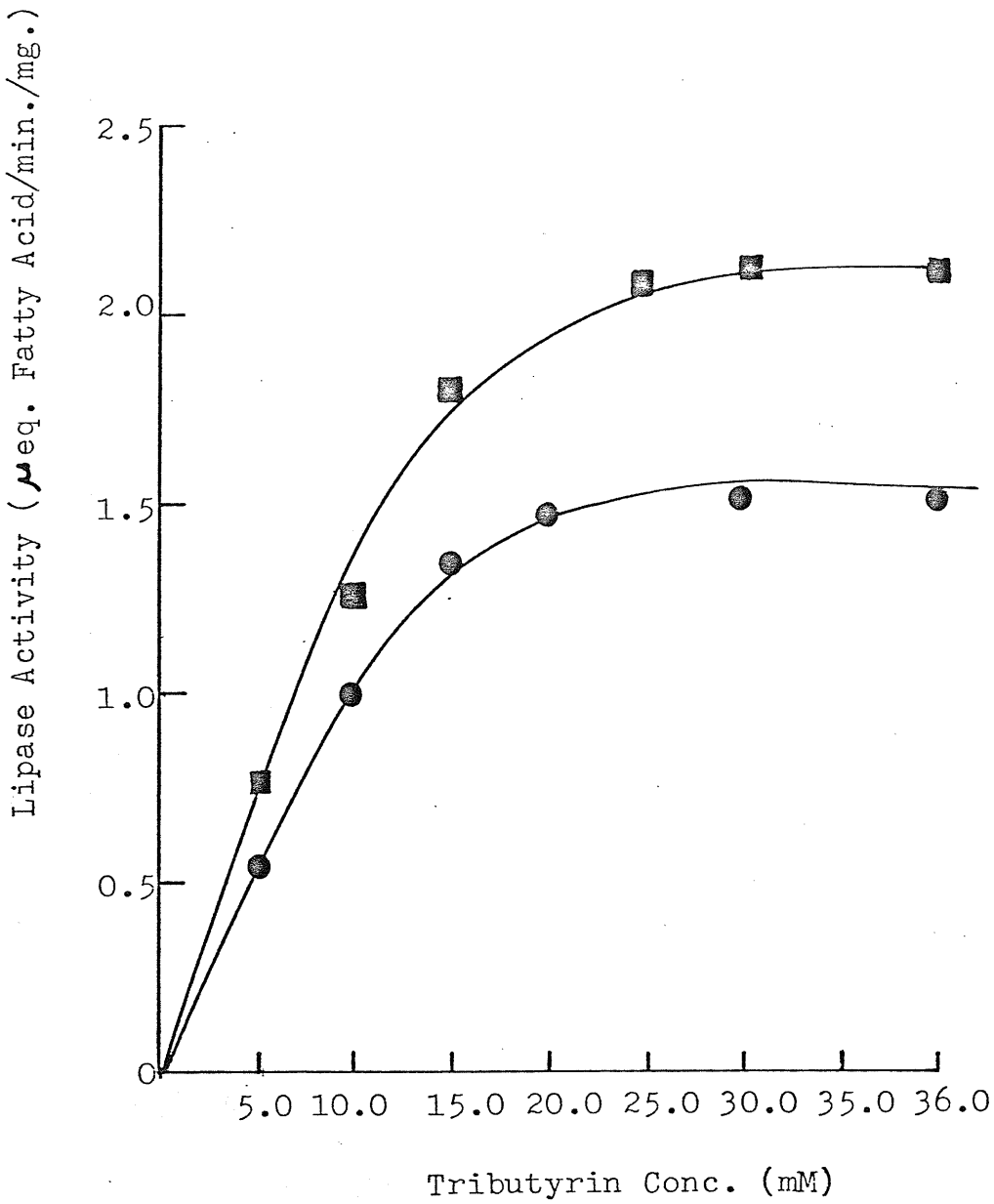
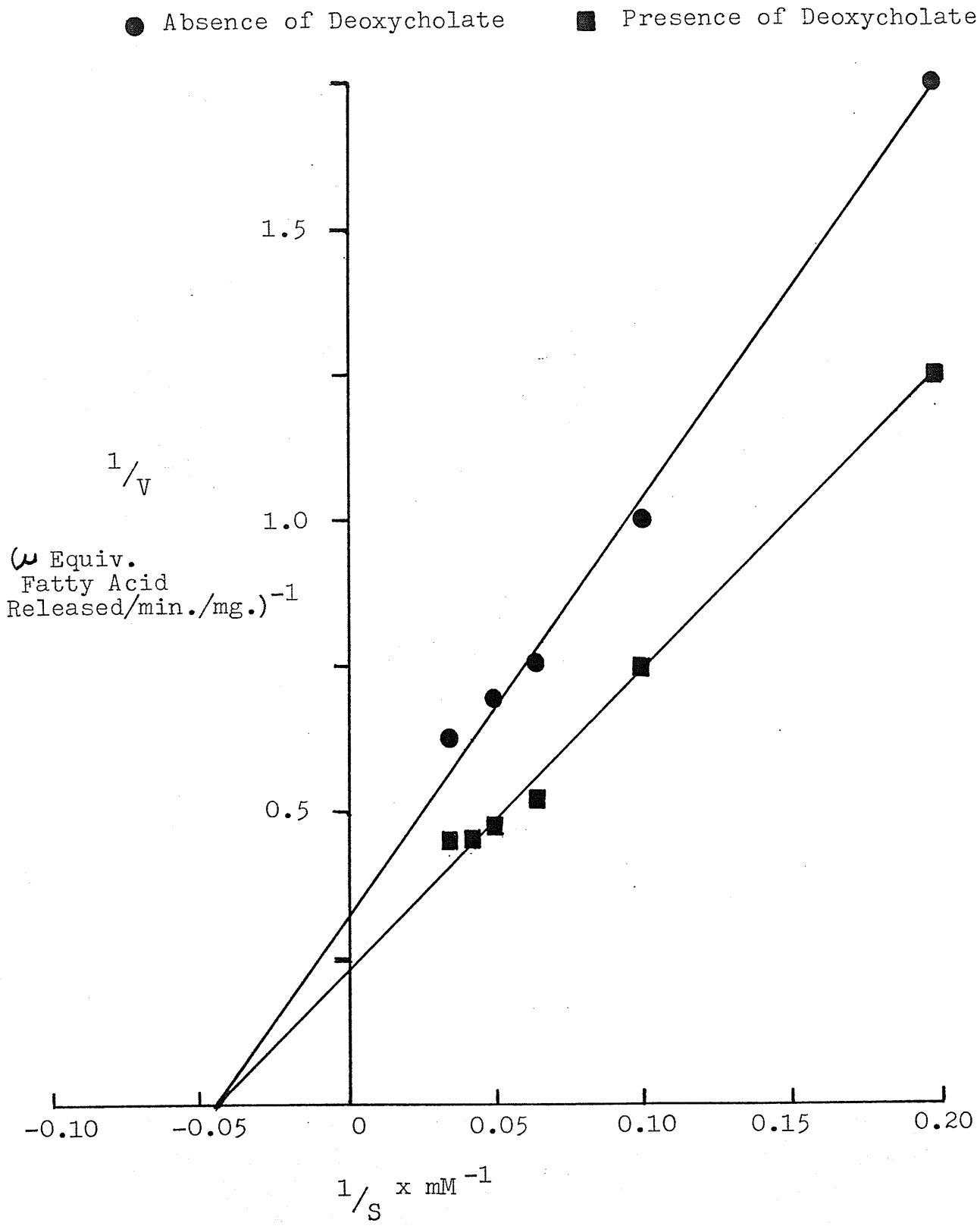


FIGURE 8

LINEWEAVER-BURK PLOT OF THE EFFECT OF SUBSTRATE CONCENTRATION ON FABA BEAN LIPASE ACTIVITY



constant (Km) has been calculated as 22.2 mM. This value corresponds favourably with that found for Leptospiral lipase of 47.6 mM (Patel et al, 1964) and 6.0 mM for oat lipase (Martin and Peers, 1953).

4.6 pH PROFILE

The influence of pH on the activity of faba bean lipase is presented in Figure 9. The pH profile shows enzyme activity within a pH range of 7.5-10.0, with the optimum at pH 8.5. Little or no activity was exhibited by the enzyme below pH 7.5, while at high pH values instantaneous hydrolysis of the substrate in the absence of the enzyme did not permit valid measurements.

4.7 pH STABILITY

The sensitivity of the enzyme to pH is demonstrated in Figure 10, where the stability of the enzyme to various pH values at different time intervals is measured as a percentage of residual activity. The results of experiments on the pH stability of the enzyme indicate that after a 10-minute interval, the enzyme is stable over a pH of 6.5-9.0, but after 20 minutes stability declines at higher pH values of 8.0 and above, while after 30 minutes, the enzyme is most stable at pH 7.0. This indicates that the enzyme is susceptible to both acidic and alkaline denaturation at 37°C.

FIGURE 9

EFFECT OF pH ON THE ACTIVITY OF FABA BEAN LIPASE

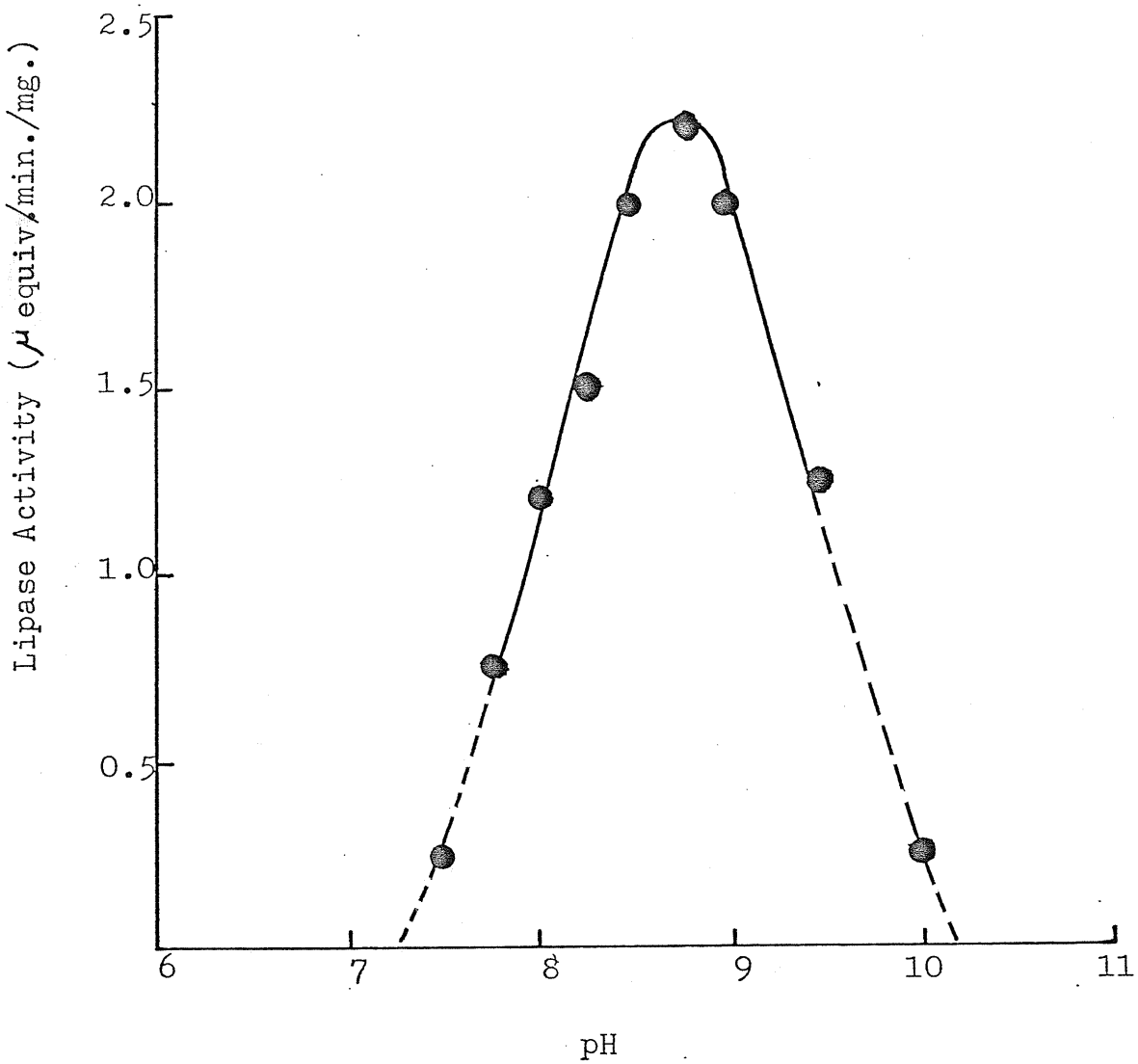
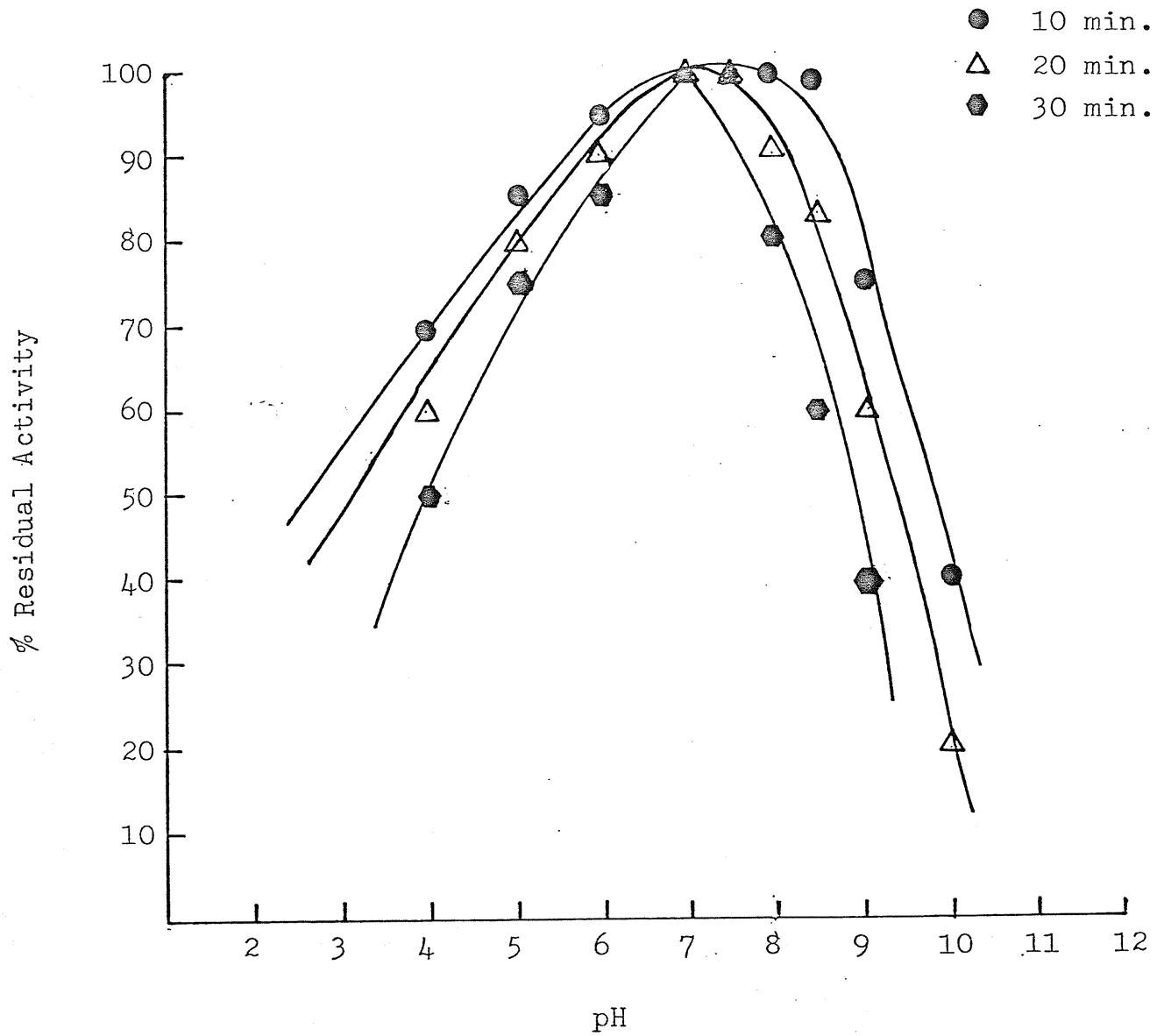


FIGURE 10

pH STABILITY OF FABA BEAN LIPASE



4.8 EFFECT OF TEMPERATURE

The activity of faba bean lipase is affected by temperature (Figure 11). Here the optimum temperature was determined to be 38°C, close to that obtained for pancreatic lipase (Sarda et al, 1964). The enzyme was not very active at or below 25°C while the activity rapidly declined at temperatures above 40°C due to enzyme denaturation. Stability of the enzyme to temperature is shown in Figure 12. Here the enzyme is subjected to preselected temperatures for various times. Figure 12 shows that the enzyme is stable at 38°C. The enzyme loses 50% of its initial activity at 51°C for ten minutes, while temperatures approaching 60°C drastically altered activity within five minutes, and almost completely destroyed after two to five minutes at 64°C. The temperature stability of the enzyme is similar to that obtained for peanut alkaline lipase (Sanders and Pattee, 1972), but is more heat-labile than castor bean lipase which lost 20% of its activity during 30 minutes at 60°C (Ory, 1960). Faba bean lipase is completely destroyed upon boiling, while loss in activity in crude and partially-purified extracts was noticeable in ten hours at room temperature, but each extract could be stored for two weeks at 0-4°C without any appreciable loss in activity.

4.9 ACTION OF SODIUM CHLORIDE

The effect of sodium chloride on the reaction rate of faba bean lipase is shown in Figure 13. The assay conditions are those of the standard assay except for the salt con-

FIGURE 11

EFFECT OF TEMPERATURE (OPTIMUM TEMPERATURE) OF FABA BEAN LIPASE

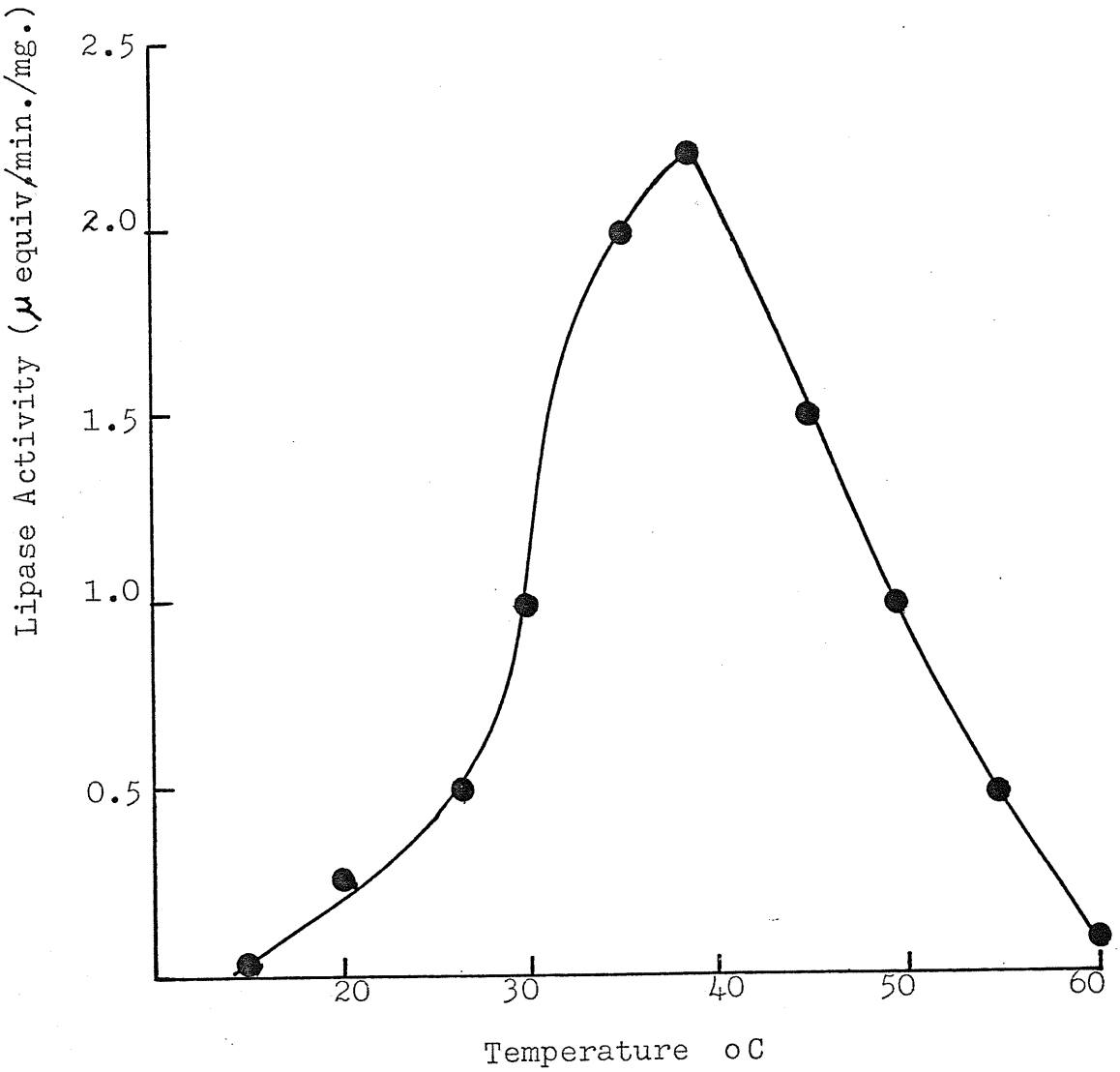


FIGURE 12

TEMPERATURE STABILITY OF FABA BEAN LIPASE

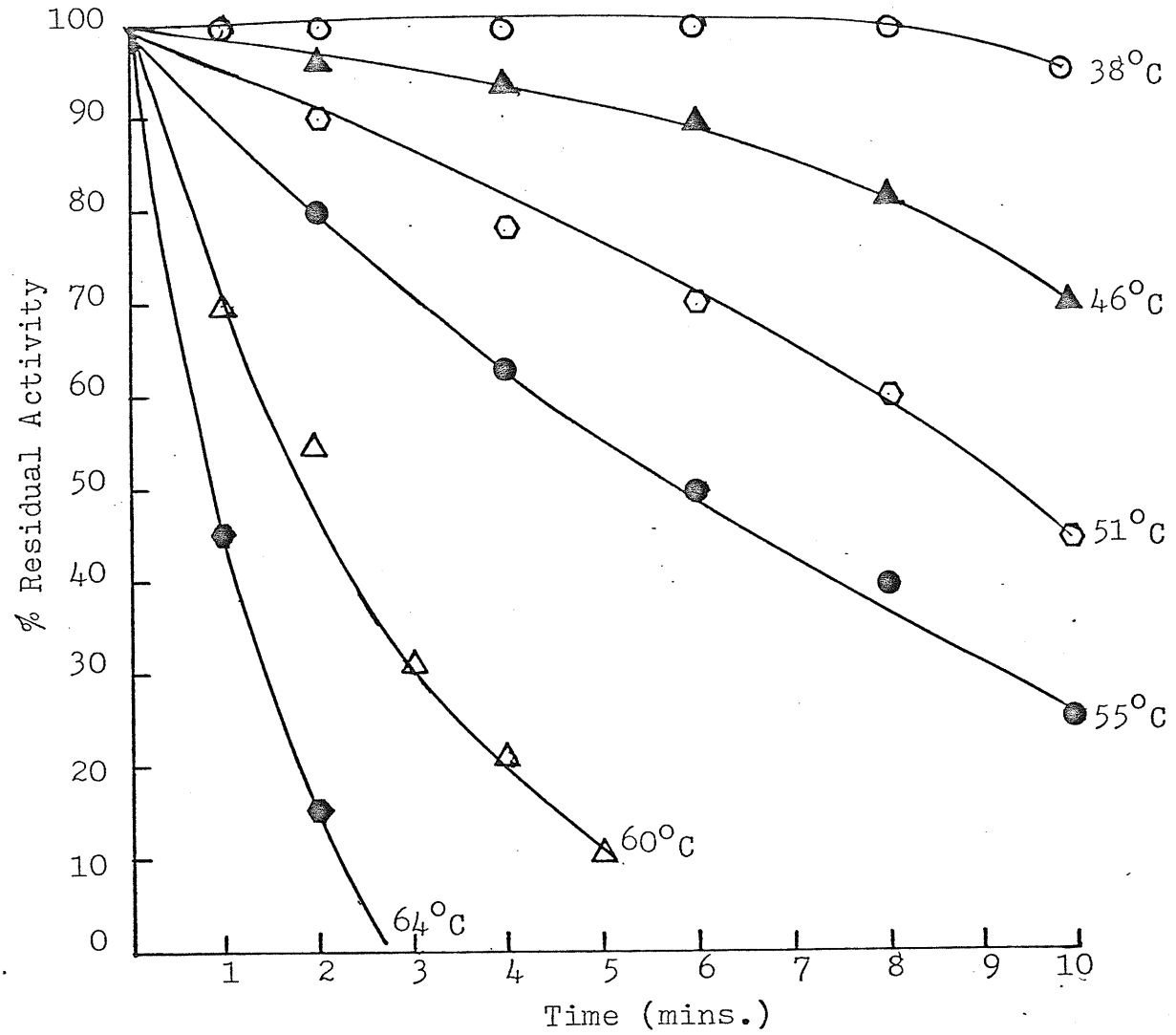
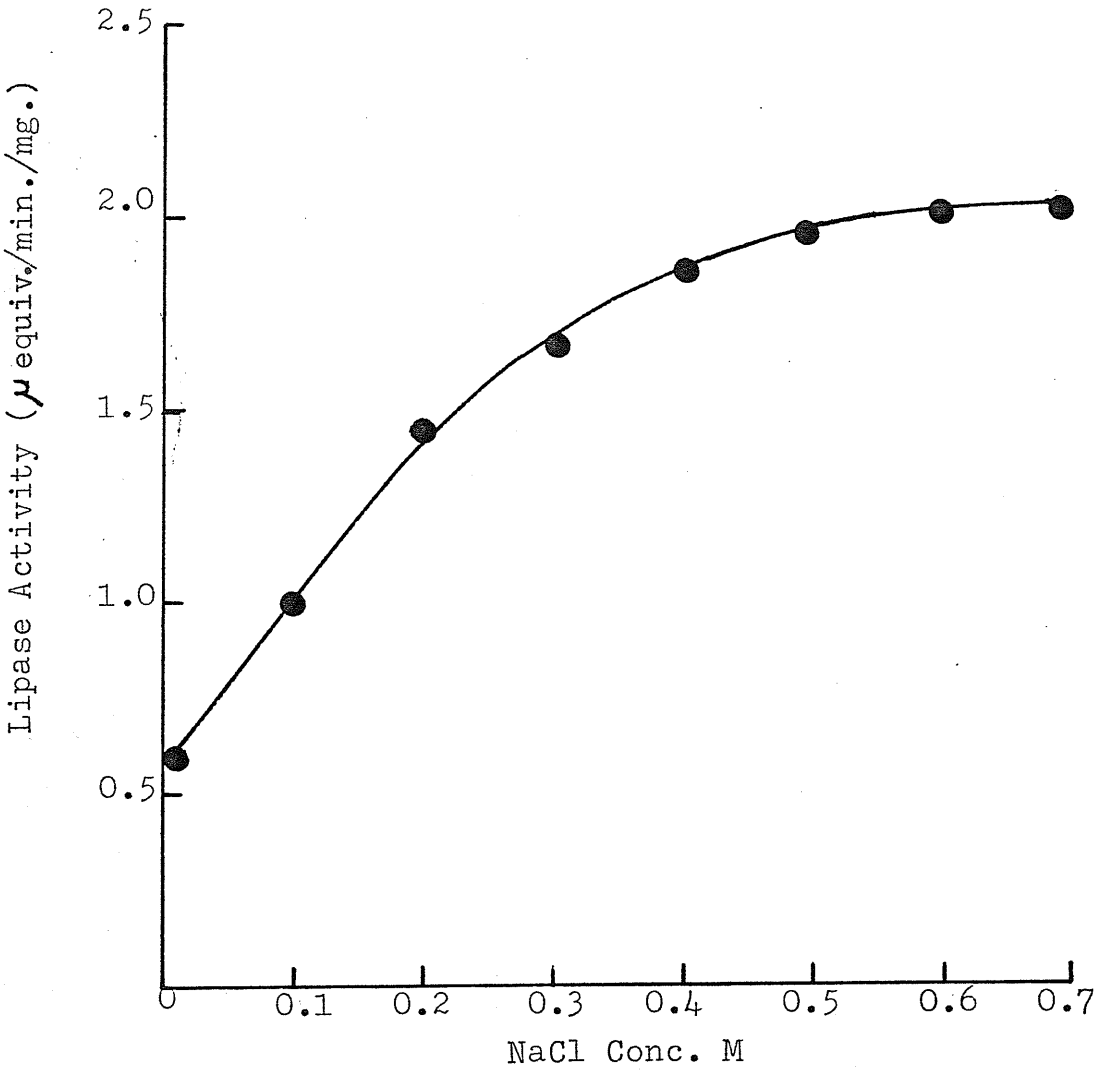


FIGURE 13

EFFECT OF NaCl on FABA BEAN LIPASE



centration. The enzyme is slightly active at low ionic strength (no NaCl in the medium). Activity is greatly increased with the addition of low concentrations of salt up to a maximum at 0.6 M NaCl, and then levels off at this value. This agrees with the values obtained by Benzonana and Desnuelle (1968) for pancreatic lipase, with a maximum activity attained in the presence of 70 mM sodium chloride.

4.10 ACTION OF $CaCl_2$ AND $MgCl_2$

The effect of calcium and magnesium chlorides on the activity of faba bean lipase is presented in Figure 14. Magnesium chloride shows a slight inhibitory effect at the higher concentrations employed. Calcium chloride, a known activator of pancreatic lipase, does not affect the activity of the faba bean enzyme up to a concentration of 0.3 M.

4.11 ACTION OF BILE SALTS

Variations of the bile salts (sodium deoxycholate and sodium taurocholate) concentration in the assay media induce the changes in the reaction rates shown in Figure 15, all other components being similar to those of the standard assays. The influence of the bile salts on faba bean lipase is to promote greater activity with the addition of low concentrations of bile salts being necessary. Sodium deoxycholate results in a greater activation of lipase activity than taurocholate, although enzyme activity attained a maximum value at about 12.0 mM concentration of the bile salts. No inter-

FIGURE 14

EFFECT OF $CaCl_2$ and $MgCl_2$ ON FABA BEAN LIPASE

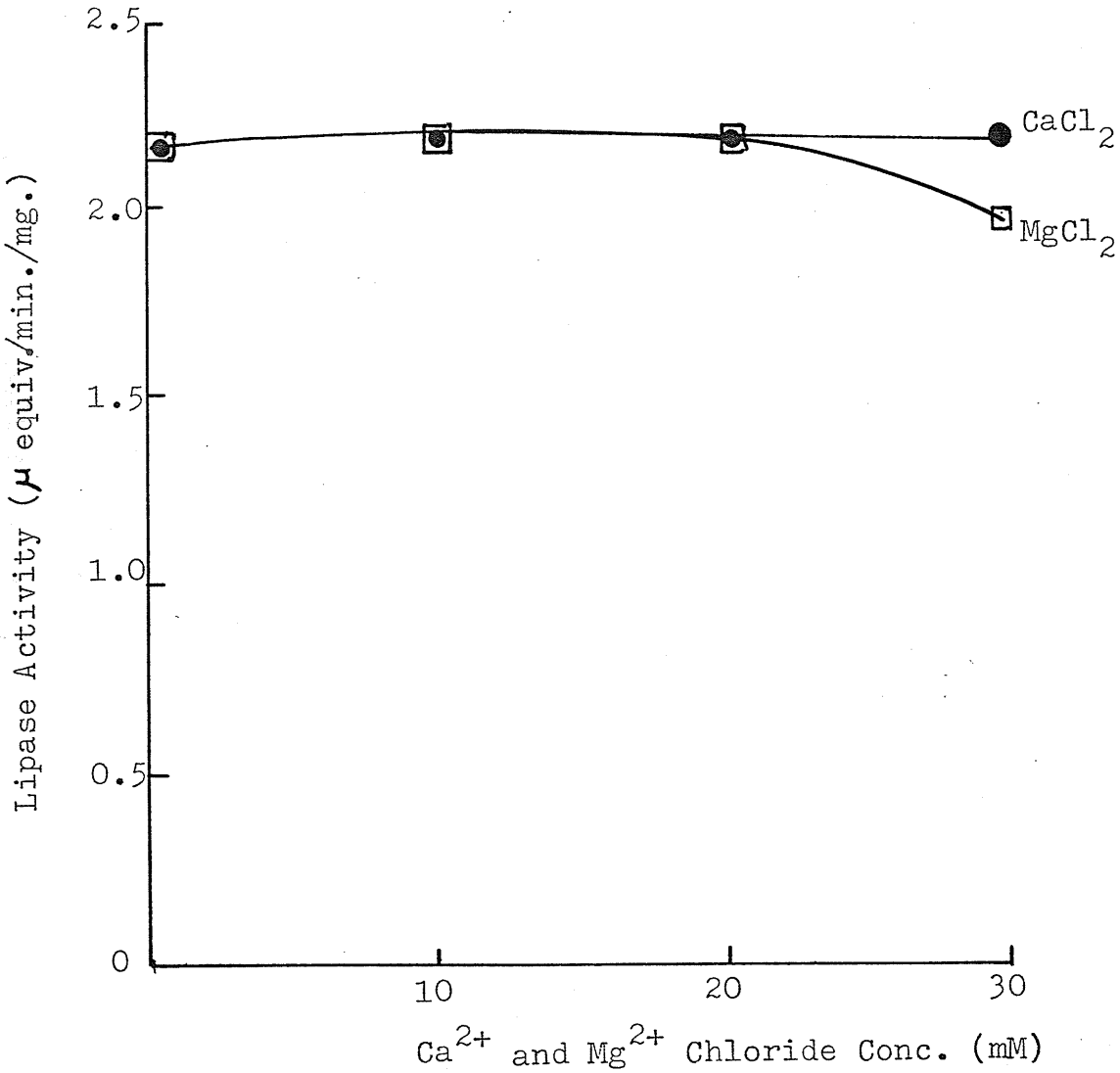
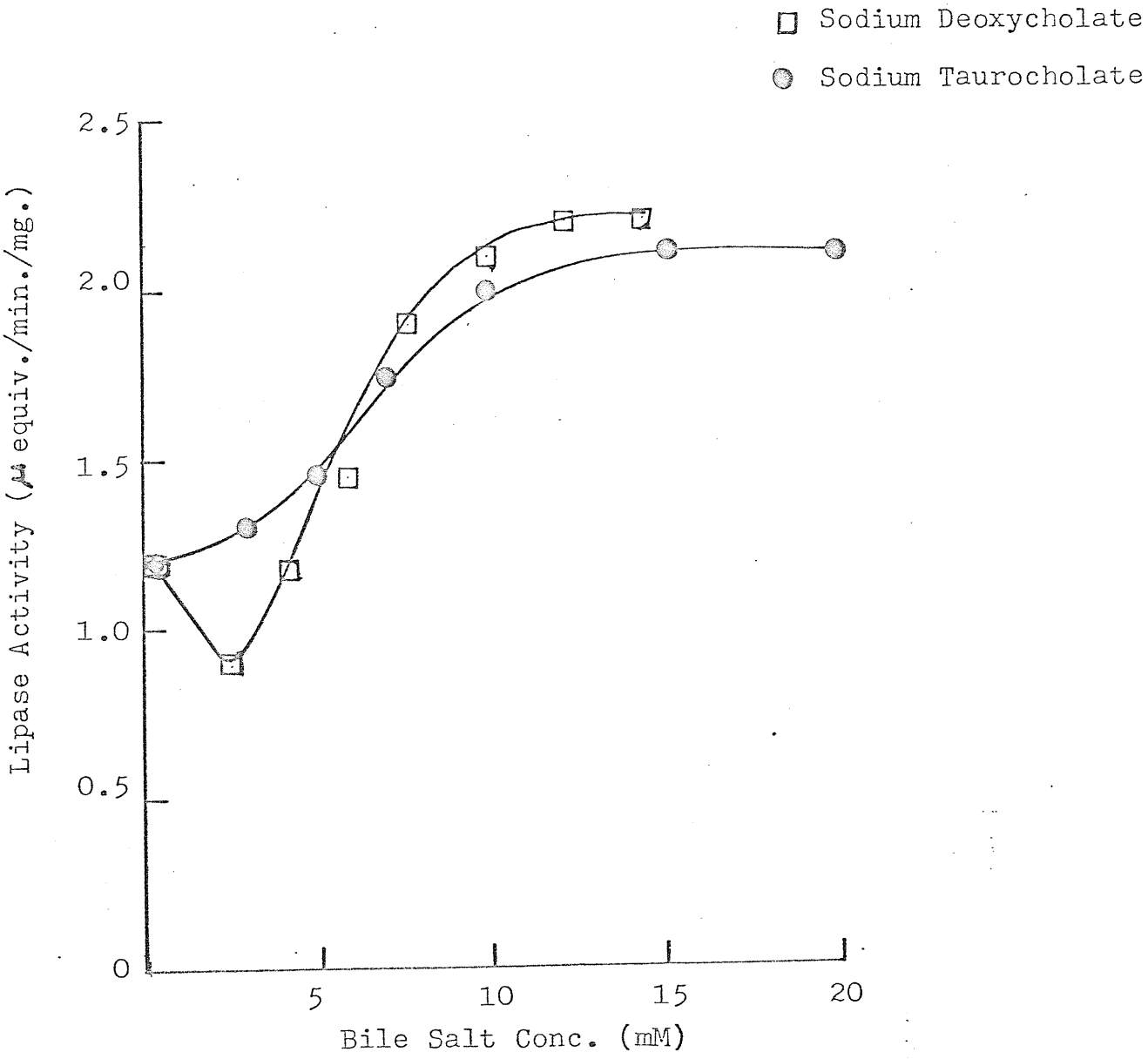


FIGURE 15

EFFECT OF BILE SALTS ON FABA BEAN LIPASE



pretation can be made regarding the initial decrease in activity shown in the addition of 2.0 mM of deoxycholate, although this decrease was reproducible in all experiments (carried out in triplicate). The activation of faba bean lipase by low concentrations of bile salts agrees with the results obtained for pancreatic lipase (Borgstrom, 1964) in the presence of 10.60 mM sodium deoxycholate.

4.12 EFFECT OF INHIBITORS

4.12.1 Mercuric Chloride Inhibition

The activity of faba bean lipase was inhibited by mercuric chloride, with over 60% of the original activity being inhibited by 5.0 mM mercuric chloride (Figure 16.) This agrees with the degree of lipase inhibition by mercuric ions obtained for peanut alkaline lipase with a value of 0.15 mM $HgCl_2$ (Sanders and Pattee, 1972), 0.50 mM $HgCl_2$ for castor bean lipase (Ory *et al*, 1960) and 0.11 mM $HgCl_2$ for Leptospiral lipase (Patel *et al*, 1964).

The mode of inhibition exhibited by mercuric ions on faba bean lipase was determined by the use of a double reciprocal plot of the data from Figure 16. The Lineweaver-Burk plot (Figure 17), shows that inhibition by mercuric ions is noncompetitive, as both the vertical intercept and slope of the reciprocal plot are affected and converge on the abscissa.

4.12.2 p-Chloromercuribenzoate Inhibition

Faba bean lipase was inhibited very slightly by a high concentration of *p*-chloromercuribenzoate, possibly in-

FIGURE 16

THE EFFECT OF $HgCl_2$ on FABA BEAN LIPASE

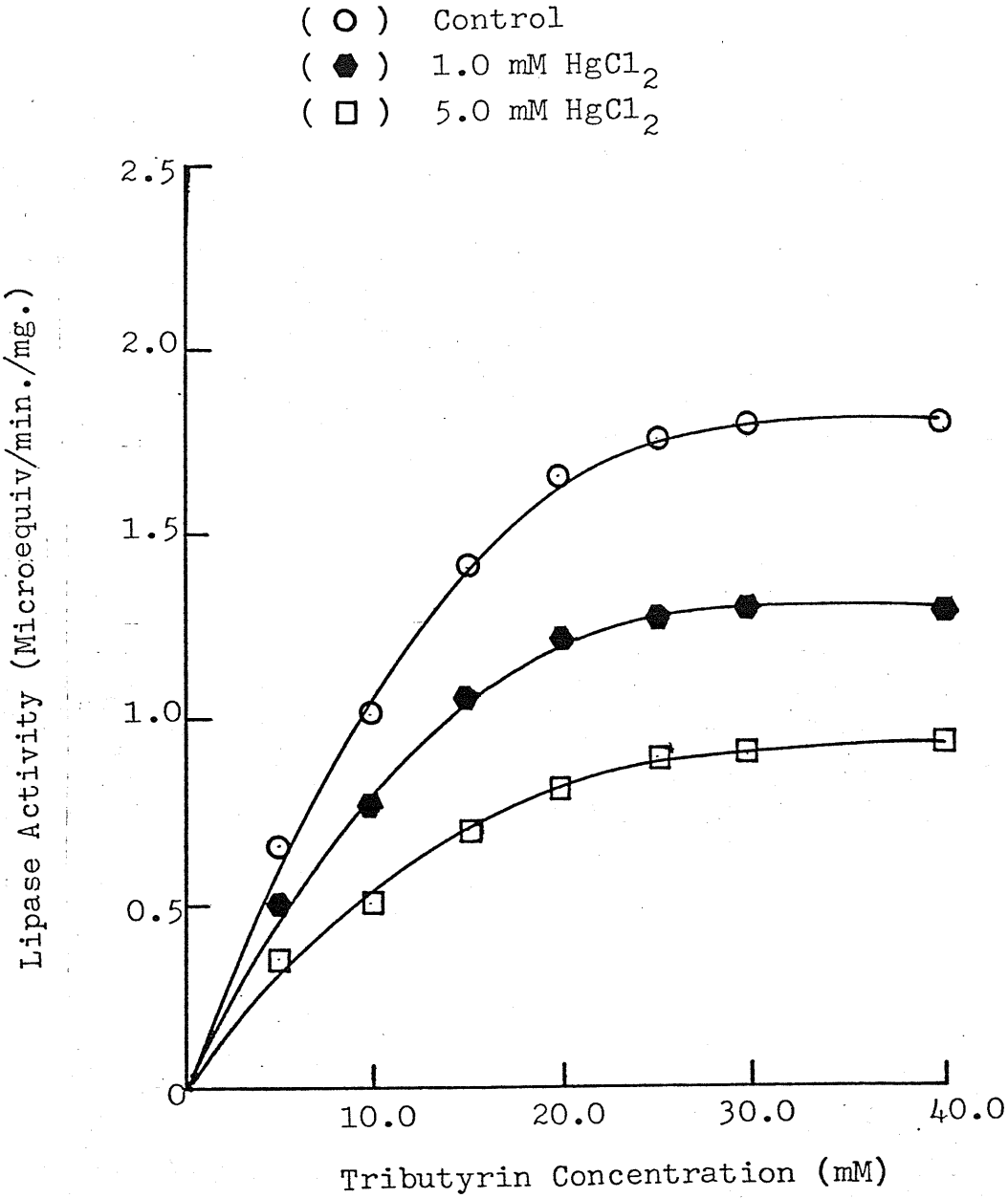
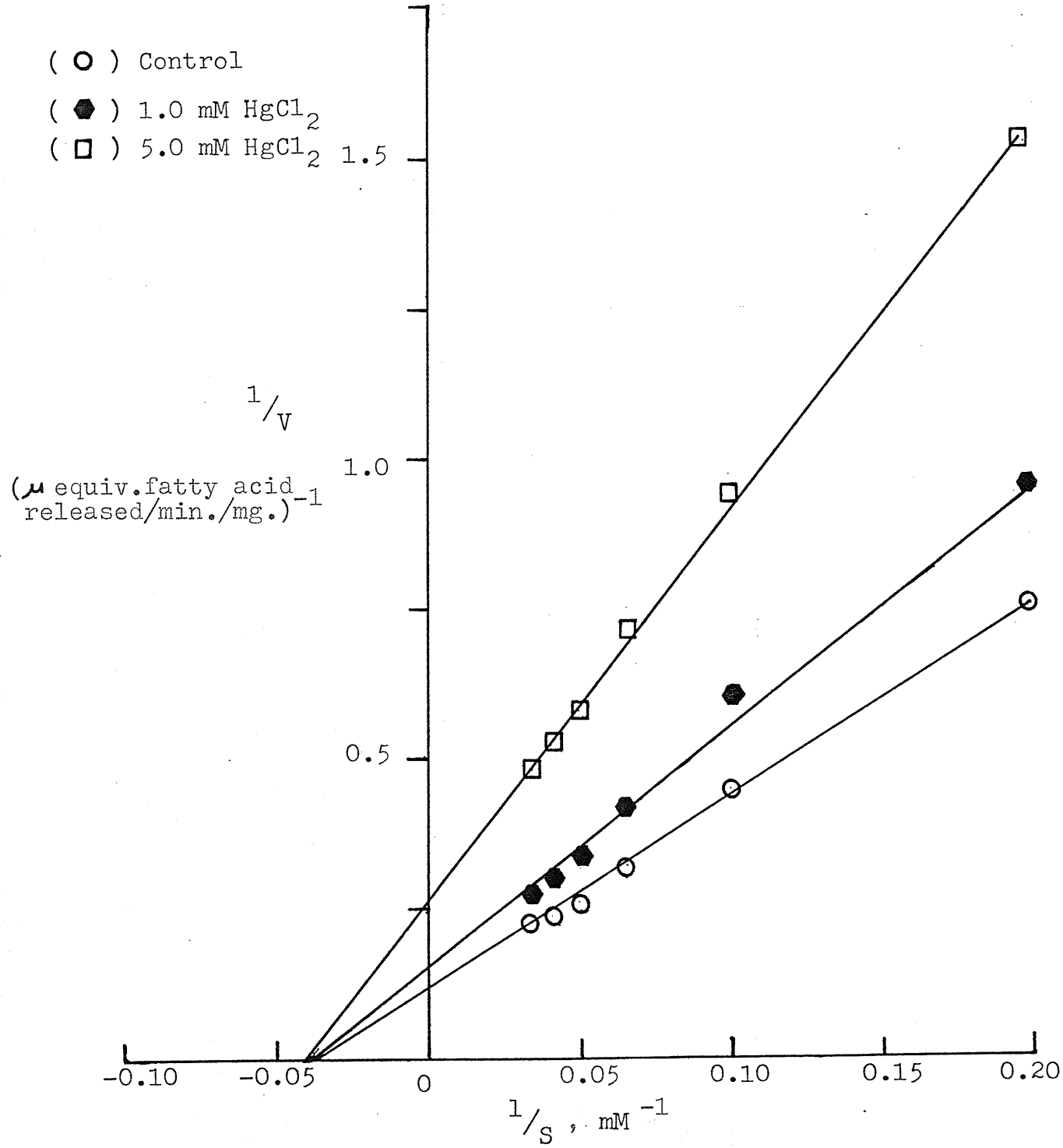


FIGURE 17

THE LINEWEAVER-BURK PLOT OF THE EFFECT OF $HgCl_2$ ON FABA BEAN LIPASE



dicating the presence of sulphydryl groups in the enzyme molecule. A 10.0 mM concentration of p-chloromercuribenzoate reduced the enzyme activity by 20% of the uninhibited reaction (Figure 18). This corresponds with the findings of other workers who concluded that lipase treated with a relatively high concentration of p-chloromercuribonzoate, which would completely block all -SH groups, lost between 20-40% of its original activity. This has been shown in lipase from Puccinia graminis where a 0.01 mM concentration of p-CMB reduced enzyme activity to 20% (Knoche and Horner, 1970).

The addition of high concentrations of ethylenediamine-tetraacetate (10.0 mM) to the inhibited lipase preparations did not reactivate lipase activity.

The mode of inhibition exhibited by p-chloromercuribenzoate on faba bean lipase was determined by use of a double reciprocal plot of the data from Figure 18. The plot (Figure 19) indicates that inhibition was noncompetitive.

4.13 DISC GEL ELECTROPHORESIS

The detection of lipase and proteins bands to determine the purity of the enzyme after each purification step and the isoenzyme patterns of the enzyme, was undertaken by the use of disc gel electrophoresis.

Disc electrophoresis on polyacrylamide gel at pH 9.3 of the crude extract of faba bean lipase revealed several protein bands when stained in Amido Black (Figure 20A). The protein band which corresponds to that containing lipase activity is indicated by staining obtained using esters of

FIGURE 18

THE EFFECT OF p-CMB ON FABA BEAN LIPASE

(O) Control

(⬡) 10.0 mM p-CMB

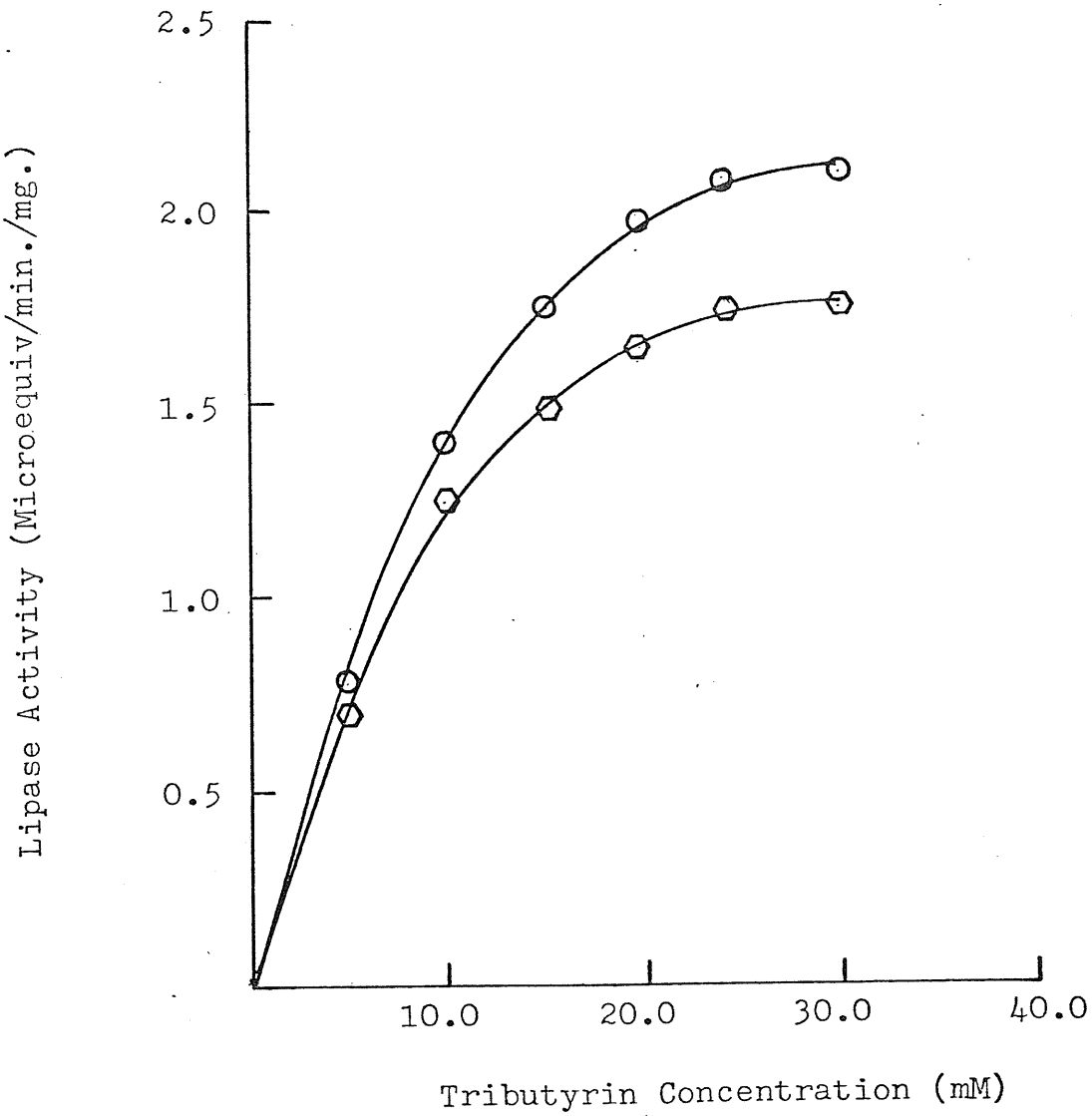


FIGURE 19

THE LINEWEAVER-BURK PLOT OF THE EFFECT
p-CMB ON FABA BEAN LIPASE

(O) Control
(⬡) 10.0 mM p-CMB

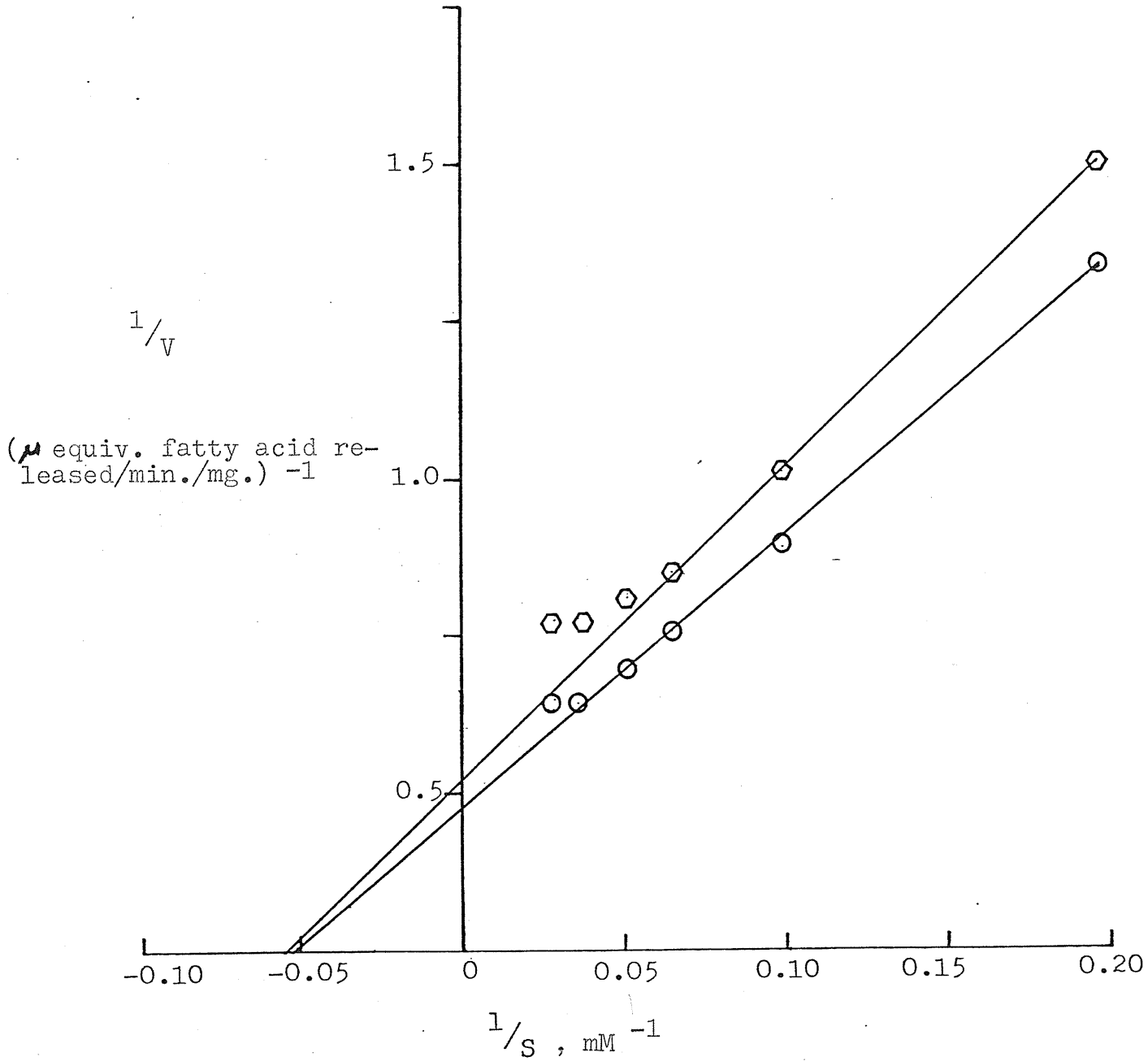
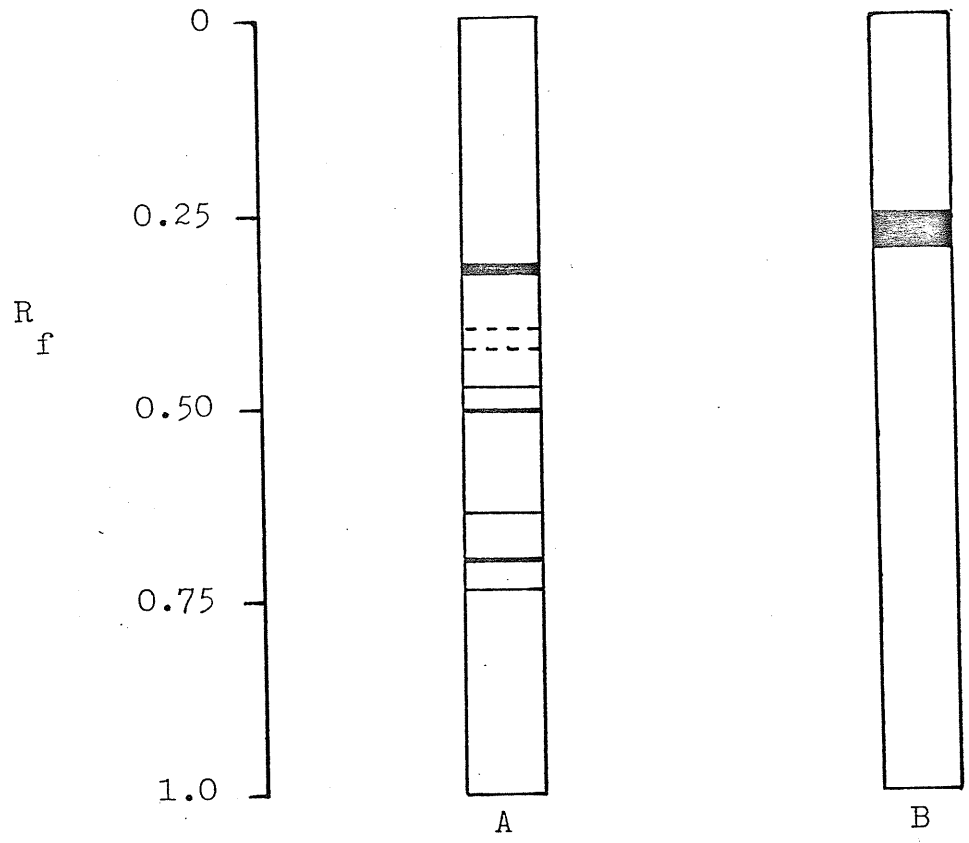


FIGURE 20

DISC GEL ELECTROPHORETOGRAMS OF CRUDE FABA BEAN LIPASE

A. Protein bands
B. Lipolytically active bands

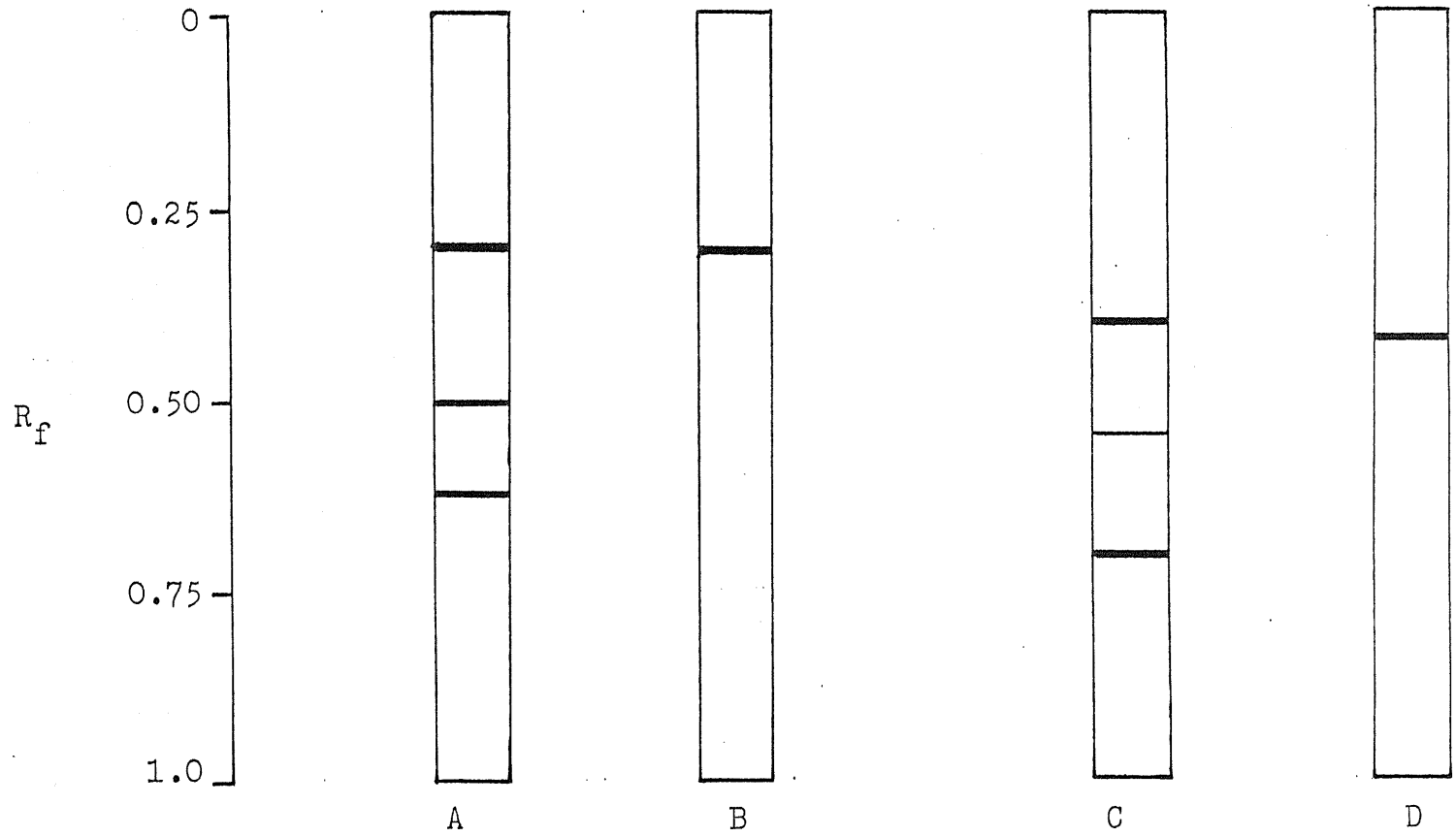


α-naphthol (Figure 20B). A major lipase band was shown to occur at R_f 0.30 while a faint lipase activity band was detected at R_f 0.74. These correspond to the protein bands at R_f 0.31 and 0.75 respectively, the other protein bands being inactive with respect to lipase. No data is presented for hog pancreatic lipase at this point, as the available commercial powder was partially-pure.

Partially-purified lipase preparations from both faba bean and hog pancreas both resulted in three protein bands determined by gel electrophoresis (Figure 21). The three protein bands for faba bean lipase were located at R_f 0.31, 0.50 and 0.62 (Figure 21A), while the lipase activity band was detected at R_f 0.31 (Figure 21B). The faba bean lipase used here was concentrated by ethanol fractionation. In comparison to that of hog pancreatic lipase, where the protein bands were detected at R_f 0.38, 0.53 and 0.68 (Figure 21C), while the activity band was located at R_f 0.36 (Figure 21D), it is seen that the movement of the proteins in hog pancreatic lipase was further down the gel, while the faba bean lipase fractions were located near the top of the gel, thus indicating a greater mobility on the part of hog pancreatic lipase proteins.

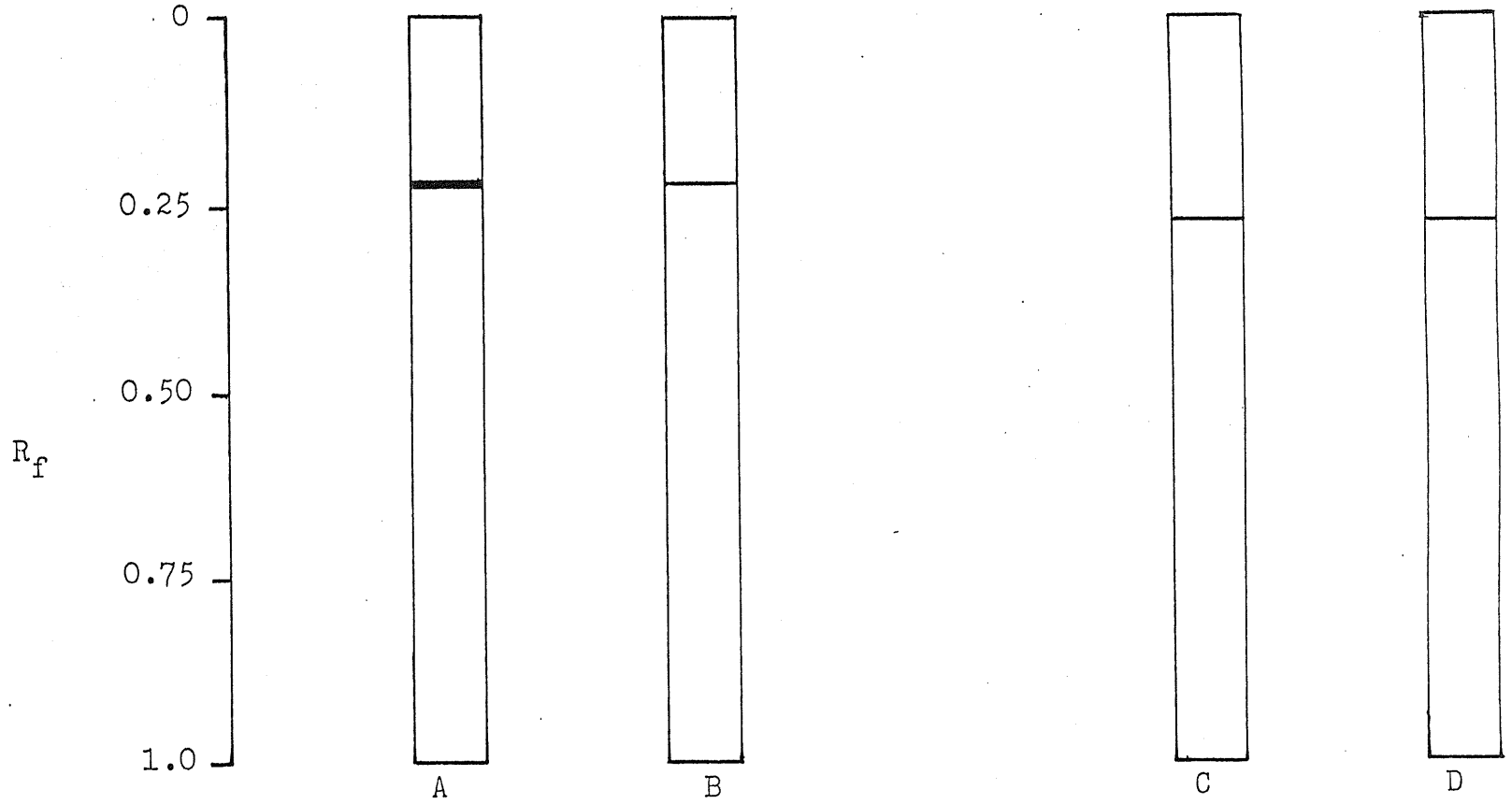
Lipase preparations purified through Sephadex G-100 resulted in the detection of a single protein and lipase activity peak for faba bean lipase (Figure 22A and B). The protein and lipase activity bands were both located at R_f 0.25. In comparison, the single protein peak for hog pancreatic

FIGURE 21



DISC GEL ELECTROPHORETOGRAMS OF PARTIALLY PURIFIED FABA BEAN LIPASE
(A. Protein bands B. Lipolytically active bands)
and commercial porcine pancreas lipase
(C. Protein bands D. Lipolytically active bands)

FIGURE 22



DISC GEL ELECTROPHORETOGRAMS OF SEPHADEX G-100 EXTRACTS OF FABA BEAN LIPASE
(A. Protein bands B. Lipolytically active bands)
and commercial porcine pancreas lipase
(C. Protein bands D. Lipolytically active bands)

lipase obtained from Sephadex G-100 filtration was located at R_f 0.30 (Figure 22C), and the activity band was also located at R_f 0.30 (Figure 22D).

The presence of a single protein and enzyme activity band in the Sephadex fractions indicate that no isoenzymes of lipase exist in either of these faba bean or hog pancreatic lipase preparations.

4.14 DETERMINATION OF THE ISOELECTRIC POINT

The isoelectric point was determined by isoelectric focusing on 7.5% polyacrylamide gel, using an LKB Ampholine carrier ampholyte no. 8141 (pH 3-10). Figure 23 illustrates the isoelectric focusing profile obtained with faba bean lipase. As a result the lipase band appeared in a single peak at pH 4.8 representing the estimated isoelectric point (pI) of faba bean lipase. No result was obtained for hog pancreatic lipase as no band was detected, but the isoelectric point of 4.8 for faba bean lipase compares with that estimated for pancreatic lipase of pI 5.2 (Marchis-Mouren *et al*, 1952), and that of *Corynebacterium acne* lipase of pI 3.8 (Hassing, 1971).

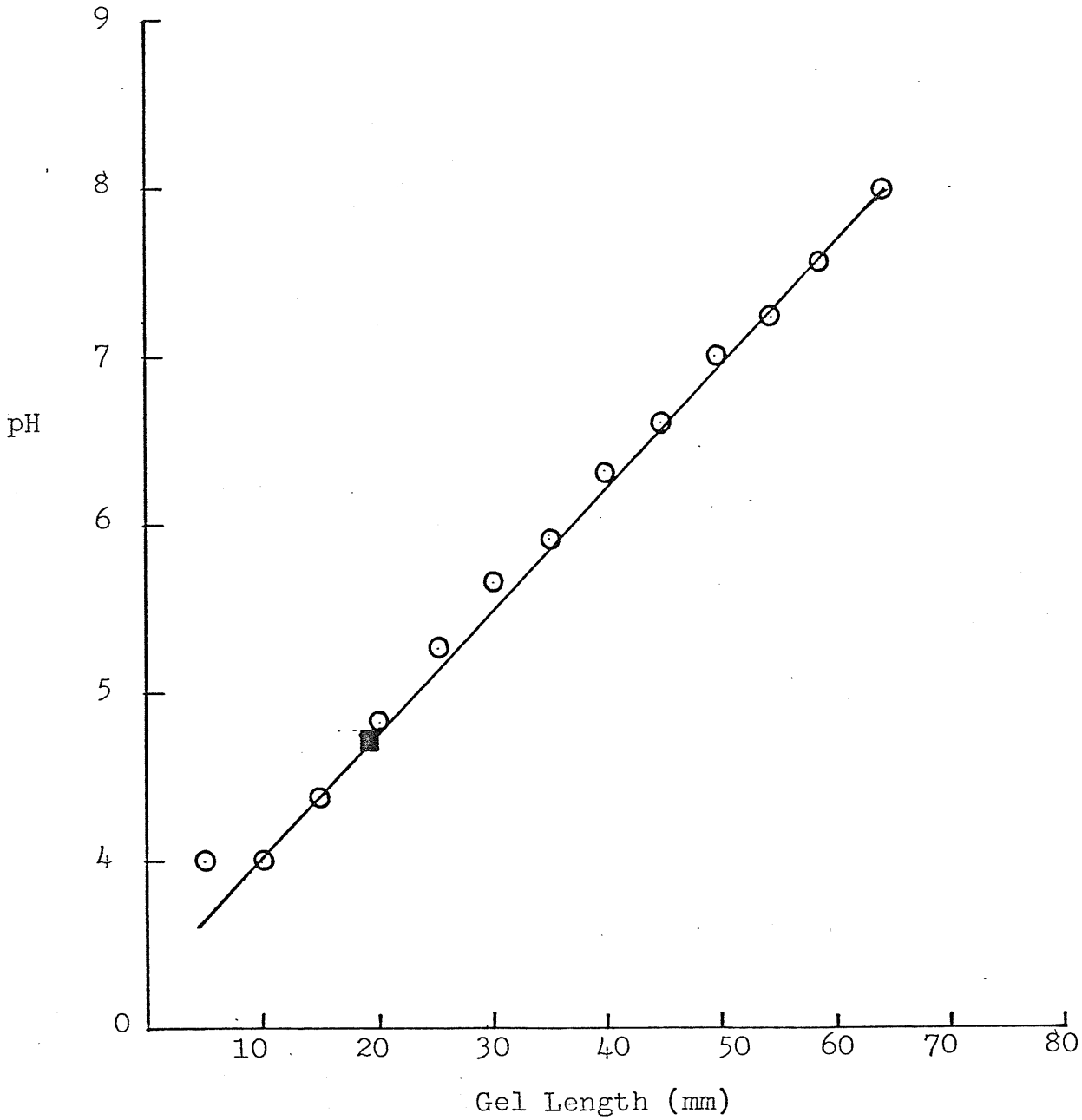
4.15 MOLECULAR WEIGHT DETERMINATION

The molecular weight of the enzyme was determined by disc gel electrophoresis (5% polyacrylamide gel, pH 7.2) in the presence of 0.1% sodium dodecyl sulphate, utilizing standard proteins of which the relative mobilities were cal-

FIGURE 23

THE DETERMINATION OF THE ISOELECTRIC POINT OF FABA BEAN LIPASE BY DISC GEL ELECTROPHORESIS

(■) Faba Bean Lipase

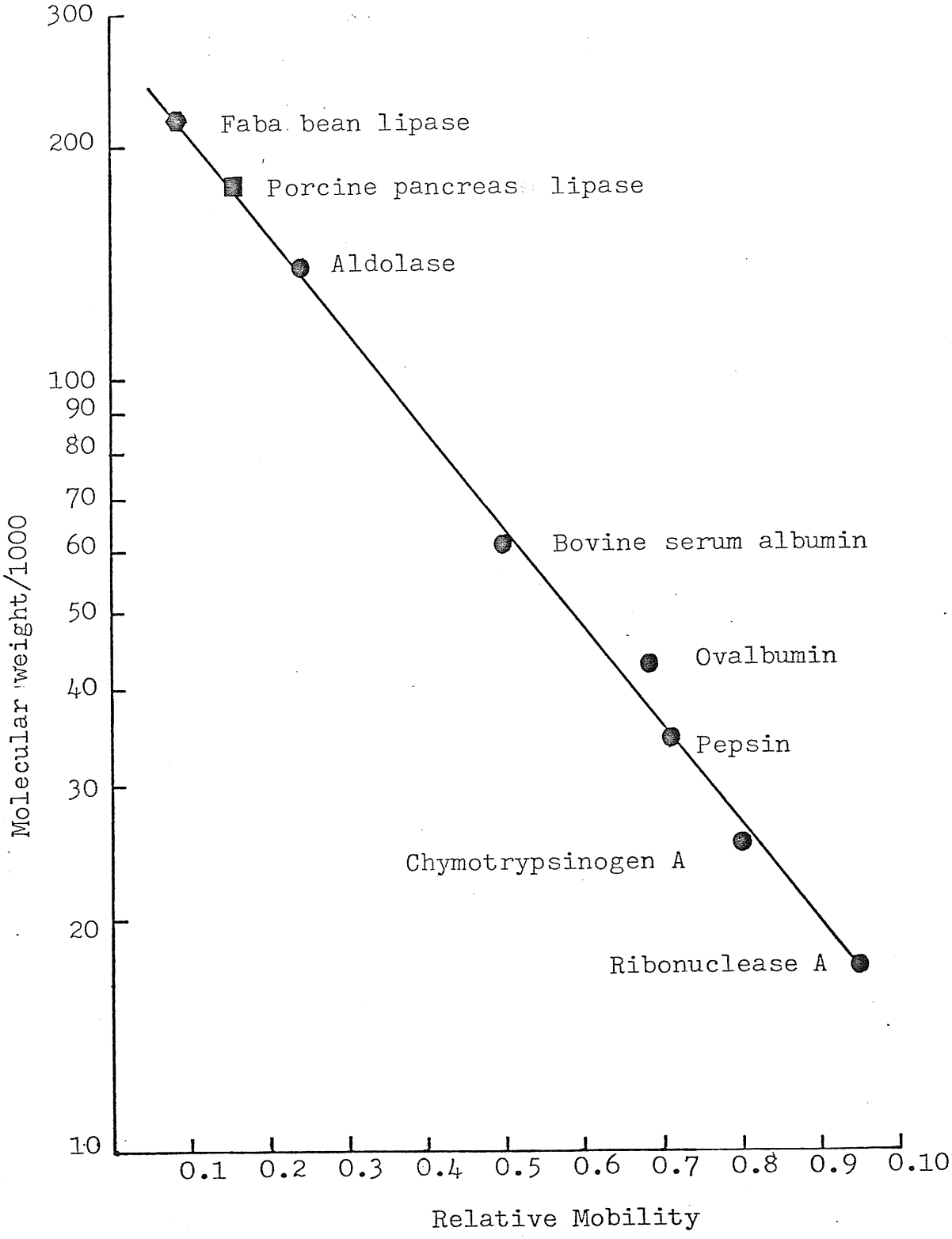


and plotted against molecular weight, this representing the standard molecular weight calibration curve (Figure 24). Lipase fractions obtained from gel filtration on Sephadex G-100 columns were used, with the faba bean lipase band being calculated to give a molecular weight of 210,000 ± 20,000 (4 determinations). The value for the molecular weight of hog pancreatic lipase was calculated as 180,000 ± 5,000 (4 determinations), (Figure 24), which corresponds exactly with the value obtained by Downey and Andrews (1965).

This high molecular weight calculated for faba bean lipase would suggest that the single lipase entity isolated is the "fast lipase", and corresponds with a value associated with lipase activity in Vernonia anthelmintica with a molecular weight in excess of 200,000 (Olney et al, 1968).

FIGURE 24

CALIBRATION CURVE FOR THE DETERMINATION OF FABA BEAN LIPASE AND PORCINE PANCREAS LIPASE MOLECULAR WEIGHT BY S.D.S. ELECTROPHORESIS



DISCUSSION

an approximately 40-fold purification of a faba bean lipase preparation was achieved by the procedure outlined previously in this thesis. The concentration of lipase by ethanol precipitation is one of the few methods that may be applied in the isolation of lipase from plant and microbial sources, as the use of pH precipitation and ammonium sulphate fractionation techniques resulted in unsatisfactory lipase isolation. This is believed to be attributed to the fact that "fast" lipase, the form of lipase isolated during the course of this work, is a multimolecular aggregate of enzyme molecules with strong associations with lipids, especially phospholipids (Brockerhoff and Jensen, 1974). This complex is easily denatured or inactivated by the application of isoelectric point or salt precipitation techniques through the disruption of the hydrophobic bonds of the enzyme aggregate.

The purification of the enzyme by Sephadex G-100 filtration resulted in the enzyme being eluted immediately after the void volume, the enzyme being detected as a fast moving band as shown in the elution profiles of figures 3 and 4. The exclusion of the enzyme from the column indicates that its molecular weight exceeds 150,000, the upper fractionation range of the Sephadex G-100 gel, and may correspond to the "fast lipase" of rat pancreas (Morgan et al, 1968), hog pancreas (Downey and Andrews, 1965) and Micrococcus (Lawrence et al, 1967). It was shown in Figure 3 that enzyme purifi-

cation obtained from gel filtration with a sodium chloride gradient resulted in the enzyme fraction being recovered with an accompaniment of great amounts of protein. The use of sodium deoxycholate to convert "fast lipase" into "slow lipase" has been applied on Sephadex gel filtration (Chorvath and Fried, 1970), with the possible action of the bile salt being the disaggregation of the multimolecular "fast lipase". The application of sodium deoxycholate to the elution buffer in the chromatography of faba bean lipase on Sephadex G-100 runs resulted in an apparent decrease in the total protein of the first peak which contains lipase activity. This indicates that the action of bile salt was in the removal of inactive protein material resulting in two protein peaks, one containing lipase and the other without lipase, which produced a 40-fold purification of the enzyme in comparison to an 18-fold purification over the original by gel filtration in the absence of the bile salt. Also in figure 4, the enzyme peak was removed from the column directly after the void volume, indicating that the enzyme was still excluded from the gel column and was of a higher molecular weight than the gel's upper fractionation limit. This indicates that the enzyme aggregates or lipid fractions may be tightly bound together to form the high-molecular weight faba bean lipase.

Gel electrophoresis at pH 9.3 of the partially purified faba bean lipase by gel filtration revealed a single esterase which hydrolysed esters of α-naphthol. This single lipase band is apparently identical with the lipase enzyme of hog

pancreas, as the electrophoretic patterns of both the protein bands and lipase activity were similar. It has been demonstrated (figure 21), that partially-pure extracts contain single fractions at R_f 0.30 for faba bean lipase and at R_f 0.38 for hog pancreas lipase. The detection of a single hydrolytic band by gel electrophoresis of partially-purified lipase preparations from faba bean lipase suggests that only one enzyme was involved in the hydrolysis of its emulsified substrates, which supports the conclusion that a single lipase is isolated which exhibits a general specificity towards its emulsified substrates, rather than several isoenzymes with different substrate specificity (Brockerhoff, 1973).

An indication of the purity of the different preparations subjected to gel electrophoresis was estimated by the determination of the isoelectric point of the enzyme. The determination of the isoelectric point of the enzyme from the gel filtration procedure was attempted by isoelectric focusing. The isoelectric homogeneity of the enzyme sample was demonstrated by the detection of a single protein band by gel electrofocusing (figure 23), revealing a calculated isoelectric point of the faba bean enzyme of pH 4.8. This value for faba bean lipase compares well with the isoelectric point of pH 5.2 for porcine pancreatic lipase (Marchis-Mouren et al, 1959), and falls between the values of the low molecular form (lipase B) of Chromotobacterium viscosium of pH 6.9 (Sigiura and Isobe, 1974) and the high molecular

form lipase of Corynebacterium acnes with a pI value 3.8 (Hassing, 1971).

Molecular weights of the faba bean and commercial hog pancreatic lipase preparations were determined by gel electrophoresis in the presence of sodium dodecyl sulphate. After standard proteins were run and the relative mobilities calculated and plotted, the molecular weight of faba bean lipase was estimated as 210,000 ± 20,000 (4 determinations), while that of hog pancreatic lipase was found to be 180,000 ± 5,000 (4 determinations). The use of gel electrophoresis to determine the molecular weight of the enzyme demonstrated that the value estimated for hog pancreatic lipase corresponded with that of one of the forms of "fast lipase" obtained from Sephadex G-200 molecular weight determination (Downey and Andrews, 1965). The estimated value of 210,000 ± 20,000 as the molecular weight of faba bean lipase corresponds with that estimated for most lipases separated from plant and microbial sources such as Vernonia anthelmintica seed lipase estimated in excess of 200,000 (Olney et al, 1968), and micrococcus lipase estimated at approximately 250,000 (Lawrence et al, 1967).

Due to the simple instrumentation and low substance requirements the accuracy of molecular weight determinations may be increased by many determinations thus obtaining a statistical average. Only a few percent of deviation have to be expected for the low molecular weight range, while for molecular weights above 100,000, the error is frequently

somewhat greater. For this reason, the estimated value for the molecular weight of faba bean lipase is regarded as an approximate figure and must be regarded as an apparent value. Thus the molecular weight of faba bean lipase is given as a mean value of 210,000 $\pm$ 20,000, this being obtained from four determinations.

The application of gel filtration and the analysis of the recovered lipase fractions from faba beans, by disc gel electrophoresis, gel electrofocusing and molecular weight determination by gel electrophoresis provides evidence that lipase obtained from faba bean is a single esterase, isolated as a high-molecular weight form, probably being an aggregate known in the literature as "fast lipase".

The lipase-catalyzed hydrolysis of natural insoluble esters (lipolysis), is a function of the "concentration" of the emulsion of the substrate, the reaction occurring at the oil-water interface. In lipolysis, the enzyme has to seek out the substrate, as the latter is partly immobilized in a larger matrix, this being regarded as an association between the substrate molecules to form aggregates surrounded by water. The term "supersubstrate" (S_s) has been proposed (Brockerhoff, 1974) to describe the matrix in which a substrate molecule is embedded. In lipolytic reactions, this matrix may be the surface of a triglyceride droplet and thus consists of an aggregate of many substrate molecules, or it may be such a surface modified by the inclusion of nonsubstrates, such as anions and cations. Lipolysis is a

one-substrate reaction, and at infinite substrate concentration, the velocity (v) becomes Vmax, the maximum velocity of the reaction. In lipolytic reactions, which are not usually subjected to substrate inhibition, Vmax is easily determined. This is done by measuring the initial velocity v at several substrate concentrations, with v being expressed as specific activity, for example, as micromoles of substrate converted by 1.0 mg. of enzyme in one minute. The experimental data shown in figure 7 are consistent with the assumption that lipase normally acts at the triglyceride-water interface of the emulsion. The activity curves, typical rectangular hyperbolas, can be regarded as an adsorption isotherm in the sense that it illustrates the progressive adsorption of the enzyme at an interface of increasing concentration. When the concentration of the interface is small, as in diluted emulsions, few enzyme molecules are adsorbed and the rate is low. The rate increases with the interface concentration until all enzyme molecules are simultaneously adsorbed, and this upper limit corresponds to the maximal rate of lipolysis (V_{max}) for a given enzyme amount. The concentration, expressed as a molarity of the substrate, is not regarded as a weight, but as the amount of substrate in the emulsion (Brockerhoff and Jensen, 1974). When an enzyme depends on an activator, the initial rates of reaction may be affected. The use of the bile salt, sodium deoxycholate (figure 7), results in a higher V_{max} than is shown in the curve in the absence of deoxycholate, which indicates that the V_{max} obtained is an apparent value for lipase.

Lipolytic reactions, as have been shown, can formally be treated so that they follow Michaelis-Menten kinetics. In lipolysis the formation of the Michaelis complex is preceded by the adsorption of lipase to the oil-water interface which is the supersubstrate (S_s) in which the substrate is embedded (Brockerhoff, 1974).

$$E + S_s \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E + S_{\text{absorbed}} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P$$

If the difference between S_s and S is neglected, this equation describes formally a one-substrate enzymic reaction with two intermediate complexes. It can be resolved for $v = dp/dt$ (Mahler and Cordes 1971):

$$v = \frac{(k_1k_3k_5\ s - k_2k_4k_6\ p)e_o}{k_2k_5 + k_2k_4 + k_3k_5 + k_1(k_3 + k_4 + k_5)s + k_6(k_2+k_3+k_4)p}$$

In the beginning of the steady state reaction, $p = 0$, and

$$v = \frac{k_1k_3k_5\ s\ e_o}{k_2k_5+ k_2k_4+ k_3k_5+ k_1(k_3k_4k_5)s}$$

This equation can be converted into the Michaelis-Menten equation by the appropriate substitution:

$$\frac{k_3k_5\ e_o}{k_3+ k_4+ k_5} = V$$

$$\frac{k_2k_5+ k_2k_4+ k_3k_5}{k_1(k_3+ k_4+ k_5} = km$$

$$v = Vs/(Km + S).$$

The Km of the reaction may be defined as the interface concentration for which the rate is $V_{max}/2$.

The apparently high Km value of 22 mM obtained for faba bean lipase may be an indication that the degree of emulsification used was not great enough to provide complete access of the substrate to the enzyme. The Km value obtained compares with those of 0.26mM for peanut alkaline lipase (Sanders and Pattee, 1972) and 47.6 mM for leptospiral lipase (Patel et al, 1964).

Faba bean lipase, like all lipases, hydrolyzes short-chain triglycerides at a much faster rate than long-chain natural triglycerides. Faba bean lipase was more active towards tributyrin than to the olive oil and corn oil substrates.

The pH optimum of faba bean lipase was found to be 8.5, this value concurring with the pH optima of most lipases which occur within the range of pH 8.0 - 9.0 (Wills, 1965; Desnuelle, 1972; Brockerhoff, 1973). The effect of pH on the rate of hydrolysis by lipase is a resultant of its effects not only on the enzyme itself, but also on the emulsified substrate and the properties of the substrate: aqueous phase interface. The substrate emulsion requires an alkaline pH and at pH levels below 8.0, less and less fatty acid may be titrated with base, while at pH values greater than 10.0, spontaneous breakdown of the substrate is likely to occur, resulting in inadequate assay conditions. Faba bean lipase was stable from pH 6.0 to 8.0 for 30 minutes,

while for ten minutes the enzyme was stable from pH 5.0 to 9.0. This indicates that the enzyme is highly sensitive to pH changes.

The optimum temperature of faba bean lipase was found to be 38°C, which corresponds with the temperature range of 30-40°C within which lipases are most active (Wills, 1965; Desnuelle, 1972; Brockerhoff and Jensen 1974). It was noted that lipase was not very active below 25°C while the activity decreases very sharply at temperatures greater than 40°C. The stability of the enzyme to heat is similar to that obtained for peanut alkaline lipase (Sanders and Pattee, 1972). With the temperature approaching 50°C the activity of lipase was drastically altered, while at 60°C the faba bean lipase lost 90% of its original activity after five minutes. This indicates the degree of heat-lability of the enzyme. Many substances have been known as activators of lipases, the best known being the bile salts, while sodium and calcium chlorides can influence the course of lipolysis (Wills, 1965; Desnuelle, 1972; Brockerhoff and Jensen, 1974).

Variations in the level of bile salts in the assay media influence the activity of faba bean lipase (figure 15). Both sodium salts of deoxycholate and of taurocholate effected maximum activation at a concentration of 12.0 mM, with deoxycholate resulting in a higher rate of activation than taurocholate. Bile salts appear to prevent the inhibition of the lipolysis caused by the liberated long-chain fatty acid products (Benzonana and Desnuelle, 1968), and thus

it seems that the bile salts are not true activators but enable the reactions to proceed at a zero-order rate.

Faba bean lipase was hardly active in the absence of sodium chloride on the assay emulsion stabilized with deoxycholate (figure 13). The increase in reaction rate, due to the addition of sodium chloride, reaches a maximum at 0.5-0.6 M concentration, which correlates with that of 70 mM obtained for pancreatic lipase (Benzonana and Desnuelle, 1968). It is believed that the cations suppress the pH gradient at the interface and lower the apparent pK_a of the fatty acids which can then be more quantitatively titrated (Mattson and Volpenhein, 1966). Whether the activation of faba bean lipase by sodium chloride is caused by an increase in the degree of ionization of the liberated fatty acids, is not known, but it is believed that the action of this activator is not directly on the enzyme, but on the substrate emulsion (Desnuelle 1972; Brockerhoff and Jensen, 1974).

In contrast to pancreatic lipase, for which calcium ions are necessary to promote the removal of unionized fatty acids from the interface through the formation insoluble calcium soaps, faba bean lipase does not appear to be affected by calcium ions. The substitution of calcium by magnesium ions, which was believed to be an irreplaceable activator, also does not produce any significant activation of faba bean lipase activity. This is believed to be due to the fact that thepresence of sufficient calcium ions in the partially-pure enzyme preparation would negate the effect of the added salts and does not necessarily mean that the

faba bean enzyme is not activated by calcium chloride.

The activity of faba bean lipase was inhibited in the presence of $HgCl_2$, the heavy metal salt resulting in significant inhibition of the enzyme at a concentration of 0.01 M. This compares with mercuric chloride inhibition of peanut alkaline lipase where activity was inhibited completely by 0.15 mM $HgCl_2$ and over 60% by 160.0 mM $HgCl_2$ concentration (Sanders and Pattee, 1972), and with that of castor bean lipase which was inhibited completely by 0.5 mM $HgCl_2$. It is shown from the double reciprocal plot of the $HgCl_2$ inhibition studies that the inhibition was linear noncompetitive, which indicates that $HgCl_2$ inhibition of faba bean lipase occurs irrespective of the substrate concentration.

A 0.01 M concentration of p-chloromercuribenzoate (p-CMB) reduced faba bean lipase activity by less than 20% of the uninhibited reaction, thus suggesting the possible involvement of a sulphydryl group near the active site of the enzyme. The inhibition pattern displayed by p-chloromercuribenzoate as determined by a Lineweaver-Burk plot revealed a linear noncompetitive type of inhibition.

In this thesis, the main objective, that is the detection and isolation of a lipase in the small faba bean, has been successfully attained, and the physical and chemical properties which have been determined have been shown to conform generally with plant, animal and microbial lipases so far investigated.

Additional research is warranted on the lipase of faba bean, such as extended studies on lipase isolation and puri-

fication, as the purification technique of ethanol fractionation used throughout this study has many drawbacks. The lyophilization of faba bean extracts to concentrate lipase is one area of investigation, although the application of this method as a commercial source of lipase is unlikely to rival that from pancreatic sources because of the low concentration of lipase in the beans. Of great importance is the deaggregation of the high-molecular weight "fast lipase" species isolated from faba bean, to obtain the monomolecular weight "slow lipase", and this may be attempted by the application of second-step chromatography involving DEAE and CM-cellulose ion exchangers. The reduction of the "fast lipase" form to a monomeric species of seed lipase is important and may prove essential to the biochemist as analytical and synthetic tools. Other areas of further investigation are the relationship of lipase activity with seed maturation, the detection of an acid lipase such as that of castor bean this requiring an assay method capable of detecting activity at acid pH, and the observation of the positional specificity of faba bean lipase with respect to triglycerides.

The main concern of the food scientist for the seed lipase is the control of lipolysis of the triglyceride fraction in processed beans in the form of faba bean flour and protein concentrate. From the studies carried out on faba bean lipase, it would appear that the most suitable method for lipolysis control is a heat-treatment stage [of the beans] before or after processing. As shown in this study, the enzyme is heat-labile when in solution, being inactivated

at a temperature of 65°C in less than five minutes. Further studies are needed, relating to the amount of heat necessary to inactivate the enzyme in raw or processed beans. In the incorporation of a heat-treatment phase in the processing of faba beans, consideration should be given as to whether a heat-treatment of the faba bean flour or concentrate may not result in a deterioration of quality in the form of the development of bitter taste and discoloration. A mild toasting of the whole beans before processing might be more feasible.

The use of chemical inhibitors to inactivate the enzyme is not recommended, as it was shown from this study that the enzyme is susceptible only to high levels of the chemical inhibitors employed.

SUMMARY

1. Lipase from faba bean (Vicia faba L var. minor) was partially purified and characterized.

2. Due to the properties of the crude extracts of faba bean lipase, ammonium sulphate precipitation was unsatisfactory for the concentrating and/or purification of the enzyme. Ethanol fractionation resulted in a nine-fold purification of the enzyme over the crude extract.

3. An 18-fold purification over the original was obtained by the gel filtration of enzyme extracts on a Sephadex G-100 column employing an NaCl gradient. Approximately 45% of the original activity was recovered.

4. Comparison of the elution patterns of faba bean lipase and commercial hog pancreas lipase from Sephadex G-100 gel filtration showed good correlation in the detection of the lipase peaks.

5. The purification of the enzyme by Sephadex gel filtration resulted in elution of the enzyme immediately after the void volume, indicating that the molecular weight of lipase was greater than 150,000, the upper frationation range of the gel column and thus, may be a high-molecular weight "fast lipase" form.

6. The use of sodium deoxycholate incorporated in the eluting buffer to convert "fast lipase" into "slow lipase" resulted in an apparent decrease in the total protein

of the active lipase peak, but not in the deaggregation of the "fast lipase" form. A 40-fold purification of the enzyme over the original was obtained, with a 25% recovery, from the application of sodium deoxycholate.

7. Comparison of the electrophoretic patterns between purified faba bean and commercial hog pancreas lipase showed good correlation between both enzymes at various purification stages. The electrophoretic technique provided good correlation between protein bands and lipolytically-active zones. Both enzymes revealed one lipolytically-active zone after filtration through Sephadex G-100 gel column.

8. The isoelectric point of the enzyme was estimated from a disc gel isoelectric focusing technique to be 4.8. The detection of a single lipase band from isoelectric focusing, indicates that the enzyme obtained from Sephadex G-100 was greatly purified.

9. A calibration curve for the estimation of the molecular weight of lipase was plotted using standard markers. Molecular weight was determined by sodium dodecyl sulphate gel electrophoresis.

10. The molecular weight of faba bean lipase was estimated to be 210,000 $\pm$ 20,000 while that of commercial hog pancreas lipase was estimated to be 180,000 $\pm$ 5,000. The high-molecular weight lipase form isolated from faba bean indicates that a "fast lipase" was purified.

11. A linear reaction with time for enzyme concentration against the rate of hydrolysis was obtained for extracts of faba bean lipase.

12. Faba bean lipase exhibited greater activity towards the short-chain triglyceride substrate (tributyrin), than the long-chain triglycerides, corn oil and olive oil.

13. A high tributyrin concentration, with the Km value determined to be 22.0 mM was necessary to obtain a zero-order rate of lipolysis.

14. The faba bean lipase exhibited lipolytic activity against an emulsified substrate over a pH range of 7.5-9.5 with an optimum at pH 8.5.

15. The pH stability range after a ten-minute exposure at assay temperature was 6.5 to 9.0 for the faba bean lipase.

16. In solution, the faba bean lipase lost all lipolytic activity after two minutes' exposure to $64^{o}C$. The optimum temperature for maximal activity was $38^{o}C$.

17. NaCl was confirmed as being necessary for a maximum rate of lipolysis. In the absence of this activator, faba bean lipase-catalyzed hydrolysis was minimal.

18. The bile salts, sodium deoxycholate and taurocholate were shown to cause activation at low concentrations.

19. Calcium and magnesium chlorides had no apparent effect on faba bean lipase, as no activation of the enzyme was evident up to high concentrations of the salts.

20. High concentrations of both mercuric chloride and p-chloromercuribenzoate inhibited the lipolytic activity of faba bean lipase. Mercuric chloride strongly inhibited the enzyme, while less than 20% of the original activity was inhibited by 0.01 M p-chloromercuribenzoate.

21. Lipase purification by second phase chromatography may be attempted to further characterize this enzyme from faba bean.

22. A heat-treatment stage is recommended as the probable method of lipase control for bean processing.

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