NOTICE

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

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

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

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

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

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

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

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

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

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

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

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

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

E. coli Lipase and Lysophospholipase

by

Guy Nantel

A thesis submitted in partial fulfillment of the

requirements for the degree

of

Doctor of Philosophy

in the

School of Graduate Studies

University of Ottawa

Ottawa, Canada

May 1976

à Marie-Christine

ACKNOWLEDGEMENTS

My thanks are extended to Professor Pierre Proulx for the opportunity to work in his laboratory and for the benefit from his knowledge and research experience.

Gratitude is also expressed to all members of the department of Biochemistry for their courses, advice, and encouragement which have been very helpful.

I acknowledge with thanks the technical assistance of Carmen Lantaigne and Georgia Baraff.

My thanks also to Mrs. V. Kneisly for the typing of this thesis.

Financial support during the first three years of my studies in the form of a Graduate Fellowship from the Province of Ontario is gratefully acknowledged. Assistance from the Medical Research Council, grant No MT 2632 to Professor Proulx is greatly appreciated.

SUMMARY

Part One of the thesis describes the characterization, partial purification and substrate specificity of a lipolytic enzyme from E. coli. Under conditions favouring phospholipase A1 activity, the crude lipolytic enzyme also displayed lipase activity which was found to readily attack mono- and diglycerides, and to hydrolyze tripalmitin slowly. A survey of the optimal conditions for lipase activity revealed a requirement for calcium, detergent, and an alkaline pH. Under the optimal conditions the crude enzyme hydrolyzed both triolein and tricaprylin, but the latter substrate was not degraded by the partially purified enzyme. Lipase activity was sought and found in E. coli B (ATCC 11303), 015, K12, and Bfad, each grown to the late log phase.

A protocol was established based on the work of Scandella and Kornberg (176) for the partial purification of coliform lipase. In the absence of detergent the enzyme was found to be insoluble at all stages of purification, as is the case for phospholipase A1. Lipase copurified with phospholipase A; in addition, both activities occurred in a single band following electrophoresis on SDS gels. The enzyme extracted from these gels was resolved into four bands on urea gels, but no activity could be recovered from them.

E. coli lipase was shown to attack all three positions of medium chain triglycerides, although activity towards the central ester was slower than towards the peripheral

esters. Because the sn-1 and sn-3 positions of triglycerides are attacked at similar rates, it could be concluded that the lipase activity is not stereospecific. The purified enzyme attacked diglyceride at the fastest rate, triglycerides being attacked somewhat more slowly. Monoglycerides were degraded at a rate of approximately 1/10 that of triglycerides. Other esters such as methyl oleate and cetyl palmitate were readily broken down, but not cholesterol oleate or the water-soluble ester p-nitrophenylacetate. Coliform lipase carries out a transacylation reaction in the presence of 40% ethanol as acceptor and phosphatidyl ethanolamine or triolein as donors.

Part Two of the thesis presents some results on the characterization of a cytosol lysophospholipase of E. coli extracts. This enzyme manifests itself between about pH 5.5 and 9, and is not stimulated by detergents but is inhibited by positively charged detergents. High concentrations of Triton X-100 also inhibit the enzyme. Some preliminary work was carried out on the purification of lysophospholipase which revealed its partial resistance to heat. Gel filtration on Sephadex G-200 provided a rough estimate of its molecular weight of 35,000.

TABLE OF CONTENTS

LIST OF SCHEMES

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

FA	Fatty acid
MG	Monoglyceride
DG	Diglyceride
TG	Triglyceride
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PG	Phosphatidyl glycerol
L-PC	Lysophosphatidyl choline
L-PE	Lysophosphatidyl ethanolamine
SDS	Sodium dodecyl sulfate
EDTA	Ethylenediaminetetracetic acid
TLC	Thin layer chromatography

GENERAL INTRODUCTION

This thesis is concerned mainly with the discovery, the isolation, and the properties of E. coli lipase, phospholipase A and lysophospholipase. To situate this study in its proper context, one is compelled to review first the present state of knowledge pertaining to the occurrence, the properties, and the functions of these enzymes from different organisms, and then to focus on the coliform enzymes in greater detail. Although the introduction is structured in this manner, the author has had to cope with the over abundant information on the mammalian enzymes and the comparatively skim knowledge regarding the bacterial enzymes.

Particular attention has been given to the pancreatic enzymes since these are generally representative of analogous classes of enzymes from other sources; when not entirely representative, they serve for comparison. Also, some of the pancreatic enzymes have been highly purified and studied in great detail. Information relating their structures and catalytic activities cannot be dismissed since it provides basic understanding of lipolytic phenomena. On the other hand, descriptions of enzymes from other sources are not so detailed, but do take into account variations in catalytic requirements and modes of action.

In this review of the literature the author has underlined evidence indicating that certain enzymes are misnamed. The question of whether a phospholipase A1 is an enzyme distinct from lipase is revived, and whatever results have been accumulated to answer this question are also critically examined.

Finally, regarding E. coli, a detailed description of the metabolism of phosphoglycerides is given, with particular emphasis on breakdown, and on this basis the roles of various lipolytic enzymes are evaluated.

REVIEW OF THE LITERATURE

A. GENERAL OCCURRENCE AND PROPERTIES OF PHOSPHOLIPASES A.

Many organisms possess both phospholipase A1 and A2 activities. However, much more work has been done with the A2 species, probably because it is readily available and can be more easily purified from various snake venoms (1).

1. PHOSPHOLIPASE A1

Recent work on phospholipase has been well reviewed (1-3). The first evidence for a phospholipase A1 came from Lloveras et al. (4) who showed that splenic extracts preferentially hydrolyzed the saturated fatty acids from the 1-position of purified egg yolk lecithin. Other phospholipases were then investigated in rat lung and liver homogenates (5,6) and in pancreatic tissue, where a heat-stable phospholipase A2 and a heat-labile phospholipase A1 were identified (7). Other mammalian tissues in which phospholipases A1 have been identified include post-heparin human plasma (8-10), rat and calf brain (11-12), and bovine adrenal medulla (13). Most of the mammalian tissue phospholipases A1 are localized in the lysosomal or microsomal fractions, usually possess an acid pH optimum, and are either unaffected or inhibited by Ca^{++} (1-3).

The mammalian phospholipases A1 seem to be considerably different from that of E. coli which is described in a later section. Two phospholipases A1 which have been purified some 50-fold from rat and calf brain (12) are both stimulated by neutral and anionic detergents, have an optimal pH of 4.0, and show no Ca++ requirement. Both preparations have a low lysophospholipase activity; in addition, the calf brain enzyme displays a lipase activity. Human brain phospholipases A1 and A2 have optimal pH's near 5, and require no Ca++ (14). A phospholipase A1 from rat liver lysosomes was inhibited by Ca++ and EDTA, while its A2 functional analogue showed only a partial inhibition (15). A guinea pig pancreatic phospholipase A1 which is more active towards phosphatidyl inositol than lecithin, with an optimal pH of 6.0, is also inhibited by Ca++ but not by EDTA (16). This enzyme, which is present in both mitochondrial and microsomal fractions, is also heat labile.

On the other hand, rat liver microsomes and plasma membranes (17) as well as myocardial microsomes (18) have phospholipases A1 which are activated by Ca++ at optimal pH's near 9.

Waite et al. (19,20) studied a phospholipase A1 which is solubilized by heparin from plasma membranes of rat liver, and which catalyzes the hydrolysis of mono- and diglyceride, lyso PE, and is able to carry out a transacylation

of the acyl group from position 1 of these glycerides to an acceptor monoglyceride.

As will be discussed in greater detail later, lipases usually display phospholipase A1 activity, and for almost ten years now the question has been posed as to whether or not phospholipase A1 is not just a lipase with broad substrate specificity. Very recently, Van den Bosch et al. (21) were able to purify pancreatic phospholipase A1 activity to near homogeneity by subjecting acid treated tissue extract to sequential chromatography on CM-cellulose, DEAE-cellulose, and Sephadex A-50. The purified enzyme hydrolyzes pure phosphoglycerides at position 1 only, whereas 2-lyso-phosphoglycerides are hydrolyzed at a rate 10 times less than 1-lyso analogues. In the presence of lysolecithin the enzyme behaves as a phospholipase B and hydrolyzes both acyl ester bonds of lecithin before releasing the products. The lysophospholipase activity is inhibited by bile salts. Interestingly, the enzyme appears to be the same as the non-specific esterase previously isolated by this group (22,23), which had a strong lipolytic activity towards tributyrin. But the results of this paper are presented and interpreted in a way that minimizes the importance of this lipase activity, whereas, in fact, this activity is greater than both lyso-phospholipase and esterase activities together. It is unfortunate that this ambiguity exists, since the question of

distinction between phospholipase A1 and lipase is very much a problem of the day. This pancreatic enzyme is therefore a non-specific esterase possessing phospholipase A1 and B, lysophospholipases A1 and A2, lipase, and other esterolytic activities. Its discovery may provide an important stepping stone for the understanding of lypolytic activity.

2. PHOSPHOLIPASE A2

The most fully characterized phospholipase A2 to date is that from porcine pancreas (24). A number of such enzymes from various venoms have been studied quite in detail and are found to resemble the porcine pancreas enzyme in their characteristics. The pancreatic phospholipase A2 is stable to boiling, and resists denaturation with 8 M urea (24). It requires Ca^{++}, which interacts with the enzyme independently of the substrate, and it is able to attack a molecularly dispersed substrate, although at a slow rate (25). Its optimal pH is near 8 in the presence of anionic detergents, but drops to 5.8 in their absence (25).

This phospholipase seems to attack its substrate after having penetrated between the substrate molecules at the interface, since activity is inhibited by excess surface pressure in a monolayer. It is also stimulated by anionic detergents and negatively charged phospholipid (26). These substances seem to favour a wider spacing between long chain

phospholipid molecules, and thereby permit an easier penetration of the enzyme. The stimulating effects of n-alcohols, cholesterol, fatty acid ester unsaturation (27,28), and Triton X-100 (29), which have been studied with various phospholipases A, allow the same conclusion.

Crotalus adamanteus phospholipase A2 (30) as well as the porcine pancreatic enzyme (24) are stereospecific. The venom enzyme, however, is more active toward zwitterionic phospholipids (31) whereas the acidic species are the preferred choice of the pancreatic enzyme.

The minimum requirement in order that a substance be a substrate for pancreatic phospholipase A2 is that one fatty acid ester bond be in a position adjacent to the alcohol-phosphate ester function, with the acyl chain occupying a correct stereochemical orientation (32,33). The phosphate moiety can be replaced by a phosphonate or sulfonate group, and a sulfonyl ester instead of an acyl ester bond will not give rise to any inhibition.

The complete amino acid sequence of pancreatic phospholipase A2 has been elucidated by de Haas et al. (34). It contains 123 amino acids in a single polypeptide chain, with an additional seven amino acids on the N-terminal for its zymogen (35), and six disulfide bridges (36). Zymogen precursors also exist for this enzyme in human (37) and rat (38) pancreas.

The phospholipases A2 of venoms are a fairly diverse group of proteins which show considerable variation in molecular weights (from 14,500 to 30,000) (39-42) and in their amino acid composition, in the known cases (40-49). Even isoenzymes from the same venom show variations: for example, two isoenzymes from Agkistrodon halys blomhoffi venom have isoelectric points of 10.0 and 4.0 (43). A salient feature of phospholipases A2 seems to be their thermostability which is likely due to their large content of disulfide bonds (40,41).

B. GENERAL OCCURRENCE AND PROPERTIES OF LYSOPHOSPHOLIPASES

Lysophospholipases are widely occurring and have been observed in various animal tissues, insects, plants, bacteria, algae, yeast, molds, fungi, amoeba, and mycoplasma (see introductions to refs. 82 and 84). Until recently, only partial purifications of this enzyme had been reported (44,45).

Lysophospholipases from various sources vary considerably in their substrate specificity, pH optimum, and their response to Ca^{++} and detergents. It is possible that a number of these enzymes are simply carboxylesterases with wide substrate specificities, particularly since their substrates, lysophospholipids, are slightly to completely soluble.

The enzyme from Penicillum notatum which has been purified to homogeneity (46) and which includes 30% neutral sugars, is observed to also possess a phospholipase B activity. Van den Bosch et al. (22) purified to homogeneity a beef pancreas phospholipase which, as mentioned previously, seems very similar to the phospholipase A1 recently purified from pancreas extracts by another procedure (21). The substrate specificity of this enzyme extends over a wide range and is more characteristic of a carboxylesterase; it hydrolyzes triacetin, tributyrin, p-nitrophenyl acetate and

p-nitrophenyl caprylate (23) as well as 1- and 2- acyl lysophospholipids and diacyl phospholipids (21).

The beef pancreatic lysophospholipase activity (22) as well as that from ox pancreas (44) and rat liver (47-49) have optimal pH's from 6.5 to 7.0. But this is not the general rule, since this enzyme from rat brain (45), Mycoplasma (50), microsomes of mosquito larvae (51), rat aortae (52), and E. coli (53) exhibit broad pH optima in the alkaline pH range.

Many lysophospholipases have no Ca++ requirement, such as the enzymes from beef pancreas (22), horsefly (54), rat intestinal mucosa (55), brain (45), and Mycoplasma (50), and most are inhibited by various detergents which stimulate phospholipases A1, such as deoxycholate (22,45,47,50,55,57), sodium dodecyl sulfate (54,56,58) and Triton X-100 (45,50).

The lysophospholipases which also possess phospholipase A1 activity, have ion and detergent requirements which are quite different from the two activities. If the same site of the enzyme is responsible for both activities then it is possible that the difference between the presence of one or two esters in phospholipid must be compensated by ions and detergents in order to make the interface functionally similar in both cases. However, different sites for each activity remains a very likely possibility.

C. GENERAL OCCURRENCE AND PROPERTIES OF LIPASES

1. DEFINITION OF LIPASES

The classification and nomenclature of enzymes depend on the nature and type of reactions catalyzed. In this respect the classification of carboxyl ester hydrolases is difficult to organize since there are two types of enzymes, esterases and lipases, hydrolyzing closely related substrates. Lipases and esterases have been differentiated on the basis of various inhibitors (59), and on the length of the fatty acyl moiety or the nature of the alcohol moiety (59). But since lipases are rather unspecific enzymes, these categories are misleading. The most valid approach to the classification seems to be that of Desnuelle who took into account the physical state of the substrate (60), that is, whether it is soluble or not. However, even this criterion is not entirely reliable since an increase in concentration of an esterase substrate such as triacetin, for example, to oversaturation of the bulk phase will bring about the formation of emulsified particles which can then be hydrolyzed by lipases (61). Furthermore, the addition of increasing amounts of short chain glycerides to a fixed volume of 0.1M NaCl brings about two mesomorphic transitions. The first is an aggregated form of the glyceride, between a molecularly dispersed solution and heterogeneous

emulsion which remains isotropic. The second transition following saturation is characterized by the appearance of a second phase forming relatively large emulsified particles. Pancreatic lipase is able to act against both these forms (62).

The second edition of the Report of the Enzyme Commission of the International Union of Biochemistry (63) considers lipases (EC 3.1.1.3) as enzymes hydrolyzing glycerol esters according to the following equation:

triglyceride + H_2O ⟶ diglyceride + a fatty acid ion.

It is mentioned that pancreatic lipase acts only at an ester-water interface with a preference for the external links in triglyceride molecules. Very appropriately, Desnuelle (60) has criticized this definition on many points. The equation given is incorrect since most lipases hydrolyze glycerides beyond the diglyceride stage, and sometimes up to the release of free glycerol (64). Some lipases hydrolyze triglycerides under acidic conditions where the released fatty acids are not fully ionized. Furthermore, lipases will often hydrolyze ester substrates other than glycerides, such as methyl esters for example.

Lipases are classified as hydrolases, and in most cases the equilibrium is on the side of hydrolysis because of the high molar concentration of water. But the

participation of lipases in transfer reactions in which molecules other than water act as the acceptor for fatty acyl radicals has been demonstrated (60). Furthermore, since lipase activity occurs at an hydrophobic interface, this could restrict the concentration of water vicinal to the active site in such a way as to promote synthetic processes. This has been observed (65,66).

A classification of lipases must take into consideration the type of lipase concerned since there are lipases known to be specific for monoglycerides, diglycerides, and even for classes of fatty acyl groups. Furthermore, considerable variations are observed in the extent to which water-soluble substrates are attacked; to resolve this difficulty it may be necessary to define lipases according to their activity against a series of standard substrates.

2. DISTRIBUTION OF LIPASES IN LIVING ORGANISMS

The distribution of lipases has been reviewed quite extensively by Wills (67). They have been found in most animals, plants, or microorganisms checked for this enzyme. Of the animal lipases, pancreatic lipase seems to have received the most attention followed closely by a variety of lipoprotein lipases. Lipases from microorganisms have more recently been investigated because of possible medical and industrial application, particularly since many of them are excreted into the culture medium, and are therefore more easily extractable; several have been purified and carefully investigated (60).

There are three main groups of lipases in mammals; those discharged into the digestive tract by specialized organs, such as pancreatic, gastric, and intestinal lipases, and those present in tissues and milk.

A large number of tissues are reported to contain lipases; blood, most organs of the mammalian body, heart, brain, arteries, lungs, adrenals and suprarenals, ovaries, white and brown adipose tissues, and skin are among these (see review, ref 60). Their physiological functions are not always known although in adipose tissue, lipase is very responsive to energy requirements of the body, and under second messenger control will function to increase the free fatty acid supply (68-70).

Milk lipases have long been suspected of being an important factor in causing spoilage of milk flavour during storage. Thus both practical and theoretical reasons have prompted an extensive study of this enzyme (60).

The intracellular distribution of these enzymes is quite often cytosolic although they have been found in mitochrondria and microsomes (see review, ref 67).

Plants have not been studied extensively with respect to tissue distribution of lipase, but the enzyme has been found in many seeds and their flour, and also in fruit. Castor bean lipase is perhaps exceptional to the others of this group in that it has been quite well characterized (see reviews, ref 60 and 67).

Bacteria, yeasts and fungi all possess lipases. Many of these are intracellular lipases, but the majority studied are extracellular, being discharged through the external membrane into the culture medium. These enzymes are often inducible by various lipids and surfactants, depending on the microorganisms (see review, ref 60).

3. PANCREATIC LIPASE

(a) PURIFICATION

Early attempts at the purification of pancreatic lipase go as far back as the early 1920's, but they were not successful. The first complete purification procedure was published in 1957 by Sarda et al. (71) and was later improved to the point of obtaining a chromatographically and electrophoretically pure preparation (72). A short time later Basky et al. (73) obtained a preparation which was homogeneous according to ultracentrifugation and free boundary electrophoresis but which contained two inactive components on the basis of starch gel electrophoresis and DEAE cellulose chromatography. Recombination of the purified pancreatic lipase obtained from the step just prior to the DEAE cellulose chromatography could restore 90% of the activity when added to the protein obtained as the second component.

Other purification methods were sought to improve the yield, since quantities isolated by early procedures were

insufficient for structural studies. One approach by Sarda et al. (74) involved the addition of a partially purified preparation to a Sephadex G-200 column, through which the lipase ran with the void volume. The enzyme, in this case, was in the form of an undefined high molecular weight complex and was designated as the "fast lipase". With this method most of the protein was retained by the column so that a substantial purification was achieved. The "fast lipase" was then converted to a "slow lipase" by a treatment typical of those disrupting lipoproteins, and had a molecular weight of 38,000. A later paper by this group, however, reported that there were two components in this slow enzyme preparation (75).

Another "fast lipase" was obtained by Kimura et al. (76) when bile was added to human pancreatic juice. A low molecular weight lipase could be obtained by extraction with acetone or phospholipase A digestion, and could also be reconverted back to the high molecular weight form by incubating with bile, lecithin, and phosphatidyl serine. Both these forms were immunologically identical. These authors believe that a low molecular weight lipase is synthesized in the pancreas and is modified in the duodenum by a process involving the presence of bile, to confer greater stability.

Vandemeers et al. (77) purified rat pancreatic lipase to homogeneity as judged by disc electrophoresis, and

were the first to submit a preparation to amino acid analysis. Their purified enzyme also contained 14% lipid.

Desnuelle's group separated two active components, LA and LB, from porcine pancreas, with essentially the same purification procedure reported earlier by them, but involving a longer DEAE cellulose column (75). The two forms have slightly different isoelectric points, the LB form being slightly more basic than the LA form. Both forms were electrophoretically and ultracentrifugally pure, and had the same specific activities. Since similar results could be obtained by starting the purification from pancreatic juice, the difference between these two forms is not likely due to any lipid bound to one or the other. The molecular weights were estimated to be 48,000. Amino acid analyses revealed a very similar residue composition between these isoenzymes, with a difference only in the isoleucine content. The amino acid composition also showed a high degree of homology with Vandemeers' rat pancreatic lipase mentioned previously.

(b) PANCREATIC COLIPASE

As already stated, Basky et al. (73) reported that purified porcine pancreatic lipase lost its activity against a bile salt-stabilized triglyceride substrate after having been passed through a DEAE cellulose column. But the lipase activity was recoverable after addition of a

boiled pancreatic extract. Other studies with rat pancreatic juice fractionations further confirmed these findings: only 51% of lipase activity was recovered from rat pancreatic juice run on Sephadex G-100 and G-75 columns, but the full activity was present when the columns were run in the presence of sodium taurodeoxycholate at levels above its micellar concentration (78). It was rationalized that the absence of the bile salt permitted a separation of a small molecular weight lipase activator.

This small protein, called colipase, was purified to homogeneity on the basis of electrophoresis and ultracentrifugation analyses (79). It was found to contain neither methionine nor tryptophan. On the other hand, its thermal stability is well accounted for by the existence of five disulfide bridges. Erlanson et al. (80) found colipase to consist of a single polypeptide chain with a molecular weight near 10,000, as determined by several techniques (79). An amino acid analysis revealed 21 acidic and 10 basic residues, in agreement with the isoelectric point of this protein which is 5.0. This relatively large percentage of polar residues, in addition to the five disulfide bridges, seem to indicate a rather compact tertiary structure. This is further confirmed by the low frictional ratio of colipase ($f/fo=1.19$), and agrees well with the heat stability of this protein.

Borgstrom et al. (81,82) carried out some experiments with rat pancreatic juice to understand the function and physiological importance of pancreatic colipase. They found lipase free of colipase and bile salts to be optimally active at pH 9.0. When 1mM sodium taurodeoxycholate was added in the absence of colipase, the activity was almost completely inhibited. If colipase was added in the absence of bile salt, only a small stimulation, in the order of 1.3 to 1.4 was noted (82). However, a most pronounced effect was observed when both bile salts and colipase were added to the lipase: the activity was considerably stimulated and the optimal pH was shifted to 6-7.

Inhibitory effects of taurodeoxycholate, taurocholic acid, and of glycine conjugates of cholic and deoxycholic acid, in the absence of colipase, were found to occur at concentrations in the vicinity of their critical micelle concentrations (82,83). Other detergents such as sodium dodecyl sulfate, N-dodecanoyl glycine, N-decanoyl taurine, and lysolecithin were inhibitory even in the presence of colipase, but these inhibitions could be reversed if conjugated bile salts were added. Desnuelle (84) was recently able to conclude that lipase inhibition by taurodeoxycholate at pH 6 was of the competitive type since the addition of 2 moles of colipase per mole of lipase raised the K_i from 0.15 mM to 3.2 mM.

Molecular weight determinations from gel filtration experiments with different combinations of lipase and colipase, and with or without bile salts, showed that in bile salt solution colipase forms a dimer, and that in the lipase-colipase complex these compounds are present in a ratio of 1:2 (82).

Colipases from human, rat and porcine pancreas were found to be interchangeable, and complemented the lipases from all three sources (82). Porcine and bovine colipases are also interchangeable (85).

(c) STRUCTURAL PROPERTIES RELATED TO CATALYTIC ACTIVITY

Garner and Smith (86) examined the two forms of pancreatic lipase, LA and LB, for the presence of carbohydrate residues, since other pancreatic enzymes have been found to be glycoproteins. After having identified the presence of carbohydrates by staining lipases in acrylamide gels, these workers ran a 75% pure preparation of lipase on an agarose column containing covalently linked concavalin A. This phytohemagglutinin binds terminal α-D-glucopyranosyl, α-D-fructopyranosyl, and α-D-mannopyranosyl residues of the carbohydrate moieties of glycoproteins (87). 99% of the lipase activity was bound, and both forms of lipase were recovered after eluting with α-methyl mannoside. Two carbohydrate components were identified by gas chromatography as mannose and N-acetyl glucosamine. These were bound in proportions of

4 moles and 3 moles respectively per mole of lipase; both forms of the enzyme were found to be similar. Another group partly confirmed the results of Garner and Smith but found other sugars as well (88).

In another approach, Verger et al. (75) reduced both lipases with 2-mercaptoethanol, followed by an alkylation with acrylonitrile to cleave and stabilize the disulfide bonds against disulfide interchange reactions, and then subjected these preparations to tryptic digestion. The peptides produced were not all soluble, but 89 to 94% of the reducing sugars were found to be in the soluble fraction. Fractionation of the tryptic products revealed three glucopeptides for lipase LA and one for lipase LB. All peptides were similar in amino acid sequence, but different in their carbohydrate residues.

Pancreatic lipase was examined for the presence of reactive thiol groups. Both molecular species, LA and LB, were each found to contain 2 SH groups by titration with 5,5'-dithiobis-(2-nitrobenzoic acid), p-mercuribenzoate, and N-ethylmaleimide (89). However, only one SH group reacted in the native enzyme. The second reacted in the presence of 0.3% sodium dodecyl sulfate or 8M urea. It seems therefore that one thiol group is at the surface of the enzyme and, furthermore, in an hydrophobic environment, since no reaction was observed with alkylating reagents such as iodoacetate and iodoacetamide.

Both SH groups react with phenylmercuric chloride, and advantage was taken of this reaction to prepare two well defined S-substituted lipase derivatives: mono-5-thio-2-nitrobenzoic acid (TNB) lipase, in which the SH_1 group was selectively blocked by a TNB radical, and a diphenyl mercuric (DPM) lipase in which the SH_1 and SH_2 groups were both blocked by the phenylmercuric radical (90). These reactions were fully reversible in the presence of excess thiol. The TNB lipase had a Km value of 10 fold higher than the native enzyme whereas the Vmax was unaffected, while the DPM lipase showed a 40% decrease in Vmax without a change of Km. These results suggest that the SH_1 group is at or near the site of attachment of the enzyme for the hydrophobic interface, whereas the SH_2 group is near the catalytic site. This would put both groups in an hydrophobic region of the molecule.

Since the enzyme does not require the SH groups for catalysis, it seemed plausible to search for a threonine or serine residue which would become acylated to make the acyl-lipase intermediate in catalysis. But low concentrations of diisopropylphosphofluoridate not only have been found not to inhibit the enzyme even in the presence of bile salts, but have often been used to stabilize the enzyme against proteolytic contaminants. Higher concentrations of this organophosphate were found to bind to a tyrosine residue not involved in activity (91). In contrast, pancreatic lipase

was found to be inhibited by either an emulsion or a solution of diethyl p-nitrophenyl phosphate (DNP), but only in the presence of at least 0.05% bile salts. It seems therefore that this reagent has to be included into micelles before the inhibition can take place. The inactivation was found to be pH dependent, with an optimum pH of 6.0, and running parellel to the release of 1 mole of p-nitrophenol for 1 mole of inhibited enzyme. By using ^{32}P-labelled DNP followed by various proteolytic treatments it was possible to isolate a radioactive peptide containing a phosphorylated serine. These results are therefore fully consistent with the view that lipase is active exclusively on substrates in an emulsified or micellar state, and that a serine is involved in the activity of the enzyme, very possibly as an acyl acceptor.

A histidine residue has also been found to be involved in lipase activity (92). The pH dependence of the reaction with tributyrin is consistent with the participation of one essential ionizable group with a pK of 5.8 which must be unprotonated; only the imidazole ring of histidine ionizes in this pH range. Furthermore, a strong correlation was established between the rate of enzyme inactivation and the rate of oxidation of a histidine residue in photooxidative assays; three other residues were also photooxidized, but without such a good correlation as that obtained with histidine.

It seems likely therefore, that pancreatic lipase, like a number of esterases and proteases with esterolytic activity, is a "serine-histidine" enzyme.

Dufour et al. (93) investigated the role of the carboxyl groups in the activity of the enzyme. They based their studies on a report by Hoare and Koshland (94) who worked with other carboxyl esterases and proteases. This approach consists in reacting the proteins with a carbodiimide, followed by condensation with a nucleophile. In this way 14 carboxyl groups reacted rapidly with N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) and glycine ethyl ester. A more bulky carbodiimideand nucleophile, 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide p-toluene sulfonate (CMC) and norleucine methyl ester, reacted with only 5 carboxyl groups. In both cases lipase activity was completely abolished, suggesting that at least one of these carboxyls is essential. However, this group must have a structural role since the enzyme with 5 modified groups was fully titratable with diethyl-p-nitrophenyl phosphate in the same way that the catalytic site of the native enzyme is titratable. The 5-modified-group-enzyme was also more rapidly denatured in 8M urea than the native enzyme, again supporting a structural role for the essential carboxyl(s).

Since a maximum of 14 carboxyls reacted with EDC out of a total of 41, the majority of carboxyls must be "buried" in the lipase molecule. The 5 groups reacting with CMC must be less sterically hindered than the others.

Work on the primary structure of pancreatic lipases LA and LB shows that the molecules consist of single polypeptide chains with serine at the N-terminal and a half cystine at the C-terminal.

(d) GENERAL REQUIREMENTS AND SUBSTRATE SPECIFICITY

(i) OPTIMAL pH AND COFACTOR REQUIREMENTS

Lipase activity is stimulated by various ions and bile salts, which, in some cases, bring about shifts in the optimal pH. Borgstrom (95) studied how the optimal pH of lipase activity of rat pancreatic juice is affected in the presence of varying concentrations of taurocholic acid. The pH optimum of lipase without detergent is situated at about pH 8. This optimum shifts downward with increasing taurocholate concentrations, to its lowest at pH 6 in the presence of 0.2% taurocholate. At higher concentrations of detergent a second pH optimum develops at pH 9.0.

Without calcium salts pancreatic lipase is reported to hydrolyze triolein only as far as diolein (96). The addition of large concentrations of this cation permits lipolysis to progress to the monoglyceride stage. Furthermore, calcium ions have been found to stabilize this enzyme

against heat denaturation (97). In the presence of deoxycholate lipase is inactive unless calcium is also present (98). Since the Km is found to vary proportionately to the reciprocal value of the Ca++ concentration while the Vmax remains constant, it is likely that the Ca++ is bound by the enzyme rather than by the substrate. Therefore calcium is postulated as being necessary for the adsorption of the enzyme at the triglyceride-water interface in the alkaline pH range, to compensate for the electrostatic repulsion existing between the negatively charged lipase molecule and the anionic group of the bile salts at this interface.

With a simple system composed of two reactants, triglyceride and water (stabilized by NaCl and a trace of oleate), Benzonana et al. (45) showed that the addition of Ca++ and deoxycholate did not appreciably increase the initial rate of lipolysis, but rather prevented the inhibitory effect of long chain soaps; in the absence of deoxycholate the reaction rate drops after a few seconds, but in its presence the rate remains constant for several minutes. On the other hand, high concentrations of this bile salt are inhibitory, possibly by a competitive effect exerted by bile salt micelles.

The presence of NaCl is essential to pancreatic lipase activity in a system of water and triglycerides (99), and is observed to affect the initial rate of the reaction.

Although this NaCl effect is not clearly understood, it is suggested that it may be due to a minimum ionic strength requirement of the aqueous phase before lipase can become active. The optimal concentration of NaCl was found to be 7 mM.

(ii) FATTY ACID CHAIN AND ALCOHOL MOIETY SPECIFICITIES

In studying the specificity of pancreatic lipase towards different fatty acid chains of triglycerides one has to keep in mind various problems which complicate the interpretation of data; for example, optimal concentration of bile salts, calcium ions, and hydrogen ions vary considerably with the length of the acyl chain (100,101). Also, the glycerides used may be liquid or solid at the assay temperature so that the reaction site would be a liquid/liquid interface in one case, and a solid/liquid interface in the second case (102,103).

It is in the light of this that one must weigh the results indicating that shorter chains in triglycerides are preferentially hydrolyzed by pancreatic lipase at an increasing rate from C_2 to C_4. Assays with mixed triglycerides show similar rates of hydrolysis for saturated chains from C_{12} to C_{18} as well as for oleoyl and linoleoyl chains (104,105).

Mattson and Volpenheim (103) have studied how the hydrolysis rate is affected with esters of primary n-alcohols containing 1 to 18 carbons esterified to fatty acids with 2 to 18 carbons. They found that the speed of hydrolysis is influenced independently by both the acyl and the alkyl chains. Regardless of the fatty acid moiety, the rate of hydrolysis decreased as the alcohol chain length increased from ethanol to pentanol, methanol being a less suitable substrate than ethanol, and then increased until the highest rate was obtained with heptanol. For the fatty acid component there was a progressive increase in the rate of hydrolysis with C_3 and C_4 fatty acid chains, a large drop of activity at C_5, followed by a gradual increase from this point on to C_{12}. The highest activity was obtained with the C_{12} chain, the C_4 chain's activity following closely. According to this data the highest rate for any of these esters should be with heptyl dodecanoate, and indeed, this was observed (103).

Branching of the aliphatic chain in the neighbourhood of the carboxyl, such as with 2,2-dimethyl stearic acid esters, causes resistance to lipolysis, probably because of steric hindrance (106,107). The polyenoic acids of marine oils which have double bonds in position 4 or 5 (108) or trans-3-enoic esters of Grindelia oxylepis seed oil (109) are all fairly resistant to lipase hydrolysis.

Brockerhoff (110) has carried out a most comprehensive study with monoenoic, polyenoic, methyl-branched, ω-cyclohexyl, and ω-phenyl acids esterified to p-chlorobenzyl alcohol or 2-fluoroethanol. He essentially confirmed the above-mentioned findings, and further found that multiple branching of the fatty acid moieties introduced greater resistance, with almost no activity remaining when an ester of cyclohexylacetic acid was the substrate. The inhibition observed with ω-phenyl groups disappeared gradually as the chain was lengthened to C_6, with an abnormally high rate of hydrolysis being obtained with a 8 carbon chain. The enzyme also attacked formates rapidly. The author concludes that steric hindrance is responsible for inhibitions up to C_5, in the case of substituted acyl residues.

(iii) POSITIONAL AND STEREOSPECIFICITY

Much work has been carried out and many methods have been used to determine the positional specificity of pancreatic lipase on glycerides. The literature pertaining to this work has been reviewed by Desnuelle and Savary (102). It is now well understood that the ester bonds in the α-positions of triglycerides are hydrolyzed first, and that the enzyme is not stereospecific since both positions 1- and 3- are attacked at the same rate (111). α,β-diglycerides formed are broken down at a slower rate than triglycerides and release β-monoglycerides. Glycerol can be released very slowly from

α-monoglycerides which may form by isomerization from β-monoglycerides (102,112,113).

(iv) THE PHOSPHOLIPASE A1 ACTIVITY OF PANCREATIC LIPASE

Porcine pancreatic lipase was found to hydrolyze the fatty acid ester bond at the 1-position of synthetic phosphatidylcholine, and to be nonstereospecific towards this substrate since it also hydrolyzed the ester in position 3- of a sn-2,3-diacyl phosphatidylcholine (114). A more detailed study of this phospholipase A1 activity has been carried out by Slotboom et al. (115) in which they found this enzyme to hydrolyze exclusively the 1-position, regardless of the chain length at position 2- or the distribution of saturated and unsaturated fatty acid moieties between positions 1- and 2-.

Contrary to most phospholipases A1 which are stereospecific, the phospholipase A1 activity of pancreatic lipase is not stereospecific, which is in accord with the non-stereospecificity displayed towards glycerides (115). Furthermore, anionic phosphoglycerides were found to be degraded more rapidly than zwitterionic phosphoglycerides, whereas lysophosphoglycerides were almost inactive as substrates (115).

If position 2- of a 2-deoxy-phospholipid is occupied by an aliphatic chain, there results no lipolytic activity; but an ether linkage in this position renders the ester of position 1- liable for 50% of the activity observed with the corresponding phospholipid (115). The compound, 1,3-diacyl-sn-glycero-2-phosphorylcholine is not hydrolyzed by pancreatic lipase, even if two fatty acid ester linkages at primary hydroxyls are available; however, the removal of the negative charge by substituting the phosphatidate with two methyl groups favours attack by the enzyme (115).

(e) PHSICO-CHEMICAL ASPECTS RELATED TO SUBSTRATE REQUIREMENTS

The concept that pancreatic lipase acts only at an oil-water interface, and would consequently be adapted to this function, has been questioned by Entressangles et al. (62) since they observed enzymatic activity on micellar triacetin in the presence of 0.1M NaCl. Brockerhoff (116) later found that the hydrolysis of oleic acid esters depended on the nature of the alcohol, the rate of the reaction being influenced by inductive effects and steric hindrance. Lipolysis could start with a nucleophilic group of the enzyme attacking the carbonyl carbon of the substrate. If this is the rate-limiting step, the reaction should accelerate when the carbon is made more electrophilic. This was indeed found to be the case; the aliphatic and hydroxyl groups

kept the activity low, whereas alkoxy, halogens, cyanide, phenyl, and NO_2 substituents increased the activity. Therefore, the result of activation or deactivation by neighbouring ester or hydroxyl groups explains the accumulation of diglycerides and monoglycerides during pancreatic hydrolysis. This theory also explains the slowness with which the α-bound fatty acid in lecithin is attacked, although the structure of the lecithin micelle may also be partially responsible for this effect. Since the phosphate group has a high electron density and negative charge it deactivates the ester group. Removal of the negative charge on the phosphate, such as in the compound diphenyl phosphatidate, diminishes this inhibition.

This inductive effect does not explain why lypolysis does not start at the β-ester of triglyceride, the carbonyl group of which must be even more electrophilic than those in the α-positions, since it is flanked by two activating groups. The reason given is an inhibition through steric hindrance; this is further supported with the substitution of the two methyl groups of isopropyl oleate, an inactive substrate, with fluorine [$(F_3C)_2$-CHO-], which should have a highly activating effect on this substrate, and yet does not. However, 1,3-difluoroisopropyl oleate is definitely split by lipase (116). A similar situation arises with phenyl oleate which is not hydrolyzed as compared to p-nitrophenyl oleate

which is.

Since the effects of the substitutents are not additive, it must be supposed that these substrates impose their characteristics on the structure of the lipid-water interphase. Therefore steric hindrance may not be the only factor causing deviations from the inductive effect theory (116).

Whether or not lipase will be active against an ester seems to depend primarily on the hydrophobicity of the molecule rather than the chain length or the structure of the alcoholic moiety (117). For example, when the substrate tripropionin is emulsified in a solution of an inhibitor such as triacetin, the inhibitor will distribute itself between the two phases according to its lipophilic and hydrophobic character. It will not only dilute that emulsion, but if it is more hydrophilic than the substrate, it will concentrate in the interface and displace the substrate. If this interpretation is correct, any substance that can be absorbed by the emulsified substrate should inhibit lipolysis, and such is found to be the case; diethylether is found to be a better inhibitor than the completely water-soluble dimethyl ether and diethylene glycol. Among alcohols tested, butanol is the most lipophilic and the best inhibitor.

The use of monolayers to study lipolytic activity provides a simple system which is stable in the absence of tensioactive compounds, and in which all substrate molecules are in contact with the solvent containing the enzyme (118). Accordingly, the technique has been used to study the effect of surface pressure on lipase hydrolysis of ester monolayers (119). The substrates 1,3-dihexanoyl-2-butyl ether and trihexanoyl glycerol were used. A considerable activity increase was found from 10 to 20 dynes/cm, with a sharp maximum at 23 dynes/cm, followed by a rapid drop at higher pressures. Interestingly, this maximal lipase activity for both substrates was attained at nearly the same surface pressure which, however, represents widely different surface densities (1.84×10^{14} and 1.42×10^{14} molecules/cm^2 respectively) and molecular areas (54 Å^2 respectively) for these substances. Therefore activity of a lipase on a substrate monolayer is not controlled by the surface density but rather by other parameters closely related to surface pressure. This could come about by the existence of reversible transconformations of the lipase molecules leading to at least three states: a native and inactive state favoured by high film pressure, a slightly modified and active state favoured by intermediary values of pressure and energy, and finally a denatured and therefore inactive state favoured by low pressure.

This hypothesis is further supported by results showing an irreversible inactivation of lipase in systems

containing a hydrocarbon-water interface where the free interfacial energy can be expected to be especially high (i.e. a low pressure) (120). Kinetic studies show that taurocholate and albumin can prevent but cannot reverse the unfolding of the enzyme; this is how these agents can have an "accelerating" effect on lipolysis.

Bile salts may also prevent the blocking of oil-water interfaces by denatured protein, since they have been shown to be able to even clear an interface that is already blocked (121). In addition, bile salts form mixed micelles with fatty acid and soaps, and therefore accelerate the diffusion of these compounds from an hydrophobic interface into water (122).

At physiological concentrations bile salts inhibit lipolysis, probably by blocking the interface themselves. The substrate displacement effect is possibly combined with electrostatic repulsion of the enzyme since conjugated bile acids, which are much stronger acids, are also much better inhibitors (99).

It is generally accepted that esterolysis catalyzed by enzymes is in principle reversible but that usually the excess of water is so high as to preclude any significant resynthesis. However, there have been a number of accounts in the past concerning the synthesis of triglycerides by lipase (60). This may be related to the comparatively low

concentration of water near the interface of the emulsified oil droplets. An acidic pH with unionized fatty acids may further help this phenomenon.

4. OTHER MAMMALIAN LIPASES

There are a multitude of other lipases which have been identified in most organs of mammals. Their distribution and their properties have been reviewed by many authors (60, 67,102,123) and will be summarized only briefly here.

Monoglyceride lipases have now been observed in a number of mammalian tissues (124-129). Some have been shown to be without effect on long chain di- and triglycerides (130) or to be devoid of esterase activity (131), but may attack methyl and ethyl esters and short chain di- and triglycerides (130). A few of these enzymes have been partially purified (130,132,133).

Lipoprotein lipase from rat (134) and human (135) postheparin plasma has been purified to homogeneity. It requires the presence of lipoprotein, with maximumal stimulation provided by high density lipoprotein, to be active against triglycerides; however, it will hydrolyze di- and monoglyceride without this cofactor. This enzyme is able to break down glycerides to fatty acids and glycerol, although the degradation of monoglyceride is slow (134). Some authors have found this lipase to attack triglycerides

preferentially at position 1-, to release sn-2,3-diglycerides (136). This may be an important step to avoid the stimulation of phospholipid biosynthesis.

There are also a profusion of other lipases in post-heparin plasma as well as in most other tissues some of which are under second messenger control (see reviews, ref 60,67, 102,123).

5. PLANT LIPASES

Among plant lipases, castor bean lipase from dormant castor beans has been studied the most, and only this lipase will be considered here.

(a) FUNCTION, ISOLATION AND PROPERTIES OF CASTOR BEAN LIPASE

This enzyme has only been partially purified (137, 138), mainly because it is insoluble in water. Electron microscopy shows that this enzyme is bound to membranes of the oil-containing organelles of the seed, the spherosomes (138,139). This lipase hydrolyzes short and long chain triglycerides at an optimal pH of 4.2 (138). The assays require no added emulsifiers or fatty acid acceptors (140,141) because it contains a natural one, a cyclic tetramer of ricinoleic acid (142) (which is extractable by dry butanol from a lyophilized powder). Other amphipaths can stimulate the activity of this lipase, but not as well.

A protein cofactor has also been found for this enzyme (143). It can be obtained by centrifugation of a self-digest preparation at pH 6.0, and purified from the supernatant with DEAE cellulose chromatography. This cofactor is a small heat-stable glycoprotein which appears to be related to some of the castor allergens (142).

(b) SUBSTRATE SPECIFICITY OF CASTOR BEAN LIPASE

Castor bean lipase was originally believed to be devoid of substrate positional specificity since mono- and diglycerides do not accumulate as products of triglyceride hydrolysis (144). The enzyme was later thought not to cleave secondary ester bonds (145) and such release of fatty acids from position 2 was attributed to isomerization of the mono- and diglycerides prevailing at acid pH. It has recently been found, however, that 1,2-diglycerides do not isomerize appreciably under these conditions, and that both ester bonds of 1,2-diglycerides are hydrolyzed at the same rate (146). The enzyme also attacks the esters of 1- and 2-monoglycerides. The appearance of 1,3-diglycerides under certain conditions in the hydrolysis products was found to occur by a synthesis of 1,3-diglyceride from 1-monoglyceride and fatty acid. Hydrolysis finally runs to completion because the reaction is irreversible after the release of free glycerol.

It is interesting to see how this enzyme has its own emulsifier in addition to a protein cofactor. In this respect, although this is a plant lipase, the enzyme acts in a way not very different from pancreatic lipase. However, there is one major difference in that this lipase is extremely insoluble, whereas pancreatic lipase is a soluble enzyme. Their structures, therefore, must be quite different.

6. LIPASES FROM MICROORGANISMS

A number of microbial lipases have been purified to homogeneity, some of which have been crystallized (see review, ref 60). Since many of these are extracellular, their purification from cell-free culture media has been possible with conventional techniques used for soluble enzymes.

(a) STRUCTURAL PROPERTIES OF SOME MICROBIAL LIPASES

Present knowledge concerning the structure of microbial lipases is very incomplete. Many extracellular lipases are glycoproteins, or otherwise contain a polysaccharide moiety which is not covalently bound to the protein. The lipase from Rhizopus arrhizus is linked to a sugar moiety which accounts for 8,500 out of the total molecular weight of 43,000 (147). However, this sugar moiety is not covalently bound and can be separated from the protein by heating or by cold 5% trichloracetic acid precipitation. The sugar-free form of the enzyme is as

active as the native enzyme, so that the sugar moiety does not constitute a structural requirement for activity.

The lipase from Torulopsis ernobii, with an isoelectric point of 2.95 and a molecular weight of 43,000 consists of a single peptide chain of 306 residues (148) comprising 3 half cystines; no free SH groups were reported, however. There is a sugar moiety associated with this enzyme which consists of 36 moles of mannose per mole of enzyme. An extracellular lipase produced by Candida cylindracea, which has been purified to homogeneity (194) was found to have a high content of hydrophobic residues and to contain the sugars mannose and xylose (150).

A lipase from Rhizopus delemar was recently purified and other minor contaminating lipase components were eliminated (151,152). This lipase, with a molecular weight of 41,000, was shown to have a high content of apolar residues, suggesting the presence of a hydrophobic region capable of interaction with water-soluble and lipid-soluble reagents. Various amino acid residues were therefore modified according to their degree of disclosure in an oil or in a water phase; this procedure led to the finding of two essential tryptophan residues, one modified from the olive oil side at the interface of the emulsion, and the other, thought to be the binding site, was modified from either the water or oil side (152). These residues seem to be buried

in the lipase molecule since they are not reactive unless substrate is added and the enzyme-substrate complex is formed.

The lipase of Geotrichum candidum, purified to homogeneity (153), and with a molecular weight of 27,500, contains 18.9% α-helix, as estimated by circular dichroism (154). Amino acid analysis revealed 224 residues comprising six half cystines per molecule. Neither sugar nor lipid could be detected. The enzyme is stable up to 60°C, and is not further stabilized by Ca++. The addition of 8M urea is inhibitory, and is observed to induce a distinct change in the circular dichroism spectrum, indicating a considerable alteration in the enzyme structure (154). However, upon decreasing the urea concentration by dilution, the enzyme is able to revert back to its active form.

Oi et al. (155) crystallized two extracellular lipases from Penicillum crustorum, lipase I and II, with molecular weights of 29,000 and 32,000 respectively. Lipase I hydrolyzed substrates more typical of lipases such as olive oil, while lipase II hydrolyzed tributyrin more efficiently. Both enzymes were activated by Ca++ and inhibited by sodium lauryl sulfate. These two enzymes are interesting because they are interconvertible with respect to their substrate specificity. Lipase I changed to lipase II after prolonged incubation with Ca++, or treatment with sodium deoxycholate,

EDTA, or p-chloromercuribenzoate; exposure of lipase II to hydrogen peroxide or sodium borohydride brought about a conversion to lipase I. The mechanism underlying these transformations is not yet understood.

(b) GENERAL REQUIREMENTS OF SOME MICROBIAL LIPASES

Perhaps the most interesting aspect concerning microbial lipases studied so far is their substrate specificities. These show considerable species variation, and in some cases differ substantially from mammalian lipases. Alford et al. (156) have carried out a survey of 83 microorganisms for the presence of extracellular lipases. They retained 13 of these as possessing sufficient lipolytic activity to warrant further investigation. In this group, the enzymes from Pseudomonas fragi, Pseudomonas fluorescens, Pseudomonas geniculata, Candida lipolytica, Phycomyces nitens, Mucor sufu, Penicillum roqueforti, Rhizopus oligosporus, Chaetostylum fresnei, and Thamnidium elegans all displayed substrate specificity characteristics of pancreatic lipase, and attacked primarily the 1-position of triglycerides (156). The lipases of Staphylococcus aureus and Aspergillus flavus were found to have no positional specificity towards triglycerides, hydrolyzing the esters at the 2-position at about the same rate as for the 1-position (156). Neither of these two enzymes were inhibited by diisopropyl fluorophosphate, an inhibitor specific for esterases, and without

effect on lipases (156). And yet, the S. aureus enzyme attacked water-soluble substrates such as Tween-20 and -80 (156).

The thermophilic fungus Humicola langinusa S-38, whose lipase has also been purified to homogeneity (153), has no apparent positional specificity with either triglycerides or phospholipids (156). In a homologous series of triglycerides with chain lengths from C_2 to C_{18} trilaurin was hydrolyzed at the fastest rate. Methyl laurate and ethyl myristate were also the favoured substrates in a series. The esters of both positions of phosphatidylethanolamine were degraded. In addition, the water-soluble substrates methyl butyrate, Span-20 and Tween-20 were attacked. The activity was inhibited by n-alcohols, fatty acids and other surfactants, with anionic amphipaths being more potent inhibitors than neutral detergents (160). The n-alcohol inhibitions were reversible by an addition of more substrate, but not by enzyme. On the other hand, fatty acid and bile salt inhibitions could be overcome by the addition of Ca^{++}, but apparently, not by substrate.

The three ester bonds of long chain triglycerides are hydrolyzed by the lipase from Candida cylindracea, with a preference for palmitic and oleic esters over stearic esters when the three are present in the same glyceride (64). A hydrolysis of the ester in the 2-position of 1,3-dihexadecyl ether-2-oleoyl glycerol was used to indicate that position 2

is hydrolyzed in the absence of isomerization. The pH optimum of 5.2 of this lipase increases to 7.2 in the presence of an emulsifier such as polyvinyl alcohol. Sodium taurocholate is stimulatory only when polyvinyl alcohol is also present; alone, this bile salt is inhibitory.

The lipase of Geotrichum candidum is unusual in that it has a high degree of specificity for unsaturated fatty acid esters of the cis-9 and cis,cis-9,12 types, but has no positional specificity (156,161). Petroselinate (cis-6), vaccenate (cis-11) or trans isomers were not hydrolyzed by this enzyme (162). A series of triacylglycerols containing, in approximately equal amounts, chains 12:0, 14:0, 16:0, and $\Delta^{9,12}$ 18:2, randomly mixed, and only one positional isomer of 18:1 (with unsaturation varied from Δ^{2} to Δ^{16}), were tested as substrates for this lipase (163). Analysis of the products revealed that only small quantities of any of the 18:1 isomers were released, except in the case of the Δ^{9} 18:1 containing triglyceride, which was rapidly hydrolyzed to yield the unsaturated acid. The $\Delta^{9,12}$ 18:2 ester was also hydrolyzed at the same rate as the Δ^{9} 18:1. This more detailed study confirmed the remarkable specificity of this lipase for cis-9 fatty acid unsaturation. This enzyme also discriminated between cis,cis and trans,trans $\Delta^{9,12}$ 18:2 geometric isomers, with a preference for the cis,cis; however, a mixture of cis,trans and trans,cis isomers were attacked at the same rate (164).

Rhizopus arrhizus produces a lipase which has a substrate specificity very similar to that of pancreatic lipase, hydrolyzing only the external chains of triglycerides (165), as well as the ester of position 1- of phosphoglycerides (115). In fact, this enzyme has been found suitable for the analysis of fatty acid distribution in most glycerolipids since it also attacks sulfolipids, digalactosyl diglycerides, di- and triglucosyl diglycerides and phosphoglucolipids (166).

The extracellular lipase of Pseudomonas fragi, which is very unstable, has been purified to homogeneity in a very low yield (167). This enzyme, with an optimal pH of 8.6, hydrolyzes the esters of positions 1- and 3- of mono-, di- and triglycerides, but is without effect on methyl esters and lecithin.

The homogeneously pure (168,169) enzyme from Mucor javanicus with an optimal pH of 7.0 and an isoelectric point of 3.8, is inhibited by taurocholate and NaCl (169). Tricaprylin is most rapidly hydrolyzed among triglycerides, with tricaprin and trilaurin next in order (168). But the interesting point concerning this enzyme is that it hydrolyzes a serum-activiated oil emulsion 10 times faster than one without serum; it therefore behaves much like a lipoprotein lipase (169). Narasaili et al. (17) have also purified from Pseudomonas M-12-33 a lipase which acts as a lipoprotein lipase.

Many of the lipases from microorganisms are very different in their characteristics, and, often, the work carried out with them is rather fragmentary, so that comparisons are difficult.

The sugar moieties that many of these enzymes possess, if they are not related to catalytic activity, as one example suggests, may be necessary for their discharge from the cell after synthesis. Their structures which include a high content of hydrophobic residues imply that they function at an interface in a way similar to other mammalian lipases. However, the wide variations in substrate specificities from one species to another show that the basic mechanism for lipolytic activity must be quite easily adaptable.

D. THE CATABOLISM OF PHOSPHOGLYCERIDES IN E. COLI

Enzymes for the complete degradation of phosphoglycerides have been found in E. coli. Their sequential activities are shown in Scheme I.

1. PHOSPHOLIPASES A AND LYSOPHOSPHOLIPASES

The presence in E. coli preparations of a phospholipase A with broad substrate specificity was reported in 1969 by independent workers (53,58). Shortly after, the positional specificity and general requirements of this phospholipase activity were described by Proulx and Fung (56). It appeared from these earlier studies that E. coli was equipped with at least two enzymes each attacking a different acyl ester bond of phosphoglycerides according to the following reactions (cf Scheme I, reactions 1 and 2):

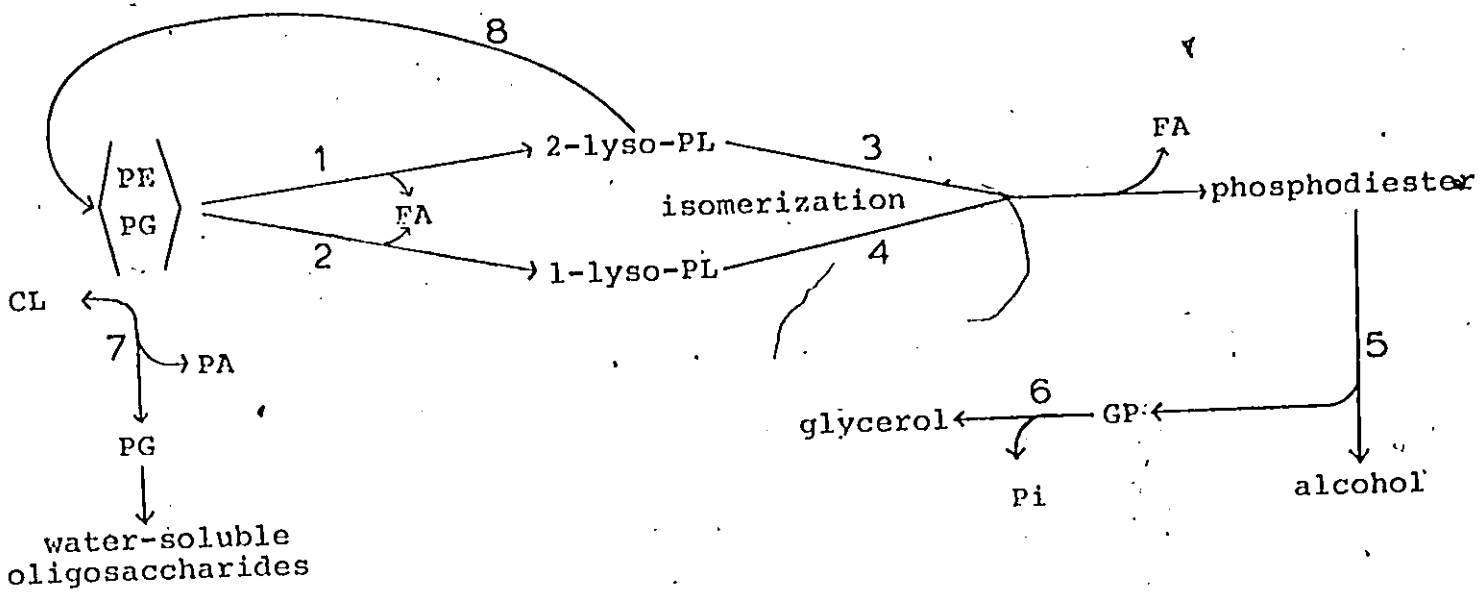
$$\text{phosphoglyceride} \xrightarrow{\text{Phospholipase A1}} \text{2-lyso derivative}$$

$$\text{phosphoglyceride} \xrightarrow{\text{Phospholipase A2}} \text{1-lyso derivative}$$

The predominant activity displayed was that of phospholipase A1. Both activities required Ca++, an anionic detergent, and an alkaline pH. A less pronounced activity was detectable at pH 4.5 - 6 in the absence of detergents (53), but was absent when sodium lauryl sulfate was added (53) or when phosphate buffer was replaced by malate (171). In E. coli W spheroplast preparations, addition of deoxycholate did not abolish the acid phospholipase A1 activity, but shifted

SCHEME I

Catabolism of Phosphoglycerides in E. coli



its optimum to a lower pH (172). It is difficult to conclude on the basis of the evidence at hand, whether there are both an acid and an alkaline phospholipase A1, or whether certain buffer-detergent-cation combinations in the assay medium would not result simply in an inhibition of a single enzyme between pH 6 and 7.5.

Lysophospholipase activity (cf Scheme I, reaction 3 or 4) has also been detected in E. coli (173). The enzyme(s) has an alkaline pH optimum, or cation requirement, is inhibited by anionic detergents, and is present in both the cytosol and the particulate fraction (53,174).

Working with E. coli 0118, Bernard et al., (175) assigned the positional specificity of phospholipase A to position 2 of phosphoglycerides mainly on the basis that the hydrolysis of sn-2[1-^{14}C-oleoyl]-PE resulted in the appearance of labelled lyso-PE as product. It is difficult to reconcile their results with those obtained by other workers using various E. coli strains (53,172,176), and very likely, the position of the label in their substrate was incorrectly assessed.

Since these earlier studies, several types of coliform phospholipases A have been reported, some of which resemble those already described. A detergent-resistant phospholipase A present in the 105,000 x g pellet of E. coli K12 was observed to be heat-stable, stimulated by organic

solvents, and to catalyse transacylation reactions (174). This enzyme is likely the same as the phospholipase A1 mentioned previously (53,56,58). In addition, a detergent-sensitive phospholipase A was identified which was inhibited by various detergents, organic solvents, heat treatment, and showed no ion requirement for activity (174). The detergent-resistant enzyme, active in the alkaline pH range, attacked both phosphatidyl glycerol and phosphatidyl ethanolamine, while the detergent-sensitive enzyme with an optimal pH at 6.5 - 7.0 was active only against phosphatidyl glycerol. Surely these two phospholipases must be different since a mutant which had no detergent-resistant activity did possess a normal detergent-sensitive phospholipase A. There is also genetic evidence indicating that phospholipase A1 and A2 together with at least one lysophospholipase are linked to a single enzyme, since in mutants lacking detergent-resistant activity (177), the two phospholipase A activities disappear together with much of the lysophospholipase activity. Also in normal strains, phospholipases A1 and A2 show similarities in optimum pH (56), localization in the membrane fraction (178), and susceptibility to trypsin treatment (177).

(a) PURIFICATION AND PROPERTIES OF E. COLI PHOSPHOLIPASE A1

From the foregoing presentation one may surmise that although several types of phospholipase A activities

have been reported, most of these can probably be ascribed to a single enzyme with broad specificity. However, this idea cannot be convincingly defended in the light of other results obtained by Scandella and Kornberg (176) who purified E. coli phospholipase A1 to near homogeneity. Their highly purified enzyme, which requires Ca++ and has an optimal pH of 8.4, hydrolyzes only the 1-acyl ester of phosphatidyl glycerol, phosphatidyl ethanolamine and cardiolipin at comparable rates for each substrate. Since the D-isomer of phosphatidyl choline was not attacked, the enzyme seems to be stereospecific. Furthermore, triolein was not hydrolyzed under their incubation conditions, so that this enzyme was considered to be a true phospholipase A1 as has been postulated for other organisms (12,14-16).

The fact that the catalytic properties of this purified enzyme were studied under rather limited incubation conditions may be a point to consider if one is to attempt an eventual reconciliation between conclusions derived from genetic and sub-cellular studies and those of Scandella and Kornberg. However, at the moment the better evidence favours the existence of phospholipase A1 and A2 as separate enzymes. Again, whether lipase activity is associated with coliform phospholipase A1 remains to be further determined. Studies entertaining this idea are described elsewhere in this thesis.

This phospholipase A1 which is very tightly bound to the membrane, could be purified to near homogeneity only after solubilization of the whole membrane in a buffer containing EDTA, sodium dodecyl sulfate and butanol (176). Unlike the crude enzyme, purified phospholipase A1 is inhibited by SDS and yet is stable in its presence since the activity can be recovered by washing off the SDS with butanol at room temperature. The enzyme tends to aggregate in aqueous solutions and shows a strong affinity for phospholipid in a way similar to other structural proteins (179, 180), so that it may have a structural as well as a catalytic role.

In addition to its phospholipase A1 activity, the purified enzyme exhibits a strong lysophospholipase activity towards 1-acyl lysophosphatides (176). A similar situation is found in the case of phospholipase A2 from Vipera palestinae (purified to homogeneity, as judged by electrophoresis), which also possesses a lysophospholipase activity (181).

(b) SUBCELLULAR DISTRIBUTION OF PHOSPHOLIPASES A AND LYSOPHOSPHOLIPASES; POSITIONAL SPECIFICITIES OF LYSOPHOSPHOLIPASES.

Results already described indicated that phospholipases A, depending on the type, may be found in the cytosol or may be particulate-bound. Lysophospholipase is also partly soluble. More detailed studies by White et al.

(182) and Bell et al. (183) have indicated that whereas most of the enzymes synthesizing phosphoglycerides are present on the inner membrane of E. coli, phospholipase A1 is located on the exterior layer of the envelope. Recently, Albright et al. (178) were able to separate three subcellular fractions of E. coli, the cell wall, the inner membrane, and the cytosol, and to localize various phospholipid-hydrolyzing activities within them. They found phospholipase and lysophospholipase A1 activities (cf Scheme I, reactions 2 and 4) which seem to correspond to Scandella's enzyme, and are located in the cell wall fraction. In addition, these workers observed a phospholipase A2 activity in the cell wall with properties very similar to the A1 activity with respect to calcium and detergent requirements, pH optimum, and heat stability. A strong lysophospholipase A2 activity (cf Scheme I, reaction 3) was detected in the inner membrane, in addition to a low level of another lysophospholipase A1 activity present in both the inner membrane and the cytosol. The latter activity was distinguishable from that found in the wall on the basis of its heat lability and different optimal conditions.

(c) IS PHOSPHOLIPASE A1 A PHOSPHOLIPASE B?

Interestingly, the highly purified coliform phospholipase A1 (176), like its recently isolated pancreatic homologue (21), is very active as a lysophospholipase.

Unfortunately, the E. coli lysophospholipase activity of phospholipase A1 was characterized only with 1-acyl lyso derivatives. Nevertheless, it is clear from the foregoing discussion that E. coli possesses a phospholipase A2 activity having a subcellular distribution and assay requirements very similar to phospholipase A1. Albright et al. (178) even suggest that the same enzyme may be involved with both these activities. On this basis it would seem reasonable to propose the renaming of phospholipase A1 as phospholipase B, implying that it catalyzes the following reaction:

$$\text{phosphoglyceride} \xrightarrow{\text{phospholipase B}} \text{phosphodiester} + 2 \text{ fatty acids}$$

The hydrolysis of the second ester may, however, require an acyl migration to position 1. The term phospholipase B as it was used by Dawson (184) and Beare and Kates (185) to describe an enzyme present in P. notatum (and not as used by some authors to describe lysophospholipase) can no longer be automatically considered an unresolved mixture of phospholipase A and lysophospholipase. Often, detergents are added to a preparation to inhibit lysophospholipase, and phospholipase A then appears as a distinct activity. However, selective inhibition by a detergent cannot be taken as valid proof for the existence of two enzymes since only one of the activities of a single enzyme may be abolished in this way. This is precisely the case for purified pancreatic phospholipase A1, the lysophospholipase activity of which is selectively inhibited by bile salts (21).

2. DEGRADATION OF WATER-SOLUBLE PRODUCTS

Once phosphoglycerides are degraded by the combined action of phospholipase A and lysophospholipase of phospholipase B, water-soluble phosphodiesters are produced. These phosphodiesters can be further broken down by cytosolic phosphodiesterases and phosphatases (18,178) according to the reactions 5 and 6 of Scheme I:

$$\text{glycerophosphoryl-X} \xrightarrow{\text{phosphodiesterase}} \text{glycerophosphate} + \text{X}$$

$$\text{glycerophosphate} \xrightarrow{\text{phosphatase}} \text{glycerol} + \text{phosphate}$$

Thus it appears that E. coli is equipped with enzymes capable of completely degrading phospholipids. Whether these enzymes actually operate in the normal turnover of phosphoglycerides in viable cells will be discussed shortly.

3. OTHER LIPOLYTIC ENZYMES

Phospholipase C, reported to occur in some E. coli strains (58,175,186) is not found in most others studied and is therefore not an enzyme typical of this organism (193).

A phosphatidate phosphohydrolase requiring Mg^{++} and detergent has been characterized in the particulate fraction of E. coli K 12 (187). It hydrolyzes both phosphatidic acid and its lyso analogue. The function of this enzyme is unclear at the moment since synthesis of phosphoglycerides in E. coli does not apparently depend on diglycerides (188). Furthermore, diglycerides represent a metabolically inactive pool in normally growing cultures of this organism (189).

A lipase has also been found in E. coli (190), the detailed description of which is presented in this thesis.

A cardiolipin-specific phospholipase D (cf Scheme I, reaction 7) requiring Mg^{++} has been found in Haemophilus parainfluenzae by Ono and White (191). Until recently, however, the occurrence of this enzyme was not, or possibly could not be extended to other bacterial species. This may have been due to a lack of ATP in the assay medium. Recently Cole et al. (192) detected a cardiolipin-specific phospholipase D in cell-free extracts and cytosolic preparations of E. coli. In freshly prepared extracts, the enzyme required only Mg^{++}, whereas in preparations that had been frozen and thawed several times or stored frozen for prolonged periods, there was an additional requirement for ATP. The catalytic and physiological properties of this enzyme are being further studied in our laboratory.

4. PHYSIOLOGICAL ROLE OF LIPOLYTIC ENZYMES IN E. COLI

Although the presence of lipolytic enzymes can be readily demonstrated in E. coli extracts, the physiological role of some of these enzymes remains unclear. A detailed study of phosphoglyceride turnover in cold-shocked and normally growing E. coli 015 cells (193) failed to indicate a sparing of acyl moieties relative to phosphorus. Also a parallel decrease in labelling of acyl chains at positions

1 and 2 was noticed when labelled cells were chased under these conditions. Phospholipid turnover was accompanied by an accumulation of only very small amounts of free fatty acids, and no lysophosphoglycerides were detected as intermediate breakdown products. On this basis, involvement of phospholipase A (or perhaps phospholipase B) activity in phosphoglyceride turnover could not be readily ascertained although this enzyme is known to be very active in cells subjected to adverse conditions (176,192).

On the other hand, Aibara et al. (195) observed that in cells grown at 40°C and then chased at 20°C, there resulted an increase in the levels of cis-vaccenic acid in PE and PG. Although most of their data could be explained in terms of a de novo synthesis of more highly unsaturated species, the authors concluded that a deacylation-reacylation cycle, as depicted in Schemes I and II, reactions 1 or 2 and 8, occurred:

Scheme II

phosphoglyceride $\xrightarrow{\text{phospholipase A}}$ fatty acid + lysophosphoglyceride

Scheme I, reactions 1 or 2

lysophosphoglyceride $\xrightarrow{\text{acyltransferase}}$ phosphoglyceride

Scheme I, reaction 8

This cycle could then account in part for the increase in cis-vaccenic acid in position 1 of PG. The conclusion of Aibara et al. assigns a significant role to both phospholipase A1 and a lysophosphoglyceride acylating enzyme also present in E. coli (58,173,176) but is incompatible with the results obtained by Bright-Gaertner and Proulx (193).

Possibly a shift-down in temperature constitutes a condition which activates phospholipase A in whole cells; however, it seems that one consequence of cold exposure is to decrease polyglycerophosphatide turnover in ^{14}C-labelled and ^{32}P-labelled cells (193). There is no obvious explanation for the differences in results obtained by these two groups, except that manipulation of the cells in one case may have caused more damage to the membranes than in the other.

The proper detection of phospholipase A activity in ^{14}C-acetate-labelled, growing cells would likely be impaired by an induced oxidation of fatty acids released during the chase. This point was examined in detail by Audet et al. (196) who used, in addition to wild E. coli B, two mutant strains, B fad and K 19, lacking β-oxidation enzymes. Phospholipase A was active in at least one of the strains tested, i.e. E. coli B fad. This strain, however, possessed a defective cell envelope. During growth it released lypopolysaccharide, lipids, and proteins, including phospholipase A1 into the medium. The normal B strain was

characterized by a much lower level of phospholipase A activity *in vivo*, if any, and a much less pronounced tendency to release envelope materials during growth. Strain K 19 displayed no apparent phospholipase activity and a completely stable cell envelope under chase conditions.

This study and another (176,194) indicate that under usual culture conditions cells possessing normal envelopes do not apparently depend on phospholipase A for lipid turnover. This conclusion invites the important question: What then is the role of phospholipase A in *E. coli*?

The presence of phospholipase A1 and A2 at the exterior surface of the cell (178), and the resistance of these enzymes as well as *E. coli* itself to bile salts are probably not inconsequent to the natural habitat of this organism, the gut, where it thrives symbiotically. There, these enzymes likely act on phospholipid-bile salt micelles and procure for the cell fatty acids that it would otherwise synthesize at the expense of energy, or alternatively, that can serve as an energy source. In consort with phospholipase A activity, the lysophosphoglyceride acylating enzyme may serve to incorporate exogenous fatty acids or appropriate lyso derivatives into phosphoglycerides of the envelope. At any rate, there is as yet no convincing evidence favouring the involvement of a deacylation-reacylation cycle acting on

endogenous lipids, irrespective of which phospholipase A may be implicated. The role of phospholipase A in E. coli would then appear to be similar to that of its lysosomal homologue, but bringing about a digestion of extrinsic material at the envelope surface rather than inside the cell.

If phospholipase A is not implicated in the normal turnover of E. coli lipids, how can one explain the loss of counts in the polyglycerophosphatide fractions noticed during chase of labelled cells (193,197-199)?

The turnover of PG could be largely accountable by its conversion to cardiolipin. However, a turnover independent of this conversion has been proposed. Recent studies in this laboratory by Audet and also by Ballesta et al. (200) revealed that in ^{14}C-glycerol-labelled cells, the unacylated glycerol moiety of PG turns over at a rate about twice that of its acylated counterpart. Ballesta et al. also showed that ^{14}C-glycerol readily incorporates into the distal glycerol of PG in a mutant lacking glycerokinase. They proposed a simple exchange mechanism, as indicated in the following reaction:

$$PG + \text{glycerol}^* \longrightarrow PG^* + \text{glycerol}$$

The authors did not, however, rule out an alternative pathway implicating the reversal of cardiolipin synthesis:

$$\text{Cardiolipin} + \text{glycerol}^* \longrightarrow PG + PG^*$$

Also to be considered is that the sum of the cardiolipin synthase and the cardiolipin-specific phospholipase D activities is equivalent to a phospholipase D attack on PG. In wild type strains possessing the glycerokinase, the uneven turnover of the glycerol moieties in PG could then be at least partly explained by a net phospholipase D breakdown of PG and some reincorporation of the PA product into PG. These points need further confirmation.

The ultimate catabolic drain in polyglycerophosphatide metabolism could again involve cardiolipin-specific phospholipase D. One product of this reaction, PA, can be degraded further to diglyceride and to fatty acid, or converted to PE, according to reactions outlined in Scheme I.

E. AIMS OF THE PRESENT INVESTIGATION

1. To further characterize certain lipolytic enzymes of E. coli, notably a phospholipase A1, a lipase, and a lysophospholipase.

2. To isolate the lipase and study its properties with respect to substrate and positional specificities as well as stereospecificity.

3. To begin work on the purification of lysophospholipase and establish some of its properties.

PART ONE

E. COLI LIPASE

SECTION I THE CHARACTERIZATION OF LIPASE ACTIVITY IN E. COLI; PROPERTIES OF THE CRUDE ENZYME

1. MATERIALS

Escherichia coli 015 was obtained from the Department of Microbiology of the University of Ottawa, while Escherichia coli Bfad and K19 were a generous gift from Dr. J.E. Cronan. The strains, K12 (ATCC 25404), and B (ATCC 11303 and ATCC 23226) were purchased as lyophilized pellets from the American Type and Culture Collection. Escherichia coli B (ATCC 11303) was also bought from General Biochemicals as frozen sediments of cells cultured to the mid log or late log phase. Stock cultures of these strains were maintained on nutrient agar slants.

The substrates tri[1-^{14}C-palmitoyl]-glycerol, tri[1-^{14}C-oleoyl]-glycerol, and tri[1-^{14}C-capryloyl]-glycerol were purchased from New England Nuclear Corporation; their purity was ascertained by thin layer chromatography. 1-^{14}C-palmitic acid and 1-^{14}C-linoleic acid were bought from Amersham and Searle Corporation, or New England Nuclear Corporation.

The detergents were procured from various sources: hexadecylpyridinium chloride from Eastman-Kodak Co., Triton X-100, from Canadian Laboratory Supplies Ltd., sodium dodecyl sulfate from Fisher Scientific Co., sodium

glycocholate from K and K Laboratories Inc., and sodium taurocholate (ox bile extract) from Sigma Chemical Co. The taurocholate was purified by dissolving in hot ethanol and by decolorizing with activated charcoal. The almost colourless solution was evaporated to dryness, and the residue was used for the enzyme studies. A purer grade of sodium taurocholate was also purchased from K and K Laboratories.

The cofactors CoA and ATP, as well as the lyophilized Crotalus adamanteus snake venom were bought from Sigma Chemical Co.

Unlabelled phosphatidyl ethanolamine and phosphatidyl choline were extracted from rat liver and quantified as described for radioactive analogues in the methods section following.

2. METHODS

(a) PREPARATION OF E. COLI CELLS

E. coli cultures were prepared in shallow flasks containing one litre of nutrient broth and incubated at 37°C with shaking. The cultures were started with 3-5 ml broth inoculums. The nutrient medium consisted of 15 g bacto-peptone, 1 g yeast extract, 5 g NaCl, and 20 g glucose, per litre, autoclaved 20 minutes at 121°C. The glucose was autoclaved separately. The cells were grown 7 hours, which coincided with the late log phase.

The purity of the cultures was verified by Gram stain and by streaking cells onto an agar plate from which eight individual colonies were transferred to a MacConkey plate. A cloudy red appearance in the area of cell growth indicated enteric bacteria. A streak from the colonies on a citrate slant differentiated between Aerobacter, which causes a colour change from green to blue, and E. coli, which does not grow in this medium.

The cells were harvested by centrifugation at 8,000 x g for 15 min. at 4°C, resuspended in either buffer or water, and stored at -20°C when not used immediately. Commercial preparations were kept at this same temperature.

(b) PREPARATION OF HOMOGENATES FOR ASSAY OF LIPOLYTIC ENZYMES

Cell suspensions were submitted to ultra sonic oscillation in ice with a Biosonik II cell disruptor for two to four periods of 5 minutes at 70 kcycles. The temperature of the sonicate was maintained below 22°C.

The protein concentration of the sonicated cell preparation was determined according to the method of Lowry et al. (202). The sonicates were then used as such or else further diluted to known concentrations of protein. The pH values of the homogenates were adjusted as required for the assays.

(c) EXTRACTION OF LIPIDS

Lipids were extracted according to the method of Bligh and Dyer (203). This involved first the addition of a mixture of redistilled chloroform - methanol (1:2) so that the final proportions were water - chloroform - methanol, 0.8:1.0:2.0. This solution was stirred for about 1 minute. One volume of chloroform was then added, and stirring was continued another minute before the final addition of 1 volume of water and a few drops of 1N HCl. The latter has the effect of lowering the pH and preventing ionization of the fatty acids, which assures their quantitative extraction. After another minute of stirring the tubes were centrifuged at 2,000 rpm for 5 minutes to separate two phases. The lower chloroform phase was removed with a Pasteur pipet. The remaining water - methanol phase was re-extracted with 1 volume of chloroform, and pooled with the first extract. The solvent was evaporated under reduced pressure, at 35-40°C, and the remaining lipids were stored under nitrogen at -20°C when necessary.

(d) SEPARATION OF LIPIDS

Lipids were routinely separated by thin layer chromatography (TLC) on 20 x 20 cm or 5 x 20 cm plates coated with 0.5 mm silica gel G. The slurry was prepared by mixing thoroughly 50 g of silica gel G with 100 ml of water. After drying at room temperature the plates were activated 1 to 2 hours at 110°C and stored in a desiccator up to a maximum of 24 hours.

Phospholipids were separated with the solvent system chloroform - methanol - water, 65:25:4 (system A); the Rf values of various standards are given in table 1. Neutral lipids were separated with three systems. Systems B and C consisted of petroleum ether (b.p. 60°-90°) - ether - formic acid, in the proportions 75:25:1.5 and 65:35:1.5, respectively. System D was made of petroleum ether (b.p. 60°-90°) - ether - acetic acid, 90:10:1. The Rf values for lipids separated in these systems are listed in table 2. Other chromatographic systems periodically used are described for each experiment when applicable.

(e) QUALITATIVE AND QUANTITATIVE ANALYSES

Lipid components on TLC plates were located by exposure to iodine vapour, most of which was subsequently removed by aeration. Radioactive lipids were located by scanning the plates with a Nuclear Chicago Actigraph III radioscanner. For preparative work, nitrogen-containing phospholipids were detected by spraying with Ponceau red (0.005% Ponceau red and 0.1% uranyl nitrate in .01N HCl); the stain was then removed by the Bligh and Dyer extraction.

Phospholipids were quantified by determining the amount of phosphorus present, with either of two methods. In the first procedure, aliquots of phospholipid were digested in concentrated perchloric acid to oxidize the organic material, then neutralized with 12N NaOH, and brought to a known volume. The amount of phosphorus present was estimated

TABLE 1

Rf Values for Thin Layer Chromatography of Phosphoglycerides

phospholipid	Rf values*
cardiolipin	.74
phosphatidyl ethanolamine	.46
phosphatidyl glycerol	.37
phosphatidyl choline	.25
lysophosphatidyl ethanolamine	.20
lysophosphatidyl glycerol	.15
lysophosphatidyl choline	.10

*solvent system A: chloroform - methanol - water, 65:25:4. Plates were coated with silica gel G.

TABLE 2

Rf Values for Thin Layer Chromatography of Neutral Lipids

neutral lipids	Rf values*		
	B	C	D
triglyceride	.75	.85	.40
methyl esters	.73	.79	.61
fatty acids	.50	.61	.23
1,3-diglyceride	.18	.45	-
1,2-diglyceride	.06	.40	-
monoglyceride	.03	.09	-
dialkylmonoacyl glycerol	-	-	.61

*Systems B and C: petroleum ether (B.P. 60°-90°)-ether-formic acid, 75:25:1.5, and 65:35:1.5, respectively. System D: petroleum ether (B.P. 60°-90°)-ether-acetic acid 90:10:1. All plates were coated with silica gel G.

by the method of Fiske and Subbarow (204). If the amount of material was small, digestion was carried out in 10N sulfuric acid, and phosphorus was determined according to the method of Bartlett (205).

(f) LIQUID SCINTILLATION COUNTING

A Beckman LS 133 liquid scintillation counter was used to estimate the radioactivity present in various samples. The scintillation mixture consisted of 0.5% 2,5-diphenyl oxazole (PPO) in toluene, to which 100 ml of methanol and 3.5 ml of glacial acetic acid were added per litre. The mixture was suitable for counting material not previously eluted from silica gel (206). Counting was performed by the external standard method in the earlier work, and by channels' ratio for the rest. Quench curves were prepared either from our own chloroform-quenched samples or from Amersham and Searle or Beckman quenched standards.

(g) PREPARATION OF FATTY ACID-LABELLED PHOSPHOGLYCERIDES

1-[1-^{14}C-palmitoyl]-phosphatidyl ethanolamine, and 2-[1^{14}C-linoleoyl]-phosphatidyl choline were prepared by first sonicating over a salt-ice mixture during 5 to 10 minutes 25 µCi of 1-^{14}C-palmitic acid or 1-^{14}C-linoleic acid in 5 ml of a buffer containing 0.125M KCl, 0.002M $MgCl_2$, 0.2M Tris, 75 mg ATP, 2.5 mg CoA, and 1N HCl, to pH 7.4. The sonicate was then added to 3 ml of a rat liver homogenate prepared by homogenizing 2.5 g of liver from a young rat (about 150-200 g) in 10 ml of the Tris-KCl buffer. This preparation

was incubated at 37°C for 45 to 60 minutes. The reaction was stopped by adding the solvents for Bligh and Dyer extraction (203), and following extraction, lipids were separated on silica gel G plates with system A.

The nitrogen-containing phospholipids, PE and PC, were identified by spraying the TLC plates with a solution of Ponceau red. The lipids were eluted and rechromatographed a few times until chromatographically pure.

The distribution of label in the ester positions of PE and PC were determined by a modification of White and Tucker's method (207). A phospholipase A2 solution was prepared by adding 3-4 mg/ml of lyophilized venom from Crotalus adamanteus to 0.1M Tris buffer at pH 7.2, containing 0.01M Ca++, and heating at 60° for 10 minutes to destroy a lysophospholipase which may be present. Aliquots of the phospholipids were dissolved in 2 ml ether, to which 30 µl of the phospholipase A2 solution were transferred, followed by incubation with agitation at room temperature for up to 10 hours. At the end of the incubation period the mixtures were evaporated to dryness. The lipids were separated by TLC in system A, and individual species were identified by radioscanner. If any unhydrolyzed phospholipid remained, it was eluted and subjected to another period of degradation, since results of only a partial breakdown of the phospholipids may reflect the substrate specificity of venom phospholipase A2 and mask the true isotope distribution.

With this method, 1-^{14}C-palmitic acid ester was found to be distributed in the 1-position of 1-[1-^{14}C-palmitoyl]-phosphatidyl choline and 1-[1-^{14}C-palmitoyl]-phosphatidyl ethanolamine in proportions of 91% and 94% respectively. 2-[1^{14}C-linoleoyl]-phosphatidyl choline checked in a similar manner had 90% of the label in position 2-. Specific activities were estimated by counting an amount of lipid assessed by determining lipid phosphorus (204).

(h) PREPARATION OF FATTY ACID-LABELLED GLYCERIDES

1-[1^{14}C-palmitoyl]-2-acyl glycerol and 2-[1-^{14}C-linoleoyl]-1-acyl glycerol were prepared by hydrolysis of 1-[1-^{14}C-palmitoyl]-PE and 2-[1-^{14}C-linoleoyl]-PC with phospholipase C from Bacillus cereus. Phospholipase C was prepared according to the method of Chu (208), from the cell-free incubation medium of Bacillus cereus. The culture medium (209) consisted of 10 g peptone, 10 g yeast extract, 5 g NaCl, and 0.4 g NaH_2PO_4, adjusted to pH 7.2 with 0.1N NaOH. After having grown the bacteria for 18 hours, the medium was clarified at 4°C by a 20 minute centrifugation at 18,000 x g. 60 g of $(NH_4)_2SO_4$ were added per 100 ml of medium and this was stirred at 4°C for 16 hours. The precipitate was collected at 40°C by a 20 minute centrifugation at 18,000 x g, and dissolved in 2 ml 0.1M Tris buffer at pH 7.2. The enzyme could be stored 3 - 4 months or more at -20°C.

The PE was dissolved in 5 ml ether, to which was added 3 ml of 0.1M Tris containing 0.01M Ca++ at pH 7.4, and 1 ml of the phospholipase C preparation. Incubation was carried out for 4 hours at 37°C. The ether was then evaporated and a Bligh and Dyer extraction was carried out with the remaining water phase. The lipids were separated on TLC plates prepared from a slurry of silica gel G made with a 0.4M boric acid solution instead of water, and run in a system of chloroform - acetone, 96:4, according to the method of Thomas et al. (210). The presence of boric acid in the silica gel prevents acyl migration known to occur on TLC plates. This permitted a maximal recovery of sn-1,2-diglycerides. This solvent system is also very useful for separation of sn-1,2- and sn-1,3-diglyceride isomers (Rf values - .36 and .52 respectively). The specific activity and percentage isotope distribution of these glycerides was determined on the parent PE, as described previously.

1-[1-^{14}C-palmitoyl]-glycerol was prepared from 1-[1-^{14}C-palmitoyl]-PC which was sequentially subjected to phospholipase A2 and phospholipase C treatments, as outlined above. The specific activity was again measured from the parent phospholipid.

3. RESULTS AND DISCUSSION

(a) THE FIRST OBSERVATIONS OF LIPASE ACTIVITY

Initially, E. coli 015 homogenates were tested for lipase activity under assay conditions suitable for phospholipase A1 (53,56). These assay conditions were chosen on the supposed basis that both activities were due to a single enzyme. A number of glycerides were tried as potential substrates for this enzyme, but they were not all attacked. These results, shown in table 3, also include the percentage of fatty acid produced by degradation of 1-[1-^{14}C-palmitoyl]-PE to show that phospholipase A1 was indeed active under these conditions.

It is rather remarkable that despite the prolonged incubation times used, triglycerides were not hydrolyzed, except perhaps tripalmitin, which was attacked very slowly. On the other hand, diglycerides and monoglycerides were readily hydrolyzed. The results with 2-[1-^{14}C-linoleoyl]-diglyceride could indicate an attack at both the 1- and 2-positions since labelled fatty acid and monoglyceride products were obtained. However this result had to be interpreted with caution because the product of an attack at position 1 of diglycerides is a 2- monoglyceride which can isomerize to a 1- monoglyceride under alkaline conditions (113). Furthermore, the hydrolysis rates at either position could not be deduced from such prolonged incubation conditions and other inadequacies of the preliminary procedure used. In the light of this, the 1- positional specificity suggested by the

TABLE 3

Hydrolysis of Various Lipids by E. coli Homogenates

Substrate	Percent dpm recovered as product		
	FA	DG	MG
tri-[1-^{14}C-palmitoyl]-glycerol	6	2	4
tri-[1-^{14}C-oleoyl]-glycerol	0	0	0
tri-[1-^{14}C-capryloyl]-glycerol	0	0	0
1-[1-^{14}C-palmitoyl]-2-acyl glycerol	31	-	0
1-acyl-2-[1-^{14}C-linoleoyl]-glycerol	32	-	12
1-[1-^{14}C-palmitoyl]-glycerol	32	-	-
1-[1-^{14}C-palmitoyl]-phosphatidyl ethanolamine	49	-	-

The incubations contained the following amounts of substrate: 0.65 nmoles tri-[1-^{14}C-palmitoyl]-glycerol (sp. act. = 21.7 µCi/µm); 0.34 nmoles tri-[1-^{14}C-oleoyl]-glycerol (sp. act. = 38 µCi/µm); 1.3 nmoles tri-[1-^{14}C-capryloyl]-glycerol (sp. act. = 8.43 µCi/µm); 36.5 nmoles 1-[1-^{14}C-palmitoyl]-2 acyl-glycerol (sp. act. = 6.7 x 10^5 dpm/µm); 25,000 dpm 1-acyl-2-[1-^{14}C-linoleoyl]-glycerol (underdetermined sp. act.); 280 nmoles of 1-[1-^{14}C-palmitoyl]-glycerol (sp. act. = 5 x 10^4 dpm/µm); 36.5 nmoles of 1-[1-^{14}C-palmitoyl]-phosphatidyl ethanolamine (sp. act. = 6.7 x 10^5 dpm/µm). The lipids were dissolved in 0.3 ml ether and mixed with 2 ml of incubation mixture comprising 1 ml of E. coli 015 homogenate (32 mg protein), 5 mM Ca^{++}, and 0.6% sodium dodecyl sulfate (w/v), in 0.1M Tris, pH 8.0. The incubations were carried out at 37°C for 4 hours, with shaking.

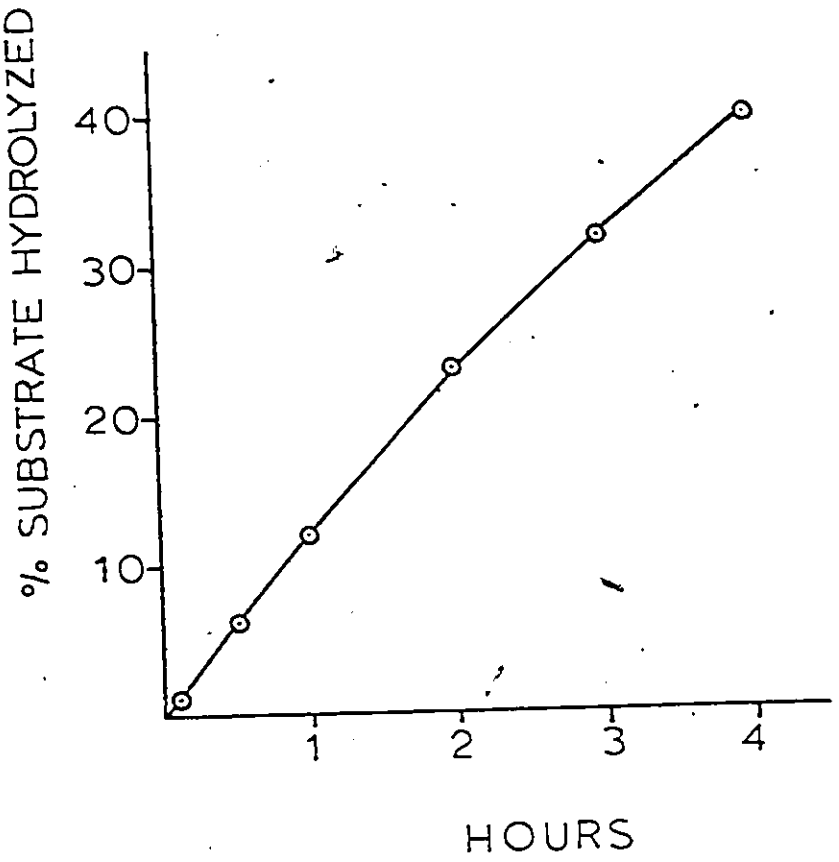
results obtained with substrates labelled in position 1- is neither contradictory nor necessarily exclusive.

These preliminary results did suggest that mono- and diglyceride, but not triglyceride, constitute the real substrates for coliform lipase. This would not have been an ususual case since a number of well characterized mammalian lipases hydrolyze preferentially mono- and diglycerides, with little activity against triglyceride (211). It will be seen later, however, that E. coli lipase can attack triglycerides under proper assay conditions.

Since the lipase activity revealed at this time was directed towards either diglycerides or monoglycerides, a possibility existed that these substrates were transformed to phosphoglycerides prior to attack. Diglyceride phosphokinase activity has been shown to occur in E. coli (212), and endogenous ATP levels could be sufficient to allow this conversion. To test this possibility and to further define the positional specificity of coliform lipase, the time course of the reaction with 1-[1-^{14}C-palmitoyl]-diglyceride was studied. Results in figure 1 reveal that at no time during the incubation were labelled monoglyceride or phospho-glyceride detected, although such products were systematically sought. Consequently, the diglyceride was hydrolyzed directly, without formation and degradation of an intermediate phosphoglyceride by the combined action of several enzymes. Also the results do strongly suggest that lipase activity is directed mainly towards the 1- position of diglycerides,

FIGURE 1

Time study of the hydrolysis of 1-[1-^{14}C-palmitoyl]-2 acyl glycerol. The reaction conditions were the same as those described under table 3 for this substrate.


% SUBSTRATE HYDROLYZED
40
30
20
10
1
2
3
4
HOURS

although small amounts of labelled 1- monoglyceride may have been formed and then rapidly degraded. The results could also be explained on the basis that both ester positions are hydrolyzed prior to release of the deacylated product from the enzyme. This would be a very unusual mode of action for a lipase although it is the case for phospholipase B activity on phosphoglycerides. This point will be treated in greater detail in a later section.

Under similar assay conditions, E. coli B was shown to attack diglycerides and PE in a manner similar to that seen with E. coli 015. Since this strain is most common and can be purchased as large scale cultures, it was chosen for our later purification studies.

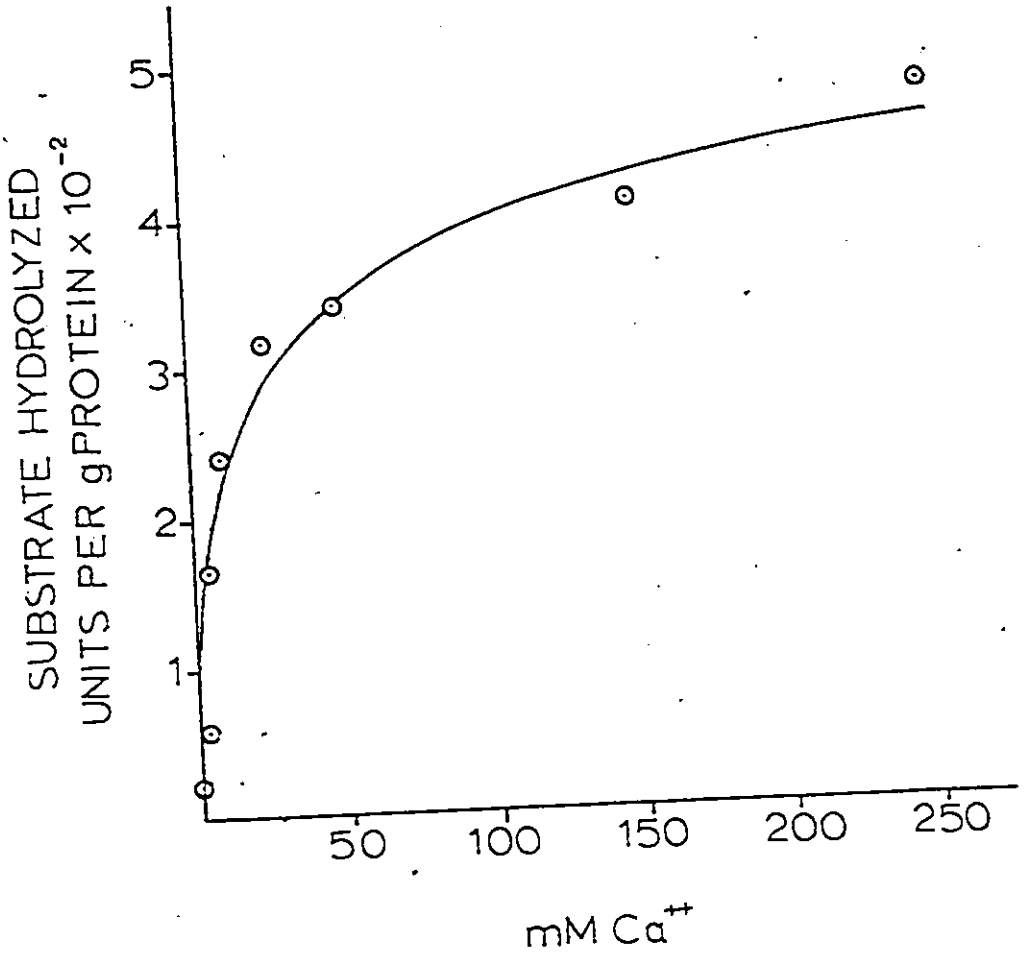
(b) THE EFFECT OF CALCIUM

At this stage a systematic examination of the optimal conditions for lipase activity was carried out. In preliminary experiments a number of additives were varied to see whether triglycerides could be hydrolyzed by E. coli preparations. Among the conditions found to be important for optimal lipase activity with these substrates was the Ca++ concentration and the type of detergent used.

As can be seen in figure 2, there was little or no basal activity in the absence of added Ca++. Stimulation by this cation was intense up to a concentration of 50 mM and progressed slowly thereafter. A concentration of at least 50 mM was adopted for most of our subsequent studies.

FIGURE 2

The effect of calcium concentration on hydrolysis of tri[1-^{14}C-oleoyl]-glycerol. The incubation mixture contained, in a total volume of 2 ml, 70 nmoles of tri-[1-^{14}C-oleoyl]-glycerol (sp. act. = 1.4 x 10^6 dpm/μm), 5 mg protein from an E. coli B (ATCC 11303) homogenate, 2.5% sodium taurocholate, varied amounts of Ca++, and 0.025M Tris buffer, pH 8.4. Incubations were agitated for 30 min. at 37°C.


SUBSTRATE HYDROLYZED
UNITS PER gPROTEIN x 10^{-2}
1
2
3
4
5
50
100
150
200
250
mM Ca^{++}

It is doubtful that the high Ca^{++} concentration reflects an entirely specific catalytic requirement of the enzyme. The high ionic strength of the medium may have secondary effects on the enzyme conformation or on the type of substrate aggregation which favours activity. Also, a more effective binding of Ca^{++} with the fatty acid product may be a contributing factor which reduced product inhibition. At any rate, little hydrolysis of triglyceride occurs when the Ca^{++} concentration is below 10 mM, and one can therefore conclude that our initial assay medium was inadequate with respect to Ca^{++} concentration.

(c) THE EFFECT OF DETERGENTS

Results in table 4 reveal the effect of various detergents on the hydrolysis of triolein. Only anionic detergents were stimulatory; no activity was obtained with either neutral or cationic surfactants. At concentrations of 0.5% (w/v) the highest rate of hydrolysis was obtained with sodium dodecyl sulfate (SDS). Higher concentrations of SDS caused the formation of calcium precipitates and results became variable. Problems of this type were also encountered with sodium desoxycholate.

Sodium glycocholate, and more effectively so, sodium taurocholate could be used up to concentrations of 2.5% (w/v). The effect of increasing taurocholate concentrations is illustrated in figure 8. On the basis of the results obtained

TABLE 4

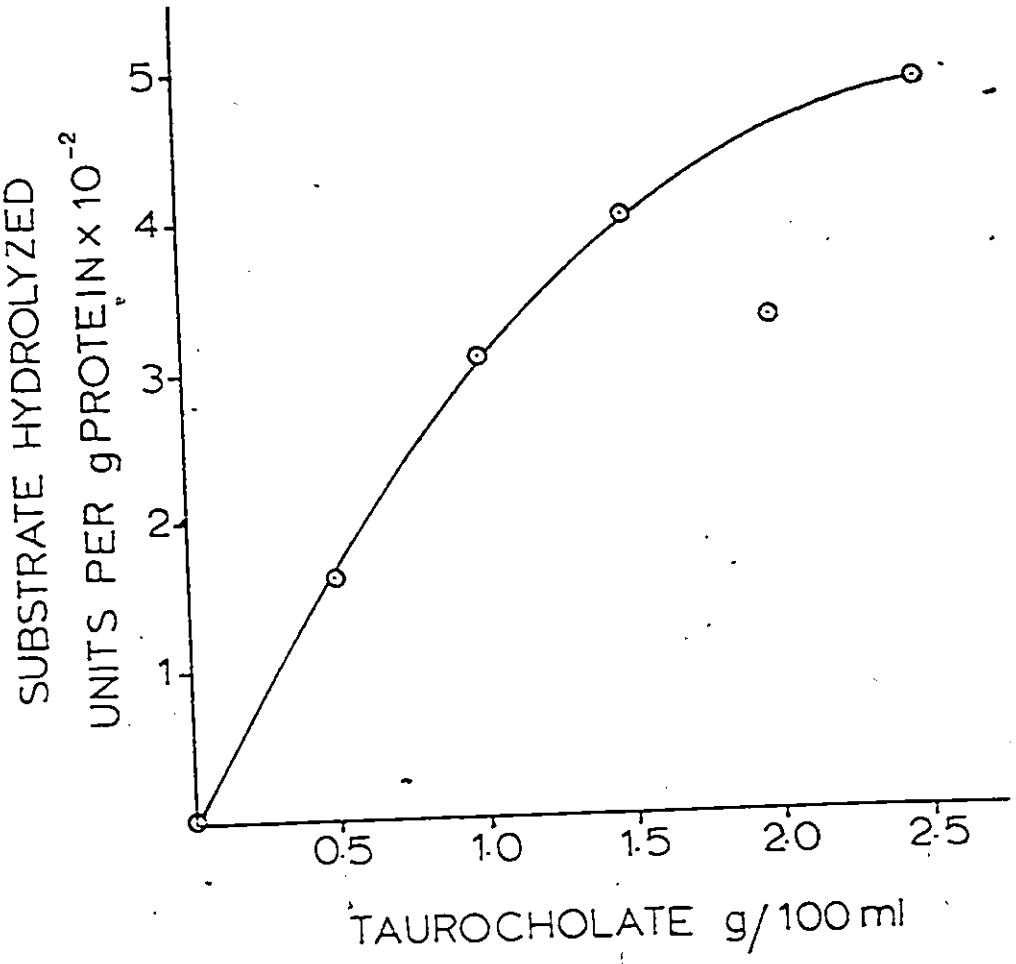
The Effect of Various Detergents on Lipase Activity

detergent	concentration g/100 ml	μmoles of tri-[1-^{14}C-oleoyl]-glycerol hydrolyzed/g protein/min
none	-	0
hexadecyl pyridinium chloride	0.5	0
Triton X-100	0.5	0
Sodium dodecyl sulfate	0.5	21.6
Sodium glycocholate	0.5	10.3
Sodium taurocholate	0.5	16.1

The incubation conditions were as stated for Figure 2, except that 0.25M Ca++ was used, and the concentration and type of detergent was varied.

FIGURE 3

Effect of taurocholate concentration on hydrolysis of tri[1-^{14}C-oleoyl]glycerol. The incubation conditions were as stated for figure 2, except that 0.25M Ca++ was used, and the taurocholate concentration was varied.


SUBSTRATE HYDROLYZED
UNITS PER g PROTEIN x 10^{-2}
1
2
3
4
5
0·5
1·0
1·5
2·0
2·5
TAUROCHOLATE g/100 ml

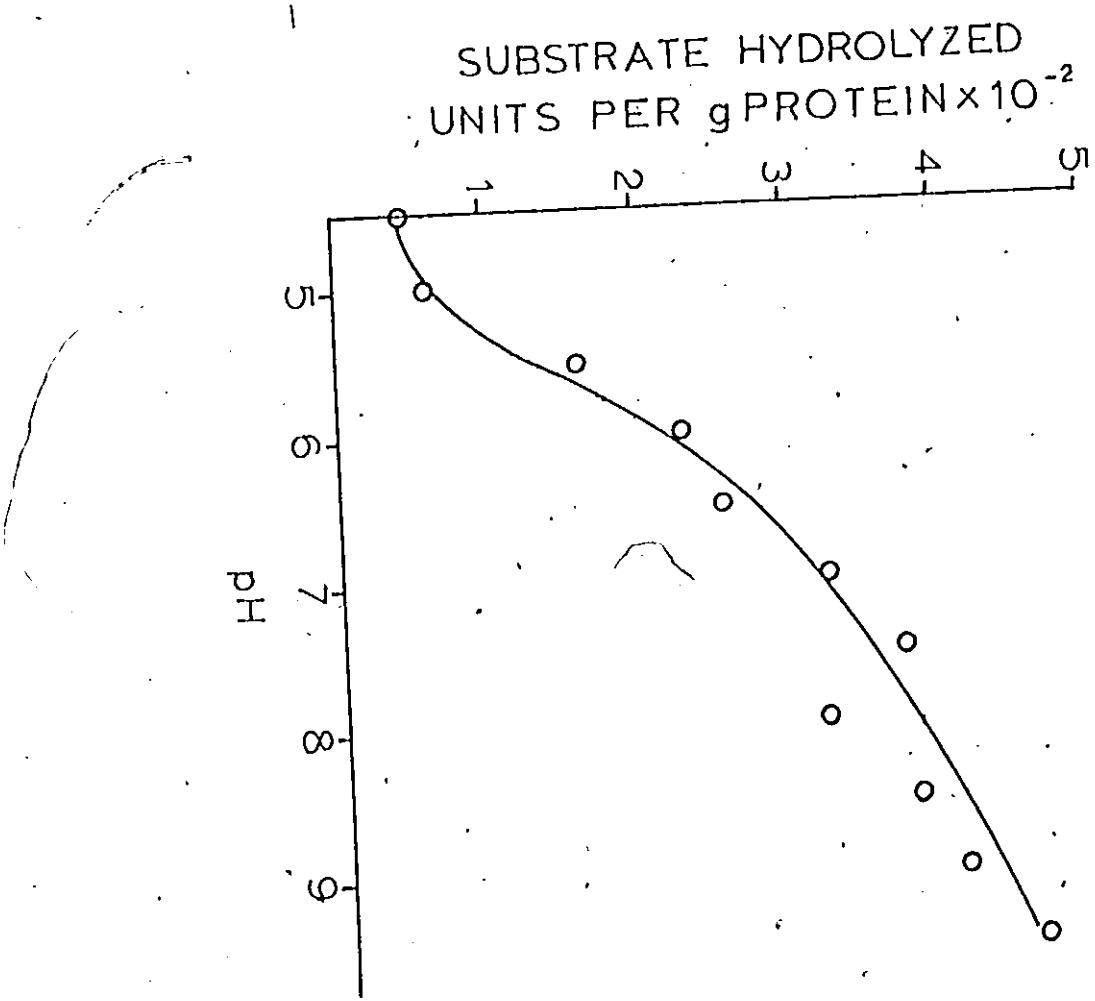
a level of 2.5% taurocholate was adopted as a condition for most of our studies. However, such a high concentration of this detergent caused certain difficulties. The detergent aggregated with part of the diglyceride and triglyceride to form a tenacious complex. It is not clear how the complex formed - perhaps it formed during application of the lipid extract on the silica gel plates. This material remained at the origin and initially was mistaken as monoglyceride. Later work showed however that it could be dissociated by Bligh and Dyer extraction from the silica gel, after which the di- and triglycerides ran to their expected positions on TLC plates. The amount of complex formed was variable but was usually within 10% of the fixed total glyceride counts added. Initial measurements of lipase activity based on triglyceride disappearance varied accordingly, but were reproducible within 10-20%. Much less variation was noticed if enzyme activity was calculated on the basis of fatty acids released, for these do not form stable complexes with detergents, as control experiments did show. At any rate, this is the most common method of expressing lipase activity, and was consequently adopted for most of our later work. The variation in this case was always within 10%.

(d) THE EFFECT OF pH

A study of the effect of pH on lipase in the range from 5.5 to 10.5 revealed maximal activity at alkaline pH (c.f. figure 4). No optimum could be found, with the activity increasing continuously up to pH 10.5, the highest tested.

FIGURE 4

The effect of pH on the hydrolysis of tri-[1-^{14}C-oleoyl]-glycerol by E. coli Lipase. The incubation mixtures contained, in a total volume of 2 ml, 70 µmoles of tri[1-^{14}C-oleoyl]-glycerol (sp. act. = 1.4 x 10^6 dpm/µm), 5 mg protein from an E. coli B (ATCC 11303) homogenate, 2.5% taurocholate, 0.25M Ca++, buffered as follows: pH 5.5 - 6.5, with 0.05M histidine monohydrochloride-NaOH; pH 7.0 - 9.0, with 0.05M Tris-HCl; pH 9.5, with 0.05M glycine-NaOH. Incubations were agitated for 30 min. at 37°C.


SUBSTRATE HYDROLYZED
UNITS PER g PROTEIN × 10⁻²
1
2
3
4
5
pH
5
6
7
8
9

Past this point the activity could no longer be measured accurately because of the occurrence of interfering non-enzymatic hydrolysis of the substrate which became considerable. Because an optimal pH could not be found, and since a glycine-NaOH buffer was used from pH 9.5 to 10.5, a possible stimulatory effect produced by glycine was investigated by examining the activity in that same pH range with the use of a $NaHCO_3$-Na_2CO_3 buffer. This confirmed the first results, showing that the activity was indeed very marked up to that pH, and that the glycine buffer was without enhancing effect. Assays were nevertheless routinely carried out at pH 8.4 which is the optimal pH for phospholipase A1.

(e) Vmax AND Km

Since lipases act against insoluble substrates, kinetic data must be interpreted differently than with enzymes acting on soluble substrates. Benzonana and Desnuelle (213) found that the initial rate of lipolysis in an emulsion stabilized by deoxycholate is controlled by the area of the glyceride-water interface in one volume unit of emulsion. All other factors being constant, this "interface concentration" plays the same role as the substrate concentration in ordinary aqueous solutions; when it is increased for a fixed amount of lipase, more enzyme molecules are adsorbed at the interface and catalyze the reaction. The reaction rate increases to a maximal value (Vmax)

corresponding to complete adsorption. Similarly, a Km (emulsion) of lipase can be defined as the concentration at the interface for which the rate is Vmax/2. The concentration at this interface can vary not only according to the amount of substrate added, but also to the degree of dispersion. In a number of preliminary experiments with different substrates the dispersion was accomplished by detergent addition followed by sonication for various times. However, with the high concentration of taurocholate there appeared to be no requirement for additional dispersion by sonication. Consequently, this step was omitted and variations in lipase activity which did occur within 10% were attributed to factors other than the degree of dispersion, such as recovery of fatty acid product, which was within 90-95%. The intercepts of the 1/V and -1/Km axes were determined by regression analysis.

Approximate Vmax and Km values for tri-[1-^{14}C-oleoyl]-glycerol and tri-[1^{14}C-capryloyl]-glycerol were determined for E. coli B (ATCC 11303) lipase by Lineweaver-Burke plots (figures 5 and 6) under the optimal assay conditions just established. The values obtained for both substrates were sufficiently alike (triolein : Vmax = 3.2 x 10^{-8} units/g protein, and Km = 4.2 x 10^{-6} moles/litre; tricaprylin : Vmax = 2.3 x 10^{-8} units./g protein, and Km = 5.0 x 10^{-6} moles/litre) to indicate similar rates of hydrolysis and equal affinities of the enzyme for both types of emulsion.

FIGURE 5

Lineweaver-Burk plot of E. coli B (ATCC 11303) lipase activity on tri-[1-^{14}C-oleoyl]-glycerol. The incubation mixtures contained, in a total volume of 2 ml, varying amounts of tri[1-^{14}C-oleoyl]-glycerol (sp. act. = 33.4×10^6 dpm/μm), 2.9 mg protein from an E. coli B (ATCC 11303) homogenate, 2.5% taurocholate, and 0.05M Ca++ in 0.025M Tris buffer, pH 8.4. The incubations were agitated at 37°C for 30 min. The Vmax was found to be 3.2×10^{-8} units/g protein, and the Km was 1.2×10^{-6} moles/litre.

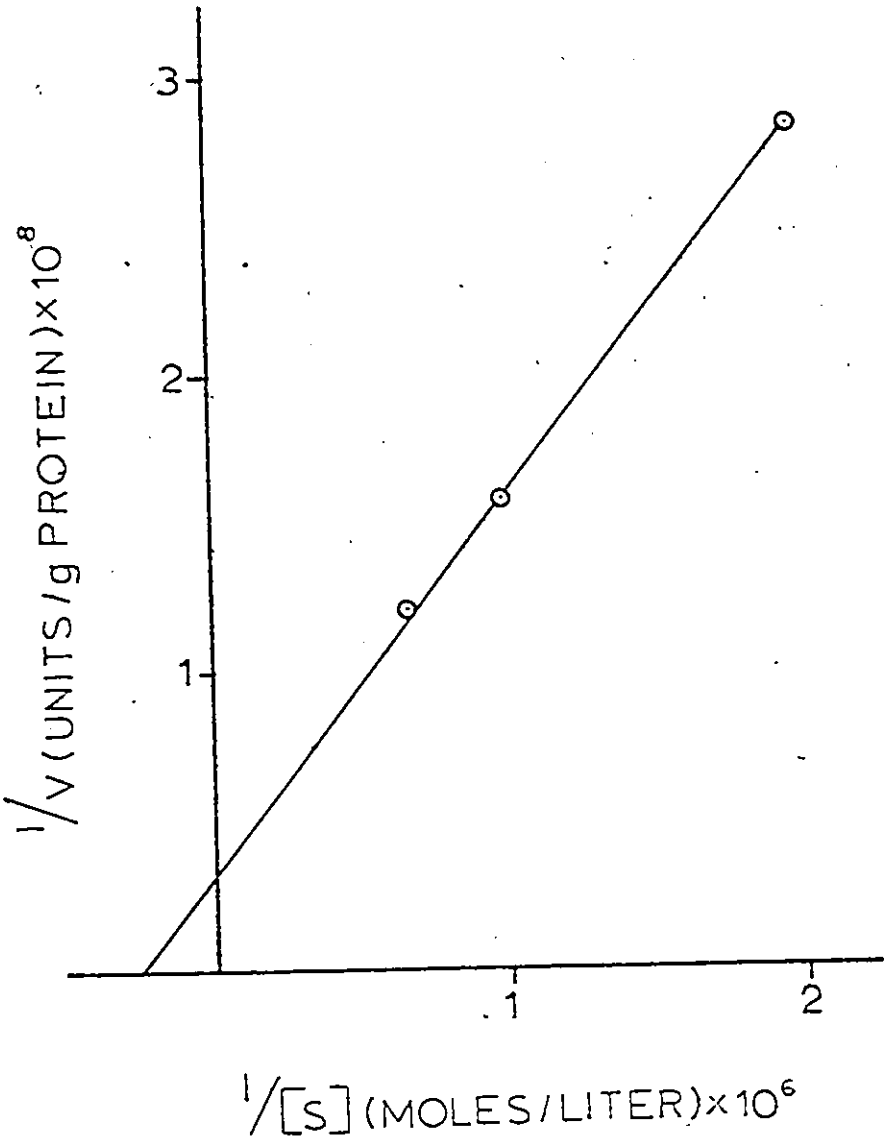
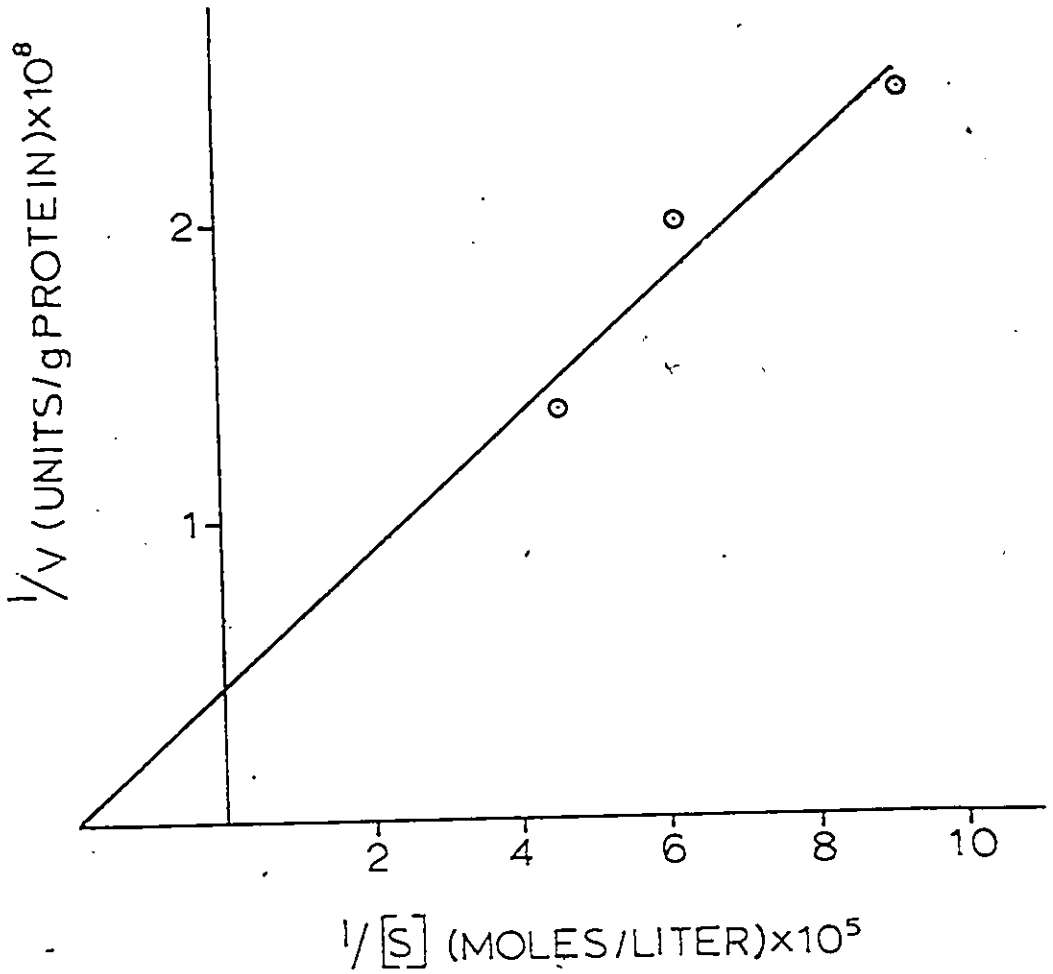

3
2
1
.1
2
1/V (UNITS/g PROTEIN)×10⁸
1/[S] (MOLES/LITER)×10⁶

FIGURE 6

Lineweaver-Burk plot of *E. coli* B (ATCC 11303) lipase activity on tri[1-^{14}C-capryloyl]-glycerol. The incubation conditions were the same as those described under figure 5, except that the substrate was tri-[1-^{14}C-capryloyl]glycerol (sp. act. = 18.1 x 10^6 dpm/µm). The Vmax was found to be 2.3 x 10^{-8} units/g protein, and the Km was 5.0 x 10^{-6} moles/litre.


1/V (UNITS/g PROTEIN)X10⁸
2
1
2
4
6
8
10
1/[S] (MOLES/LITER)X10⁵

(f) OCCURRENCE OF LIPASE IN VARIOUS E. COLI STRAINS

Approximate values of Vmax were determined for lipase from various strains of E. coli B by using a concentration of substrate approximately 10 times the Km value established with E. coli B (ATCC 11303) and other optimal conditions stated previously, except for the calcium concentration which was increased to 0.25M. For this purpose, homogenates of each strain were prepared in an identical manner from batches of E. coli B (ATCC 11303), 015, K12 and Bfad, each grown to the late log phase, and diluted to give approximately equal concentrations of protein. The results, reported in table 5, do not show great differences in the levels of lipase amongst these strains, the activities being only slightly higher in E. coli B (ATCC 11303) and 015. The Bfad strain, a mutant incapable of β-oxidation, had the lowest activity, i.e., half that found in E. coli B (ATCC 11303).

(g) SUBCELLULAR DISTRIBUTION OF LIPASE ACTIVITY

The subcellular distribution of lipase activity was examined with E. coli 015. Cells re-suspended in water (pH 5.5) were sonicated 10 min. The homogenate was then centrifuged at 100,000 x g during 90 minutes, separated into supernatant and pellet fractions, and assayed. 85% of the activity was found associated with the pellet. Furthermore, the protein obtained after precipitation with a saturating concentration of ammonium sulfate from a cell-free culture medium, followed

TABLE 5

Lipase Activity in Different Strains of E. coli

E. coli strain	μmoles of tri-[1-^{14}C-oleoyl]glycerol hydrolyzed/g protein/min
B (ATCC 11303)	46.0
015	37.0
K 12	28.4
B fad	23.0

The incubation conditions were the same as that given under figure 2, except that various strains of E. coli were used as indicated, and 0.25M Ca++.

by dialysis, showed no lipase activity. This enzyme is therefore not excreted normally into the culture medium, in comparison to a number of other microbial lipases (156).

SECTION II - THE PARTIAL PURIFICATION OF E. COLI LIPASE

1. MATERIALS

Acrylamide, N,N'-methylenebisacrylamide and Coomassie Brilliant Blue were obtained from Bio-Rad Laboratories, the N,N,N',N'-tetramethylenediamine (Temed) from Eastman Kodak, and β-mercaptoethanol, ammonium persulfate, trichloroacetic acid, glycerol, and bromophenol blue from Fisher Scientific Co. The acrylamide solution was filtered through a Nalge disposable filter unit with a 0.2 micron membrane.

Other chemicals used have previously been listed under "Materials" in the previous section.

2. METHODS

(a) THE ASSAYS

The assays for phospholipase A, were carried out in a 2 ml volume, with conditions similar to those of Scandella and Kornberg (176); this included 150 nmoles of 1-[1-^{14}C-palmitoyl]-PE (specific activity = 2 x 10^5 dpm/μmole), with 50 mM Ca++ and 0.05% Triton X-100 in 0.025M Tris-HCl buffer at pH 8.4.

The lipase assay conditions, also in a 2 ml volume, consisted of 55 nmoles of tri-[1-^{14}C-oleoyl]-glycerol (specific activity = 1.4 x 10^6 dpm/μmole) with 0.25M Ca++, 1.5% taurocholate, and 0.025 M Tris-HCl buffer at pH 8.4. The

taurocholate was used at this concentration rather than at its optimal concentration of 2.5% to further reduce the complex with bile salt which tends to form at the origin of TLC plates.

The incubations were carried out for 30 minutes at 37°C. The assay conditions are summarized in table 6. The amount of protein added to each assay depended on the particular purification fraction being assayed. The assays were repeated when necessary until a protein concentration range was found that gave less than 15% of the total radioactivity as fatty acid counts. Under these conditions the extent of hydrolysis was linear with protein concentration and with time for at least 40 minutes.

In later work, the assays were carried out by finding Vmax values for a given amount of protein of each fraction, with Lineweaver-Burk plots. Initial velocities representing less than 15% hydrolysis for a given time were again used. As will be discussed in the next section on substrate specificity, lipase activity was found to be higher against methyl-1-^{14}C-oleate than against tri[1-^{14}C-oleoyl]-glycerol. This substrate was then used to assay the lipase since it permitted the use of much less enzyme. Furthermore, methyl oleate was hydrolyzed in the presence of Triton X-100, so that the same buffer could be used for both phospholipase A1 and lipase assays.

TABLE 6

Assay Conditions Used for Phospholipase A1 and Lipase During Purification

Phospholipase A1	Lipase
150 nmoles 1-[1-^{14}C-palmitoyl]-PE	55 nmoles tri[1-^{14}C-oleoyl]-glycerol
50 mM Ca	250 mM ca
0.05% Triton X-100	1.5% taurocholate
0.025M Tris-HCl, pH 8.4	0.025M Tris-HCl, pH 8.4
various protein concentrations	various protein concentrations
30 minutes at 37°C	30 minutes at 37°C
strong agitation	strong agitation

(b) PROTEIN DETERMINATIONS

The protein concentrations of the various fractions were determined by the method of Lowry (202), but only after prior precipitation with cold trichloroacetic acid to remove any interfering compounds, such as Tris for example (214, 215). The protein was left to precipitate out at 0°-4°C for 30-45 minutes and centrifuged 10 minutes at 25,000 x g, or kept at 4°C overnight and centrifuged 15 minutes at 3,000 x g. After discarding the supernatant, 1 ml NaOH was added and the tubes were heated in a boiling water bath for 10-15 minutes to dissolve the protein. The Lowry protein determination was then performed as usual, except that a 2% Na_2CO_3 solution without NaOH was used instead of the usual 2% Na_2CO_3 in 0.1N NaOH.

(c) ELECTROPHORESIS

(i) BUFFERS AND GELS

The buffers used for electrophoresis on SDS gels and the solutions involved in the preparation of the gels were those used by Scandella and Kornberg (176) who modified the Tris-glycine discontinuous buffer system of Jovin et al. (216). The difference was simply in the addition of 0.1% SDS in the gel and running buffer. Also, stacking (upper) gels were not made.

The composition of the solutions and buffers used are given in tables 7 and 8; the gel solutions were never stored more than two months, while the ammonium persulfate was prepared fresh everytime. The solutions used in the preparation of the SDS gels (table 8) were taken out of refrigerated storage and left to warm to room temperature so that they could be de-aerated by vacuum as efficiently as possible; this is important because oxygen inhibits proper polymerization, and air bubbles may become trapped in the gels.

The composition of urea gels (table 9) was adapted for our purposes from the system of Widnell and Unkeless (217). The solutions used in the preparation of these gels were also allowed to warm to room temperature prior to de-aeration The gels were polymerized 30-45 minutes after layering with 75% acetic acid. Pre-electrophoresis was carried out for 2 hours at 3 mA/gel in a solution of 35% acetic acid in order to eliminate artifacts. Here urea was not added to the pre-electrophoresis solution because the urea in the gels is not mobile.

(ii) SAMPLE PREPARATION

The samples for SDS gels were prepared by adjusting the protein concentration to a maximum of 4 mg/ml (200 µg/50 µl). For each gel to be run 50 µl aliquots of protein were incubated at 37°C for 1 hour with 50 µl of 2% SDS at pH 7.0. 40 µl of

TABLE 7

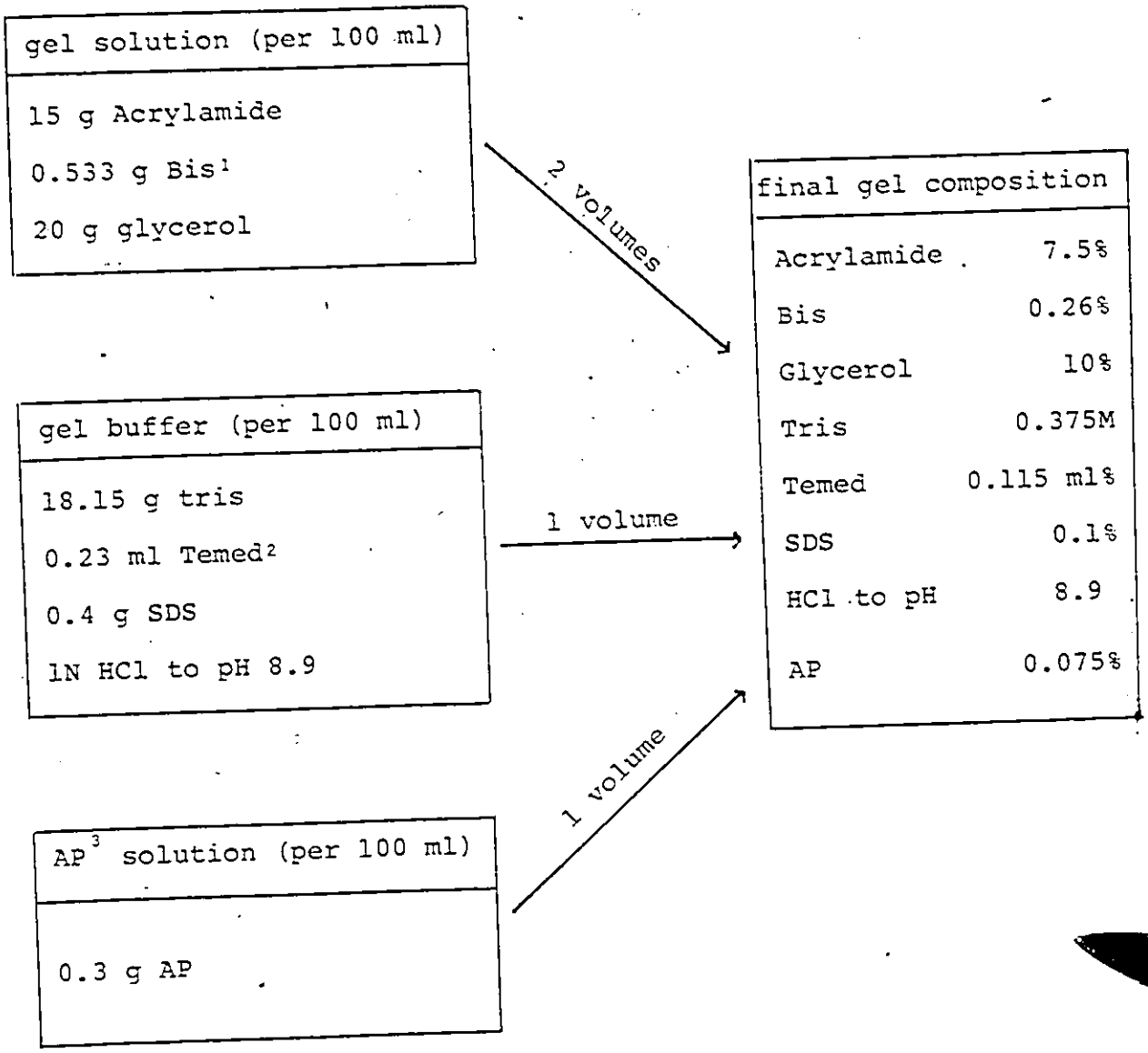
Composition of Electrophoresis Buffers

Running buffers		Pre-electrophoresis buffer
Upper	Lower	
2.88 g glycine (38.4 mM) 0.6 g tris (5.5 mM) 1.0 g SDS (0.1%)	60 ml 1N HCl (.06N) solid tris to pH 8.1	45.4 g tris (0.375M) 1.0 g SDS (0.1%) 10N HCl to pH 8.9

The amounts given are per litre.

TABLE 8

Composition of the SDS Gel Solutions and Final Gel



[1] Bis = N,N'-methylenebisacrylamide

[2] Temed = N,N,N',N'-tetramethylenediamine

[3] AP = ammonium persulfate

TABLE 9

Composition of the Urea Gel Solutions and Final Gel

gel solution (per 100 ml)
10 g Acrylamide
0.267 g Bis[1]
48 g urea
46.7 ml glacial acetic acid
0.8 ml Temed[2]
H_2O to 100 ml

AP solution (per 100 ml)
1.5 g AP[3]
48 g urea

final gel composition	
Acrylamide	7.5%
Bis	0.267%
Acetic acid	35%
Urea	8M
Temed	0.6%
AP	0.375%

[1] Bis = N,N'-methylenebisacrylamide

[2] Temed = N,N,N',N'-tetramethylenediamine

[3] AP = ammonium persulfate

upper gel buffer were then added, along with 5 µl of 0.05% bromophenol blue (a tracer dye which moves with the solvent front), and 1 drop of glycerol. These were mixed thoroughly, added onto the gels (170 µl/gel), and layered with upper running buffer, to the top of the tubes. The chambers of the apparatus were filled with their respective buffers, and the gels were run at room temperature, at 2 mA/gel, until the dye reached the edge of the tubes (3-4 hours).

The samples for urea gels were prepared by dissolving the lyophilized protein to approximately 5 mg/ml in phenol-acetic acid - H_2O (2:1:1, W, V, V) containing 2M urea. 20 to 30 µl of this protein solution (100-150 µg protein) were added to each gel and layered with 75% acetic acid. The gels were run with 10% acetic acid in both chambers for 3 hours at 3 mA/gel, with the cathode in the bottom, as opposed to the connections for the SDS gels.

(iii) STAINING THE GELS

Both types of gels were removed from the tubes with a 22 G - 1½ in. needle syringe, and cut at their dye front. Those gels from which the bands were to be extracted for assays were frozen at -20°C in individual sealed tubes. The other gels were fixed immediately in 12.5% trichloroacetic acid at 65°C for 30 minutes, then stained under similar conditions with a solution containing 0.2% Coomassie brilliant blue, 45% ethanol, and 10% acetic acid. The background dye

was removed with a solution of 25% ethanol and 10% glacial acetic acid, destained once during 20 minutes, and again for 30 minutes at 65°C. The destaining process was then pursued at the same temperature by changing a solution of 10% acetic acid every 30 minutes, until the background dye was washed out. The gels were then stored refrigerated in 3% acetic acid.

(iv) EXTRACTION OF PROTEIN FROM THE GELS

Portions of the unstained gels were cut with a razor blade according to the Rf values found for the stained bands. Equivalent fractions were pooled, and their protein was extracted by homogenizing the gel pieces twice in cold 5% butanol, centrifuging 5 minutes at 3,000 x g, and lyophilizing the pooled supernatants. SDS, which is inhibitory to purified phospholipase A1, was removed from the samples by washing with 3-4 ml of butanol; the protein was collected as a precipitate and dissolved in a fixed volume of water.

All solutions used in the preparation of gels were filtered with Whatman No 1 filter paper, except the acrylamide solution which was passed through a Nalge disposable filter unit with a 0.2 micron plain membrane prior to the addition of glycerol. Proportional volumes of each solution were

measured (table 8) and both buffer and gel solutions were mixed; this was cooled on ice, along with the ammonium persulfate just prior to adding them together to prevent as much as possible the dissolution of air which occurs more rapidly in the cold. The refrigeration of the solutions on ice was necessary to slow down the polymerization process sufficiently to allow enough time for the pouring and layering of the gels with a few milliliters of water; this last step is required for the obtention of a flat surface. The tubes used for the gels measured about 7.5 cm long, with a 0.5 cm internal diameter. The polymerization process was left to proceed during 45 to 60 minutes, and the gels were then run immediately in pre-electrophoresis buffer (in both upper and lower chambers of the apparatus) at 3 mA/gel for 2 hours, to remove residual peroxides which can cause artifacts; Scandella and Kornberg (176) found this step essential. The gels were then stored in pre-electrophoresis buffer for a maximum of two days before using.

3. PROCEDURE 1

The procedure used by Scandella and Kornberg (176) for phospholipase A1 purification is summarized in Scheme III. The initial steps of this procedure appeared to have general applicability for extraction of SDS-resistant membrane proteins such as the lipase described in this study. In initial attempts to purify lipase, therefore, this procedure

SCHEME III

Summary of the Purification for *E. coli* Phospholipase A1 According to Scandella and Kornberg - Procedure 1

1 lb of *E. coli* B cells (ATCC 11303) (late log) resuspended in 1 ℓ of 0.1M Tris, pH 7.5

STEP I — Homogenized in a Manton-Gaulin homogenizer; centrifuged at 8,000 x g for 30 minutes.

↓

pellet

STEP II — Resuspended in 500 ml water and autolyzed 1 hr. at 20°C; centrifuged at 8,000 x g for 30 minutes.

↓

pellet

STEP III — Resuspended in 500 ml of 0.03M Tris pH 8.4; pH readjusted to 8.4 by adding solid Tris base;

EDTA and SDS added to final concentration of 1 mM and 10 mg/ml, respectively; 30 min. stirring at 20°C, then chilled to 0°C, and butanol added to saturation (0.15 volume); Centrifuged at 30,000 x g for 1 hour

↓

supernatant (500 ml)

(SCHEME III cont'd)

STEP IV

Added 1 ml of 1M $MgCl_2$ and 50 ml of 1M CH_3COONa, pH 5.2 (final conc. of 2 mM and 91 mM respectively;
Stirred at 2°C for 1 hr and centrifuged at 8,000 x g for 15 minutes.

supernatant

Added 750 ml acetone (-15°C) over a 5 min interval, chilled preparation to -15°C; stirred 30 min. and centrifuged at 8,000 x g for 15 min.

pellet (acetone paste); may be stored at -15°C.

STEP V

Resuspended in 40 ml of 0.03M Tris pH 8.4, containing 1mM EDTA and 10 mg/ml SDS, and saturated with butanol;
Stirred 30 minutes at 0°C, centrifuged at 8,000 x g for 10 minutes.

supernatant

STEP VI

Added .08 ml 1M $MgCl_2$ + 4 ml 1M CH_3COONa (final conc. of 2 mM and 91 mM respectively);
Cooled to -3°C.

(SCHEME III cont'd)

STEP VI (continued)

Fractionated with acetone by peristaltic pump at 0.3 ml/min.

12 ml acetone — FRACTION I
10 ml acetone — FRACTION II
12 ml acetone — FRACTION III

Stirred 20 min. at 3°C after each addition; centrifuged each fraction at 8,000 x g for 5 min.

pellet

STEP VII

Resuspended in 1 ml $\Delta H_2 0$, lyophilized;

Dispersed finely in 10 ml butanol and incubated 1 hr. at 20°C;

Centrifuged at 5,000 x g for 15 minutes.

pellet

STEP VIII

Residual butanol removed by vacuum;

Pellet resuspended in 1 ml $\Delta H_2 0$

Up to 0.5 mg protein incubated for 60 minutes at 37°C in 0.1 ml of 1% SDS, then layered onto 10% acrylamide gels, and run for 3 hrs. at 20°C at 2 mA/gel.

Gels fractionated and extracted with 5% butanol;

Extract lyophilized and washed with butanol to remove the SDS.

purified enzyme.

served as general guideline. The method was adapted for 50 - 100 g of E. coli cells.

The first step involved cell disruption until a decrease of 95% in OD660 was obtained. In the original procedure this necessitated two passages through a Manton-Gaulin homogenizer. Homogenates of this type could not be prepared in our laboratory for lack of required equipment, so that in our early work modifications were introduced to disrupt the cells. E. coli cells grown to the mid log phase and purchased as a frozen sediment were thawed in 0.1M Tris buffer, pH 7.5. This suspension was sonicated during two to four periods of 5 minutes each at 70-80 Kcycles in a salt-ice mixture such that the temperature did not rise above 22°C. A maximum of 73% decrease in OD660 was obtained. The procedure was then carried through to step IV without modification. Results summarized in table 10 indicate some degree of purification; by contrast, these first four steps were reported by Scandella and Kornberg to increase the specific activity of phospholipase A1 some 135 fold. It is interesting to note however, that the bulk of the lipase activity was recovered in the acetone paste (step IV). This prompted our continued use of the Scandella-Kornberg procedure, at least as a model system for lipase purification. Further modifications of step one were tried in an attempt to adequately disrupt the cells. Accordingly, suspensions of commercial cells were prepared as described earlier. Lysozyme (1 mg/ml)

TABLE 10

Purification of *E. coli* Lipase by a Method Based on the Procedure of Scandella and Kornberg

Fraction	Lipase Specific Activity (units/g protein)	Purification Factor	Percentage of Total Units
1 - homogenate	6.3×10^{-2}	1	100
2 - 8,000 x g pellet	9.5 "	1.5	97
3 - 8,000 x g pellet after autolysis	9.6 "	1.5	88
4 - acetone paste*	104.6 "	17	62

*values calculated from another preparation.

Details are given in the text; one unit is defined as one μmole of fatty acid ester hydrolyzed per gram of protein per minute.

and EDTA (20 mM) were added to the suspension and incubated 30 min. at 37°C prior to sonication for different times. Results in table 11 indicate a maximal 84% decrease of OD660 after 20 minutes of sonication. The resulting homogenate became very viscous upon standing briefly but could be clarified by adding 50 µg/ml of DNAse and 5 mM $MgCl_2$ followed by stirring for 30 minutes at room temperature. The procedure monitored at the initial and final steps for both lipase and phospholipase A1 indicated that most of the activity could be recovered in the particulate fraction after centrifuging at 30,000 x g x 30 min. However, when steps II to VI were followed through without modification, the degree of purification of either enzyme was not as impressive as that obtained by Scandella and Kornberg for phospholipase A1. Saliant results are summarized in table 12.

Most of either activity was recovered in the first acetone fraction of step VI. By contrast Scandella and Kornberg obtained the bulk of the phospholipase A1 activity in acetone fraction A II. Several attempts to reproduce their acetone fractionation were unsuccessful. Also, the steps prior to the acetone fractionation were not entirely reproducible since substantial losses of phospholipase A1 resulted from the addition of $MgCl_2$ and acetate to the material obtained from step V (cf Scheme III). 10% of the phospholipase A1 purified some 30 fold was recovered

TABLE 11

Effect of Lysozyme Treatment and Sonication on the Optical Density of an E. coli Cell Suspension

Description of Treatment	OD at 660 nm	Percent Decrease in OD
Resuspended cells	.540	0
EDTA - lysozyme	.328	39
5 min. sonication	.260	52
10 " "	.175	68
15 " "	.096	82
20 " "	.088	84

An EDTA - lysozyme treatment was carried out during 20 minutes at pH 7.5 with 20 mM EDTA and 1 mg/ml of lysozyme. The sonication was performed by a Biosonyk II cell disruptor at 70-80 kcycles over a salt-ice mixture. The readings were taken on aliquots diluted 50 times.

TABLE 12

Purification Factors and Recovery of *E. coli* Phospholipase A1 and Lipase for Procedure 1

	Phospholipase A1			Lipase		
Fraction	Specific Activity units/g Protein	Purification Factor	Recovery Percentage	Specific Activity units/g Protein	Purification Factor	Recovery Percentage
Homogenate	0.36	1	100	0.022	1	100
Acetone precipitate	13.26	37	11	-	-	-
Step VI						
acetone pptate AI	19.6	54	32	0.47	21	13
AII	4.0	11	3.7	0	-	-
AIII	0.14	0.4	0	0	-	-
AIV	0.13	0.4	0	0.084	4	0.2

Details of the procedure are given in the text; assays were performed as given in table 6; one unit of enzyme, for both enzymes, is defined as one μmole of fatty acid ester hydrolyzed per minute per gram of protein.

in the precipitate resulting from these additions. It was clear from these initial studies that the Scandella and Kornberg procedure would require further modifications before a substantial purification of lipase resulted. Why steps II onwards could not be reproduced in the phospholipase A1 purification remains unclear. It is perhaps fortuitous that the authors, whose procedure was used in these studies, chose Manton-Gaulin homogenization as an initial step since alternative methods of cell disruption almost equally effective in terms of decrease in OD660 did not yield material that could be appropriately processed in subsequent steps.

For later studies involving purification of lipase and possible copurification of phospholipase A1, arrangements were made with the Grain Processing Corporation, Iowa, to treat freshly grown cells in a manner identical to that used by Scandella and Kornberg.

4. PROCEDURE 2

STEP I

Late log cells passed twice through a Manton-Gaulin homogenizer prior to freezing were purchased from the Grain Processing Corporation, Iowa, as a frozen paste. Portions of the preparation were suspended in 0.1M Tris buffer, pH 7.5, and sonicated at 90 Kcycles during the thawing out process and for an additional 5 to 10 minutes while the temperature was maintained below 22°C.

STEP II

A pellet was collected at 8,000 x g x 30 minutes, resuspended in water (15-20 mg protein/ml) and incubated 30 minutes at 37°C to allow autolysis. The particulate material was recovered by centrifugation at 8,000 x g for 30 min. and was resuspended to half the homogenate volume in 0.03M Tris, pH 8.4. The pH was adjusted to 8.4 with solid Tris base when required.

STEP III

EDTA and SDS were then used in various portions in an attempt to solubilize the entire cell envelope with minimal inactivation of the lipase or phospholipase A1. In preparation A, 0.8 mM EDTA and 0.75% SDS were added to the suspension; in preparation B, 2.1 mM EDTA and 1.2% SDS, and in preparation C, 26 mM EDTA and 1.2% SDS. This was followed by stirring for 30 minutes at room temperature to permit complete dissolution of the material. After cooling the solubilized material to 0-2°C on ice, saturating amounts of butanol cooled to -15°C were added (0.15 volumes), and stirring was continued for another 30 minutes. The suspension, which at this point usually became quite viscous was clarified by sonication for a few seconds at 90 Kcycles. Inactive proteins which had precipitated were removed by centrifugation at 38,000 x g for 40 minutes, and most of the activity was recovered in the supernatant.

STEP IV

More protein was removed from this fraction by adding 1M $MgCl_2$ and 1M sodium acetate, pH 5.2, to final concentrations of 2 mM and 91 mM respectively. This step produces conditions of high ionic strength and low pH, which in the presence of saturating amounts of butanol causes proteins of low lipid affinity to precipitate. Scandella and Kornberg reported a 90% decrease in protein concentration and only a 62% loss of phospholipid at this step. The precipitate was removed by centrifugation at 15,000 x g for 20 minutes.

STEP V

The supernatant protein was recovered by precipitation with 1.5 volumes of acetone precooled to -15°C, added over a 5 minute interval, while the temperature of the preparation was gradually lowered to -15°C and stirred for 30 minutes. The precipitate was collected by centrifugation at 8,000 g for 15 min.

STEP VI

The acetone paste was washed twice with distilled water previously adjusted to pH 7. It was found that about 75% of the protein in the acetone paste was water-soluble and contained enzyme of low specific activity.

Scheme IV summarizes the steps involved for procedure 2 (Preparation C) which gave the highest purification factor.

SCHEME IV

Summary of the Purification Procedure for *E. coli* Lipase - Procedure 2

Fresh *E. coli* B (ATCC 11303) cells (late log) homogenized by Manton-Gaulin homogenizer, and frozen.

STEP I — Sonicated during thawing out process plus an additional 5-10 minutes, in 0.1M Tris, pH 7.5; centrifuged at 8,000 x g for 30 minutes.

pellet

STEP II — Resuspended in water and autolyzed 30 minutes at 37°C; centrifuged at 8,000 x g for 30 minutes.

pellet

Resuspended in 0.03M Tris, pH 8.4; pH readjusted to 8.4 by adding solid Tris base.

STEP III — Solubilized particulate material by addition of 26 mM EDTA and 1.2% SDS; Part of the protein precipitated by addition of butanol (at -15°C) to saturation and removed by centrifugation at 38,000 x g for 40 min.

Supernatant

(SCHEME IV cont'd)

STEP IV

Added 1M $MgCl_2$ (0.2 ml/100 ml) and
1M CH_3COONa, pH 5.2 (10 ml/100 ml)
to final concentrations of 2 mM and
91 mM respectively.
Stirred at 0-2°C for 1 hr., and
centrifuged at 12,000 x g for 10 min.

↓

Supernatant

STEP V

Added 1.5 volumes of acetone, precooled
to -15°C, over a 5 min. interval;
Stirred 30 min. at 0°C and centrifuged
at 8,000 x g for 15 min.

↓

pellet (acetone paste)

STEP VI

Resuspended in 40 ml water and
centrifuged at 30,000 x g for
30 min.

↓

partially purified enzyme (pellet)

5. RESULTS AND DISCUSSION

(a) DISCUSSION OF THE PROCEDURES

Results in table 13 reveal the extent of purification obtained with the several modified procedures used. The latter are essentially very similar to the Scandella-Kornberg procedure and therefore not all steps were monitored for both lipase and phospholipase A1 activities. It is only with preparation B that a systematic examination of the various steps for lipase activity was made. It may be underlined, however, that little purification, or in our case, loss of phospholipase A1 activity occurs prior to Step IV (196). This was also the case with our own procedures monitored for lipase activity.

Step V allows a recovery of 70-90% of the lipase enzyme in the acetone paste with a purification factor of about 50 fold. Washing the acetone paste with distilled water increases the purification by at least two fold, but there is some loss of enzyme with low specific activity in the washings. Also, this step may cause some inactivation as well since not all the remaining activity is recovered in the washed precipitate (Preparation B, table 13).

The high concentration of EDTA used in procedure C did not permit proper assay of lipase activity in steps prior to V. It is seen, however, that once the acetone paste is washed with water the resultant material (Step VI) is

TABLE 13

Lipase Activities and Recoveries in Various Preparations at Different Stages of Purification

Preparation and Fraction	Lipase Specific Activities (units g/protein)	Purification Factor	Percentage of Total Units
PREPARATION A			
homogenate	0.044	1	100
washed acetone paste	1.50	34	6.4
PREPARATION B			
homogenate	0.018	1	100
butanol precipitate (discarded)	0.006	-	5.3
acetone paste	0.89	49	73
washed acetone paste	1.87	104	35
washing from acetone paste	0.27	10	20
PREPARATION C			
homogenate	0.033	1	100
washed acetone paste	8.50	258	24

Details of the procedure are given in the text; Preparation A had 0.8 mM EDTA and 0.75% SDS added to its solubilization buffer, whereas Preparation B had 2.1 mM and 1.2%, and Preparation C had 26 mM and 1.2%, respectively; assays were performed under conditions given in table 6; one unit of enzyme had been defined under table 9.

purified some 260 fold with respect to lipase activity. The recoveries in this fraction were lower than for Preparation B. The increased concentrations of SDS and EDTA used in step III of Preparations B and C were advantageous. This is likely because of an increased solubilization of envelope material and greater dissociation of hydrophilic proteins from hydrophobic proteins which can then be separated in subsequent steps.

Scandella and Kornberg make no mention of freezing any of the fractions prior to processing up to step V. In our case the Manton Gaulin homogenate was of necessity frozen prior to processing, and this could possibly result in artificial aggregations of proteins requiring more drastic dissolution methods.

In all of the final fractions (isolated in Step VI) both lipase and phospholipase A1 activities were detected. This was not surprising since our procedure was only slightly different from that of Scandella and Kornberg. Results in table 14 do in fact reveal a copurification of phospholipase A1 and lipase activity for both Procedures 1 and 2. The ratio of phospholipase A1/lipase remains about the same in the homogenates and in the final fractions. In fact, in all fractions assayed for both enzymes throughout the various purification studies, the rates of activities remained relatively constant. On this basis it can be tentatively proposed that there is at least one enzyme in E. coli

TABLE 14

Ratio of Phospholipase A1 to Lipase Activity at Various Stages of Purification

Fraction	Specific Activities (units/g protein)		Ratio (phospholipase A1/lipase)
	Phospholipase A1	Lipase	
PART A			
1 - homogenate	0.36	0.022	1.6 x 10
2 - 8,000 x g pellet	0.67	0.032	2.1 "
3 - 8,000 x g to 30,000 x g pellet	1.32	0.026	5.1 "
4 - Acetone fractionation, fraction I	19.58	0.472	4.2 "
PART B			
PREPARATION B			
5 - Washed acetone paste	91.00	1.87	4.7 "
PREPARATION C			
6 - homogenate	1.50	0.033	4.5 "
7 - washed acetone paste	361.00	8.500	4.2 "

Part A shows some fractions obtained from the purification of phospholipase A1 according to Procedure 1, whereas Part B lists fractions from lipase purification of two preparations, according to Procedure 2. Assays were performed under the conditions given in table 6, except that 1,3-^{14}C-glycerol PE (sp.act. = 2.1 x 10^6 dpm/µm) and 1-^{14}C-acetate labelled PE (sp.act. = 2.6 x 10^6 dpm/µm) were used for phospholipase A1 assays; all activities in Part B were determined by Lineweaver Burk plots; one unit has been defined in table 11.

possessing both lipase and phospholipase A1 activity. In our case at least, the recoveries of activities after step V were low and not all losses were accounted for. Thus it is possible that enzymes exist which possess only one of the activities and are lost or inactivated in steps prior to V.

Attempts to further purify the enzyme obtained from Procedure 2, Preparation C, by the acetone fractionation procedure of Scandella and Kornberg (cf Scheme III, steps V and VI) were without success. As with earlier preparations, most of the lipase and phospholipase activities precipitated in the first acetone fraction (Scheme III, step VI, Fraction I), together with the bulk of the protein, and little or no further purification was obtained.

(b) ELECTROPHORESIS STUDIES ON PARTIALLY PURIFIED LIPASE

The enzyme preparation from Procedure 2, Preparation C was delipidated by resuspending the precipitate in a minimal volume of water and freezing this suspension as a thin film. After lyophilization, 10 ml of butanol were added to the dry protein. The mixture was vortexed and given short bursts of sonication with a small probe to obtain the finest dispersion possible. After 1 hour incubation the delipidated protein was recovered by centrifugation at 5,000 x g for 5 minutes. The residual butanol in the precipitate was removed under vacuum. The samples were prepared and applied on a series

of gels as described in the methods section. Results shown in figure 7 reveal that the semipurified preparation of Preparation C contained 5 major components which stained with Coomassie blue. Figure 7 also includes a tracing of the densitometric scan of this separation. The peak observed at 0 centimeters did not correspond to a protein band and was an artifact. The unstained gels, 1 cm in parallel, were cut into slices and similar fractions were homogenized twice with 1 ml per slice of 5% cold butanol; after centrifugation the supernatants were collected, lyophilized, and washed with 2 ml butanol at room temperature to remove the remaining SDS in the extracts. The residual butanol was then removed under vacuum. The eluted material from each fraction was assayed for both lipase* and phospholipase activities. The bulk of both activities was recovered in a single band (band d, figure 7) with an Rf value between 0.70 and 0.80 (c.f. table 15). The fractions above and below this band had low levels of either activities, but this was thought to arise from a slight overlapping of the active band into these fractions due to unavoidable inaccuracies in cutting the gels. In all runs no

---

*A modified lipase assay procedure was used (c.f. methods, Section III, paragraph (b). Studies described later indicated that purified coliform lipase was more active against methyl oleate in buffer containing Triton X-100 than against triolein in buffer containing taurocholate. (The Vmax for methyl oleate was about 13 times greater than that for triolein). Since the amount of enzyme in each sample was small, the activity was measured against methyl oleate in Triton X-100.

FIGURE 7

SDS polyacrylamide gel of coliform lipase and its densitometric scan. The details of the gel preparation are given in methods and in the text. The gel and densitometric scan show the protein bands obtained by electrophoresis of the washed acetone paste of Preparation C (table 13) which was obtained by following purification Scheme IV. The activity was recovered in band d. The peak observed at 0 centimeters did not correspond to a protein band and was an artifact.

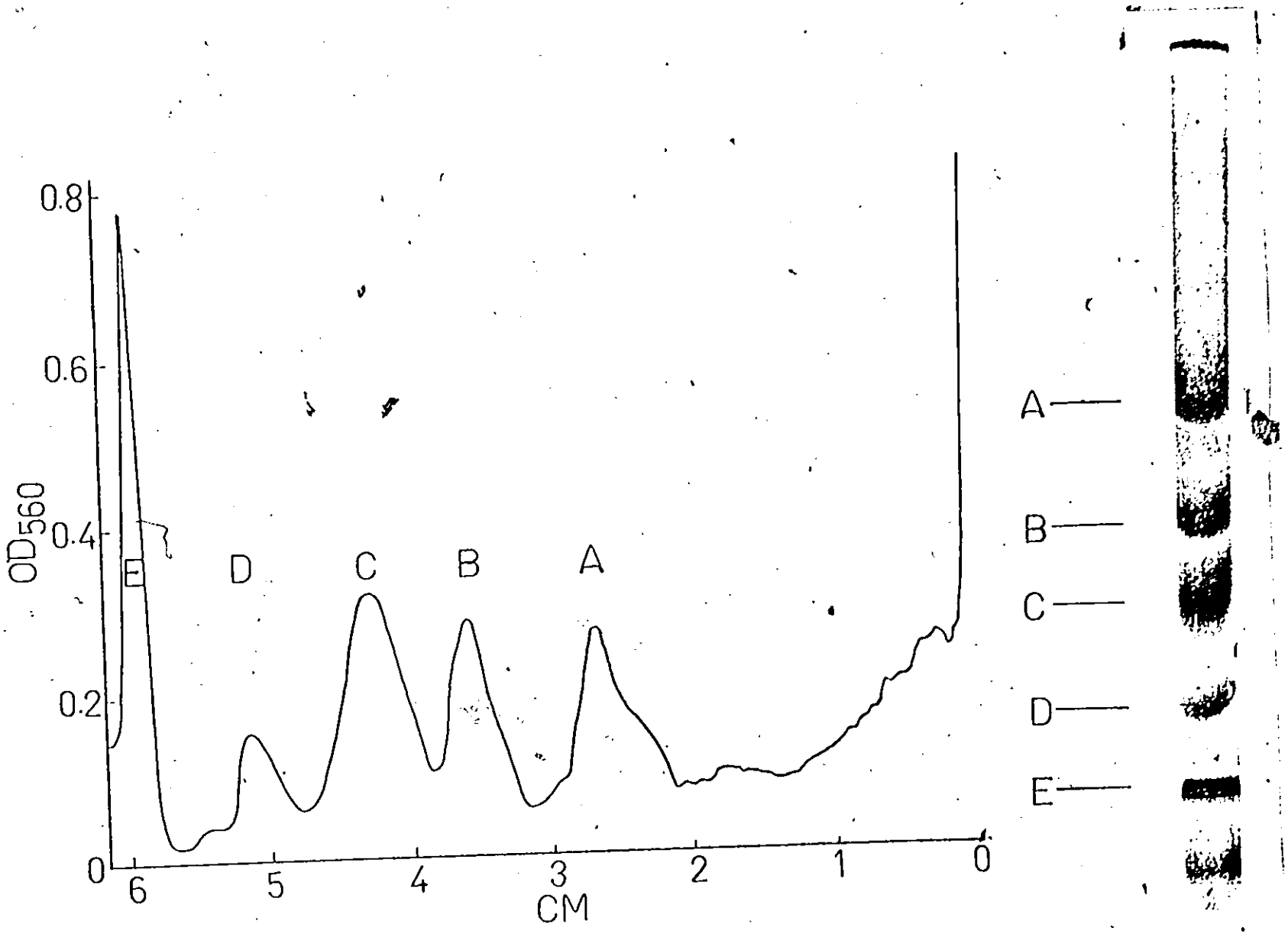

0.8
0.6
0.4
0.2
0
OD560
E
D
C
B
A
6
5
4
3
2
1
0
CM
A
B
C
D
E

TABLE 15

Phospholipase A1 and Lipase Activities in SDS Disc Gel Electrophoresis Fractions

Rf values	Counts per min. of Product	
	[1,3-^{14}C-glycerol]-lyso PE from [1,3-^{14}C-glycerol]-PE	1-^{14}C-oleic acid from methyl-1-^{14}C-oleate
.57 - .70	106	733
.70 - .78	1424	2087
.78 - .84	427	506

The incubation medium contained 19.4 nmoles [1,3-^{14}C-glycerol]-PE (sp.act. = 2.06 x 10^6 dpm/µm) or 7.1 nmoles methyl-1-^{14}C-oleate (sp. act. = 5.6 x 10^6 dpm/µm), some of the protein extracted from each fraction, 0.05M Ca^{++}, 0.05% Triton X-100 and 0.025M Tris, pH 8.4. The incubations were carried out at 37°C with shaking during 30 minutes.

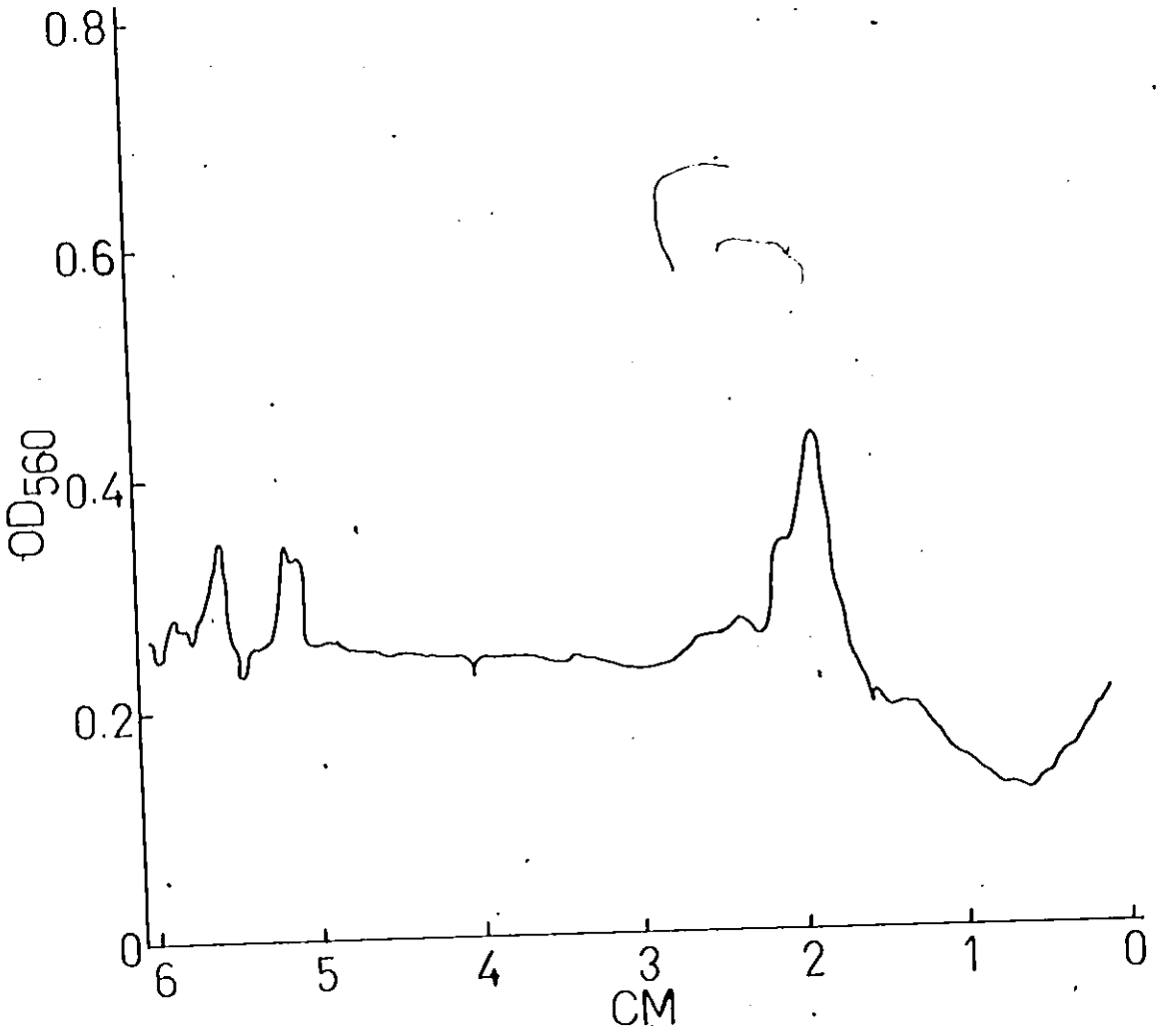
activity was found above an Rf of .53.

Part of the protein extracted from the active band (band D, fig. 7) was run on urea gels prepared as described in methods. Four bands were obtained close together, the fourth, being the largest. The densitometric scan of this gel, reproduced in figure 8, permits identification of the bands. The peaks appearing between 5 and 6 centimeters in figure 8 were artifacts since no Coomassie blue stain corresponded with them. The pattern obtained shows striking similarities to the results of Scandella and Kornberg (176), which suggests the enzyme in band d on SDS gels could have reached a stage of purification close to that obtained by these workers. Urea gel electrophoresis irreversibly destroyed all enzymatic activity, thus preventing identification of the band containing the lipase.

The specific activity of the phospholipase A1 and lipase in band D (figure 7) could not be accurately determined, unfortunately. Standard methods for protein determination such as the Lowry method, the biuret method, or methods based on direct measurement of light absorption at 280 mμ were not satisfactory. Materials eluted from the gels interfered with colour development or contributed to the absorption at 280 mμ such that Beer's Law was not followed; sometimes the results indicated that more protein was recovered in this Rf .75 fraction than had been added to the entire gel. Because of this the protein was determined by densitometric scanning

FIGURE 8

Densitometric scan of a urea gel with coliform lipase. The details of the gel preparation are given in methods and in the text. Because of the dense background tint of the gel, it could not be reproduced here, and only the densitometric scan has been included. The densitometric scan shows the bands obtained after extracting band d (c.f. figure 7) from SDS gels and running on urea gels; the enzyme added to urea gels was irreversibly inactivated. The peaks obtained between 5 and 6 centimeters were artifacts since no Coomassie blue stain corresponded with them.


0.8
0.6
0.4
0.2
0
OD_{560}
6
5
4
3
2
1
0
CM

of the Coomassie blue stain (cf fig. 7) and weighing the areas of the paper corresponding to the peaks. The protein was estimated from albumin standards subjected to the same electrophoretic and staining procedures. Linearity was displayed only above 3 µg of albumin; thus the method was not sensitive enough to give an accurate estimate of the amount of protein in the peak containing the enzyme. Furthermore, some proteins tend to absorb more Coomassie blue dye than others so that the relationship of colour density to protein concentration is not always linear. We have not tried to verify this situation with any of the proteins separated on these gels.

By comparing the weight of the active peak from the densitometric scan of the gel (cf fig. 7) to the weight total of all the peaks combined, it was estimated that the semipurified enzyme was about 7% pure. This estimate, however, could not serve to calculate accurately the amount of protein in the eluted sample since the efficiency of elution is undeterminable. This efficiency is bound to vary with each type of protein. Scandella and Kornberg, for the same procedure, indicate a 50% - 100% recovery of activity for the combined electrophoresis and elution steps. This is quite a wide range of variation and it is uncertain whether the authors imply in their statement, enzyme inactivation or incomplete elution of the enzyme. On the basis of a similar densitometric

measurement of protein these authors obtain an extra 4-fold purification of their phospholipase A1 enzyme, which is attributed to a gel electrophoresis method which we have followed literally. The authors do not describe any difficulties with the protein determination involved; however, gross inaccuracies are inherent in their method of protein assay. Estimates of specific activity in our case, based on the densitometric assay procedure, indicated a decrease to about half the value found prior to electrophoresis for both lipase and phospholipase A1.

At this point, attempts to further purify the lipase - phospholipase A1 preparation were discontinued. In summary, it can be concluded that the first five steps of the procedure described by Scandella and Kornberg can be applied to the purification of lipase activity after relatively minor modifications. These steps afford a 200-250 fold copurification of lipase and phospholipase A1 activities and one can therefore suggest that both activities are catalyzed by a single enzyme; indeed more extensive purification is required to validate such a conclusion. It is unfortunate that Scandella and Kornberg did not test their more highly purified phospholipase A1 preparations for lipase activity under varied conditions. The type of detergent used and the substrate chosen appear to be important factors even for the detection of coliform lipase activity. Nevertheless, our own results do

indicate that lipase and phospholipase have similar electrophoretic properties which again favours their association with a single enzyme.

The acetone fractionation used by Scandella and Kornberg could not be adapted for lipase purification. In our hands at least, this procedure did not work for phospholipase A1 purification either. The first acetone fraction precipitating most of the protein also precipitated most of the phospholipase A1 such that little purification occurred.

SECTION III: SOME PROPERTIES OF SEMIPURIFIED COLIFORM LIPASE

1. MATERIALS

The radiochemicals cholesterol-1-^{14}C-oleate (sp. act. = 53.5 μCi/μmole), 1-^{14}C-acetate (sp. act. = 2.2 μCi/μmole and 1,3-^{14}C-glycerol (sp. act. = 57.5 μCi/μmole) were purchased from New England Nuclear. Cetyl alcohol and p-nitrophenylacetate were obtained from Sigma Chemicals, methyl oleate from Nutritional Biochemicals Corporation, and 1,3-dipalmityl glycerol diether and sn-1,2-diacyl glycerol from Serdary Research Laboratories. Pancreatic lipase containing 230 units/mg was bought from Worthington Biochemical Corporation.

The reagents used in synthesizing esters, namely trifluoroacetic acid, palmitoyl chloride, and trifluoroacetic anhydride were Eastman Kodak products purchased from Fisher Scientific.

Other chemicals and reagents used in these experiments have been described in a previous section.

2. METHODS

(a) PREPARATION OF SUBSTRATES

1-^{14}C-acetate-labelled PE and [1,3-^{14}C-glycerol]-PE were prepared by growing cultures of *E. coli* B in the presence of appropriate radiochemicals. 250 ml of medium (described

previously) were autoclaved with 250 μCi of 1-^{14}C-acetate (sp. act. = 2.2 μCi/μmole), or 250 μCi of 1,3-^{14}-glycerol (sp. act. = 57.5 μCi/μmole), inoculated with a 5 ml broth culture and grown for 7 hours at 37°C with shaking. The cells were collected by 15 minute centrifugation at 10,000 x g, and resuspended in a known volume of water. Their lipids were extracted by the method of Bligh and Dyer (203) (described previously), and separated by TLC with silica gel G and solvent system A (cf methods, Section I). Caution was observed not to overload the plates to permit a good separation of PG and PE. The percentage distribution of the 1-^{14}C-acetate label in PE prepared in this way is equal in both acyl positions, as was previously determined in our laboratory (193). 1-^{14}C-acetate-labelled sn-1,2-diglyceride was obtained by the action of B. Cereus phospholipase C on the 1-^{14}C-acetate-labelled PE, as has been previously described (c.f. methods, Section I).

Methyl-1-^{14}C-oleate was prepared by carrying out alkaline methanolysis of tri-[1-^{14}C-oleoyl]-glycerol by the method of Marshall and Kates (220). This consisted in dissolving the lipid material in 0.5 ml $CHCl_3$-methanol (2:3), and mixing with 0.5 ml of 0.2M dry methanolic NaOH. After standing at room temperature for 15 minutes, 1 ml $CHCl_3$-methanol (4:1) and 0.9 ml water were added. This mixture was stirred, then centrifuged to separate out a methanol-water

phase (on top) and a chloroform phase, as in the Bligh and Dyer extraction. The lipid material was collected from the $CHCl_3$ phase. The methyl-1-^{14}C-oleate formed was checked by TLC and found to be pure. The required specific activity was obtained by diluting with non-labelled methyl oleate.

2-[1-^{14}C-palmitoyl]-1,3-dipalmityl glycerol diether, 2-[1^{14}C-palmitoyl]-1,3-dipalmitoyl glycerol, and sn-3[1-^{14}C-palmitoyl]-1,2-diacyl glycerol were synthesized by the method of Bourne et al. (221). This consisted in reacting 1 ml of trifluoroacetic anhydride during some 10 minutes with 4 µmoles of 1-^{14}C-palmitic acid which had been diluted to the required specific activity with unlabelled palmitic acid. The mixture containing the mixed anhydride was added to 2 µmoles of either 1,3-dipalmitoyl glycerol diether, 1,3-dipalmitoyl glycerol (dipalmitin), or sn-1,2-diacyl glycerol (from pig liver). The 1,3-dipalmitoyl glycerol was prepared by purifying the 1,3-isomer of a mixture of commercial 1,2 and 1,3-dipalmitin by TLC in system C (cf methods, Section I). The reaction was allowed to proceed for 1 hour at 35-40°C with occasional shaking in a vessel plugged with an anhydrous $CaCl_2$ tube. The contents were then added to 4 ml 0.1M $NaHCO_3$, and the lipids were extracted twice with diethyl ether. The products were isolated by chromatography in systems B, C and/or D (cf methods, Section I) and were radiochemically pure.

Cetyl-1-^{14}C-palmitate and 1-stearoyl-2-oleoyl-3-[1-^{14}C-palmitoyl]-glycerol were synthesized by reacting cetyl alcohol or 1-stearoyl-2-oleoyl-glycerol with 1-^{14}C-palmitoyl chloride.

The 1-^{14}C-palmitoyl chloride synthesis (222) involved refluxing 1-^{14}C-palmitic acid, diluted to a known specific activity with unlabelled palmitic acid, in excess (about 1 ml) thionyl chloride during one hour at about 77°C in a wax bath. 2 ml of toluene (b.p. 110-111°C) were then added to the reaction mixture and distillation was carried out until all the thionyl chloride and most of the toluene were gone. The addition of toluene permitted the complete removal of thionyl chloride. The contents were then diluted to a measured volume with toluene, and stored at -20°C for future use.

20 µmoles of cetyl alcohol were dissolved in 2 ml trifluoroacetic acid, and this was added to 60 µmoles of 1-^{14}C-palmitoyl chloride (from which toluene had been evaporated with a stream of nitrogen). The reaction flask was stoppered with a $CaCl_2$ tube, and the reaction was allowed to proceed for 3½ hours at 40°C. The reaction mixture was then added to 5 ml of 0.1M $NaHCO_3$ and extracted twice with $CHCl_3$. The product was purified with TLC system B, until radiochemically pure cetyl-1-^{14}C-palmitate was obtained.

(b) PHOSPHOLIPASE A1 AND LIPASE ASSAYS

Vmax in both cases were determined by Lineweaver-Burk plots. The assay conditions for these as well as for time course

studies were similar, except for the substrate concentrations.

Phospholipase A1 assays were carried out essentially according to the method described earlier with 1,3-^{14}C-glycerol PE (sp. act. = 2.06 x 10^6 dpm/µm). The activities were calculated from the amount of PE which disappeared during the incubation. Any non-enzymatic loss of PE was corrected from two recovery controls performed for each experiment. The incubation media contained varying amounts of substrate, with 0.05M Ca++ and 0.05% Triton X-100 in a 0.025 M Tris buffer at pH 8.4. The tubes were incubated 15 minutes at 37°C with shaking.

The lipase assay media contained substrate and protein in various concentrations as indicated in each of the tables, and 0.25M Ca++, 1.5% taurocholate, and 0.025M Tris, pH 8.4, in 1 or 2 ml final volumes. The incubations were carried out with shaking at 37°C for various periods of time.

The esterase assay with p-nitrophenylacetate was carried out essentially according to the method of Huggins and Lapides (223), with the same incubation medium as that used for the lipase assays just described, except that the pH was lowered to 8 in order to reduce the rate of non-enzymatic hydrolysis which takes place at alkaline pH. Hydrolysis was measured by continuous reading of the change in optical density, with a Carry 15 Spectrophotometer at 405 µm, using

tandem cells (cf figure 9). The arrangement permitted an automatic subtraction of the non-enzymatic hydrolysis of p-nitrophenylacetate which occurs at alkaline pH.

In order to be able to dissolve the p-nitrophenylacetate, a stock solution 100 times more concentrated was prepared in 10 ml methanol. Prior to incubation, 1 ml of this was added to 99 ml of distilled water by placing the tip of the pipet under the water surface. This ensures a rapid mixing which prevents the formation of a substrate precipitate on the surface of the water.

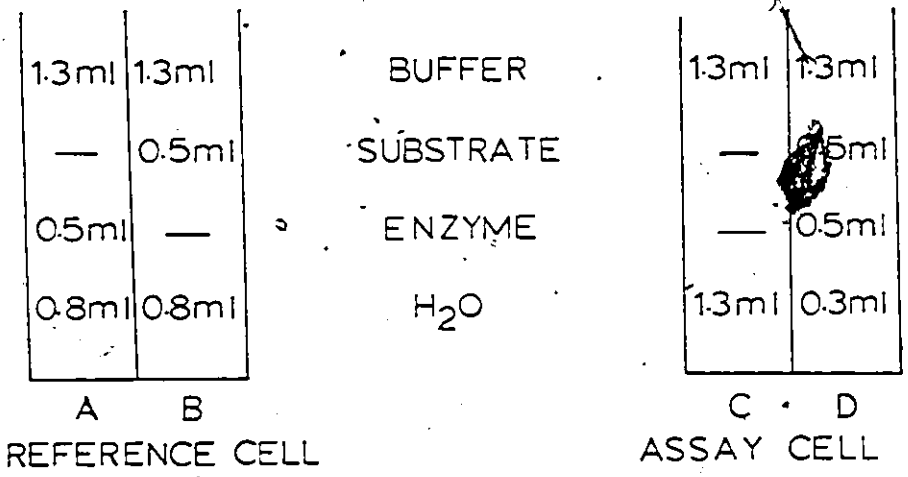
3. RESULTS AND DISCUSSION

(a) GLYCERIDE POSITIONAL SPECIFICITY

In the first section of this thesis on the identification and properties of E. coli lipase, some preliminary information was obtained on the positional specificity of coliform lipase. The results in table 3, Section I, show an attack at position 1 since no labelled monoglycerides were produced from 1-[1-^{14}C-palmitoyl]-2 acyl glycerol. Upon incubation of the crude lipase with 2-[1-^{14}C linoleic]-1-acyl glycerol, however, both labelled fatty acid and monoglyceride were observed, thus suggesting a possible attack at position 2. However, interpretation of the results was complicated by the possibility of acyl migration in the substrate, particularly with the long incubation periods used, and this prevented a definite conclusion regarding positional specificity.

FIGURE 9

Tandem cell system to measure esterase activity. The incubation medium (cell D) contained 36 or 144 nmoles p-nitrophenyl acetate, 85 µg protein (from acetone fraction I of Step VI, according to purification Scheme III), 0.25M Ca++, 1.5% taurocholate, 0.025M Tris at pH 8.0, and water to complete the volume to 2.6 ml. Incubations were carried out for 30 minutes at room temperature, with continuous reading in a Carry 15 double beam spectrophotometer, at 405 nm.


1.3ml
1.3ml
—
0.5ml
0.5ml
—
0.8ml
0.8ml
BUFFER
SUBSTRATE
ENZYME
H_2O
1.3ml
1.3ml
—
0.5ml
—
0.5ml
1.3ml
0.3ml
A
B
REFERENCE CELL
C
D
ASSAY CELL

A time study with 1-[1-^{14}C-palmitoyl]-2-acyl glycerol (figure 1, Section I) indicated hydrolysis only at position 1 since no labelled MG product could be detected at any time. However, this result obtained with whole cell homogenate is also explainable on the basis of a hydrolysis at both ester positions prior to release of products from the enzyme. A second possibility allowing attack at position 2 would imply the presence of a distinct, very active MG lipase in homogenates, preventing accumulation of monoglycerides. A third possibility exists where the positional specificity is linked to fatty acid specificity; in this case attack of position 2 would occur only if the proper acyl group was the substituent.

Results in figure 10, with a partially purified lipase, indicate an approximately equal rate of FA and MG production from ^{14}C-acetate-labelled DG. This preparation quite obviously lacked a very active monoglyceride lipase. The results also indicate that the products can be released from the enzyme when only one of the ester positions has been attacked.

Results in figure 11 indicating the kinetics of 2-[1-^{14}C-palmitoyl]-1,3-dipalmitoyl glycerol hydrolysis by pancreatic lipase were entirely as expected. Since pancreatic lipase attacks only the outer ester positions of TG, the substrate used did contain label only in position 2; otherwise labelled FA production should have been noted.

FIGURE 10

Time study of E. coli lipase activity on 1-^{14}C-acetate-labelled diglyceride. The incubation medium contained 16 nmoles 1-^{14}C-acetate-labelled sn 1,2-diglyceride (sp. act. = 2.45 x 10^6 dpm/μm) and 0.085 mg protein (from acetone fraction I of Step VI according to purification Scheme III), 0.25M Ca++, 1.5% taurocholate, and 0.025M Tris, pH 8.4, in a total volume of 2 ml. The incubations were shaken at 37°C for the indicated times.

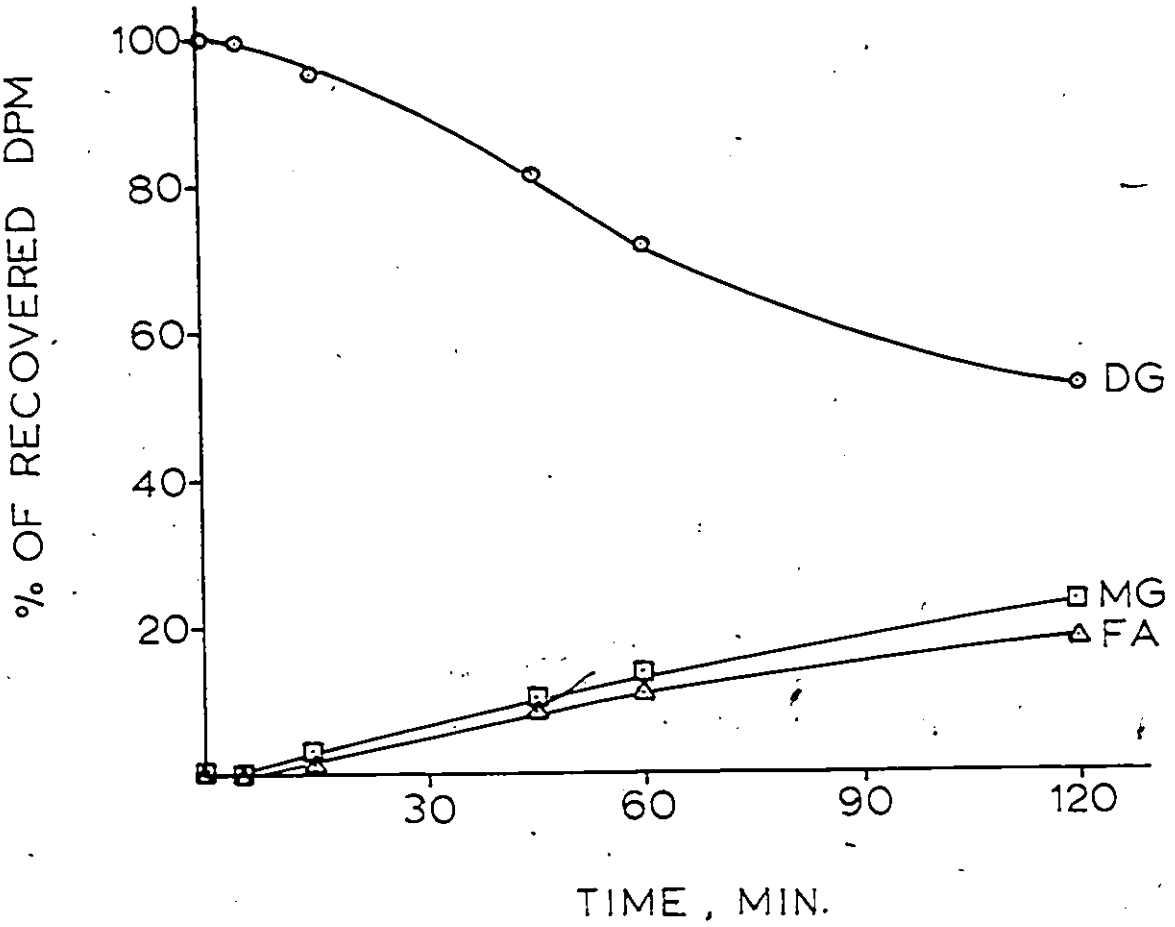
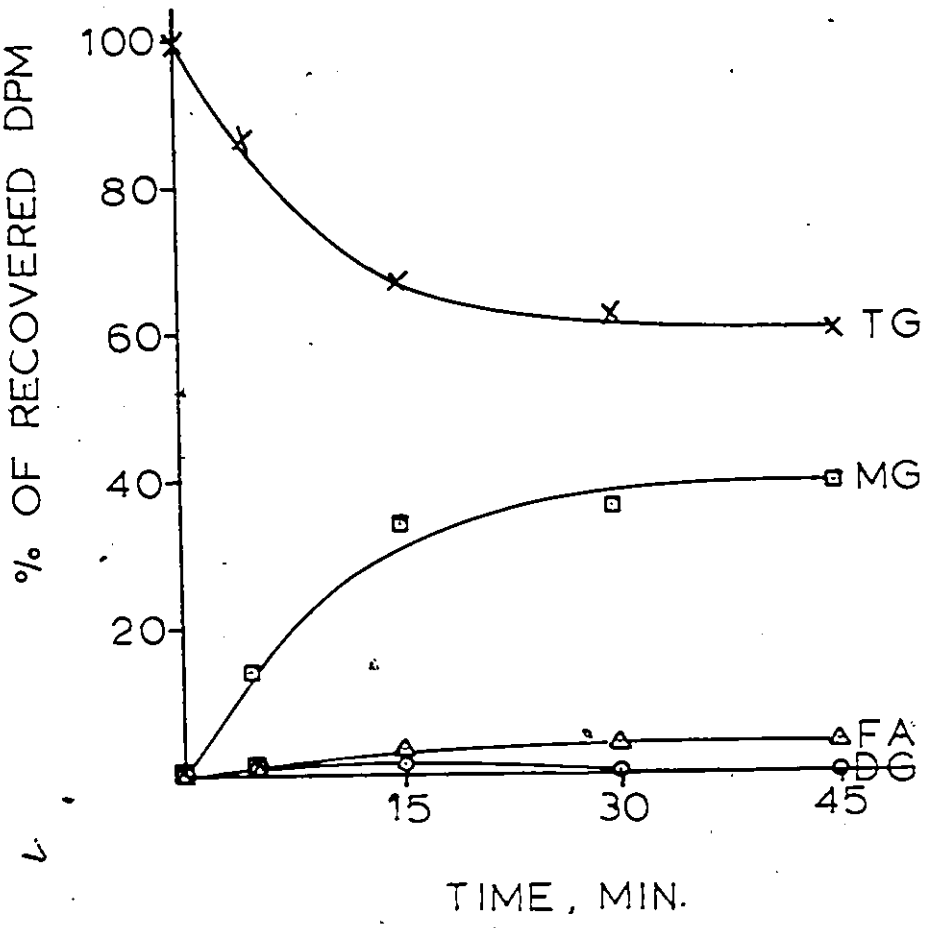

% OF RECOVERED DPM
100
80
60
40
20
DG
MG
FA
30
60
90
120
TIME , MIN.

FIGURE 11

Time study of pancreatic lipase on 2-[1-^{14}C-palmitoyl]-1,3-dipalmitoyl glycerol. The incubation medium contained 1 mmole of 2-[1-^{14}C-palmitoyl-1,3-dipalmitoyl]glycerol (sp. act. = 15.4 x 10^6 dpm/μm), 0.043 mg pure pancreatic lipase (230 units/mg), 0.05M Ca++, 1.5% taurocholate, and 0.025M Tris, pH 8.4, in a 2 ml volume. The incubations were shaken at 37°C for the indicated times.


% OF RECOVERED DPM
100
80
60
40
20
TG
MG
FA
DG
15
30
45
TIME, MIN.

Results established in figures 12 and 13 with this substrate reveal that initially the main product of triglyceride hydrolysis was labelled DG, but there was at that time a significant production of labelled fatty acid as well. Within 2 hours, however, DG levels fell substantially, and both MG and FA accumulated.

Thin layer chromatography analysis of the diglyceride produced in the initial stages of the hydrolysis of tri-[1^{14}C-oleoyl]-glycerol under identical conditions indicated that it consisted mainly of the 1,2 species, but some 1,3 species (about 5 percent of the total diglyceride) was also detected (cf figure 14). The Vmax obtained for 2-[1-^{14}C-palmitoyl]-1,3-dipalmitoyl glycerol (0.23 units/g protein; cf figure 15) showed that hydrolysis of the labelled ester proceeded at a considerably slower rate than 1/3 of the Vmax for tri-[1^{14}C-palmitoyl]-glycerol (Vmax = 4.4 units/g protein/min.; cf figure 16) which would have been expected had the hydrolysis rate been equal for all positions.

These results indicate that the main activity of the lipase is exerted at the 1 (3) position of tri- or di-glycerides but the 2 position is also attacked at a slow rate. This hydrolysis at position 2 likely involves a direct action of the lipase rather than an indirect mechanism proceeding only after non-enzymatic isomerization of labelled 1,2-diglycerides to labelled 1,3-diglycerides. Under similar incubation conditions (pH 8 instead of 8.4 used in our case)

FIGURE 12

Time study of E. coli lipase activity on 2-[1-^{14}C-palmitoyl]-1,3-dipalmitoyl glycerol. The incubation medium contained 1 nmole 2-[1-^{14}C-palmitoyl]-1,3-dipalmitoyl glycerol (sp. act. = 15.4 x 10^6 dpm/μm), 0.08 mg protein (165 units/g protein), 0.25M Ca++, 1.5% taurocholate, and 0.025M Tris, pH 8.4, in a 2 ml volume. The incubations were shaken at 37°C for the indicated times.

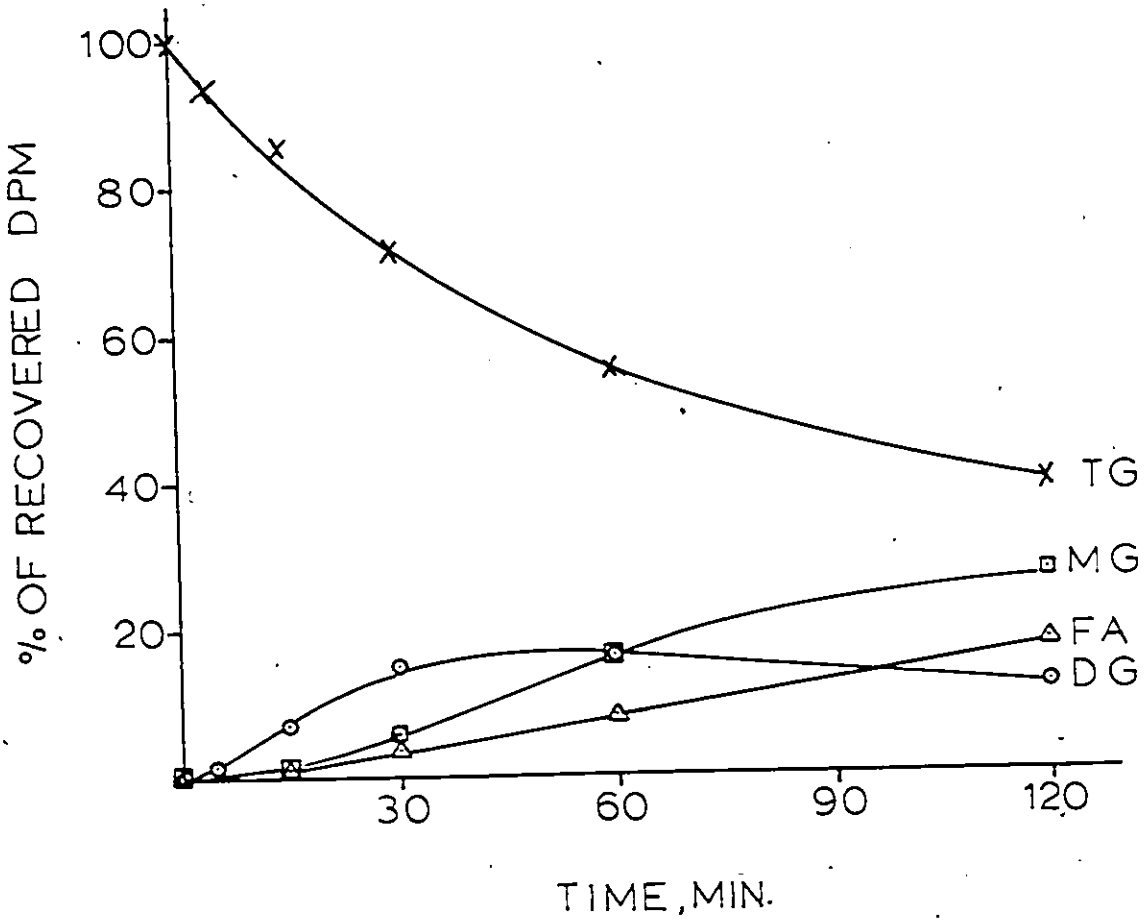

% OF RECOVERED DPM
100
80
60
40
20
30
60
90
120
TIME, MIN.
TG
MG
FA
DG

FIGURE 13

Time study of E. coli lipase activity on 2-[1-^{14}C-palmitoyl]-1,3-dipalmitoyl glycerol, with results expressed as percentage of products. The incubation conditions were the same as given under figure 12.

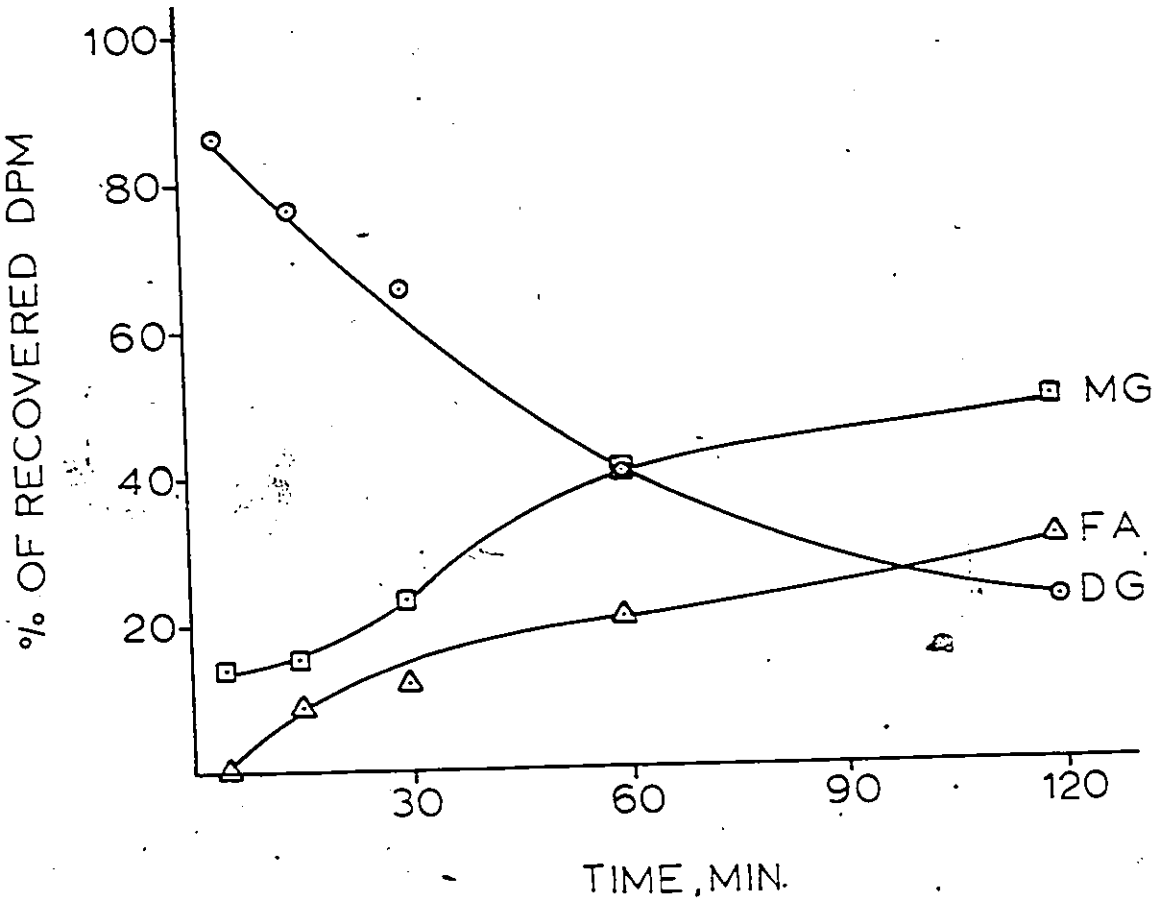

100
80
60
40
20
% OF RECOVERED DPM
MG
FA
DG
30
60
90
120
TIME, MIN.

FIGURE 14

Radiochromatogram of products obtained after 30 minutes incubation of coliform lipase activity with tri[1-^{14}C-oleoyl]-glycerol. The incubation medium contained 15 nmoles tri-[1-^{14}C-oleoyl]-glycerol (sp. act. = 5.9 x 10^6 dpm/μm), 34 μg protein (228 units/g protein), 0.25M Ca^{++}, 1.5% taurocholate, and 0.05M Tris, pH 8.4, in a total volume of 2 ml. The incubation was shaken 30 min. at 37°C. The extracted lipids were chromatographed on a borate-impregnated silica gel G plate developed in System C (cf methods, Section I). The lipid species were identified from standards run concurrently on the same plate.

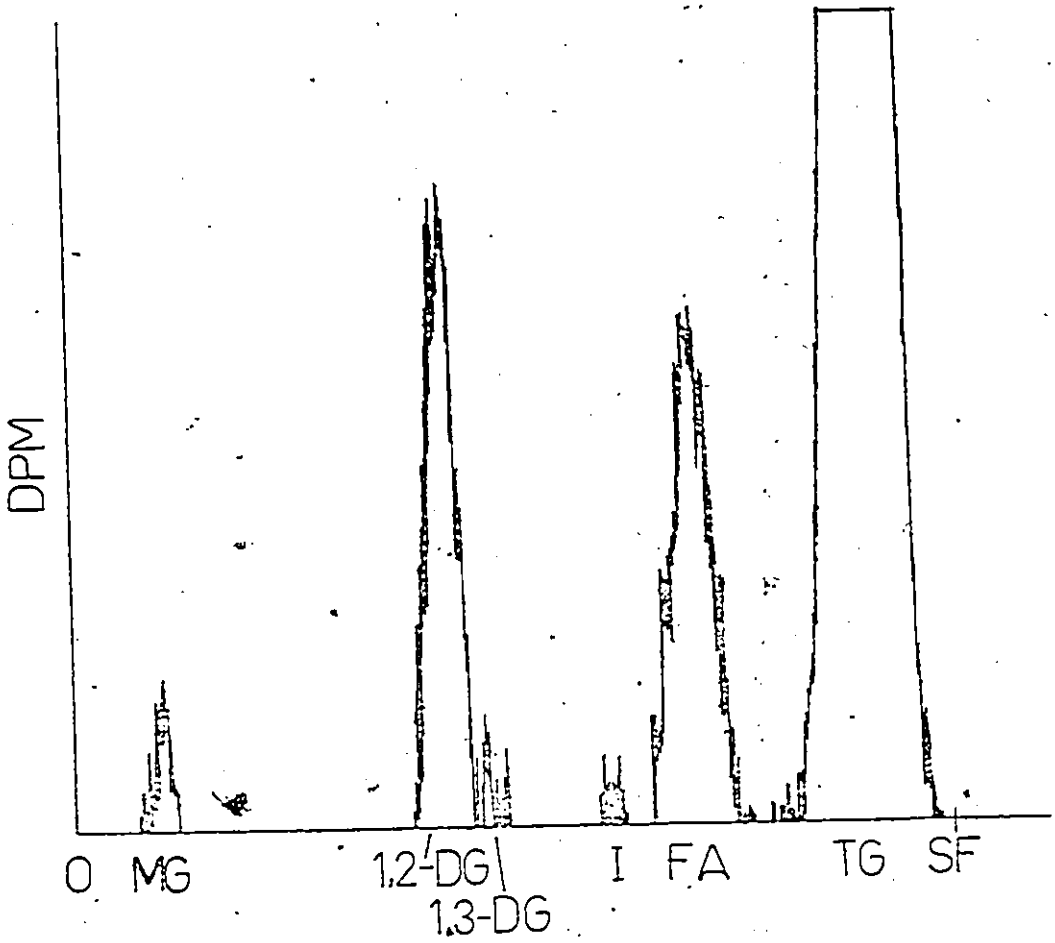

DPM
O MG
1,2-DG
1,3-DG
I FA
TG
SF

FIGURE 15

Lineweaver-Burk plot of *E. coli* lipase activity on 2-[1-^{14}C-palmitoyl]-1,3-dipalmitoyl glycerol with 1.5% taurocholate. The incubation media contained from 0.4 to 2.4 nmoles 2-[1-^{14}C-palmitoyl]-1,3-palmitoyl glycerol (sp. act. = 15.4 x 10^6 dpm/μm), 0.047 mg protein (228 units/g protein), 0.25M Ca++, 1.5% taurocholate, and .025M Tris, pH 8.4, in a 1 ml volume at 37°C. The Vmax found was 0.23 units/g protein.

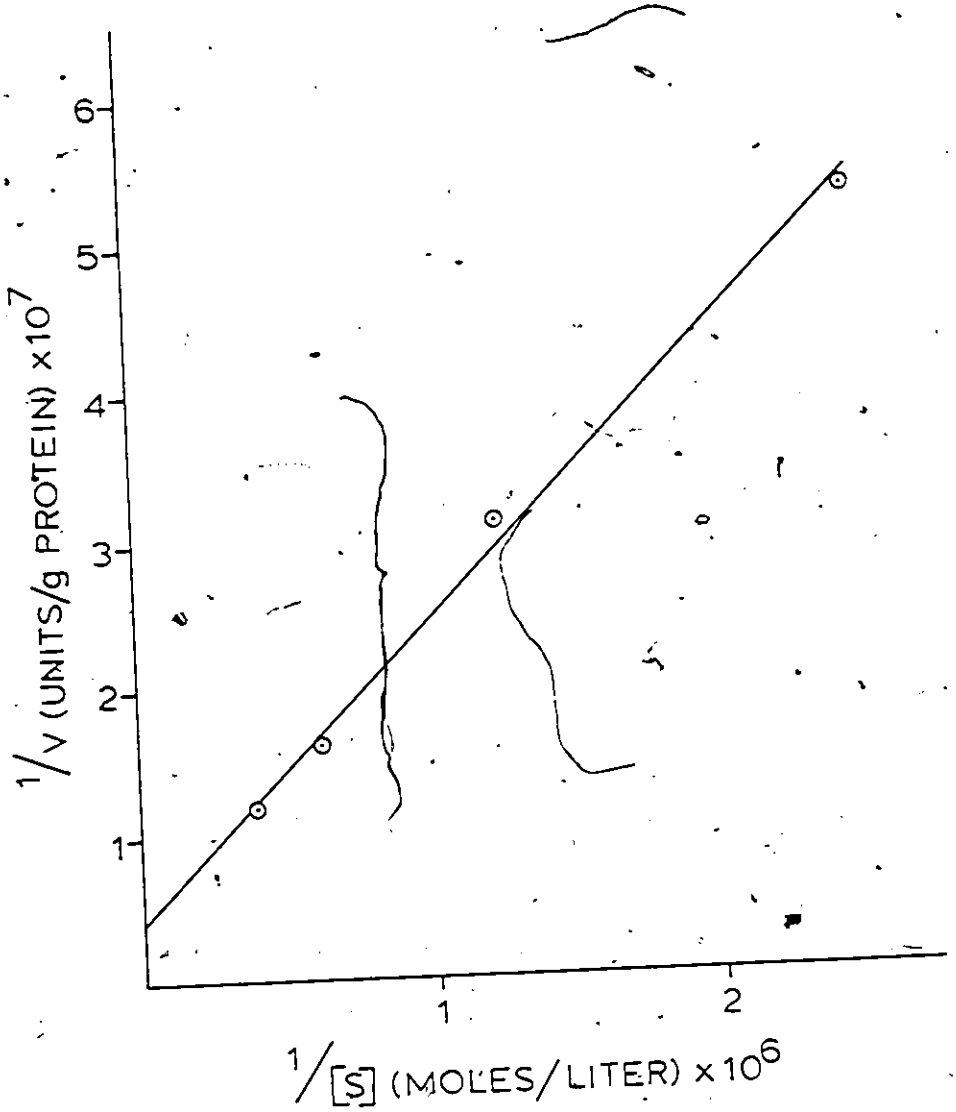
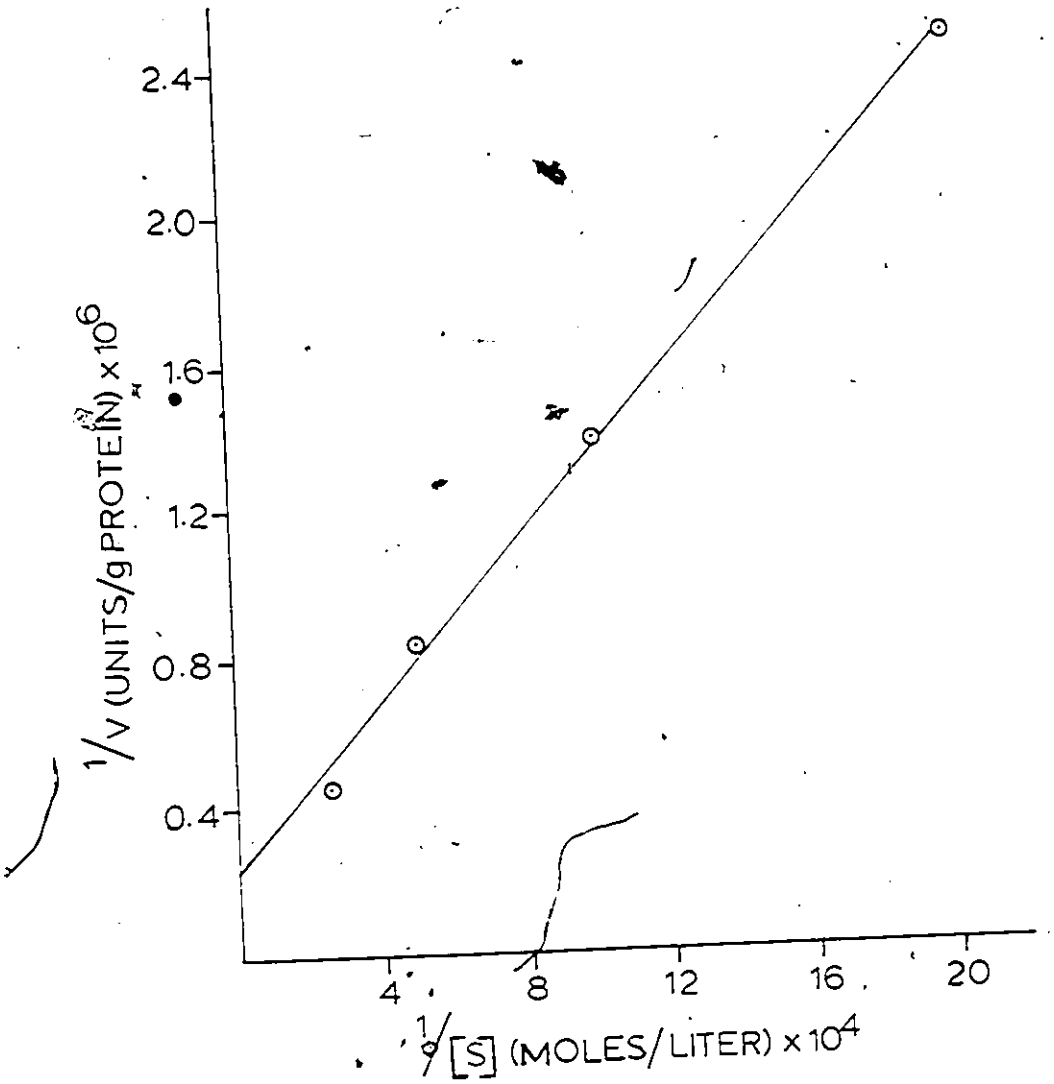

1/V (UNITS/g PROTEIN) x10^7
6
5
4
3
2
1
1
2
1/[S] (MOLES/LITER) x10^6

FIGURE 16

Lineweaver-Burk plot of E. coli lipase activity on tri-[1-^{14}C-palmitoyl]-glycerol with 1.5% taurocholate. The incubation conditions were as given under table 18. The Vmax found was 4.40×10^{-6} moles/g protein.


2.4
2.0
1.6
1.2
0.8
0.4
1/V (UNITS/g PROTEIN) x 10^6
4
8
12
16
20
1/[S] (MOLES/LITER) x 10^4

Entressangles et al. (62) observed no more than 6% isomerization of 1,2-diglycerides after 45 minutes of incubation. These same conditions however, did lead to considerable isomerization of 2-acyl MG.

Additional proof of hydrolysis at position 2 of triglycerides was sought with the use of 2-[1-^{14}C-palmitoyl]-sn-1,3-dipalmityl glycerol diether as substrate. The advantage of using this type of substrate is that the products of the reaction are stable, and this allows clearer conclusions concerning the positional specificity of the enzyme. One has to concede, however, the possibility that the enzyme may discriminate against or change its specificity towards the ether type of substrates. Using such a substrate, Mattson and Volpenheim (219) showed that the 2 position of TG was definitely not attacked by pancreatic lipase whereas Benzonana and Esposito (64) indicated that the lipase of Candida cylindracea which lacks stereospecificity, readily attacks the 2 position of TG.

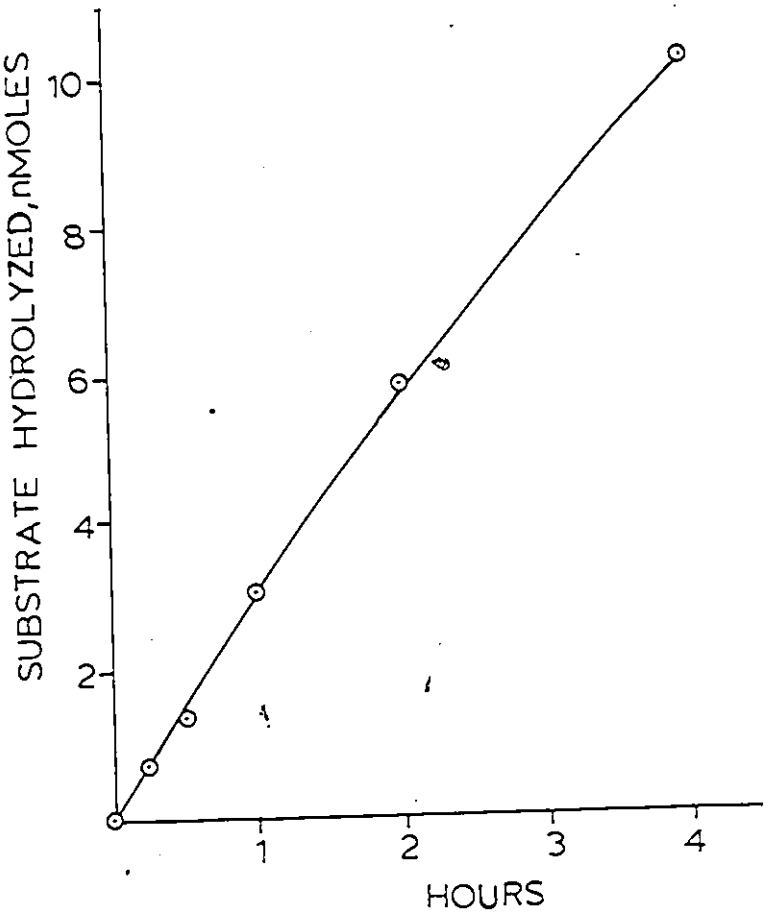
Results with E. coli lipase definitely indicated hydrolysis at position 2 of 2-[1-^{14}C-palmitoyl]-sn-1,3-dipalmityl glycerol diether (cf figure 17).

(b) STEREOSPECIFICITY

Studies with labelled glycerides have indicated so far that the coliform lipase is slightly different from pancreatic lipase since it has the ability to hydrolyze the

FIGURE 17

Time study of E. coli lipase on 2-[1-^{14}C-palmitoyl]-sn-1,3-dipalmityl glycerol diether. The incubation medium contained 17.8 nmoles of 2-[1-^{14}C-palmitoyl]-sn-1,3-dipalmityl glycerol diether (sp. act. = 2.25 x 10^6 dpm/μm) and 0.085 mg protein (from acetone fraction I of Step VI, according to purification Scheme III), 0.25M Ca++, 1.5% taurocholate, and 0.05M Tris buffer, pH 8.4, in a 2 ml volume. The incubations were shaken at 37°C for the indicated times.

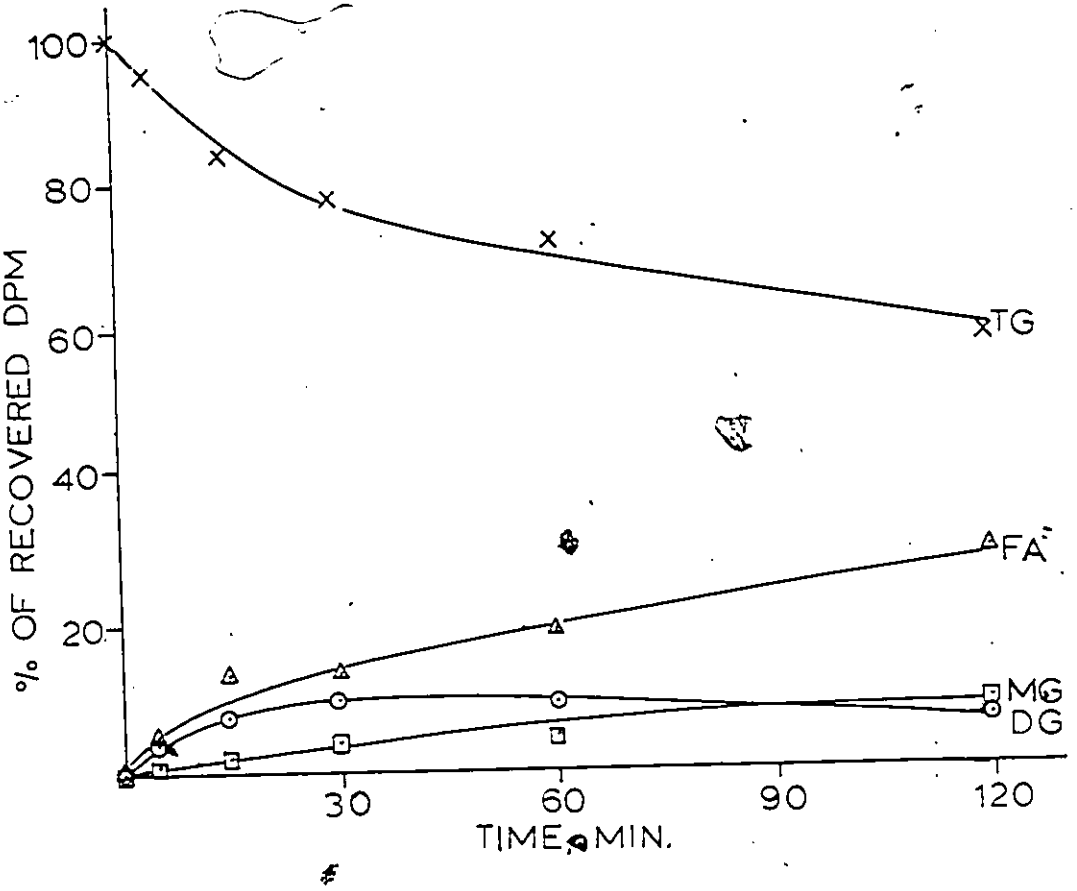

SUBSTRATE HYDROLYZED, nMOLES
10
8
6
4
2
1
2
3
4
HOURS

ester at position 2 of glycerides, but this at a rate slower than that for the esters of the outer positions. The pancreatic lipase lacks stereospecificity since it hydrolyzes both the 1 and 3 positions of triglycerides.

To see whether coliform lipase attacked position 3 of triglycerides, a mixture of hepatic 1,2-diglycerides was acylated with palmitoyl chloride (cf Methods for, details of preparation) and the sn-3 fatty acid-labelled triglyceride species resulting were used as substrate. Results of figure 18 show an accumulation of radioactive FA right from the onset of incubation and one must conclude therefore that position 3 is attacked. Initially, the labelled FA and DG productions are approximately equal, which means that the 1 and 3 positions are hydrolyzed at similar rates. One may recall that the main DG species produced initially is 1,2-DG. After prolonged incubation the labelled DG production falls and there is an accumulation of labelled MG. The type of MG produced was not characterized; however it is likely a 3-monoacyl glycerol since the thermodynamics of acyl migration favour 9 to 1 the formation of species with a peripheral ester (224). The last result therefore further reveals that coliform lipase can catalyze a hydrolysis at position 2 of glycerides. Also one can suggest from the time course study that MG hydrolysis proceeds at a slower rate than the other glycerides since it did accumulate.

FIGURE 18

Time study of E. coli lipase activity on sn-3-[1-^{14}C-palmitoyl]-1,2-diacyl glycerol. The incubation medium contained 0.8 nmoles sn-3-[1-^{14}C-palmitoyl]-1,2-diacyl glycerol (sp. act. - 15.1 x 10^6 dpm/µm) with 0.075 mg protein (228 units/g protein), 0.25M Ca++, 1.5% taurocholate and 0.025M Tris, pH 8.4, in a 2 ml volume. The incubations were shaken at 37°C for the indicated times.


100
80
60
40
20
% OF RECOVERED DPM
TG
FA
MG
DG
30
60
90
120
TIME, MIN.

The sn-3 fatty acid-labelled substrate used in this last study was a mixture of triglycerides, some of which may not have been optically active. However, when 1-stearoyl-2-oleoyl-3-[1^{14}C-palmitoyl]-glycerol was used as substrate, there resulted an accumulation of labelled palmitic acid at a rate of approximately 1.1 units/g protein, whereas a rate of 4.4 units/g protein was noted when tri-[1-^{14}C-palmitoyl]-glycerol was used. If we consider that the latter substrate can release three moles of labelled fatty acids per mole of substrate, this again indicates that hydrolysis at positions 1 and 3 proceed at approximately similar rates.

On the basis of these results, therefore, it seems that coliform lipase is not stereospecific.

(c) SUBSTRATE SPECIFICITY

(i) GLYCERIDE SPECIFICITY

Perhaps the first question that comes to mind in terms of substrate specificity of a lipase is whether it hydrolyzes glyceride species other than triglyceride. This has been already partially answered for E. coli lipase, particularly in terms of positional specificity.

In the present study the relative initial rates of hydrolysis for similar concentrations of labelled triglyceride, diglyceride and monoglyceride by E. coli lipase are compared (cf table 16). The triglyceride and monoglyceride are of

TABLE 16

Comparison of Initial Rates of Hydrolysis of *E. coli* Lipase Activity Against Various Glycerides

Substrate	moles FA released per g protein after 10 min.	after 20 min.
1-[1-^{14}C-oleoyl]-glycerol	2 x 10^{-6}	6 x 10^{-6}
1-^{14}C-acetate-labelled diglyceride	33 "	61 "
tri-[1-^{14}C-oleoyl]-glycerol	20 "	46 "

The incubation medium contained 7.65 nmoles 1-[1-^{14}C-oleoyl]-glycerol (sp. act. = 2.0 x 10^6 dpm/μm) or 8.15 nmoles of 1-^{14}C-acetate-labelled diglyceride (sp. act. = 2.4 x 10^6 dpm/μm) or 7.65 nmoles tri-[1-^{14}C-oleoyl]-glycerol (sp. act. = 5.9 x 10^6 dpm/μm), 34 μg protein (228 units/g protein), 0.25M Ca^{++}, 1.5% taurocholate and 0.25M Tris, pH 8.14, in a total volume of 1 ml. The incubations were shaken at 37°C for the indicated times.

the oleoyl ester type whereas the diglyceride originated from E. coli PE as a product of phospholipase C degradation. Preferably, labelled diolein should have been used, but attempts to synthesize this substrate by the action of pancreatic lipase on labelled triolein were not very successful, the yields being very poor. Results in table 18 show that triolein was hydrolyzed at a rate 1.5 times faster than tripalmitin. E. coli PE contains a majority of C_{16} fatty acids and therefore the value found here would have been expected to be higher with diolein than that with diglyceride reported in table 16. It can be tentatively concluded therefore that diglyceride was the most rapidly hydrolyzed of the glycerides.

It is also interesting to note in the results of table 16 that there is a hydrolysis of MG proceeding at a rate approximately 1/10 the rate found for triglyceride. One can conclude therefore, that our semipurified lipase prefers as substrate diglyceride, followed by triglyceride and then monoglyceride. Since the crude homogenate readily degraded monoglyceride (cf table 3, Section I) it is likely that our purification procedure eliminated a MG lipase.

(ii) ACYL CHAIN LENGTH SPECIFICITY.

Although an extensive study of acyl chain length specificity was not made, it appears that the purified coliform lipase prefers long chain esters since the tricapryloyl species is not attacked by the enzyme (cf table 17).

TABLE 17

Range of Substrates Hydrolyzed by E. coli Lipase

Substrate	detergent used	active	inactive
tri-[1-^{14}C-palmitoyl]-glycerol	1.5% taurocholate	✓	
tri-[1-^{14}C-oleoyl]-glycerol	"	✓	
tri-[1-^{14}C-capryloyl]-glycerol	"		✓
cetyl-1-^{14}C-palmitate	"	✓	
methyl-1-^{14}C-oleate	"	✓	
methyl-1-^{14}C-oleate	0.05% Triton X-100	✓	
p-nitrophenylacetate	1.5% taurocholate		✓
cholesterol-3β-1-^{14}C-oleate	"		✓
[1,3-^{14}C-glycerol]-phosphatidyl-ethanolamine	0.05% Triton X-100	✓	

In all cases the incubation media contained 0.025M Tris, pH 8.4, except with p-nitrophenylacetate, where the pH was 8.0, the amounts of detergents indicated in the table, 0.25M Ca++, except with PE where there was 0.05M Ca++, and various concentrations of protein (2 to 85 µg) (228 units/g protein, except for p-nitrophenylacetate which was obtained from acetone fraction I of Step VI according to purification Scheme III). The substrate was tested at the following concentrations: tri[1-^{14}C-palmitoyl]-glycerol (sp. act. = 6 x 10^6 dpm/µm), 5 to 40 nmoles; tri[1-^{14}C-oleoyl]-glycerol (sp. act. = 5.9 x 10^6 dpm/µm), 2.5 to 20 nmoles; tri[1-^{14}C-capryloyl]-glycerol (sp. act. = 17.2 x 10^6 dpm/µm), 2.6 and 6.4 nmoles; acetyl-1-^{14}C palmitate (sp. act. = 2.25 x 10^6 dpm/µm), 13 to 106 nmoles; methyl-1-^{14}C-oleate (sp. act. = 2.8 x 10^6 dpm/µm and 5.7 x 10^6 dpm respectively), 4 to 64 nmoles; p-nitrophenylacetate (non radioactive) 36 and 144 nmoles; cholesterol-3β-1^{14}C-oleate (sp. act. = 107 x 10^6 dpm/µm), 0.6 nmoles; [1,3-^{14}C-glycerol]-phosphatidyl ethanolamine (sp. act. = 2.06 x 10^6 dpm/µm), 3 to 22 nmoles. The incubations were shaken at 37°C in 1 ml volumes, except with p-nitrophenylacetate which was incubated at room temperature in a 2.6 ml volume and not shaken; the incubation times were 30 minutes, except for PE, where they were 15 minutes.

TABLE 18

Summary of Vmax for *E. coli* Lipase Activity with Various Substrates

Substrate	Detergent	Vmax (units/g protein)
tri[1-^{14}C-palmitoyl]-glycerol	1.5% taurocholate	4.40
tri-[1-^{14}C-oleoyl]-glycerol	"	6.49
cetyl-1-^{14}C-palmitate	"	23.6
methyl-1-^{14}C-oleate	"	227
methyl-1-^{14}C-oleate	0.05% Triton X-100	84.8
[1,3-^{14}C-glycerol]-phosphatidyl ethanolamine	"	361

The incubation conditions were as given in table 17.

Crude homogenate however did hydrolyze tricaprylin (cf figure 6, Section I), and very likely another enzyme was involved in this case or else the purification of this lipase brought about some changes in its properties. Possibly a type of protein or specific cofactor (a colipase) may have been eliminated.

(iii) ESTER TYPE SPECIFICITY

The coliform lipase was found to be quite active against single esters such as methyl-1-^{14}C-oleate and cetyl-1-^{14}C-palmitate, but was inactive against cholesteryl-1-^{14}C-oleate or the water-soluble ester, p-nitrophenylacetate (cf tables 17 and 18 and figures 16, and 19-23). It thus behaved as a true lipase with no cholesterase activity.

Vmax values for methyl-1-^{14}C-oleate have been compared with 1.5% taurocholate and 0.05% Triton X-100 as emulsifiers (cf table 18 and figures 21 and 22). With Triton X-100 one finds the Vmax about one third lower. Under these detergent conditions no activity was detectable against triolein in the presence of 0.05% Triton X-100. These results point to the role of detergents in organizing and orienting insoluble substrates and products at the micelle water interface with the enzyme. The enzyme may have a preference for negatively charged micelles.

As seen from the results in table 18, the semipurified lipase preparation hydrolyzed PE at a rate 1.6 to 4 times faster than that for methyl oleate, depending on the detergent

FIGURE 19

Lineweaver-Burk plot of E. coli lipase activity on tri-[1-^{14}C-oleoyl]-glycerol with 1.5% taurocholate. The incubation conditions were as given in table 18. The Vmax found was 6.49×10^{-5} units/g protein.

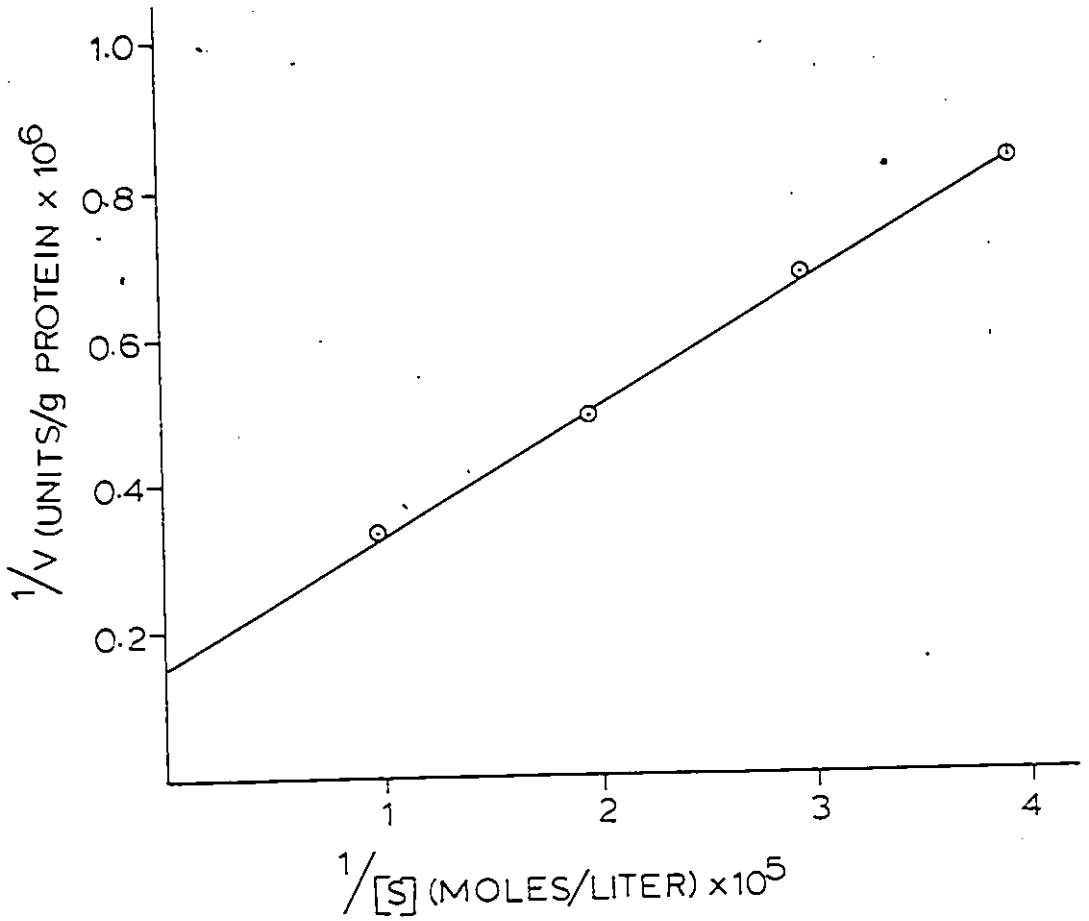

1.0
0.8
0.6
0.4
0.2
1/V (UNITS/g PROTEIN x 10^6
1
2
3
4
1/[S] (MOLES/LITER) x10^5

FIGURE 20

Lineweaver-Burk plot of E. coli lipase activity on cetyl-1-^{14}C-palmitate with 1.5% taurocholate. The incubation conditions were as given in table 18. The Vmax found was 2.36×10^{-5} units /g protein.

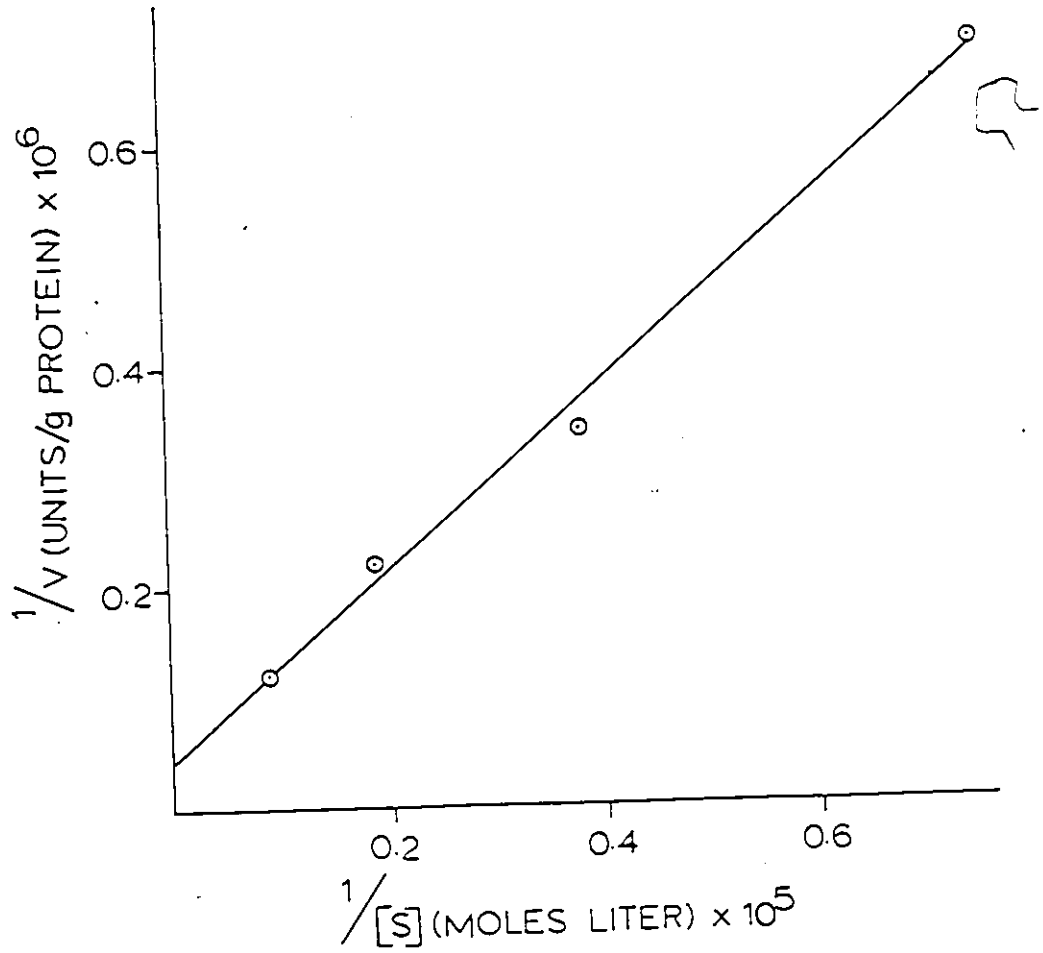

1/V (UNITS/g PROTEIN) x 10^6
0.6
0.4
0.2
0.2
0.4
0.6
1/[S] (MOLES LITER) x 10^5

FIGURE 21

Lineweaver-Burk plot of E. coli lipase activity on methyl-1-^{14}C-oleate with 1.5% taurocholate. The incubation conditions were as given in table 18. The Vmax found was 2.27×10^{-4} units/g protein.

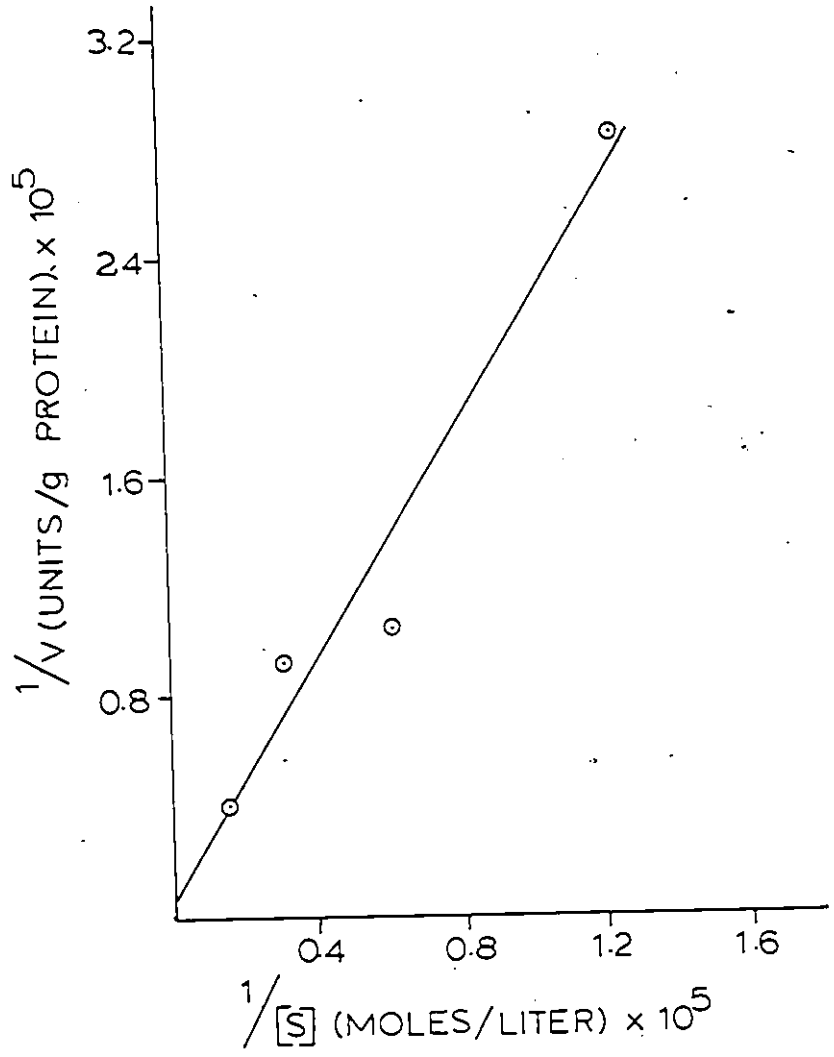

3.2
2.4
1.6
0.8
1/V (UNITS/g PROTEIN) x 10^5
0.4
0.8
1.2
1.6
1/[S] (MOLES/LITER) x 10^5

FIGURE 22

Lineweaver-Burk plot of E. coli lipase activity on methyl-1-^{14}C-oleate with 0.05% Triton X-100. The incubation conditions were as given in table 18. The Vmax found was 9.52×10^{-5} units/g protein.

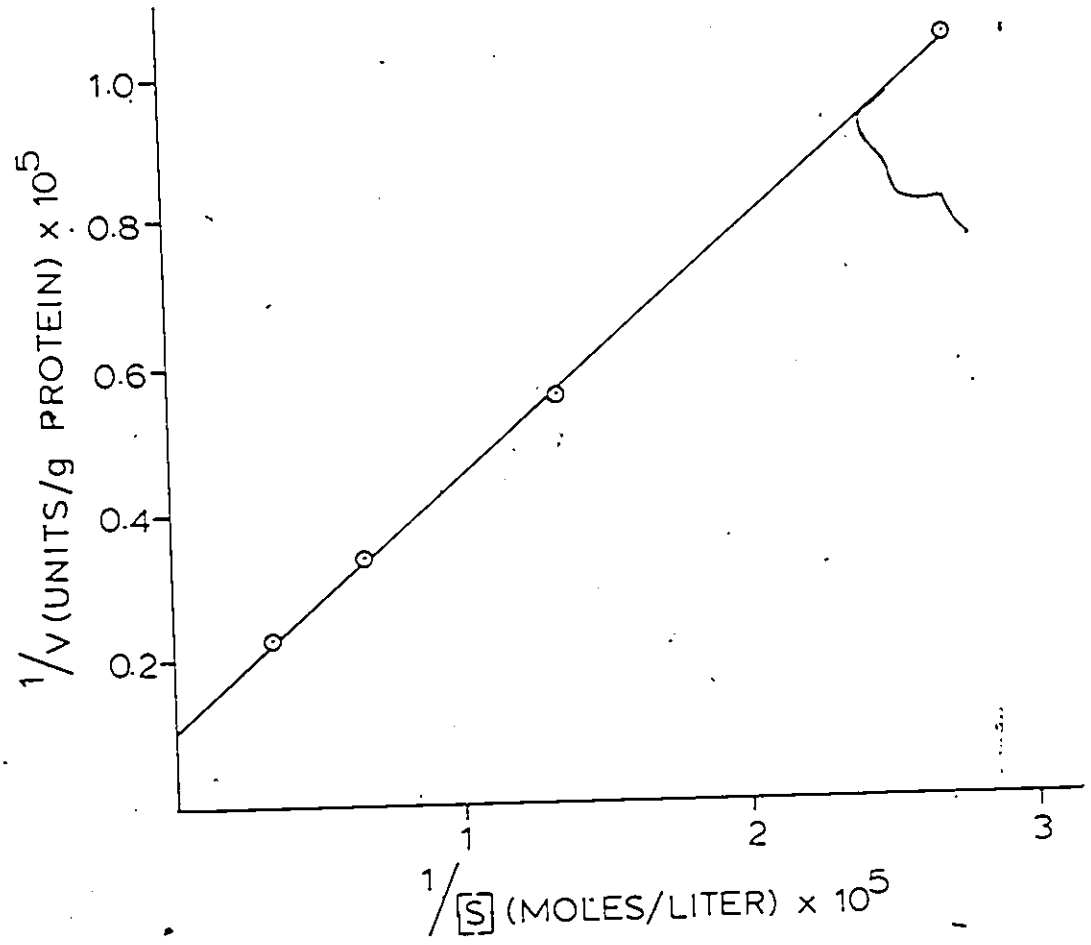
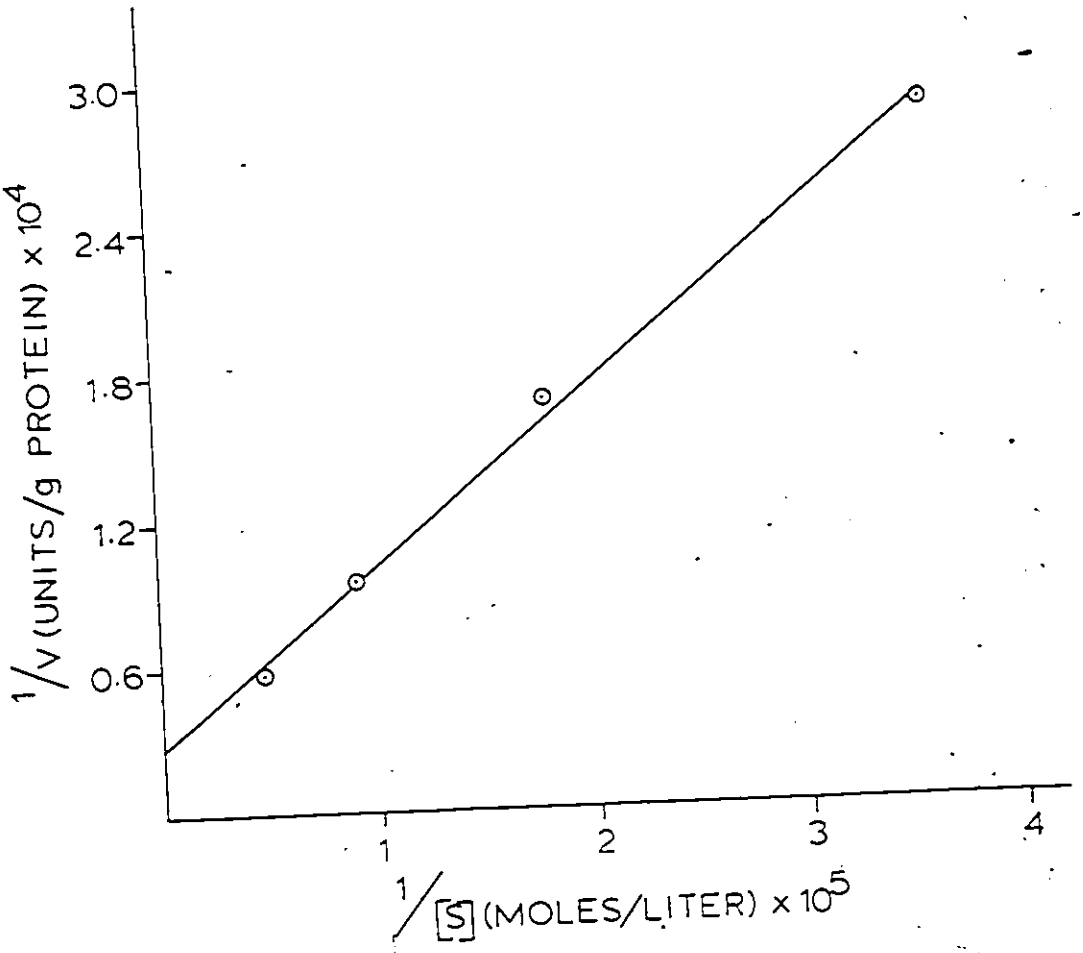

1.0
0.8
0.6
0.4
0.2
1/V (UNITS/g PROTEIN) x 10^5
1
2
3
1/[S] (MOLES/LITER) x 10^5

FIGURE 23

Lineweaver-Burk plot of E. coli lipase activity on 1,3-^{14}C-glycerol-PE with 0.05% Triton X-100. The incubation conditions were as given in table 18. The Vmax found was 3.61 x 10^{-4} units/g protein.


3.0
2.4
1.8
1.2
0.6
1/V (UNITS/g PROTEIN) x 10^4
1
2
3
4
1/[S] (MOLES/LITER) x 10^5

conditions used. Assuming there is only one enzyme, one can ask at this stage whether it is appropriate to designate our enzyme preparation a lipase or a phospholipase A. Probably the latter designation would be more appropriate, although it conveys the idea of a much more specific enzyme than is actually found in E. coli. The assumption that only one enzyme is involved for both lipase and phospholipase activities is substantiated to some extent by the fact that both activities copurify 50-200 fold, depending on the procedure used, and following SDS electrophoresis both activities are recovered in a single band.

(iv) EFFECT OF pH

The effect of pH on semipurified coliform lipase with methyl-1-^{14}C-oleate as substrate produced results essentially similar to those observed with the homogenate (cf figure 4, Section I), but with one significant difference: this time an optimum was found near pH 9.0 (cf figure 24). Although incubation conditions were not exactly the same, none of these differences seemed obviously conducive to a change in optimum pH.

(v) OTHER ACTIVITIES OF COLIFORM LIPASE

Quantitative results in table 19 indicate the ability of coliform lipase to transacylate when ethanol is the acceptor and either 1-[1-^{14}C-palmitoy]-PE or tri[1-^{14}C-oleoyl]-glycerol are the donors. This is indicative that an

FIGURE 24

The effect of pH on the hydrolysis of methyl-1-^{14}C-oleate by partially purified coliform lipase. The incubation medium contained 6.1 nmoles methyl-1-^{14}C-oleate (sp. act. = 5.7×10^6 dpm/μm), 0.023 mg protein (228 units/g protein), 0.25M Ca^{++}, 0.05% Triton X-100 and .025M histidine HCl-NaOH at pH 6.0, 0.025M Tris at pH 7.0, 7.5, 8.0, 8.5, 9.0, and 0.025M glycine-NaOH at pH 10.0, in 1 ml volumes. The incubations were shaken at 37°C for 30 min.

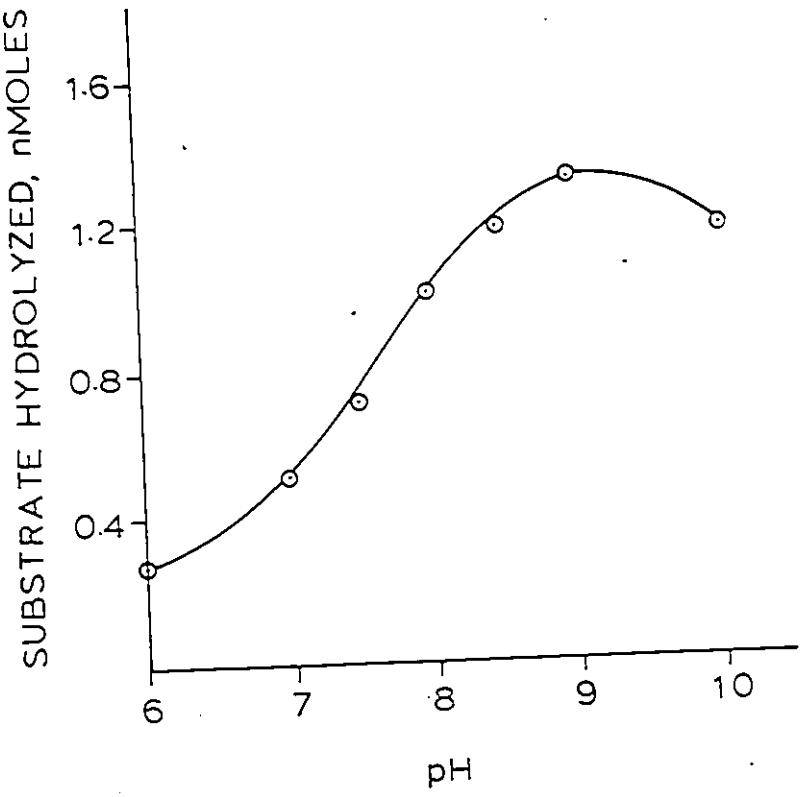

SUBSTRATE HYDROLYZED, nMOLES
1.6
1.2
0.8
0.4
6
7
8
9
10
pH

TABLE 19

Ethyl Ester Formation by Partially Purified *E. coli* Lipase in the Presence of 40% Ethanol

Substrate	nmoles methyl esters produced
1-[1-^{14}C-palmitoyl]-PE	14.3
tri-[1-^{14}C-oleoyl]-glycerol	1.4

The incubation mixtures contained 0.15 µmoles 1-[1-^{14}C-palmitoyl]-PE (sp. act. = 0.2 x 10^6 dpm/µm), 0.05M Ca++, 0.05% Triton X-100, and 0.025M Tris, pH 7.5, or 27.4 nmoles tri-[1-^{14}C-oleoyl]-glycerol (sp. act. = 1.14 dpm/µmole), pH 7.5. Both systems contained 0.5 mg protein (from acetone fraction I of step VI, according to purification Scheme III), and the total volumes were 2 ml. The incubations were shaken 2 hours at 37°C.

acyl-enzyme intermediate is formed during hydrolysis of phosphatidyl ethanolamine or triglycerides.

PART TWO

E. COLI LYSOPHOSPHOLIPASE

1. INTRODUCTION

An accumulation of lysophosphoglycerides in a cell can bring about its lysis. Lysophospholipase is believed to play an important role in the maintenance of the integrity of the cell by hydrolyzing lysophospholipids to non lytic products. In accord with this function this enzyme is involved in the complete breakdown and turnover of phospholipids. Despite its importance, lysophospholipase has not received much attention; in fact, at the time this work was carried out there were only two reports describing its purification. Proulx and Fung (56) and also Doi et al. (174) reported some of its characteristics in several strains of E. coli. The present work characterizes further this enzyme in E. coli B (ATCC 11303), and describes our attempts at its purification. It is now known that phospholipase A1, purified to near homogeneity (176), possesses strong lysophospholipase activity; however, the latter enzyme being strongly membrane-bound, is found exclusively in the particulate material after high speed centrifugation. The present section describes our attempt to purify the 100,000 x g supernatant lysophospholipase which is devoid of phospholipase A1 activity.

Upon studying lysophospholipase activity, we encountered an acyltransferase activity which could reincorporate fatty acid liberated from lysophosphoglyceride into diacylphosphoglyceride. The latter enzyme has been reported earlier (225). We examined briefly a few of the properties of this acyltransferase, but our aim was really to eliminate its activity since it interfered with proper lysophospholipase assay.

2. MATERIALS

The solvents heptane and isopropyl alcohol, and the phosphonic acid used for the Dole and Meinertz (226) fatty acid extraction procedure, and the reagent dimethylformamide, used for affinity chromatography, were purchased from Fisher Scientific. The CNBr-activated Sepharose 4B was obtained from Pharmacia. The source of the other materials used was given in previous sections.

3. METHODS

(a) SUBSTRATE PREPARATION

The substrate 1-[1-^{14}C-palmitoyl]-lyso lecithin was prepared by the action of Crotalus Adamanteus venom on 1-[1-^{14}C-palmitoyl]-lecithin (the preparation of the latter compound has been described in a previous section), according to an adaptation of the methods of White and Tucker (207) and Hildebrand and Law (227), as previously described for the analysis of the distribution of label in the fatty ester positions of PC and PE.

(b) LYSOPHOSPHOLIPASE ASSAYS

The assays for lysophospholipase were carried out in 2 ml volumes comprising a final concentration of 0.05M phosphate or Tris buffer, pH 7.2, unless otherwise specified, with two different concentrations of the enzyme to be assayed, in a range giving linear hydrolysis with protein concentration for at least 30 minutes. The substrate 1-[1-^{14}C-palmitoyl]-lyso-PC, with specific activities of 5 or 8 x 10^4 dpm/µm, was added in concentrations of 0.25 µmoles per assay. The incubations were stopped after 30 minutes by the addition of lipid extraction solvents. The homogenates assayed were prepared by sonication, as previously described; their protein concentrations were in the range of 25-32 mg/ml.

(c) LIPID EXTRACTIONS

In some experiments the lipids were extracted by the method of Bligh and Dyer (203), chromatographed by TLC, and scanned by radioscanner, as described previously. When it was not necessary to examine the products of the reaction on TLC, and particularly to simplify the assays for the numerous fractions obtained dufing the purification procedures, the Dole and Meinertz procedure (226) was used to extract the fatty acids. This consisted in adding 10 ml of an extraction mixture made up of 40 volumes of isopropyl alcohol, 10 volumes of heptane, and 1 volume of 1.23M H_3PO_4, to the

2 ml assay mixtures. After stirring and waiting 4-5 minutes, 4 ml distilled water and 6 ml heptane were added and stirred over the vortex. As soon as the phases had separated (after about 1 minute), the upper heptane phase containing the fatty acids was removed as completely as possible with a Pasteur pipet, and added into scintillation vials containing scintillation fluid. This procedure was found to extract 85% ± 2.4% of 1-^{14}C-palmitic acid, and no 1-[1-^{14}C-palmitoyl]-lyso PC.

(d) IDENTIFICATION OF LYSOPHOSPHOLIPASE ACTIVITY IN COLUMN CHROMATOGRAPHY FRACTIONS

In order to determine which fractions from column chromatography contained activity prior to the determination of their specific activities, 0.4 ml of each fraction were incubated 30 minutes at 37°C with 1.6 ml of 0.05M phosphate buffer, pH 7.15. The fatty acids were then extracted by the Dole and Meinertz procedure just described, and 1 ml aliquots of the heptane phases were removed and counted. This permitted a relatively rapid identification of the fractions containing the activity.

(e) AFFINITY CHROMATOGRAPHY

Affinity chromatography was attempted once only in a batch procedure. 0.57 g of CNBr activated Sepharose 4B were swollen in distilled water for 1½ hours, while some pre-swollen Sepharose 2B was used in a parallel experiment as a

control to account for the non specific binding of protein to this type of gel; both gels were subjected to identical preparative treatments. Since the activated Sepharose is packaged with dextran and lactose as stabilizers, these had to be washed away before proceeding with the coupling. This was done by washing each gel solution with 114 ml each of 10^{-3}M HCl. Then 5 ml of the coupling solution, made up of 144 µmoles of L-PE and 1 volume of a solution containing 0.2M $NaHCO_3$ and 1.0M NaCl, and 1 volume of dimethylformamide were added to the activated Sepharose 4B, while the same coupling solution free of L-PE was added to the Sepharose 2B. The gels were stirred constantly in this reagent for two hours at room temperature, then washed twice with the binding solution free of L-PE to remove the excess L-PE from the Sepharose 4B. The remaining active groups were blocked by reacting the gels for 1½ hours in 5 ml of 1M ethanolamine at pH 8.0. The gels were then washed 3 times with 5 ml of 0.15M acetate buffer, pH 5.0, at 0°C.

Once prepared, these Sepharose gels were reacted 15 minutes at 0°C with aliquots from a 100,000 x g supernatant in 0.15M acetate buffer, pH 5.0. This pH was selected because lysophospholipase is not very active at pH 5.0; in this way the L-PE bound to the gel would not be hydrolyzed. Of course, success would depend on whether the enzyme is able to bind L-PE at that pH. The solutions were then centrifuged to

separate the Sepharose with its bound protein from the lysophospholipase which remained in the supernatant. This unbound lysophospholipase was assayed after dialysis against 0.05M phosphate buffer, pH 7.15. The amount of enzyme bound to Sepharose was found by calculating the decrease in the specific activity of lysophospholipase remaining in solution, as compared to that of the control with Sephadex 2B.

(f) PREPARATION OF DIALYSIS TUBING

Prior to use, dialysis tubes were treated to remove heavy ions which are sometimes bound to them and inhibit enzymes. The procedure consisted in boiling the tubes for 10 minutes in a solution containing 9 g $NaHCO_3$ and 1.4 g EDTA per liter, followed by two 10 minute boiling periods in distilled water. The tubes were rinsed well with distilled water between each boil. After this treatment they were stored refrigerated in the buffer to be used.

4. RESULTS AND DISCUSSION

I - ACYLTRANSFERASE

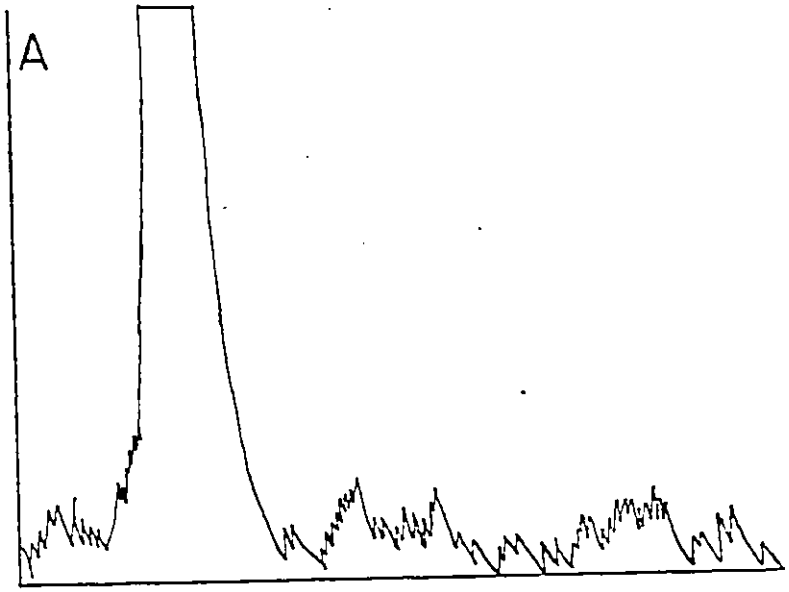
Upon assaying lysophospholipase with the substrate 1-[1-^{14}C-palmitoyl]-lysolecithin at various pH's and at relatively high concentrations of enzyme, radioactive products other than 1-^{14}C-palmitic acid were observed. These were identified by TLC as PC and PE mainly (cf figure 25).

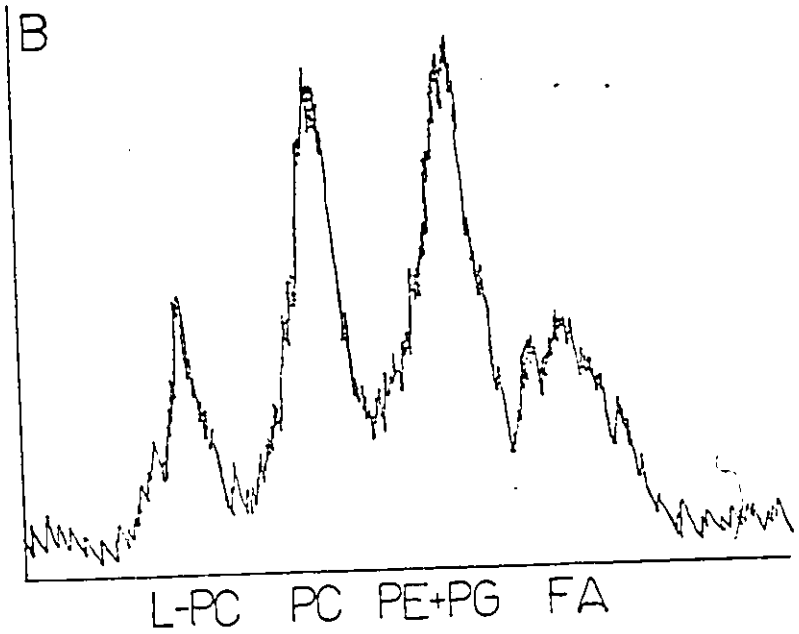
FIGURE 25

(A) control: inactive enzyme + ATP + CoA.

(B) *E. coli* B homogenate + ATP + CoA.

Transacylase activity in *E. coli* B. The incubation mixtures contained 0.4 nmoles of 1-[1-^{14}C-palmitoyl]-lyso PC (sp. act. = 5 x 10^4 dpm/μm), 25-32 mg protein from an *E. coli* B (ATCC 11303) homogenate, and 0.05 M phosphate buffer, pH 6.0. In addition, the incubation mixture of (A) contained 2 ml of methanol-chloroform (25:1) to inactivate the enzyme, and that of (B) contained 0.6 mg CoA and 15 mg ATP. The TLC plates were developed in chloroform - methanol - water 12:6:1.


A


B
L-PC
PC
PE+PG
FA

Such results were attributed to the presence of an acyltransferase activity which could acylate endogenous lysophosphoglyceride as well as the added substrate. Both E. coli B (ATCC 11303) and 015 were found to give similar results. However, Strain B seemed to contain endogenous ATP and CoA which were not strictly required as additives for acylation, but which markedly increased PE (and PG) formation, in E. coli 015 extracts.

This acyltransferase seems to be active over a wide pH range, since we observed it both at pH's 6.0 and 8.0, although it was more prominent at the latter pH. Sodium dodecyl sulfate, at pH 8.0, was able to inhibit completely this activity.

Since this acyltransferase activity was present over most of the pH range of lysophospholipase, it was important to determine to what extent it might interfere with our lysophospholipase assays. It was soon realized, however, that this acyltransferase activity was much lower than that of lysophospholipase. Thus, at relatively low protein concentrations, lysophospholipase could be assayed without interference from the acyltransferase.

II - LYSOPHOSPHOLIPASE

(a) PROPERTIES

(i) SUBCELLULAR DISTRIBUTION

The first aspect of the properties of lysophospholipase examined was its subcellular distribution. Now that it is known that phospholipase A1, a strongly membrane-bound enzyme has lysophospholipase activity, the picture provided in table 20 is more revealing; the lysophospholipase activity due to phospholipase A1 remained in the 100,000 x g pellet, while a "soluble" lysophospholipase was recovered in the supernantant. Furthermore, all the sedimentable lysophospholipase could actually be removed by a centrifugation of 50,000 x g; the supernatant from such a centrifugation was also devoid of phospholipase A1 activity. Therefore, any particulate material removed by centrifugation at speeds above 30,000 x g and up to 100,000 x g (the highest used) afforded a small purification factor for this enzyme, while it removed phospholipase A1.

(ii) EFFECT OF pH

The pH curve obtained (cf figure 26) with the substrate 1-[1-^{14}C-palmitoyl]-lyso PC and a homogenate of *E. coli* B (ATCC 11303) shows a broad optimum between pH 5.5 and 9.0. A slightly different pH curve of *E. coli* 015 lysophospholipase was obtained by Proulx and Fung (56) and

TABLE 20

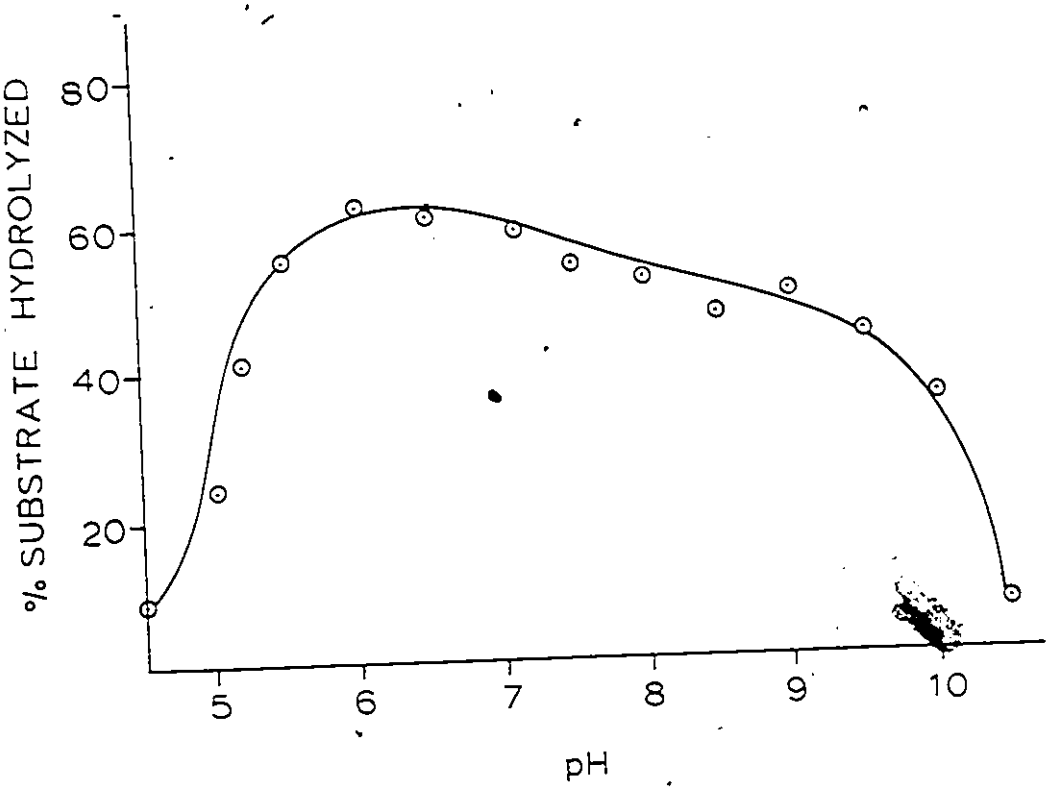
Subcellular Distribution of Lysophospholipase

Fraction	Specific Activity (units/min)	Purification Factor	Percentage of Total Units
homogenate	6.7	1	100
100,000 x g supernatant	9.2	1.4	64.3
100,000 x g pellet	5.0	.7	39

The specific activities were determined by incubating 0.25 µmoles 1-[1-^{14}C-palmitoyl]-lysolecithin (sp. act. = 8 x 10^4 dpm/µm) in 2 different concentrations of protein for each fraction in a range where the hydrolysis was linear with protein concentration for at least 30 minutes, and 0.025M Tris, pH 8.0. The incubations were shaken at 37°C for 30 min.

FIGURE 26

The effect of pH on the hydrolysis of 1-[1-^{14}C-palmitoyl]-lysolecithin by E. coli lysophospholipase. The incubation medium contained, in a 2 ml volume, 0.5 µmoles of 1-[1-^{14}C-palmitoyl]-lysophosphatidyl choline (sp. act. = 5 x 10^4 dpm/µm), 25-32 mg protein from an E. coli B homogenate, and 0.025M of the following buffers: pH 4.5 to 5.5 with acetate, pH 6.0 to 7.0 with carbonate; pH 7.5 to 9.0 with Tris, and pH 9.5 to 10.5 with carbonate. The incubations were shaken at 37°C during 2 hours.


% SUBSTRATE HYDROLYZED
80
60
40
20
5
6
7
8
9
10
pH

also by U. Rapp (228) using 1-[1-^{14}C-stearoyl]-lyso-PE instead of lysolecithin as substrate; under their conditions, one optimum appeared at pH 10, with activity being prominent approximately from pH 5.0 to at least 11 (the highest pH tested). On the other hand, Doi et al. (174) obtained an optimum between 9.5 and 9.7 with washed E. coli K12. Also with the lyso-PE substrate, Albright et al. (178) found three pH optima for this enzyme, depending on which subcellular fraction of E. coli was used; these included optima at pH 8.5, 9.0, and 10.

For most of our experiments, the incubation media were buffered at pH 7.2 which was within the optimal range when lyso PC was used as substrate.

(iii) DETERGENT EFFECTS

The effects of various detergents on lysophospholipase activity in homogenates were then examined. Proulx and Fung (56) had previously found a 95% inhibition of lysophospholipase with 0.5% deoxycholate or sodium lauryl sulfate. Table 21 shows that neutral detergents such as Triton X-100 and Cutscum are not inhibitory at 0.01 and 0.1% concentrations whereas a cationic detergent such as cetyltrimethylammonium chloride inhibits the activity even at a concentration of 5 mg/100 ml (0.01%). Although not shown in table 21, we have also observed a 92% inhibition of the

TABLE 21

Effect of Cationic and Neutral Detergents on Lysophospholipase Activity

Detergent	Concentration (mg/100 ml)	Percent hydrolysis of 1-[1^{14}C-palmitoyl]-lysolecithin
none	-	35.5
cetyltrimethyl ammonium chloride	5	29.4
	25	24.5
	50	18.6
Triton X-100	5	38.8
	25	41.2
	50	35.7
Cutscum	5	39.8
	25	41.8
	50	37.0

The incubation media contained 0.5 μmole of 1-[1^{14}C-palmitoyl]-lysolecithin (sp. act. = 5 x 10^4 dpm/μm), 25-32 mg protein from an E. coli B homogenate, 0.25M phosphate buffer, pH 6.5, and the detergents listed in the table, in a 2 ml volume. The incubations were shaken at room temperature for 2 hours.

lysophospholipase activity present in a 30,000 x g supernatant in the presence of 6% Triton X-100.

(iv) ION REQUIREMENTS

Our own results as well as those of others reveal that lysophospholipase, an enzyme with a high specific activity, can be detected in E. coli in the absence of added ions. The ion effects were further tested with an enzyme preparation which had been precipitated with ammonium sulfate, followed by dialysis. As shown in table 22, the enzyme had no apparent cation requirement.

(b) PURIFICATION OF LYSOPHOSPHOLIPASE

(i) INTRODUCTION

Having established some of the properties of coliform lysophospholipase, we proceeded with its purification. From an examination of the subcellular distribution of this enzyme (cf table 20), we observed that 39% of its activity remained in a 100,000 x g pellet. Since we know from the work of Scandella and Kornberg (176) that phospholipase A1 is predominantly a lysophospholipase, it is not surprising to find considerable activity residing in the high speed pellet. Considering these results we decided to approach the purification of the supernatant enzyme as with soluble enzymes.

TABLE 22

Effect of Various Cations on Lysophospholipase Activity

Ions	Concentration (mM)	Specific Activity (units/g protein)
none	-	66
Ca^{++}	1	64
	10	69
	100	65
Mg^{++}	1	63
	10	66
	100	67
K^{+}	1	61
	10	67
	100	67
NH_4^{+}	1	67
	10	58
	100	67

The incubation media contained 0.25 μmole 1-[1-^{14}C-palmitoyl]-lysolecithin (sp. Act. - 8 x 10^4 dpm/μm), 8.8 mg protein of a partially purified fraction (details in text), .025M Tris, pH 7.2, and the cations listed in the table, in a 2 ml volume. The incubations were shaken at 37°C for 30 minutes.

(ii) ACID PRECIPITATION

54 g of E. coli B (ATCC 11303) were resuspended in water to yield a volume of 80 ml in 1 mM EDTA, and sonicated during four periods of 5 minutes each over a salt-ice mixture. 20 ml of glycerol were then added (20% glycerol) as a protective measure for the enzyme. The preparation was subjected to a 1 hour autolysis at 37°C to reduce its viscosity. 5 ml aliquots were removed and subjected to acid precipitation by slowly stirring in 1M acetic acid until the pH's 4.0, 4.5, 5.0, and 5.5 were reached. All volumes were then completed to 10 ml with 1M acetate buffers at their corresponding pH's. Each solution was centrifuged at 100,000 x g during 90 minutes, the fractions separated, the pellets were resuspended in distilled water, and all samples were dialyzed against distilled water before assaying. All the pellets were practically devoid of activity, whereas interesting information was obtained from the supernatants; the latter results are shown in table 23 and suggest that the pk of the enzume is close to 5.0. Below this value, at pH 4.0, a 7-fold purification was obtained, but with a very poor yield.

(iii) EFFECT OF HEATING

Preliminary experiments indicated that part of the lysophospholipase was relatively heat stable, but this property could not be exploited with much advantage. An

TABLE 23

Acid Precipitation of Lysophospholipase

Fraction	Specific Activity (units/g protein)	Purification Factor	Percentage of Total Units
homogenate	6.2	1	100
100,000 x g - 90 min. supernatant			
pH 4.0	45.3	7.3	11
pH 4.5	26.4	4.3	10
pH 5.0	1.2	0.2	1
pH 5.5	8.2	1.3	12

The different pH's were obtained by slowly stirring 1M acetic acid into aliquots of supernatant until the desired pH was reached, and 1M acetate buffer at the corresponding pH's was added to fixed volumes. The incubation media were as described under table 20, except that 0.025M phosphate buffer, pH 7.2 was used instead.

homogenate was prepared by resuspending 30 g of E. coli B (ATCC 11303) paste in 100 ml of distilled water, and sonicating during four periods of 5 minutes over a salt-ice mixture. The preparation was then incubated at 37°C for 1 hour to allow autolysis.

The homogenate was then heated in a water bath at 88°C for 5 minutes, and then allowed to cool at room temperature for 1 hour, since slow cooling is known to favour renaturation of some heat-denatured proteins. A 100,000 x g - 90 minute supernatant was recovered from this preparation and assayed. From the results in table 24, this supernatant showed a 4-fold purification factor, with 44% yield. Considering both the purification factor and the yield, this method seemed to be most promising as an initial purification; however, it could not be combined satisfactorily in a purification scheme, since the yields and purification factors obtained by procedures following this one were particularly low.

(iv) AMMONIUM SULFATE FRACTIONATION

Fractionation with $(NH_4)_2SO_4$ as a means of purification was tested. A 100,000 x g, 90 minute supernatant in 0.05M phosphate buffer, pH 7.15, was used for these experiments. In a first attempt, precipitates were collected by centrifugation after salt additions at 0°C representing 25%, 50% and 75% saturation. The pellets were resuspended in distilled water

TABLE 24

Effect of Heat as a Purification Step for Lysophospholipase

Fraction	Specific Activity (units/g protein)	Purification Factor	Percentage of Total Units
A - homogenate	20.2	1	100
B - homogenate autolyzed 1 hr at 37°C	20.2	1	100
C - homogenate heated 5 min. at 88°C	7.4	0.4	37
D - 100,000 x g - 90 min supernatant from fraction C	80.8	4	44

The assay conditions were the same as given under table 20 except that 0.025M phosphate buffer, pH 7.2 was used instead.

and, along with the supernatant from the 75% saturation, were dialyzed against distilled water before assaying. The results obtained from these fractions are listed in table 25, Part A. They show that the activity fell with the precipitates over a range between 25 to 75% saturation, with three times more enzyme distributed in the 50-75% range. This suggests first that lysophospholipase precipitated in a range of saturation which overlapped between the fractions, arbitrarily chosen (cf table 25, Part A, fractions D and E), and secondly that the enzyme is precipitated in the same range as most of the proteins from this supernatant, since the acceptable 43% recovery for fraction E (cf table 25, Part A) is matched by only a 1.3 fold purification factor. We therefore pooled fractions D and E, added phosphate buffer, pH 7.25, to a 0.05M concentration, and attempted another $(NH_4)_2SO_4$ fractionation. This time precipitates were collected at 45%, 55%, 65%, and 100% saturation. However, the activity was found scattered among two fractions which precipitated between 45-55% and 65-100% saturation. (cf table 25, Part B). A 3.7 fold purification was obtained from the 65% to 100% saturation fraction, with a yield of only 14% of the total units.

The results as a whole indicated that ammonium sulfate is not particularly useful in the fractionation of this lysophospholipase, since a very limited purification was obtained at sizeable losses in activity.

TABLE 25

Ammonium Sulfate Fractionation of Lysophospholipase from a 100,000 x g supernatant

Fractions	Specific Activity (units/g protein)	Purification Factor	Percentage of Total Units
PART A			
A - homogenate	8.2	1.0	100
B - 100,000 x g supernatant	10.6	1.3	67
C - 0-25% $(NH_4)_2SO_4$ pellet	0	0	0
D - 25-50% " "	8.4	1.0	15
E - 50-75% " "	10.5	1.3	43
F - 75% $(NH_4)_2SO_4$ supernatant	0	0	0
PART B			
G - Pooled fractions D & E	9.9	1.2	57
H - 0-45% pellet from G	5.7	0.7	10
I - 45-55% " " "	16.2	2.0	8
J - 55-65% " " "	0	0	0
K - 65-100% " " "	30.2	3.7	14

PART A: Fractionations obtained directly with the 100,000 x g supernatant.

PART B: Fractionations obtained after pooling fractions D & E of Part A.

The incubation conditions were the same as given in table 20, except that 0.25M phosphate buffer, pH 7.2, was used instead.

Fraction D from table 24 which had been obtained from heating a homogenate, was subjected to ammonium sulfate fractionation after carrying out a delipidation with 1-butanol, but without success; very little activity remained, and no significant increase in specific activity was observed. An acid precipitation on a supernatant similar to that of fraction D, table 24, was equally unsuccessful.

(v) AFFINITY CHROMATOGRAPHY

Affinity chromatography was briefly examined as a potential method for the purification of lysophospholipase. A lyso PE-substituted Sepharose 4B was used in a batch procedure for this experiment, the details of which have been described under "Materials and Methods". The results were obtained by measuring the decrease in specific activity of lysophospholipase from a high speed supernatant after having exposed it to the substituted Sepharose for 15 minutes at pH 5.0. Since the Sepharose was substituted with a substrate rather than a competitive inhibitor (none of the latter were available at the time), this acid pH, at which lysophospholipase is inactive, was chosen to prevent the hydrolysis of the substituent. Approximately 25% of the enzyme became bound to the substituted Sepharose, when compared to a control of Sepharose 2B. However, a large amount of non specific binding of protein also occurred, so that the decrease in the specific activity of the supernatant was not significant.

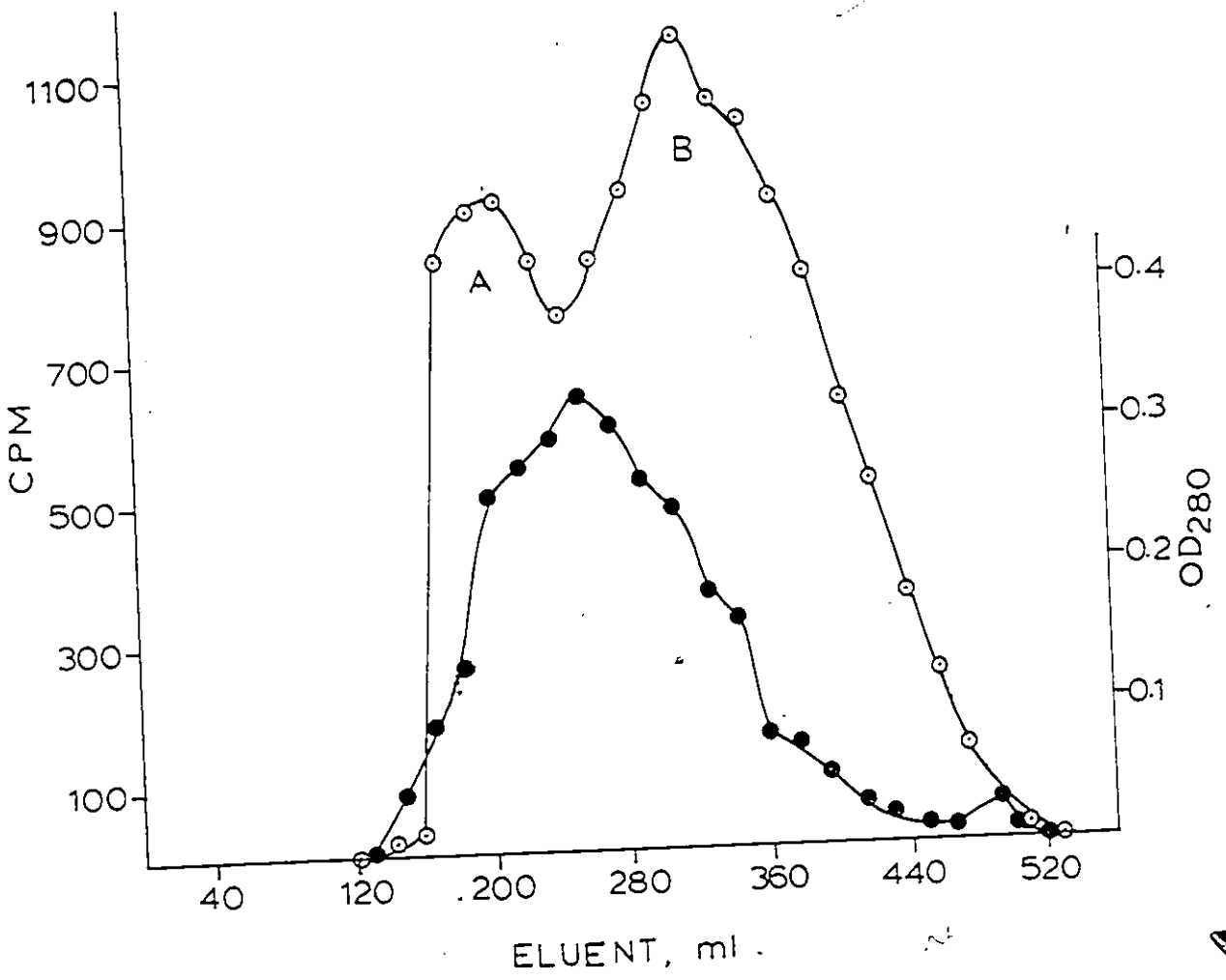
The method did not seem helpful for purifying lysophospholipase, and was not further explored.

(vi) GEL FILTRATION

Since none of the conventional methods worked as initial steps, gel filtration was tried on crude high speed supernatant fractions. 25-30 g E. coli B (ATCC 11303) were resuspended to 100 ml in distilled water, and subjected to freezing and thawing three times, followed by 10 minutes of sonication (4 periods of 2½ minutes each) over a salt-ice mixture. The 100,000 x g - 60 minute supernatant from this preparation was then concentrated from 90 to 18 ml in an Amicon cell with a PM-10 membrane. To 15 ml of this concentrate were added 5 ml of 0.2M phosphate buffer, for a final concentration of 0.05M phosphate, pH 7.15. 15 ml of this preparation were added onto a 2.5 cm chromatography column containing 83 cm of Sephadex G-200 fine, for a total volume of 407.5 cm^3. The column was eluted at a flow rate of 8.8 ml/hr., controlled by a Perpex peristaltic pump, with a 0.05M phosphate buffer at pH 7.15; fractions were collected every hour. The void volume (Vo) of the column was determined at 167 ml with blue dextran. The empty circles of figure 27 represents the elution profile of the lysophospholipase activity obtained with this column. Peak A (cf figure 27) was eluted with the void volume, thus indicating a molecular weight of 800,000 or greater, the excluded molecular weight

FIGURE 27

Elution profile of lysophospholipase on Sephadex G-200. The enzyme preparation added to the column was a 100,000 x g - 60 minute supernatant obtained from an E. coli B homogenate. The assays were performed with 0.25 µmoles of 1-[1-^{14}C-palmitoyl]-lyso PC (sp. act. = 8 x 10^4 dpm/µm) to which was added 1 ml of 0.05M phosphate buffer, pH 7.2, and 1 ml from each fraction. The incubations were shaken at 37°C during 30 min. The protein determinations were according to the method of Lowry (202) and the OD was read at 280 nm. ⊙——⊙ , lysophospholipase activity profile; ●——● , protein concentration profile.


1100
900
700
500
300
100
CPM
A
B
0.4
0.3
0.2
0.1
OD280
40
120
200
280
360
440
520
ELUENT, ml

for Sephadex G-200. Since a second peak, peak B (cf figure 27), with a somewhat greater activity was also present, it was reasoned that peak A could have consisted of a lipoprotein complex of sorts, perhaps containing the same enzyme as found in peak B. In fact, the eluate of peak A was somewhat turbid and contained much more lipid than that from peak B, as judged by thin layer chromatography of the lipid material extracted by the method of Bligh and Dyer (203).

The specific activity of peak B was only 1.5 times that found for the homogenate. In addition, although the total units recovered from this chromatography were not calculated, it is obvious from the distribution of the recovered activity shown as peaks A and B (cf figure 27) that each accounts for a considerable portion of the total activity.

There exists also the possibility that peaks A and B represent two quite different lysophospholipases. This possibility was not eliminated, but some aspects of this problem were examined. Possibly peak A represented small fractions of membrane which might contain phospholipase A1 and the lysophospholipase activity that is associated with this enzyme. Accordingly, eluate was tested from each peak for coliform phospholipase A1 with two assay procedures; the first involved the method of Proulx and Fung (56) with the substrate 1-[1-^{14}C-palmitoyl]-lecithin (specific activity = 7.7×10^5 dpm/μm), and the second, that of Scandella and

Kornberg (176) with 1-[1-^{14}C-palmitoyl]-phosphatidyl ethanolamine (specific activity = 6.6 x 10^5 dpm/μm). No hydrolysis was observed in either case. The lysophospholipase activity of phospholipase A1 was therefore not implicated in either of these peaks. Attempts were made to convert the cytosol lysophospholipase into a single form. This was carried out by lyophilizing a 100,000 x g - 90 minute supernatant obtained from 27 g of E. coli B (ATCC 11303) and extracting the material twice with 1-butanol which had been precooled to -20°C, to remove most of the lipid. The residual butanol was washed off with acetone at -20°C, and in turn, any residual acetone was removed under reduced pressure. The dry protein was resuspended in 0.05M phosphate buffer, pH 7.15, and an aliquot from this preparation was subjected to chromatography on Sephadex G-200 in a way similar to the one just described, with the exception that the column was run upside down in the hope of improving the resolution. The column dimensions were 2.5 cm in width, with 81.5 cm of Sephadex G-200 fine, for a total volume of 400 cm^3. The void volume, Vo, was determined at 173 ml with blue dextran. The flow rate this time was 9.9 ml/hr., and fractions were again collected every hour.

This time less protein had been applied to the column, again in an effort to increase the resolution. The two peaks obtained did separate better. The elution profile

for this chromatography is shown in figure 28. It is quite noticeable that peak A was considerably diminished whereas peak B remained. It is unknown whether peak A was converted to peak B since results in table 26 indicated that butanol treatment caused a loss of nearly 50% of the activity from the 100,000 x g supernatant, with a concomitant decrease in the specific activity. On the other hand, the fractions collected under peak B showed a specific activity nearly twice that of the homogenate or the butanol-treated preparation, but with only 28% of the total units remaining.

Because of the better separation obtained with the butanol extracted enzyme, it was possible to make a rough estimate of the molecular weight of peak B (cf figure 28) by calculating the Kav with the equation $Kav = \frac{Ve-Vo}{Vt-Vo}$, where Ve (306.9 ml) is the elution volume of Peak B, Vt (400.1 ml) the total column volume, and Vo (173.0 ml) is the void volume. The Kav for peak B (cf figure 28) was calculated at 0.59, which, according to a graph of the relationship between elution behaviour and molecular weight for Sephadex G-200 (229), corresponds to a molecular weight of 35,000. It seems therefore that the cytosolic lysophospholipase is a relatively small molecule, although this figure is only very approximate.

No further attempts to purify the cytosol lysophospholipase were made.

FIGURE 28

Elution profile of lysophospholipase on Sephadex G-200, after delipidation with 1-butanol. The enzyme preparation added to the column was a 100,000 x g - 60 minute supernatant from an E. coli B homogenate which was lyophilized and delipidated with 1-butanol prior to running (see text for details). The assays and protein determinations were performed as described under figure 27. ⊙——⊙ , lysophospholipase elution profile; ●——● , protein concentration profile.

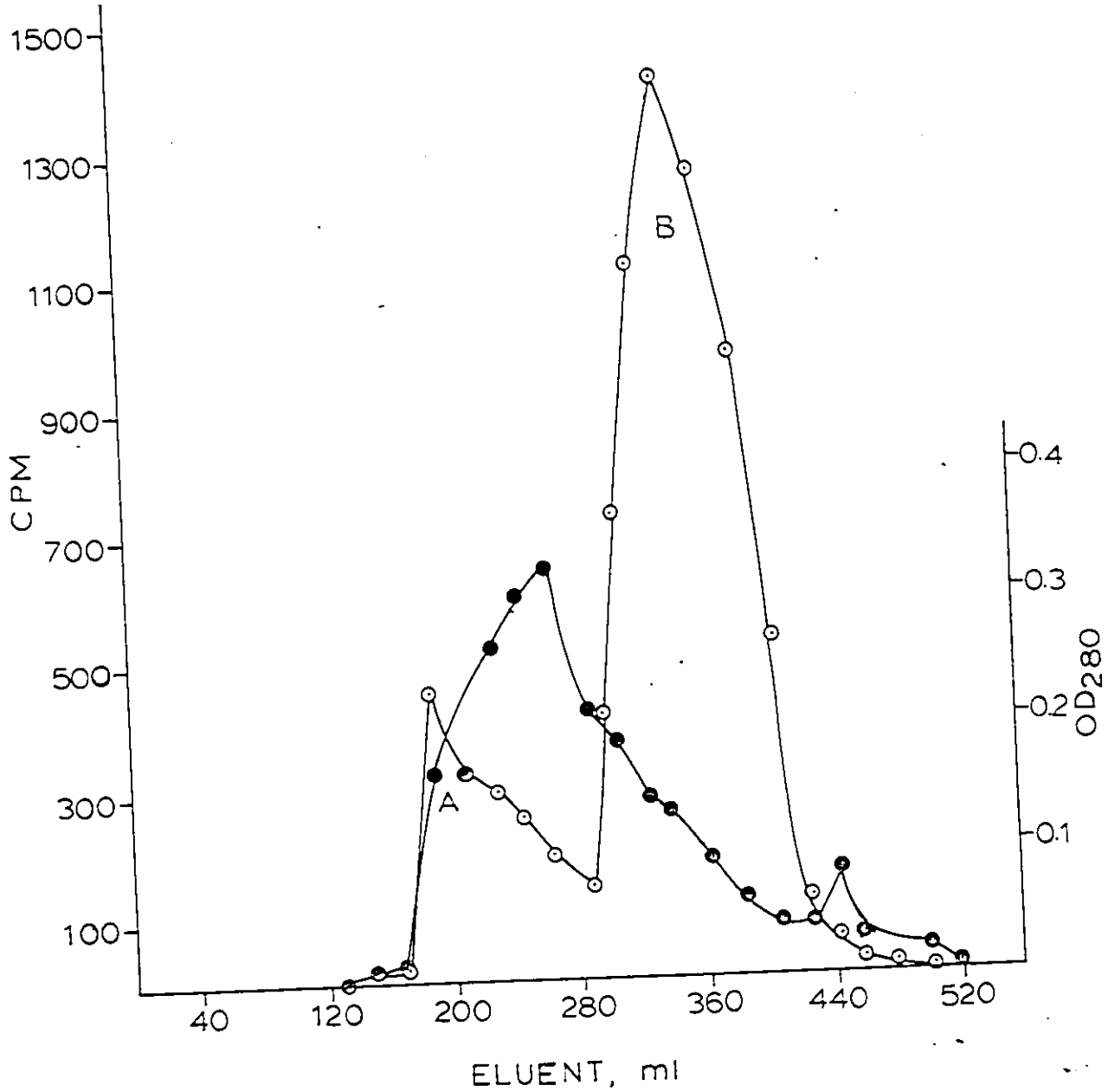

1500
1300
1100
900
700
500
300
100
CPM
0.4
0.3
0.2
0.1
OD280
A
B
40
120
200
280
360
440
520
ELUENT, ml

TABLE 26

Effect of 1-Butanol Delipidation Followed by Sephadex G-200 Chromatography on Lysophospholipase

	Specific Activity (units/g protein)	Purification Factor	Percentage of Total Units
homogenate	7.1	1	100
100,000 x g - 90 min. supernatant	12.0	1.7	64
butanol-treated supernatant	6.9	1	37
peak B, figure 28	13.6	1.9	28

The assay conditions were the same given under table 20, except that 0.025M phosphate buffer, pH 7.2 was used instead.

GENERAL DISCUSSION

A. LIPASE

1. COPURIFICATION OF LIPASE AND PHOSPHOLIPASE A1

When this study began, E. coli had already been shown to possess phospholipase A1 as a predominant lipolytic enzyme. Since in many species this enzyme is also associated with triglyceride hydrolysis, a search for lipase activity in cell-free extracts of E. coli seemed an appropriate research course to follow. The initial studies described in this thesis, did unequivocally demonstrate the presence of lipase activity in several E. coli strains as well as the general properties of the crude enzyme in one of the strains. This lipase displayed activity on tri-, di-, and monoglycerides when proper conditions for assay were applied. A requirement was found for detergents, bile salts being most suitable, and calcium ions. An alkaline pH was also needed but no definite optimum was revealed. The enzyme seemed to have a preference for the outer ester positions of glycerides, although the 2 position was also attacked more slowly. In most respects the E. coli enzyme resembled the pancreatic lipase, now so well characterized.

At this point the purification of coliform lipase was envisaged with the aim of establishing its properties more clearly and seeing if a copurification of phospholipase A1 would result. Just as this study was getting underway,

Scandella and Kornberg (176) published a method for the purification of E. coli phospholipase A1. Their conclusion regarding substrate specificity of this enzyme was that it was devoid of lipase activity. On the basis of this conclusion one had to accept that coliform lipase was indeed a distinct enzyme. Yet the conditions employed to demonstrate lipase activity in the purified phospholipase A1 preparation (176) were very limited; triolein was the only substrate tried and Triton X-100 was the only solubilizing agent used. Results presented in this thesis did clearly indicate that crude lipase was active against triglycerides only if proper detergents were employed, such as taurocholate.

The research aim at this point was revised slightly. The intention was now to repeat the purification of Scandella and Kornberg and check whether the enzyme isolated possessed both phospholipase A1 and lipase activities when proper assay conditions were used for each. It was reasoned that if indeed distinct enzymes were involved, separation of the activities would unavoidably result at some stage of the purification. However, since both lipase and phospholipase A1 were membrane bound and detergent-resistant, at least some of the initial steps used by Scandella and Kornberg might also serve for purification of lipase. Many difficulties were met in attempting to repeat the procedure of Scandella and Kornberg. However, the results of this thesis indicate

clearly that insofar as their method was reproducible, a copurification of phospholipase A1 and lipase occurred.

Step one of their procedure called for Manton-Gaulin homogenization which we could not perform in our laboratory. The result of this process was to reduce the optical density of the suspension by 95%. Attempts to replace this type of homogenization by combining ultra-sonication and enzymatic hydrolysis resulted in substantial decreases in optical density of the medium (85). However, further steps in the isolation procedure could not blend with this modification and no substantial purification occurred. Cells that had been passed through a Manton-Gaulin homogenizer were subsequently purchased from Grain Processing Corporation, Iowa, and were received as a frozen paste. Whether the freezing was a cause or not, the Scandella-Kornberg procedure, unmodified, could not be successfully applied to these homogenates. It is not clear whether frozen homogenate was used in the original procedure (176).

One aspect that appears to be very critical in the purification is the degree of envelope disruption brought about, not only by mechanical means, but by chemical agents such as EDTA and SDS (cf Step III of Purification Procedure 2, Scheme IV). In the original procedure (176) 1 mM EDTA and 1% SDS were used whereas increasing these concentrations to 26 mM and 1.2% respectively, greatly improved the purification

in our case, very likely because of better dissolution of the envelope. The implication here is that much of the envelope protein is complexed together with non-proteinacious material via cation bridging, and hydrophobic disruptive agents are insufficient per se to dissociate the constituents. Once metal chelation occurs, the detergents together with butanol can extract out the enzyme from the other envelope material.

After obtaining an acetone paste (Step V, Scheme IV) according to the original method (176) and washing this precipitate with water, much of the soluble protein was eliminated, and the remaining insoluble protein containing the phospholipase A1 - lipase activities became purified some two fold further. This step had not been described previously. Up to this point the procedure of Scandella and Kornberg was found to be reproducible but only with some significant modifications. These investigators were able to increase their purification factor beyond this point some 17-fold by first dissolving the acetone precipitate in an EDTA - SDS - butanol-containing buffer, and removing much of the unwanted protein by precipitation with 0.002M $MgCl_2$ and 0.09M sodium acetate. The resulting supernatant was fractionated into three parts by gradual addition of cold acetone. The further purified material was found in the second acetone fraction. We were totally unsuccessful in attempting to repeat this part of the procedure. A loss of

up to 40% of the activity resulted with the $MgCl_2$ - acetate precipitation, and the activity remaining in the supernatant was precipitated in the first acetone fraction with most of the protein, and little or no further purification.

Disc gel electrophoresis was carried out on the material obtained after washing the acetone paste (Step V, Scheme IV). The lipase and phospholipase A1 activities were both present in a single protein band (Rf value, 0.75) which indicated very strongly that both these activities were associated with the same enzyme. However, definite proof in this respect is lacking because this active material, when extracted from the gel and rerun on urea gels, produced one major and three minor protein bands. Thus the material isolated from the SDS gel was not pure. Nevertheless our protein separation patterns on both the SDS gels and the urea gels were very similar to those obtained by Scandella and Kornberg for which they claimed purification of phospholipase A1 to near homogeneity.

It is unfortunate that the common protein estimation methods were inadequate for the material extracted from the gels. It appeared that contaminants extracted from the gels interfered with proper colour development in the common spectrometric methods available. Scandella and Kornberg used a densitometric method for estimating protein in the gel based on the intensity of staining of the proteins by Coomassie

blue. We adopted this method but found it to be quite crude. When protein was estimated in this way the specific activity of the final fraction obtained after SDS gel electrophoresis showed a 5 fold loss rather than a gain. Such a result may have been due to incomplete extraction of the enzyme from the gel (whereas the protein was estimated directly on the gel), or to inactivation during electrophoresis.

The lipase activity in the material eluted from SDS gels was assayed with methyl oleate because the enzyme was found to be some 10-15 times more active against this substrate than against triolein. In fact, however, no lipase activity in the eluted material was detected with triolein, likely because there was a loss of enzyme activity during electrophoresis. The results do indicate semi-quantitatively that phospholipase A is the predominant activity associated with our purest fraction obtained although considerable lipase activity is also seen when simple esters such as methyl oleate are used. In the fraction representing a 200 fold purification of phospholipase A, there was lipase activity against methyloleate only some 3-4 fold less, and against triolein, some 50 times less. On the basis of the evidence presented in this thesis, and since the work of Scandella and Kornberg also clearly showed that their purified material was more active as a lysophospholipase than a phospholipase A1, one can propose that there exists in

E. coli an enzyme with general lipolytic activity attacking acyl esters from phosphoglycerides and neutral lipids. Further work by other workers in this laboratory with material prepared by Procedure 2 (cf Scheme IV) and carried through the electrophoresis step further showed that phospholipase A1 (with 1-[1-^{14}C-palmitoyl]-PE as substrate), phospholipase A2 (with 2-[-^{14}C-oleoyl]-PE as substrate), lysophospholipase (with 1-[-^{14}C-palmitoyl] lysolecithin as substrate), and lipase (with methyl-1-^{14}C-oleate, but not triolein, as substrate) were all associated with the same band. When ^{32}P-PE was used as substrate some labelled water-soluble products were found which indicated either phospholipase B activity or sequential hydrolysis of both acyl ester positions of PE. Since ^{32}P-lyso PE appeared as a major product in this hydrolysis the latter conclusion seems most appropriate.

Since the recoveries of phospholipase and lipase activities in the prepared material were low it is possible that enzymes with more specific lipolytic activities were elminated. In the case of lipase this may well be, especially since discarded non-active fractions were assayed with triolein only.

Confirmation of the presence of a lipase activity in E. coli, hydrolyzing mono-, di- and triglycerides, which is related to detergent-resistant phospholipase A1 has been

reported by Doi and Nojima (231); they observed a total absence of triglyceride-hydrolyzing activity in an E. coli mutant deficient in detergent-resistant phospholipase A1, and a reduced activity against mono- and diglycerides. This also suggested that more than one enzyme in E. coli can attack the latter glycerides. Genetic evidence also exists favouring the occurrence of both phospholipase A1 and A2 together since both activities are absent in mutants lacking detergent-resistant phospholipase activity (177). Bernard et al. (175) found a membrane bound phospholipase A2 activity in E. coli 0118 which seemed similar to phospholipase A1.

2. SUBSTRATE REQUIREMENTS FOR E. COLI LIPASE ACTIVITY

Perhaps the most interesting feature of coliform lipase is its activity against a wide range of neutral lipid substrates which are attacked under various incubation conditions. Under assay conditions for phospholipase A1 in homogenates, established by Fung and Proulx (53,56), with 10 mM SDS and 10 mM Ca++ at pH 8.4, tripalmitin was slowly hydrolyzed, but not triolein or tricaprylin (cf table 3, section I). But after establishing optimal conditions for lipase with 2.5% taurocholate and 0.25M Ca++, triolein and tricaprylin were attacked at similar rates, but no activity could be observed against tripalmitin. On the other hand, diglycerides and monoglycerides were hydrolyzed under both sets of conditions.

Once the lipase had been purified up to the washed acetone paste, activity against tricaprylin was no longer present, and monoglyceride hydrolysis was slower than di- or tryglyceride breakdown. The latter result suggested that another lipase attacking short chain triglycerides - possibly a monoglyceride lipase, may have been eliminated from this preparation.

Upon testing various substrates with the partially purified coliform lipase we also found activity against methyl oleate in the presence of either taurocholate or Triton X-100, whereas triolein was not hydrolyzed in the presence of the last detergent. Furthermore, methyl oleate was hydrolyzed some 3 times more rapidly with taurocholate as detergent. These results are difficult to explain and point the inherent difficulties in interpreting kinetic data of lipid (hydrophobic) systems.

The literature reveals a number of partially purified phospholipase A1 preparations which also hydrolyze triglycerides at a slower rate than phospholipids (12,14,16), and not all under the same optimal conditions. A possibly more dramatic example of such a situation occurred when Van den Bosch et al. (21) purified a pancreatic phospholipase A, and a non-specific esterase (22,23) by two different methods, only to later realize that both activities were the result of the same enzyme. Changes in the relative

rates of hydrolysis of PG and PE have also been observed with phospholipase A1 from B. megaterium spores when a neutral or ionic detergent was used (230); PG was attacked 400 times more rapidly than PE in the presence of Triton X-100, but this difference fell to twofold in the presence of sodium taurocholate. It would appear in this case that a net negative charge at the surface of the micelles was one factor influencing the rate of phospholipase A activity. A similar situation seems to arise in the case of E. coli activity since hydrolysis of neutral lipids is favoured by conferring negative charge to the micelles with anionic detergent addition.

It may be appropriate to note also that coliform phospholipids are all acidic and equally well attacked by phospholipase A1. Phosphatidyl choline, however, is also a good substrate for coliform phospholipase A (53) although there is at the moment no precise data indicating whether or not hydrolysis of this substrate is favoured by anionic detergents or whether it is as extensive as that of other negatively charged substrates.

E. coli lipase is interesting with respect to substrate positional specificity. Our results in figures 12 and 13, and the hydrolysis found against 2-[1-C^{14}palmitoyl]-1,3-dipalmityl glycerol diether indicate a slow but direct hydrolysis of position 2 of triglycerides. This is interesting

in comparison to the positional specificity of pancreatic lipase which hydrolyzes exclusively the peripheral esters of triglycerides (102,111 and figure 11). Brockerhoff (116) found the rate of hydrolysis of esters by pancreatic lipase to be influenced by inductive effects, and postulated steric hindrance as preventing an attack at position 2 of triglycerides. Clearly, if this hypothesis is correct, E. coli lipase must have a different active site structure than pancreatic lipase because it is able to hydrolyze position 2. Nevertheless, Brockerhoff's postulate that steric hindrance plays a role may still be valid since activity against this position is slower than at position 1 even though it is flanked by esters. According to Brockerhoff's theory, the hydrolytic reaction is accelerated when the carbon being attacked is made more electrophilic. This occurs most strongly at the central ester of triglycerides because of the electrophilic properties of the neighbouring ester groups. Steric hindrance may therefore exert an influence on E. coli lipase by slowing down the reaction rate at position 2 of triglycerides.

Scandella and Kornberg reported phospholipase A1 to be stereospecific since it hydrolyzed only 2% of D- isomer of PC. The authors unfortunately do not state to what extent hydrolysis of the L- isomer occurred under their conditions. At any rate theirs is the only study to date indicating stereospecificity for a phospholipase A1. The rate of hydrolysis

at position 3 of 3-[1-C^{14}-palmitoyl]-1-stearoyl-2-oleoyl glycerol was approximately 1/3 that found for the release of 1-C^{14}-oleic acid from tri-[1-C^{14}-oleoyl]-glycerol. Also studies on the rate of hydrolysis of 3-[1-C^{14}-palmitoyl]-1,2-diacyl glycerol indicate that labelled DG does not accumulate as final product, and that the initial rates of production of labelled DG and FA are equal. Thus hydrolysis of the exterior positions must have been equivalent. These results are further supported by the time study with 2-[1-C^{14}-palmitoyl]-1,3-dipalmitoyl glycerol which indicated no accumulation of diglyeride as final product. It is evident from this that the purified lipase has no stereospecificity. This could be taken as an indication that triglyceride lipase is indeed different from phospholipase A1 but our own evidence with a partially purified 200 fold purified enzyme does not support this conclusion. Our results with the lipase activity invite a reinvestigation of the stereospecificity of phospholipase A1 of E. coli.

Slotboom et al. (115) studied a similar situation with lipases from pork pancreas and from the mold Rhizopus arrhizus. They found both enzymes to completely hydrolyze racemic mixtures of phospholipids, which confirmed their lack of stereospecificity found against triglycerides (113,165).

3. THE ROLE OF LIPOLYTIC ENZYMES IN E. COLI

White et al. (182) and Bell et al. (183) have found the enzymes of the inner cytoplasmic membrane involved in the biosyntheses of PA, CDP-diglyceride, PE, PG and di-PG, to have specific activities at least ten times those observed in the cell wall. Thus synthesis of lipid occurs mainly in the inner membrane, but the phospholipid composition and content of inner and outer membranes seems similar (232), which implies a highly dynamic translocation of phospholipids between both membranes of the cell (233). On the other hand, the catabolic enzyme phospholipase A1 shows the highest specific activity in the outer membrane (183). Lipase activity, however, has not yet been identified with a particular membrane although it is an envelope enzyme. In a study of the enzymes involved in the catabolism of phospholipids in three subcellular fractions of E. coli, the cell wall, the inner membrane, and the cytosol, Albright et al. (178) found phospholipase A1, lysophospholipase A1 and phospholipase A2 activities associated with the cell wall. The inner membrane possessed a strong lysophospholipase A2 activity in addition to a low lysophospholipase A1 activity. An activity similar to the latter was also present in the cytosol, but with different heat stability and optimal conditions. Upon examination of the substrate specificities reported by others for these enzymes, these authors surmised that the phospholipid

degradation must be complex.

Phospholipases do not seem to be involved in release of phage λ since mutants devoid of detergent-resistant and detergent-sensitive phospholipase A1 release the phage particles with the same burst size as wild-type host cells (234). However, the detergent-resistant activity seems to be involved in the release of fatty acids upon infection since the mutants did not release any fatty acids.

A study of phosphoglyceride turnover in cold-shocked and normally growing E. coli 015 cells (193) failed to clearly implicate phospholipase A1 because the turnover of acyl moieties was equal to that of phosphorus. Furthermore, the turnover rate of both acyl positions of phospholipids was found equal, no lysophospholipids were detected as intermediate products, and very little free fatty acids accumulated.

Because induced β-oxidation in E. coli could mask the release of fatty acids by phospholipase A1, Audet et al. (196) examined two mutant strains devoid of β-oxidation enzymes, B fad and K 19, for evidence of phospholipase activity. B fad was found to possess an active phospholipase A, but this was attributed to a defective cell envelope since liposaccharide, lipids and proteins, including phospholipase A1, were released into the culture medium during growth of this strain; K 19, on the other hand, displayed no apparent

phospholipase activity. Other studies as well (176,194) have indicated that cells with normal envelopes growing under usual culture conditions do not use phospholipase A for their lipid turnover.

The evidence discussed to date does not clarify the role of phospholipase A or lipase in E. coli. At best it shows a lack of involvement of phospholipase A in normal phospholipid turnover in E. coli. Considering the work of Doi and Nojima with detergent-resistant phospholipase A-deficient mutants mentioned previously (231), as well as the data presented in this thesis, all of which tend to show a physical association of lipase and phospholipase A1 activities, one is compelled to propose the existence of a non-specific lipolytic enzyme acting at the exterior of the cell. Such an enzyme, stimulated by bile salts present in the gut, the natural habitat of E. coli, is likely involved in procuring for the cell exogenous fatty acids and phospholipid breakdown products. These can be used for energy purposes or for resynthesis of phosphoglycerides compatible with the structural requirements of the envelope.

B. LYSOPHOSPHOLIPASE

Part of the results of this thesis have concerned the further characterization of a cytosol lysophospholipase

of E. coli extracts. Recently, Doi and Nojima (235) published their results on a 500-fold purification of the same enzyme and confirmed and extended the preliminary results observed in this study.

The cytosol lysophospholipase is an enzyme which remains in the post-100,000g supernatant. When this supernatant was added to a Sephadex G-200 column two peaks of activity (cf figure 27) appeared, the first being excluded from the Sephadex gel. This first peak activity (peak A) was possibly a lipoprotein complex of sorts resembling peak B in catalytic requirements. After delipidating a lyophilized high speed supernatant with cold 1-butanol a decrease in peak A activity relative to B occurred, but because this treatment caused a loss in total activity, an actual conversion of the activities from peak A to B could not be concluded. There is no evidence, therefore, to preclude the possible presence of two lysophospholipases in this high speed fraction.

Doi and Nojima also found lysophospholipase to be active over a wide pH range, from about pH 5.0 to at least 11, with an optimum at approximately pH 9.5 when 1-acyl PE was the substrate. Similar results were obtained by U. Rapp in our laboratory (unpublished results). The activity against lysolecithin, on the other hand, does not show a

sharp optimum as such, but is equally high over the whole range from pH 5.5 to 9.5.

Low concentrations of ionic detergents were found to inhibit lysophospholipase activity, whereas neutral ones at the same concentrations were without effect. However, a 6% concentration of Triton X-100 inhibited 92% of lysophospholipase activity. Doi and Nojima found a twofold stimulation with 23 mM sodium cholate, although sodium dodecyl sulfate at 2 mM was totally inhibitory with their purified enzyme. They also found Triton X-100 inhibiting activity gradually with increasing concentration.

Lysophospholipase purified by Doi and Nojima hydrolyzed both 1-acyl and 2-acyl-GPE, but did not hydrolyze phospholipids in the absence of detergent or in the presence of 50% methanol (incubation system for detergent-resistant phospholipase A) or 5% sodium taurocholate. We also did not observe activity against phospholipids in the presence of either Triton X-100 or sodium dodecyl sulfate at the concentrations used for phospholipase A1, assays.

A highly interesting characteristic of Doi and Nojima's purified enzyme was that it also hydrolyzed 1 acyl glycerol, but not di- and triglycerides. Again, one sees the involvement of a phospholipid catabolic enzyme in neutral lipid metabolism; the significance of this is hard to assess

at the moment. In the case of phospholipase A1 - lipase activities, the site of action involved seems to be at the exterior surface of the cell where exogenous lipid degradation would occur. The interior location of lyso-phospholipase - monoglyceride lipase implies a function in relation to endogenous lipid metabolism yet none of the lipid turnover studies with isotope labelled cells seems to support the involvement of any of these enzyme activities in the normal metabolism of E. coli.

REFERENCES

1. McMurray, W.C., and Magee, W.L., Ann.Rev.Biochem. 41 (1972) 129.

2. Lennarz, W.J., Ann.Rev.Biochem. 39 (1970) 359.

3. Hill, E.D., and Lands, W.E.M., in Lipid Metabolism, S.J. Wakil, ed., Academic Press, N.Y., p.185, (1970).

4. Lloveras, J., Douste-Blazy, L., and Valdiguié, L., Compt.Rend. 256 (1963) 1861.

5. Van den Bosch, H., and van Deenen, L.L.M., Biochim. Biophys.Acta, 106 (1965) 326.

6. Van den Bosch, H., and van Deenen, L.L.M., Biochim. Biophys.Acta, 84 (1964) 234..

7. Van den Bosch, H., Postema, N.M., de Haas, G.H., and van Deenen, L.L.M., Biochim.Biophys.Acta, 98 (1965) 657.

8. Vogel, W.C., Ryan, W.G., Koppel, J.L. and Alwin, J.H., J.Lipid Res. 6 (1965) 335.

9. Vogel, W.C., and Bierman, E.L. , J.Lipid Res. 8 (1967) 46.

10. Infante, R., Koumanov, K., and Polonovski, J., Biochim. Biophys.Acta, 164 (1968) 436.

11. Gatt, S., Barenholz, Y., and Roitman, A., Biochem. Biophys.Res.Comm. 24 (1969) 169.

12. Gatt, S., Biochim.Biophys.Acta, 159 (1968) 304.

13. Winkler, H., Smith, A.D., Dubois, F., and Van den Bosch, H., Biochem.J., 105 (1967) 38c.

14. Cooper, M.F.; and Webster, G.R., J.Neurochem. 17 (1971) 1543.

15. Fransome, R., Waite, M., and LaVia, M., Biochem. 10 (1971) 1942.

16. White, D.A., Pounder, O.J., and Hawthorne, J.N., Biochim.Biophys.Acta, 242 (1971) 99.

17. Newkirk, J.D., and Waite, M., Biochim.Biophys.Acta, 225 (1971) 224.

18. Weglicki, W.B., Waite, M., Sisson, P., and Sohet, S.B., Biochim.Biophys.Acta, 231 (1971) 512.

19. Waite, M., and Sisson, P., J.Biol.Chem., 248 (1973) 7985.

20. Waite, M., and Sisson, P., J.Biol.Chem., 248 (1973) 7201.

21. Van den Bosch, H., Aarsman, A.J., and van Deenen, L.L.M., Biochim.Biophys.Acta, 348 (1974) 197.

22. Van den Bosch, H., Aarsman, A.J., de Jong, J.G.N., and van Deenen, L.L.M., Biochim.Biophys.Acta, 296 (1973) 94.

23. de Jong, J.G.N., Van den Bosch, H., Aarsman, A.J., and van Deenen, L.L.M., Biochim.Biophys.Acta, 296 (1973) 105.

24. de Haas, G.H., Postema, N.M., Nieuwenhuizen, W., and van Deenen, L.L.M., Biochim.Biophys.Acta, 159 (1968) 103.

25. de Haas, G.H., Bonsen, P.P.M., Pietersen, W.A., and van Deenen, L.L.M., Biochim.Biophys.Acta, 239 (1971) 252.

26. Verger, R., Mieras, M.C.E., and de Haas, G.H., J.Biol. Chem. 248 (1973) 4023.

27. Jain, M.K., and Cordes, E.H., J.Membrane Biol. 14 (1973) 101.

28. Jain, M.K., and Cordes, E.H., J.Membrane Biol. 14 (1973) 119.

29. Dennis, E.A., J.Lipid Res., 14 (1973) 152.

30. Slotboom, A.J., de Haas, G.H., and van Deenen, L.L.M., Chem.Phys. Lipids 1 (1967) 192.

31. de Haas, G.H., Bonsen, P.P.M., and van Deenen, L.L.M., Biochim.Biophys.Acta, 116 (1966) 114.

32. van Deenen, L.L.M., and de Haas, G.H., Biochim.Biophys. Acta, 70 (1963) 538.

33. Bonsen, P.P.M., de Haas, G.H., Pietersen, W.A., and van Deenen, L.L.M., Biochim.Biophys.Acta, 270 (1972) 364.

34. de Haas, G.H., Slotboom, A.J., Bonsen, P.P.M., van Deenen, L.L.M., Maroux, S., Puigserver, A., and Desnuelle, P., Biochim.Biophys.Acta, 221 (1970) 31.

35. de Haas, G.H., Postema, N.M., Nieuwenhuizen, W., and van Deenen, L.L.M., Biochim.Biophys.Acta, 159 (1968) 118.

36. de Haas, G.H., Slotboom, A.J., Bonsen, P.P.M., Nieuwenhuizen, W., van Deenen, L.L.M., Maroux, S., Dlouha, D., and Desnuelle, P., Biochim.Biophys. Acta, 221 (1970) 54.

37. Belleville, J., and Clément, J., Bull.Soc.Chim.Biol., 50 (1968) 1419.

38. Arnesjo, B., Barrowman, J., and Borgstrom, B., Acta Chem. Scand., 21 (1967) 2897.

39. Wu, T.W., and Tinker, D.O., Biochem., 8 (1969) 1558.

40. Currie, B.T., Oakley, D.E., and Broomfield, C.A., Nature, 220 (1968) 371.

41. Wells, M.A., and Hanahan, D.J., Biochem., 8 (1969) 414.

42. Callewaert, G.L., Cottrell, R.C., Doonan, S., Vernon, C.A., and Banks, B.E.C., Eur.J.Biochem., 20 (1971) 459.

43. Kawanchi, S., Iwanaga, S., Samejima, Y., and Suzuki, T., Biochim.Biophys.Acta, 236 (1971) 142.

44. Shapiro, B., Biochem.J., 53 (1953) 663.

45. Leibovitz, Z., and Gatt, S., Biochim.Biophys.Acta, 164 (1968) 439.

46. Kawasaki, N., and Saito, K., Biochim.Biophys.Acta, 296 (1973) 426.

47. Van den Bosch, H., Aarsman, A.J., Slotboom, A.J., and van Deenen, L.L.M., Biochim.Biophys.Acta, 164 (1968) 215.

48. Dawson, R.M.C., Biochem.J., 64 (1969) 192.

49. Erbland, J.F., and Marinetti, G.V., Biochim.Biophys. Acta, 106 (1965) 128.

50. van Golde, L.M.G., McElhaney, R.N., and van Deenen, L.L.M., Biochim.Biophys.Acta, 231 (1971) 245.

51. Rao, R.H., and Subrahmanyam, D., J.Lipid Res. 10 (1969) 636.

52. Eisenberg, S., Stein, Y., and Stein, O., Biochim.Biophys. Acta, 164 (1968) 205.

53. Fung, C.K., and Proulx, P., Can.J.Biochem. 47 (1969) 371.

54. Kumar, S.S., Millay, R.H., and Bieber, L.L., Biochem., 9 (1970) 754.

55. Subbaiah, P.V., and Ganguly, J., Biochem.J., 118 (1970) 233.

56. Proulx, P., and Fung, C.K., Can.J.Biochem. 47 (1969) 1125.

57. Magee, W.L., Gallai-Hatchard, J.J., Sanders, H., and Thompson, R.H.S., Biochem.J., 83 (1962) 17.

58. Okuyama, H., and Nojima, S., Biochim.Biophys.Acta, 176 (1969) 120.

59. Aldridge, W.N., Biochem.J., 53 (1953) 110, and 57 (1954) 692.

60. Desnuelle, P., in The Enzymes, P.D. Boyer, ed., Academic Press, N.Y., 1972, vol. VII, p.575-616.

61. Sarda, L., and Desnuelle, P., Biochim.Biophys.Acta, 30 (1958) 513.

62. Entressangles, B., and Desnuelle, P., Biochim.Biophys. Acta, 159 (1968) 285.

63. Florkin, M., and Stotz, E.H., eds., Report of the Enzyme Commission of the International Union of Biochemistry, rev. ed., Elsevier, Amsterdam, 1964.

64. Benzonana, G., and Esposito, S., Biochim.Biophys.Acta, 231 (1971) 15.

65. Borgstrom, B., Acta Physiol.Scand., 25 (1952) 328.

66. Bergstrom, S., and Borgstrom, B., Acta Soc.Med.Upsalien, 58 (1953) 331.

67. Wills, E.D., Adv.Lipid Res., 3 (1965) 197.

68. Butcher, R.W., Ho, R.J., Meng, H.C., and Sutherland, E.W., J.Biol.Chem., 240 (1965) 4515.

69. Huttunen, J.K., Steinberg, D., and Mayer, S.E., Biochem. Biophys.Res.Comm., 41 (1970) 1350.

70. Huttunen, J.K., Steinberg, D., and Mayer, S.E., Proc.Natl. Acad.Sci., U.S., 67 (1970) 290.

71. Sarda, L., Marchis-Mouren, G., Constantin, M.J., and Desnuelle, P., Biochim.Biophys.Acta, 23 (1957) 264.

72. Marchis-Mouren, G., Sarda, L., and Desnuelle, P., Biochem. Biophys.Acta, 41 (1960) 358.

73. Basky, B., Klein, E., and Lever, W.F., Arch.Biochem. Biophys., 102 (1963) 201.

74. Sarda, L., Maylié, M.F., Roger, J., and Desnuelle, P., Biochim.Biophys.Acta, 89 (1964) 183.

75. Verger, R., de Haas, G.H., Sarda, L., and Desnuelle, P., Biochim.Biophys.Acta, 188 (1969) 272.

76. Kimura, H., Kitamura, T., and Tsuji, M., Biochim. Biophys.Acta, 270 (1972) 307.

77. Vandermeers, A., and Christope, J., Biochim.Biophys. Acta, 154 (1968) 110.

78. Morgan, R.G.H., Barrowman, J., and Borgstrom, B., Biochim.Biophys.Acta, 175 (1969) 65.

79. Maylié, M.F., Charles, M., Gache, C., and Desnuelle, P., Biochim.Biophys.Acta, 229 (1971) 286.

80. Erlanson, C., and Borgstrom, B., Biochim.Biophys.Acta, 271 (1972) 400.

81. Borgstrom, B., and Erlanson, C., Biochim.Biophys.Acta, 242 (1971) 509.

82. Borgstrom B., and Erlanson, C., Eur.J.Biochem., 37 (1973) 60.

83. Morgan, R.G.H., and Hoffman, N.E., Biochim.Biophys.Acta, 248 (1971) 143.

84. Desnuelle, P., in Dietary Lipids and Postnatal Development, C. Galli, G. Jacimi, and A. Pecicle, ed., Raven Press, N.Y., 1973, p.73-76.

85. Julien, R., Canioni, P., Rathelot, J., Sarda, L., and Plummer, Jr., T.H., Biochim.Biophys.Acta, 280 (1972) 215.

86. Garner, C.W., Jr., and Smith, L.C., J.Biol.Chem., 247 (1972) 561.

87. Goldstein, I.J., So, L.L., Yang, Y., and Callies, Q.C., J.Immunol., 103 (1969) 695.

88. Plummer, T.H., Jr., and Sarda, L., J.Biol.Chem., 248 (1973) 7869.

89. Verger, R., Sarda, L., and Desnuelle, P., Biochim. Biophys.Acta, 207 (1970) 377.

90. Verger, R., Sarda, L., and Desnuelle, P., Biochim. Biophys.Acta, 242 (1971) 580.

91. Maylié, M.F., Charles, M., and Desnuelle, P., Biochim. Biophys.Acta, 276 (1972) 162.

92. Sémériva, M., and Desnuelle, P., Biochem., 10 (1971) 2143.

93. Dufour, C., Sémériva, M., and Desnuelle, P., Biochim. Biophys.Acta, 327 (1973) 101.

94. Hoare, D.G., and Koshland, Jr., D.E., J.Biol.Chem., 242 (1967) 2447.

95. Borgstrom, B., Biochim.Biophys.Acta, 13 (1954) 149.

96. Desnuelle, P., Naudet, M., and Constantin, M.J., Biochim.Biophys.Acta, 5 (1950) 561.

97. Wills, E.D., Biochim.Biophys.Acta, 40 (1960) 481.

98. Benzonana, G., Biochem.Biophys.Acta, 151 (1968) 137.

99. Benzonana, G., and Desnuelle, P., Biochim.Biophys.Acta, 164 (1968) 47.

100. Wills, E.D., Biochem.J., 57 (1954) 109.

101. Entressangles, B., Pasero, L., Savary, P., Sarda, L., and Desnuelle, P., Bull.Soc.Chim.Biol., 43 (1961) 581.

102. Desnuelle, P., and Savary, P., J.Lipid Res., 4 (1963) 369.

103. Mattson, F.H., and Volpenheim, R.A., J.Lipid Res., 10 (1969) 271.

104. Mattson, F.H., and Beck, L.W., J.Biol.Chem., 219 (1956) 735.

105. Savary, P., and Desnuelle, P., Biochim.Biophys.Acta, 21 (1956) 349.

106. Bergstrom, S., Borgstrom B., Tryding, N., and Westoo, G., Biochem.J., 58 (1954) 604.

107. Tryding, N., Acta Physiol.Scand., 40 (1957) 232.

108. Bottino, N.R., Vandenburg, G.A., and Reiser, R., Lipids, 2 (1967) 489.

109. Kleiman, R., Earle, F.R., and Wolf, I.A., J.Am.Oil Chem. Soc., 42 (1965) 147 A.

110. Brockerhoff, H., Biochim.Biophys.Acta, 212 (1970) 92.

111. Tatrie, N.H., Bailey, R.A., and Kates, M., Arch.Biochem. Biophys., 78 (1958) 319.

112. Desnuelle, P., Adv.Enzymol., 23 (1961) 129.

113. Entressangles, B., Sari, H., and Desnuelle, P., Biochim. Biophys.Acta, 125 (1966) 597.

114. de Haas, G.H., Sarda, L., and Roger, J., Biochim. Biophys.Acta, 106 (1965) 638.

115. Slotboom, A.J., de Haas, G.H., Bonsen, P.P.M., Burbach-Westerhuis, G.J., and van Deenen, L.L.M., Chem. Phys.Lipids, 4 (1970) 15.

116. Brockerhoff, H., Biochem.Biophys.Acta, 159 (1968) 296.

117. Brockerhoff, H., Arch.Biochem.Biophys., 134 (1969) 366.

118. Bangham, A.D., and Dawson, R.M.C., Biochem.J., 75 (1960) 133.

119. Esposito, S., Sémériva, M., and Desnuelle, P., Biochim. Biophys.Acta, 302 (1973) 293.

120. Brockerhoff, H., J.Biol.Chem., 246 (1971) 5828.

121. Fraser, G.P., and Nicol, A.D., Clin.Chim.Acta, 13 (1966) 552.

122. Benzonana, G., Biochim.Biophys.Acta, 176 (1969) 836.

123. Hubscher, G., in Lipid Metabolism, S.J. Wakil, ed., Academic Press, N.Y., 1970, p.279-370.

124. Tidwell, H.C., and Johnston, J.M., Arch.Biochem. Biophys., 89 (1960) 79.

125. DiNella, R.R., Meng, H.C., and Park, C.R., J.Biol. Chem., 235 (1960) 3076.

126. Senior, J.R., and Isselbacher, K.J., Biochem.Biophys. Res.Comm., 6 (1961) 274.

127. Belfrage, P., Biochim.Biophys.Acta, 98 (1965) 660.

128. Strand, O., Vaughan, M., and Steinberg, D., J.Lipid Res., 5 (1964) 554.

129. Gorin, E., and Shafrir, E., Biochim.Biophys.Acta, 84 (1964) 24.

130. Pope, J.L., McPherson, J.C., and Tidwell, H.C., J.Biol. Chem., 241 (1966) 2306.

131. Kupiecki, F.P., J.Lipid Res., 7 (1966) 230.

132. Wallach, D.P., J.Lipid Res., 9 (1968) 200.

133. Katocs, A.S., Calvert, D.N., and Lech, J.J., Biochim. Biophys.Acta, 229 (1971) 608.

134. Fielding, C.J., Biochim.Biophys.Acta, 178 (1969) 499.

135. Fielding, C.J., Biochim.Biophys.Acta, 206 (1970) 109.

136. Morley, N., and Kuksis, A., J.Biol.Chem., 247 (1972) 6389.

137. Ory, R.L., St. Angelo, A.J., and Altschul, A.M., J.Lipid Res., 3 (1962) 99.

138. Ory, R.L., Lipids, 4 (1969) 177.

139. Ory, R.L. , Yatsu, L.Y., and Kircher, H.W., Arch. Biochem.Biophys., 123 (1968) 255.

140. Ory, R.L., and Altschul, A.M., Biochim.Biophys.Res. Comm., 7 (1962) 370.

141. Ory, R.L., and Altschul, A.M., Biochem.Biophys.Res. Comm., 7 (1962) 375.

142. Ory, R.L., Barker, R.H., and Boudreaux, G.J., Biochem., 3 (1964) 2013.

143. Ory, R.L., Kircher, H.W., and Altschul, A.M., Biochim. Biophys.Acta, 147 (1967) 200.

144. Savary, P., Flanzy, J., and Desnuelle, P., Bull.Soc. Chim.Biol., 40 (1958) 637.

145. Ory, R.L., Kiser, J., and Pradel, P.A., Lipids, 4 (1969) 261.

146. Noma, A., and Borgstrom, B., Biochim.Biophys.Acta, 227 (1971) 106.

147. Sémériva, M., Benzonana, G., and Desnuelle, P., Biochim. Biophys.Acta, 191 (1969) 598.

148. Yoshida, F., Motai, H., and Ichishima, E., Biochim. Biophys.Acta, 154 (1968) 586.

149. Tomizuka, N., Ota, Y., and Yamada, K., Agr.Biol.Chem., 30 (1966) 576.

150. Tomizuka, N., Ota, Y., and Yamada, K., Agr.Biol.Chem., 30 (1966) 1090.

151. Tsujisaka, Y., Kagaku to Seibutsu, 8 (1970) 315.

152. Chiba, H., Hisatake, M., Hirose, M., and Sigomoto, E., Biochim.Biophys.Acta, 327 (1973) 380.

153. Liu, W.-H., Beppu, T., and Arima, K., Agr.Biol.Chem., 37 (1973) 349.

154. Liu, W.-H., Beppu, T., and Arima, K., Agr.Biol.Chem., 37 (1973) 2493.

155. Oi, S., Sawada, A., and Satomura, Y., Agr.Biol.Chem., 31 (1967) 1357.

156. Alford, J.A., Pierce, D.A., and Suggs, F.G., J.Lipid Res., 5 (1964) 390.

157. Sarda, L., Marchis-Mouren, G., and Desnuelle, P., in Enzymes of Lipid Metabolism, P. Desnuelle, ed., Pergamon Press, N.Y., 1961, p.20-21.

158. Mates, A., Experientia, 28 (1972) 1464.

159. Liu, W.H., Beppu, T., and Arima, K., Agr.Biol.Chem., 37 (1973) 1349.

160. Liu, W.H., Beppu, T., and Arima, K., Agr.Biol.Chem., 37 (1973) 2487.

161. Jensen, R.G., in Progress in the Chemistry of Fats and Other Lipids, R.T. Holman, ed., Pergamon Press, N.Y., 1971, p.347.

162. Marks, T.A., Quin, J.G., Sampugna, J., and Jensen, R.G., Lipids, 3 (1968) 143.

163. Jensen, R.G., Gordon, D.T., Heinermann, H., and Holman, R.T., Lipids, 7 (1972) 738.

164. Jensen, R.G., Gordon, D.T., and Scholfield, C.R., Lipids, 8 (1973) 323.

165. Sémériva, M., Benzonana, G., and Desnuelle, P., Bull. Soc.Chim.Biol., 149 (1967) 71.

166. Fisher, W., Heinz, E., and Zeus, M., H-S.Z.Physiol. Chem., 354 (1973) 1115.

167. Lu, J.Y., and Liska, B.J., Appl.Microbiol., 18 (1969) 104.

168. Ogiso, T., and Sugiura, M., Chem.Pharm.Bull., 17 (1969) 1025.

169. Saiki, T., Takagi, Y., Suzuki, T., Narasaki, T., Tamura, G., and Arima, K., Agr.Biol.Chem., 33 (1969) 414.

170. Narasaiki, T., Saiki, T., Tamura, G., and Arima, K., Agr.Biol.Chem., 31 (1967) 993.

171. Bernard, M-C., Brisou, J., Denis, F., and Rosenberg, A-J., Biochimie, 54 (1972) 261.

172. Patriarca, P., Beckerdite, S., and Elsbach, P., Biochim.Biophys.Acta, 260 (1972) 593.

173. Proulx, P.R., and van Deenen, L.L.M., BBA, 125 (1966) 591.

174. Doi, O., Ohki, M., and Nojima, S., Biochim.Biophys. Acta, 260 (1972) 244.

175. Bernard, M-C., Brisou, J., Denis, F., and Rosenberg, A-J., Biochimie, 54 (1972) 297.

176. Scandella, C.J. and Kornberg, A., Biochemistry, 10 (1971) 4447.

177. Doi, O., and Nojima, S., J.Biochem., 74 (1973) 667.

178. Albright, F.R., White, D.A., and Lennarz, W.J., J.Biol. Chem., 248 (1973) 3968.

179. MacLennan, D.H., J.Biol.Chem., 245 (1970) 4508.

180. Goldberger, R., Smith, A.L., Tisdale, H., and Bornstein, R., J.Biol.Chem., 236 (1961) 2788.

181. Shiloah, J., Klibanski, C., de Vries, A., and Berger, A.J., Lipid Res., 14 (1973) 267.

182. White, D.A., Albright, F.R., Lennarz, W.J., and Schnaitman, C.A., Biochim.Biophys.Acta, 249 (1971) 636.

183. Bell, R.M., Mavis, R.D., Osborn, M.J., and Vagelos, P.R., Biochim.Biophys.Acta, 249 (1971) 628.

184. Dawson, R.M.C., Biochem.J., 68 (1958) 352.

185. Beare, J.L., and Kates, M., Can.J.Biochem., 45 (1967) 61.

186. Proulx, P.R., and van Deenen, L.L.M., Biochim.Biophys. Acta, 144 (1967) 171.

187. Van den Bosch, H., and Vagelos, P.R., Biochim.Biophys. Acta, 218 (1970) 233.

188. Cronan, J.E., and Vagelos, P.R., Biochim.Biophys.Acta, 265 (1972) 25.

189. Chang, Y-Y., and Kennedy, P.R., J.Biol.Chem., 242 (1957) 516.

190. Nantel, G., and Proulx, P., Biochim.Biophys.Acta, 316 (1973) 156.

191. Ono, Y., and White, O.C., J.Bacteriol., 103 (1970) 111.

192. Cole, R., Benns, G., and Proulx, P., Biochim.Biophys. Acta, 337 (1974) 325.

193. Bright-Gaertner, E., and Proulx, P., Biochim.Biophys. Acta, 270 (1972) 40.

194. Bennet, J., Glavenovitch, J., Liskay, R., Wulff, D.L., and Cronan, J.E., Jr., Virology, 43 (1971) 516.

195. Aibara, S., Kato, M., Ishinaga, M., and Kito, M., Biochim.Biophys.Acta, 270 (1972) 301.

196. Audet, A., Nantel, G., and Proulx, P., Biochim. Biophys.Acta, 348 (1974) 334.

197. Okuyama, H., Biochim.Biophys.Acta, 176 (1969) 125.

198. Kanamesa, Y., Kamatsu, Y.A., and Nojima, S., Biochim.Biophys.Acta, 144 (1967) 382.

199. Kanfer, J., and Kennedy, E.P., J.Biol.Chem., 38 (1963) 2919.

200. Ballesta, J.P., Cundiffe, E., Daniels, M.J., Silverstein, J.L., Susskind, M.M., and Schaechter, M., J.Bacteriol., 112 (1972) 195.

201. Lambert, M., van Golde, G., Schulman, H., and Kennedy, E.P., Proc.Natl.Acad.Sci., U.S.A., 70 (1973) 1368.

202. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J., J.Biol.Chem., 193 (1951) 265.

203. Bligh, E.G., and Dyer, W.J., Can.J.Biochem.Physiol. 37 (1959) 911.

204. Fiske, C.H., and Subbarow, Y., J.Biol.Chem., 81 (1929) 169.

205. Bartlett, G.R., J.Biol.Chem., 234 (1959) 466.

206. Cunningham, H.M., and Leat, W.M.F., Can.J.Biochem., 47 (1969) 1013.

207. White, D.C., and Tucker, A.N., J.Lipid Res., 10 (1969) 220.

208. Chu, H.P., J.Gen.Microbiol., 3 (1949) 255.

209. Op den Kamp, J.A.F., Houtmueller, U.M.J., and van Deenen, L.L.M., Biochim.Biophys.Acta, 106 (1965) 438.

210. Thomas, A.E., Scharoum, J.E., and Ralston, H., J.Am. Oil Chem.Soc., 42 (1965) 789.

211. Biale, Y., Gorin, E., and Shafrir, E., Biochim.Biophys. Acta, 152 (1968) 28.

212. Pieringer, R.A., and Kunnes, R.S., J.Biol.Chem., 240 (1965) 2833.

213. Benzonana, G., and Desnuelle, P., Biochim.Biophys.Acta, 105 (1965) 121.

214. Peters, M.A., and Fouts, J.R., Anal.Biochem., 30 (1969) 299.

215. Gregory, J.D., and Sajdera, S.W., Science, 169 (3940) (I970) 97.

216. Jovin, T., Chrambach, A., and Naughton, M.A., Anal. Biochem., 9 (1964) 351.

217. Widnell, C.C., and Unkeless, J.C., Proc.Nat.Acad.Sci. U.S., 61 (1968) 1050.

219. Mattson, F.H., and Volpenheim, R.A., J.Lipid Res., 9 (1968) 79.

220. Marshall, M., and Kates, M., Biochim.Biophys.Acta, 260 (1972) 558.

221 Bourne, E.J., Stacey, M., Tallow, J.C., and Tedder, J.M., J.Chem.Soc., (1949) 2976.

222. Vogel, Practical Organic Chemistry, section IV, paragraph 184, "Synthesis of p-nitrobenzoyl chloride" 1956.

223. Huggins, C., and Lapides, J., J.Biol.Chem., 170 (1947) 467.

224. Lands, W.E.M., and Mertel, I., J.B.C. 238 (1963) 898.

225. Pieringer, R.A., Bonner, H., Jr., and Kunnes, R.S., J.Biol.Chem., 242 (1967) 2719.

226. Dole, V.P., and Meinertz, H., J.Biol.Chem., 235 (1960) 2595.

227. Hildebrand, J.B., and Law, J.N., Biochem., 3 (1964) 1304.

228. Rapp, U., personal communication.

229. _____, Sephadex-Gel Filtration in Theory and Pactice Pharmacia AB, Uppsala, Sweden, p.13.

230. Raybin, D.M., Butsch, L.L., and Kornberg, A., Biochemistry, 11 (1972) 1754.

231. Doi, O., and Nojima, S., Biochim.Biophys.Acta, 369 (1974) 64.

232. White, D.A., Lennarz, W.J., and Schnaitman, C.A., J. Bacteriol., 109 (1972) 686.

233. Van den Bosch, H., Ann.Rev.Biochem., 43 (1974) 243.

234. Sakakibara, Y., Doi, O., and Nojima, S., Biochem.Biophys. Res.Comm., 46 (1972) 1434.

235. Doi, O., and Nojima, S., Biochim.Biophys.Acta, 369 (1974) 64.