RADIOACTIVE PHOTOREACTIVE FATTY ACID ANALOGUES: SYNTHESIS, BIOLOGICAL UTILIZATION AND TOOLS FOR THE STUDY OF FATTY ACID TRANSPORT

By

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RADIOACTIVE PHOTOREACTIVE FATTY ACID ANALOGUES
Radioactive Photoreactive Fatty Acid Analogues: Synthesis, Biological Utilization and Tools for the Study of Fatty Acid Transport

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Abstract

The present study focused on the development, synthesis and investigation of the biological activity of a radioactive photoreactive homologous series of fatty acid analogues. The carbene precursor m-diazirinophenol was chosen because of its known photolytic properties. In the course of its synthesis according to published procedures, it was found that chlorinated isomers were produced. The latter contributed in lowering the yield of the desired product. Addition of pyridine in the t-butyl hypochlorite oxidation step generated higher yields of the desired protected m-diazirinophenol, free of chlorine.

A synthesis of an homologous series of omega tritiated omega hydroxy fatty acids was developed based on the oxidation of commercially available olefins with catalytic amounts of OsO₄ in presence of NaIO₄ to produce the precursor semialdehydes. Reduction of the latter with [3H]NaBH₄ afforded the corresponding omega hydroxy fatty acids. Subsequently, the pure m-diazirinophenol was coupled to the omega hydroxy group to yield the corresponding radioactive photoreactive fatty acid analogues.

The photoreactive homologous series was found to be biologically active both in vitro and in vivo. Using rat
liver microsomes it was possible to compare Michaelis-Menten constants with those observed for normal substrates for the production of acyl-CoA derivatives. Furthermore, these derivatives were converted to phosphatidylcholine and phosphatidylethanolamine when the corresponding lysophospholipids were present in the incubation mixture. In vivo investigations consisted of incubating L-cells, a fibroblastic cell line, in culture with the radioactive photoreactive fatty acid analogues. Analyses of the lipid fraction of these cells showed that these analogues were present mostly in phosphatidylcholine and in phosphatidylethanolamine. These phospholipids form the major components of L-cells membranes. The photolysis at 360 nm of cells grown in presence of these analogues resulted in the radioactive labelling of a large number of proteins presumed to be integral membrane proteins. In a collaborative effort, it was found that upon infection of these cells with vesicular stomatitis virus (VSV), followed by isolation and photolysis of the virions, the viral membrane glycoprotein G was intensely labelled. Prior to photolysis it was demonstrated that radioactivity attached to the G protein corresponded to the non-photolyzed fatty acid.

Use of these fatty acids as photoaffinity fatty acid analogues, permitted the identification of an Escherichia
coli 30 kD membrane protein with high affinity for [11-^3H]11-diazirinophenoxyundecanoate. When the photolytically labelled 30 kD protein was boiled in SDS its polyacrylamide gel electrophoresis pattern shifted and it behaved as a 43 kD protein.

Finally, attempts were made to develop a fatty acid diffusion assay consisting of encapsulating BSA inside lipid vesicles as a trapping agent. Its potential suitability as a fatty acid transport reconstitution assay is discussed.
List of Abstracts and Publications

The work presented in this thesis has been presented in part in the following abstracts and publications:

ABSTRACTS:


Publications:


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<th>Description</th>
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<tbody>
<tr>
<td>ACS</td>
<td>Aqueous Counting Scintillation liquid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine 5'-triphosphatase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide 3-chlorophenylhydrazone</td>
</tr>
<tr>
<td>CDI</td>
<td>Carbonyl diimidazole</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CoA (CoASH)</td>
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<tr>
<td>cpm</td>
<td>Counts per minute</td>
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<tr>
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<td>Dalton</td>
</tr>
<tr>
<td>DAP</td>
<td>Diazirinophenoxy</td>
</tr>
<tr>
<td>DATD</td>
<td>N,N'-diallyltartramide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAase/DNase</td>
<td>Deoxyribonuclease</td>
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</table>
DTT  Dithiothreitol
E. coli  Escherichia coli
EDTA  Ethylenediaminetetraacetate
g  Gram
g  Acceleration due to gravity
h  Hour
HCl  Hydrochloric acid
HEPES  4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HMPA  Hexamethyl phosphorotriamide
HPLC  High performance liquid chromatography
Hz  Cycles per second
kD  Kilo Dalton
Kd  Dissociation constant
Km  Michaelis constant
m  Meter
M  Molar (moles/liter)
MeOH  Methanol
mm  Millimeter
mg  Milligram
min  Minute
mL  Milliliter
mM  Millimolar
(millimoles/liter)
<table>
<thead>
<tr>
<th>Symbol</th>
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<tbody>
<tr>
<td>mol</td>
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</tr>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino] propanesulfonic acid</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar (micromoles/liter)</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NaIO₄</td>
<td>Sodium metaperiodate</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>O.D.</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ODS</td>
<td>Octadecyl(dimethyl)silane</td>
</tr>
<tr>
<td>OsO₄</td>
<td>Osmium tetroxide</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-Diphenyloxazole</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAase/RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>Rf</td>
<td>Retardation factor</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>S.A.</td>
<td>Specific activity</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethyl ethylenediamine</td>
</tr>
<tr>
<td>TID</td>
<td>3-(trifluoromethyl)-3-(m[125I]iodophenyl)diazirine</td>
</tr>
<tr>
<td>TLC/tlc</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V\textsubscript{max}</td>
<td>Maximum velocity</td>
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I. Introduction.

1.1 Biological membranes.

The cells constituting all organisms are defined by an external membrane referred to as the plasma membrane in eukaryotes and the external and the cytoplasmic membranes in prokaryotes. This membrane isolates each cell from its surroundings and acts as a selective barrier permitting the entry of necessary nutrients and excluding potentially toxic substances (Bretscher & Raff, 1975). It is also through this external membrane that a cell can communicate with its neighbors. In addition, eukaryotic cells possess internal membranes (DePierre & Ernster, 1977) that permit the separation of cellular functions.

The plasma membrane is involved in important cellular processes. It provides the cell with specific receptors for numerous compounds such as neurotransmitters and hormones (Helmreich, Zenner, Pfeuffer, & Cori, 1976; Kahn, 1976). The plasma membranes of specialized cells, such as T-cells, possess specific receptors that permit the immune system to distinguish self from non-self (Lindstrom, 1979; Reinherz & Schlossman, 1980; Triplett, 1962). In bacteria the plasma membrane is also the site of energy generation (Gromet-Elhanan, 1977; Racker & Stoeckenius, 1974).
Because of the important role played by the plasma membrane many investigators sought to study its structure in order to better understand the mechanisms involved in the various functions it can perform. Several approaches have been used, most of which are based on physical techniques and methods (Bretscher & Raff, 1975; Brulet & McConnell, 1975; Chapman & Dodd, 1971). The goal of this thesis was to develop an organo-chemical approach to the study of biological membrane structure and functions.

I.1 a) Structure and functions of biological membranes.

The early work of Gorter and Grendel (Gorter & Grendel, 1925) on red blood cells led to the proposal that plasma membranes are composed of a lipid bilayer. Today, it is known that biological membranes are composed of phospholipids, proteins (termed membrane proteins), and sometimes glycolipids and cholesterol (Bretscher & Raff, 1975; Singer & Nicolson, 1972). The task of determining the arrangements and interactions of these components in the membranes was facilitated by the advent of physicochemical techniques such as X-ray crystallography, electron microscopy, nuclear magnetic resonance, electron spin resonance, infra red spectroscopy, and the use of fluorescent probes (Chapman & Dodd, 1971; Oseroff, Robbins & Burger, 1973).

Several models of membrane structure have been put forward following the results of the early investigations of
biological membranes (Bretscher & Raff, 1975; Jost, Waggoner, & Griffith, 1971). The model that conforms best to the physical data that were gathered is the fluid mosaic model proposed by Singer and Nicolson (1972). As shown in Figure 1, the phospholipids making up the membrane are arranged as a closed lipid bilayer. The hydrophobic portion of the bilayer serves as solvent for the proteins making up the membrane. Further elucidation of the arrangements of membrane components came from the studies of their physical properties.

I.1 b) Physical properties of biological membranes.

According to the fluid mosaic model (Singer & Nicolson, 1972), the lipids are arranged as a continuous phospholipid bilayer in which proteins are embedded to various extent. The use of physical tools allowed investigators to recognize that the phospholipids in the bilayer were free to diffuse in the plane of the leaflet at a rate of 120 μm per second (Bulet & McConnell, 1975; Hong & Hubbell, 1972; Hubbell & McConnell, 1971; Kornberg & McConnell, 1971a). However, using the same techniques, sometimes with the help of the phospholipid exchange protein, it was also found that exchange across the bilayer occurs only once every 6 hours or so (Kornberg & McConnell, 1971b; Rothman & Davidowicz, 1975; Hughes & Zilversmit, 1975). Thus, membranes are relatively non-rigid structures where lipids provide a medium for membrane proteins which are insoluble in
Figure 1. Representations of the structure of typical biological membranes. All these representations are based on the fluid mosaic model of Singer and Nicolson (1972). (A) Schematic representation of the phospholipid bilayer and membrane proteins. (B) Representation of some of the more commonly found components of biological membranes: a) lipids, b) proteins, c) carbohydrates. This figure does not include all forms of attachment of proteins to the cell membrane (see Low and Saltiel (1988) and Jennings (1989) and references therein). (C) Representation of biological membranes from various organisms. Figures reproduced from Jain (1988).
aqueous solvents and serve as a barrier to water soluble compounds.

Several probes have been developed in order to obtain information about the interactions occurring in the lipid bilayer of membranes (Chapman & Dodd, 1971). For example, the use of electron spin resonance probes such as the nitroxide labelled phospholipids yielded information on their immediate environment (Hubbell & McConnell, 1971; Jost, Waggoner, & Griffith, 1971). It was determined that phospholipids are more rigid near the carboxylic end of the component fatty acid and more fluid toward the methyl end. The relative fluidity of the lipid bilayer was found to be perturbed when cholesterol (a major component of mammalian plasma membranes) was added, rendering the carboxylic half more rigid but leaving the terminal methyl groups in a fluid state.

It could be argued that the nitroxide probes used in these studies may not access certain protein-lipid interaction sites due to their bulkiness. Consequently, the probe would not report much information about highly ordered regions adjacent to a protein. To alleviate this problem, partial success has been achieved by attaching the nitroxide probe to the protein of interest (Landgraf & Inesi, 1969). Suggestive evidence for the occurrence of specific conformational changes in the protein under study were reported. Similar findings were reported with the less invasive technique of nuclear magnetic resonance spectroscopy.
1.2 Use of chemical probes to study biological membranes.

Most physical methods used to study biological membranes provided important information about the major components comprising the membrane (Chapman & Dodd, 1971; Hubbell & McConnell, 1971; Jost, Waggoner, & Griffith, 1971). While detailed information about the general arrangements of the lipids and proteins comprising the membrane could be obtained, only limited information could be gained about specific groups involved in lipid-lipid, lipid-protein and protein-protein interactions. The development of photoreactive probes provided an organo-chemical approach to the study of such phenomena. These photolytically generated crosslinking agents permitted the study of the topography of biological membranes (Brunner, 1981; Chakrabarti, & Khorana, 1975; Klip & Gitler, 1974).

The development of photoactivatable probes stemmed from the need of protein chemists to identify the amino acid groups involved at the reactive sites of certain enzymes (Knowles, 1972; Singh, Thornton, & Westheimer, 1962; Vaughan and Westheimer, 1969). However, the labelling of specific amino acid groups on proteins has been achieved in at least two different types of protein structure elucidation without the use of photoreactive agents. First, non-specific reagents such as the suberates (Lutter, Orlander, & Fasold, 1974; Traut, Bollen, Sun, Hershey, Sundberg, & Pierce, 1973), were
known to react with lysine side chains, and allowed the measurement of the distance between certain amino acid residues in native proteins. Second, suicide inhibitors were introduced to elucidate the nature of the active sites of enzymes (Coulson, Knowles, Priddle, & Offord, 1970; Knowles, 1972). The latter approach was found to provide direct information about the nucleophile(s) catalyzing the enzymic reaction(s) under study.

Suicide inhibitors were well suited for specific group identification. Work on various sensitive reagents attached to potential inhibitors lead to the development of photoreactive groups. A brief look at typical studies will illustrate this point. The enzyme triose phosphate isomerase of chicken muscle was incubated with bromohydroxy acetone phosphate instead of the normal substrate, hydroxy acetone phosphate (Coulson et al., 1970). This resulted in the labelling of a tyrosine side chain. When the inhibited protein was reduced before further processing, however, it was found that the actual group being labelled was a glutamic acid residue and that the tyrosine labelling was caused by migration of the group via a nucleophilic attack (Coulson et al., 1970).

Others have used diazo derivatives of potential substrates of the protein or enzyme of interest (DelPierre & Fruton, 1966; Hamilton, Spona, & Crowell, 1967; Rajagopalan, Stein, & Moore, 1966; Riehm & Scheraga, 1965). Using
diazoacetylornleucine methyl ester, Rajagopalan, Stein, and Moore (1966) reported that they could inactivate the enzyme pepsin when cupric ions were present in the mixture. This inhibition could be reversed by incubation of the inhibited protein with 2 M ammonium hydroxide at pH 9.5 and 40°C for nine hours. The latter was taken as evidence for the hydrolysis of esters formed during the inactivation process. Similar results were reported when 1-diazo 4-phenylbutanone-2 (Hamilton, Spona, & Crowell, 1967) and the L- and D-isomers of 1-diazo-4-phenyl-3-tosylamidobutanone (DelPierre & Fruton, 1965) were used. In each case only one molecule of the inhibitor was inserted per molecule of pepsin in presence of cupric ions. When copper was absent from the solution, inhibition was slower and could be competed by the presence of substrate, suggesting that the inhibition was taking place at the active site. Combining this approach with reduction of the ester it was possible for Riehm and Scheraga (1965) to identify a specific aspartate residue (Asp. 53) in ribonuclease using the inhibitor diazo-acetoglycinamide. Only one molecule of the inhibitor, was inserted per molecule of enzyme.

As can be expected from the nature of the probes used in the above studies, only nucleophiles could be identified. This was not a hindrance since very few electrophiles have been found at the active centers of proteins (Knowles, 1972). However, a more indiscriminate approach had to be developed
for the study of the hydrophobic pockets of enzymes and for investigating the hydrophobic environment of membranes and their proteins. Biological membrane proteins sometimes lack reactive sites and in many cases, minimal information is available about the nature of their biological functions.

The first steps toward the development of an undiscriminating reagent are attributable to Westheimer and his colleagues (Singh, Thornton, & Westheimer, 1962) who recognized that the diazo group described above could be photolyzed at wavelengths above 300 nm to generate a reactive carbene species. Carbenes were known for their indiscriminate reactivity towards the carbon hydrogen bond (C-H insertions) (Doering, Butley, Laughlin, & Chaudhuri, 1956; Doering & Prinzbach, 1959; Frey & Kistiakowsky, 1957).

Before exploring in greater detail the work of Westheimer and others it would be of interest to explore the types of reagents that were available to investigators interested in labelling proteins independently of the groups present on them. Two reactive groups were known for their ability to insert themselves into the unreactive C-H bonds of alkanes, the carbenes and the nitrenes.

I.2 a) Properties of Carbenes

The simplest carbene reagent is the methylene group that can be obtained from the photolysis or heat decomposition of diazomethane or ketene (Doering et al., 1956). Methylene has been found to react rather
indiscriminately with the C-H bonds of pentane (Doering et al., 1956) regardless of temperature. Furthermore, contrary to dichlorocarbene (Doering & Hoffman, 1954), methylene does not preferentially react by addition across double bonds when the latter are present. Methylene will react with primary, secondary, tertiary, allylic, and vinylic C-H bonds according to their statistical distribution in the sample. This non-discriminating nature is altered when the carbene is alpha to a carboxylic ester (Doering, & Knox, 1956). It was found that photolysis of diazoacetic esters yielded a carbene product that reacted 1.65 and 1.84 times faster with secondary and tertiary C-H bonds than with primary C-H bonds. In a more extensive study of this selectivity effect, it was found that the presence of carboxylic groups on both sides of the carbene heightened its affinity towards tertiary C-H bonds (Doering & Knox, 1961). The latter results were tentatively explained by the order of stability of carbonium ions, tertiary ions being the most stable and primary ions the least stable. Other substituents such as the phenyl group in phenylcarbene increased the rate of insertion into secondary C-H bonds by six fold compared to the rate of insertions into primary C-H bonds (Gutsche, Bachman, & Coffey, 1962).

The insertion reaction seems to proceed without the involvement of an intermediate. Thus, Doering and Prinzbach (1959) found that incubation of 2-methyl propene-1-14C with
methylene yielded mostly 2-methyl but-1-en-1-\textsuperscript{14}C implying direct insertion into the C-H bond as the likely reaction mechanism.

Carbenes are subject to undesirable rearrangements (Chaimovich, Vaughan, & Westheimer, 1968; Weygand, & Bestmann, 1960). This is exemplified in early attempts at labelling chymotrypsin's hydrophobic sites (Shafer, Baronowsky, Laursen, Finn, & Westheimer, 1966; Singh, Thornton, & Westheimer, 1962). The photoreactive group p-nitrophenyl diazoacetate was attached to chymotrypsin by transesterification, then photolyzed at wavelengths above 300 nm to avoid damaging the protein. Upon transesterification a loss in enzyme activity was observed followed by 75% recovery of activity immediately after photolysis. The incomplete recovery suggested 25% C-H insertions (Singh, Thornton, & Westheimer, 1962). The major photolysis products that were recovered were glycolic acid, active enzyme and modified enzyme. The large amount of glycolic acid represented reaction with water and thus suggested that the active site's environment was not hydrophobic. The characterization of the modified enzyme revealed that some groups were more or less labeled according to the solvent's pH (Shafer et al., 1966). Thus, a high yield of carboxymethyl tyrosine was obtained at low pH suggesting that lowering the pH resulted in chymotrypsin dimer formation. Other photolysis products, such as carboxymethyl serine resulted from a Wolf type
rearrangement of the reactive group and consequently no C-H insertion products (hydrophobic labelling) were recovered.

In order to gain more information on the lack of C-H insertion, Westheimer's group studied the reaction products of various diazoacyl esters and found that upon photolysis in water or methanol the major product reflected an attack by the solvent on a ketene intermediate that resulted from a Wolf type rearrangement (Chaimovich, Vaughan, & Westheimer, 1968; Weygand, & Bestmann, 1960). Substituting an ethyl ester or an amido group for a phenyl ester produced a greater rate of rearrangement. Although they could not explain this enhancement, they found that the presence of a trifluoroacetyl group alpha to the diazo group, as in trifluoroacetyl diazoacetate ethyl ester, lead, upon photolysis, to C-H insertions into the solvent without rearrangements (this was also reported by Weygand, Worschak, Koch, & Koustas, 1961).

The first successful labelling of an enzyme's hydrophobic binding site using a carbene group was reported by Vaughan and Westheimer (1969) who used the diazo malonyl derivative of trypsin. In the latter case a yield of 1-3% insertion into the methyl side chain of a nearby alanine residue was reported. The low yield of successful insertions combined with the problems with rearrangement products and the failure to recognize the potential solution afforded by the presence of a trifluoroacetyl group lead investigators
towards the use of nitrenes as labelling tools.

I.2 b) Properties of Nitrenes

Nitrenes are capable of undergoing the same reactions available to carbenes (Knowles, 1972), however, unlike carbenes, phenyl nitrenes can undergo ring expansion to the corresponding substituted azepines when obtained from phenyl azides (Doering, & Odum, 1966). As alluded to earlier, nitrenes are more selective than carbenes as illustrated by carbethoxynitrene which was found to react 36 times faster with tertiary C-H bonds than with primary C-H bonds (Lwowski, & Maricich, 1964). Compared to the corresponding carbethoxycarbene it represents a 10-fold greater selectivity. This selectivity problem is more pronounced in aromatic nitrenes where it has been postulated that appearance of a formal negative charge on the nitrogen would reduce the nitrenes reactivity towards sites of high electron density (Reiser & Leyshon, 1970). Further studies by Hall, Hill, and Tsai (1965) demonstrated that aryl nitrenes show a 100-fold greater reactivity towards tertiary C-H bonds than towards primary ones. They believed that resonance stabilization with the aromatic ring favored this selectivity process.

Despite the high selectivity of the generated species, investigators interested in protein structure elucidation did not depend as much as membrane chemists on the undiscriminating ability of carbenes. Thus, Fleet, Porter,
and Knowles (1969) reported the successful photolabelling of the heavy and light chain of an antibody raised against the 4-azido-2-nitrophenyl hapten. Incubations with other antibodies did not result in labelling and similarly pre-incubation with non-radioactive reagent prevented labelling. Similar results were reported with [3H]dinitrophenyl glycine diazo ketone by Converse and Richards (1969). These early successes paved the way for the use of nitrene precursors to study the topology of biological membranes.

I.3 Photoreactive reagents to study biological membranes

From the above discussion it can be concluded that both carbenes and nitrenes can insert themselves in the C-H bonds. While both species have a certain preference of reactivity for $3^\circ > 2^\circ > 1^\circ$ C-H bonds, carbenes were found to be less selective. However, the imperfect nature of both types of reagents led investigators toward the use of several types of precursors that would be suitable for the study of biological membranes (Bayley, 1983; Brunner, 1981). Thus, precursors for both types of reagents appeared in basically two forms 1) the hydrophobic labelling agents and 2) the phospholipid analogue labelling agents. In both cases the reactive species were the product of photolysis. The basic criteria (Bayley, 1983) that had to be met by these probes in order to be useful for membrane work were the following: i) the probe should penetrate the membrane bilayer, ii) the
insertion of the probe into the membrane should introduce few if any perturbations on the biological system under study, iii) the photoreactive group should be stable under physiological conditions, iv) the reactive species should be generated by photolysis at wavelengths above those absorbed by the components of the membranes and v) the reactive species (i.e., carbene or nitrene) should be unselective and produce crosslinks on the basis of proximity rather than availability. As will become apparent in the following discussion, a limited number of probes proved to be practically useful and capable of meeting these requirements. A more extensive treatment of the use of photoreactive probes and of their pitfalls has been presented by Bayley (1983).

I.3 a) Hydrophobic nitrene precursors

Although alkyl nitrenes are several fold more reactive than the aryl nitrenes, alkyl nitrene precursors (alkyl azides) could not be photolyzed under conditions where no damage could be observed (Knowles, 1972; Stoffel, 1979 and reference therein). This is exemplified in the work of Stoffel (1979) where prolonged photolysis of membranes containing fatty acyl azides as replacement for the normal fatty acids in their phospholipids lead to labelling of components known not to be associated with the membranes. With increasing photolysis times more species became labelled which would render the results difficult to interpret in
undefined systems.

Possibly the first report of successful membrane labelling using hydrophobic probes was made by Klip and Gitler (1974). They introduced 1-azidonaphthalene and 1-azido-4-iodobenzene as potential membrane probes. To demonstrate the capacity of both compounds to react with the protein components of membranes they performed photolyses in presence of bovine serum albumin (BSA) and successfully labelled it (using the radioactive derivatives). It is interesting to note that BSA is a soluble protein and that the compounds used were totally insoluble in water, suggesting that the probe partitioned into hydrophobic sites of the protein. Nonetheless, both photoreactive groups could partition into artificial lipid bilayers. These probes were used to successfully label two membrane proteins of the sarcoplasmic reticulum (Klip & Gitler, 1974). One of these protein had a molecular weight greater than 150,000 and the other appeared to be the 100,000 dalton calcium dependent ATPase. In a later study, Karlish, Jorgensen and Gitler (1977) labelled an hydrophobic segment of the Na\(^+\), K\(^+\) ATPase of the canine outer renal medulla. They made use of the more hydrophobic compound 5-[\(^{125}\)I]-iodonaphthyl-1-azide (INA). Again, it is interesting to note that only a small fragment of this protein was labelled, although it has several turns in the membrane.

The use of phenyl nitrene as a membrane probe was
carefully investigated by Bayley and Knowles (1978a) who demonstrated that C-H insertions of such probes paralleled the degree of unsaturation and the number of doubly allylic methylene groups in the fatty acyl chains, suggesting that labelling occurred mostly in those regions.

Furthermore, addition of the scavenging agent glutathione in the hydrophilic phase resulted in an 8 fold decrease in insertions suggesting that the reactive nitrene could "travel" to the aqueous surface of the lipid bilayer. Bayley and Knowles (1978a) pointed out the electrophilic nature of the generated phenylnitrene and its capacity to react with nucleophiles such as water, buffer components, surface residue of peripheral membrane proteins and the extramembranous portion of integral membrane proteins. They suggested that experiments using 5-iodonaphthyl-1-azide or other hydrophobic nitrene precursors were subject to the same criticisms and that labelled segments could be resting near the surface of the bilayer. In answer to these criticisms, Wells and Findlay (1979) demonstrated that 1-azido-4-iodo\(^3\)H benzene could crosslink to the major sialoglycoprotein in erythrocyte membranes. They avoided the use of a scavenger and noted that if the probe were capable of moving towards the surface they should have observed labelling of the outermost amino acids of the segment that was effectively labelled.

Possibly the best example of the problems encountered
with nitrene precursors in the study of biological membrane was reflected by the results reported by Hoppe, Friedl, & Jorgensen (1983). In their study of the F₁Fₒ components of the ATP-synthase of E. coli with iodonaphthyl azide, they labelled only a cysteine residue on the β subunit of the protein. The cysteine residue happens to reside on the unique hydrophobic segment of this subunit which is known not to be extensively embedded in the membrane. On the other hand the major subunits α and γ whose extent of penetration into the membrane were known to be substantial were weakly labelled at best. These results prompted Hoppe and his colleagues (Hoppe, Friedl, & Jorgensen, 1983) to conclude that iodonaphthylazide was "unsuitable" for the study of membrane topography.

An added drawback of reagents containing azido groups is the ease with which they can be reduced under physiological conditions (Staros, Bayley, Standring, & Knowles, 1978). Dithiothreitol is the most effective reductant therefore, experiments requiring reducing agents for the preservation of biological activities would benefit from the use of monothiols such as mercaptoethanol or glutathione. The latter products were found to be much less effective at reducing the azido groups to the corresponding amines (Staros et al., 1978; Takagaki, Gupta, & Khorana, 1980).

I.3 b) Carbene Precursors

Despite his successes with nitrene probes in the
labelling of antibodies binding sites, Knowles (1972) realized that the poor reactivity of arylnitrenes could prevent the generation of meaningful results in membrane studies. Consequently he introduced the aryldiazirines (Smith & Knowles, 1973, 1975) which are stable under acidic and basic conditions and are unaffected by the presence of cupric ions. Upon photolysis, aryldiazirines tend to undergo isomerisation to the linear diazo compound or decomposition to the desired phenyl carbene (Figure 2; Smith, & Knowles, 1973, 1975). The probability of either event occurring appears to be equal and presents some problems since the linear diazo isomer is sensitive to nucleophiles. Thus, sufficient generation of nucleophile-sensitive species in the membrane could result in labelling unrelated to photolysis.

As an improvement on the diazo carbene precursors Chowdhry, Vaughan, and Westheimer (1976) introduced the 2-diazo-3,3,3-trifluoropropionyl chloride group. The latter showed less Wolf type rearrangement as evidenced by the 85% -OH insertion upon photolysis in methanol, the remaining products being the result of Wolf rearrangements. When attached to fatty acids composing phospholipid bilayers it proved to be capable of C-H insertions but it was reported to be sensitive to dithiothreitol, 2-mercaptoethanol, cysteine and glutathione, thus reducing its applicability in the study of biological systems (Takagaki, Gupta, & Khorana,
Figure 2. Photolysis of phenolic diazirine. Fate of the intermediate and generation of linear diazoisomer. Upon photolysis (hv), an activated intermediate (*) is thought to be generated and it decomposes to the desired carbene or undergoes rearrangement to the linear diazoisomer. The latter can react with nucleophiles and must be avoided to prevent nonphotolysis related labelling. This can be achieved by using a high energy source of light as is the case in the present work. (Adapted from Smith and Knowles (1973)).
1980). In addition, its wavelength of photolysis was shown to be important as demonstrated by the lability of the photolysis product when the procedure was carried out at 254 nm (Chowdhry, Vaughan, & Westheimer, 1976).

In order to circumvent the problems associated with the diazirine rearrangement to the linear diazo isomer, Bayley and Knowles (1978b) studied the photolytic properties of a novel hydrophobic compound, the adamantane diazirine. Comparisons with phenyl diazirine demonstrated that the latter was about 20 times more efficient at labelling saturated lipids and 10 times more efficient at labelling unsaturated lipids. Possibly the adamantilidene may rearrange to a less reactive intermediate.

Adamantane diazirine was used to study glycophorin and the Na^+, K^+ ATPase and to label those regions embedded in the membrane bilayer (Goldman, Pober, White, & Bayley, 1979; Farley, Goldman, & Bayley, 1980). In the case of glycophorin it was found that a large number of residues were available to the probe and that none of the crosslinking was impaired by the presence of glutathione. On the other hand the Na^+, K^+ ATPase's catalytic subunit was heavily labelled but the glycoprotein subunit was lightly labelled by the probe suggesting a form of selectivity based on hydrophobic regions in the protein (Farley, Goldman, & Bayley, 1980).

Building from the idea that insertion of a trifluoro group seemed to reduce Wolf type rearrangements (Chaimovich,
Vaughan, & Westheimer, 1968; Weygand & Bestmann, 1960; Weygand, Worschak, Koch, & Koustas, 1961), Brunner, Senn, and Richards (1980) synthesized the trifluoro carbene precursor, 3-trifluoromethyl-3-phenyldiazirine. Isomerisation to the linear diazo compound occurred upon photolysis, but the latter was unreactive towards nucleophiles and consequently could be photolyzed further to generate the desired arylcarbene. Indeed, photolysis in cyclohexane yielded only C-H insertion products and the diazirine conversion to the corresponding carbene followed first order kinetics (Brunner, Senn, & Richards, 1980).

The iodinated form of the trifluoro diazirine was used to label red blood cell membranes (Brunner & Semenza, 1981) and was found to be as selective as adamantane diazirine. Band 3 was heavily labelled, and glycophorin's labelling was estimated to be 5 times less. When the same probe (3-(trifluoromethyl)-3-(m^{125}I)iodophenyl)diazirine (TID) was used to characterize the sucrase-isomaltase complex, only the most hydrophobic segment was found to contain the inserted reagent (Spiess, Brunner, & Semenza, 1982). In contrast with the results reported by Hoppe, Friedl, and Jorgensen (1983), on the F_{1}F_{0} ATPase of E. coli, Hoppe, Brunner, & Jorgensen (1984) were capable of labelling all the subunits of the F_{0} part of the protein complex. The extent of labelling of the b subunit suggested that all the amino acid residues were available for contact with the probe.
while access seemed restricted for the $\gamma$ subunit.

The results reported so far indicate that hydrophobic probes, whether they generate carbene or nitrene upon photolysis, produce a labelling pattern that reflects the hydrophobicity and nucleophilicity of the region(s) accessible to the probes. It implies that external membrane proteins and cytosolic proteins could be labelled if the probes were to be "dissolved" in their hydrophobic segment(s). This was clearly demonstrated in the preliminary studies of Klip and Gitler (1974) where they successfully labelled BSA and in a recent study with TID (Krebs, Buerkler, Guerini, Brunner, & Carafoli, 1984) where the soluble protein calmodulin and some of its fragments were labelled.

I.3 c) Photoreactive phospholipids

The problems encountered with the simple hydrophobic probes had been anticipated to an extent by Chakrabarti and Khorana (1975) who designed a synthetic scheme to introduce photoreactive groups into the fatty acyl portion of phospholipids. They synthesized a series of azido and nitrophenyl azido fatty acids containing the photoreactive groups at various positions along the acyl chain. Photolysis of phospholipid vesicles containing nitrene or carbene precursors did result in intermolecular crosslinking and suggested that it would represent a useful tool for the study of membrane proteins (Gupta, Radhakrishnan, Gerber,
Olsen, Quay, & Khorana, 1979).

The next step for Khorana's group was to demonstrate that biological activity would not be impaired by the presence of the photoreactive group. This was accomplished by assessing the capacity of photoreactive fatty acid analogues to support growth of fatty acid auxotrophs of E. coli (Greenberg, Chakrabarti, & Khorana, 1976). It was found that most straight chain and aromatic azido fatty acids were able to support growth at 30°C with the exception of 12 azido-nitrophenoxy oleic acid. The latter may have introduced too much perturbation in the bilayer to permit survival of the bacteria. Insertion of photoreactive fatty acids into the phospholipids of the cells in vivo was pursued further by Olsen, Schaechter, and Khorana (1979). The two carbene precursors used in their study (trifluoro-2-diazopropionyl and m-diazirinophenoxy) failed to support growth of the fatty acid auxotroph K1060-B5 of E. coli. The groups were attached at the omega carbon of dodecanoate and undecanoate respectively. In contrast, azidophenoxy undecanoate (nitrene precursor) supported growth and was readily inserted into the major phospholipids of the cell. Fatty acids containing nitro aryl groups failed to support growth and were not inserted into phospholipids. The exact nature of the incapacity of certain photoreactive fatty acids to support growth and to fail to be inserted in the phospholipids was not further assessed. In the course of their investigation it
was observed that phospholipase A₂ digestion of the phospholipids resulted in excision of the fatty acids that were found to support growth. Since the 2-position on the glycerol phosphate moiety is the one cleaved by the enzyme and this position is usually occupied by unsaturated fatty acids it suggested that the analogues behaved like unsaturated fatty acids. However, growth in the presence of these fatty acids was better at elevated temperature, suggesting that they behaved like saturated fatty acids. This dual role has yet to be fully explained although it was found that presence of these analogues in the growth media appeared to have stimulated the synthesis of unsaturated fatty acids in these organisms (Olsen, Schaechter, & Khorana, 1979).

Following an improvement on the synthesis of photoreactive phospholipids (Gupta, Radhakrishnan, & Khorana, 1977) Gupta, Costello, and Khorana (1979) investigated the exact location of intramolecular crosslinking of fatty acyl chains in phospholipids containing carbene precursors. Their findings suggested that the fatty acyl chain at the 2-position of the glycerol phosphate moiety is offset by 2 to 4 carbon atoms from the fatty acyl chain in the 1-position. It is as if the chain was not fixed at a specific depth in the bilayer but could move up and down in relation to the fatty acyl chain in the 1-position.

In order to determine which type of photoreactive
group would be better suited for membrane studies, Khorana's group (Gupta et al., 1979) analyzed the photolysis products of phospholipid vesicles containing nitrene and carbene precursors. Analyzing the photolysis products using mass spectrometry, it was established that nitrenes generated from azido phospholipids significantly inserted themselves into C-H bonds only in presence of unsaturated fatty acids while carbenes, generated from the photolysis of trifluoro diazopropionyl group, demonstrated significant insertion into the C-H bonds of saturated lipids, thus establishing their suitability for membrane studies.

In spite of the work of Knowles and Khorana (Bayley & Knowles, 1978a, 1978b; Gupta et al., 1979) demonstrating the unsuitability of nitrene precursors for membrane topography studies, further attempts to use these probes continued. The group headed by Bisson pursued topographical studies on cytochrome C oxidase (Bisson, Montecucco, Gutweniger, & Azzi, 1979; Bisson, & Montecucco, 1981) using "deep" and "shallow" phospholipid analogues containing 2-azido-4-nitro phenyl groups. The "deep" probe labelled intensely subunits I, III, VI, and VII and the "shallow" probe labelled subunits I, II, III, and VII. Based on these results they produced a model of the arrangement of cytochrome oxidase in the artificial bilayers that were used. These results have to be interpreted with caution in the wake of the paper presented by Hoppe, Montecucco, and Friedl (1983) on the Fo subunit of ATP
synthetase of *E. coli*.

As an extension of their study with the hydrophobic probe iodonaphthylazide, Friedl and his colleagues (Hoppe et al., 1983) used 1-palmitoyl 2-(2-azido-4-nitro)benzoyl sn-glycero-3-[³H]phosphocholine to study the $F_o$ subunit of the ATP synthetase of *E. coli*. Upon photolysis of phospholipid vesicles containing the $F_o$ component they obtained similar results as with the hydrophobic probe, successfully labelling the $b$ subunit which does not penetrate the membrane extensively. In contrast, subunit $\alpha$, which is an integral membrane protein, and subunit $\varepsilon$, which occupies a significant portion of the bilayer, were practically not labelled. These results contrast with those obtained with the hydrophobic probe TID, used to study the same protein (Hoppe, Brunner, & Jørgensen, 1984).

Brunner and Richards (1980) used reconstituted vesicles containing gramicidin to compare the efficiency of labelling of phospholipids containing nitrene or carbene precursors. Surprisingly, in their system they found that both probes selectively labelled a tryptophan residue and they expressed doubts about the use of either probe to study membrane topography. They were concerned with the non-fixed position of the probes in the lipid bilayer. This point had been reported by Gupta, Costello, and Khorana (1979) who brought some precision to the extent of the movement of the probe in their system while Brunner and Richards did not
establish this under their conditions.

The first in vivo use of the m-diazirinophenoxy fatty acid analogues was reported by Quay, Radhakrishnan, and Khorana (1981). They found that omega-(m-diazirinophenoxy)-hexadecanoic acid was inserted in the phospholipids of the fatty acid auxotroph K1060 B-5 of E. coli. In order to identify the photolytically labelled proteins they made the phospholipids radioactive by growing the bacteria in presence of $[^{32}\text{P}]\text{H}_3\text{PO}_4$. Upon photolysis, they were able to identify 13 out of 24 known membrane proteins. Because the radioactivity was in the phospholipid head groups and due to the nature of the radioactive isotope their results were blurry and some proteins were barely labelled. When the experiment was performed with omega-(m-azidophenoxy)-undecanoic acid the results were poorer, thus confirming that carbene reagents attached to fatty acid chains were preferable for membrane studies.

Photoreactive phospholipids have been used successfully to study the two conformational forms of cytochrome b$_5$ (Takagaki, Radhakrishnan, Gupta, & Khorana, 1983; Takagaki, Radhakrishnan, Wirty, & Khorana, 1983). Upon photolysis, a broad labelling pattern was observed extending from Ser 104 to Met 130 with a bell shaped intensity of labelling peaking at residue 118. This pattern of labelling was taken as evidence for a looping back structure for the transferable form of the protein. The nontransferable form
revealed a transmembrane configuration of the protein based on the use of different labels at the outer and inner leaflets. These results point to the versatility of the approach to study membrane proteins and the conformational changes they are subjected to.

Photoreactive phospholipids can be introduced in a biological system either through in vivo or in vitro procedures. The in vivo approach has been characterized by Khorana's group where the photoreactive fatty acid analogues were added to a growing culture of bacteria and were incorporated into the phospholipids by the cells' enzymes. Two methods have been in use for incorporation of photoreactive phospholipids in vitro: the phospholipid exchange protein and the mixed micelle formation. Brunner and Richards (1980) introduced the former approach while the latter method proved successful with red blood cell ghosts (Brunner, Spiess, Aggeler, Huber, & Semenza, 1983).

Improvements on the use of photoreactive phospholipids have been attempted, mostly by Khorana's group, who described a new approach for the synthesis of phospholipids containing carbene precursors in their head groups (Burnett, Robson, Takagaki, Radhakrishnan, & Khorana, 1985). The synthetic scheme allows the investigator to tailor the new headgroup to the various protein requirements for activity. This dual approach (hydrophobic and head group labelling) is exemplified by the work of Ross, Radhakrishnan, Robson, and
Khorana (1982) with Glycophorin A. A specific glutamate residue (Glu 70) thought to be in the bilayer was identified by hydrophobic labelling and a series of surface amino acid residues were successfully labelled using the head group probe.

Recognizing the limiting condition of attaching the probe at the omega carbon of the fatty acyl chains, Erni and Khorana (1980) introduced the 3,3-bis(1,1-difluorohexyl)diazirine and the 3-(1,1-difluoroctyl)-3H-diazirine which could be inserted at any position along the fatty acyl chain. These carbene precursors showed very little if any C-H intermolecular insertion products upon photolysis. Instead, photolysis yielded mostly rearrangements and intramolecular C-H insertions.

Overall, carbene precursors proved superior to nitrene precursors in membrane topography investigations. Better information was gained from attaching the probe to the fatty acyl chain than relying on the hydrophobicity of the probe itself. In light of these conclusions the work described in this thesis focused on the synthesis of the m-diazirinophenol probe and its attachment at the omega carbon of iodo fatty acids to yield photoreactive fatty acid analogues. The lack of biological activity of similar analogues reported in the early studies of Khorana's group has also been under scrutiny from the point of view of the mammalian fatty acid metabolizing enzymes. Finally, a preliminary investigation of
the fatty acid transport mechanism in *E. coli* was undertaken.

I.4 *Transport across biological membranes*

One of the goals of this thesis was to use photoreactive fatty acids to study their transport across biological membranes. The simplicity of the bacterial system facilitated the study of biological transport. Before introducing fatty acid transport in prokaryotes a discussion of the transport of molecules across lipid bilayers in general is presented.

It has already been mentioned that membranes act as selective barriers. Consequently they are responsible for controlling the traffic of various compounds that are required by the cell for survival. Although some investigators proposed that transport of biological material across biological membranes is an artefact related to the binding of the material to internal enzymes (Degrella and Light 1980 a,b), as will be demonstrated later, a large body of evidence supporting the involvement of specific proteins in transport has been gathered.

The asymmetric distribution of sodium and potassium ions across the lipid bilayer is a good example of the selective permeability of membranes. The cells maintain the concentration of sodium inside the cell that is 10 times lower than that outside and vice versa for potassium (Sweedner & Goldin, 1980). A membrane protein, the $Na^+$, $K^+$
ATPase, is the actual "pump" involved in this process (Racker, 1976). Other transport proteins or "pumps" have been identified and successfully reconstituted in artificial lipid bilayers (Kaback, 1983; Kasahara & Hinkle, 1976, 1977; Racker & Stoeckenius, 1974) supporting the view that proteins are involved in such processes.

Protein-mediated processes show typical saturation kinetic properties. Thus, if a substrate crosses the membrane by simple diffusion a linear increase in its diffusion rate should be observed with increasing concentration without showing saturation effects. In contrast, protein-mediated transport processes (active transport and facilitated diffusion) show saturation kinetics, that is, past a certain substrate concentration there is no substantial increase in the rate of uptake of that substrate that can be observed.

Entry of substrates against their concentration gradient requires a source of energy which is now known to be derived from ATP hydrolysis and/or an electrochemical potential brought about by unsymmetric distribution of protons on both sides of the membrane (Mitchell, 1961; Racker, 1976, 1980). The electrochemical potential of the membrane as a source of energy has been proposed by Mitchell (1961). Simply stated, the separation of ionic charges (protons, or other ions) across an impermeable lipid bilayer gives rise to an electrochemical potential (made of an electrical component and a difference in proton concentration
component) which can be used as a source of energy to drive transport processes (Hamilton, 1977).

The simplicity of prokaryotic organisms has facilitated the task of investigators studying transport and other biological functions. The availability of auxotrophs for the substrate under study and the ease of obtaining mutants of such strains has made the study of transport in E. coli a lot easier. However, some transport complexes in E. coli are rather intricate and encompass more than one type of transport mechanism. The development of procedures to obtain closed membrane vesicles from such organisms (Kaback, 1971, 1974) eased the work of investigators and contributed to the evolution of new ideas on transport mechanisms.

The study of transport processes has been facilitated by development of closed lipid systems (Bangham, 1983; Deamer, & Uster, 1983; Kaback, 1971, 1974; Szoka, & Papahadjopoulos, 1978, 1980) as well as means of incorporating desired proteins into them (Racker, 1979). The reconstitution of certain membrane activities using the latter approach, and the realization that protons or sodium symport is associated with substrate movement have contributed significantly in the determination of membrane proteins' function in transport (Kaback, 1983; Wilson, 1978).

The basic strategy that is used for the study of transport can be described as follows: Typically an assay for the substrate under study exists or can be developed for the
purpose at hand. Then an attempt is made to identify the transporter using labelling with specific inhibitors, and affinity or photoaffinity analogues. Often this structural assay is used for monitoring the isolation of the transport complex although failure to ascertain transport capability of the partially purified fractions has often led to isolation of inactive proteins. Thus a functional reconstitution assay has to be developed to help the isolation process (Racker, 1979). Finally the isolated components are reconstituted in an artificial lipid bilayer where different aspects of the transport mechanisms can be scrutinized.

Two types of protein-mediated transport processes have been identified a) active transport and b) facilitated diffusion (Wilson, 1978). Active transport is often observed when a substrate is transported against its concentration gradient while facilitated diffusion is more often seen when the substrate is entering the cell down its concentration gradient. In eukaryotes both facilitated diffusion and active transport are known to operate (Wilson, 1978). Prokaryotes typically make use of an active transport system except for glycerol uptake which enters the cell down its concentration gradient (Hamilton, 1975,1977; Wilson, 1978). Various proteins are involved in the passage of substrate across lipid bilayers. In gram-negative bacteria these proteins can be found in the outer membrane, in the inner membrane, in the
periplasmic space or in the cytoplasm.

I.4 a) Prokaryotic transport mechanisms

The transport of solutes in prokaryotes is influenced by their membrane organization. In contrast to eukaryotic cells, prokaryotic organisms possess two types of external membrane. The outermost membrane (outer membrane or om) is composed of lipopolysaccharides (LPS), phospholipids, proteins and lipoproteins. The inner membrane (im) lacks LPS and lipoproteins but otherwise is similar to the outer membrane in composition. These membranes are separated by the periplasmic space where a peptidoglycan layer and periplasmic proteins (including binding proteins) are found (DiRienzo, Nakamura, & Inouye, 1978). A group of negatively charged oligosaccharides referred to as membrane-derived oligosaccharides (MDO) is also found in the periplasmic space (Schneider, Reinhold, Rumley, & Kennedy, 1979). The fixed negative charges in the periplasmic space brought about by MDO give rise to a Donnan potential (inside negative) which appears to affect the influx of negatively charged compounds (Sen, Hellman & Nikaido, 1988). It has also been demonstrated that MDO play a role in osmoregulation in E. coli (Kennedy, 1982; Stock, Rauch, & Roseman, 1977).

Transport of substrates via binding protein(s) is a characteristic of gram-negative bacteria (Hamilton, 1977; Wilson, 1978). The binding protein transport process in E.
coli is characterized by its sensitivity to osmotic shock (Berger & Heppel, 1974). Shocking the cells induces the release of almost all periplasmic proteins (and some cytoplasmic proteins) some of which show specific affinity for particular substrates (Ames Ferro-Luzzi, 1986). Periplasmic binding proteins typically exhibit $K_m$ in the range of 0.01 μM to 1 μM (Ames Ferro-Luzzi, 1986). Among the best characterized periplasmic transport systems are those for histidine and maltose (reviewed in Ames Ferro-Luzzi, 1986). As described by Ames Ferro-Luzzi (1986) the prototypical periplasmic transport system consists of an outer membrane pore and a periplasmic binding protein which can interact with up to three cytoplasmic membrane proteins. For example, maltose is believed to penetrate the outer membrane via the trimeric lambda receptor LamB. Then the empty periplasmic protein MalE (maltose binding protein) picks up maltose and undergoes changes in conformation allowing it to interact with the membrane proteins MalF, MalG and MalK (Hengge & Boos, 1983). Berger and Heppel (1974) had presented evidence that ATP or other form of phosphate energy powers this transport process. Conflicting results tend to bring into question this hypothesis (Ames Ferro-Luzzi, 1986).

The shock insensitive passage of substrate across the cytoplasmic membrane of bacteria is dependent on the presence of a membrane protein and a high energy state of the membrane derived from substrate oxidation and referred to as
chemiosmotic energy (Hamilton, 1975, 1977; Kaback 1983; Mitchell, 1961). The energy for the transport process is derived from a proton electrochemical gradient brought about by the expulsion of protons outside the cells during oxidation of suitable electron donors. A direct consequence of this is that transport of substrate can be inhibited by substances known to bring equilibration of protons across the bilayer (uncouplers) (Dills, Apperson, Schmidt, & Saier, 1980). The translocation event itself does not result in any modification of the substrate. In most if not all cases the substrate is transported against its concentration gradient which explains the energy requirement. Proline uptake is thought to occur by this mechanism (Berger, 1973). Beta-galactoside permease of E. coli is a typical model of a membrane protein-mediated transport process. In absence of energy the permease functions to facilitate diffusion of galactosides down their concentration gradient until they are in equilibrium with outside concentration. In presence of metabolic energy (proton motive force) the concentration of galactosides against their concentration gradient is catalyzed (Hamilton, 1977). This effect can be demonstrated in empty E. coli vesicles (Kaback, 1974, 1983). In the case of glycerol uptake a membrane protein is required, however, it is a rare case where the substrate is actually entering the cell down its concentration gradient (Lin, 1976). The latter process is known as facilitated diffusion and is
mediated by a membrane protein and does not require a source of energy.

The final class of transport mechanism is the group translocation process whereby the substrate is modified upon its passage across the membrane. This process is thought to permit accumulation of substrate by modifying it such that it can no longer be recognized by the permease that favored its entry and consequently can't promote the reverse reaction. The phosphotransferase system is probably the best understood and is involved in the transport of certain sugars across the membrane (Hays, Simoni, & Roseman, 1973; Simoni, Nakazawa, Hays, & Roseman, 1973; Simoni & Roseman, 1973; Hamilton, 1975). It is characterized by the attachment of a phosphate group on the sugar to produce the sugar-6-phosphate derivative. A series of enzymes have been implicated in the process and are briefly described below.

Sugars enter the cells by binding to a receptor protein (located in the cytoplasmic membrane) termed enzyme II, which acts as a complex with the peripheral membrane protein, enzyme III, in the phosphorylation step. Phosphoenol pyruvate, provides the phosphate to phosphorylate the soluble enzyme I that phosphorylates the small histidine-containing phosphocarrier protein which finally phosphorylates the peripheral membrane protein, enzyme III. The net result of this chain of events is the transport of sugars inside the cell as their phosphorylated intermediates.
The transport of fatty acids has been found to follow a similar course of events. It is often referred to as "vectorial acylation" (Klein, Steinberg, Fiethen, & Overath, 1971) and involves the modification of the incoming fatty acid to the acyl-CoA intermediate.

I.4 b) Fatty acid transport in prokaryotes

The transport of fatty acids across lipid bilayers is often compared to the passage of hydrophobic ions across these barriers. Lauger and his colleagues (Benz, Lauger, & Janko, 1976; Ketterer, Neumcke, & Lauger, 1971) have identified three steps in this process: i) adsorption of lipid-soluble ions to the membrane-solution interface, ii) passage over an activation barrier to the opposite interface, and iii) desorption into the aqueous solution (cytosol). It is with these steps in mind that fatty acid transport in E. coli will be reviewed.

In the course of their studies of the enzymes involved in fatty acid degradation, Overath and co-workers observed that E. coli possessed an inducible system for the degradation and uptake of fatty acids, the oleate degradation (old) or fatty acid degradation (fad) regulon (Klein, Steinberg, Fiethen, & Overath, 1971; Overath, Pauli, & Schairer, 1969). It was reported that induction occurred upon incubation with fatty acids of chain lengths greater than 8 carbons. Mutants constitutively expressing the fad enzymes (fadR) grow on short to medium chain length fatty acids.
Overath's group further noticed that old (fad) mutants having lesions in one or all of the enzymes involved in fatty acid degradation had a lower rate of uptake of fatty acids. Moreover, they found that a mutant lacking acyl-CoA synthetase activity (fadD) failed to take up fatty acids (Klein, Steinberg, Fiethen, & Overath, 1971). This information coupled with the observation that no efflux of the internal fatty acids was observed, suggested that uptake of fatty acids was closely related to fatty acid metabolism. It was postulated that the E. coli acyl-CoA synthetase was taking part in a group translocation process that was responsible for transport of fatty acids.

Toscano and Hartline (1973) reported the existence of a saturable process for the transport of fatty acids in Pseudomonas oleovorans, and were able to measure apparent $K_m$ and $V_{max}$ values for different chain length fatty acids. Furthermore, they reported that the fatty acids were concentrated against a concentration gradient. Similar results were reported by Ferreman and Bennett (1973) working with E. coli. They noted that oleate uptake was a saturable process with an activation energy of 6.25 kcal/mole and that the uptake process was clearly affected by arsenate and 4-pentenoate which lead them to conclude that ATP and CoA were required. The fact that [1-$^{14}$C]oleate could only be found as $^{14}$CO$_2$ or in the phospholipids of the cells was taken as evidence that the rate limiting step in the
metabolism of fatty acids was the uptake process which they postulated was catalyzed by acyl-CoA synthetase.

Nunn and Simons (1978) studied an E. coli mutant capable of acyl-CoA synthesis but unable to take up fatty acids (Hill & Angelmaier, 1972) and reported a new genetic locus, fadL, necessary for fatty acid uptake. Cells expressing the fadL gene were capable of concentrating long chain fatty acids 10-fold inside the cells against their concentration gradient (Maloy, Ginsburgh, Simons, & Nunn, 1981). Fatty acid transport was reported to be shock insensitive but was inhibited by dinitrophenol and cyanide (Maloy et al., 1981). The rate of fatty acid transport was lower in mutants lacking ompC or ompF (or both) porin gene products (Maloy et al., 1981). This energy requirement was also observed by Kameda (Kameda, Suzuki, & Imai, 1987) who determined that fatty acid uptake is coupled to proton entry. Thus, 20 μM CCCP was sufficient to inhibit fatty acid transport in normal spheroplasts and in spheroplasts that were made slightly more basic than the assay medium.

In the course of Maloy's work (Maloy et al., 1981) it was found that medium chain fatty acids entered the cells by a different mechanism than long chain fatty acids and the former could not induce the fad enzymes. Studies of the fad regulon showed that it is induced by acyl-CoA and that the repressor gene product of fadR mutants is a trans-acting protein that affects fadL gene expression to the same extent
than the other fad enzymes (Simons, Egan, Chute, & Nunn, 1980; Simons, Hughes, & Nunn, 1980).

In an attempt to identify the fadL gene product Ginsburgh, Black, and Nunn (1984) compared the membrane proteins of 16 fadL mutants with the constitutive strains. A 33,000 dalton cytoplasmic membrane protein which behaved like the other members of the fad proteins, was absent in the mutants. The expression of this protein was found to be under the control of the fadR protein and was inducible (Ginsburgh et al., 1984). Restoration of fatty acid transport in these mutants paralleled the reappearance of the 33 kD protein. Further assessment of the role of this 33 kD protein revealed that introduction of plasmids containing the fadL gene resulted in the appearance of the protein in membranes of fadL mutants (Black, Kianian, DiRusso, & Nunn, 1985). In a comparison of transport activity, fadL\textsuperscript{+} strains were found to take up fatty acids 2- to 3-fold more than fadL strains.

Comparing the capacity of fadD fadL and fadD fadL\textsuperscript{+} mutants to bind fatty acids (the fadD mutation allowed them to study binding in absence of acyl-CoA synthesis), Nunn, Colburn, and Black (1986) found evidence for higher fatty acid binding activity in strain harboring the fadL gene product. They concluded that one of the function of the 33kD protein is to be a receptor for fatty acids in the membrane. They also postulated that this membrane protein is the fatty acid permease since mutants of fatty acid uptake do not show
lesions in other membrane proteins. Interestingly it was found that upon solubilization of the membranes in SDS at 100°C as opposed to 50°C, the protein band migrated from 33 kD to 43 kD when analyzed on SDS polyacrylamide gels (Black et al., 1985). This phenomenon has been reported for other proteins such as ompA (Reithmeier & Bragg, 1974; Schnaitman, 1973). These proteins are believed to have a high content of β-structure resulting in excessive binding of SDS at room temperature thus leading to their high electrophoretic mobility.

Unaware of Nunn's work, Kameda (1986) reported the partial purification of a 26.5 kD E. coli membrane protein which bound fatty acids. The crude extract bound palmitate and oleate over caproate and laurate (Kameda, 1986). This extract was also found to facilitate transport of fatty acids when reconstituted with liposomes containing acyl-CoA synthetase. In absence of the relevant membrane extract, a significant level of acyl-CoA was synthesized indicating the high diffusion rate of fatty acids across these liposomes. The membrane extract showed a slight but significant (Kameda's conclusion) increase in the amount of acyl-CoA synthesized upon short incubation periods. With increasing incubation times the difference between the two types of liposomes vanished.

Kameda's observations coupled to Nunn's work suggested that fatty acid transport in E. coli is an energy dependent
process that required the presence of channels on the outer membrane (ompC and ompF), and of fadL in the cytoplasmic membrane. However, this picture of fatty acid transport in *E. coli* may have to change in view of the recent work from Nunn's laboratory. Nunn's group reported that FLP (the fadL gene product also known as the 33 kD or the boiled 43 kD protein) is located on the outer membrane of *E. coli* (Black, Said, Ghosn, Beach, & Nunn, 1987). This report is in sharp contrast with their previous results (Ginsburgh *et al.*, 1984) and raises questions about the role of ompC, ompF, proton symport and the apparent energy requirements associated with fatty acid transport.

Nunn's group (Black *et al.*, 1987) succeeded in isolating the 33 kD protein by selectively solubilizing the outer membrane. Also when they compared inner and outer membrane preparations they could only associate this protein with the latter using their anti-FLP serum. Finally, they observed that spheroplasts from fadL⁺ and fadL⁻ strains could transport fatty acids equally well. This prompted them to conclude that fadL had to exert its effect at the outer membrane level since a difference in transport between these strains was observed when the cells were intact (Black *et al.*, 1987). Kameda (Kameda, Suzuki, & Imai, 1987) also reported that wild type *E. coli* spheroplasts transport fatty acids with greater efficiency than the intact cells. However, since spheroplasts do not normally form the majority of the
usual *E. coli* population it is possible that other factors can explain these results.

The observation by Kameda (Kameda, Suzuki, & Imai, 1987) that shock treated cells show higher uptake of fatty acids than intact cells suggests that this treatment made the cells more permeable or that a regulatory component of transport was lost in the process. A likely regulatory component which could explain both the spheroplasts results of Kameda and Nunn's groups, is the loss of membrane derived oligosaccharides (MDO) (Kennedy, 1982). Since MDO imparts an inside negative Donnan potential across the outer membrane it is possible that the latter slows the passage of fatty acids under normal physiological conditions. Sen, Hellman and Nikaido (1988) demonstrated that permeability of negatively charged compounds is affected by the Donnan potential. It remains to be demonstrated that this is the case for the transport of fatty acids.

To explain the discrepancy in the assessment of the membrane location of the **fadL** gene product, methodological differences in Nunn's group membrane preparations (Ginsburgh et al., 1984; Black et al., 1987) were suggested although no comparative studies were reported. As they pointed out it is possible that in the course of the membrane preparation, FLP "scrambled" and co-migrated with the outer membrane fraction (Ishidate, Creeger, Zrike, Deb, Glauner, MacAlister, & Rothfield, 1986) implying that it could be located at
attachment sites between the outer and the inner membranes.

The solubilization of the fadL protein with 0.1% Triton X-100, and its behavior upon boiling support the view that it resides in the outer membrane (Black et al., 1987; Reithmeier & Bragg, 1974; Schnaitman, 1973). It has been reported that 2% Triton X-100 can solubilize both inner and outer membranes completely, but in presence of magnesium the inner membrane is selectively solubilized leaving the outer membrane morphologically intact (Filip, Fletcher, Wulff, & Earhart, 1973; Lugtenberg & van Alphen, 1983; Schnaitman, 1971). Other detergents such as 2% Brij 58 and 0.5% sarcosyl affect the inner membrane preferentially (Filip et al., 1973). The presence of FLP in the outer membrane also suggests a possible association with the porins ompC and ompF to facilitate the passage of long chain fatty acids across the outer membrane.

The overall picture that emerges from these studies can be related to the three steps described by Lauger (Benz, Lauger, & Janko, 1976; Ketterer, Neumcke, & Lauger, 1971) for the transport of hydrophobic ions. Thus, adsorption of fatty acids to the membrane solution interface may be favored by FLP (or one of the porins) and the membrane phospholipids (fatty acids do partition in the lipid bilayer). Then, an unreported cytoplasmic membrane protein may be responsible for the permeation of the fatty acids to the cytoplasmic side where the acyl-CoA synthetase would be the potential
desorption agent as well as producing the required acyl-CoA derivatives.

In the course of the work presented in this thesis attempts were made to radioactively photolabel this fatty acid receptor of *E. coli*.

I.5 Rationale and Objectives

The development of photoreactive probes to study biological membranes offered a new tool to investigators interested in the study of lipid-lipid and lipid-protein interactions. Despite demonstrations that nitrenes are unsuited for the task their easy access and utilization made them prime starting materials. The early attempts demonstrated the potential information that could be gained from such probes. Recognition of the superiority of carbenes to study the hydrophobic core of membranes lead to the development of so called "hydrophobic probes" bearing little relationship structurally with membrane components. The development of photoreactive fatty acids containing the phenolic diazirine at their omega carbon was a major step forward although they could not be used in *in vivo* situations.

In order to shed some light on the underlying problems involved in this lack of biological activity and in order to improve tracking of the photolysis products the synthesis of an homologous series of radioactive fatty acid analogues containing the phenolic diazirine was designed. The added
advantage of the series would permit attachment of a fatty acid of different chain length at the second position of various phospholipids for \textit{in vitro} and \textit{in vivo} study of the degree of embedding of different membrane proteins. The potential use for the radioactive photoreactive fatty acids in the identification of integral membrane proteins and in the identification of proteins involved in fatty acid metabolism made the synthesis of the series the more interesting. Consequently the goals of the present work were as follows: i) the synthesis of the photoreactive carbene precursor m-diazirinophenol described by Smith and Knowles (1975) and utilized by Khorana's group; ii) the synthesis of a radioactive homologous series of fatty acid analogues having the photoreactive group m-diazirinophenol attached at their omega carbon; iii) the study the biological activity of these probes in light of the failures reported by Khorana's group. The priority was set on fatty acid metabolizing enzymes such as the rat liver microsomes acyl-CoA synthetase and \textit{in vivo} studies of incorporation into the phospholipids of cells in cultures; iv) the use of these probes as photoreactive substrate analogues to label an \textit{E. coli} membrane protein thought to be involved in the transport of fatty acids, and finally v) the use of the fatty acid analogues in the development of an assay for fatty acid diffusion across lipid bilayers.
II. Materials and Methods

This section contains the names and sources of chemicals used in the course of the present work as well as a list of the equipment used to carry out some of the analyses. The second part of this section consists of detailed descriptions of the experiments.

II.1 Materials

Bromophenol blue, (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid), HEPES, (3-[N-morpholino] propanesulfonic acid), MOPS, Trizma base, polyoxyethylene 20 cetyl ether (Brij 58), dl-dithiothreitol (DTT), bovine serum albumin (BSA) fraction V, m-hydroxybenzaldehyde and folic acid Ciocalteu's phenol reagent 2 N were purchased from Sigma. Sodium dodecyl sulfate (SDS) USP grade, N,N'-methylene-bis-acrylamide, N,N'-diallyltartramide (DATD), N,N,N',N'-tetramethylethylenediamine TEMED, Bio-Beads SM-2 20-50 mesh for detergent removal and Triton X-100 were obtained from Bio-Rad. Anhydrous CaCl₂ and 2,5-diphenyloxazole (PPO) were obtained from BDH. The sources of acrylamide used for polyacrylamide gel electrophoresis were Eastman and Serva. [³H]NaBH₄ was supplied by New England Nuclear and/or Amersham. Other dry chemicals such as
ammonium persulfate, NaCl and sucrose were reagent grade chemicals purchased from the following suppliers, Fisher, J.T. Baker, Caledon and BDH.

Chloromethyl methyl ether was purchased from Aldrich. Solvents such as chloroform, diethyl ether, petroleum ether, dioxane, diglyme, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), hexamethyl phosphorotriamide (HMPA) and 88% formic acid were obtained from Caledon and/or J.T. Baker. Reagent grade 95% ethanol was supplied by McMaster University. Ammonia gas was purchased as a lecture bottle from Canadian Liquid Air. Amersham's aqueous counting scintillation liquid (ACS) was used for liquid scintillation counting.

Sepharose 4-B, Sephadex LH-20 and LH-60 were obtained from Pharmacia. Thin layer chromatography was performed on silica plates IB2 and IB2-F from J.T. Baker. For preparative thin layer chromatography Whatman's PLK5 linear-K preparative silica gel plates were used.

For fluorography and autoradiography X-Ray films from Eastman Kodak were used. Liquid scintillation counting was performed on a Beckman LS 7800 liquid scintillation counter. When needed, solvents were removed from samples using the Savant Speed Vac. concentrator. Column chromatography eluates were collected with a Gilson fraction collector. Analytical and preparative high performance chromatography were
performed on a Beckman HPLC apparatus.

The UV spectra were recorded on a Varian/Cary 210 spectrophotometer. Infra red spectra were obtained from neat films using a Perkin-Elmer instrument. The 60 and 90 MHz $^1$H-NMR spectra were recorded on Varian T-60 and EM-390 spectrometers respectively. The 250 MHz $^1$H-NMR spectra were obtained and analyzed by Dr. Don Hughes on a Bruker WM-250 spectrometer. The elemental analyzes were performed by Galbraith Laboratories, Knoxville, Tennessee.

A Beckman HPLC apparatus was used both for analytical and preparative work. The analytical column was an Altec Ultrasphere ODS column (0.46 x 25 cm) and the preparative column was an Altec Ultrasphere ODS column (0.92 x 25 cm). The solvents used were H$_2$O (solvent A) and methanol (solvent B).

Millipore's GSWP and SCWP type nitrocellulose filters were respectively used for solute trapping and amino acid transport measurements.

Rats were housed in the animal room facilities of McMaster University. L-cells were obtained from the American Tissue Culture Collection by the laboratory of Dr. Hara P. Ghosh. These cells were maintained in liquid culture in Dulbeco's minimal essential medium containing 5% newborn calf serum. For the work with E. coli the strain MC1060 developed by Michel Casadaban (Casadaban, Martinez-Arias, Shapira, & Chou, 1983) was provided by Dr. Bialkoska-Hobranska of the
Cancer Research Group at McMaster University.

II.1 a) General procedures for the syntheses of m-diazirinophenol (6) and the photoreactive fatty acids (VII.1-VII.4)

All reagents were of commercial grade unless specified otherwise. Sodium m-periodate (NaIO₄), m-hydroxybenzaldehyde, oleic acid, vaccenic acid, erucic acid and nervonic acid were all purchased from Sigma. Chloromethyl methyl ether and OsO₄ (2.5% in 2-methyl-2-propanol) were obtained from Aldrich and were used without further treatment. μ-Bondapack C18 was a product of Waters Associates. Amersham and New England Nuclear were the sources of [³H]NaBH₄. Triphenyl phosphite methiodide was prepared by Dr. Gerhard E. Gerber according to the protocol described by Verheyden and Moffat (1970).

Lecture bottles of ammonia gas were purchased from Canadian Liquid Air. Methanol, DMF, and HMPA were of HPLC grade and were purchased from Caledon and/or J.T. Baker. Diethyl ether and petroleum ether (30-60°C) were reagent grade form J.T. Baker.

Silica plates IB2 and IB2-F from J.T. Baker were used for thin layer chromatography (TLC). Whatman's PLK5 linear-K preparative silica gel plates were used for the purification of the radioactive intermediates III.1-III.4, IV.1-IV.4, V.1-V.4, VI.1-VI.4, and VII.1-VII.4.

The TLC solvent systems consisted of: solvent system
A, diethyl ether/petroleum ether 1:1 (v/v), solvent system B, diethyl ether/petroleum ether 1:4 (v/v), solvent system C, diethyl ether/petroleum ether 3:2 (v/v), solvent system D, diethyl ether/petroleum ether 1:2 (v/v), solvent system E, diethyl ether/petroleum ether 19:1 (v/v), and solvent system F, diethylyl ether/petroleum ether 4:1 (v/v).

II.2 **Synthesis of photoreactive probe (6)**

The synthesis of the photoreactive probe m-diazirinophenol (6) and of the intermediates 2, 4 and 5 was adapted from previously published procedures (Smith & Knowles, 1975; Gupta, Radhakrishnan & Khorana, 1977). Smith and Knowles (1975) reported the successful oxidation of 1,3,5-triphenyl-2,4,6-triazabyclo[3.1.0]hexane with t-butyl hypochlorite to generate 3-phenyl-3H-diazirine. This particular approach was unsuccessful with para substituted phenyl groups (Smith & Knowles, 1975). Khorana’s group (Gupta, Radhakrishnan & Khorana, 1977) successfully synthesized the m-diazirinophenol (6), albeit in low yield, using the t-butyl hypochlorite oxidation route. In the course of repeating the synthesis reported by Gupta et al., (1977) it was found that the low yield of 6 could be explained by the generation of chlorinated isomers (Leblanc & Gerber, 1984). The addition of pyridine during the t-butyl hypochlorite oxidation alleviated the generation of these chlorinated products and resulted in improved yields.
II.2 a) *Synthesis of methoxymethylene ether of m-hydroxy benzaldehyde* (2).

The procedure used to derive the intermediate 2 has been modified from the one used by Gupta et al., 1977 (personal communication of R. R. Radhakrishnan to G. E. Geíber). To a cooled (0°C) stirred suspension of NaH (26.4 g, 1.1 mol) in DMF (400 mL), a solution of m-hydroxybenzaldehyde (122.12 g, 1 mol) in DMF (600 mL) was slowly added. After the cessation of H₂ evolution, an excess of chloromethyl methyl ether (83.5 mL, 1.1 mol) was carefully added and the reaction allowed to proceed for 2 h.

The mixture was then warmed to room temperature, the solvent removed under reduced pressure and the residual paste was partitioned between H₂O (500 mL) and diethyl ether/petroleum ether 3:2 (v/v) (500 mL). The aqueous phase was further extracted with diethyl ether/petroleum ether 3:2 (v/v) (6 x 500 mL). The pooled organic extracts were washed with H₂O (500 mL), dried (CaCl₂) and removed under reduced pressure. The oily residue was dissolved in a minimal volume of diethyl ether to which petroleum ether was added until cloudiness appeared. A typical solution consisted of diethyl ether/petroleum ether 9:1 (v/v). The solution was then subjected to chromatography on silica gel (5 x 30 cm, column) using the same solvent used to dissolve the crude oil.
Fractions enriched for the desired product ($R_f = 0.44$ in solvent system A) were pooled and the solvent evaporated under reduced pressure. The resulting oil was then dissolved in a solvent containing a higher percentage of petroleum ether in diethyl ether and subjected to chromatography with that same solvent as described above. After each chromatography, the resulting oil's solubility to solvent containing higher percentages of petroleum ether in diethyl ether increased significantly. The process of chromatography with increasing percentages of petroleum ether in diethyl ether was repeated until the oily residue could be dissolved in diethyl ether/petroleum ether 0.9:4 (v/v). Upon storage of this solution at $-20^\circ$C a white precipitate formed which left the mother liquor significantly enriched with compound 2. The pure methoxy methylene ether of m-hydroxybenzaldehyde (2) was obtained by vacuum distillation; yield: 123 g (74%); bp 125-127°C at 4 Torr (1 Torr = 133.3 Pa);

TLC: $R_f = 0.44$ in solvent system A;

$^1$H-NMR (60 MHz, CCl$_4$, TMS): $\delta = 3.23$ (s, 3H, CH$_3$-O-), 4.97 (s, 2H, -O-CH$_2$-O-), 6.87-7.27 (m, 4H, aromatic), 9.53 (s, 1H, -CHO).

II.2 b) Synthesis of 1,3,5-tri-(m-methoxymethylene phenyl ether)-2,4,6-triazabicyclo-[3.1.0]hexane (4)

Methanol (280 mL) was cooled to $-78^\circ$C with dry ice and stirred with a magnetic stirrer bar. Liquid NH$_3$ (70 mL, 2.81
mol) was added, followed by the careful addition of a freshly prepared solution of t-butylhypochlorite (Mintz & Walling, 1969) (0.30 mol) in t-butyl alcohol (37 mL) to generate chloramine. The m-methoxymethylenebenzaldehyde (2) (50 g, 0.30 mol) was added neat to the chloramine solution over a period of 20 min. The solution was allowed to warm to room temperature during a reaction time of 36 to 40 h. NH₃ was evaporated and the remaining solvent was removed under reduced pressure yielding an oily residue (4) that was not further characterized.

II.2 c) Synthesis of 3-(m-methoxymethylene phenyl ether)-3H-diazirine (5)

The diazirine 5 was synthesized in presence and absence of pyridine. For the synthesis of 5 in presence of pyridine, the triazabicyclohexane (4) was generated using 0.3 mol (50 g) of the m-methoxymethylenebenzaldehyde (2). When 5 was synthesized in absence of pyridine 0.18 mol (30 g) of 2 were used.

Synthesis of 5 in presence of pyridine: The crude triazabicyclohexane (4) was resuspended in methanol (60 mL) and pyridine (41 mL, 0.51 mol) was added to trap any HCl generated during the previous step. The stirred suspension was cooled (0°C) and freshly prepared t-butyl hypochlorite (Mintz & Walling, 1969) (0.17 mol) in t-butyl alcohol (20 mL) was added over a period of 10 min. The reaction was allowed
to proceed for 2 h at 0°C.

The mixture was carefully transferred to a 10% solution of sodium metabisulfite (1 L) and extracted with petroleum ether (4 x 1 L). The organic phase was washed with H₂O (1 L) and the solvent removed under reduced pressure. The oily residue was dissolved in DMF (40 mL) and extracted with petroleum ether (5 x 20 mL). The petroleum ether extracts were washed with H₂O (100 mL), dried (CaCl₂) and evaporated under reduced pressure. The oily residue was dissolved in a solution of 5% diethyl ether in petroleum ether (100 mL) and the sample was applied on a silica gel column (3.5 x 17.0 cm) and eluted with the same solvent. Fractions enriched in the desired diazirine (5) (Rf = 0.50 in solvent system C) were pooled and the solvent removed under reduced pressure. When required, the 3-(m-methoxymethylene phenyl ether)-3H-diazirine (5) was purified by preparative HPLC using an isocratic solvent system consisting of 55% methanol in H₂O. Fractions with absorbance at 360 nm were extracted with diethyl ether and the ethereal solution dried (CaCl₂). Upon removal of the solvent under reduced pressure, a pale yellow-green oil was obtained; yield: 4.30 g (24% from 2).

**Synthesis of 5 in absence of pyridine:** The synthesis of 5 in the absence of pyridine during the oxidation of the crude 4 was repeated starting with less of the benzaldehyde (2) (30 g, 0.18 mol). After following the same isolation
procedure as described above, a yellow-green oil was recovered; yield 1.95 g (18% from 2). Analysis of this product by HPLC using 65% methanol in water as solvent and a flow rate of 1.5 mL/min showed the presence of 4 components absorbing at 360 nm. The elution times of the products were 7.7, 11.5, 14.5 and 17.5 min respectively and the products were named 5a, 5b, 5c and 5d respectively, according to their elution times. Product 5a was shown to be the desired diazirine 5. Each product was isolated by preparative HPLC using 65% methanol in water as solvent. Fractions with absorbance at 360 nm were pooled, extracted with diethyl ether, dried (CaCl₂) and the solvent removed under reduced pressure to yield yellow-green oils. The yields, and the physical and spectral properties of compounds 5a, b, c and d are summarized as follows:

<table>
<thead>
<tr>
<th>Product</th>
<th>Yield (from 2)</th>
<th>TLC (Solvent, Rf)</th>
<th>HPLC*</th>
<th>UV cyclohexane λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.30 g, (24%) C 0.50 7.7 min</td>
<td>362 (ε 360)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>240 mg, (2%) C 0.50 7.7 min</td>
<td>362 (ε 360)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5b</td>
<td>560 mg, (4.4%) C 0.50 11.5 min</td>
<td>365 (ε 236)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5c</td>
<td>270 mg, (2.1%) C 0.50 14.4 min</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5d</td>
<td>700 mg, (5.5%) C 0.50 17.5 min</td>
<td>357 (ε 149)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Analytical using 65% methanol in H₂O and 1.5 mL/min flow rate.

<sup>1</sup>Synthesized in presence of pyridine using 0.30 mol of 2.
The infrared data of 5a, b, and d are summarized below:

Product

5a \( \nu_{\text{max}} \) (neat) = 1605, 1580, 1490, 990, 690 cm\(^{-1}\).

5b \( \nu_{\text{max}} \) (neat) = 1620, 1600, 1580, 1489, 980, 625 cm\(^{-1}\).

5c N/A

5d \( \nu_{\text{max}} \) (neat) = 1620, 1600, 1571, 1475, 920, 640 cm\(^{-1}\).

Lit. (Gupta et al., 1977) for

6 \( \nu_{\text{max}} \) (film) = 1580 (N=N) cm\(^{-1}\).

Lit. (Smith & Knowles, 1975) for 3-phenyl-3H-diazirine
\( \nu_{\text{max}} \) (film) = 1580 (N=N), 987 cm\(^{-1}\).

The \(^1\)H-NMR analyses are summarized below:

Product

5a (90 MHz, CCl\(_4\), TMS): \( \delta \) = 1.98 (s, 1H, diazirine ring),
3.38 (s, 3H, CH\(_3\)-O-),
5.03 (s, 2H, -O-CH\(_2\)-O-),
6.37-6.53 (m, 2H, aromatic),
6.83-7.23 (m, 2H, aromatic).

5b (250 MHz, CCl\(_4\), TMS): \( \delta \) = 2.03 (s, 1H, diazirine ring),
3.52 (s, 3H, CH\(_3\)-O-),
5.20 (s, 2H, -O-CH\(_2\)-O-),
6.44 (dd, 1H, J\(_{3,4}\) (ortho) = 8.2 Hz, J\(_{1,4}\) (meta) = 2.0 Hz, aromatic),
6.75 (d, 1H, J\(_{1,4}\) (meta) = 2.0 Hz, aromatic),
7.20 (d, 1H, J\(_{3,4}\) (ortho) = 8.2 Hz, aromatic).

5c (250 MHz, CCl\(_4\), TMS): \( \delta \) = 2.54 (s, 1H, diazirine ring),
3.50 (s, 3H, CH\(_3\)-O-),
5.24 (s, 2H, -O-CH\(_2\)-O-),
5.93 (dd, 1H, J\(_{2,3}\) (ortho) = 6.8 Hz, J\(_{2,4}\) (meta) = 2.5 Hz, aromatic),
7.09 (dd, 1H, J\(_{3,4}\) (ortho) = 8.3 Hz, J\(_{2,4}\) (meta) = 2.5 Hz, aromatic),
7.12 (dd, 1H, J\(_{2,3}\) (ortho) = 6.8 Hz,
J\textsubscript{3,4} (ortho) = 8.3 Hz, aromatic.

5d (250 MHz, CCl\textsubscript{4}, TMS): \(\delta = 2.45 \) (s, 1H, diazirine ring),
3.39 (s, 3H, CH\textsubscript{3}-O-),
5.02 (s, 2H, -O-CH\textsubscript{2}-O-),
5.90 (d, 1H, J\textsubscript{1,2} (meta) = 2.9 Hz, aromatic),
6.88 (dd, 1H, J\textsubscript{2,3} (ortho) = 8.8 Hz, J\textsubscript{1,2} (meta) = 2.9 Hz, aromatic),
7.55 (d, 1H, J\textsubscript{2,3} (ortho) = 8.8 Hz, aromatic).

Elemental analyses confirmed that compounds 5b and 5d were chlorinated: 5b Anal. calcd for C\textsubscript{9}H\textsubscript{9}N\textsubscript{2}ClO\textsubscript{2}: Cl 16.67; found Cl 17.78;
5d Anal. calcd for C\textsubscript{9}H\textsubscript{9}N\textsubscript{2}ClO\textsubscript{2}: Cl 16.67; found Cl 17.05.

II.2 d) Synthesis of m-diazirinophenol (6)

The unchlorinated 3-[(m-methoxymethylene phenyl ether)-3H-diazirine (5) (3.03 g, 17 mmol) was dissolved in glacial acetic acid (51 mL) followed by the addition of 1N aqueous HCl (9.58 mL). The reaction was allowed to proceed at room temperature for 2-3 h until complete, as assessed by TLC using solvent system B (R\textsubscript{f} of 5 and 6 were 0.36 and 0.24, respectively).

The solution was poured into 1 M NaHCO\textsubscript{3} (1.8 L,) and the crude m-diazirinophenol was recovered by extraction with diethyl ether (3 x 600 mL). The diethyl ether extracts were washed with H\textsubscript{2}O (300 mL), dried (CaCl\textsubscript{2}) and the solvent removed under reduced pressure. The residue was dissolved in diethyl ether/petroleum ether 5:95 (v/v) (100 mL) and subjected to chromatography on a silica gel column (5.5 x 17
cm) in that solvent system. Fractions containing 6 (Rf = 0.24 in solvent system B) were pooled, dried (CaCl₂) and the solvent removed under reduced pressure.

Further purification was achieved by preparative HPLC using 25% methanol in H₂O as solvent. The m-diazirinophenol (6) (150 mg) was dissolved in methanol (150 μL) followed by addition of H₂O until the sample showed some cloudiness (final volume never exceeded 2 mL). The solution was aspirated in the injection loop (2 mL) and the sample was slowly injected (1 mL/min) for the first 5 min. Then, a flow gradient to a final rate of 5 mL/min was established over a period of 5 min. Fractions were collected every minute.

Fractions absorbing at 360 nm were pooled, extracted with diethyl ether, dried (CaCl₂) and the solvent removed under reduced pressure. These steps were repeated until all of the crude 6 was purified as an oil; yield: 3.0 g (90%); TLC: Rf = 0.24 in solvent system B;
UV (cyclohexane): λmax = 362 nm (ε 360); 
Lit. (Gupta et al., 1977) UV (cyclohexane): λmax = 358 nm (ε 265); 
Lit. (Smith & Knowles, 1975) for 3-phenyl-3H-diazirine UV (hexane): λmax = 253 nm (ε 294), 259 nm (ε 314), 265 nm (ε 371), 272 nm (ε 274), 276 nm (ε 220), 344 nm (ε 179), 354 nm (ε 230), 362 nm (ε 299), 373 nm (ε 230), 382 nm (ε 245); 
¹H-NMR (250 MHz, CDCl₃, TMS): δ = 1.98 (s, 1H, diazirine
5.47 (s, broad, 1H, aromatic –OH),
6.32 (dd, 1H, J₁,₂ (meta) = 1.6 Hz, J₁,₄ (meta) = 2.6 Hz, aromatic),
6.49 (ddd, 1H, J₂,₃ (ortho) = 7.7 Hz, J₁,₂ (meta) = 1.6 Hz, J₂,₄ (meta) = 1.0 Hz, aromatic),
6.76 (ddd, 1H, J₃,₄ (ortho) = 8.1 Hz, J₁,₄ (meta) = 2.6 Hz, J₂,₄ (meta) = 1.0 Hz, aromatic),
7.16 (t, 1H, J₂,₃ = J₃,₄ (ortho) = 7.9 Hz, aromatic);
Lit. (Gupta et al., 1977) ¹H-NMR (CDCl₃): δ = 2.4 (s, 1H, diazirine ring),
5.6–7.2 (m, 5H, one proton loss upon D₂O shake);
Lit. (Smith & Knowles, 1975) for 3-phenyl-3H-diazirine ¹H-NMR (DMSO): δ = 1.75 (s, 1H, diazirine ring),
6.68 (d, 2H, irregular, aryl),
7.05 (t, 3H, irregular, aryl).

II.3 Synthesis of homologous series of radioactive photoactive fatty acids (VII.1–VII.4)

The synthetic approach consisted of oxidizing unsaturated fatty acids containing a double bond at the desired position, followed by reduction with [³H]NaBH₄ and coupling of the phenolic oxygen of m-diazirinophenol (6) at the omega carbon of the tritiated omega hydroxy fatty acids. Oleic acid, vaccenic acid, erucic acid and nervonic acid were used as precursors of the homologous series.
Typical syntheses consisted of using \(^{3}H\)NaBH\(_{4}\) (100 mCi) of varying specific activities from 5 to 60 Ci/mmol. The resulting radioactive photoreactive fatty acids (VII.1-VII.4) therefore varied in specific activity. The latter was established using the extinction coefficient found for the phenyl diazirine at 362 nm. The specific activity of intermediates III to V was estimated based on the initial specific activity of \(^{3}H\)NaBH\(_{4}\). All the radioactive intermediates were subjected to preparative TLC, detected by autoradiography and eluted with diethyl ether. The yields were recorded in terms of radioactivity recovered after elution from the plates. When the fatty acid semialdehydes (II.1-II.4) were analyzed by TLC they were detected by iodine vapour.

The characterization of the homologous series of radioactive photoreactive fatty acid methyl esters (VI.1-VI.4) by HPLC was done using an Altec Ultrasphere ODS column (0.46 x 25 cm). The solvents used were H\(_{2}\)O (solvent A) and methanol (solvent B). The column was equilibrated in 80% solvent B and the analysis performed using a linear gradient from 80% to 95% solvent B over 25 min at a flow rate of 2 mL/min. Fractions (200 µL) were collected and the radioactivity detected by liquid scintillation counting.

II.3 a) **Synthesis of fatty acid semialdehydes (II.1-II.4)**

In a round bottom flask (250 mL), the olefin
(I.1-I.4) (1 mmol) was dissolved in dioxane/water 4:1 (v/v) (10 mL) followed by the addition of aqueous NaOH (1.1 mL, 1.1 mmol). The reaction was started by the addition of a 2.5% solution of OsO₄ (0.64 mL, 2.0 mmol) in butanol. Solid NaIO₄ (2 g, 9.4 mmol) was added to the black solution followed by another 2 g addition 5 min later. The flask was capped and the biphasic solution placed on a shaker (vigourous shaking) away from light. After an hour, another portion of NaIO₄ (2 g, 9.4 mmol) was added and the reaction allowed to continue for a total of 3 h.

Then the solvent was evaporated under reduced pressure, and the residue resuspended in H₂O (5 mL) and transferred (4 x 5 mL) to a Teflon-lined screwcap tube (30 mL volume). The aqueous suspension was extracted with petroleum ether (5 x 10 mL). The petroleum ether extracts which contained the desired semialdehyde and the toxic OsO₄ were dried carefully under reduced pressure and the residue resuspended in H₂O (3 mL). The solution was made alkaline with 2 M Na₂CO₃ (1 mL) and extracted with petroleum ether (3 x 2 mL). The remaining aqueous solution was adjusted to pH 3.0-4.0 with aqueous HCl (4 mL, 4 mmol) and extracted with petroleum ether/diethyl ether 1:1 (v/v) (4 x 2 mL). The ether phase was washed with H₂O (2 x 1 mL) and the desired semialdehyde (II.1-II.4) recovered by removing the solvent under reduced pressure; (II.1) yield: 117 mg (66%); ¹H-NMR
(60 MHz, CCl₄, TMS) δ: 1.2-1.7 (m, 10H -CH₂- of C-3 to C-7),
2.2-2.6 (m, 4H, -CH₂- of C-2 and C-8), 9.45 (s, 1H, -CHO),
10.75 (s, 1H, -COOH);

(II.2) yield: 140 mg (70%); ¹H-NMR (60 MHz, CCl₄, TMS) δ:
1.2-1.7 (m, 14H -CH₂- of C-3 to C-9), 2.2-2.6 (m, 4H, -CH₂-
of C-2 and C-10), 9.45 (s, 1H, -CHO), 10.75 (s, 1H, -COOH);

(II.3) yield: 194 mg (85%); ¹H-NMR (60 MHz, CCl₄, TMS) δ:
1.2-1.7 (m, 18H -CH₂- of C-3 to C-11), 2.2-2.6 (m, 4H, -CH₂-
of C-2 and C-12), 9.45 (s, 1H, -CHO), 10.75 (s, 1H, -COOH);

(II.4) yield: 197 mg (77%); ¹H-NMR (60 MHz, CCl₄, TMS) δ:
1.2-1.7 (m, 22H -CH₂- of C-3 to C-13), 2.2-2.6 (m, 4H, -CH₂-
of C-2 and C-14), 9.45 (s, 1H, -CHO), 10.75 (s, 1H, -COOH).

II.3 b) Reduction of semialdehydes (II.1-II.4) with

[³H]NaBH₄

The reaction was routinely performed on a 100 mCi scale using specific activities in the range of 5 to 60 Ci/mmol of [³H]NaBH₄. The sealed ampoule of [³H]NaBH₄ (100 mCi) was opened and rinsed with a solution of semialdehyde (II.1-II.4) (1.1 equivalents to the amount of [³H]NaBH₄, titrated to pH 10 with 1 N NaOH) in ethanol (100 μL). Further additions of similar amounts of semialdehyde (II.1-II.4) were performed at 30 min intervals until no bubbling could be observed upon transfer of an aliquot to an acidic solution of diethyl ether.

The reduced product was recovered by acidification
with 1 N HCl to pH 2.0 and extraction into diethyl ether (4 x 5 mL). The extracts were washed with H₂O (5 mL) and the solvent evaporated under a stream of N₂. The residue was then subjected to preparative chromatography on silica gel plates using solvent system E; (III.1) yield: 50 mCi; (III.2) yield: 71 mCi; (III.3) yield: 65 mCi; (III.4) yield: 66 mCi.

II.3 c) Esterification of [ω⁻³H]-ω-hydroxy fatty acids

(III.1-III.4)

The [ω⁻³H]-ω-hydroxy methyl ester (IV.1-IV.4) was obtained by reacting the [ω⁻³H]-ω-hydroxy fatty acid (III.1-III.4) with an excess of diazomethane in diethyl ether. Typically, 1-methyl-3-nitro-1-nitrosoguanidine (1 g, 6.8 mmol) was resuspended in H₂O/diethyl ether 1:1 (v/v) (10 mL) and cooled to 0°C. The slow addition of aqueous NaOH (2 mL, 10 mmol) generated the yellow diazomethane which was trapped in the diethyl ether upper phase. The diazomethane was then distilled into a tube containing cooled (0°C) diethyl ether (5 mL). The diazomethane solution (5 mL) was then added to the [ω⁻³H]-ω-hydroxy fatty acid (III.1-III.4) that had been dried at the bottom of a tube with a stream of N₂. The reaction was allowed to proceed at room temperature, away from light, for 5-10 min.

The solvent and diazomethane were removed under a stream of N₂ and the desired methyl ester (IV.1-IV.4) was purified by preparative chromatography on silica gel plate
using solvent system C; (IV.1) yield: 45 mCi; TLC (preparative): \( R_f = 0.14 \) in solvent system C; (IV.2) yield: 64 mCi; TLC (preparative): \( R_f = 0.16 \) in solvent system C; (IV.3) yield: 59 mCi; TLC (preparative): \( R_f = 0.25 \) in solvent system C; (IV.4) yield: 59 mCi; TLC (preparative): \( R_f = 0.24 \) in solvent system C.

II.3 d) Synthesis of \([\omega-^3H]-\omega\)-iodo fatty acid methyl esters (V.1-V.4)

The dry methyl ester (IV.1-IV.4) was mixed with a 10-fold excess of triphenyl phosphite methiodide in dry DMF (1 mL/mmol of triphenyl phosphite methiodide) and the reaction allowed to proceed for 4 h at room temperature shielded from light.

The solution was then transferred to a sealed column (1.5 x 10 cm) containing 200 mg of \( \mu \)-Bondapack C18. Water was added to the column until the solution became 25% DMF in \( H_2O \). The seal was broken and the column eluted extensively with \( H_2O \). The desired \([\omega-^3H]-\omega\)-iodo fatty acid methyl ester (V.1-V.4) was eluted with a solution of methanol/diethyl ether 1:1 (v/v) or when dealing with very high specific activity material the methyl ester was eluted only with diethyl ether. Fractions containing the bulk of the radioactivity were pooled, the solvent removed under a stream of \( N_2 \) and the residue subjected to preparative thin layer chromatography on silica gel using solvent system C; (V.1)
yield: 37 mCi; TLC (preparative): \( R_f = 0.62 \) in solvent system C; \(^1\text{H-NMR}\) (90 MHz, CCl\(_4\), TMS): \( \delta = 1.25-1.84 \) (m, 12H, \(-\text{CH}_2-,\) C-3 to C-8), 2.15 (t, 2H, \( J = 7.5 \text{ Hz}, \) \(-\text{CH}_2-,\) C-2), 3.1 (t, 2H, \( J = 6.75 \text{ Hz}, \) \(-\text{CH}_2-,\) C-9), 3.55 (s, 3H, \(-\text{COOCH}_3\));

(V.2) yield: 57 mCi; TLC (preparative): \( R_f = 0.65 \) in solvent system C; \(^1\text{H-NMR}\) (90 MHz, CCl\(_4\), TMS): \( \delta = 1.25-1.84 \) (m, 16H, \(-\text{CH}_2-,\) C-3 to C-10), 2.15 (t, 2H, \( J = 7.5 \text{ Hz}, \) \(-\text{CH}_2-,\) C-2), 3.1 (t, 2H, \( J = 6.75 \text{ Hz}, \) \(-\text{CH}_2-,\) C-11), 3.55 (s, 3H, \(-\text{COOCH}_3\));

(V.3) yield: 51.6 mCi; TLC (preparative): \( R_f = 0.73 \) in solvent system C; \(^1\text{H-NMR}\) (90 MHz, CCl\(_4\), TMS): \( \delta = 1.25-1.85 \) (m, 20H, \(-\text{CH}_2-,\) C-3 to C-12), 2.15 (t, 2H, \( J = 7.5 \text{ Hz}, \) \(-\text{CH}_2-,\) C-2), 3.1 (t, 2H, \( J = 6.75 \text{ Hz}, \) \(-\text{CH}_2-,\) C-13), 3.55 (s, 3H, \(-\text{COOCH}_3\));

(V.4) yield: 53 mCi; TLC (preparative): \( R_f = 0.72 \) in solvent system C; \(^1\text{H-NMR}\) (90 MHz, CCl\(_4\), TMS): \( \delta = 1.25-1.85 \) (m, 24H, \(-\text{CH}_2-,\) C-3 to C-14), 2.15 (t, 2H, \( J = 7.5 \text{ Hz}, \) \(-\text{CH}_2-,\) C-2), 3.05 (t, 2H, \( J = 6.75 \text{ Hz}, \) \(-\text{CH}_2-,\) C-15), 3.5 (s, 3H, \(-\text{COOCH}_3\)).

II.3 e) Coupling of m-diazirinophenol (6) at the omega carbon of iodo fatty acid methyl esters (V.1-V.4)

Based on the expected theoretical specific activity of the iodo fatty acid methyl ester (V.1-V.4) a solution of the sodium salt of the phenolic diazirine (6) (1.1 equivalents) in HMPA (10 mL/mmol of phenolic diazirine 6) was added to the \([\omega-^3\text{H}]-\omega\)-iodo fatty acid methyl ester (V.1-V.4) that had been dried at the bottom of a tube. The reaction was allowed
to proceed for 1 h at room temperature shielded from light.

The solution containing the DAP-fatty acid ester (VI.1-VI.4) was transferred to a sealed column (1.5 x 10 cm) containing 200 mg of μ-Bondapack C18. The solution was made 25% HMPA in H2O and the column was eluted extensively with H2O. The desired DAP-fatty acid ester (VI.1-VI.4) was eluted with diethyl ether and fractions containing the bulk of the radioactivity were pooled and the solvent removed under a stream of N2. The residue was subjected to preparative thin layer chromatography using solvent system C; (VI.1) yield: 24 mCi; S.A. 2000 mCi/mmole; HPLC (analytical): \( t_R = 7.6 \text{ min} \); UV (cyclohexane): \( \lambda_{\text{max}} = 362 \text{ (360), 381 (250)} \); \(^1\text{H-NMR (90 MHz, CCl}_4, \text{TMS)}: \delta = 1.1-1.75 \text{ (m, 12H, } -\text{CH}_2-, \text{ C-3 to C-8)}, 1.80 \text{ (s, 1H, diazirine ring)}, 2.1 \text{ (t, 2H, } J = 7.5 \text{ Hz, } -\text{CH}_2-, \text{ C-2)}, 3.5 \text{ (s, 3H, } -\text{COOCH}_3), 3.72 \text{ (t, 2H, } J = 6.0 \text{ Hz, } -\text{CH}_2-, \text{ C-9)}, 6.1-7.10 \text{ (m, 4H, aromatic)}; \)

(VI.2) yield: 52 mCi; S.A. 994 mCi/mmole; HPLC (analytical): \( t_R = 12.8 \text{ min} \); UV (cyclohexane): \( \lambda_{\text{max}} = 362 \text{ (ε360), 381 (ε250)} \); (Lit. (Gupta et al., 1977) UV (cyclohexane): \( \lambda_{\text{max}} = 358 \text{ nm (ε286)} \); \(^1\text{H-NMR (90 MHz, CCl}_4, \text{TMS)}: \delta = 1.05-1.75 \text{ (m, 16H, } -\text{CH}_2-, \text{ C-3 to C-10)}, 1.80 \text{ (s, 1H, diazirine ring)}, 2.1 \text{ (t, 2H, } J = 7.5 \text{ Hz, } -\text{CH}_2-, \text{ C-2)}, 3.5 \text{ (s, 3H, } -\text{COOCH}_3), 3.75 \text{ (t, 2H, } J = 6.0 \text{ Hz, } -\text{CH}_2-, \text{ C-11)}, 6.1-7.10 \text{ (m, 4H, aromatic)}; \)

(Lit. (Gupta et al., 1977) \(^1\text{H-NMR (CDCl}_3): \delta = 3.5 \text{ (s, 3H), 3.8 (t, 2H, } J = 7 \text{ Hz), 5.65 (d, 1H, } J = 2 \text{ Hz), 6.3-7.2 (m, }\)
3H));

(VI.3) yield: 47 mCi; S.A. 436 mCi/mmol; HPLC (analytical):
\( t_r = 16.9 \ \text{min}; \) UV (cyclohexane): \( \lambda_{\text{max}} = 362 (\varepsilon 360), 381 (\varepsilon 250); \)
\( ^1H-\text{NMR} (90 \ \text{MHz}, \ \text{CDCl}_3, \ \text{TMS}): \delta = 1.1-1.80 \ (m, \ 20H, \)
\(-\text{CH}_2-, \ \text{C-3 to C-12}, \ 1.85 \ (s, \ 1H, \ \text{diazirine ring}), \ 2.15 \ (t, \)
\(2H, \ J = 7.5 \ \text{Hz}, \ -\text{CH}_2-, \ \text{C-2}, \ 3.52 \ (s, \ 3H, \ -\text{COOCH}_3), \ 3.80 \ (t, \)
\(2H, \ J = 6.0 \ \text{Hz}, \ -\text{CH}_2-, \ \text{C-13}, \ 6.15-7.18 \ (m, \ 4H, \ \text{aromatic}); \)

(VI.4) yield: 40 mCi; S.A. 485 mCi/mmol; HPLC (analytical):
\( t_r = 22.2 \ \text{min}; \) UV (cyclohexane): \( \lambda_{\text{max}} = 362 (\varepsilon 360), 381 (\varepsilon 250); \)
\( ^1H-\text{NMR} (90 \ \text{MHz}, \ \text{CDCl}_3, \ \text{TMS}): \delta = 1.1-1.80 \ (m, \ 24H, \)
\(-\text{CH}_2-, \ \text{C-3 to C-14}, \ 1.85 \ (s, \ 1H, \ \text{diazirine ring}), \ 2.2 \ (t, \)
\(2H, \ J = 7.5 \ \text{Hz}, \ -\text{CH}_2-, \ \text{C-2}, \ 3.52 \ (s, \ 3H, \ -\text{COOCH}_3), \ 3.82 \ (t, \)
\(2H, \ J = 6.0 \ \text{Hz}, \ -\text{CH}_2-, \ \text{C-15}, \ 6.15-7.20 \ (m, \ 4H, \ \text{aromatic}). \)

II.3 f) Saponification of \( ^{1-2H}_3 \omega-\text{DAP fatty acid methyl esters (VI.1-VI.4)} \)

The DAP-fatty ester (VI.1-VI.4) was dried at the bottom of a 15 mL screw cap tube and an excess of KOH (10 \ 
\( \mu \text{mol}/\mu \text{mol of VI.1-VI.4} \)) in 95% ethanol (10 \ \mu\text{L}/\mu\text{mol of KOH}) was added. The saponification was allowed to proceed at room temperature for 8 h (shielded from light). The solution was then acidified by adding aqueous HCl (2 equivalents/equivalent of KOH). The solvent was evaporated with a stream of N\(_2\) (this was omitted when dealing with high specific activity material > 10 Ci/mmol), the residue resuspended in H\(_2\)O (2 mL) and the solution extracted with
diethyl ether (4 x 2 mL). The ether extracts were washed with H$_2$O (2 mL) and removed under a stream of N$_2$. The desired [ω-3H]-ω-DAP fatty acid (VII.1-VII.4) was purified by preparative TLC using solvent system F; (VII.1) yield: 13.5 mCi; S.A. 2000 mCi/mmol; TLC (preparative): R$_f$ = 0.47 in solvent system F; (VII.2) yield: 34.0 mCi; S.A. 994 mCi/mmol; TLC (preparative): R$_f$ = 0.54 in solvent system F; (VII.3) yield: 33 mCi; S.A. 436 mCi/mmol; TLC (preparative): R$_f$ = 0.68 in solvent system F; (VII.4) yield: 20 mCi; S.A. 485 mCi/mmol; TLC (preparative): R$_f$ = 0.67 in solvent system F.

II.4 Detection of radioactivity

The high levels of tritium (>10 mCi) used during the synthesis of the homologous series were detected directly by autoradiography. A film (Kodak X-AR-5) was placed on top of the dry preparative or thin layer silica gel plate and enclosed in a film holder. The film was exposed at -20°C or -70°C for periods of time as short as 2 h or as long as 12 h. The position of the origin and the solvent front were identified with radioactive ink spots made after chromatography. Detection of low levels of radioactivity on thin layer chromatography was by the fluorographic method developed by Randerath (1970). Briefly the method consisted in dipping TLC plates in a 7% (w/v) solution of 2,5-diphenyloxazole in diethyl ether, followed by placing a film (Kodak X-AR-5) on top of the dried plate and placing
them in a film holder. The film holder was then sandwiched between 2 wood panels (15 x 15 cm) that were pressed together with metal clamps. The films were exposed at -70°C for various periods of time.

The quantitative determination of radioactivity of silica gel scrapings, filters, dissolved gels and liquid samples was carried out using ACS (Amersham) scintillation fluid and a Beckman LS 7800 liquid scintillation counter. Volumes of scintillation fluid varied between 6 and 18 mL per sample. Typically liquid aliquots of less than 1 mL were mixed with 6 mL of ACS, all larger volumes were dissolved in 15 mL of ACS.

II.4 a) Determination of radioactivity from polyacrylamide gels

Polyacrylamide gels were prepared as described by Laemmli (1970) with the exception that N,N'-diallyltartramide DATD was used as crosslinker instead of bis-acrylamide. Radioactive samples were applied on the gels and electrophoresis proceeded until the tracking dye (bromophenol blue) reached the bottom of the gel. At this point the gels were either stained with Coomassie brilliant blue (followed by destaining) or with silver reagent. The region(s) of interest was (were) sliced and allowed to dissolve in 1 mL 10% periodic acid. Once the gel slices had been dissolved the radioactivity was determined by liquid scintillation counting
using 15 mL ACS per sample.

Alternatively the gel was prepared in accordance with Laemmli's procedure and after electrophoresis the gel was soaked in 20 volumes of DMSO for 30 min on an agitator (Laskey & Mills, 1975). Then, fresh DMSO was added for another 30 min incubation period. At this point the gel was soaked in a DMSO solution containing 22.5% (w/v) of 2,5 diphenyloxazole (PPO) for a period of 3 h. After removal of the PPO/DMSO solution the gel was swollen in water on an agitator for 1 h and then dried in a gel drier. A film (Kodak X-AR-5) was placed on top of the dried gel and then both were placed in a film holder which was put between 2 wood plates as described above. The film was exposed at -70°C for various periods of time.

II.5 Preparation of microsomal fractions

Microsomes were obtained by the procedure described by Suzue and Marcel (1972). Rats (Wistar or Sprague-Dawley) were fasted for 12 h before being sacrificed. The livers were rapidly removed and cooled (0-4°C) in 0.25 M sucrose. The livers were subsequently sliced, drained, and then homogenized using a Potter-type homogenizer (three strokes) in four volumes of 0.25 M sucrose. The homogenate was centrifuged for 20 min at 20,000 x g. The supernatant was collected and centrifuged at 100,000 x g for 60 min. The microsomal pellets were resuspended in 0.25 M sucrose and
recenterfuged at 100,000 x g for 1 h. The washed microsomes were resuspended in 0.25 M sucrose containing dithiothreitol (1 mg/mL). Aliquots (1 mL) were kept at -20°C under nitrogen. Protein determination was performed as described later in this section.

II.6 Assay of acyl-CoA synthetase

The assay, which was developed by Dole (1956) for the measurement of non-esterified fatty acids in plasma, has been first described by Samuel and Ailhaud (1969) for the detection of acyl-CoA synthetase acitivity in bacteria. It is based on the insolubility of acyl-CoA derivatives in organic solvents such as diethyl ether and heptane. The standard assay incubation contained the potassium salts of the [3H]fatty acids (25 \text{ \mu M}) in 150 mM Tris-HCl buffer (pH 7.4), 200 mM KCl, 2 mM dithiothreitol, 15 mM MgCl$_2$, 8 mM ATP, 2.4 mM CoA, and 1 mg/mL Triton X-100. After a brief preincubation (2 min) at 37°C, the reaction was started by the addition of microsomes (14 \mu g of microsomal protein/mL) and the incubation continued at the same temperature. The reaction was terminated by the transfer of an aliquot (100 \mu L) to a silanized tube containing 500 \mu L of extraction medium (isopropyl alcohol/heptane/1 N H$_2$SO$_4$, 40:10:1 by volume). Phase separation was obtained by the addition of 300 \mu L of heptane and 300 \mu L of water. The tubes were agitated using a vortex mixer and the organic layer was discarded. Four
additional extractions were performed with 1 mL of diethyl ether. The resulting aqueous phase was transferred directly to a scintillation vial and the radioactivity was determined by liquid scintillation counting. Control incubations in the absence of either ATP or CoA were carried out with each experiment.

To verify that the aqueous phase contained the freshly synthesized acyl-CoAs it was subjected to paper chromatography and thin-layer chromatography as described by Seubert (1960). An aliquot of the aqueous phase was applied to Whatman 3 MM paper and developed using the solvent system n-butanol/acetic acid/water (5:2:3 by volume). The entire paper was cut into 1-cm strips and the radioactivity was determined by liquid scintillation counting. Alternatively, aliquots of the aqueous phase were applied to the preadsorbant on Whatman silica TLC plates, developed using the same solvent system, and the radioactivity was visualized by fluorography and quantitated as described above. A marker of authentic palmitoyl-CoA was run in each case and visualized under ultraviolet light.

II.7 Phospholipid extraction

The extraction procedure was adapted from the one described by Bligh and Dyer (1959). A typical extraction on a 1 mL aqueous volume consisted of adding 3.75 mL of a solution of methanol/chloroform (2:1) with mixing. This yielded a
single phase solution which was subsequently centrifuged in order to pellet the precipitated proteins present in the solution. The supernatant was transferred to another tube and then 4.75 mL of methanol/chloroform/water (2:1:0.8) were added to the pellet to extract remaining lipids. After mixing and centrifugation the supernatant was added to the previous one followed by addition of 2.75 mL of chloroform and 2.75 mL of water to effect a phase separation. At this point the organic layer was transferred to another tube and washed with 9.75 mL of water with 200 μL 2.5 N NaCl. After centrifugation the organic layer was kept on ice while the aqueous supernatant was kept for further washings. To the aqueous layer obtained during the phase separation 5.25 mL of chloroform were added and the mixture mixed and centrifuged. The resulting organic layer was washed with the aqueous supernatant that was kept aside and after mixing and centrifugation the organic layer was added to the former one. The organic solvent pool was then dried and the residue dissolved in a solution of methanol/chloroform (1:1) and kept at -20°C under nitrogen.

II.8 Methanolation of phospholipid samples

Typically an aliquot of phospholipid or protein extract was dried in a silanized tube and dissolved in 0.1 M methanolic KOH and allowed to remain at room temperature for 30 min. To ensure completion of the reaction aliquots were
withdrawn at various time intervals and chromatographed on thin layer silica gel plates in diethyl ether petroleum ether (1:4). The methyl esters were detected by iodine vapor and their position circled. The plates were allowed to discolor and the circled area were scraped and transferred into scintillation vials containing ACS, for determination of radioactivity. The reaction was deemed complete when no increase in radioactivity in the methyl ester region was observed.

Upon completion of the reaction the methyl esters were recovered by three extractions with an equal volume of petroleum ether. The ethereal extracts were dried and the residue dissolved in methanol and kept at -20°C for further analyses.

II.9 Isolation of E. coli vesicles

An inoculum from frozen stock of E. coli MC1060 was grown to late log phase in minimal E medium (below) containing 5 mM potassium oleate, 5 mg/mL Brij 58, and 5 µg/mL thiamine. The cells were diluted 80-fold into fresh medium containing the above mentioned additives, allowed to grow to late log phase and diluted 80-fold in fresh medium before being used for membrane preparation. Growth was monitored by measuring the optical density of an aliquot of the culture at 600 nm using fresh medium as a blank.

The minimal E medium was prepared as described by
Vogel and Bonner (1956). A 50 X stock was made by dissolving successively the following in distilled water (670 mL): MgSO\(_4\).7H\(_2\)O, 10 grams, citric acid·H\(_2\)O, 100 grams, K\(_2\)HPO\(_4\) anhydrous, 500 grams, and NaNH\(_4\)HPO\(_4\).4H\(_2\)O, 175 grams for a final volume of 1 liter. Chloroform (10 mL) was added as a preservative. For use in culture the stock was diluted 50-fold and then sterilized. For growth using potassium oleate the medium was adjusted to pH 7.4 using 10 N KOH, prior to sterilization. Note that none of the additives such as the carbon source, thiamine or Brij 58 were present in the medium to be autoclaved. The latter were filter sterilized as a 10% Brij 58 solution in minimal E medium. The stock solution was made by mixing Brij 58 and oleic acid into sterilized minimal E medium. The solution was brought to pH 7.4 (the solution turned from milky to transparent) and then filtered using the smallest pore size sterile filter. Thiamin could be added to the stock or made as a filter stock of its own.

The late log phase culture was centrifuged at 16,000 x g for 30 min and the pellet washed twice in 10 mM Tris-HCl pH 8.0 at 0°C. Alternatively, the pellet could be washed first by resuspending the latter in fresh medium containing Brij 58 (without oleate and thiamine) to remove free fatty acids. This pre-wash cycle would be repeated twice to remove free oleate followed by the usual wash in Tris-HCl pH 8.0. The
washed pellet was resuspended at room temperature in 30 mM Tris-HCl pH 8.0 containing 20% sucrose at a concentration of 1 g wet cells per 80 mL and mixed using a magnetic stirrer bar. An aliquot of 25 mg/mL lysozyme in 0.5 M potassium EDTA pH 7.0 was added to a final concentration of 0.5 mg/mL and 10 mM respectively, and the suspension was incubated at room temperature for 30 min. The spheroplasts obtained from the latter procedure were isolated by centrifugation at 16,000 x g for 30 min at 0°C. The pellet was resuspended in the smallest volume of 0.1 M KH₂PO₄ pH 6.6 containing 20% sucrose and 20 mM MgSO₄ and was homogenized with a teflon pestle at 0°C. The homogenization was facilitated by addition of DNAase I to a final concentration of 5 mg/mL.

The spheroplasts were lysed by transferring the homogenate to 500 volumes of 50 mM KH₂PO₄ pH 6.6 at 37°C and continuing the incubation for 15 min at 200 rpm in the incubator. Then, 0.5 M potassium EDTA pH 7.0 was added to a final concentration of 10 mM and incubated for 15 min with shaking at 37°C. Finally an aliquot of 1 M MgSO₄ was added to a final concentration of 15 mM and the suspension incubated as above for another 15 min. The crude membranes were isolated by centrifugation at 16,000 x g for 30 min at 0°C. The pellet was resuspended in 0.1 M KH₂PO₄ pH 6.6 containing 10 mM MgSO₄ using a 15 gauge needle. This step was reported to yield better membrane preparations in terms of enzymic
activity than the earlier procedure described by Kaback (1971).

The resuspended membranes were spun at 800 x g for 30 min in an SS34 Sorval rotor. The supernatant was carefully decanted and centrifuged at 45,000 x g for 30 min. The 800 x g pellet was resuspended in the same buffer using the 15 gauge needle and subjected to centrifugation at 800 x g. The supernatant was centrifuged at 45,000 x g as described earlier. To maximize the yield of membranes the 800 x g pellet was rehomogenized until its supernatant yielded no significant pellet upon centrifugation at 45,000 x g. The 45,000 x g pellets were washed by resuspension in 0.1 M KH$_2$PO$_4$ buffer pH 6.6 containing 10 mM MgSO$_4$, using the 15 gauge needle and centrifuged at 800 x g. The supernatant was then subjected to another centrifugation at 45,000 x g. The pellets were washed until very little material sedimented at 800 x g.

The final pellet was resuspended in 0.1 M KH$_2$PO$_4$ pH 6.6 to a final concentration of 10 mg/mL and 500 µL aliquots were kept frozen in liquid N$_2$. The amino acid transport activity was found to be stable for an indefinite period by Kaback (1971). In my hands the activity was stable for at least 3 months.

II.10 Assay of amino acid transport in E. coli vesicles

In a small silanized tube 25 µL of vesicles (stock 10
mg/mL) were mixed with 25 μL of 20 mM MgSO₄, and incubated at 37°C for 1 min. Then 10 μL of 140 mM sodium D-lactate were immediately added followed by 10 μL of 70 μM [¹⁴C]valine (280 mCi/mmol in 600 μL of 50 mM KH₂PO₄ pH 6.6 - 6.8) and the incubation continued at 37°C. The incubations were stopped by adding 2 mL of 0.11 M LiCl and the solution was immediately filtered on pre-wetted SCWP nitro cellulose filters followed by a rinse with another 2 mL of 0.11 M LiCl. Blanks consisted of adding 2 mL of 0.11 M LiCl after the one minute incubation, followed by addition of 10 μL of 70 μM [¹⁴C]valine (280 mCi/mmol) (note that no lactate was added in blanks) and immediate filtration. After the second 2 mL of 0.11 M LiCl had filtered through, the chimney of the filter apparatus was removed and the sides of the filter were rinsed with 2 mL of 0.11 M LiCl (in a Pasteur pipette) and the filter was quickly removed and put in a 15 mL scintillation vial, and allowed to dissolve in ACS. After each filtration the filter apparatus was rinsed with water and 95% ethanol (3 times). The vacuum was off when placing the filter in position and then turned on only when the sample was ready for filtration.

All samples (blanks and time points) were done in duplicate. The assay was usually linear for 2 minutes. Typical time points were 15 s, 30 s, 45 s, 60 s, 90 s, 120 s. II.11 Photolysis conditions
Photolysis was carried out at 360 nm in a silanized quartz cell (1-cm path length) located in a metal cell holder at the focal point of a 1000-watt xenon/mercury lamp (Schoeffel, model LPS 255HR). The light was passed through a filter (Corning 7-51) in a water-filled optical glass cell (5 x 5 cm), and a second filter (Corning 7-51) was attached to the window of the metal cell housing. The cell was flushed with a constant flow of N₂. Samples were photolyzed for time periods varying between 5 s and 10 min as indicated. Most photolysis were of 20 s duration.

II.12 NMR analyses

The samples were dried from organic solvents and dissolved in carbon tetrachloride or deuterated chloroform at a concentration range of 30 to 200 mg/mL. Samples were then analyzed using a 60 MHz or a 90 MHz nuclear magnetic resonance spectrometer. Analyses of samples at 250 MHz were performed by Dr. Don Hughes.

II.13 HPLC analysis of homologous series

The methyl esters of the fatty acids were prepared either by mixing an ethereal solution of the acids with diazomethane or by methanolysis of a phospholipid extract obtained by the Bligh Dyer procedure described above. The samples were dissolved in methanol and aliquots (20-100 μL) were analyzed on a Beckman HPLC system. The analytical column consisted of an Altec Ultrasphere ODS column (0.46 x 25 cm).
The solvents used were H₂O (solvent A) and methanol (solvent B). The column was equilibrated in 80% solvent B and the analysis performed using a linear gradient from 80% to 95% solvent B over 25 min at a flow rate of 2 mL/min. Fractions were collected every minute and the radioactivity determined by liquid scintillation counting as described.

II.14 Preparation of lecithin in MOPS

The dry soybean lecithin was weighed and 10 mM MOPS pH 7.4 added to obtain the desired final concentration (50 to 120 mg/mL). The solution was kept under N₂ and the lipids allowed to hydrate in the cold for 4 h. The mixture was then sonicated until clear in a bath type sonicator, and freeze/thawed once. Liquid N₂ was used for freezing and the lipids were thawed in a water bath at room temperature. Stock lipids were kept at -20°C under N₂.

II.15 Filter assay of encapsulation

Nitrocellulose filters were pre-wetted in distilled water. The assay consisted of mixing a known amount of freeze/thawed lipids with a fixed amount of [³H]sucrose (S.A. 0.1 Ci/mmol) and/or [¹⁴C]BSA (S.A. 12.2 mCi/mmol) and a final concentration of 150 mM NaCl in 10 mM MOPS pH 7.4 followed by freezing in liquid N₂. To perform the assay the frozen mixture was thawed in a water bath equilibrated at room temperature, sonicated 5 s followed by dilution to 2 mL with 10 mM MOPS pH 7.4. At this point the solution was filtered
and the filter rinsed with 2 mL 10 mM MOPS pH 7.4, the chimney removed and the filter washed with 2 mL of buffer to remove material trapped under the chimney. The filter was immediately removed and allowed to dissolve in aqueous counting scintillation liquid (ACS). Radioactivity was determined by liquid scintillation counting on a Beckman LS 7800 scintillation counter.

II.16 Determination of internal volumes of freeze/thawed vesicles

The internal volume of freeze/thawed vesicles was determined according to the concentration of radioactive sucrose and/or radioactive BSA present in the solution. The amount of material trapped inside the vesicles was determined according to the known specific activities of the radioactive species. Thus the radioactive material trapped inside the liposomes should be present at its known concentration and consequently knowing the amount present inside the vesicles indicated the volume of liquid encapsulated. The equation used was:

\[ V_i = \frac{\text{Substrate (inside)}^*}{\text{Substrate concentration}} \]

* where Substrate inside is expressed in mg or in mol.

II.17 Isolation of BSA-loaded vesicles on Sepharose 4-B

Lipid vesicles prepared as described in section II.15 were mixed with a known concentration of BSA in 10 mM MOPS pH 7.4 containing 150 mM NaCl and then subjected to a
freeze/thaw cycle. After thawing, the solution was sonicated for 5 s in a bath type sonicator and then 1 mL was allowed to percolate on a Sepharose 4-B column (30 x 1 cm) equilibrated in 10 mM MOPS pH 7.4 containing 150 mM NaCl and 1 mL fractions were collected. The most opaque fraction (typically fraction 8 or 9) was used for diffusion assays.

II.18 Separation of photolytically labelled BSA from photolyzed fatty acids

The following procedure was applied for quantification of BSA crosslinking either during diffusion assays using liposomes or just using mixtures of BSA and photoreactive [9-3H]9-DAP-nonanoate and/or [11-3H]11-DAP-undecanoate. After photolysis the samples were transferred to a silanized tube (13 x 100 mm) with two rinsings of the appropriate buffer, followed by the addition of 100 μL of 2% Triton X-100 and the subsequent removal of solvents using the speed Vac. The dry residues were resuspended in 500 μL 88% formic acid, sonicated and then applied on LH-60 columns equilibrated in 95% ethanol/88% formic acid (70/30). Fractions (500 μL) were collected and their radioactive content determined by liquid scintillation counting using ACS (aqueous counting scintillation liquid from Amersham).

II.19 Protein determination

The method used was an adaptation of the one described by Lowry, Rosebrough, Farr, and Randall (1957). The first
reagent consisted of a mixture of 0.5 mL 0.5% CuSO₄·5H₂O and 25 mL 2% Na₂CO₃, 0.02% Na,K tartrate in 0.1 N NaOH. One mL of the above reagent was added to a sample or standard mixture of 200 μL (containing 20 μL of 10% deoxycholate) and allowed to stand at room temperature for 30 min. Then 100 μL of a 50% aqueous dilution of Folin-Ciocalteu reagent was added and the samples kept at room temperature for 60 min. The absorption of standard and samples was performed at 660 nm using blanks obtained from using water instead of protein in the sample. The standard curve was constructed using BSA concentrations ranging from 0 to 25 μg.

II.20 Polyacrylamide gel electrophoresis

All gels were prepared according to the procedure described by Laemmli (1970) using a stacking gel on top of the separating gel. Essentially the procedure consisted in preparing a 12.5% polyacrylamide separating gel from a stock solution made of 30% w/v acrylamide, 0.8% w/v N,N' bis acrylamide dissolved in water. The final solution contained 0.375 M Tris-HCl pH 8.8 and 0.1% SDS. The mixture was degassed for 10 min and then polymerization was effected by addition of TEMED and ammonium persulfate to a final concentration of 0.25% and 0.08% respectively. The solution was poured between two glass plates and before polymerization was complete, water was carefully layered on top of it to permit even polymerization. A 5% stacking gel was prepared
in a similar fashion with the difference that the final solution contained 0.125 M Tris-HCl pH 6.8 and 0.1% SDS. The solution was degassed and polymerized as above followed by its addition to the separating gel. Note that the water on top of the separating gel was removed prior to pouring the stacking gel. A 10 or 12 well "comb" was inserted between the glass plates before polymerization of the stacking gel was complete. The electrode buffer consisted of 0.025 M Tris, 0.192 M glycine and 0.1% SDS.

Typically samples (50 to 75 μL) were prepared by addition of 150 μL of sample buffer made of the following: 0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue. When L-cells were used they were spun down in an Eppendorf tube and the pellet was resuspended in 25 μL of solubilizing buffer containing phospholipase A₂ (2 mg/mL), DNAase I (1 mg/mL), 0.01 M CaCl₂ and 0.01 M Tris-HCl pH 7.4. After a 45 min incubation at 37°C, the solubilized pellets were mixed with 150 μL of sample buffer. E. coli samples were solubilized by incubating the pellet with 10 μL of lysozyme (2 mg/mL) dissolved in 0.01 M Tris-HCl pH 8.0 and incubation at room temperature for 30 min. Then 15 μL of phospholipase A₂ (2 mg/mL), DNAase I (1 mg/mL) in 0.01 M Tris-HCl pH 7.4 were added and the incubation continued for another 30 min at 37°C prior to addition of sample buffer.
II.21 Reductive methylation of molecular weight markers

When radioactive samples were used in polyacrylamide gel electrophoresis, radioactive molecular weight markers were subjected to electrophoresis in adjacent lanes. These markers were prepared according to the procedure described by Jentoft and Dearborn (1983). Typically 100 μg of protein dissolved in 0.05 M HEPES pH 7.0 was mixed with 500 μmol of NaCNBH₃ dissolved in 0.05 M HEPES pH 7.0 containing 10 mM NiCl₂. To this mixture, 500 nmol of [¹⁴C]formaldehyde (50 mCi/mmol) was added and the reaction allowed to proceed at room temperature for 2 h or overnight in the cold. The methylated protein was freed of unreacted formaldehyde by extensive dialysis against 500 volumes of 0.05 M HEPES pH 7.0 containing 0.1 M NaCl. Solutions of desired specific activity were obtained by dilution with nonradioactive protein. The following proteins were subjected to the above procedure: bovine serum albumin (BSA, 68,000 D), ovalbumin (43,000 D), aspartate transcarbamylase (34,000 D subunit), carbonic anhydrase (30,000 D), chymotrypsinogen (26,000 D), and aspartate transcarbamylase (17,000 D subunit). These standards were used in fluorographs presented in this work unless specified otherwise.

II.22 Evaluation of [³H]NaBH₄ reducing capacity

[³H]NaBH₄ was dissolved in anhydrous diglyme (5 mL) and the amount of radioactivity determined by liquid
scintillation counting. Its reducing capacity was established by titration with m-hydroxybenzaldehyde. In a final volume of 600 μL, aliquots (0 to 60 μmol) of m-hydroxybenzaldehyde (0.1 M in ethanol) were mixed with 1.5 equivalents of aqueous NaOH followed by the addition of 25 μL [3H]NaBH₄ (estimated to represent 50 microequivalents). The final volume was adjusted with ethanol. The reaction was allowed to proceed overnight and stopped by addition of 100 μL of 6 N HCl to quench unreacted [3H]NaBH₄. An aliquot was dried to remove trapped H₂ gas and the radioactivity determined by liquid scintillation counting.
III. Results and Discussion

The main thrust of the work presented below consisted in the synthesis and investigation of the biological activity of the photoreactive probe, m-diazirinophenol (6), and its use in membrane studies. A synthesis scheme was designed to attach the probe at the omega carbon of an homologous series of radioactive omega hydroxy fatty acid methyl esters (IV.1-IV.4). The remainder of the work focussed on the study of the biological activity of these photoreactive fatty acid analogues and on their use in labelling an E. coli membrane protein putatively involved in the transport of fatty acids.

III.1 Synthesis of the photoreactive probe m-diazirinophenol(6)

Smith and Knowles (1973, 1975) showed that it was possible to obtain phenolic diazirines by different oxidation procedures of a triazabicyclodiaziridine intermediate such as compound 4 in Figure 3. They reported their best yields using t-butyl hypochlorite as an oxidizing agent. However, they found that para-substituted triazabicyclophenolicdiaziridines were refractory to this approach (Smith & Knowles, 1975). In order to attach a phenolic diazirine at the omega carbon of fatty acids Gupta, Radhakrishnan and Khorana (1977) succeeded in obtaining m-diazirinophenol (6) via the oxidation with t-butyl hypochlorite. As shown in Figure 3 the approach that
Figure 3. Scheme for the synthesis of m-diazirinophenol.
was used in the course of this thesis paralleled the one described by Smith and Knowles (1973, 1975) and Gupta, Radhakrishnan and Khorana (1977) with the exception that their approach did not include the use of pyridine during the oxidation step with t-butyl hypochlorite. Both groups reported relatively low yields of the desired diazirine ranging from 2-5%. The phenolic diazirine (6) synthesized according to the scheme developed in the course of this work was obtained with yields ranging from 24 to 30%.

III.1 a) Synthesis of protected benzaldehyde (2)

As initially described by Gupta et al., (1977) the phenolic group was protected with chloromethyl methyl ether. The latter group can be removed under mild acidic conditions. Purification of the desired methoxymethylene ether of m-hydroxybenzaldehyde (2) permitted the elimination of undesired contaminants present in the starting material. Initially the desired product was isolated by silica gel chromatography but it later was found that prolonged contact with silica gel eliminated the protective group. To alleviate this problem chromatography was performed to remove the bulk of the contaminants and then vacuum distillation yielded the pure material in good yields.

During the course of the synthesis the purity of some of the synthons (compounds 2, and 5 in Figure 3) was monitored by nuclear magnetic resonance (NMR) spectroscopy. For example Figure 4 represents the $^1H$-NMR of the
Figure 4. $^1$H-NMR (60 MHz) of methoxymethylene ether of m-hydroxybenzaldehyde (compound 2 in Figure 3). The synthesis and purification were as described in materials and methods. A solution of 150 mg/mL in carbon tetrachloride was prepared and transferred to an NMR glass tube. The field was adjusted using tetramethysilane as standard. The sweep offset was from 500 to 700 Hz and the proton resonance is 11 ppm.
methoxymethylene ether of m-hydroxybenzaldehyde (compound 2 Figure 3). The methyl group is found to resonate at 3.4 ppm and the methylene group at 5.2 ppm. The broad multiplet between 7.0 and 7.5 ppm represent the aromatic protons while the offset aldehydic proton resonates at 11 ppm thus demonstrating that the desired intermediate had been synthesized.

III.1 b) Synthesis of 3-(m-methoxymethylene phenyl ether)-3H-diazirine (5)

Initial attempts at producing the desired m-diazirinophenol (6) resulted as reported by others in poor yields (Smith & Knowles, 1975; Gupta, Radhakrishnan & Khorana, 1977). Some of the clues explaining these results appeared during 1H-NMR analyses of these early reaction products. Figure 5A represents the NMR spectrum of the pure diazirine (compound 5 in Figure 3) and Figure 5B represents the NMR spectrum of an early synthesis of diazirine. As can be observed in Figure 5A the resonance of the former aldehydic proton (11 ppm), now the diazirine proton, was observed around 2 ppm while the methyl and methylene protons resonance was unaffected. The diazirine proton in the low yield product was shifted to lower field and resonated at 2.2 ppm (Figure 5B). Moreover, it is noteworthy to point out the differences in the aromatic protons resonance between Figure 5A and 5B. Integration of these spectra revealed the loss of one aromatic proton in the compound analyzed in Figure 5B.
Figure 5. $^1$H-NMR (60 MHz) analysis of pure 3-(m-methoxyethylene ether phenyl)-3H-diazirine (5) (A) and the reaction product of earlier synthetic attempts (B). The samples were prepared for analysis as described in Figure 4. The synthesis was performed as described in the Materials and Methods section.
Furthermore, the resonance of one of the aromatic protons in the latter product was shifted to higher field (5.7 ppm) and the resonance of the methyl methyl ether group was slightly shifted to higher fields (3.2 and 4.8 ppm for the methyl and methylene groups respectively).

The loss of an aromatic proton as well as the shifts in resonances of the diazirine proton, the methylmethyl ether protons and of the aromatic ring protons suggested that the desired product was modified during the course of the synthesis. Since the first intermediate (compound 2 in Figure 3) showed no sign of a loss of a proton of its aromatic ring (Figure 4) then the focus of attention was turned to the oxidation reaction. It has been noted in the past (Mintz & Walling, 1969) that oxidation using t-butyl hypochlorite could generate chlorine in the presence of hydrochloric acid. Since hydrochloric acid is a by-product of the chloramine generation step (Figure 3) it was thought that chlorination of the phenyl ring was responsible for the difference in NMR spectral properties observed. In addition it was thought possible to eliminate the chlorination of the ring by removing hydrochloric acid as the pyridinium hydrochloride salt.

Aliquots of the oxidation reaction were analyzed using high performance liquid chromatography (HPLC) (Figure 6). These analyses revealed that more than one product showing the characteristic absorption at 360 nm were generated. When
Figure 6. High performance liquid chromatographic analyses of the oxidation products (5 in Figure 3) in the absence (a) and the presence (b) of pyridine. The reaction conditions were as outlined in materials and methods for the oxidation of the triazabicyclohexane. An aliquot (10 μL) of the reaction mixture was injected after 45 min, on an Altec Ultrasphere ODS HPLC column (0.46 x 25 cm) using 65% methanol in water as an eluting solvent at a flow rate of 1.5 mL/min. The eluates were monitored using an ISCO detector equipped with a 340-365 nm filter.
pyridine was added to the medium prior to the addition of t-butyl hypochlorite only one product absorbing at 360 nm was observed (Figure 6b). This supported the hypothesis that the generation of chlorine in situ was responsible for the abnormal NMR spectrum observed previously (Figure 5B). Thus, the presence of pyridine, prevented the generation of side products absorbing in the 360 nm range.

III.2 Analysis of 3-(m-methoxymethylene phenyl ether)-3H diazirine (5) synthesized in absence and presence of pyridine

In order to demonstrate that the diazirine material analyzed in Figure 5B was indeed a mixture of chlorinated isomers, the materials eluting at 7.5, 11.5 and 17.5 minutes (Figure 6a) were collected and analyzed. Elemental analyses confirmed the presence of one chlorine in each of the diazirines eluting at 11.5 min and 17.5 min. The ultraviolet absorption spectra of the chlorinated diazirines differed significantly from the pure diazirine (Figure 7 and Figure 8). The pure compound 5 (Figure 3) has major absorption maxima (in decreasing order) at 362 nm, 382 nm, 373 nm and 345 nm. Its molar extinction coefficient for the absorption maxima at 362 nm was found to be 360 in cyclohexane. The spectrum of the product eluting at 11.5 min (Figure 8b) revealed maxima (decreasing order) at 365 nm, 376 nm, 385 nm and 347 nm and its molar extinction coefficient at 365 nm was found to be 236. Maxima (decreasing order) at 357 nm, 352 nm
Figure 7. UV spectrum of pure 3-(m-methoxymethylene ether phenyl)-3H-diazirine (5). The spectrum of 3-(m-methoxy methylene ether phenyl)-3H-diazirine (5) (0.21 mg/mL) was recorded at two different sensitivity levels to permit detection at lower wavelengths. (a) range of absorbance 0 to 0.5; b) range of absorbance 0 to 2.0).
Figure 8. Ultraviolet spectra of pure (a) and chlorinated 3-(m-methoxymethylene ether phenyl)-3H-diazirine (5). Spectra (b) and (c) represent material eluting at 11.5 and 17.5 min, respectively in the chromatogram shown in Figure 6 (a). The samples were isolated by silica gel chromatography and reverse phase HPLC as described in Figure 6. The oily products were dissolved in cyclohexane ((a) 0.3 mg/mL; (b) 0.85 mg/mL; (c) 1 mg/mL) and the spectra obtained by scanning the samples over a wavelength range of 300 to 400 nm.
(shoulder), 340 nm, 370 nm (shoulder), and 379 nm were found for the compound eluting at 17.5 min. The latter product had a very distorted spectrum compared to the pure product and its molar extinction coefficient at 357 nm was the lowest with a value of 149. It is interesting to note that the molar extinction of coefficient of the pure diazirine (5) (Figure 7 and Figure 8a) is higher at 382 nm higher than at 373 nm. This relationship is reversed in the chlorinated isomer shown in Figure 8b (the molar extinction coefficient at 376 higher than at 385). This feature served as a quick reference point in later syntheses of phenolic diazirines.

$^1$H-NMR analyses at 250 MHz (Table I) permitted the identification of the position of the chlorine atom on the phenyl ring of the chlorinated diazirines $5b$, $5c$ and $5d$. Chlorination of the aromatic ring had an effect on the chemical shifts and on the coupling constants of the protons of the ring and those adjacent to the substituted position. As observed in Figure 5B and in Table I, the chlorinated isomers of compound 5 (compounds $5b$, $5c$ and $5d$ according to their elution order in Figure 6a) had their diazirine ring proton resonance at lower field than the pure compound $5a$. This effect was not as prominent in compound $5b$ because the chlorine substitution occurred at a position para to the diazirine ring. Substitutions at the two ortho positions (compounds $5c$ and $5d$) resulted in a drastic shift of the diazirine ring proton to lower field due to the deshielding
Table I

$^1$H nuclear magnetic resonance shifts (ppm) of the chlorinated and unchlorinated diazirines.

\[
\text{\begin{tabular}{lcccccc}
\text{Compounds} & \text{R}^3 & \text{Aromatic protons} \\
\hline
\text{H} & \text{N} & \text{CH}_3\text{=O-CH}_2\text{=H} & \text{H} & \text{R}^1 & \text{R}^2 & \text{R}^3 & \text{R}^4 \\
\text{5a} & 1.38 & 3.20 s, 2H & - & 6.37 s & 6.53 s & 6.37 s & 6.37 s \\
 & & 5.02 s, 2H & - & 7.23 (4H, m) & 7.23 (4H, m) & 7.23 (4H, m) & 7.23 (4H, m) \\
\text{5b} & 2.93 & 3.52 s, 2H & - & 6.75 d & \text{Cl} & 7.28 d & 6.44 dd \\
 & & 5.30 s, 2H & - & (J_{1,4} = 2.0) & & (J_{2,4} = 8.3) & (J_{2,4} = 8.3) \\
& & & & & & & (J_{2,4} = 2.0) \\
\text{5c} & 2.54 & 3.50 s, 2H & - & 5.93 dd & \text{Cl} & 7.12 dd & 7.58 dd \\
 & & 5.24 s, 2H & - & (J_{1,4} = 6.0) & & (J_{2,4} = 6.0) & (J_{3,5} = 8.3) \\
& & & & (J_{2,4} = 2.0) & & (J_{3,5} = 8.3) & (J_{3,5} = 2.5) \\
\text{5d} & 2.45 & 3.38 s, 2H & - & 5.40 d & 6.68 dd & 7.29 d & \text{Cl} \\
 & & 5.02 s, 2H & - & (J_{1,4} = 2.9) & (J_{1,4} = 2.9) & & (J_{2,4} = 8.0) \\
& & & & & & & (J_{2,4} = 8.0) \\
\text{6a} & 1.98 & - & 5.47 & 6.22 dd & 6.49 dd & 7.14 & 6.75 dd \\
 & & & & (J_{2,4} = 2.6) & (J_{2,4} = 2.6) & (J_{3,5} = 7.4) & (J_{3,5} = 7.4) \\
& & & & (J_{2,4} = 1.6) & (J_{2,4} = 1.6) & (J_{3,5} = 7.7) & (J_{3,5} = 7.7) \\
& & & & (J_{2,4} = 1.0) & (J_{2,4} = 1.0) & (J_{3,5} = 6.1) & (J_{3,5} = 6.1) \\
\end{tabular}}
\]

\[^5\text{a}, \text{b}, \text{c}, \text{d} \text{ are products from oxidation in absence of pyridine.}\]

\[^6\text{a} \text{ is derived from oxidation in presence of pyridine.}\]
effect of the chlorine. When positions R¹ and R² were chlorinated (compounds 5b and 5c) the resonance of the methyl methyl ether group (R⁵) was shifted to lower field while substitution at position R⁴ (compound 5d) had no effect.

The shift to lower field brought about by the chlorine atom at different positions on the ring facilitated the assignment of the aromatic proton resonance of the pure m-diazirinophenol (Figure 9). Thus the hydrogen at position R¹ is the most shielded and consequently resonated at higher field than the other aromatic protons while the hydrogen at position R³ was deshielded and showed resonance at very low field (7.16 ppm). The hydrogen at position R² was shielded by the phenolic hydroxyl group and consequently its shift was assigned at 6.49 ppm while the hydrogen at position R⁴ was slightly deshielded by the diazirine ring and was assigned at 6.76 ppm.

The inclusion of pyridine during the t-butyl hypochlorite oxidation successfully avoided the generation of chlorinated diazirines and improved the yield of desired phenolic diazirine (6). As illustrated in Table I, the chlorination of the phenolic diazirines caused a significant effect on the resonance of the diazirine ring proton (1.98 ppm 5a to 2.54 ppm for compound 5c). It is noteworthy that the diazirine ring proton resonance previously reported was 2.4 ppm (Gupta, Radhakrishnan, & Khorana, 1977) as opposed to 1.98 ppm as reported here. It is equally important to point
Figure 9. $^1$H-NMR (250 MHz) spectrum (CDCl$_3$) of the unchlorinated m-diazirinophenol (6). The sample was prepared as described in Figure 4.
that the pure protected diazirine was found to be rather volatile and some loss of material occurred during partial purification. This loss may have contributed to an enrichment in chlorinated diazirines in previous syntheses since during purification the solvents were removed under reduced pressure. The synthesis of phenolic diazirines described by others did not include pyridine during the oxidation step; thus it is likely that the absence of biological activity of fatty acid analogues containing such a group (Olsen, Schaechter & Khorana, 1979) was caused by its chlorination.

III.3 Synthesis of radioactive photoreactive homologous series of fatty acid analogues (VII.1-VII.4)

As discussed in the introduction several drawbacks were observed when fatty acids containing nitrene precursors were used to study the hydrophobic environment of biological membranes. In the hope of alleviating these problems the synthesis of a radioactive photoreactive homologous series of fatty acid analogues containing the phenolic diazirine at their omega carbon was designed (Figure 10). It was thought that the radioactivity would facilitate the monitoring of metabolism and the identification of crosslinked material (upon photolysis). Furthermore, these fatty acid analogues could be attached at one or the other position of the phospholipid backbone (in vitro or in vivo) and consequently could be used as "yardstick" of the extent of embedment of specific membrane proteins in the lipid bilayer.
Figure 10. Synthetic scheme for the synthesis of the photoreactive homologous series. The values for m and n can be found in Table II. The bold face hydrogen represent the site of the radioactive label.
The synthetic scheme shown in Figure 10 outlines the strategy that was followed. The approach consisted of oxidizing available olefins (Table II) to generate semialdehydes that could be reduced with $[^{3}\text{H}]\text{NaBH}_4$ to generate the corresponding omega tritiated hydroxy fatty acids. Conversion to the iodo derivatives followed by coupling with the phenolic diazirine and subsequently followed by saponification yielded the homologous series.

III.3 a) Synthesis of semialdehydes (II.1–II.4)

The oxidation reaction was essentially as described by Johnson and colleagues (Pappo, Allen, Lemieux & Johnson, 1956) and consisted of a solid phase reaction between the sodium salt of the olefin, OsO$_4$ and solid NaIO$_4$ in aqueous dioxane. Initial attempts at using soluble components failed. The only conditions that were favorable were those described in the Materials and Methods section where solid NaIO$_4$ was added to the OsO$_4$ olefin complex. Due to the nature of the reaction it was difficult to determine at which point it was complete. This determination became important when it was realized that prolonged exposure of the desired semialdehyde to the reaction mixture lead to further oxidation to the dicarboxylic acid. Thus to evaluate the extent to which the reaction had proceeded, aliquots were withdrawn and reduced with $[^{3}\text{H}]\text{NaBH}_4$. The assay is described in Figure 11 where panel A demonstrates the use of 3-OH benzaldehyde to assess the reductive potential of the $[^{3}\text{H}]\text{NaBH}_4$ solution and panel B
Table II

List of precursors and products used and synthesized in this study.

*Tritiated diazirinophenoxy fatty acids*

The boldface letter indicates the site of tritium labelling. R represents the m-diazirinophenyl group as shown in Fig.10

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Tritiated product</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1 Oleic acid ( m = 7 ) ( n = 7 ) ( \text{(cis-9-octadecenoic acid)} )</td>
<td>VII.1 ([9-^3H]9)-DAP-nopanoic acid</td>
</tr>
<tr>
<td>I.2 Vaccenic acid ( m = 5 ) ( n = 9 ) ( \text{(cis-11-octadecenoic acid)} )</td>
<td>VII.2 ([11-^3H]11)-DAP-undecanoic acid</td>
</tr>
<tr>
<td>I.3 Erucic acid ( m = 3 ) ( n = 11 ) ( \text{(cis-13-octadecenoic acid)} )</td>
<td>VII.3 ([13-^3H]13)-DAP-tridecanoic acid</td>
</tr>
<tr>
<td>I.4 Nervonic acid ( m = 7 ) ( n = 13 ) ( \text{(cis-15-tetracosenoic acid)} )</td>
<td>VII.4 ([15-^3H]15)-DAP-pentadecanoic acid</td>
</tr>
</tbody>
</table>
Figure 11. Assay of olefin oxidation using $[^3\text{H}]\text{NaBH}_4$. An aliquot of the oxidation reaction was reduced with $[^3\text{H}]\text{NaBH}_4$ of known reducing capacity. A Evaluation of the reducing capacity of $[^3\text{H}]\text{NaBH}_4$. Duplicate aliquots (0 to 60 $\mu$mol) of an ethanolic solution of 0.1 M 3-OH benzaldehyde were mixed with aqueous NaOH (1.5 equivalents) and 25 $\mu$L of $[^3\text{H}]\text{NaBH}_4$ (about 50 microequivalents) was added to the mixture. After an overnight incubation at room temperature the reduction was stopped by the addition of aqueous HCl (100 $\mu$L, 600 $\mu$mol) to use up unreacted $[^3\text{H}]\text{NaBH}_4$. An aliquot of the sample was dried to remove trapped $[^3\text{H}]\text{H}_2$ and the radioactivity determined by liquid scintillation counting. B Reduction of aliquots obtained from the oxidation of oleic acid. Several oxidation reactions were carried out as described in section II.3 a) using oleic acid (50 nmol) in Teflon-capped tubes. At the indicated time points the reaction was stopped by addition of H$_2$O (1 mL) and aqueous HCl (100 $\mu$L, 600 $\mu$mol). To an aliquot (200 $\mu$L) of the stopped reaction aqueous NaOH (50 nmol) was added and reduced with an excess of $[^3\text{H}]\text{NaBH}_4$ of known reducing capacity (as determined in A). The reduction proceeded 4 to 6 h and was stopped by adding aqueous HCl (100 $\mu$L, 600 $\mu$mol). The yield of oxidized material was estimated based on the level of radioactivity found in the reduced sample as described in A. The results are presented as percent of expected radioactivity (aldehyde and semialdehyde).
shows the results obtained using aliquots from the oxidation of oleic acid with OsO₄. It was thus possible to determine that the reaction was terminated after 3 hours.

A typical NMR spectrum of the desired semialdehyde is shown in Figure 12. In many of the early attempts it was difficult to observe the aldehydic proton triplet at 9.45 ppm suggesting that some of the product was further oxidized to the dicarboxylic acid derivative. The remaining portion of the spectrum corresponds to the expected structure. The carboxylic proton resonated at 10.75 ppm, the methylene groups of carbon atoms 2 and 8 resonated at 2.2 to 2.6 ppm, while the protons of carbon atoms 3 to 7 inclusively resonated at 1.2 to 1.7 ppm.

III.3 b) Synthesis of the homologous series of photoreactive tritiated fatty acids (VII.1-VII.4)

The omega hydroxy fatty acids were converted to the corresponding iodo derivatives using Rydon's reagent (Verheyden & Moffat, 1970) (Figure 13) to facilitate the coupling with m-diazirinophenol. Rydon's reagent proved very mild and afforded the desired products in virtually quantitative yields. The ¹H-NMR spectrum of the iodononanoyl methyl ester derivative is reproduced in Figure 14. The singlet at 3.5 ppm is characteristic of the methyl ester group, the triplet at 3.0 ppm represents the resonance of the omega methylene which is influenced (deshielded) by the iodine. The other triplet centered at 2.1 ppm represents the
Figure 12. $^1$H-NMR (60 MHz) spectrum ($\text{CCl}_4$) of the nonanoate semialdehyde (II.1) obtained from oxidation of oleic acid. The sample was prepared as described under material and methods and used as a 150 mg/mL solution in $\text{CCl}_4$. The sweep offset was from 500 Hz to 800 Hz and the proton shifts are 9.43 and 10.75 ppm respectively.
Figure 13. Proposed mechanism for the conversion of the hydroxyl group of the methyl ester of the hydroxy fatty acids (Verheyden & Moffat, 1970). (TPM, Triphenyl phosphite methiodide).
Figure 14. $^1$H-NMR (90 MHz) spectrum (CCl$_4$) of the iodononanoyl methyl ester. The sample was obtained as described under materials and methods and prepared for analysis as indicated in Figure 4.
resonance of the methylene group at the alpha carbon and the broad multiplet ranging from 1 ppm to 1.8 ppm is characteristic of the envelope protons of the carbon chain. The $^1$H-NMR analyses were consistent with the proposed structures of the desired synthetic intermediates (V.1 - V.4).

Finally, the coupling of the photoreactive group was effected in good yield in HMPA. The reaction between the sodium salt of m-diazirinophenol (6) and the iodo derivative was practically quantitative. The $^1$H-NMR spectrum of the methyl ester of [11-$^3$H]11-DAP-undecanoate (VI.2) is represented in Figure 15. It is interesting to note the shift in resonance of the omega methylene group from 3.0 ppm (Figure 14) to 3.8 ppm. The integration of the resonance of the aromatic group and of the diazirine proton at 1.9 ppm showed that they were present at a ratio of one with the protons from the fatty acid chain and the methyl ester.

The homologous series was further characterized by UV spectroscopy and high performance liquid chromatography (Figures 16 and 17). The UV spectra of [11-$^3$H]11-DAP-undecanoate (VII.2) and [13-$^3$H]13-DAP-tridecanoate (VII.3) were very similar. They showed the characteristic maximum at 362 nm of the phenolic diazirine and based on the molar extinction coefficient of the latter (360 at 362 nm) their specific activities were determined. The values of specific activities obtained using the latter approach
Figure 15. $^1H$-NMR (90 MHz) spectrum ($CCl_4$) of the diazirinophenyl undecanoyl methyl ester (VI.2). The sample was obtained as described under materials and methods and prepared for analysis as indicated in Figure 4.
Figure 16. UV spectra of [9-\textsuperscript{3}H]9-DAP-nonanoate (VII.1) (206 nmol (S.A. 2 Ci/mmol)) (left) and [13-\textsuperscript{3}H]13-DAP-tridecanoate (VII.3) (263 nmol (S.A. 436 mCi/mmol)) (right). Samples were prepared as in materials and methods, and dissolved in cyclohexane for analysis. The scans were from 300 to 400 nm.
Figure 17. Characterization of the methyl esters of the fatty acids by reverse-phase high performance liquid chromatography. The methyl esters of the tritiated omega-DAP derivatives of nonanoate, undecanoate, tridecanoate, and pentadecanoate (compounds VI.1, VI.2, VI.3, VI.4 in Table II) were dissolved in methanol and an aliquot was applied to a column (0.46 x 25.0 cm) of Ultrasphere-ODS previously equilibrated in 80% of solvent B (A, water; B, methanol). The analyses were performed using a linear gradient from 80% to 95% solvent B over 25 min at a flow rate of 2 mL/min. Fractions (200 µL) were collected and the radioactivity detected by liquid scintillation counting as described in materials and methods.
compared very well with values obtained by means of the weight of the iodo derivatives.

The methyl esters of this homologous series was free of impurities or cross contamination as revealed by reverse-phase high performance liquid chromatography analyses (Figure 17). The successful separation of the methyl esters by reverse phase HPLC served as a useful assay of their metabolism in later studies.

During the synthesis of the iodo derivatives and the coupling of the m-diazirinophenol it was found that traces of organic solvents hindered the isolation of the products. DMF and HMFA had deleterious effects on the chromatographic pattern of the desired synthetic intermediates, which complicated their isolation (Figure 18A) and the interpretation of their NMR spectra. In order to effectively remove these solvents prior to preparative thin layer chromatography, a reverse phase manipulation was developed. The strategy consisted of partitioning the particular iodomethyl ester or omega diazirino methyl ester into μ-Bondapack C18 reverse phase packing material followed by extensive washings with water to remove the solvent. The desired products were then eluted with diethyl ether. The difference in chromatographic properties are reproduced in Figure 18B.

The synthetic scheme that was adopted led to the successful purification of an homologous series of
Figure 18. Autoradiographs of preparative TLC of iodononanoyl methyl ester (V.1) in the presence (A) and absence (B) of DMF. The synthesis was carried out as described in materials and methods. The iodo derivative was isolated by preparative thin layer chromatography in the solvent system described in the method section. A shows the typical pattern when the DMF solution was applied directly to the plate, allowed to "dry" followed by chromatography. B shows the chromatographic pattern after removal of DMF as described in section II.3 d).
radioactive photoreactive fatty acids analogues. The next step consisted in determining if these probes had biological activities both *in vitro* and *in vivo*.

III.4 **Biological utilization of the homologous series of radioactive photoreactive fatty acids**

Khorana and co-workers (Olsen, Schaechter & Khorana, 1979) reported that fatty acid auxotrophs of *E. coli* failed to grow when DAP-undecanoate (VII.2) was used as a carbon source, while other phenolic fatty acid analogues appeared to be suitable carbon sources (Table III). As can be concluded from Table III most azido unsaturated fatty acids either supported growth and/or were incorporated to a significant extent in the bacterial phospholipids. Khorana's group reported that m-azido phenoxy fatty acids were able to support growth and were incorporated in the phospholipids of *E. coli*. However, the corresponding nitrophenoxy, nitro azido phenoxy and m-diazirinophenoxy fatty acids failed to stimulate growth and were not incorporated in the phospholipids of *E. coli* auxotrophs. This prompted the study of the metabolism of the homologous series, synthesized in this thesis, in order to identify the step or steps at which they were not recognized as normal substrate.

III.4 a) **Utilization of radioactive photoreactive fatty acids by rat liver microsomes**

The first step in the metabolism of fatty acids consists of a transformation to the corresponding acyl-
Table III.

Capacity of certain fatty acid analogues to support growth of \textit{E. coli} auxotrophs. Table taken from Olsen, Schaechter and Khorana (1979).

\begin{tabular}{llll}
\hline
Fatty acid & Growth response & \multicolumn{2}{c}{Incorporation of analog into phospholipids} \\
& & With Casamino Acids supplement & \% of total fatty acids \\
\hline
7-Azido-16:0 & - & & \\
8-Azido-16:0 & + & + & 53.5 \\
9-Azido-16:0 & + & + & 53.9 \\
& & - & 45.8 \\
9-Azido-18:0 & + & + & 47.5 \\
& & - & 34.2 \\
10-Azido-18:0 & + & + & 35.0 \\
& & - & 27.4 \\
12-Azido-18:0 & + & + & 59.8 \\
& & - & 38.5 \\
11-Phenyl-11:0 & - & + & 0 \\
& & - & 0 \\
11-Phenoxy-11:0 & \pm & + & 1.6 \\
& & - & 4.1 \\
11(m-Azidophenoxy)-11:0 & + & + & 72.0 \\
& & - & 42.0 \\
11(m-Nitrophenoxo)-11:0 & - & + & 0 \\
& & - & 0 \\
11(2-Nitro-4-azidophenoxy)-11:0 & - & + & 0 \\
& & - & 0 \\
13-Phenyl-13:0 & + & + & 49.0 \\
& & - & 46.6 \\
13-Phenyl-12-tridecanoic acid & + & + & 28.0 \\
& & - & 28.0 \\
n12(Trifluoro-2-diazopropionyl)-12:0 & - & + & 0 \\
n11(m-Diazirinophenoxy)-11:0 & - & + & 0 \\
n11-Azidoformyl-18:0 & - & + & 0 \\
cis-\Delta^9-18:1 (Oleic acid) & + & + & 71.9 \\
& & - & 68.0 \\
\hline
\end{tabular}
Coenzyme A derivatives (Figure 19). In E. coli this step is apparently linked to the uptake of fatty acids (Klein et al., 1971) while in mammalian cells both processes occur in different compartments.

Rat liver microsomes were used as a source of enzyme(s) to study acyl-CoA synthesis with the homologous series. Using the assay developed by Dole (1956), the rate of formation of acyl-CoA between palmitate and DAP-nonanoate was compared (Figure 20). Early results indicated that [9-3H]9-DAP-nonanoate (VII.1) was an excellent substrate for the acyl-CoA synthetase of rat liver microsomes. The reaction was also found to be CoASH and ATP dependent. These results established that a key mammalian enzyme involved in lipid metabolism was able to recognize [9-3H]9-DAP-nonanoate (VII.1) as a normal substrate. It further indicated that any block preventing their insertion into phospholipids had to be at another step.

III.4 b) Determination of Michaelis-Menten constants for the process of acyl-CoA synthesis in rat liver microsomes

Further studies of the kinetic parameters involving the homologous series required that a standard set of conditions be used. One key component in this system was to find the exact detergent to protein ratio which is characteristic of many membrane proteins (Figure 21). This was established by measuring the acyl-CoA synthetase activity
Figure 19. Activation of fatty acids to the CoA derivatives in rat liver microsomes. For almost all metabolic pathways involving fatty acids, such as chain elongation, oxidation, unsaturation, and esterification, they must be activated to the acyl thiol ester of Coenzyme A.
Figure 20. Time course of $[9,10-^3\text{H}]$palmitate and $[9-^3\text{H}]9$-DAP-nonanoate (VII.1) utilization by rat liver microsomes. The potassium salts of the fatty acids (200 µM) were incubated in presence of 3.0 mM ATP, 2.4 mM CoA, 50 mM MgCl$_2$ in a buffer containing 150 mM Tris-HCl pH 7.4, and 60 µg of microsomal protein in a final volume of 100 µL. The reaction was stopped by the addition of 500 µL of Dole's reagent (isopropyl alcohol : heptane : 1 N H$_2$SO$_4$ (40:10:1)). Phase separation was achieved by adding 300 µL of heptane followed by 175 µL of H$_2$O. The heptane layer was removed and the remaining solution extracted 6 more times by addition of 800 µL of heptane. The results are expressed in percent utilization of substrate as a function of time. $[9-^3\text{H}]9$-DAP-nonanoate (VII.1) in the presence (▲) and in the absence (△) of CoA; $[9,10-^3\text{H}]$palmitate in the presence (●) and in the absence (○) of CoA.
Figure 21. Acyl-CoA synthetase activity as a function of varying detergent to protein ratio. The assay was performed as described in Figure 20 with the addition of Triton X-100 (1 mg/mL). The microsomal protein concentration was varied from 2 μg/mL to 40 μg/mL.
as the concentration of Triton X-100 was kept constant and varying amounts of microsomal proteins were added. A protein to detergent ratio of 0.014 was found to be optimum for the acyl-CoA synthetase reaction (Figure 21).

The assay conditions were linear over a 3 minute period for all members of the homologous series (Figure 22). Under these conditions, initial velocities were determined over a range of substrate concentrations. At very large substrate concentrations compared to the amount of enzyme the initial concentration of product is considered negligible (Suelter, 1985). Therefore, the initial velocity of the enzyme reaction can be described by the Michaelis-Menten equation \( v = V_{\text{max}} [S]/(K_m + [S]). \)

From the initial rate values, the \( K_m \) and \( V_{\text{max}} \) of the homologous series was determined (Figure 23 and Table IV). A study of Table IV revealed that \( [11-^3\text{H}]11-\text{DAP-undecanoate} (\text{VII.2}) \) had the lowest \( K_m \) of the series as well as the lowest \( V_{\text{max}}, \) suggesting that it would function almost as an inhibitor of the acyl-CoA synthetase. As the chain length increased however, the \( K_m \) and \( V_{\text{max}} \) increased. \( [9-^3\text{H}]9-\text{DAP-nonanoate} (\text{VII.1}) \) had the highest \( K_m \) and \( V_{\text{max}} \) although the values never approached those observed for palmitate. The values reported for palmitate in the present study differ from those reported by Bar-Tana, Rose and Shapiro (1971) \( (K_m = 42 \ \mu\text{M}, \ V_{\text{max}} = 0.30 \ \mu\text{mol.h}^{-1}.\text{mg}^{-1}) \) and Tanaka et al. (1979) \( (K_m = 13 \ \mu\text{M}, \ V_{\text{max}} = 28 \ \mu\text{mol.h}^{-1}.\text{mg}^{-1}) \) or of those reported by
Figure 22. Acyl-CoA synthesis in rat liver microsomes. The potassium salts of the $[^{3}H]$fatty acids (25 μM) were incubated with rat liver microsomes as described in Figure 20 according to the conditions established in Figure 21. At the times indicated, aliquots of 100 μL were withdrawn and processed as in Figure 20. The results shown are for palmitic acid (S.A. 2 Ci/mmol) (●) and the omega $[^{3}H]$-DAP derivatives of nonanoate (VII.1) (S.A. 2 Ci/mmol) (◇), undecanoate (VII.2) (S.A. 994 mCi/mmol) (■), tridecanoate (VII.3) (S.A. 436 mCi/mmol) (□), and pentadecanoate (VII.4) (S.A. 485 mCi/mmol) (▲).
Figure 23. Determination of $K_m$ and $V_{max}$ values for each member of the homologous series for acyl-CoA synthetase of rat liver microsomes. Assays were performed as described in Figure 20 with the addition of Triton X-100 (1 mg/mL) and a final microsomal protein to detergent ratio of 0.014. The concentration of the potassium salts of the fatty acids were varied between 0.70 and 30 $\mu$M. The incubations were done in duplicate for time periods of up to 2 min. $1/V$ is expressed in $(\mu$mol/h/mg of microsomal protein)$^{-1}$ and $1/S$ as $(\mu$M)$^{-1} \times 10^{-3}$. The results shown are for palmitic acid (●) and the omega-DAP derivatives of nonanoate (VII.1) (○), undecanoate (VII.2) (■), tridecanoate (VII.3) (□), and pentadecanoate (VII.4) (▲).
Table IV

Michaelis constants and $V_{\text{max}}$ values of the homologous series for acyl-CoA synthetase of rat liver microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ $\mu$M</th>
<th>$V_{\text{max}}$ $\mu$mol$h^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitate</td>
<td>23.2</td>
<td>11.4</td>
</tr>
<tr>
<td>[9-$^3$H]9-DAP-nonanoate (VII.1)</td>
<td>9.2</td>
<td>5.2</td>
</tr>
<tr>
<td>[11-$^3$H]11-DAP-undecanoate (VII.2)</td>
<td>3.6</td>
<td>0.7</td>
</tr>
<tr>
<td>[13-$^3$H]13-DAP-tridecanoate (VII.3)</td>
<td>5.7</td>
<td>0.8</td>
</tr>
<tr>
<td>[15-$^3$H]15-DAP-pentadecanoate (VII.4)</td>
<td>8.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Noy and Zakim (1985) (Table V). In the latter study the fatty acids were presented as dispersions in soya bean lecithin as opposed to the salt and no detergent was used. Noy and Zakim (1985) studied the substrate specificity of rat liver microsomes. Although, their experimental approach differed from the one reported in this work the values for $K_m$ and $V_{max}$ that were found can be analyzed in terms of the trends that were observed with the homologous series.

It is interesting to note that with increasing chain length the $K_m$ values increased as was observed for [11-$^3$H]11-DAP-undecanoate (VII.2), [13-$^3$H]13-DAP-tridecanoate (VII.3) and [15-$^3$H]15-DAP-pentadecanoate (VII.4), while [9-$^3$H]9-DAP-nonanoate (VII.1) was exceptional having a very high $K_m$. In their study it was found that inclusion of a double bond resulted in lower $K_m$ values than for comparable saturated substrate, and the value was lower when the double bond was in cis than when it was in trans (Noy & Zakim, 1985).

III.4 c) Synthesis of radioactive photoreactive phosphatidylcholine by rat liver microsomes

The next step in lipid metabolism consists in the transfer of the activated fatty acid to the 2- position of lysophospholipids to generate the desired phospholipids. To measure the capacity of the newly generated acyl-CoA's to be inserted into phosphatidylcholine by the endogenous acyl-CoA transferase(s) of rat liver microsomes, lysophosphatidylcho-
Table V

Michaelis constants and $V_{\text{max}}$ values of fatty acids for acyl-CoA synthetase of rat liver microsomes as determined by Noy and Zakim (1985).*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>$K_m$ (mol fatty acid/mol microsomal phospholipids)</th>
<th>$V_{\text{max}}$ (µmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanoate</td>
<td>0.0023</td>
<td>0.10</td>
</tr>
<tr>
<td>Laurate</td>
<td>0.0041</td>
<td>2.2</td>
</tr>
<tr>
<td>Myristate</td>
<td>0.0090</td>
<td>1.15</td>
</tr>
<tr>
<td>Palmitate</td>
<td>0.014</td>
<td>1.0</td>
</tr>
<tr>
<td>Stearate</td>
<td>0.038</td>
<td>0.5</td>
</tr>
<tr>
<td>Oleate</td>
<td>0.011</td>
<td>0.83</td>
</tr>
<tr>
<td>Elaidate</td>
<td>0.020</td>
<td>0.85</td>
</tr>
</tbody>
</table>

*Note the $K_m$ values reported by Noy and Zakim are expressed in terms of mol fatty acid/mol microsomal phospholipids while the $K_m$ results presented in Figure 23 and Table IV are expressed as µmol of fatty acids. Furthermore, the fatty acids in Noy and Zakim's study were presented dispersed in soya bean lecithin in absence of additional detergent as was the case in Figure 23.
line was added to the incubation mixture (Figure 24). The corresponding phosphatidylcholines were successfully produced and the enzyme demonstrated the same substrate specificity as acyl-CoA synthetase. Consequently, \([9-^3\text{H}]9\text{-DAP-nonanoate (VII.1)}\) was the preferred substrate followed by \([11-^3\text{H}]11\text{-DAP-undecanoate (VII.2)}\), \([13-^3\text{H}]13\text{-DAP-tridecanoate (VII.3)}\) and \([15-^3\text{H}]15\text{-DAP-pentadecanoate (VII.4)}\). Furthermore, the rate of synthesis of phosphatidylcholine from \([9-^3\text{H}]9\text{-DAP-nonanoate (VII.1)}\) was similar to the rate observed with palmitate.

Overall the homologous series of radioactive photoreactive fatty acid analogues was recognized by the key enzymes involved in phospholipid synthesis. These observations further suggested that the lack of biological activity observed by others may have been caused by chlorination of the phenolic diazirine group. The following step in the investigation of their biological activity consisted in studying their behavior \textit{in vivo} such that entry into the cells could be tested. The simplest system available at the time this work was in progress consisted of using tissue culture cells \((L\text{-cells})\).

\textbf{III.4 d) Incorporation of} \[^{3}\text{H}1\text{DAP-fatty acids into L\text{-cells}}\]

\textbf{phospholipids}

Numerous reports have been published on the incorporation of fatty acids in cultured cells (e.g. Horwitz, Hatten and Burger 1974; Williams \textit{et al.}, 1974; and Ferguson et
Figure 24. Synthesis of phosphatidylcholine from lysophosphatidylcholine in rat liver microsomes. The potassium salts of the [³H]fatty acids (25 μM) were incubated in 150 mM Tris-HCl pH 7.4, 200 mM KCl, 2 mM dithiothreitol, 15 mM MgCl₂, 8 mM ATP, 2.4 mM CoA and 5 mM lysophosphatidylcholine. The reaction was started by the addition of microsomes (3 mg/mL). At the times indicated an aliquot (100 μL) was transferred to a solution containing 375 μL of methanol:chloroform (2:1) for a Bligh-Dyer extraction. After the extraction was completed the organic layer was dried and resuspended in 100 μL methanol:chloroform (1:1). An aliquot (20 μL) was subjected to chromatography on silica gel using chloroform:methanol:water (65:25:4). The radioactivity visualized by fluorography was quantified as described in the methods. The results shown are for palmitic acid (●) and the [³H]DAP derivatives of nonanoate (VII.1) (○), undecanoate (VII.2) (■), tridecanoate (VII.3) (□), and pentadecanoate (VII.4) (▲).
al., 1975). As noted by most investigators the presence of endogenous lipids in the serum could affect the uptake of fatty acids. Incubations in presence of the salts of the respective fatty acids were attempted. The serum effect was observed with L-cells and is demonstrated in Figure 25. As expected, less [9-3H]9-DAP-nonanoate (VII.1) was incorporated in PC and PE in presence of serum than in its absence. Consequently, to investigate the uptake of the homologous series by L-cells, the experiment was carried out in serum free media.

As shown in Figure 26, when this experiment was performed it was found that contrary to the results observed with rat liver microsomes, [11-3H]11-DAP-undecanoate (VII.2) appeared to be more readily incorporated in phosphatidylcholine and phosphatidylethanolamine than [9-3H]9-DAP-nonanoate (VII.1). [11-3H]11-DAP-undecanoate (VII.2) and [9-3H]9-DAP-nonanoate (VII.1) were incorporated to a similar extent and showed a slightly better incorporation than palmitate into phosphatidylcholine. [15-3H]15-DAP-pentadecanoate (VII.4) appeared to be a better substrate than [13-3H]13-DAP-tridecanoate (VII.3) which contrasts with the results observed with rat liver microsomes where the order observed was [9-3H]9-DAP-nonanoate (VII.1), [11-3H]11-DAP-undecanoate (VII.2), [13-3H]13-DAP-tridecanoate (VII.3) and [15-3H]15-DAP-pentadecanoate (VII.4). In the case of incorporation into phosphatidylethanolamine,
Figure 25. Effect of serum in the growth media on the incorporation of [9-3H]9-DAP-nonanoate (VII.1) in L-cells phospholipids. L-cells were grown to near confluence on 60 mm plates in minimal essential medium containing 5% newborn calf serum. Before adding [9-3H]9-DAP-nonanoate (VII.1) the plates were rinsed with serum free minimal essential medium and then they were incubated at 37°C in 5 mL of serum free or serum containing minimal essential medium containing a 180 µM concentration of [9-3H]9-DAP-nonanoate (VII.1) (S.A 2 Ci/mmol). At the indicated time points the cells were collected, washed 3 times with phosphate-buffered saline and extracted by the Bligh-Dyer procedure. A portion of each extract corresponding to 20,000 cells was analyzed on a silica thin layer plate (CHCl₃/MeOH/H₂O; 65:25:4). The spots corresponding to each phospholipid were visualized by fluorography and recovered, and the radioactivity was determined by liquid scintillation counting. (O) phosphatidylcholine; (Δ) phosphatidylethanolamine. Filled circles represent values obtained in presence of serum; PC is phosphatidylcholine; PE is phosphatidylethanolamine.
Figure 26. Incorporation of $[^3\text{H}]$DAP-fatty acids (VII.1 - VII.4) into L-cells phospholipids. L-cells were grown to near confluence on 60 mm plates in minimal essential medium containing 5% newborn calf serum. Before adding the $[^3\text{H}]$DAP-fatty acids (VII.1 - VII.4) the plates were rinsed with serum free minimal essential medium and then they were incubated at 37°C in 5 mL of serum free minimal essential medium containing 180 μM of photoreactive fatty acid or palmitic acid. At the indicated time points the cells were collected, washed 3 times with phosphate-buffered saline and extracted by the Bligh-Dyer procedure as described in materials and methods. A portion of each extract corresponding to 20,000 cells was analyzed on a silica thin layer plate (CHCl₃/MeOH/H₂O; 65:25:4). The spots corresponding to each phospholipid were visualized by fluorography and recovered, and the radioactivity was determined by liquid scintillation counting. The figure shows the extent of incorporation of radioactivity into phosphatidylcholine (a) and phosphatidylethanolamine (b). The fatty acids used were the tritiated omega-DAP derivatives of nonanoate (VII.1) (S.A. 2 Ci/mmol) (●), undecanoate (VII.2) (S.A. 994 mCi/mmol) (○), tridecanoate (VII.3) (S.A. 436 mCi/mmol) (▲), pentadecanoate (VII.4) (S.A. 485 mCi/mmol) (Δ), as well as $[^3\text{H}]$palmitate (S.A. 2 Ci/mmol) (■).
[11-^{3}H]11-DAP-undecanoate (VII.2) was by far the best substrate followed by [15-^{3}H]15-DAP-pentadecanoate (VII.4), [9-^{3}H]9-DAP-nonanoate (VII.1), palmitate and [13-^{3}H]13-DAP-tridecanoate (VII.3). Again the latter results differ from those obtained using rat liver microsomes. The origin of the cells (L-cells are mouse fibroblasts) being different than the rat liver microsomes and the nature of the experiment may account for these differences. However, these results further established the biological activity of the homologous series and it remained to be demonstrated that they were capable of photolytically labelling integral membrane proteins.

III.4 e) Labelling of L-cells membrane proteins using in vivo insertion of [9-^{3}H]9-DAP-nonanoate (VII.1) into the major phospholipids

The homologous series proved to be biologically active both in vitro and in vivo. The next step consisted of establishing that once inserted in the phospholipid bilayer these probes could report on the presence of integral membrane proteins. L-cells grown in presence of [9-^{3}H]9-DAP-nonanoate (VII.1) were analyzed on polyacrylamide gel electrophoresis before and after photolysis (Figure 27; lanes e-h). Before photolysis some bands were labelled suggesting that they represent fatty acylated membrane proteins as reported by others (Schmidt, Bracha & Schlesinger, 1979; Schlesinger, Magee & Schmidt, 1980). Upon photolysis at 360 nm, a different pattern emerged where some
Figure 27. Photolysis of L-cells labelled with $[9^{-3}H]9$-DAP-nonanoate (VII.1). Cells were labelled for 4 h with $[9^{-3}H]9$-DAP-nonanoate (VII.1) (S.A. 2 Ci/mmoll) as described in Figures 25 and 26. For $[^{35}S]$methionine labelling, L-cells grown as described for $[9^{-3}H]9$-DAP-nonanoate (VII.1), were incubated for 2 h in methionine-free minimal essential medium containing 2% dialyzed newborn calf serum and $[^{35}S]$methionine (20 μCi/mL). Cells ($2 \times 10^6$) were harvested, washed 3 times with cold phosphate-buffered saline and resuspended in 4.0 mL of phosphate-buffered saline. Aliquots of 1.0 mL were subjected to photolysis as described under materials and methods. The cells were recovered by centrifugation and suspended in 25 μL of a solution containing 10 mM Tris-HCl, pH 7.6, 2 mM CaCl$_2$, 1 mM phenyl methyl sulfonyl fluoride, phospholipase A$_2$ (100 μg/mL), and deoxyribonuclease (50 μg/mL) and were incubated for 10 min at 37°C. An equal volume of 2 X sample buffer (see materials and methods) was then added and the samples were subjected to electrophoresis on a 10% polyacrylamide gel and the radioactivity detected by fluorography as described in materials and methods. Lanes a-d, $[^{35}S]$methionine-labelled L-cells photolyzed for 0, 2, 4 and 10 seconds respectively; lanes e-h, $[9^{-3}H]9$-DAP-nonanoate-labelled L-cells photolyzed for 0, 2, 4, and 10 seconds respectively. The molecular weight markers indicated are the viral proteins L, G, N, and M ($M_r$= 190,000, 69,000, 50,000, and 29,000, respectively) present in $[^{35}S]$methionine-labelled vesicular stomatitis virus.
of the fatty acylated bands were more intensely labelled and some new bands appeared. Photolysis of cells grown in presence of $[^{35}S]$methionine demonstrated that the photolytic process did not generate membrane damages as no change in electrophoretic patterns could be observed (Figure 27; lanes a-d). These results confirmed that the photolytic group was still intact after being incubated with L-cells and further demonstrated its utility for in vivo studies in the labelling of integral membrane proteins.

III.4 f) **Labelling of the integral membrane protein of vesicular stomatitis virus**

The successful labelling of L-cells membrane proteins prompted a study to demonstrate that a known integral membrane protein could be labelled using this approach. The vesicular stomatitis virus (VSV) is an enveloped virus which buds from the plasma membrane of the cells it infects (Wagner, 1991). One of the proteins it codes for is known to be glycosylated and inserted in the host's membrane and is referred to as G-protein. The G-protein of VSV was reported to be normally acylated with palmitate (Schlesinger, Magee & Schmidt, 1980; Schmidt, Bracha & Schlesinger, 1979) as seems to be the case for a number of membrane proteins. In collaboration with John Capone, VSV was used to infect L-cells grown in presence of $[9^{-3}H]9$-DAP-nonanoate (VII.1). It was found that the G-protein was fatty acylated and after
photolysis the intensity of the labelling increased dramatically (Figure 28; lanes d-f). Photolysis of virions obtained from L-cells grown in presence of palmitate did not enhance the labelling of the G protein (Figure 28; lanes a–c). It established that the labelling observed in lane f was the result of the photolysis of phospholipids containing $[^{3}H]9$-DAP-nonanoate (VII.1). While other bands were observed having apparent molecular weights higher than G only one of these bands (band I) reacted with anti-G antibodies suggesting it was a G-G dimer. Since the other bands failed to react with antibodies to G, M and N respectively, they must reflect the presence of host membrane proteins present in the budding membrane (Lodish & Porter, 1980; Capone, Leblanc, Gerber & Ghosh, 1981). These results supported the hypothesis that M and N are not integral membrane proteins. Acylation with $[^{3}H]9$-DAP-nonanoate (VII.1) did not favor contact of G with either of these proteins. Two minor bands having lower molecular weights than G were also observed (bands V and VI migrating as 55–66 kD and 24 kD respectively). These proteins showed no affinity for the anti-G serum. The 24 kD protein may represent a host-derived protein appearing in low numbers in budding virions (Lodish & Porter, 1980) while the nature of the 55–66 kD protein remained unknown. Thus photoreactive phospholipids helped identify integral membrane proteins and allowed the detection of minor transmembrane proteins. It is important to note that
Figure 28. Photolysis of vesicular stomatitis virus obtained from L-cells grown in presence of [9-\(^3\)H]9-DAP-nonanoate (VII.1). As reported by Capone et al., (1981) virus samples were diluted to 400 \(\mu\)L with a buffer containing 0.01 M Tris-HCl (pH 7.5) and 0.1 M NaCl and were purged with water-saturated nitrogen for 45 min. The samples were then photolyzed for 30 s, and analyzed by SDS-polyacrylamide gel electrophoresis. Photolysis was carried out as described in materials and methods. Lanes a and d, [\(^3\)S]methionine-labelled (20 \(\mu\)Ci/mL) VSV; lanes b and c, [\(^3\)H]palmitate-labelled VSV (180 \(\mu\)M; S.A. 17 Ci/mmol) before and after photolysis respectively; lanes e and f [9-\(^3\)H]9-DAP-nonanoate-labelled VSV (180 \(\mu\)M; S.A. 2 Ci/mmol) before and after photolysis, respectively. Bands I-IV represent the irradiation-induced products of molecular weights greater than G having apparent molecular weights of 140,000, 107,000, 100,000, and 90,000, respectively. Bands V and VI correspond to molecular weights of 60,000 and 24,000, respectively. (Figure reproduced from Capone, Leblanc, Gerber & Ghosh, 1981).
Stoffel (1979 and references therein) using radioactive azido fatty acids, reported similar results but upon prolonged photolysis, protein bands corresponding to M and N were also observed. The latter suggested that damages were caused during photolysis and that possibly other phenomena were responsible for the labelling process.

As shown in Figure 29, methanolysis of purified unphotolyzed G protein, followed by HPLC analysis of the resulting methyl esters, revealed that 30% of the radioactivity associated with this protein was as the \([9^{-3}H]9\text{-DAP-nonanoate (VII.1)}\) (Capone et al., 1981). Elongation products corresponding to \([11^{-3}H]11\text{-DAP-undecanoate (VII.2)}\), \([13^{-3}H]13\text{-DAP-tridecanoate (VII.3)}\) and \([15^{-3}H]15\text{-DAP-pentadecanoate (VII.4)}\) were also observed and they made up for 16, 10 and 17% respectively of the radioactivity associated with the protein. The remainder of the radioactivity eluted as shorter and longer chain methyl esters that were not characterized.

By analogy with other systems such as the microsomes of rat small intestine and rat brain, the elongation products appear to be the results of the action of microsomal enzymes (Cook, 1982; Christiansen, Rortveit, Norum & Thomassen, 1986). In these particular examples the lengthening of the fatty acids chains by 2 carbons has been shown to be dependent on ATP and inhibited by CoASH.

It was concluded that the homologous series could be
[9-\textsuperscript{3}H]9-DAP-nonanoate metabolites

(cm x 10\textsuperscript{-2})

TIME (min)

\% Methanol
Figure 29. High performance liquid chromatographic analysis of radioactivity associated with G protein after labelling with [9-\textsuperscript{3}H]9-DAP-nonanoate (\textit{VII.1}). The G protein obtained by VSV infection of L-cells, grown in presence of [9-\textsuperscript{3}H]9-DAP-nonanoate (\textit{VII.1}) (Capone \textit{et al.}, 1981) as in Figure 28, was extracted by the Bligh-Dyer procedure. The fatty acid remaining covalently attached to the delipidated G protein was transesterified by treatment with 0.1 M methanolic KOH as described in the methods section. The resulting fatty acid methyl esters were analyzed by reverse phase high pressure liquid chromatography on an Ultrasphere ODS column using the gradient shown (A, water; B, methanol). The flow rate was 2 mL/min and 0.5 min fractions were collected and the radioactivity determined. The solid line represents the methyl ester derivatives obtained from delipidated G protein by transesterification. The dashed line represents the elution profile of the starting methyl ester derivative of [9-\textsuperscript{3}H]9-DAP-nonanoate (\textit{VII.1}). The arrows represent the elution positions of radioactive markers corresponding to the methyl ester derivatives of DAP nonanoate, undecanoate, tridecanoate, and pentadecanoate in order of increased retention time, respectively. (Figure reproduced from Capone, Leblanc, Gerber & Ghosh, 1981).
used to identify integral membrane proteins and, that all the members of the series were biologically active, although the \[9-^{3}\text{H}]9-DAP-nonanoate (VII.1) and \[11-^{3}\text{H}]11-DAP-undecanoate (VII.2) seemed to behave as best substrate for most applications. These biologically active radioactive photo-reactive fatty acid analogues were used as photoaffinity analogues of the recently described \textit{E. coli} fatty acid binding protein FLP (the \textit{fadL} gene product reported by Black et al., 1987).

III.5 \textbf{Labelling of a fatty acid binding protein in \textit{E. coli}}

Initial studies have demonstrated that transport of fatty acids in bacteria is an energy linked process (Frerman & Bennett, 1973; Klein et al. 1971; Maloy et al., 1981). There is no evidence that transported fatty acids are found in their free state once inside \textit{E. coli}. Most (if not all) of the fatty acids are found as the acyl-CoA derivatives or as other metabolites, thus supporting the idea that fatty acids are transported across the membrane by a group translocation process. As discussed in the introduction, Overath (Klein et al., 1971) has observed a relationship between transport of fatty acids and acyl-CoA synthetase activity. The idea that the latter enzyme was directly involved in the transport process was proposed by Frerman and Bennett (1973). However, Nunn and co-workers isolated mutants with normal acyl-CoA synthetase activity but that failed to grow on long chain fatty acids as a carbon source (Nunn &
Simons, 1978). Nunn's group (Ginsburgh, Black & Nunn, 1984) reported that these mutants that failed to grow on oleic acid as a sole carbon source lacked an inner membrane protein of approximate molecular weight of 30,000. Since there was evidence that a membrane protein was necessary for transport of fatty acids in E. coli it became a likely target for the use of DAP-fatty acids.

At the time these investigations were undertaken it had not been established that the E. coli 30 kD protein is located in the outer membrane (Black et al., 1987). Consequently, the results presented below represent preliminary experiments that were not designed to address the issue of the exact membrane location (inner or outer) of the 30 kD protein. As will be seen, a 30 kD protein was identified with [11-3H]11-DAP-undecanoate but its identity with the protein reported by Nunn's group (Nunn et al., 1986; Black et al., 1987) was not established.

III.5 a) Photolysis of E. coli vesicles with [11-3H]11-DAP-undecanoate (VII.2)

To establish the presence of the FLP protein described by Nunn's group (Black et al., 1985; Black et al., 1987; Nunn et al., 1986;) in E. coli membranes, empty E. coli vesicles, obtained by the procedure of Kaback (1971), were incubated in the presence of [11-3H]11-DAP-undecanoate (VII.2). As shown in Figure 30, photolysis of this mixture resulted in labelling of a protein band of about 30 kD. The labelling of
Figure 30. Incubation of *E. coli* enveloped vesicles with [11-\(^3\)H]11-DAP-undecanoate (VII.2). Enveloped vesicles were prepared by the procedure of Kaback as described in the method section. Enveloped vesicles (100 µg of proteins) were incubated with 2 µM of [11-\(^3\)H]11-DAP-undecanoate (VII.2) (S.A. 1.0 Ci/mmol) in a final volume of 50 µL of 0.1 M KH\(_2\)PO\(_4\) buffer pH 6.8. The reaction was started by addition of the photoreactive fatty acid and was performed at 37°C in siliconized quartz cuvettes. The reaction was stopped by photolysis at 360 nm for 20 s. After photolysis, the samples were transferred to siliconized 1.5 mL eppendorf tubes and the cuvettes rinsed twice with 2 X sample buffer (50 µL aliquots). The samples were then subjected to electrophoresis on 12.5% SDS-polyacrylamide gel. The radioactivity was detected by fluorography using a 19 days exposure at -70°C. Lanes 1-5 represent incubations of 0.5, 2, 5, 10, and 15 min, respectively.
a protein band around 26 kD was sometime observed and its presence appeared to depend on the membrane preparations. Longer incubation time with [11-\textsuperscript{3}H]11-DAP-undecanoate (\textbf{VII.2}) prior to photolysis did not affect the labelling pattern (Figure 30). These vesicles were not assessed as to their degree of composition of inner and outer membrane. However, based on the results reported by Black et al., (1987), it is likely that a significant contamination of outer membrane was present in this preparation.

Subsequent to this experiment, Nunn's group reported that upon boiling in SDS, the FLP protein band, putatively corresponding to the 30 kD protein in this study, was observed to shift to 42-43 kD (Black et al., 1985). This effect was investigated by incubating \textit{E. coli} vesicles with [11-\textsuperscript{3}H]11-DAP-undecanoate (\textbf{VII.2}) and subjecting the photolyzed products to boiling. As can be observed in Figure 31, boiling the samples prior to electrophoresis resulted in the appearance of an intensely labelled 42-43 kD protein band. Concomitantly there was a disappearance of a diffuse band at about 30-33 kD. The difference in labelling intensity between the boiled 42-43 kD protein and the unboiled labelling intensity of the 30-33 kD protein is difficult to explain since not all the protein bands showed a major intensity increase.

The difference in electrophoretic mobility may be explained by an incomplete denaturation of the protein
Figure 31. Effect of boiling photolyzed E. coli vesicles labelled with [11-3H]11-DAP-undecanoate (VII.2). The experiment was carried out essentially as described in Figure 30. E. coli vesicles (100 μg of proteins) obtained as described in materials and methods were incubated in a siliconized quartz cuvette with 2 μM [11-3H]11-DAP-undecanoate (VII.2) (20 Ci/mmol) in a final volume of 50 μL of 0.1 M KH2PO4, pH 6.8 at 37°C. After a 5 min incubation the samples were photolyzed for 20 s at 360 nm. After photolysis, the samples were transferred to siliconized 1.5 mL eppendorf tubes and the cuvettes rinsed twice with 2 X sample buffer (50 μL aliquots). Prior to electrophoresis on 12.5% SDS-polyacrylamide gel one sample (lane 2) was boiled for 5 min while the other sample (lane 1) was kept at room temperature. Radioactivity was detected by fluorography using a 21 days exposure at -70°C.
resulting in the maintenance of some secondary structure which would make the protein migrate as a lower molecular weight species (Schnaitman, 1973; Reithmeier & Bragg, 1974). As pointed out by Black et al., (1987) this phenomenon is often seen with E. coli outer membrane proteins. According to Kaback (1971), it is not surprising to observe a significant level of outer membrane contaminants with certain strains of E. coli other than ML, K12, W and B derivatives. The strain MC1060 used in this study was falsely believed to be a K-12 derivative which may explain the presence of an FLP-like protein in the membrane preparation. A Western blot analysis, using the anti-FLP serum developed by Nunn's group (Black et al., 1987), should confirm the identity of the labelled protein with the fadL gene product.

At the time these experiments were performed it was believed that the 30 kD (42-43 kD boiled) protein was an inner membrane protein involved in fatty acid transport. Therefore, it seemed logical to devise a crude fatty acid transport assay to further assess the function of this protein.

III.6 Attempts to develop an in vitro fatty acid transport assay

A reconstitution assay allowing the determination of fatty acid transport activity should help identify which protein(s) is (are) necessary for fatty acid permeation. This assay would permit characterization of the fatty acid binding
capacity of the protein as well as its ability to drive fatty acid transport across the lipid bilayer under various energetic conditions.

Inherent to transport assays is the problem of distinguishing between diffusion and transport. With fatty acids, an added problem is their propensity to partition into the lipid bilayer, thus making accurate measurements of uptake difficult. To bypass this problem an assay was designed around the use of an internal trapping agent inside lipid vesicles. Incubation of these vesicles with photoreactive fatty acids for increasing periods of time followed by photolysis and determination of radioactivity crosslinked to the trapping agent would reveal if an increase in labelling could be observed with time. This effect should be dependent on the presence of a reconstituted membrane fraction containing the desired protein(s). Since BSA was known to have fatty acid binding sites (Spector, 1975) it appeared well suited for use as a fatty acid trapping agent.

First, it had to be demonstrated that lipid vesicles could be "loaded" with a known substrate and that the vesicles would be sealed. Many approaches have been described for the encapsulation of proteins, nucleic acids and other substrate inside lipid vesicles (Deamer, & Uster, 1983; Szoka & Papahadjopoulos, 1978, 1980). However, the simple method of freeze/thawing described by Hinkle (Kasahara & Hinkle, 1977) was appealing because of its speed, ease of execution and the
relative mild conditions involved. Simply described, encapsulation was achieved by incubating preformed unilamellar lipid vesicles with the substance of interest. The mixture was then frozen to liquid N\textsubscript{2} temperature and then allowed to thaw in a water bath at room temperature. The extent of encapsulation was measured by isolation of these vesicles by chromatography or by a simple filtration assay.

It is believed that freezing of the solution breaks the vesicles which then form lipid sheets. Upon thawing, the sheets join each other to form larger vesicles and in the process a certain amount of liquid is "trapped" inside the newly formed vesicles. Thus, any substance dissolved in the solution bathing the vesicles prior to freezing will be encapsulated in the new vesicles (Kasahara & Hinkle, 1977).

III.6 a) Encapsulation of [\textsuperscript{3}H]sucrose and [\textsuperscript{14}C]BSA inside lipid vesicles

Encapsulation of [\textsuperscript{3}H]sucrose as measured by the filtration assay is demonstrated in Figure 32. As the concentration of soybean lecithin was increased in the pre-freeze/thaw mixture a corresponding increase in encapsulated volume was observed. Because BSA may interact with phospholipids in a manner that could prevent perfect resealing of the vesicles, the efficiency of encapsulating sucrose in the presence of BSA was determined. The amount of sucrose being encapsulated was not significantly affected when BSA was present during the freeze/thaw process (Figure 33). The internal volumes
Figure 32. Trapping of sucrose inside lipid vesicles. Aliquots from a stock (163 mg/mL) of soybean lecithin S-II solution in 10 mM MOPS pH 7.4 were mixed to give the final concentrations indicated. The assay mixture contained 150 mM NaCl and 12 µM [³H]sucrose (S.A. 0.1 Ci/mmol). The samples were frozen in liquid nitrogen followed by thawing (5 min) at room temperature. The solution was transferred to a borosilicate tube, sonicated for 5 seconds at the center of a sonicator water bath, and then 50 µL aliquots were diluted into 1.5 mL of cold (0°C) 0.1% BSA in 10 mM MOPS pH 7.4, 150 mM NaCl and 12 µM sucrose present in the filtration apparatus. Immediately after the dilution was effected the vacuum was applied and the filtration was started. The assay was performed in duplicate. The results are plotted as volume of sucrose trapped inside the vesicles.
Figure 33. Trapping of sucrose and BSA inside lipid vesicles. Aliquots from a stock (163 mg/mL) of soybean lecithin S-II solution in 10 mM MOPS, pH 7.4 were mixed to give the final concentrations indicated. The assay mixture contained 150 mM NaCl and 12 μM [3H]sucrose (S.A. 0.1 Ci/mmol) (continuous line), or [14C]BSA (S.A. 12.2 mCi/mmol) with 12 μM sucrose (dotted line). The samples were frozen in liquid nitrogen followed by thawing (5 min) at room temperature. Processing of the samples was as described in Figure 32. The assay was performed in duplicate as outlined in materials and methods. Results are plotted as percent of material trapped inside the vesicles.
that were found using sucrose or BSA were comparable (e.g.
found 0.40 μL(+/-0.05)/mg phospholipids with [3H]sucrose and
0.37 μL(+/-0.05)/mg phospholipids with [14C]BSA when 30 mg/mL
of lecithin were used). Encapsulation of BSA paralleled the
encapsulation of sucrose and increased linearly with
increasing lipid concentration (Figure 33).

To increase the amount of BSA being encapsulated
inside the vesicles, an investigation was made to determine
if increasing the size of the lipid vesicles prior to
freeze/thawing would be effective. This was achieved by
subjecting small unilamellar vesicles to an initial
freeze/thaw cycle before mixing them with the substance to be
encapsulated. The results presented in Figure 34 support the
idea that a single freeze/thaw cycle dramatically increased
the amount of sucrose encapsulated. This approach was
subsequently used for all other preparations of lipids. Thus,
an optimum concentration of soybean lecithin (90 mg/mL) was
hydrated and sonicated until clear and then subjected to a
freeze/thaw cycle in absence of the material to be
encapsulated. Vesicles obtained with higher concentrations of
soybean lecithin tended to clog the assay filters and were
difficult to isolate by column chromatography.

For assay purposes the loaded vesicles had to be iso-
lated free from unencapsulated BSA by sieving chromatography
on Sepharose 4-B (Figure 35). As demonstrated in Figure 35,
capsulated [3H]sucrose and [14C]phosphatidylcholine, added
Figure 34. Effect of a pre-freeze/thaw cycle on sucrose encapsulation. Lipids (soybean lecithin S-II) (45 mg/mL) were suspended in 10 mM MOPS, pH 7.4 and the mixture was sonicated in a sonicator tube located at the center of a sonicator water bath until a clear solution was obtained (15 to 30 min). A portion of this stock solution was subjected to a freeze/thaw cycle (----) prior to being mixed with 12 μM [3H]sucrose (S.A. 0.1 Ci/mmol), 150 mM NaCl while the remainder of the lipid solution was used as described in Figure 32 (-----). After the "loading" freeze/thaw cycle, the assay was performed in duplicate as described in Figure 32. Results are expressed in terms of encapsulated sucrose.
Figure 35. Elution of $[^3\text{H}]$sucrose-loaded vesicles, $[^{14}\text{C}]$BSA and $[^{14}\text{C}]$phosphatidylcholine on Sepharose 4-B. Vesicles were prepared by mixing 250 nmol (25 μCi) of $[^3\text{H}]$sucrose (S.A. 0.1 Ci/mmol) (○) with 81 mg of lecithin (pre-freeze/thawed at a concentration of 90 mg/mL) containing 0.1 μCi of $[^{14}\text{C}]$phosphatidylcholine (PC; ×•×•×•) in a final volume of 1.25 mL of 10 mM MOPS pH 7.4, containing 150 mM NaCl. After the freeze/thaw cycle, the solution was sonicated for 5 s at the center of a sonicator water bath and 1 mL of solution was applied on a 20 mL Sepharose 4-B column equilibrated in the above buffer containing 0.02% NaN₃. Fractions of 500 μL were collected. In a separate elution, 8.25 mg of $[^{14}\text{C}]$BSA (S.A. 12.2 mCi/mmol) was added and analyzed in a similar way (♦•♦•). Radioactivity was determined by liquid scintillation counting using ACS.
as a tracer of the vesicles, co-eluted at the void volume. Unencapsulated $[^{3}\text{H}]$sucrose (and $[^{14}\text{C}]$lysophosphatidylcholine present as a contaminant of the tracer amount of $[^{14}\text{C}]$phosphatidylcholine) eluted at the bed volume. Unencapsulated $[^{14}\text{C}]$BSA eluted near the bed volume thus allowing the separation of the vesicles from the unencapsulated material.

The internal volumes determined using $[^{3}\text{H}]$sucrose and/or $[^{14}\text{C}]$BSA were in agreement suggesting that no BSA aggregates were trapped in the process. As observed in the filtration assay BSA did not prevent proper resealing of the vesicles (Figure 36). Typical internal volumes were of the order of 1.5-2.9 $\mu$L/mg of lipids which is comparable to the values determined by the filtration assay (1.2-1.9 $\mu$L/mg lipids) using the pre-freeze/thawed lipids in both cases. The lower values obtained with the filtration assay may reflect breakage of vesicles which would in effect reduce the amount of material trapped. These values are in agreement with those reported by Hinkle (Kasahara & Hinkle, 1977) who used a single freeze/thaw cycle (no pre-loading cycle) and reported values up to 0.98 $\mu$L/mg of lipids. This value is much higher than the one that was found in absence of the pre-freeze/thaw cycle (0.40 $\mu$L/mg of lipids). Technical differences such as length of exposure of the vesicles to the vacuum during filtration as well as handling of the filtered vesicles (extent of washings) could account for this discrepancy.

For transport and/or diffusion assay purposes, frac-
Figure 36. Determination of the internal volume of lipid vesicles freeze/thawed in presence of [\(^3\)H]sucrose and [\(^{14}\)C]BSA. Vesicles were prepared by mixing 250 nmol (25 μCi) of [\(^3\)H]sucrose (S.A. 0.1 Ci/mmol) with 8.25 mg of [\(^{14}\)C]BSA (S.A. 12.2 mCi/mmol) and with 81 mg of lecithin (pre-freeze/thawed as described in Figure 35) in a final volume of 1.25 mL of 10 mM MOPS, pH 7.4 containing 150 mM NaCl, followed by a freeze/thaw cycle and a 5 s sonication as described in Figure 35. One mL of the thawed material was applied on a 30 mL Sepharose 4-B column equilibrated in the described buffer containing 0.02% NaN\(_3\). One mL fractions were collected and the radioactivity determined by liquid scintillation counting using ACS.
tions containing the vesicles were pooled or more often the most turbid fraction was used. The latter typically contained a total of 190 μg of BSA resulting in the presence of 28 μg of BSA per assay point.

The possibility that some BSA could remain on the outside of the vesicles was considered as a potential contribution to non-specific labelling by photoreactive fatty acids. To study the extent of BSA binding to vesicles isolated by chromatography, [14C]BSA was added after the loading freeze/thaw cycle (Figure 37). It was determined that 0.2 to 0.5 μg of BSA per mg of lipids clung to the vesicles which represented less than 0.1 to 0.4% of the BSA encapsulated. In view of this small contribution no attempts were made to remove it. From these findings it was concluded that BSA could be used as a fatty acid trapping agent inside lipid vesicles. The next step consisted in separating photolytically labelled BSA from photolyzed DAP-fatty acids.

III.6 b) Study of binding of photoreactive fatty acids to fatty acid binding sites on BSA

The second step of the assay consisted in successfully separating photolytically labelled BSA from photolyzed fatty acids. This was achieved by separating both components by chromatography on LH-60 in 95% ethanol/88% formic acid (70/30) (Figure 38). In the latter experiment [9-3H]9-DAP-nonanoate (VII.1) was photolyzed, dried and resuspended in 88% formic acid prior to chromatography on LH-60. Similarly
Figure 37. Determination of the amount of BSA sticking on the outside of the freeze/thawed vesicles. Vesicles were prepared by mixing 250 nmol (25 μCi) of [3H]sucrose (S.A. 0.1 Ci/mmol) with 81 mg of freeze/thawed lecithin (as described in Figure 35) in a final volume of 1.0 mL of 10 mM MOPS pH 7.4 containing 150 mM NaCl, followed by a freeze/thaw cycle. After freeze/thawing the vesicles, 8.25 mg of [14C]BSA (S.A. 402 dpm/μg) were mixed and 1 mL was chromatographed on a Sepharose 4-B column as described in Figure 36. One mL fractions were collected and the radioactivity determined by liquid scintillation counting using ACS.
Figure 38. Separation of $[^{14}\text{C}]$BSA and photolyzed $[9-{^3}\text{H}]$9-DAP-nonanoate (VII.1) on Sephadex LH-60. $[9-{^3}\text{H}]$9-DAP-nonanoate (VII.1) (55 nmol) (S.A. 170 mCi/mmol) or 3.34 mg of $[^{14}\text{C}]$BSA (S.A. 12.2 mCi/mmol) were photolyzed in a final volume of 500 µL of phosphate buffered saline. Photolysis was carried out at room temperature as described in materials and methods. Each solution was dried under vacuum and the dried material was resuspended with 500 µL of 88% formic acid. The acidic solutions were assayed separately by chromatography on a 30 mL LH-60 column equilibrated in 95% ethanol/88% formic acid (70/30). One mL fractions were collected and the radioactivity determined by liquid scintillation counting using ACS. $[^{14}\text{C}]$BSA (•—•), photolyzed $[9-{^3}\text{H}]$9-DAP-nonanoate (VII.1) (Δ---Δ).
[14C]BSA was photolyzed, the solution dried and the residue resuspended in 88% formic acid followed by chromatography. The net result was that [14C]BSA eluted at the void volume while photolyzed [9-3H]9-DAP-nonanoate (VII.1) eluted near the bed volume.

When photolyzed [9-3H]9-DAP-nonanoate (VII.1) was mixed with photolyzed [14C]BSA followed by drying and chromatography on LH-60, some binding of [9-3H]9-DAP-nonanoate (VII.1) was observed (Figure 39A). This non specific binding of photolyzed [9-3H]9-DAP-nonanoate (VII.1) could be alleviated by the addition of Triton X-100 during the drying process (Figure 39B).

Double labelling experiments established that incubation of [14C]BSA with [9-3H]9-DAP-nonanoate (VII.1) followed by photolysis resulted in substantial crosslinking of the latter to BSA (Figure 40). Interestingly about 50% of the total tritium in the sample was found associated with BSA after photolysis. These results established that it was possible to label the fatty acid binding sites present on BSA.

The presence of Triton X-100 could conceivably have affected the chromatographic pattern of [14C]BSA. To attest that no effect on the elution pattern resulted from this addition, [14C]BSA was mixed with Triton X-100 and processed as described in Figure 39. As expected the elution pattern of BSA was unaffected under these conditions (Figure 41).
Figure 39. Binding of photolyzed [9-^3H]9-DAP-nonanoate (VII.1) to BSA. [9-^3H]9-DAP-nonanoate (VII.1) (S.A. 170 mCi/mmol; Δ--Δ) and 3.34 mg of [^14C]BSA (S.A. 180 nCi/mg; +----+) were photolyzed separately as described in Figure 38 in a final volume of 500 μL. The solutions were mixed and dried under vacuum followed by resuspension in 88% formic acid (500 μL) and chromatography on LH-60 in 95% ethanol/88% formic acid (70/30). Fractions of 1 mL were collected (A). In (B) the same procedure was followed using non-radioactive BSA. The samples were (Δ--Δ) or were not (+----+) mixed with 70 μmoles of Triton X-100 prior to drying. The dried samples were treated as described above. Radioactivity was determined by liquid scintillation counting using ACS.
Figure 40. Photolysis of [14C]BSA in presence of [9-3H]9-DAP-nonanoate (VII.1). In 500 µL of phosphate buffered saline, 55 nmol of [9-3H]9-DAP-nonanoate (VII.1) (S.A 170 mCi/mmol) were mixed with 3.34 mg of [14C]BSA (S.A. 12.2 mCi/mmol). Photolysis was carried out at room temperature as described in materials and methods. After photolysis the samples were dried in presence of 70 µmol of Triton X-100 and resuspended in 88% formic acid (500 µL) followed by chromatography on LH-60 in 95% ethanol/88% formic acid (70/30). Fractions of 1 mL were collected and the radioactivity was determined by liquid scintillation counting using ACS.
Figure 41. Final conditions for the separation of [9-3H]9-DAP-nonanoate (VII.1) crosslinked to BSA from photolyzed [9-3H]9-DAP-nonanoate (VII.1). Non radioactive BSA (3.34 mg) was mixed with 55 nmol [9-3H]9-DAP-nonanoate (VII.1) (170 mCi/mmol) and photolyzed as described in Figure 38. Triton X-100 (70 µmol) was added to all photolyzed samples (unless indicated otherwise) before drying. The elution profiles from an LH-60 column of photolytically labelled BSA (•—•), of photolyzed [9-3H]9-DAP-nonanoate (VII.1) mixed with Triton X-100 (Δ--Δ), of photolyzed [14C] BSA mixed with Triton X-100 (▲---▲), and of photolyzed [9-3H]9-DAP-nonanoate (VII.1) in absence of Triton X-100 (○—○) are shown. The eluant used was 95% ethanol/88% formic acid (70/30). Fractions of 1 mL were collected and the radioactivity was determined as in Figure 40.
Similarly the effect of Triton X-100 on photolyzed and unphotolyzed \([9-^{3}H]9\text{-DAP-nonanoate}\) was studied. No change in chromatographic pattern was observed, thus eliminating the possibility that large mixed micelles were formed in the process (Figure 41).

III.6 c) Titration of fatty acid binding sites on BSA

The successful separation of photolyzed \([9-^{3}H]9\text{-DAP-nonanoate}\) from labelled BSA permitted the determination of the extent of labelling of the latter upon increasing the concentration of \([9-^{3}H]9\text{-DAP-nonanoate}\). The results of this experiment are reported in Figure 42. It was observed that with increasing concentration of \([9-^{3}H]9\text{-DAP-nonanoate}\) a corresponding increase in labelling of BSA resulted. Pre-incubation of BSA with the lipid mixture used during the freeze/thawing procedure did not affect labelling to a significant extent. This further validated the use of BSA as a fatty acid trapping agent.

In previous sections it was demonstrated that BSA could be encapsulated inside lipid vesicles (Figures 33 and 36) and that under these conditions fatty acid binding sites remained available for binding \([9-^{3}H]9\text{-DAP-nonanoate}\) (Figure 42). Thus the next step in the investigation of these vesicles for use in reconstitution assays consisted in performing diffusion experiments to determine the extent of labelling of BSA trapped inside the vesicles.
Figure 42. Titration of BSA with $[9-^3H]9$-DAP-nonanoate (VII.1). BSA (---) (334 µg) was mixed with increasing concentrations of $[9-^3H]9$-DAP-nonanoate (VII.1) (S.A. 170 mCi/mmol) in a final volume of 50 µL phosphate buffered saline as indicated. In one case (○-○) BSA had been obtained from the supernatant of a freeze/thaw experiment where the lipid concentration was 64.8 mg/mL and the BSA concentration was 6.6 mg/mL. After a 30 s incubation at room temperature, photolysis was carried out as described in materials and methods. After photolysis 70 µmol of Triton X-100 were added and the samples dried under vacuum. The dried samples were resuspended in 88% formic acid (500 µL) and were subsequently analyzed as described in Figure 41.
III.6 d) Assay of fatty acid diffusion using BSA-loaded vesicles

To evaluate the BSA loaded vesicles for diffusion assay purposes, they were incubated with [9-\(^3\)H]9-DAP-nonanoate (VII.1). The results reported in Table VI suggest that the lipids did not act as a valid barrier for fatty acids given the large extent of labelling observed. Reduction of the temperature of incubation in the hope that the lipids would be in a more rigid conformation did not improve the situation. However, when concentrations of fatty acids in the range of 0.05 \(\mu\)M to 1.0 \(\mu\)M were used, a linear relationship was established between the extent of labelling and the decrease in concentration. Thus, for a 10 fold diminution in concentration a 10 fold decrease in BSA labelling was observed (Table VII). The source of this BSA labelling had to be differentiated between diffusion and the presence of residual BSA on the outside of the vesicles. As evidenced from the results reported in Table VIII, the extent of BSA labelling that was observed likely reflected the amount of BSA on the outside of the vesicles. Although it was previously determined that negligible amounts of BSA was left on the outside of the vesicles, the results of Table VII and VIII suggest that some vesicles lysed upon standing or in presence of [9-\(^3\)H]9-DAP-nonanoate (VII.1). This would lead to increased levels of BSA on the external side of the vesicles.

To limit the extent of vesicle lysis the photoreactive
Table VI

Diffusion Assay using BSA loaded vesicles isolated on Sepharose-4B*

<table>
<thead>
<tr>
<th>Incubation Time (min.)</th>
<th>Labelling (dpm) at 37°C</th>
<th>Labelling (dpm) at 0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>68,000</td>
<td>56,000</td>
</tr>
<tr>
<td>1.0</td>
<td>88,000</td>
<td>140,000</td>
</tr>
<tr>
<td>5.0</td>
<td>93,000</td>
<td>116,000</td>
</tr>
<tr>
<td>15.0</td>
<td>89,000</td>
<td>133,000</td>
</tr>
<tr>
<td>30.0</td>
<td>87,000</td>
<td>105,000</td>
</tr>
</tbody>
</table>

*BSA-loaded vesicles were prepared essentially as described in Figure 35 using non-radioactive BSA. The BSA-loaded vesicles were separated from unencapsulated BSA by chromatography on a 30 mL Sepharose 4-B column (Figure 35) and fraction 8 was used. The vesicles were equilibrated at the appropriate temperature in a water bath and the assay was started by the addition of [9-3H]9-DAP-nonanoate (VII.1) (S.A. 1.45 Ci/mmol) to give a final concentration of 100 μM. At the indicated time points 150 μL aliquots** were transferred to siliconized quartz cuvettes and photolyzed for 20 s at 360 nm as described in materials and methods. After photolysis, the cuvettes were rinsed with two aliquots of 200 μL 10 mM MOPS, pH 7.4 containing 150 mM NaCl. Triton X-100 (70 μmol) was added to each samples prior to removal of the solvent under reduced pressure. The dried samples were resuspended in 500 μL 88% formic acid and assayed as described in Figure 41.

**(containing about 28 μg of BSA).
Table VII

Effect of fatty acid concentration on labelling of BSA trapped inside liposomes.*

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>1 μM (dpm)</th>
<th>0.1 μM (dpm)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.42</td>
<td>20,400</td>
<td>2,400</td>
<td>0.33</td>
</tr>
<tr>
<td>1</td>
<td>24,000</td>
<td>2,800</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>24,700</td>
<td>2,500</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>27,800</td>
<td>2,900</td>
<td>10</td>
</tr>
<tr>
<td>19</td>
<td>28,000</td>
<td>3,400</td>
<td>15</td>
</tr>
</tbody>
</table>

*BSA-loaded vesicles were prepared and assayed essentially as described in Table VI. The assay was performed at 37°C and was started by addition of [9-3H]-9-DAP-nonanoate (VII.1) (S.A. 1.45 Ci/mmol). At the indicated time points an aliquot containing 28 μg of BSA was taken and processed as described in Table VI.
Table VIII

Incubation of empty freeze/thawed vesicles with increasing amounts of BSA.

<table>
<thead>
<tr>
<th>BSA</th>
<th>Phospholipids*</th>
<th>dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µg</td>
<td>+</td>
<td>80</td>
</tr>
<tr>
<td>5 µg</td>
<td>+</td>
<td>180</td>
</tr>
<tr>
<td>10 µg</td>
<td>+</td>
<td>360</td>
</tr>
<tr>
<td>28 µg</td>
<td>+</td>
<td>2000</td>
</tr>
<tr>
<td>28 µg</td>
<td>-</td>
<td>9100</td>
</tr>
</tbody>
</table>

*Phospholipid vesicles were prepared as described in Table VI in absence of BSA. Fraction 8 was used as a source of vesicles. Aliquots were incubated at 37°C for 2 min in siliconized quartz cuvettes in presence of 0.05 µM [9-³H]9-DAP-nonanoate (VII.1) (S.A. 1.45 Ci/mmol). Increasing amounts of BSA that had been in contact with phospholipids (as described in Figure 40) were added. The incubations were stopped by photolysis and the samples analyzed as described in Table VI.
fatty acids could be presented to the vesicles on a protein carrier. After photolysis, the vesicles would be isolated by chromatography on Sepharose 4-B prior to processing as described above. Alternatively, the assay could be modified such that the photoreactive fatty acids would be trapped inside the vesicles and BSA would be left outside. Post photolysis processing would then consists of isolating the BSA fractions from a Sepharose 4-B column.
IV Conclusion

The main objectives of this thesis consisted of: 1) the synthesis of the photoreactive probe m-diazirinophenol (6), 2) the synthesis of an homologous series of radioactive hydroxy fatty acid analogues, 3) the attachment of the photoreactive probe (6) to the omega carbon of the radioactive homologous series, 4) the evaluation of the biological activity of these probes both in vitro and in vivo, and 5) the evaluation of the use of these probes in the identification of integral membrane proteins.

The synthesis of the photoreactive probe m-diazirinophenol proved more complex than had originally been anticipated. Anomalous NMR spectra of m-diazirinophenol (6) suggested that an aromatic proton was lost in the course of the synthesis. HPLC analyses of the products of the t-butyl hypochlorite oxidation step required for the generation of the diazirine ring revealed the presence of multiple compounds having the characteristic diazirine absorption at 360 nm. These compounds were found to be chlorinated isomers of the desired m-diazirinophenol (6). The unexpected discovery of chlorination of the phenyl ring provided clues to improve the yield of the desired product. Thus, the inclusion of pyridine in the oxidation mixture prevented chlorination and improved significantly the yield of m-diazirinophenol.
This phenolic probe was attached at the omega carbon of an homologous series of radioactive omega hydroxy fatty acids. A synthetic approach was designed using inexpensive starting materials (olefins). Olefins were oxidized with OsO₄ and the isolated semialdehyde intermediates were reduced with [³H]NaBH₄. Following this scheme, the synthesis of an homologous series of radioactive photoreactive fatty acid analogues was achieved. In the course of the synthesis, strategies were developed to free hydrophobic compounds of aprotic solvents.

In view of reported failures (Olsen, Schaechter, & Khorana, 1979) it was of interest to establish the biological activity of these analogues. Using rat liver microsomes as a source of enzyme(s) it was found that the analogues behaved like normal substrates and were activated to the corresponding acyl-CoA derivatives. Furthermore, under proper assay conditions it was demonstrated that rat liver microsomes could incorporate these analogues into phospholipids. It was thus established that the endogenous microsomal enzymes involved in phospholipid synthesis were not affected by the photoreactive group. It was also found that L-cells in culture were capable of incorporating all the members of the homologous series into their major phospholipid classes. These results suggested that the lack of biological activity reported by others (Olsen, Schaechter, & Khorana, 1979), most likely reflected the chlorination of
the photoreactive probe used in their investigations.

The advantage of these probes in membrane studies was confirmed by the photolysis work. First it was found that L-cells processed these fatty acid analogues like the normal substrate. Thus, the \([9-^3\text{H}]\text{C}-\text{DAP-nonanoate (VII.1)}\) derivative was used to acylate normally fatty acylated proteins. Photolysis of L-cells or of VSV virions obtained from cells growing in the presence of \([9-^3\text{H}]9\text{-DAP-nonanoate (VII.1)}\) resulted in intense labelling of integral membrane proteins. The advantage of using a high energy source was demonstrated by the relatively short time required for complete photolysis (Figure 27) and in turn no photolytic damage was observed.

Incubations of \(\text{E. coli}\) membrane vesicles with \([11-^3\text{H}]11\text{-DAP-undecanoate (VII.2)}\) followed by photolysis resulted in the labelling of a protein of 30 kD (43 kD upon boiling) which behaved in a similar fashion to the FLP protein described by Nunn's group (Ginsburgh et al., 1984, Black et al., 1985, Black et al., 1987). Further comparisons with FLP would consist in using the FLP anti-serum described by Black et al., (1987) and to perform immunoprecipitations and/or Western analyses of the photolyzed membranes.

Attempts were made to develop a fatty acid diffusion assay. The major idea governing the assay consisted of inserting a trapping agent inside closed lipid vesicles. BSA was chosen for its fatty acid binding capacity (Spector, 1975). Using filtration and chromatographic techniques it was
demonstrated that vesicles containing BSA could be obtained. These vesicles appeared relatively stable and could be isolated quickly. In order to quantitate the passage of fatty acids across the lipid bilayer of these vesicles, means of separating photolytically labelled-BSA from photolyzed fatty acids were developed. BSA was found to elute at the void volume and the photolyzed DAP-fatty acids eluted near the bed volume of an LH-60 column in the solvent system 95% ethanol/88% formic acid (70/30).

Vesicles loaded with BSA were found to be mostly impermeable to [9-³H]9-DAP-nonanoate (VII.1) although a small amount of BSA labelling was observed upon photolysis. It is possible that local leakage resulting from the addition of [9-³H]9-DAP-nonanoate (VII.1) was responsible for the presence of BSA on the outside of the vesicles. An alternative form of this assay could consist in trapping the fatty acids instead of BSA. The type of lipid used in this model system may not reflect the usual composition encountered with different organisms. Therefore, it might be necessary to test different mixture of lipids under these assay conditions.

In conclusion the present work established the usefulness of these photoreactive fatty acid analogues as photoaffinity reagents and as membrane probes. They proved to be stable under in vitro and in vivo conditions and were amenable to measurement of Michaelis-Menten constants. Their
capacity to bind to hydrophobic sites on BSA, as demonstrated by the high level of crosslinking observed, further support their closeness to the natural substrate.

As membrane probes, this homologous series showed advantages to the traditional surface iodination or the use of hydrophobic photoreactive probes since the labelling of membrane proteins was dependent on nearest neighbors present in the lipid bilayer rather than on availability of reactive sites. Moreover, minor membrane components, such as observed in the case of VSV (Figure 28, band VI), were intensely labelled therefore facilitating their characterization. In the case of membrane components buried into the lipid bilayer these analogues will provide a more intense labelling than other membrane labelling approaches that rely on the random partitioning of the probes into the bilayer.
V. Directions for future research

Recent developments in the area of fatty acid transport in *E. coli* warrant a brief review of the subject. As presented in this thesis, Black et al., (1987) purified FadL (FLP) and established that it is an outer membrane protein that serve as a fatty acid binding protein. The gene coding for FadL was cloned into plasmid pN103 (*fadL*<sup>+</sup>) and its sequence has been determined (Said, Ghosn, Vu, & Nunn, 1988; Black, 1991). According to the latest corrections to the sequence (Black, 1991), the *fadL* gene extends over 2,197 base pairs with an open reading frame of 1,344 base pairs coding for a protein of 448 amino acids and a molecular weight of 48,831. This pre-FadL protein is apparently processed by signal peptidase I through the recognition sequence Ala-Trp-Ser to generate the mature 45,969 kD product. It was found that FadL shares sequence homology with the heat-modifiable outer membrane protein P1 of *Haemophilus influenzae* type b.

Transfection studies with plasmid pN103 (*fadL*<sup>+</sup>) established that FadL is involved in the sensitivity of *E. coli* to bacteriophage T2 (Black, 1988). Rare T2 resistant (*ttr*) strains of *E. coli* fail to grow in minimal media containing 5 mM oleate. Expression of FadL restored the T2<sup>S</sup> phenotype and allowed growth on oleic acid. This observation
may explain earlier results showing the dependence of fatty acid transport on the presence of OmpF since the latter is also known to be involved in T2 sensitivity (Maloy et al., 1981).

In a series of experiments repeating earlier work (Nunn, Colburn, & Black, 1986), Black (1990) rediscovered that FadL binds oleate and measured new $K_d$ values of $2.3 \times 10^{-7}$ and $8.8 \times 10^{-7}$ for oleate and palmitate respectively. Oleate binding could be inhibited with anti-FadL serum, and it was found to be pH independent. Black estimated that about 35,000 oleate binding sites are present in fadL+ strains (Black, 1990).

Kumar and Black (1991) have attempted to study the structure function relationship of FadL by linker mutagenesis. They mutagenized a total of 5 different sites (4 Hpa II and 1 Sal I) covering the whole length of the protein. The resulting mutants were at positions 41, 81, 238, 389 and 410 and are referred to as H1, H2, H3, H5, and S1 respectively. Mutants closer to the N-terminal region such as H1 and H2 had their fatty acid binding capacity affected while the carboxyl terminal mutants H5 and S1 showed a greater defect in oleate transport. S1 produced an FadL protein that was not heat modified as is typically the case for the wild type product (Kumar & Black, 1991). Thus suggesting that the N-terminal region of FadL is involved in fatty acid binding while the C-terminal region plays a role
in transport.

Is FadL sufficient for the transport of fatty acid in *E. coli*? Earlier work (Kameda, Suzuki, & Imai, 1987) suggested spheroplasts transported fatty acids with greater efficiency than intact cells therefore arguing against the need for a cytoplasmic membrane protein. Recent work by Mangroo and Gerber (1991; D. Mangroo personal communication) further support this view. However, they have observed that starved *E. coli* ML308 had a reduced uptake of fatty acids. When D-lactate, L-lactate, succinate or acetate was supplied to these bacteria, fatty acid transport was stimulated although energy dependent transport, such as proline transport, was not affected (Mangroo & Gerber, 1991). It appears that acyl-CoA synthetase activity was enhanced upon addition of these substrates. Furthermore, a study of vesicles obtained from these experiments indicated that acyl-CoA synthetase was recruited to the membrane as treatment with Triton X-100 had a 100-fold stimulation of its activity.

Future work will certainly focus on the mechanism of recruitment of acyl-CoA synthetase to the plasma membrane. This membrane association may be brought about by a change in the conformation or by hydrophobic modification such as fatty acylation. The latter hypothesis has been put forward by many investigators studying the role of protein fatty acylation (James, & Olson, 1990; Magee, Gutierrez, Marshall, & Hancock,

The availability of photoreactive fatty acids will certainly help the study of various proteins involved with the binding and metabolism of fatty acids. Thus, one can ask: does the passage of fatty acids across the plasma membrane of mammalian cells require a plasma membrane fatty acid binding protein? And what is the role of fatty acylation of proteins in cellular signaling? Hopefully these probes will help clarify the ideas surrounding these phenomena.

The radioactive photoreactive fatty acids synthesized in this thesis were used to identify integral membrane proteins in vivo using the well defined system of the G protein of VSV. It might be useful to evaluate these fatty acids for their use as membrane probes typical of the hydrophobic probes presented in the introduction. Using purified virions of VSV it would be possible to compare the efficiency of the two approaches to label integral membrane proteins. Other defined systems could also be evaluated were the integral membrane protein is known not to have specific affinity for fatty acids. One example to consider would be the labelling of the class I peptides of the major histocompatibility complex (Nathenson, Uehara, Ewsonstein, Kindt, & Colligan, 1981). This complex includes one peptide chain of 45 kD that is embedded in the membrane and a noncovalently attached peptide called β-2 microglobulin which has a molecular weight of 12,000. Recently, the human class I
HLA-A2 structure has been elucidated by X-ray crystallography and therefore it would be of interest to study the labelling patterns of the homologous series with respect to its membrane fraction (Bjorkman, Saper, Samraoui, Bennett, Strominger, & Wiley, 1987). It would be interesting to see if the latter could be labelled by hydrophobic partitioning of the DAP-fatty acids. Such information would help define the limit of such probes for membrane studies.

The work of Khorana's group on cytochrome b₅ (Takagaki, Radhakrishnan, Gupta, & Khorana, 1983; Takagaki, Radhakrishnan, Wirtz, & Khorana, 1983) demonstrated that chemically attaching such photoreactive groups at the second position of phospholipids provides information about the position of certain protein fragments in the bilayer. Thus future efforts might be aimed at synthesizing photoreactive phospholipids and study their use in reconstitution systems of interest.
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