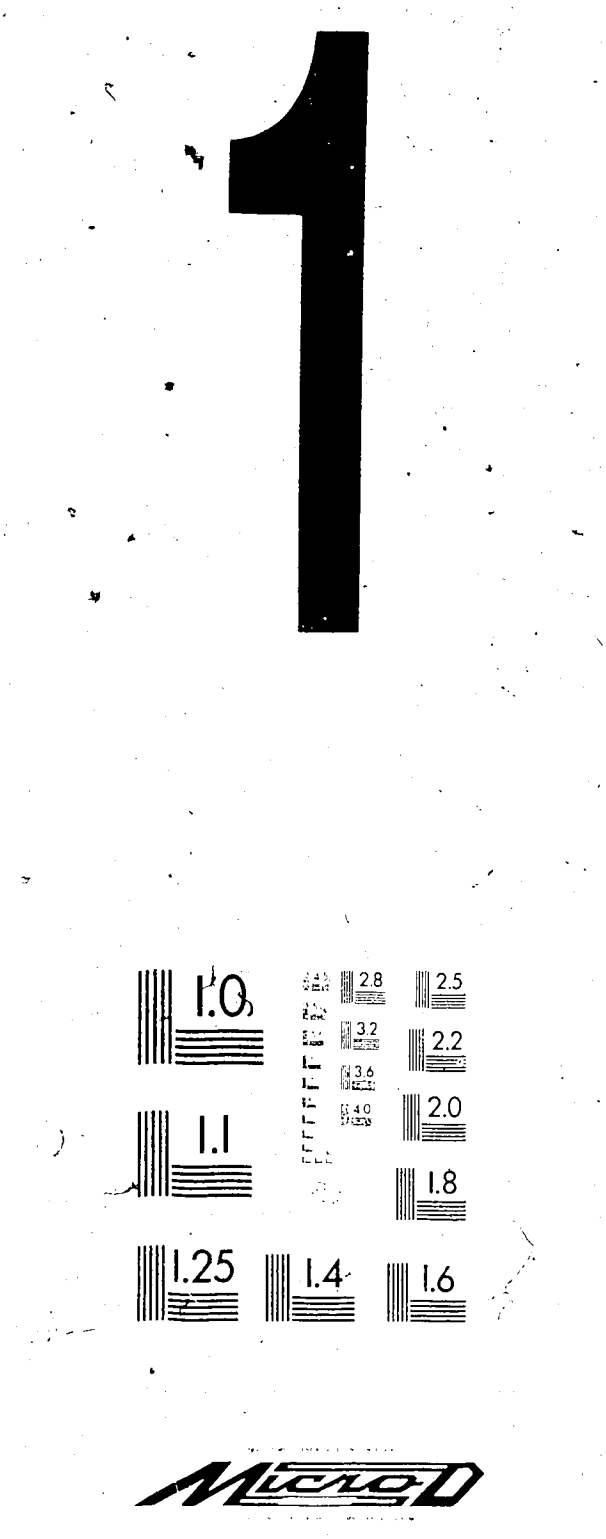
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THE STABILIZATION OF DRUGS IN LIPOSOMES:THEORY AND PRACTICE

by

© MUHAMMAD J. HABIB

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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Supervisor

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External Examiner

Date...July 6, 1987.....

DEDICATION

TO MY PARENTS AND ALL MY TEACHERS

ABSTRACT

Liposomes have been investigated for their potential to stabilize drugs with which they become associated. Acetylsalicylic acid, local anesthetics, and cefotaxime sodium were selected as model compounds for this evaluation. The kinetics of hydrolysis in liposomes were studied as a function of liposome composition, method of preparation, drug:lipid ratio, temperature, pH, and ionic strength of the medium. When ASA was incorporated initially in the lipid phase, a 25 % increase in stability was obtained in neutral dimyristoyl lecithin (DMPC) liposomes at pH 4.0 and 30°. Increasing the liposome concentration provided a proportional increase in the stabilization of ASA. The addition of stearylamine (STEAR) to the organic solvent mixture caused a rapid initial hydrolysis of ASA but once liposomes were formed the remaining ASA was stabilized at substantially higher levels. The evidence suggests that anionic ASA complexes electrostatically to the phospholipid polar groups (to the positively-charged choline groups) and is subsequently protected against hydrolytic attack.

The liposome stabilities of several local anesthetics were also measured at pH 12.2 at 30°. Increases in stability correlated with their partition coefficients and ranged from 17% to 84%, but the addition of 50 mole% of CHOL reduced the increase to about one-third of what it was in lecithin liposomes alone whereas negatively-charged

liposomes yielded markedly increased stabilization of the local anesthetics.

Cefotaxime sodium (CFX) was not stabilized in liposomes. However, unionized CFX rapidly solubilized liposomes, the rate of solubilization rapidly decreasing with an increase in pH. After solubilization, the solutions gradually returned to a turbid state and large crystals were observed microscopically. The solubilization effect is probably due to mixed micelle formation of phospholipid and CFX initiated by the surface activity of the drug.

In conclusion, the potential of liposomes to stabilize drugs in solution is dependent on both the fraction of drug associated with the liposomes and the protection of drug in the phospholipid bilayers. The partition coefficient plays an important role in the stabilization of nonionic drugs whereas electrostatic association of anionic drug, such as ASA, with phospholipid plays a major role. Overall, levels of stabilization in liposomes of 20%-80% were achieved.

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My brothers and sister for their continuous encouragement and support throughout my life.

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ASA	Acetylsalicylic Acid
CFX	Cefotaxime Sodium
CHOL	Cholesterol
CMC	Critical Micellar Concentration
DCP	Dicetylphosphate
DLPC	Dilaurylphosphatidylcholine
DMPC	Dimyristoylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DRV	Dehydration-Rehydration Vesicle
EPC	Egg Phosphatidylcholine
LUV	Large Unilamellar Vesicle
MLV	Multilamellar Vesicle
ORM	Orcinol Monohydrate
PA	Phosphatidic Acid
PG	Phosphatidylglycerol
PL	Phospholipid
PS	Phosphatidylserine
REV	Reverse-Phase Evaporation Vesicle
SA	Salicylic Acid
SPHING	Sphingomyelin
SPLV	Stable Plurilamellar Vesicle
STEAR	Stearylamine
SUV	Small Unilamellar Vesicle
Tc	Phase Transition Temperature

1. INTRODUCTION

The stability aspects of drugs formulated in dosage forms is of paramount importance if drug products are to be safe, effective and reliable. One of the main criteria of preformulation studies is to establish the chemical (and in some instances, physical) stability of a drug and hence, ascertain its potential shelf-life in the physical unit in which it is to be administered. On many occasions, the stability behavior of the drug precludes its formulation into certain types of dosage forms and the route by which it can be administered most efficiently.

The formulation of a drug necessary to maintain adequate stability under these circumstances requires careful selection of vehicle and excipients which ideally will minimize degradation of the drug in a complementary manner but at the same time present the drug in an acceptable form. The selection of the ingredients of the dosage form is generally made with consideration of the mechanisms by which the particular drug substance degrades. These degradative processes mainly include hydrolysis, oxidation, racemization, photolysis, isomerization and reactions due to microbial contamination. For example, esters and amides primarily undergo hydrolytic degradation whereas ascorbic acid, epinephrine and some steroids are subject to oxidative degradative reactions.

A major problem of many drugs is their hydrolytic degradation in aqueous solutions and various physical and chemical means have been used to improve their stabilities (1-3). For example, the addition of non-aqueous organic solvents, such as alcohol, glycerin or propylene glycol, often can reduce hydrolysis usually by lowering the dielectric constant of the vehicle (4-6). Another possibility is to reduce the solubility of a drug. Thus, the stability of penicillin in an aqueous system was improved by forming the less soluble procaine derivative and also by formulating it with citrate, dextrose, sorbitol, or gluconate (7). Complexation of drugs such as benzocaine, procaine and amethacaine has also been shown to increase the stability of these drugs against base catalyzed hydrolysis (8). A unique approach of formulating more stable drugs is their incorporation into micelles. The success of this, however, is dependent on the extent of partitioning of the drug in the micelles which is a function of several complex interactions determined by the chemical nature of the drug, the surfactant and additives which may be present (9-17). A mechanistic approach has been used (18) employing the Hammett free-energy relationship (19) to predict the role of a substituent on a allylbarbituric acid drug molecule on its overall stability. Microencapsulation (20), use of antioxidants (21,22), lipid coating (23), chelation (24,25) and the pro-drug approach (26-28) are now also fairly common

methods to improve the stability of drugs in pharmaceutical preparations.

In spite of the various approaches used to stabilize a drug, there is seldom an ideal solution to a drug stability problem, thus new, improved formulation methods are still needed. In particular, this thesis is concerned with the improvement of the stability of drugs which degrade by hydrolysis in aqueous solution. This has the potential of improving the shelf-life of a liquid formulation of a drug and improving the efficiency of absorption of the drug administered orally.

HYPOTHESIS:

Liposomes have been shown to have potential use in drug delivery systems by providing sustained release of drugs, localized drug delivery and enhanced uptake of drugs by target cells (29-34). Although phospholipids are able to spontaneously form liposomes in aqueous media (35) and entrap polar and non-polar molecules (36), liposome systems have seldom been examined for their potential for formulating more stable aqueous preparations of drugs (37-40). **The basis of this methodology lies mainly in the concept that lipid-soluble drug entrapped within the bilayer structures of liposomes are shielded from catalyzed hydrolytic attack. Also, water-soluble drugs entrapped within the aqueous core or compartments of liposomes may be**

protected from harsher external environments provided that the liposome itself remains stable and impermeable under these conditions. It is with this approach in mind that studies were conducted in detail to determine the important factors in formulating certain types of drugs in liposomes in order to increase their overall stabilities.

AIM:

The ultimate aim of this thesis was to investigate the potential of the liposome system to improve the hydrolytic stability of susceptible drugs under various conditions of composition, concentration, temperature, aqueous environment and method of preparation.

OBJECTIVES:

The objectives to be achieved include the following :

1. To prepare liposomes of various compositions containing acetylsalicylic acid (ASA), cefotaxime sodium (CFX) or benzocaine and to compare the kinetics of degradation in liposomes to aqueous solutions of these drugs as controls.
2. To quantify the kinetics of each system and to determine the important kinetic factors responsible for the stabilization of drugs in liposomes.
3. To determine the role of partition coefficient in the stabilization of drugs in liposomes using a series of

unionized local anesthetics.

4. To determine, the physico-chemical requirements of the drug-liposome system necessary to achieve a stable liposome preparation.

2. BACKGROUND

2.1 THE LIPOSOME SYSTEM

Liposomes were first prepared inadvertently in 1958 when Ottewill and Parriera (41) claimed to form micellar solutions with lecithin concentrations as low as 10^{-5}g/ml. Two years later, Robinson (42) showed, from molecular weight measurements and light scattering experiments, that the structures formed when phospholipids spontaneously disperse in water are smectic lamellae and not true micelles. Bangham and coworkers were the first to suggest that these structures could have biological implications (43) and he reported the first extensive physical characterization of liposomes in 1965 (44); he called these "Bangosomes", although eventually they became known as liposomes. Since then there has been a rapid growth of literature dealing with the physical properties of liposomes and their use as model membrane systems, and more recently, as drug delivery systems. There are now several good reviews, dealing with both their physical behavior (45-48) and their biological (49-63) properties.

2.1.1 Definition and General Description

When dry phospholipid comes in contact with excess aqueous solution the molecules spontaneously arrange themselves in what is known as the lipid bilayer vesicle or

liposome (46,64). Liposomes are closed spherical, bilayer shells separated by aqueous compartments, analogous to the multi concentric layers of an onion (Figure 1). Although the amphiphilic nature of phospholipids (Figures 1 and 2) (64) is a prime requirement for liposome formation, mixtures of phospholipid with agents such as sterols, glycolipids, organic bases or acids, proteins or artificial polymers can, at the appropriate concentrations, also be formulated into liposomes (65,67). The three-dimensional geometry (i.e.,branched chains) of the phospholipid molecules is claimed to be an important requirement for the stability and integrity of liposomes, thus, the nature of liposomes is affected by the nature and amount of additive to these systems (64). It is well known that the addition of detergents (e.g., Triton-X100) at low to medium concentration can disrupt the bilayer structure through a cooperative effect which essentially converts the molecules into the micellar state i.e., solubilization occurs. It should be noted that detergents can have similar disruptive effects on biological membranes reflecting the similar properties and characteristics of liposomes and membranes.

2.1.2 Liposome Composition

Liposomes have been formed from both synthetic and natural phospholipids. Lecithin (L-α-phosphatidyl choline), which also occurs naturally in membranes, is one of the main

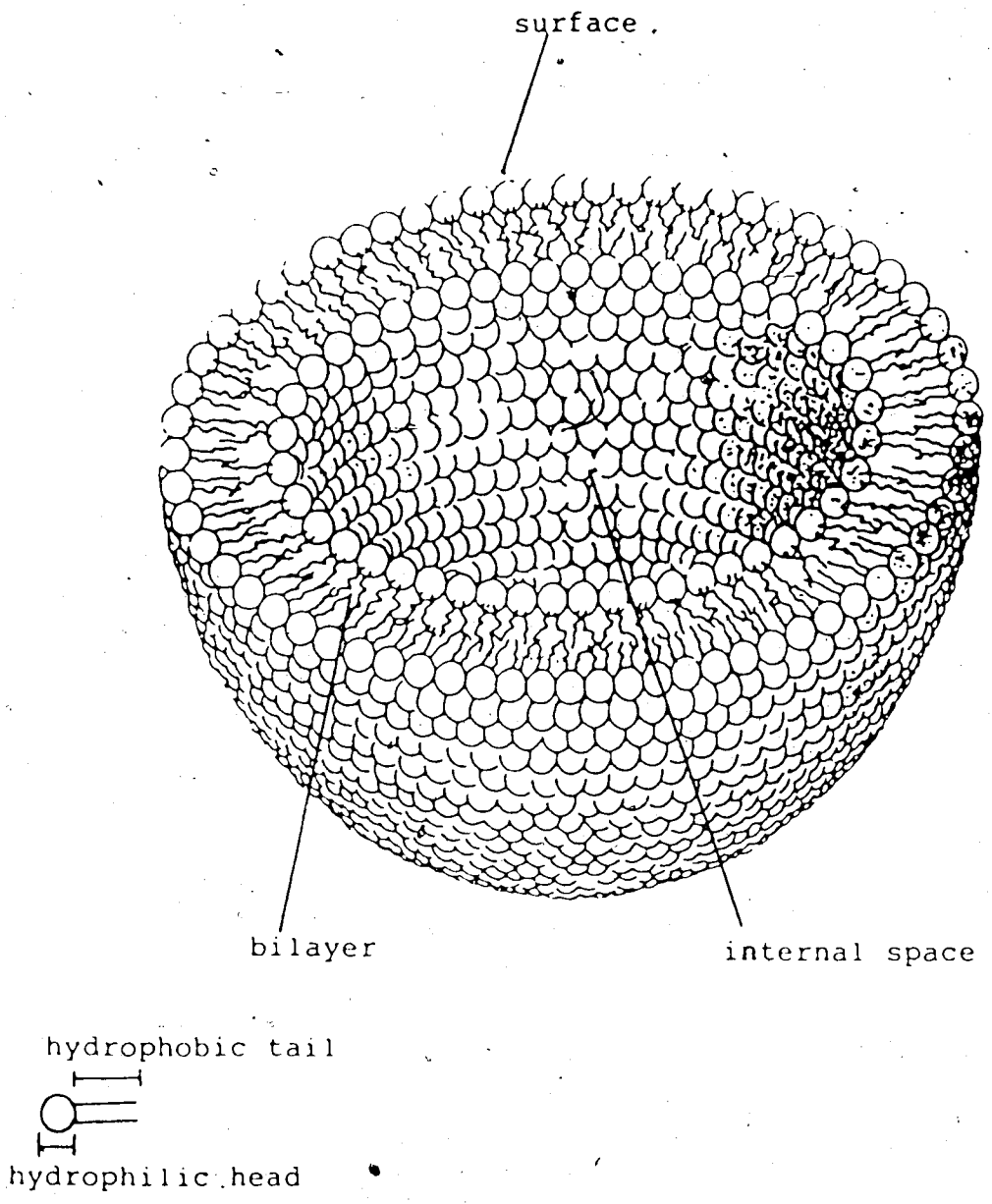
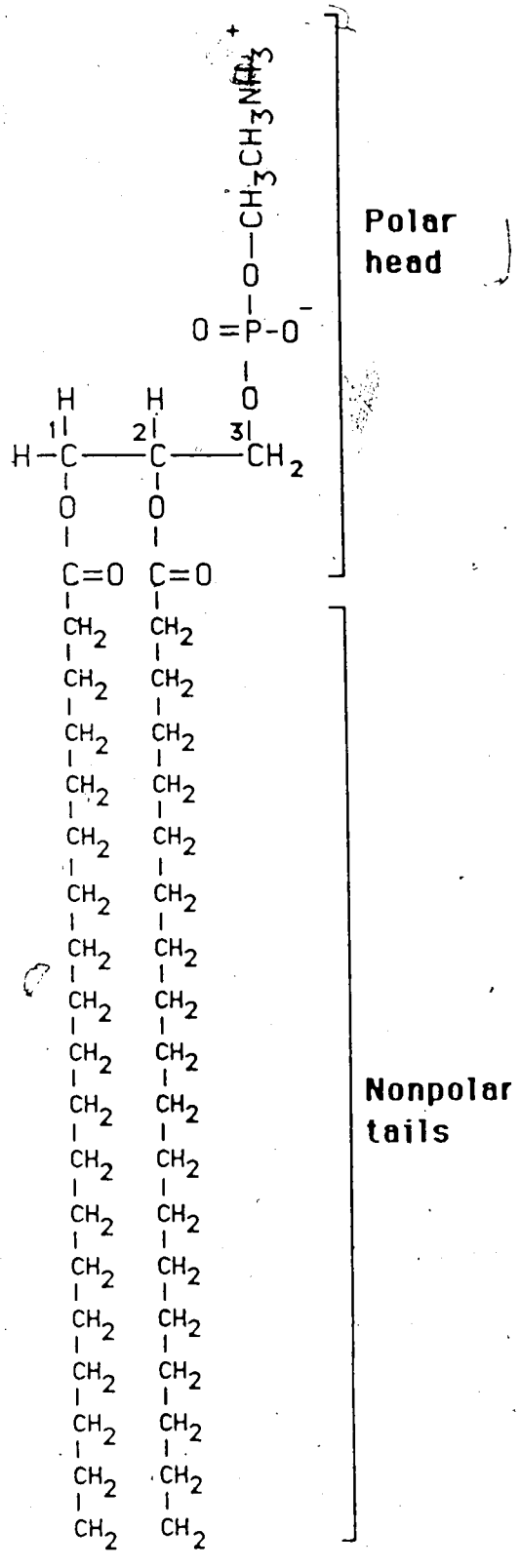


Fig. 1. Schematic Diagram of an Unilamellar Liposome.



Phosphatidyl choline

Fig. 2. Structure of Phosphatidylcholine Showing the Polar and Nonpolar regions.

phospholipids used to prepare liposomes. Of the phospholipids of erythrocyte membrane, phosphotidylcholine and sphingomyelin account for some 46-60 percent of the total lipid. Each phospholipid is found in both halves of the bilayer, but the distribution is highly asymmetric (92). In aqueous dispersion lecithin is neutral in charge due to its zwitterionic nature (between pH 1-11, (48)). Other neutral lipids employed in liposomes include the phosphatidylethanolamines and the sphingomyelins. On the other hand, liposomes possessing a net negative surface charge can be prepared using phosphatidylserine, phosphatidylinositol or phosphatidic acid alone or in combination with lecithin; dicetyl phosphate or stearylamine is commonly incorporated into lecithin liposomes to produce negatively- or positively- charged liposomes, respectively (48).

Liposomes can be prepared from a variety of lipids and lipid mixtures and many different combinations of phospholipids have been used to prepare liposomes (52,56). Cholesterol, which does not itself form liposomes, can be added in amounts upto 50 mole% (48). The different types of nonpolar side chains in the molecule impart different behaviors in bilayer fluidity and permeability. Phosphatidylethanolamine appears to be unique among the phospholipids in not forming closed bilayer vesicles at pH 7.0. In fact, liposomes cannot be formed from PE (at

neutral pH) if the mole fraction of PE is greater than 70%. Depending on the lipid compositions used for the formation of liposomes, wide variations may occur in the specific composition of the external surface of the bilayer such as lateral phase separations, and transbilayer asymmetries (48).

2.1.3 Preparation of Liposomes

An important formulation feature of the preparation of liposomes is that a variety of shapes and sizes of structures can occur. These liposomes vary in dimension from several hundred angstroms to fractions of a millimeter. Under specialized conditions of preparation they can be as large as 25 microns (50) or as small as 200 angstroms (e.g., after sonication) which is thought to be the limiting smallest size due to molecular packing and bilayer curvature constraints (46). Thus, liposomes when prepared are usually heterogeneous in nature. A light scattering technique (68), analytical ultracentrifugation (69) and NMR spectroscopy (70) are most appropriately applied to measure the size distribution of liposomes . Freeze-etch and freeze-fracture electron microscopic techniques have also been extensively used to study vesicle size and structure (71-73).

2.1.3.1 Multilamellar Vesicles (MLV)

Liposomes, also known as multilamellar vesicles (MLV), exist initially following the procedure earlier

described by Bangham (44) with subsequent refinements (46,65). Briefly, lipids of the desired composition and weight are deposited from organic solvents in a thin film on the wall of a round-bottom flask following rotary evaporation under reduced pressure. An aqueous phase of the desired composition is then added to the dry lipid film and the lipids are mixed by gentle swirling or agitation forming MLVs of varying size (0.1-5 μm diameter). This usually has to be carried out at a temperature above the phase transition temperature of the phospholipid. During this process a proportion of the aqueous phase is entrapped within the lipid bilayers which are formed along with any solutes which are contained therein. One of the strong advantages of MLVs is their simplicity of preparation.

Several additional methods of preparation of MLVs have been reported (74,75). One novel method is called the Dehydration-Rehydration Vesicle (DRV) technique (74). In this instance, MLVs are reduced to small unilamellar vesicles (SUVs) by sonication, lyophilized and then rehydrated with distilled water. The method is quite simple, employs mild conditions and the vesicles formed are capable of efficient entrapment (40-50%).

Another mode of MLV preparation produces what have been referred to as Stable Plurilamellar Vesicles (SPLV) and these also have reportedly better entrapment

efficiencies and stabilities than MLVs. In this method, the aqueous and organic phases are emulsified in a bath sonicator, the organic solvent evaporated, then the resulting cake is resuspended in buffer (75).

2.1.3.2 Small Unilamellar Vesicles (SUV)

Small unilamellar vesicles range in diameter from approximately 200 to 500 Å and consist of a single lipid bilayer surrounding an aqueous core. SUVs differ from MLVs in several respects other than size (76-78). But in particular, SUVs are osmotically insensitive whereas MLVs behave as perfect osmometers and also SUVs have been reported to be metastable in a number of instances (48,56,78).

The usual procedure for preparing SUVs is to sonicate an MLV preparation with a probe sonicator for 15-30 min depending on the concentration of lipid or with a bath sonicator for 4-24 hr (79,80). Each method has its distinct advantages and disadvantages depending on the application at hand. The bath sonication method has the advantage of non-contamination of the sample with metal fragments from the probe. On the other hand, the probe sonicator imparts greater energy to the lipid dispersion, thereby converting a very high fraction of MLV to SUV in a relatively short time. In both approaches, oxidative degradation and hydrolysis of lipids can be avoided by sonicating in an inert

atmosphere of nitrogen or argon gas and at controlled temperatures. However, sometimes, the process imposes a major difficulty especially when compounds which are prone to oxidative or heat dependent degradation are being used. Many drugs and macromolecules may be denatured or degraded due to the high energies and high local temperature produced during sonication.

Several other methods are available for the preparation of SUVs such as detergent solubilization (81-83), ether or ethanol injection (84-87) or the "French-Press" technique (58,88,89). Other procedures which produce large unilamellar vesicles include calcium induced fusion (48,90,91), reverse phase evaporation vesicle method (48) and the preparation of giant vesicle method (92,93).

The most important attribute of a SUV preparation is a narrow particle size distribution of small particles which can be readily isolated, if necessary, from larger particles by means of gel permeation chromatography or extrusion filtration (59). But this attribute may not be suitable for certain other situations such as in the stabilization of entrapped molecules where the larger internal aqueous environment is not required. On the other hand, MLV preparations are suitable due to their ability to encapsulate a variety of substances and can be made with a wide variety of

lipid compositions. Moreover, it is the simplest of all the methods available which can produce a relatively homogeneous size distribution of liposomes from batch to batch and are more stable in serum than the SUVs (48).

2.1.4 Properties of Liposomes

2.1.4.1 Phase Transitions

An important property of a phospholipid in aqueous suspension is its phase transition temperature (T_c). As the temperature is raised, the fatty acyl side-chains of the phospholipids in the bilayer of the liposome progress from a closely-packed relatively ordered array (known as the gel state) (Figure 3A) to a more loosely packed, less ordered state where the side-chains are capable of more rotational motion (known as the fluid state) (Figure 3B)(48,94). For liposomes composed of homogeneous lipids this order-disorder transition occurs over a narrow temperature range known as the thermotropic phase transition. The occurence of the phase transition can be detected by probes which measure molecular motion (ESR, NMR and fluorescence depolarization) (45) or by monitoring the sharp increase in enthalpy of the system by differential scanning calorimetry (DSC)(95).

Phospholipids have characteristic T_cs, determined in part by van der waals forces of attraction between

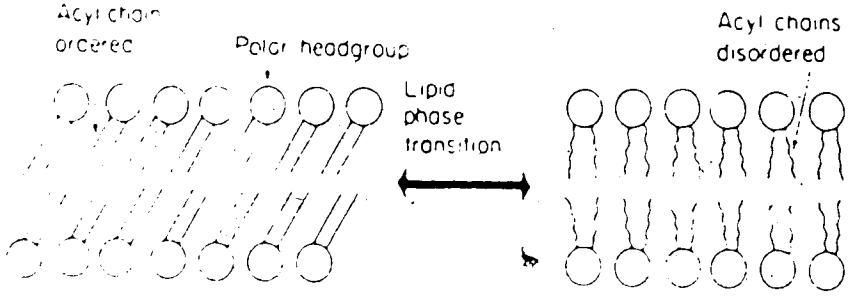


Fig. 3. States of Phospholipid Molecules below (A) and above (B) Phase Transition.

the hydrocarbon moieties of the molecules and partly by polar group interactions and extent of hydration. One key factor that influences the T_c is the degree of acyl group unsaturation. Introducing a double bond into one chain of a phospholipid produces a kink. The kink prevents an orderly packing of acyl groups as the temperature is decreased, so that the T_c is considerably lower than if the chains were totally saturated and without kinks. Other factors that influence the T_cs include the length of the fatty acid carbon skeleton, the nature of the polar head group, and the presence of other lipid soluble molecules. The addition of cholesterol to a phospholipid bilayer gradually reduces the T_c, a function of its mole fraction and at about mole%, it results in the abolition of the T_c (48,94).

The behavior and properties of liposomes is highly dependent on whether they exist in the fluid liquid crystalline state ($>T_c$) or in the gel state ($<T_c$). Thus, the T_c of the particular phospholipid(s) is important.

2.1.4.2 Permeabilities

Liposome permeability to various substances has been investigated in great detail (96-100). Permeability properties to small molecules like anions, monosaccharides and water varies directly with liposome fluidity. Liposomes have been shown to be permeable to

water, ions, and nonelectrolytes, although the permeabilities depend on the chemical composition of the liposomes. Positively-charged liposomes are essentially impermeable to cations, whereas negatively-charged liposomes are permeable to cations. In general, permeability of the bilayer increases with decreasing acyl chain length and the degree of unsaturation of the acyl chains. In liposomes composed of defined phospholipids the permeability is relatively low below the T_c, it exhibits an anomalous increase and decrease in the vicinity of the T_c, and increases further at temperatures above the T_c. When the lipid composition includes at least 33 mole% cholesterol, the permeability is decreased and the anomalous increase of permeability in the vicinity of the transition temperature is eliminated. The permeability of various molecules through liposome bilayers are also different. The intact bilayer is most permeable to nonpolar substances, which can partition into the nonpolar region of the bilayer (46). The liposomal lipids are also subject to oxidation and the products of lipid oxidation often cause increased liposome permeability. This problem can be prevented by incorporating small amounts of an antioxidant such as α-tocopherol into the liposome (101-104).

2.1.5 Incorporation of Drugs in Liposomes

Drugs and other molecules of interest may be encapsulated in the liposome. The amount of the drug that can be entrapped within liposomes depends on the hydrophobicity of the compound and the type of liposomes being prepared. The method of making the liposomes and the phospholipid composition also determine the amount of material entrapped within the liposomes (105).

2.1.5.1 Hydrophilic Drugs

Water-soluble substances are generally incorporated into the aqueous phase of the liposomes by dissolving them in the solution which is added to lipid film. The encapsulation and retention of small water-soluble drugs is a function of the internal free aqueous space of the liposome and of the lipid concentration. However, the entrapment efficiency of charged, polar drugs depends on additional factors, like the surface charge of the lipid and the ionic strength of the medium (60).

The entrapment efficiency of SUVs can be controlled to a certain degree by incorporating cholesterol into the bilayer (32.0-50.0 mole%), which leads to a significant (up to 75%) increase in vesicle size. The aqueous space between the bilayers of MLVs can be increased by up to 50% as a result of electrostatic repulsion between adjacent bilayers, when charged lipids like PS, PA, PG or charged hydrocarbons like DCP or

STEAR are included in the lipid mixture (60).

An increase in the ionic strength of the aqueous medium will result in a decreased entrapment efficiency of polar drugs, which is brought about, at least partially, by charge quenching of drug and/or lipid. Once a drug of low molecular weight is entrapped, its retention within the liposome is primarily determined by the bilayer permeability and it depends mainly on the size and charge of the drug and on the physical state of the membrane (60).

2.1.5.2 Lipophilic and Amphiphilic Drugs

Lipophilic materials can be incorporated into the lipid bilayer itself by including them in the organic solution of lipids rather than the buffer constituting the aqueous phase. The resultant lipid film contains the solute and when liposomes are formed as previously described, the lipophilic agent tends to remain in its preferred environment of the lipid bilayer due to its higher lipid-water partition coefficient. The amount of nonpolar drug that can be associated with the liposomes is usually greater by an order of magnitude or more than similar liposomes containing polar compounds (106).

Since amphiphilic molecules readily partition between lipid and water phases, such drugs can be added to preformed vesicles, thus avoiding possible deleterious effect such as sonication. Various studies

indicate that the distribution of amphiphilic drugs is related to the fluidity of the membrane and is maximal at the T_c. However, partitioning of amphiphilic molecules into the lipid bilayer will invariably alter the packing characteristics of the latter, and, hence the T_c. Also related to their strong association with the liposomal membrane, is the probability that they are less readily released. In vitro experiments have shown that after 10 min of sonication of nonpolar, drug-bearing liposomes, 50% of the drug still remains associated with the bilayer membranes, whereas polar drugs are completely released after 3-4 min of sonication (48,60).

Since lipophilic drugs are efficiently extracted by the lipid phase of liposomes, it is possible to achieve degrees of encapsulation of over 90%, often making it unnecessary to consider removal of the free drug. However, in the encapsulation of polar drugs, only a fraction of the total drug is entrapped and most of it remains as free drug in solution. The non-entrapped portion of drug can be separated, if necessary, from the liposome-entrapped drug by several methods such as dialysis (107), gel filtration (108), ultracentrifugation or ultrafiltration (109).

2.1.6 Stability of Liposomes

From a pharmaceutical point of view, clearly any liposomal formulation must have adequate stability over the time period between its preparation and ultimate use. Liposome stability is defined as the ability of the liposomal membrane to retain its structural integrity and to remain associated with the incorporated drug. It includes the chemical stability of the component materials (lipids and drugs) and the physical stability of the liposomes in terms of the integrity of the encapsulated materials and the size parameters. In addition, the liposomes should maintain their integrity *in vivo* until incorporation into the target tissue or until their sustained drug release function is completed (48).

2.1.6. *In-vitro* Stability

Although *in-vitro*, liposomes are basically stable structure since they represent the favorable thermodynamic state of phospholipids in water, however, liposomes are heterogeneous systems and as such have several *in-vitro* problems due to chemical and physical instability (34). Stability of liposomes in buffer solutions is limited by possible lipid degradation (oxidation of unsaturated lipids and hydrolysis and vesicle-vesicle fusion (34)). Sonicated liposomes stored below their respective phase transition temperatures tend to aggregate and fuse. These changes

in size of liposomes becomes a major problem during storage. The surface potential is an important parameter influencing liposomal physical stability. Decreasing the ionic strength and increasing the surface charge density, the physical stability of liposome can be increased (110). The influence of lipid composition and ionic strength on the physical stability of liposomes has been studied recently (111).

Another physical stability problem is retention of entrapped substances due to the physico-chemical changes in liposomes taking place during storage which can lead to modified permeability properties of the liposome. It depends on their size, charge, and polarity as well as on the lipid composition, the membrane fluidity and the curvature of the bilayer (48). The different formulation factors such as pH, ionic strength, buffer system, and solvent system also potentially influence the stability of liposome (112). Environmental conditions such as temperature, light, oxygen and heavy metal ions which may initiate chemical and physical reactions such as hydrolysis, oxidation, photochemical decomposition and changes in size distribution of vesicles also play an important role. These factors may be eliminated if liposomes are stored sterile, under nitrogen and at low temperature (49). In order to prevent the loss of entrapped material by passive

leakage, the vesicles can be stored in the original medium of preparation together with the nonentrapped drugs and separated from them only immediately before application.

A sterile liposomal preparation can be obtained by sterilizationor by filtration; filtration minimizing the extent of possible deterioration. Since filtration requires using filters with a pore size of 0.2μm to ensure sterility, this limits the size range of liposomes for different applications. However, when filtering liposomes at a temperature above phase transition temperature, phospholipid vesicles larger than 0.2μm can be filtered because of the increased flexibility of the phospholipid membrane in its liquid crystalline state (113,114).

2.1.6.2 In-vivo Stability in the G.I. Tract

The use of liposomes for drug delivery necessitates the examination of the effects of various biological fluids on the stability of liposomes.

For liposomes to survive in the G.I. tract and to act as protective barriers against proteolytic enzymes, they would need to be resistant to the low pH and pepsin of the stomach and to the bile salts and lipases of the duodenal milieu. It has been reported that the enzyme phospholipase A_2 had little effect on the stability of liposomes made with DMPC, DLPC or DPPC, above and below

the T_cs of the phospholipids. Hydrolysis of these phospholipids occurs only at temperatures close to the T_c (63). For oral liposomes, the phospholipid should be chosen such that their T_cs are very different than the temperature inside the gastrointestinal tract (i.e.,37°C), as at the temperature close to T_c they are vulnerable to phospholipase attack. In this respect, DPPC DLPC and DMPC with a T_cs of 0°C and 23°C respectively, would be resistant to pancreatic enzyme attack at 37°C. These predictions sometimes are not entirely reliable as the T_cs and, hence, fluidity of the liposomes may vary according to the precise phospholipid/lipid composition and the ionic strength of the medium. In particular, Ca^{++}, have been shown to change the T_cs of phospholipids especially when acid phospholipids are used. Liposomes of various composition have been tested for their oral stability which is discussed elsewhere (115,116).

2.1.6.3 In-Vivo Stability in Serum and Blood

Liposomes of certain compositions have relatively poor stabilities in blood (117-119). However, with appropriate lipid compositions, liposomes can be made to be very stable. For example, a significant increase in liposomal stability in biological fluids was obtained after cholesterol was incorporated. Optimal stability was achieved at a 1:1 mole ratio of phospholipid and

cholesterol. Depending on the lipid composition, cholesterol-rich liposomes were found to be 80%-99% intact after incubation in whole blood for 1 hour at 37°C, whereas in the absence of cholesterol more than 90% of the internal aqueous space markers were released (48,60). MLV stability in serum is usually greater than that of the unilamellar liposomes due to a larger number of bilayer shells, and the structural integrity of the inner lamellae is retained for a longer period of time. However, interaction with plasma proteins can lead to changes in solute permeability rather than a structural disintegration of the liposomes by high-density lipoproteins . The role of lipid composition in preserving liposomal stability in vivo has been studied (120).

2.1.7 Applications of Liposomes

2.1.7.1 The Liposome as a Model of Biological Membranes

Liposomes were initially used as models to study various properties of biological membranes. The presence of striking similarities in osmotic properties between liposomes and biological cells has stimulated researchers to use the liposomal system as an experimental model for biomembranes. Several results support the hypothesis that a lipid bilayer which acts as a backbone of biological membranes determines to a

large extent the barrier function of biomembranes (121,122). Therefore, the liposome system provides the opportunity of studying in simpler in vitro fashion the molecular interactions and barrier functions of isolated and selected membrane constituents. For example, the liposome has been used to determine the interaction of certain ligands with incorporated glycolipids (123). Using liposomes as a theoretical model the linear irreversible thermodynamic properties of bacteriorhodopsin were determined (124). Mechanisms involving catecholamine release have been studied using stimulants such as sodium or calcium ions entrapped within liposomes (125). Increased intracellular sodium has been shown to induce catecholamine release from the adrenal medulla by mobilizing intracellular calcium (125). Other studies include the study of phospholipid flip-flop and molecular motions within membranes (126), the interaction and binding of local anesthetics to erythrocyte membranes (127) and a freeze-etch study of the effects of polyene antibiotic on liposomes and human erythrocytes (128). The work of deGier et al gives a good description of the relationships between liposomes and biological membranes (129).

2.1.7.2 Liposomes in Drug Delivery

Liposomes have been most widely studied as potential drug delivery systems. One of the advantages

is the protection of encapsulated drugs from degradation. For example, insulin administered orally is destroyed by the gastric juices and, consequently, has to be given by injection. However, liposome encapsulated insulin when administered orally to animals has been found to decrease blood sugar levels. This is comparable to intraperitonial or intravenous routes of administration (130,131). Polynucleotides, such as immune RNA, encapsulated in liposomes were also protected from degradation and can potentiate the production of interferon. This therapy may find application in the treatment of cancer and viral infections (132).

A distinct advantage of liposomal drugs is the possibility of using smaller doses of drugs by formulating liposomes which target to specific sites and tissues, thus reducing their toxicities and side-effects. Cortisol palmitate in liposomes has been used to treat rheumatoid arthritis wherein only one-twenty fifth of the conventional therapeutic dose produced improvement in synovitis (133).

One of the earliest developments in the liposome drug-carrier concept was the use of the carrier in the treatment of cancer. It would reduce side-effects due to the action of drugs on normal tissues and also reduce premature loss of certain drugs through inactivation in

the circulation. For example, liposomes containing various porphyrins injected to mice results in remarkably more efficient tumor targeting (134). Liposomes containing lymphokines have been used to control metastases by activating the macrophages in vivo (135). The sensitivity of liposomes to pH and temperature has been used to attempt to target drugs to tumors. By incorporating pH-sensitive agents such as palmitoyl homocystein into liposomes pH-specific release can be obtained due to changes in the environment. pH liposomes proved clinically useful as they enable drugs to be targeted to areas of the body wherein the pH is less than physiological such as primary tumors and metastases (136). For example, pH-sensitive immunoliposomes significantly enhanced the cytotoxic effect of ARA-C to target cells as compared to free drugs (137). Liposomes with T_cs a few degrees above physiological temperature were reported to deliver more than four times as much methotrexate to murine tumors heated to 42°C compared to unheated control tumors. Higher ratios could be obtained by optimizing liposome size, composition, and charge. Encapsulation of drugs into temperature-sensitive liposomes, in combination with local hyperthermia, could be used to treat localized tumors without exposing the rest of the body to the toxic side-effects of anti-cancer agents (138).

Liposomes have also been used in other dosage forms, such as the topicals. Corneal penetration of penicillin G and indoxole were enhanced several fold when encapsulated in liposomes (139). Using triamcinolone as a model drug, liposomal encapsulation favourably altered the drug disposition (31). The concentration of the drug decreased at the site of its adverse effects in the systemic circulation and increased in the skin where its activity is desired.

2.1.7.3 The Stability of Drugs in Liposomes

The association of a drug with the lipid bilayer of a liposome to protect it against catalytic hydrolysis only sparsely investigated. Generally, it has been found that stabilization of an ester or amide for example is related to the depth of the reaction center of the drug molecule within the bilayer. On the other hand, electrostatic charges on the liposome surface can cause orientation of drugs which results in its degradation rate enhancement or retardation (140). Thus, the rate of hydrolysis of procaine in neutral liposomal suspension was found to be slower than in the aqueous buffer in the pH range of 8.5 to 13 (37). 2-Diethylaminoethyl p-nitrobenzoate was found to hydrolyze at a faster rate in the pH-range of 7.8 to 10 in the presence of neutral liposomes as compared to plain aqueous buffer (38). The hydrolysis rate of

Indomethacin was reduced by 80% accompanied by 80% liposomal association in neutral, negatively-charged and positively-charged liposomes (40), but the stability of cyclocytidine was unaffected by liposomes with which it did not associate (40). However, the effect of liposomes on p-nitrophenyl acetate was to increase its hydrolysis in positively-charged liposomes (40) or when the lecithin concentration in neutral liposomes was increased (39) and rate retardation in neutral or negatively-charged systems (40). The role of partition coefficient of drugs in liposomal stabilization has been indicated but a systematic study has not been conducted to show the actual relationship between partition coefficient and stability of drugs in liposomes.

In a recent investigation, it was observed that the rate enhancement of p-nitrophenyl acetate at the initial stage was due to preferential aminolysis of this ester in the presence of stearylamine-containing liposomes and not due to positive charge of the liposome (141).

Increased stability of proteins and enzymes after incorporation into liposomes has been attributed to the method of preparation of liposomes (142). Protection of β-lactam antibiotics from enzymatic inactivation following entrapment within liposomes and washed off liposomes has been observed relative to controls (143). This suggests that a greater degree of stability can be

achieved if liposome containing drugs are washed free of external drug and administered immediately.

2.2 STABILITY OF DRUGS IN MICELLAR SYSTEMS

There are striking similarities between micelles and liposomes and the stability of drugs in micellar system has been studied in detail. Micelles are formed from surfactant molecules which have both hydrophobic and hydrophilic properties and above a certain small concentration range termed the critical micellar concentration (CMC), molecules of surfactant aggregate to form micelles. The hydrophobic part of the aggregate forms the core of the micelle which is hydrocarbon in nature while the polar head groups are located at the micelle water interface. The decrease in free energy of the system which results from the preferential self-association of the hydrophobic hydrocarbon chains is the primary reason for the formation of micelles. Detailed explanation of micelle formation theories are found in a number of monographs (144-147).

It is the micelles, rather than the individual surfactant molecules, which are responsible for altering the stability of drugs in aqueous solutions of surfactants. A variety of micellar catalytic reactions have been comprehensively surveyed by Fendler and Fendler (148). An appropriate choice of surfactant, drug and other additives can lead to hydrolysis rate decreases of several-fold

compared to the same reaction in the absence of micelles.

Drugs formulated in micellar solution can be stabilized by partitioning of the drug into the hydrophobic interior of the micelles. This implies that lipophilic drugs are stabilized to a greater extent than more hydrophilic drugs. For example, cetomacrogol 1000 and polysorbate 80 reduced the rate of hydrolysis of aspirin in its unionized state but did not stabilize the ionized form of the drug as the ionized aspirin could not partition into the hydrophobic interior of micelles (15,149). Similarly, the hydrolysis of benzocaine was reduced in micellar systems (9,16,150,151). It was found that the hydrolysis rate of benzocaine in nonionic surfactants varied with the concentration of the surfactant more than with the length of the polyoxyethylene chain of the surfactant. Hydrolysis apparently occurred within the micelle as well as in the aqueous phase.

Ionized drugs can also be stabilized in the micellar solution. But the mechanism of stabilization is different in this case. It is suggested that a complex formation due to electrostatic interaction between the oppositely charged ions could sterically hinder the approach of the attacking agent and/or the complex could alter the electronic structure of the esters to render them less susceptible to the attacking reagent. A number of drugs in the ionized state was stabilized in this way. For example, the hydrolysis of anionic aspirin was suppressed by only the

cationic surfactants (13).

But there are other examples where a deviation from the expected behavior was observed. The alkaline hydrolysis of indomethacin was suppressed with nonionic and anionic surfactant but a degradation rate enhancement was observed when a cationic surfactant was used (12,152,153). It is suggested that ionized indomethacin confers a charge on the micelles of nonionic surfactants which then repels the attacking OH^- ion and thus a greater stability was obtained. For a similar reason the anionic surfactant offered protection and cationic surfactant enhanced the degradation.

Also, contrary to electrostatic theories of stabilization, the base catalyzed hydrolysis of procaine was inhibited by non-ionic, anionic and cationic micelles (10). Meakin and others have reported that CTAB decreases the rate of hydrolysis of ethyl p-amino benzoate and p-amino phenyl acetate (154), contrary to expectation, a phenomenon ascribed to the orientation of these molecules in the CTAB micelles which retards degradation. At low concentrations of CTAB, the rate of hydrolysis of benzocaine was slightly increased because the density of hydroxide ions at the micelle surface was greater. But in another study, it was found that both anionic and cationic surfactants suppressed the hydrolysis of benzocaine. In this instance, it was suggested that the polar heads of the surfactant molecules shielded the benzocaine from catalytic attack(9).

A catalytic effect of CTAB and benzalkonium chloride on the stability of cephalexin has been reported (155). At neutral pH the degradation involves intramolecular nucleophilic attack on the α-amino side chain of the β-lactam carbonyl. Above the CMCs of CTAB or benzalkonium chloride, the degradation of cephalexin increased by a factor of about 9 to 14 at pH 6.5. In contrast, cationic and nonionic micelles reduced the degradation of several penicillins by a factor of 4 to 12 in acidic media whereas anionic micelles resulted in an increased rate of degradation (156). In contrast, the acid degradation of cefazolin was not influenced by any of the surfactants.

Thus it appears that the kinetics of degradation of drugs in micellar systems is quite complex in nature and can not be generalized but two apparent dominant factors which are responsible for the stabilization are: the electrostatic interactions and the hydrophobic interactions between the micellar phase and the reactants.

2.2.1 Kinetics of Stabilization of Drugs

The aim of the kinetic analysis in micellar system was to develop equations to calculate the contribution of the micellar phase to the overall hydrolysis of the drugs. On the two-phase model of micelle formation solute will partition between the aqueous phase and the micellar phase. Using the two phase system Yamada and Yamamoto (157) derived

Eq.1 :

$$k_{obs} = \frac{1-V}{KV + (1-V)} k_W \qquad (1)$$

where k_{obs}, is the observed rate constant in surfactant solutions, k_W is the rate constant in aqueous solution, K is the apparent partition coefficient of the solute to the micelle, and V is the volume fraction of the micellar phase. The differential rate equation for the concentration change with respect to the total system is(146) :

$$\frac{dx}{dt} = k_W C_W (1-V) \frac{(C-X)}{C} \qquad (2)$$

where C is the initial reactant concentration, C_W the initial reactant concentration in the aqueous phase and X the concentration reacting in time t.

This equation on integration and rearrangement gives :

$$k_W = \frac{C}{C_W(1-V)} \frac{1}{t} \ln\frac{C}{C-X} \qquad (3)$$

and by substituting $K = \frac{C-C_W(1-V)}{C_W V}$ and by knowing $k_{obs} = \frac{1}{t} \ln\frac{C}{C-X}$

in the above equation one can get Eq.1. If the reaction partially takes place in the micellar phase an additional term must be added as the total rate constant is now the sum

of k_w and the rate constant in the micellar phase, K_m. Therefore,

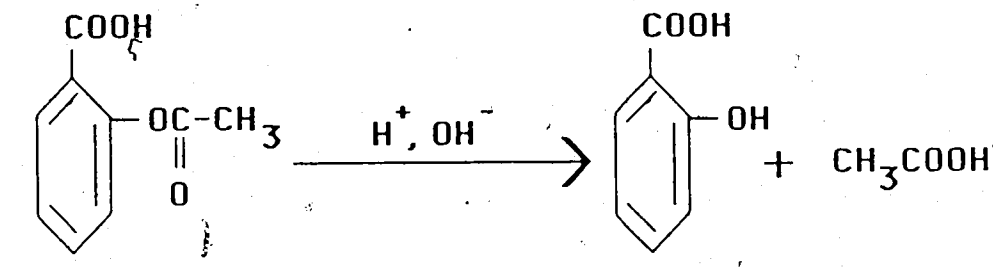
$$k_{obs} = k_m + \frac{(k_w - k_{obs})}{KV} \qquad (4)$$

The above equation can be re-arranged to a suitable form to calculate different parameters from the experimental data. Several other equations have been used in studies, the concept of which were basically the same.

2.3 SELECTION OF DRUGS

A number of drugs exhibit stability problems for several reasons. The most common cause of the decomposition of drugs is hydrolysis and the main classes of drugs in this catagory are the esters and the amides. So the drugs in these groups can be used as models for studying the effect of liposome formulation on the stability of drugs.

As a first model drug acetylsalisylic acid (ASA) has been chosen (scheme I). It is an ester drug for which there has been a renewed interest in the clinical field principally because of its usefulness in the management of thromboembolic disease (158). The acetylation of platelet cyclooxygenase, the responsible factor in the management of this disease, occurs only in the presence of acetylsalicylic acid and not in the presence of its degradation product (158). It is, therefore, important that acetylsalicylic



Acetylsalicylic Acid

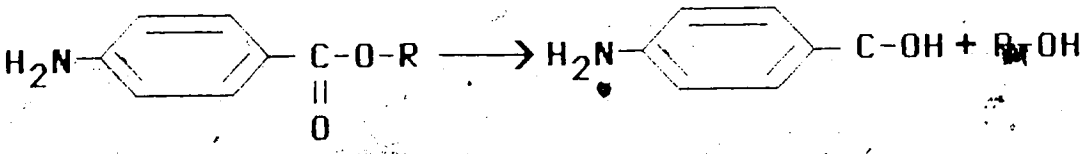
Salicylic Acid Acetic Acid

SCHEME I

acid be absorbed in the intact form into the blood stream to have any meaningful use of this drug in the management of this disease. Hence, the present study has been undertaken to determine its stability in liposomes. It is a polar drug and the studies of its stabilization in liposome would be very useful information for other such drugs. The mechanism of degradation of this drug in aqueous solution has been well documented (159-161). At all pHs it exhibits first-order hydrolysis kinetics to produce salicylic acid and acetic acid.

Secondly, a group of six local anesthetic drugs have been chosen for study. These are : N-methyl p-amino benzoate, N-propyl p-amino benzoate, N-butyl p-amino benzoate, benzocaine, procaine and tetracaine. A study of these drugs in liposome systems under various conditions offers a convenient means of determining the important factors and the role of partitioning on their stability in this type of formulation. Also, it would be possible to compare these stabilities with the stabilities in micellar system as these drugs have been studied in micellar system in great detail. The hydrolysis of these drugs in aqueous solution follows mainly first-order base-catalysed kinetics (162-164). The degradative pathways have been shown in scheme II-III.

Finally, cefotaxime sodium, a semi-synthetic third generation cephalosporin antibiotic has been selected. This



Local Anesthetic

P-Amino Benzoic Acid

1. N-Methyl P-Amino Benzoate, R = $-CH_3$

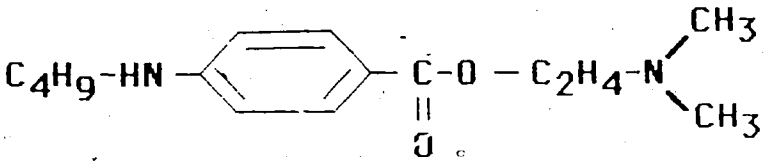
2. N-Propyl P-Amino Benzoate, R = $-C_3H_7$

3. N-Butyl P-Amino Benzoate, R = C_4H_9

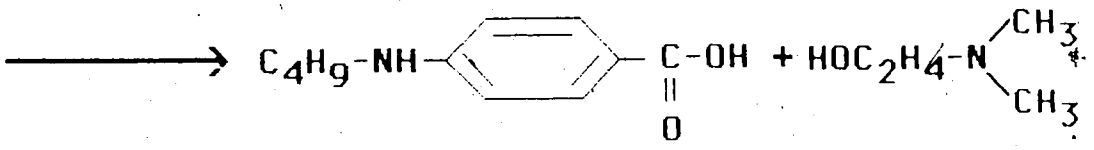
4. Benzocaine, R=$-C_2H_5$

5. Procaine, R=$-C_2H_4-N(C_2H_5)(C_2H_5)$

SCHEME II



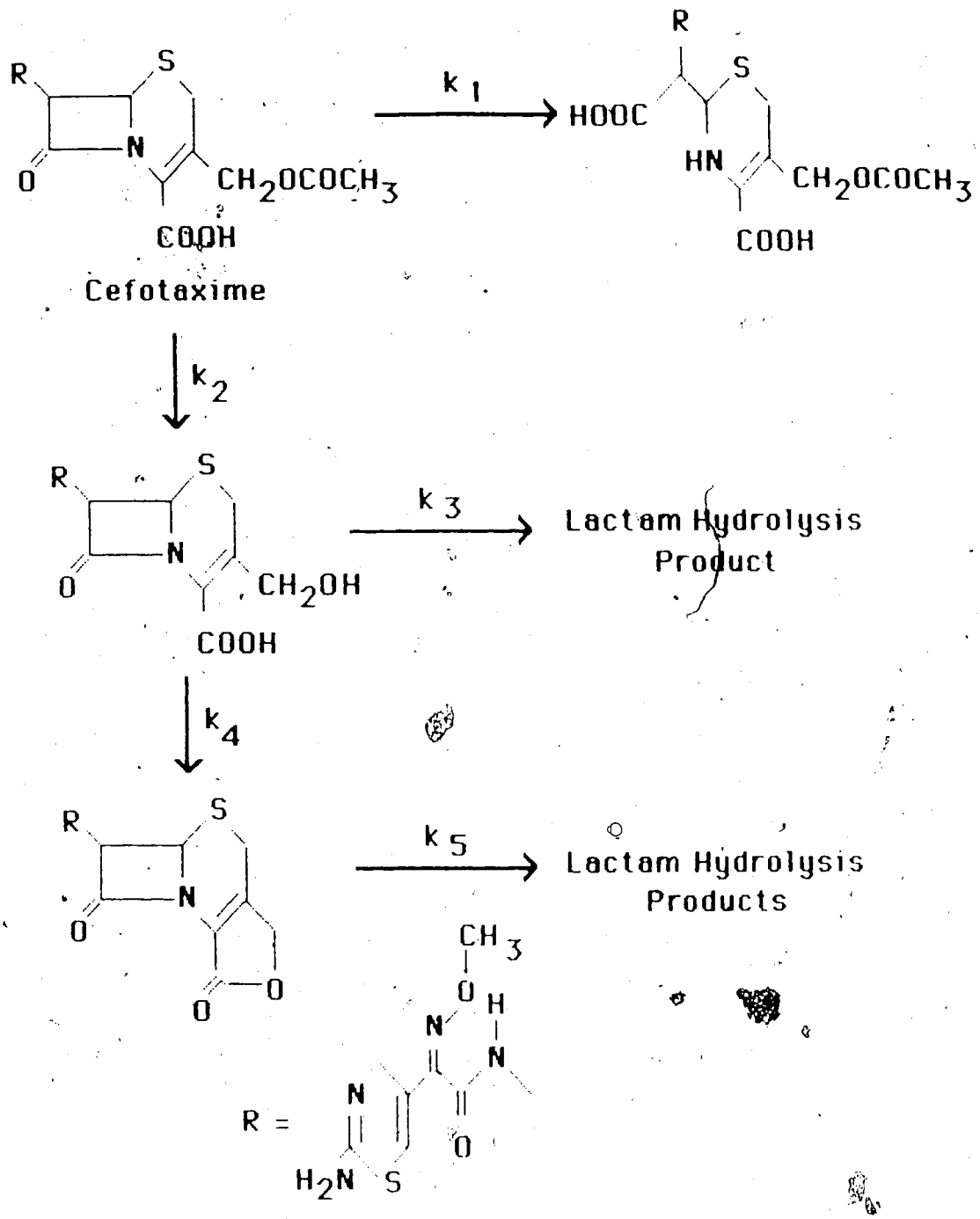
Tetracaine



Butyl p- Amino Benzoic Acid Dimethyl-Ethanol Amine

SCHEME III

drug is effective against a wide variety of micro-organisms when administered intravenously but in aqueous solution, degradation takes place according to the various degradative pathways. The rate of degradation of this drug follows first-order kinetics (165,166). At lower pH, the β-lactum moiety undergoes hydrolysis (K_1 step in scheme IV) and at higher pH (>8), the side chain undergoes hydrolysis (K_2 step). So a liposomal formulation of cefotaxime would be clinically desirable if a stable preparation can be obtained.


R
S
N
O
CH2OCOCH3
COOH
k1
HOOC
HN
Cefotaxime
k2
CH2OH
k3
Lactam Hydrolysis Product
k4
k5
Lactam Hydrolysis Products
CH3
H
R =
H2N

SCHEME IV

3. EXPERIMENTAL

3.1 MATERIALS

The drugs investigated in these studies were : acetylsalicylic acid (ASA)(99%)[1], cefotaxime Sodium[2], methyl p-amino benzoate[3], N-propyl p-amino benzoate[3] and N-butyl p-amino benzoate[3], benzocaine[4], procaine Hydrochloride[5], and indomethacin.[6] Other compounds required for use during analysis include 4-(butylamino)benzoic acid , p-aminobenzoic acid ; and salicylic acid . A variety of phospholipids and lipids were employed as follows :

L-α-dimyristoylphosphatidylcholine[6] (DMPC,98%), L-α-dipalmitoylphosphatidylcholine[6] (DPPC,99%), Egg phosphatidylcholine[6] (EPC,98%, type V-E from egg yolk), Phosphatodylserine[6] (PS), and Sphingomyelin[6] (SPHING, from bovine brain), Cholesterol[6] (CHOL), Stearylamine[6] (STEAR) and Dicetyl phosphate[6] (DCP).

All chemicals and solvents used were reagent grade but acetonitrile [7] was HPLC grade. De-ionized glass distilled water was used to prepare all aqueous solutions.

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[1]Aldrich Chemical Company, Inc., Milwaukee, WIS, USA.
[2]Roussel Canada Inc., Montreal, Canada.
[3]Pfaltz and Bauer, Inc., Waterbury, CT, USA.
[4]Baker Chemical Company, USA.
[5]Allen and Hanburys, USP.
[6]Sigma Chemical Company, St. Louis, MO, USA.
[7]Fisher Scientific Co., N.J., USA.

3.2 METHODS

3.2.1 Preparation of Buffer Solutions

Aqueous buffer solutions were prepared by dissolving the buffer ingredients in one liter of deionized, distilled water then the pH of the solution was verified by pH measurement.[a] Unless otherwise stated, the ionic strength of the buffer solutions was adjusted to 0.15 by the addition of sodium chloride and the osmolarity checked.[b] The compositions and pHs of the various buffer solutions are shown in Table 1.

3.2.2 Preparation of Liposomes

Liposomes were prepared (10 mg/ml of phospholipid) following a modified Bangham (46) procedure. Thus, the lipid components were initially dissolved in chloroform in a 1L round-bottom flask, the solvent was removed under reduced pressure using rotary evaporation at about 40°C, the resulting film on the walls of the flask was flushed with N_2 gas, then the unstoppered flask was placed in a vacuum oven at 40°C for 12-14 hr over P_2O_5. Subsequently, the lipid film was hydrated by the addition of aqueous solution, hand-shaking until all of the film was removed from the walls of the flask then vortex-mixing for 10 min thereby

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[a] Fisher "Acumet" Model 320 pH Meter, Fisher Scientific Co.,USA.
[b] Advanced Digimatic Osmometer, Advanced Instrument Inc., Needham Heights, Massachusetts, USA.

TABLE 1

Composition of Buffer Solutions

pH	Aqueous Buffer Components
1.0	HCl (0.108 M) + KCl (0.05 M)
2.0	HCl (0.012 M) + KCl (0.05 M)
2.4	Citric Acid (0.094 M) + Na_2HPO_4 (0.012 M)
2.6	Citric Acid (0.089 M) + Na_2HPO_4 (0.021 M)
3.0	Citric Acid (0.079 M) + Na_2HPO_4 (0.040 M)
4.0	Acetic Acid (0.16 M) + Sodium Acetate (0.036 M)
6.0	KH_2PO_4 (0.059 M) + Na_2HPO_4 (0.007 M)
7.4	KH_2PO_4 (0.013 M) + Na_2HPO_4 (0.054 M)
8.0	KH_2PO_4 (0.0025 M) + Na_2HPO_4 (0.064 M)
9.4	Boric Acid (0.1 M) + Sodium Carbonate (0.101 M)
10.0	Boric Acid (0.06 M) + Sodium Carbonate (0.14 M)
12.2	Sodium hydroxide solution (0.045 M)

forming MLVs. Drugs were incorporated in liposomes during formation either via the aqueous phase (AP1) or via the organic phase (OP1) (Figure 4).

3.2.3 Kinetic studies in Aqueous Buffers and in Liposomes

The kinetics of hydrolysis were determined as described for each [illegible] below.

Generally, the stock liposome preparation containing drug was divided into 2 ml portions, placed in capped vials, and maintained at 30°C in a water-bath. Samples were withdrawn at various time intervals, diluted to 25 ml with isopropyl alcohol in a volumetric flask, which yielded a clear solution, then assayed spectrophotometrically[10] or by HPLC.

3.2.3.1 Acetylsalicylic Acid:

The overall hydrolysis of ASA is given by

$$ASA + H_2O \longrightarrow SA + HAc \qquad (5)$$

although it is understood (191) that the hydrolysis proceeds through several intermediate reaction products prior to reaching the end products as shown. Salicylic acid (SA) absorbs UV light at the λ_{max} of ASA and, therefore, interferes with the analysis of ASA but ASA does not

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[10] UV/VIS Spectrophotometer, Model 25, Beckman Instruments, Irvine,Ca 92713, USA

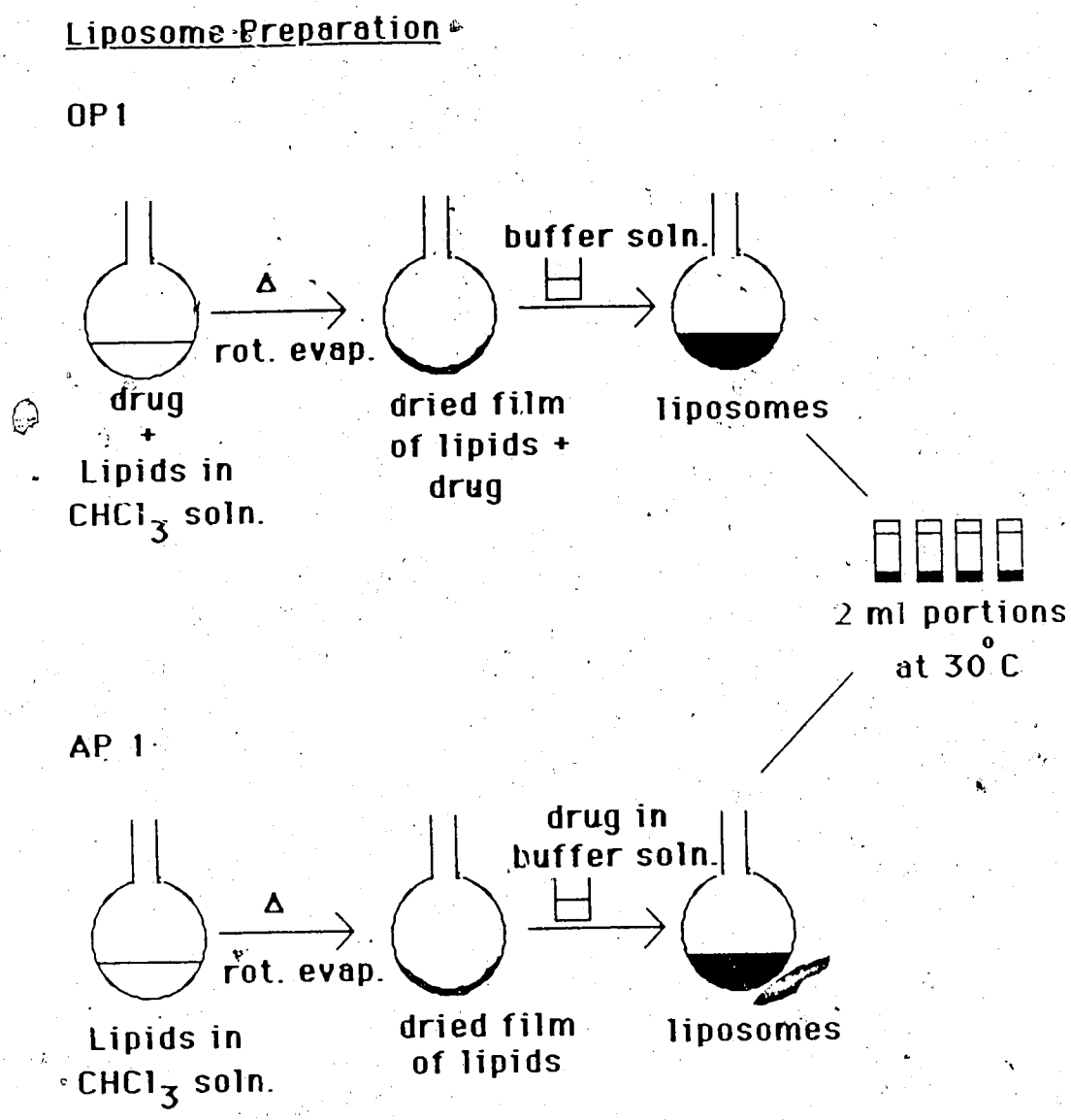

Fig. 4. Schematic Drawing of the Preparation of Liposomes Showing the Incorporation of Drugs.

interfere with the analysis of SA. Therefore, the kinetics of hydrolysis of ASA was followed by measuring the absorbance of SA in the final solution. Similar procedures have been adopted by others (13,15).

Eq.5 shows that one mole of ASA yields one mole of SA, thus at t= ∞,

$$[SA]_\infty = [ASA]_o = C_o^{ASA} \quad (6)$$

The absorbance of SA at t=∞ is given by

$$A_\infty = \epsilon_{SA} \times C_o^{ASA} \quad (7)$$

where ϵ_{SA} is the molar absorptivity of SA, and C_o^{ASA} is the initial concentration of ASA in the solution for analysis. It follows that

$$A_\infty - A_t \propto C_t^{ASA} \quad (8)$$

where A_t is the absorbance of SA at time t. The degradation of ASA followed pseudo-first order kinetics (160) and is described by :

$$C_t^{ASA} = C_o^{ASA} \cdot e^{-kt} \quad (9)$$

where k is the pseudo-first order hydrolysis rate constant

of ASA and can be obtained from a linear plot of $\log(A_\infty - A_t)$ versus time.

In addition, the degree of stabilization of ASA obtained in liposomes relative to an aqueous buffer solution of ASA was determined as follows:

$$\frac{k_B - k_{obs}}{k_B} \times 100 = \% \text{ increase in stability} \qquad (10)$$

where k_B and k_{obs} are the pseudo-first order hydrolysis rate constants in buffer solution and in liposomes respectively. Normally, standard deviations were used to evaluate the differences in the various k values.

The hydrolysis rate constant of ASA associated with the lipid phase (k_L) was also determined as follows : Liposomes were first prepared as usual (28.8 mM of DMPC), then centrifuged for 30 minutes at 30°C (135,000x g). The supernatant was carefully removed, the pellet resuspended with aqueous buffer solution and recentrifuged. In total, the liposomes were washed three times and the final DMPC concentration was made to 4.4 mM by dilution. The degradation of ASA remaining in the lipid phase was then followed as before. In these experiments the initial concentration of ASA in the liposomes must be determined by analysis. However, because spectorphotometric determination of ASA is interfered by SA, a procedure involving simultaneous equations was used. At λ_{max}=276nm for ASA, the

total absorbance (A^{276}_{Total}) is given by

$$A^{276}_{Total} = \epsilon^{276}_{ASA} \times C_o^{ASA} + \epsilon^{276}_{SA} \times C_o^{SA} \qquad (11)$$

where ϵ^{276}_{ASA} and ϵ^{276}_{SA} are the apparent molar absorptivities of ASA and SA, respectively, at 276 nm and C_o^{ASA} and C_o^{SA} are their respective concentrations.

At λ_{max}=303nm for SA, the total absorbance A^{303}_{Total} is attributed to SA only since there was no interference by ASA at 303 nm. Therefore,

$$A^{303}_{Total} = \epsilon^{303}_{SA} \times C_o^{SA} \qquad (12)$$

where ϵ^{303}_{SA} is the apparent molar absorptivity of SA. Thus the concentration of ASA can be calculated by solving simultaneously Eqs. 11 and 12. Thus

$$C_o^{ASA} = \frac{\epsilon^{303}_{SA} A^{276}_{Total} - \epsilon^{276}_{SA} A^{303}_{Total}}{\epsilon^{303}_{SA} \epsilon^{276}_{ASA}} \qquad (13)$$

The molar absorptivities of ASA and SA are determined experimentally.

3.2.3.2 Local Anesthetics

1) Calibration Curves :

Stock solutions of local anesthetics (7.6×10^{-4}M) were prepared in 0.045N sodium hydroxide solution (pH 12.2). Dilutions were then made in isopropyl alcohol to

obtain concentrations ranging from 1.5×10^{-5} to 7.5×10^{-5}M. The absorbances of the solutions were measured at the λ_{max} and a linear calibration curve for each drug was constructed. Each was confirmed subsequently by analysis of additional standard solutions and application of the regression equation for each curve. Agreement was found to be at least 99%.

2) Kinetic Analysis:

The hydrolyses of local anesthetics were measured in 0.045N sodium hydroxide (pH 12.2). These conditions have been previously used by others (151) with respect to benzocaine hydrolysis in micellar solutions to allow convenience in the kinetic studies and to ensure the constancy of the OH^- concentration during the hydrolysis reactions. In this study the UV spectra of the drugs and their respective degradation products overlapped, hence, the total absorbance of the mixture at a given wavelength A_λ, is given as

$$A_\lambda = \epsilon_1 C_1 + \epsilon_2 C_2 \qquad (14)$$

where C_1 and C_2 are the total concentration of reactants and degradation products, respectively and ϵ_1 and ϵ_2 are their respective apparent molar absorptivities at wavelength λ_{max}. Simplification of Eq.14 yields Eq.15 (192)

$$\frac{A_\lambda}{C_o} = \Delta\epsilon\frac{C}{C_o} + \epsilon_2 \qquad (15)$$

which, on rearrangement, gives Eq.16.

$$\frac{C}{C_o} = \frac{A_\lambda/C_o - \epsilon_2}{\Delta\epsilon} \qquad (16)$$

where, $C = C_t$ = concentration of drug at time t, C_o = initial concentration of drug, and $\Delta\epsilon = \epsilon_1 - \epsilon_2$. Values of ϵ_1 and ϵ_2 at λ_{max} are listed in Table 2.

3.2.3.3 Cefotaxime Sodium

Several HPLC methods of analysis of CFX have appeared in the literature (165,166) but none of these were suitable or adaptable for the determination of CFX in liposomes. Thus, an HPLC method of analysis was developed using orcinol monohydrate (ORM) as the internal standard.

The chromatographic system consisted of a solvent delivery system[11], a fixed wavelength UV detector[12] (280 nm), a Rheodyne injector [13] with a 20 µl loop, a 15 cm C_{18} column[14], and an integrator-plotter.[15] The mobile phase consisting of 10%v/v acetonitrile in pH 5.0 acetate buffer (0.07 M sodium acetate and 0.03 M acetic

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[11] Model M45, Waters Assoc. Inc., Milford, MA 01757, USA.
[12] Model 440, Waters Assoc. Inc., Milford, MA 01757, USA.
[13] Model 7125, Spectra Physics, Cotali, California, USA.
[14] "Novapac", Waters Assoc. Inc., Milford, MA, 01757, USA.
[15] Chromatopac Model C-R3A, Shimadzu Corporation, Kyoto, Japan.

TABLE 2

Structures and Spectral Characteristics of Local Anesthetics and Their Degradation Product

Local Anesthetic or Degradation Product	Structure[a]	λ (nm)	Molar Absorptivity
Benzocaine	X=C_2H_5, Y=H	291	21,552
Procaine	X=C_2H_4-N(C_2H_5)(C_2H_5), Y=H	293	22,747
Tetracaine	X=C_2H_4-N(CH_3)(CH_3), Y=C_4H_9	309	29,552
Methyl p-amino benzoate	X=CH_3, Y=H	294	18,934
n-propyl p-amino benzoate	X=C_3H_7, Y=H	294	19,235
n-Butyl p-amino benzoate	X=C_4H_9, Y=H	294	19,156
p-Aminobenzoic acid	X=H, Y=H	291 293 294	5,721 4,946 4,946
p-Butylamino-benzoic acid	X=H, Y=C_4H_9	309	5,437

[a] General structure Y-HN-C$_6$H$_4$-CO-O-X

acid) was freshly prepared and filtered through a 0.22μ filter[1] under vacuum just prior to use. The flow rate was set at 1.5 ml/min.

1) Calibration Curve :

Separate stock solutions of CFX (1mg/ml) and ORM internal standard (1.5mg/ml) were prepared in 40%v/v methanol. Dilutions were made as follows :

Solution A : 5ml CFX solution + 20 ml ORM.

Solution B : 10 ml CFX solution + 20 ml ORM

Each was diluted to 100 ml with 40%v/v methanol solution. 20 μl of solution A or solution B were injected and chromatograms obtained and peak areas quantitated. All solutions were run in triplicate and the results averaged. Confirmation of the calibration was made by injecting standard solutions as before and comparing concentrations.

2) Kinetic analysis :

A convenient concentration (3.5 mg/ml) of CFX was dissolved in buffer and equilibrated at 30°C. At different time intervals, a 2ml portion of the CFX solution was withdrawn and combined with 20 ml of internal standard stock solution and diluted to 100 ml with 40%v/v methanol then 20 μl was injected. The appropriate dilution factors were applied to the recorded concentration to obtain the actual

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[1] Millipore Corp.,Bedford, MASS, 01730, USA.

concentration of cefotaxime sodium in the samples.

When liposome preparations were analyzed, samples were first dissolved in pure methanol, the internal standard solution added then diluted to 100 ml with water such that the solvent system contained 40 %v/v methanol-in-water. This solution for analysis was subsequently analyzed as before.

3.3 CHARACTERIZATION OF LIPOSOME FORMULATIONS

3.3.1 ASA Liposomes containing Stearylamine

3.3.1.1 Synthesis and Isolation of N-Stearylacetamide

Molar equivalents of stearylamine and acetic anhydride or ASA were allowed to react overnight in chloroform solution at room temperature with constant shaking. It was then washed three times with 15 ml of cold 10% hydrochloric acid to remove the excess bases as hydrochloride salts. The chloroform layer was then washed and dried with anhydrous sodium sulfate then removed by rotary evaporation. The powdered N-stearylacetamide was then recrystallized three times from methanol and stored in powder form.

3.3.1.2 Thin-Layer Chromatography of N-Stearylacetamide

Qualitative thin-layer chromatography tests for the formation of the amide were performed on silica gel

using chloroform:methanol (9:1v/v) as the developing solvent.

TLC plates[17] were 10x10 cm, 0.2 mm thickness. Approximately 200 µg samples of stearylamine, N-stearylacetamide synthesized from acetic anhydride or from ASA dissolved in chloroform were spotted and the plate developed for 5 min. The solvent front was marked, and the plate dried. The spots were visualized using UV light[18] or by reaction with iodine vapors and then marked with a pencil.

3.3.1.3 Infra-Red Analysis of N-Stearylacetamide

Samples were analyzed by IR spectroscopy[19] using the pressed disc method (167).

Pure, dry, and finely powdered potassium bromide was intimately mixed with each sample in a Wig-L-Bug[20] at a concentration of about 1% w/w. The mixture was then compressed in a die under vacuum at room temperature and at high pressure[21] to form a solid transparent disk in a holder which was then placed in the IR spectrometer from which the spectra were produced.

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[17] Silica gel 60 F 254, E.Mark, W.Germany.
[18] Model C-60, UVP Inc., San Gabriel, Ca, USA.
[19] IR Spectrophotometer, Nicolet Instrument Corporation, Madison, Wisconsin, 53711, USA.
[20] Wig-L-Bug Amalgamator, Cresent Dental Mfg. Co., Chicago, ILL., USA.
[21] Carver Laboratory Press, Model B, Fred S. Carver Inc., N.J., USA.

3.3.2 The Liposome-Cefotaxime System

3.3.2.1 Turbidity Analysis

Liposomal suspensions of CFX and DMPC were prepared and vortexed as described under "Preparation of Liposomes" then equilibrated in a water bath. At different time intervals, a 2 ml sample was analyzed spectrophotometrically at 520 nm and the absorbance (or turbidity) recorded.

3.3.2.2 Photomicrographic Analysis

Samples of liposomal suspension with CFX were examined microscopically at x500 magnification using a binocular microscope[22] equipped with an automatic camera. A Drop of suspension on microscopic slides were photographed (1/2 to 1/15 sec. exposure time) at different time intervals using Kodak Plus-X (125 ASA) film.

3.3.3 Determination of the Fraction of Drug Associated with the Lipid Phase of Liposomes (f_L)

Determination of f_L was carried out at the beginning of the kinetic studies. Once liposomes were prepared, they were centrifuged[23] (135,000xg, 30 minutes, 30°C), the supernatant removed and analyzed for drug. The differences

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[22] Carl Zeiss Co.,Berlin, W. Germany.
[23] Model L8-55, Ultracentrifuge, Beckman Instruments, Palo-Alto, Ca, 94304,USA.

between the total amounts of drug in the liposomal suspensions (D_T) and the residual amounts of drug in the supernatants (D_B) after centrifugation divided by D_T yielded values of f_L.

3.3.4 Determination of Partition Coefficients of Local Anesthetic Drugs

3.3.4.1 Liposome-Water System

Freshly prepared DMPC liposomes, were equilibrated with drug for 30 min in a shaking water bath.[24] The pH was adjusted somewhat lower than in the but sufficiently above their pKa's to maintain a fully unionized state. The phospholipid and drug concentrations in these experiments were 2.88 mM and 7.6 mM, respectively. The liposomes were centrifuged (135,000xg, 30 min., 30°C), the supernatant carefully removed by Pasteur pipette, then diluted with isopropyl alcohol and analyzed spectrophotometrically. Concentrations of drug in the supernatants were determined from a calibration curve and the residual amounts in the phospholipid phase determined from mass balance calculations.

The molal partition coefficients, K_W^L , were determined from Eq.17:

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[24] Dubnoff Metabolic Shaking Incubator, Precision Scientific Co.,Chicago,IL 60647, USA.

$$K_W^L = \frac{(C_T - C_W)W_1}{C_W W_2} \qquad (17)$$

where C_T is the total concentration of the drug in the liposomal suspension, C_W is the concentration of the drug determined in the supernatant after equilibration, W_1 is the weight of the aqueous phase and W_2 is the weight of the lipid in the sample. Partition coefficients were validated at 1 hr equilibration time. All partition coefficients were determined at least in triplicate and the results averaged.

3.3.4.2 Octanol-Water System

Convenient volumes of aqueous phase (5ml buffer solution) containing the appropriate concentration of drugs (7.6mM) and n-octanol (0.5ml) were weighed into 25 ml round-bottom flasks and equilibrated 2 hours at constant temperature (30°C) in a shaking water-bath. Both phases had been mutually pre-equilibrated. Concentrations of drug in the aqueous phase were determined by UV spectrophotometry. The concentration of drug in the oil phase was determined by mass balance. The molal partition coefficients, K_W^O, were determined from a similar equation as Eq.17.

3.3.5 Measurement of Surface Tension of Cefotaxime Sodium

The measurement of surface tension of cefotaxime sodium solutions were made by the drop volume method using 'Agla micrometer syringe'. Different concentrations of cefotaxime

solutions were prepared at a pH of 3.0 and 24°C, and the volume of a drop from each solution was measured and the surface tension was calculated according to the following equation :

$$\gamma = \frac{r^2 \cdot \Delta\rho \cdot g}{2X^2} \qquad (18)$$

where, r=radius of micrometer tip

$\Delta\rho$=difference in densities of air and solution

g=acceleration due to gravity, and

X=correction factor for $\frac{r}{V^{1/3}}$, where V= volume of a water drop (obtained from Ref.190).

4. RESULTS

4.1 ACETYLSALICYLIC ACID

4.1.1 Ultraviolet Spectrophotometric Analysis

A linear calibration curve of salicylic acid which obeyed Beers law was obtained and is shown in Figure 5. Regression analysis of the experimental data yielded: slope = 3743.2, intercept = .0088 and correlation coefficient, r = .9999. The molar extinction coefficient, ϵ, was calculated from

$$\epsilon = A/bC \qquad (19)$$

where, A is the recorded absorbance at λ = 303 nm, b is the optical path length and is equal to 1 cm, C is the molar concentration of SA in isopropanol. The average value of ϵ was 2830 (n=9) (cf. ϵ = 3620 in aqueous solution (160)). The isosbestic point in isopropanol of SA occurred at 303 nm, allowing the determination of SA independent of pH (155,162).

4.1.2 Apparent First-Order Hydrolysis

The hydrolysis of ASA in control buffer solutions or liposome preparations was followed up to three half-lives (Figure 6) and found to obey pseudo first-order kinetics as

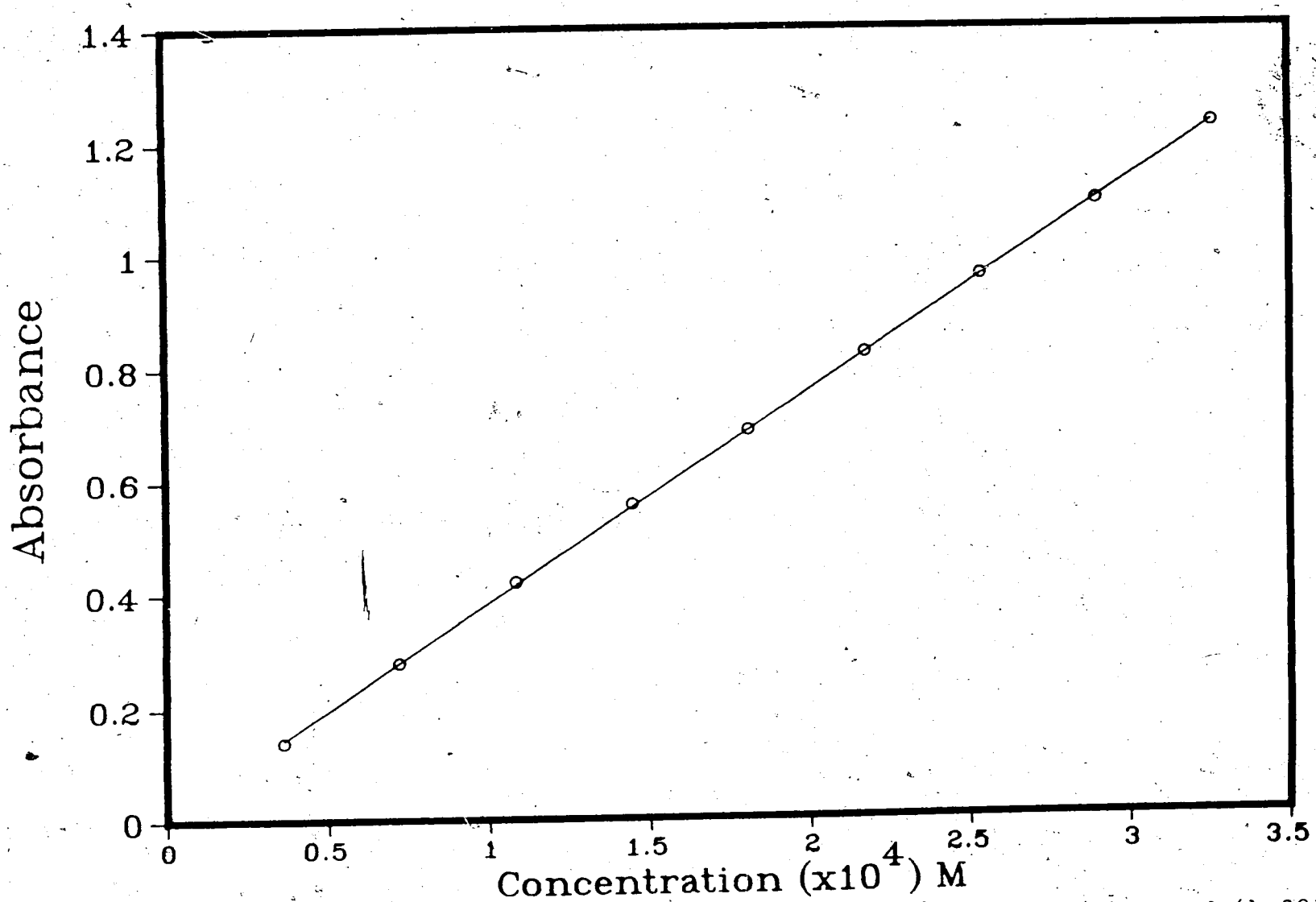


Fig. 5. Beers Plot for the Quantitation of Salicylic Acid in Isopropyl Alcohol (λ=303 nm).

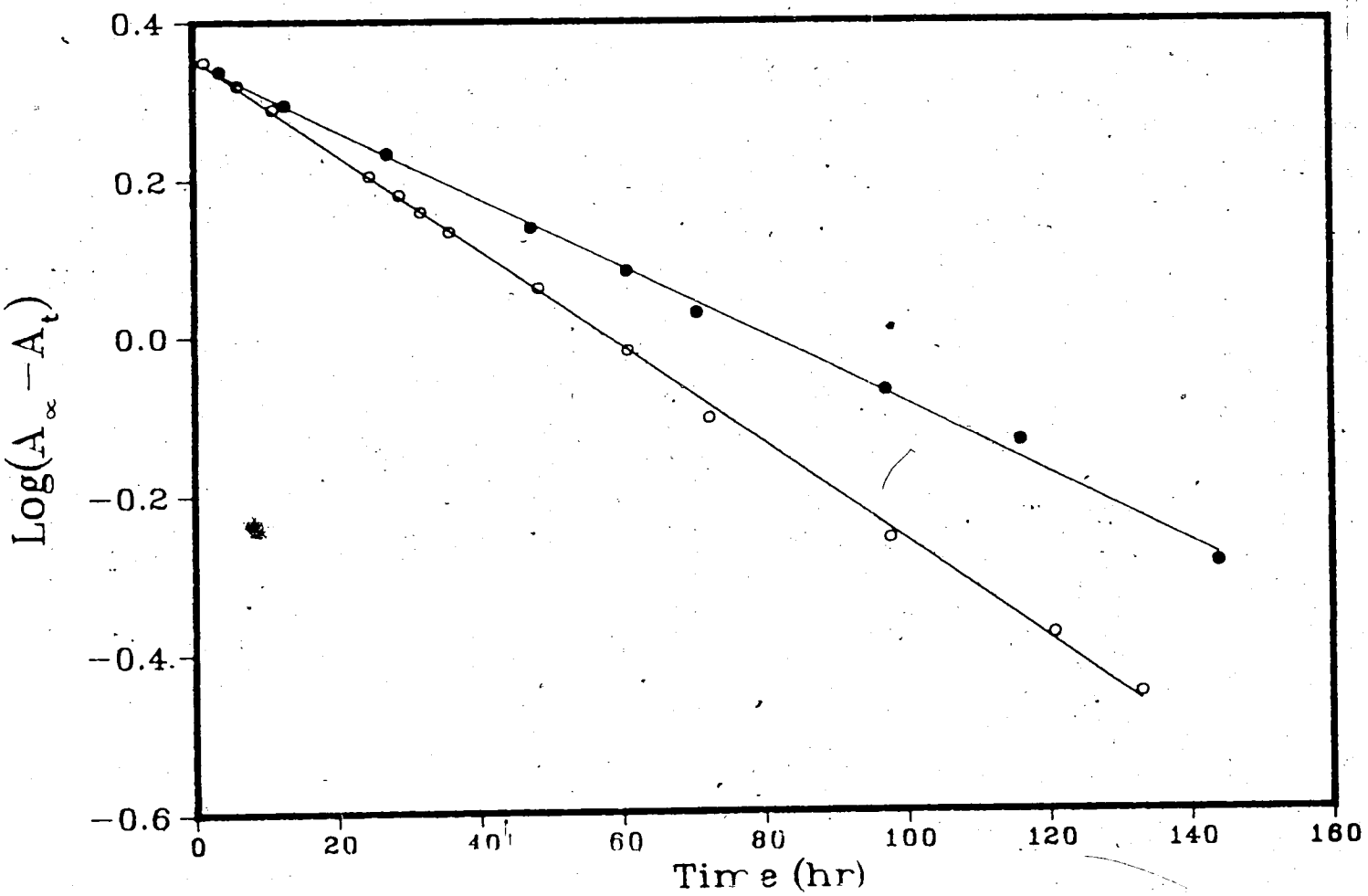


Fig. 6. Kinetics of Hydrolysis of ASA at pH 4.0 and 30°C in: (-o-) Acetic Acid-Sodium Acetate Buffer Solution, and (-•-) DMPC Liposomes. DMPC Concentration was 14.4 mM; Initial ASA Concentration was 7.5 mM.

described by,

$$C = C_o e^{-kt} \qquad (20)$$

where C_o and C are the molar concentrations of ASA initially and at time t, respectively and k is the pseudo first-order hydrolysis rate constant. For hydrolysis in aqueous buffer solution $k=k_B$ and in liposome systems $k=k_{obs}$, when ASA was incorporated via the organic phase and $k=k^x_{obs}$, when ASA was incorporated via the aqueous phase. Hydrolysis in the lipid phase (k_L) and in the external aqueous phase of liposomes (k_b) was taken to obey Eq. 20 and that $k_b = k_B$. thus,

$$k_{obs} = k_L f_L + k_B f_B \qquad (21)$$

where f_L and f_B are the fraction of drug in the lipid and aqueous phases, respectively. All rate constants were determined from linear regression analysis of $\log(A_\infty - A_t)$ versus time plots. Reproducibility of these kinetic experiments agreed to within one percent.

4.1.3 Comparative Stabilities of ASA in Liposomes

4.1.3.1 Effect of pH

The pH-rate profiles for ASA degradation in aqueous buffer solution or in liposomes as shown in Figure 7 were found to have features similar to that previously

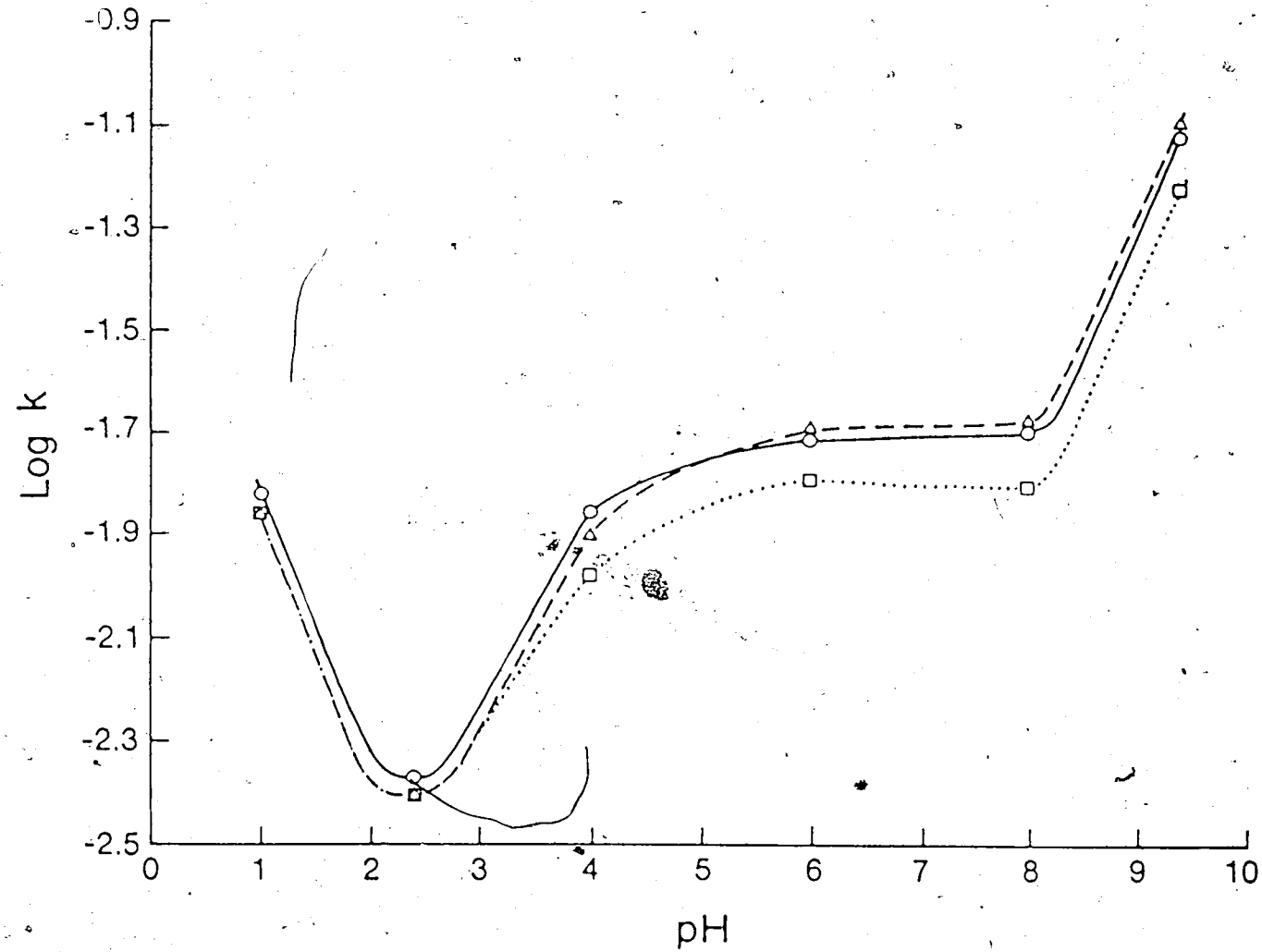


Fig. 7. pH-Rate Profile of the Hydrolysis of ASA at 30°C in Aqueous Buffer Solutions (-o-), in Liposomes in Which ASA was Incorporated Via the Aqueous Phase (-Δ-), and in Liposomes in Which ASA was Incorporated via the Organic Phase (-□-).

reported (160). Between pH 1-3 k_{obs} was slightly lower than k_B. (Student "t" test, $p< 0.10$) but at higher pH values the stability of ASA in liposomes was determined by the manner in which ASA was incorporated into the liposomes. When ASA was added initially via the organic phase, the stability of ASA was enhanced. On the other hand, when ASA was incorporated via the aqueous phase, ASA was not stabilized. These differences were not observed at pH<3. When ASA was incorporated in liposomes via the organic phase, k_{obs} at pH 4.0 and at higher pH values yielded 22-25% increase in stability ($p< 0.05$)($n=3$).

Table 3 compares the rate constants obtained at different pH values. It shows a pattern of change of k_{obs} and k_B consistent with the pH-rate profile (Figure 7).

4.1.3.2 Effect of Liposome Composition

The effect of liposome composition on k_{obs} is shown in Table 4. These results can be divided into Group A or Group B. Liposome compositions within either of the two groups did not yield significant differences in k_{obs} but Group A was significantly different than Group B ($p<0.10$). Liposome compositions in Group B yielded less fluid and more rigid bilayer structures than those in Group A either due to the existence of a gel state of the bilayers (30°C is $<T_c$ of DPPC) or to CHOL addition

TABLE 3

Comparison of the First Order Hydrolysis Rate Constant of ASA in DMPC Liposomes (k_{obs}) and Aqueous Buffer (k_B).

pH	k_{obs} (hr^{-1}) ($x10^2$)	k_B (hr^{-1}) ($x10^2$)	k_{obs}/k_B
1.0	1.39($4x10^{-5}$)	1.51($4x10^{-4}$)	0.921
4.0	1.05($5x10^{-4}$)	1.39($2x10^{-4}$)	0.755
8.0	1.57($2x10^{-4}$)	2.04($9x10^{-4}$)	0.770

[DMPC] = 14.4 mM; $[ASA]_o$=7.5 mM; 30°C.

Standard deviations (n=3) shown in brackets.

TABLE 4

Hydrolysis of ASA in Liposomes of Various Compositions at pH 4.0 and 30°C.

Liposome Composition[a]	k_{obs} (hr^{-1}) ($x10^{2}$)	k_{obs}/k_B		T_c[d] (°C)
DMPC	1.05($5x10^{-4}$)	0.755		23
EPC	1.11($6x10^{-4}$)	0.799		0
SPHING	1.12($2x10^{-4}$)	0.806	A	25-40
DMPC:DCP(2:1)	1.12($1x10^{-4}$)	0.806		-
DMPC:EPC(1:1)	1.085($2x10^{-4}$)	0.777		-
DPPC	1.16($2x10^{-5}$)	0.835		41
DMPC:CHOL(3:1)	1.21($8x10^{-5}$)	0.871	B	-

[a] Total lipid concentration =14.4 mM; mole ratios shown in brackets.
Standard deviations (n=3) shown in brackets beside k_{obs} values.

Group A and group B are significantly different at $p< 0.10$.

[d] Ref. 45

(169) and this resulted in an increased K_{obs}.

DMPC:STEAR Liposome System

The addition of STEAR to DMPC imparts a net positive surface charge to liposomes. The interaction of ASA with this lipid composition leads to both increased hydrolysis initially, followed by increased stabilization compared to the control buffer solution. The evidence of this can be seen in Table 5. As the DMPC:STEAR mole ratio increases, both the initial loss as well as the increase in stability decreases reflecting a diminishing influence of STEAR. By varying the DMPC:STEAR mole ratio, the total lipid concentration, and the initial ASA concentration, the initial loss of ASA could be minimized and the increase in stability could be maximized as seen from the results presented in Table 6. In Table 7 it can be seen that pH affects the influence of STEAR, after an initial loss in the organic solvent. At pH 1.0, although the overall stabilization was lower than at other pH values, a substantial increase in stability was obtained compared to the liposomes in the absence of STEAR.

Initially it appeared that ASA was undergoing a biexponential decay in a manner similar to p-nitro phenyl acetate (39,40). However, as shown in Figure 8, it was later discovered that ASA was degrading in the chloroform solution prior to forming the lipid film.

TABLE 5

Effect of Stearylamine on the Stability of ASA in DMPC Liposomes at pH 4.0 and 30°C.

DMPC:STEAR[a]	$k_{obs} \times 10^2 (hr^{-1})$	Initial Loss of ASA (%)	% Increase in Stability[b]
2:1	0.80±.016	34.7±1.15	42.5±1.13
5:1	1.01±.008	20.9±2.36	27.4±0.56
10:1	1.01±.009	16.6±2.49	27.4±0.60

[a] mole ratios; total lipid concentration = 14.4 mM; initial ASA concentration = 7.5 mM.

[b] using Eq. 10 and $k_B = 1.39 \pm 0.02 \times 10^{-2} hr^{-1}$; n=3.

TABLE 6

Influence of Total Lipid and Initial ASA Concentration on the Stability of ASA in DMPC:STEAR Liposomes at pH 4. and 30°C

DMPC: STEAR[a]	Total Lipid Conc. (mM)	Initial ASA Conc. (mM)	k_{obs} x10²(hr⁻¹)	Initial Loss of ASA (%)	Percent Increase in Stability[b]
2:1	14.4	7.5	0.800±.016	34.7±1.15	42.5±1.13
2:1	14.4	15.0	0.903±.010	18.2±0.46	35.2±0.80
2:1	28.7	15.0	0.710±.037	32.5±0.28	48.9±2.65
4:1	28.7	15.0	0.787±.034	21.1±1.70	43.5±2.42

[a] mole ratios
[b] using Eq. 10 and k_B=1.39±0.02x10⁻²hr⁻¹ (n=3)

TABLE 7

Effect of stearylamine on the stability of ASA in DMPC Liposomes at various PH values and 30°C

pH	k_{obs} $x10^2(hr^{-1})$	k_B $10^2(hr^{-1})$	Initial Loss of ASA (%)	Percent Increase in stability[a]
1.0	1.06±.0197	1.51±.043	36.3	29.3 (8.1)
4.0	0.80±.0160	1.39±.017	34.7	42.5 (24.5)
8.0	1.04±.0125	2.04±.091	35.9	48.2 (23.1)

Total lipid concentration = 14.4 mM; Initial ASA concentration = 7.5 mM.

DMPC:STEAR = 2:1

[a]Figure in bracket represents stability in the absence of STEAR.

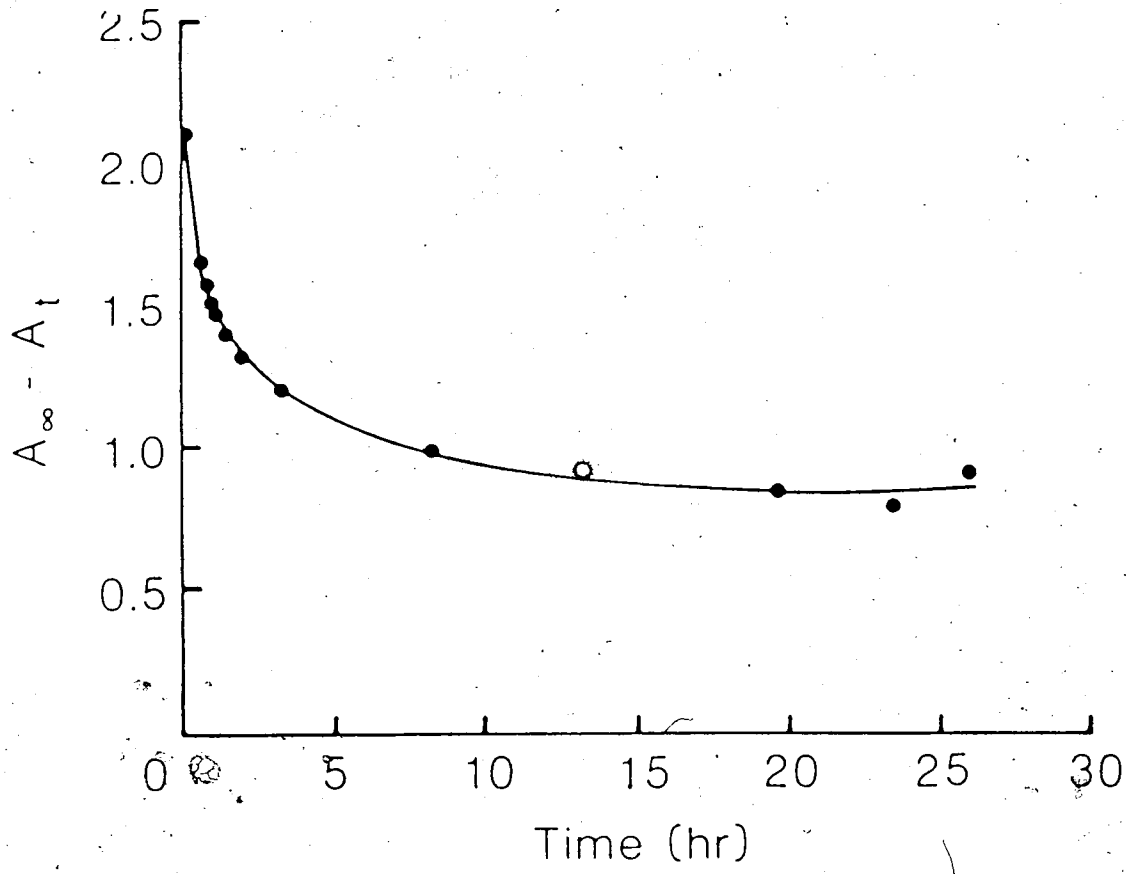
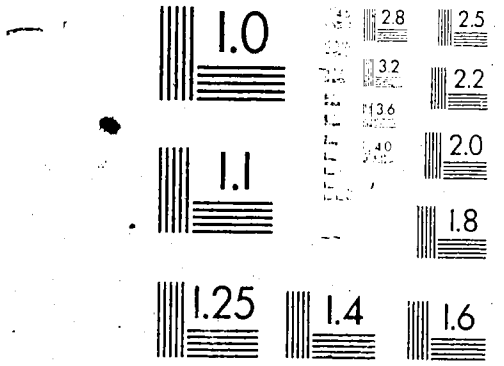


Fig. 8. Hydrolysis of ASA in a Chloroform Solution Containing DMPC:STEAR 2:1 Mole Ratio at 30°C. DMPC Concentration was 14.4 mM, Initial ASA Concentration was 7.5 mM.

Similar levels of degradation also occurred if a solvent of 9% methanol-in-petroleum ether was used instead of chloroform. However, once the dried film is dispersed in aqueous buffer solution to form liposomes, ASA was stabilized. The results shown in Tables 5 and 6 were obtained under essentially the same conditions of solution preparation and drying time to form the films. In contrast, if ASA was incorporated into the aqueous buffer solution during liposome preparation or if an aqueous solution of ASA was added to preformed liposomes no initial loss of ASA was observed but the degree of stabilization was low. This was again dependent on the pH of the medium. The kinetics of degradation of ASA in DMPC:STEAR liposomes at pH 4.0 and pH 8.0 are illustrated in Figure 9 and 10, respectively. Again, it is seen in Figure 9 that $k^{x}_{obs}=k_B$ but $k_{obs}<k_B$ following an initial rapid degradation phase. However, at pH 8.0, $k^{x}_{obs}\neq k_B$ and in contrast, the degradation curves corresponding to ASA incorporated via the aqueous phase exhibit biexponential characteristics, the biexponential behavior being more pronounced in 1:1 DMPC:STEAR liposomes than in 2:1 DMPC:STEAR liposomes. Proceeding on the assumption that ASA is undergoing reaction with STEAR in the chloroform solvent to form N-stearylacetamide, the degradation product was isolated and subjected to IR analysis. Figure 11 shows this



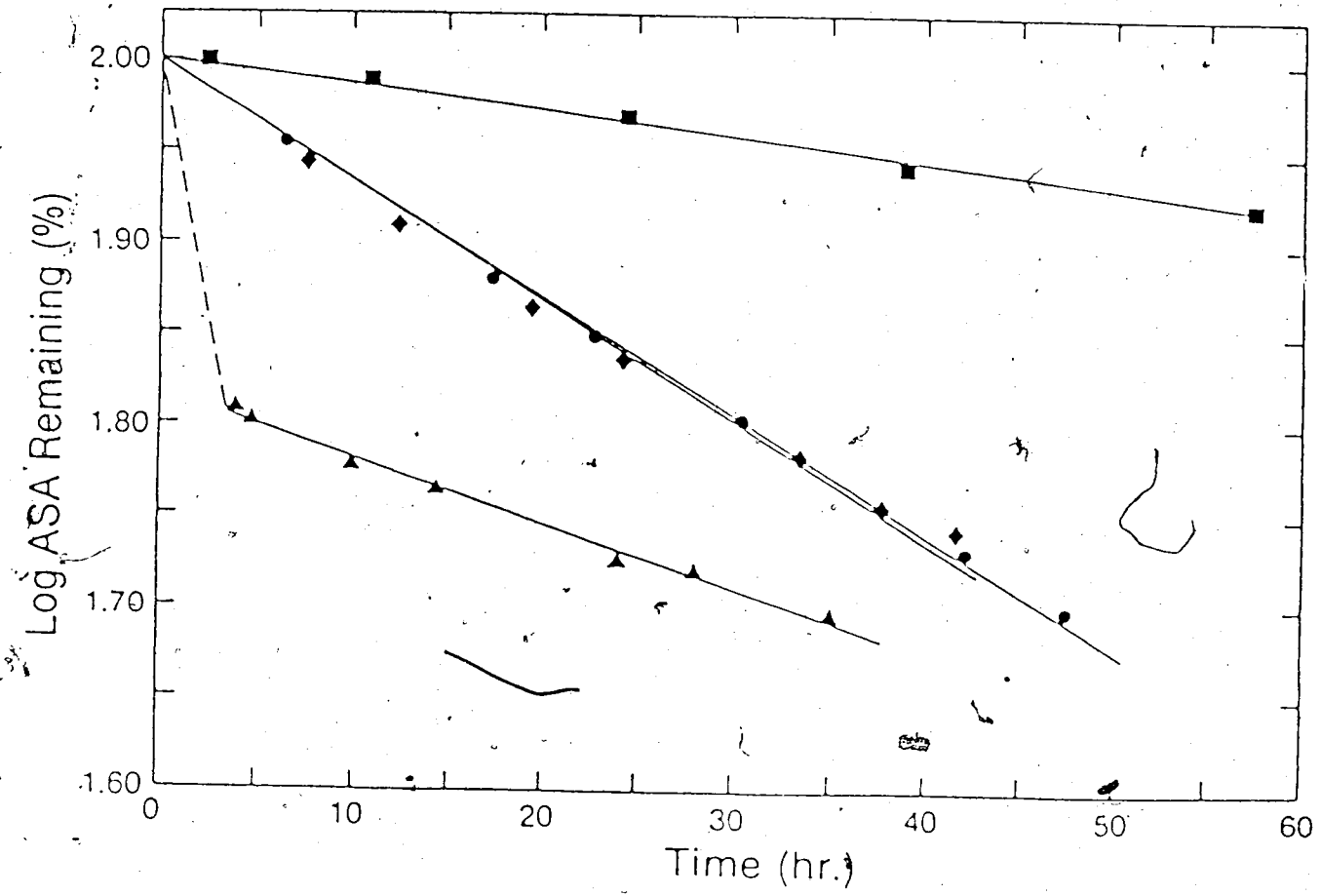


Fig. 9. Kinetics of Hydrolysis of ASA at pH 4,0 and 30°C in: (-●-), Acetic Acid-Sodium Acetate Buffer Solution, (-◆-), DMPC:STEAR (2:1) Liposomes in Which ASA was Incorporated Via the Aqueous Phase, (-▲-), DMPC:STEAR (2:1) Liposomes in Which ASA was Incorporated Via the Organic Phase, (-■-), DMPC:STEAR Liposomes in Which ASA was Incorporated Via the Organic Phase, the Non-entrapped ASA was Removed by Centrifugation Then the Liposomes Were Resuspended in the Buffer Solution.

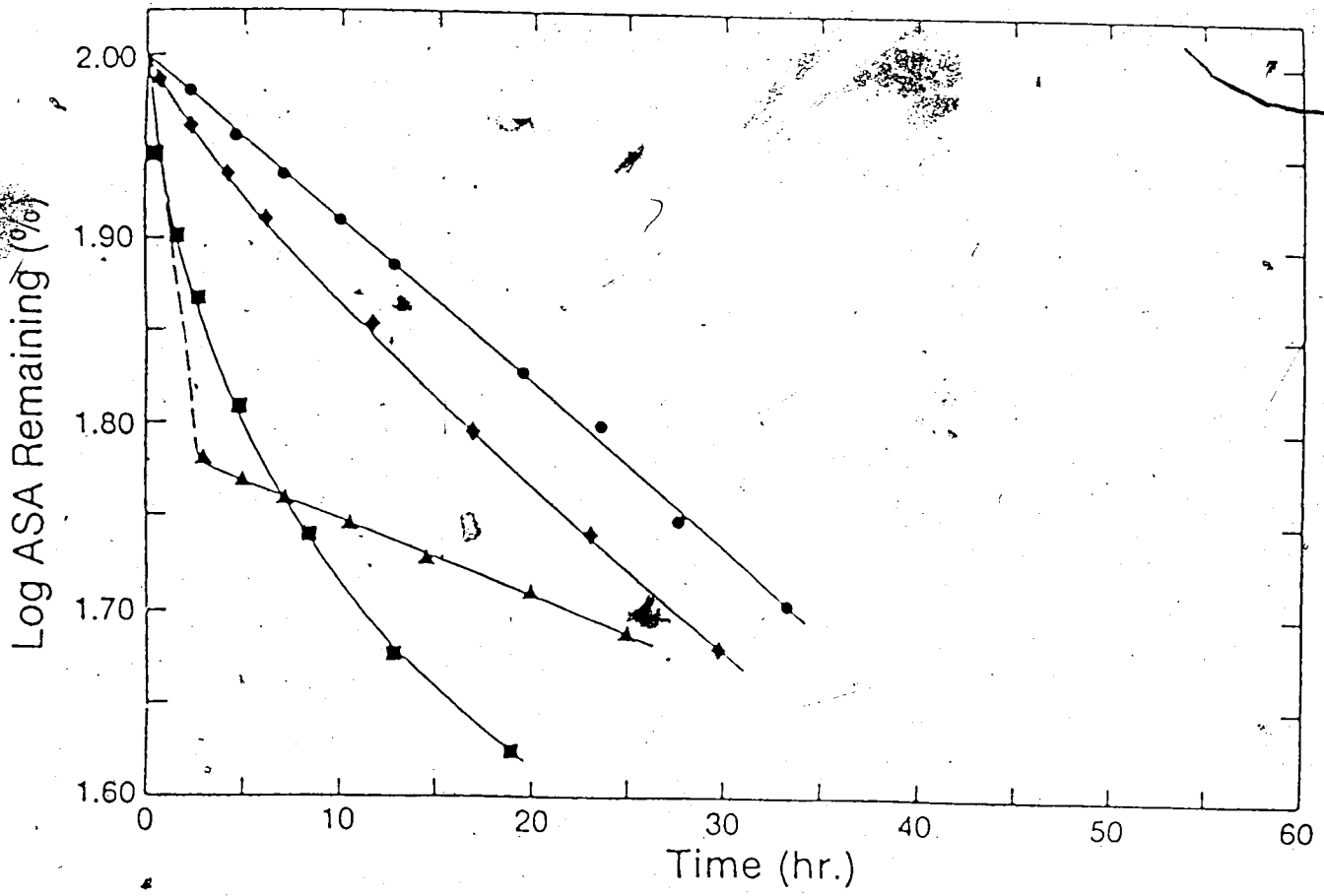


Fig. 10. Kinetics of Hydrolysis of ASA at pH 8.0 and 30°C in: (-●-), KH_2PO_4-Na_2HPO_4 Buffer Solution; (-◆-), and (-■-), DMPC:STEAR (2:1) and (1:1) Liposomes, respectively, in Which ASA was Incorporated Via the Aqueous Phase; (-▲-), DMPC:STEAR (2:1) Liposomes in Which ASA was Incorporated Via the Organic Phase.

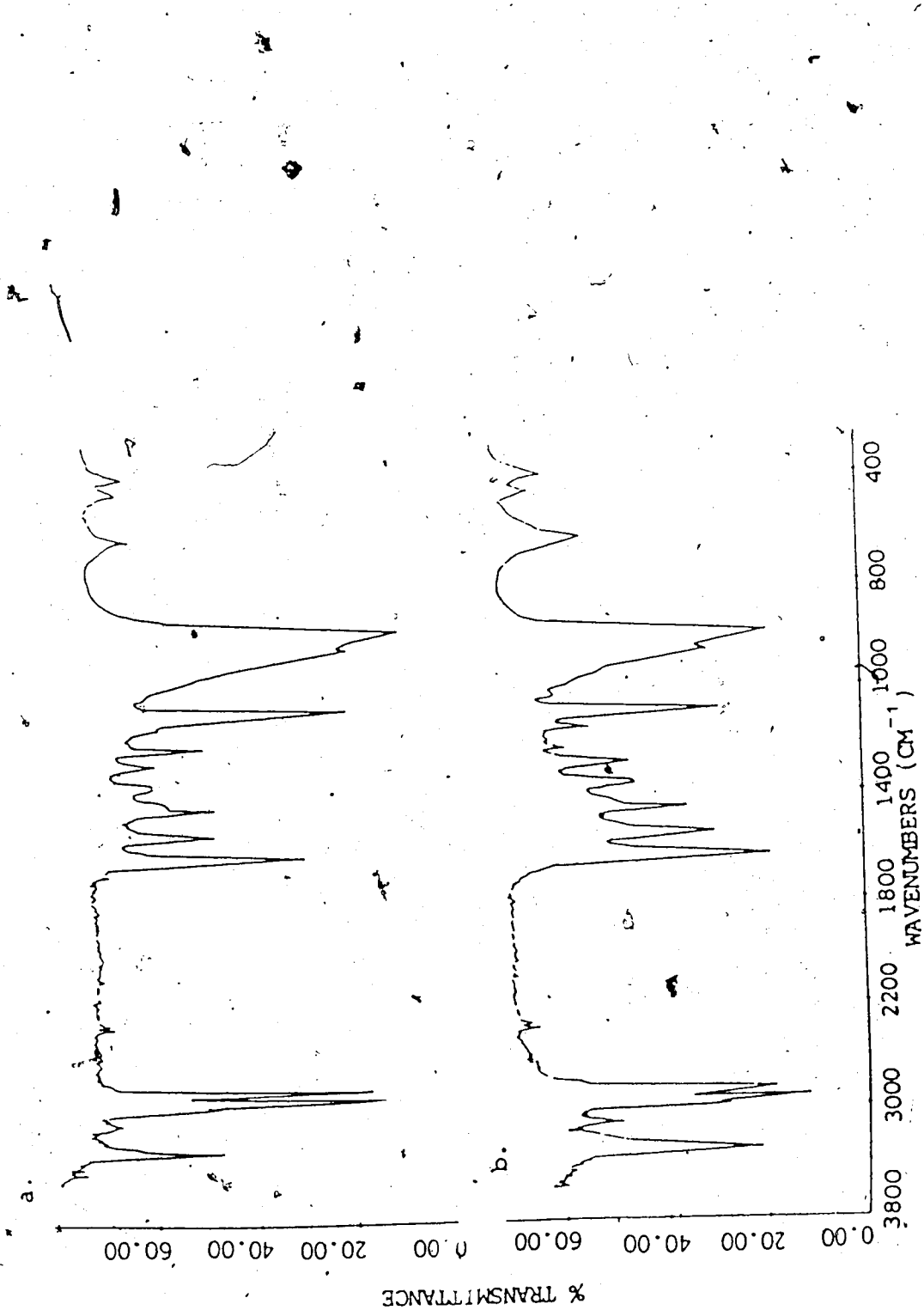


Fig. 11. Infra-Red Spectra of N-Stearylacrylamide Synthesized from: a) Acetic Anhydride and STEAR and b) ASA and STEAR. Characteristic Peaks at: 3300 cm^{-1} for N-H, ≃ 3000 cm^{-1} for C-H and 1650 cm^{-1} for C=O.

result, together with N-stearylacetamide formed from STEAR and acetic anhydride. Their similarities are notable with respect to the characteristic peaks of N-H at 3300 cm^{-1}, of C-H at ≃3000 cm^{-1}, and of C=O at 1650 cm^{-1}. Furthermore thin-layer chromatography showed that the R_f value of the reaction product of ASA and STEAR was the same as that of N-stearylacetamide (Figure 12).

In Table 8, a comparison of the effect of the presence of amines in addition to STEAR or SA on the degradation of ASA in DMPC liposomes is made. When N-stearylacetamide was included in the liposome, the initial loss of ASA in chloroform solution did not occur but an increase in ASA stability was not obtained in the final liposomes. On the other hand, when p-chloroaniline was incorporated, again an initial loss of ASA in chloroform followed by increased stabilization of the remaining ASA in liposomes was observed as it was when STEAR was used. The addition of CHOL to DMPC:STEAR liposomes reduced the stabilization in a manner that was also observed in DMPC liposomes. When excess SA was incorporated into the liposomes (as the degradation product of ASA) stabilization was reduced from 25% to 9-10%.

4.1.3.3 Effect of Phospholipid and ASA Concentration

Using DMPC as the representative phospholipid, the effect of varying its concentration on the stabilization

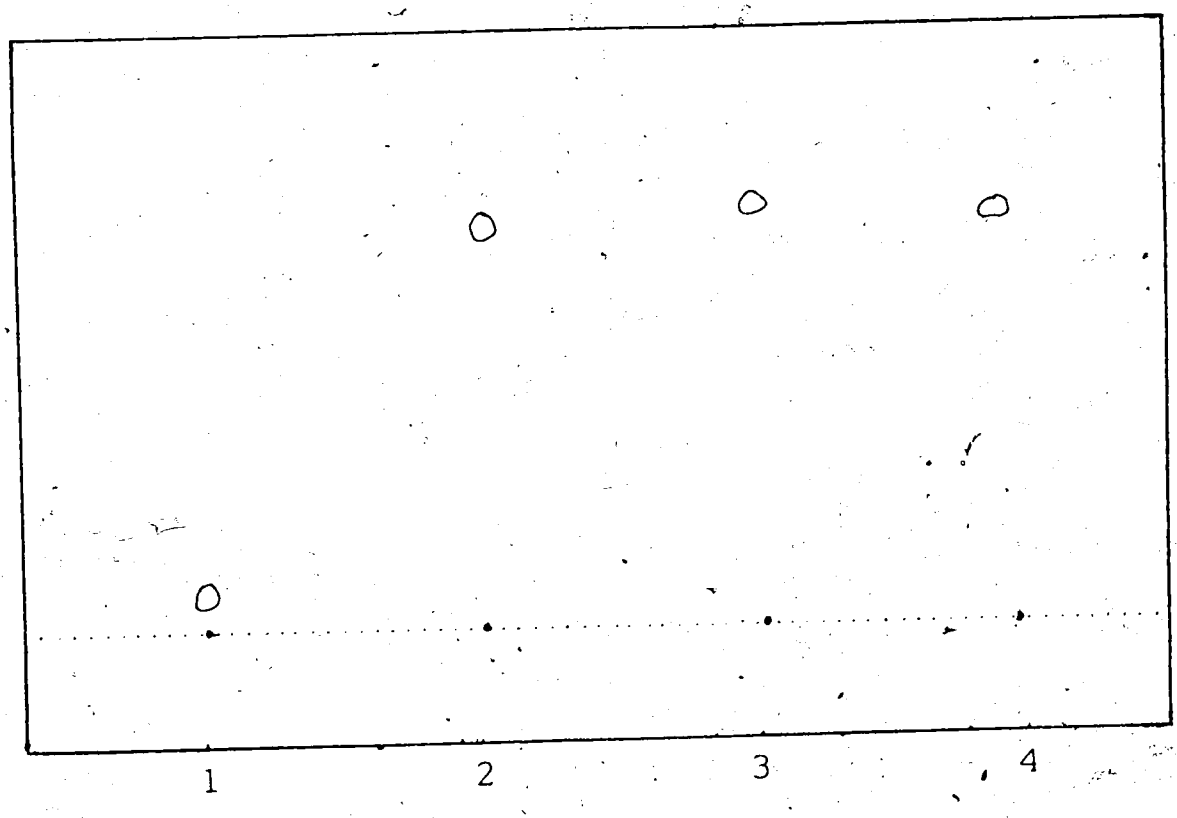


Fig. 12. Thin-Layer Chromatogram of : 1. STEAR 2. N-Stearylacetamide from Acetic Anhydride 3. N-Stearylacetamide from ASA 4. Mixed N-Stearylacetamide from 2 and 3. TLC Plate: 10x10 cm (Silica gel 60F254, 0.2 mm Thickness, E.Mark, W. Germany) Developed with Chloroform:Methanol (9:1 v/v) and Visualized Using Iodine Vapor.

TABLE 8

A Comparison of the Effect of the Presence of Amines or Salicyclic Acid on the Degradation of ASA in DMPC Liposomes at pH 7.0 and 30°C.

Lipsome Composition[a]	Initial Loss of ASA (%)[b]	% Increase in ASA Stability
DMPC	0	24.5
DMPC:STEAR (2:1)	34.7	42.5
DMPC: N-Stearylacetamide (2:1)	0	26.0
DMPC + 2.7 mM SA (5:1)	0	9.0
DMPC: N-stearylacetamide (2:1) + 2.7 mM SA	0	9.8
DMPC:p-chloroaniline (2:1)	27.4	53.0
DMPC:STEAR:CHOL (2:1:1)	25.0	30.0

[a] total lipid conc.=14.4 mM; mole ratios in brackets.
[b] degradation of ASA in the organic solvent prior to liposome formation.

of ASA was determined. The results of varying the DMPC concentration at an ASA conc. of 7.5mM are presented in Table 9. Thus, at approximately a 1:1 mole ratio of DMPC:ASA, a 15% increase in stability was obtained. When the concentration of DMPC was doubled, the percentage increased approximately two-thirds such that an increase in stability of 42.0% was obtained at a 4:1 mole ratio of DMPC:ASA (equivalent to 20 mg/ml DMPC). Therefore, increasing the concentration of DMPC liposomes yielded a linear decrease in k_{obs} (Figure 13). It is noted that extrapolation of the curve to the Y-axis, does not intersect at 1.0 indicating a non-linear dependency at the lower concentrations. In contrast, a 4-fold increase in the ASA concentration at constant DMPC concentration showed a slight decrease in the liposome stabilization of ASA (increased in k_{obs}/k_B) (Table 10).

4.1.3.4 Effect of Temperature and the Ionic Strength of the Medium

The kinetics of ASA hydrolysis were studied over the temperature range of 10° - 50° and an Arrhenius plot constructed. The buffer solutions for the temperature study were checked for pH and adjusted at each different temperatures. Figure 14 depicts these results in aqueous buffer solution and in liposomes ($r=0.999$) yielding identical energies of activation (E_a) of the

TABLE 9

Stability of ASA in Liposomes as a Function of Phospholipid Concentration at pH 4.0 and 30°C.

DMPC Conc. (mM)[a]	$k_{obs} \times 10^2 (hr^{-1})$	Percent Increase in Stability[b]
0	1.39	-
7.2	1.19	14.8
10.8	1.12	19.6
14.4	1.05	24.5
28.7	0.81	42.0

[a] initial ASA concentration = 7.5 mM.

[b] using Eq. 10 and $k_B = 1.39 \pm 0.02 \times 10^{-2}$ hr^{-1}

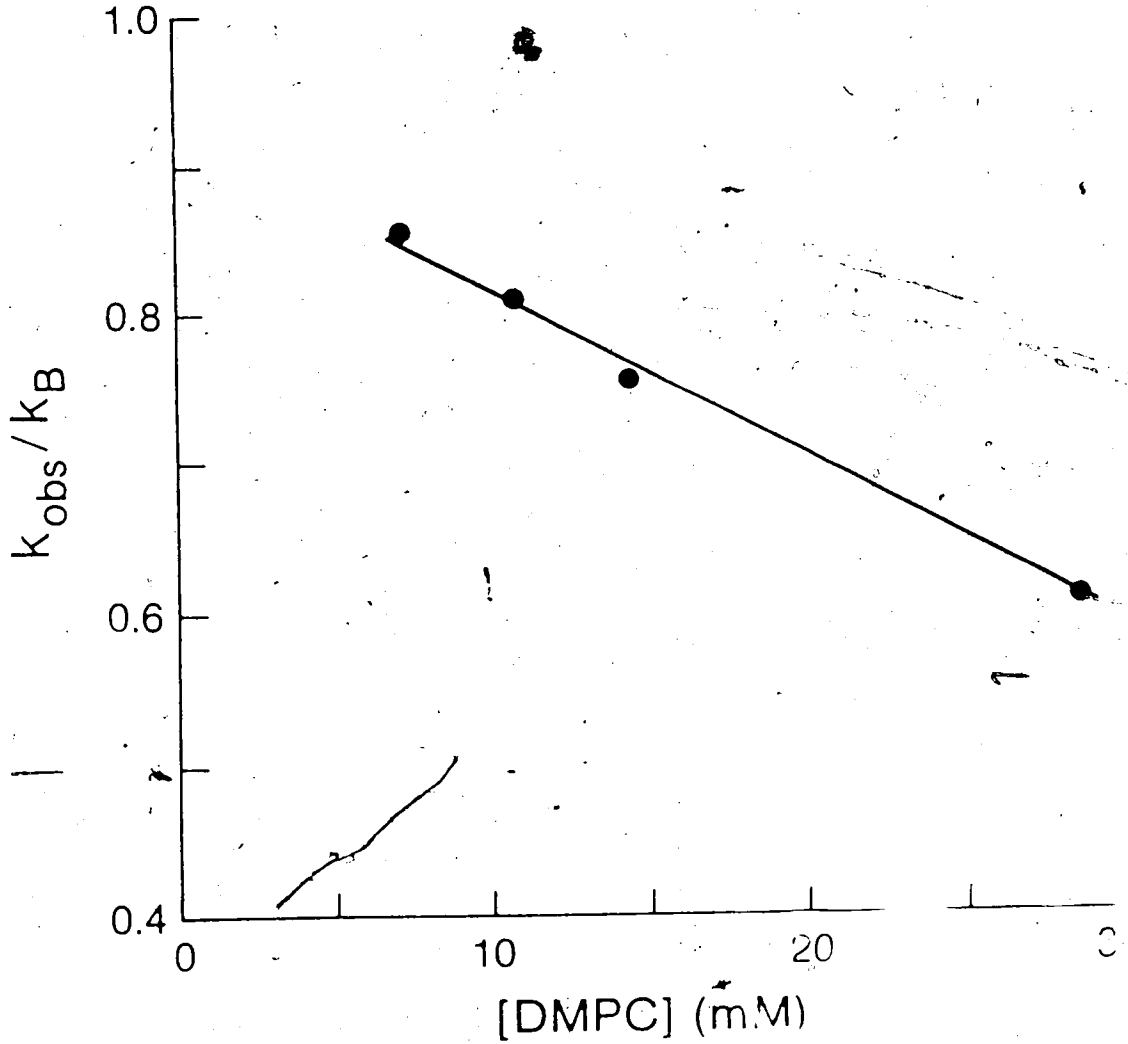


Fig. 13. Variation of ASA Stabilization in Liposomes as a Function of Phospholipid (DMPC) Concentration at pH 4.0 and 30°C.

TABLE 10

Hydrolysis of ASA in DMPC Liposomes as a Function of ASA concentration at pH 4.0 and 30°C.

ASA Conc. (mM)	k_{obs} (hr^{-1}) (x10^2)	k_B (hr^{-1}) (x10^2)	k_{obs}/k_B
3.75	1.01(2x10^{-4})	1.34(2x10^{-4})	0.754
3.75	1.01(2x10^{-4})	1.34(2x10^{-4})	0.754
7.50	1.05(5x10^{-4})	1.39(2x10^{-4})	0.755
15.0	1.20(3x10^{-4})	1.28(3x10^{-4})	0.781

[DMPC] = 14.4 mM.

Standard deviations (n=3) shown in brackets.

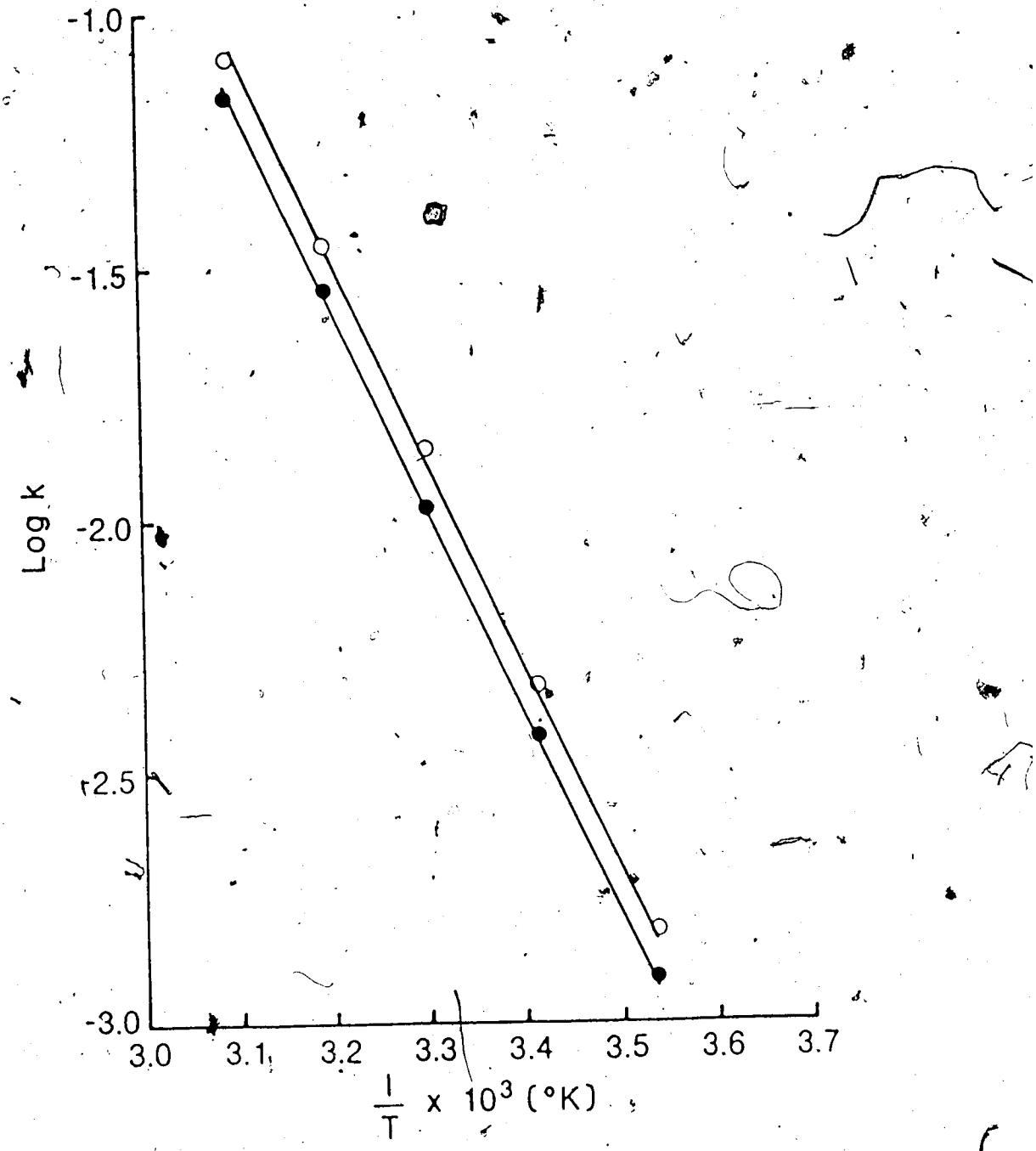


Fig. 14. Arrhenius Plots of ASA at pH 4.0 in: (-o-) Acetic Acid-Sodium Acetate Buffer Solution, and (-•-), DMPC Liposomes.

reaction equal to 18 kcal/mole in each system. This indicates that the association of ASA molecules with the PL bilayers does not alter the mechanism of degradation of ASA. Table 14 summarizes the influence of temperature on the stabilization of ASA in liposomes. Although k_{obs} and k_B increase with temperature the percent increase in stability rises to a maximum at about 30° then decreases sharply above this temperature. This type of behavior is analogous to the partitioning of some solutes in liposomes where maximum values have been observed at temperatures immediately above the T_c of the phospholipid (170). In spite of this change in trend which may be related to the different physical states of liposomes below and above the T_c of DMPC, overall these variations are only slight.

In Table 12 the effect of ionic strength of the medium on the hydrolysis of ASA in DMPC liposomes have been shown. Although at higher ionic strength a trend of decreasing k_{obs}/k_B was observed a significantly large change was not obtained over the range between 0.07 to 0.63 M.

4.1.3.5 Stability of ASA in the Lipid Phase of Liposomes

Table 13 and Figures 15,16 and 17 describe the kinetics of hydrolysis of ASA entrapped in the lipid phase only under various conditions. It can be seen in Table 13 that k_L and f_L both decreased with increase in

TABLE 11

Stability of ASA in DMPC Liposomes as a Function of Temperature at pH 4.0

Temperature (°C)	$k_{obs} \times 10^2 (hr^{-1})$	$k_B \times 10^2 (hr^{-1})$	k_{obs}/k_B
10	0.123	0.153	0.804
20	0.369	0.464	0.795
30	1.05	1.39	0.755
40	2.86	3.51	0.815
50	6.74	8.17	0.825

TABLE 12

Hydrolysis of ASA in DMPC Liposomes as a Function of Ionic Strength of the Medium at pH 4.0 and 30°C

Ionic Strength (M)	k_{obs} (hr^{-1}) ($x10^2$)	k_B (hr^{-1}) ($x10^2$)	k_{obs}/k_B
0.07	1.06($5x10^{-4}$)	1.398($2x10^{-4}$)	.758
0.15	1.05($5x10^{-4}$)	1.390($2x10^{-4}$)	0.755
0.33	0.986($8x10^{-5}$)	1.405($5x10^{-4}$)	0.702
0.63	0.99($45x10^{-5}$)	1.429($2x10^{-4}$)	0.693

[DMPC] = 14.4 mM.

Standard deviations (n=3) shown in brackets.

TABLE 13

Comparison of the First Order Hydrolysis Rate Constant of ASA in the Lipid Phase (k_L) of DMPC Liposomes, Aqueous Buffer (k_B) and of the Fraction of ASA Associated with the Lipid Phase (f_L).

pH	$k_L(hr^{-1})$ $(x10^2)$	$k_B(hr^{-1})$ $(x10^2)$	f_L	k_L/k_B
1.0	1.12(3x10^{-4})	1.51(4x10^{-4})	.325(.009)	0.742
4.0	0.30(8x10^{-5})	1.39(2x10^{-4})	.258(.013)	0.216
8.0	0.11(7x10^{-5})	2.04(9x10^{-4})	.196(.004)	0.054

[DMPC] = 14.4 mM; 30°C; standard deviations (n=3) shown in brackets

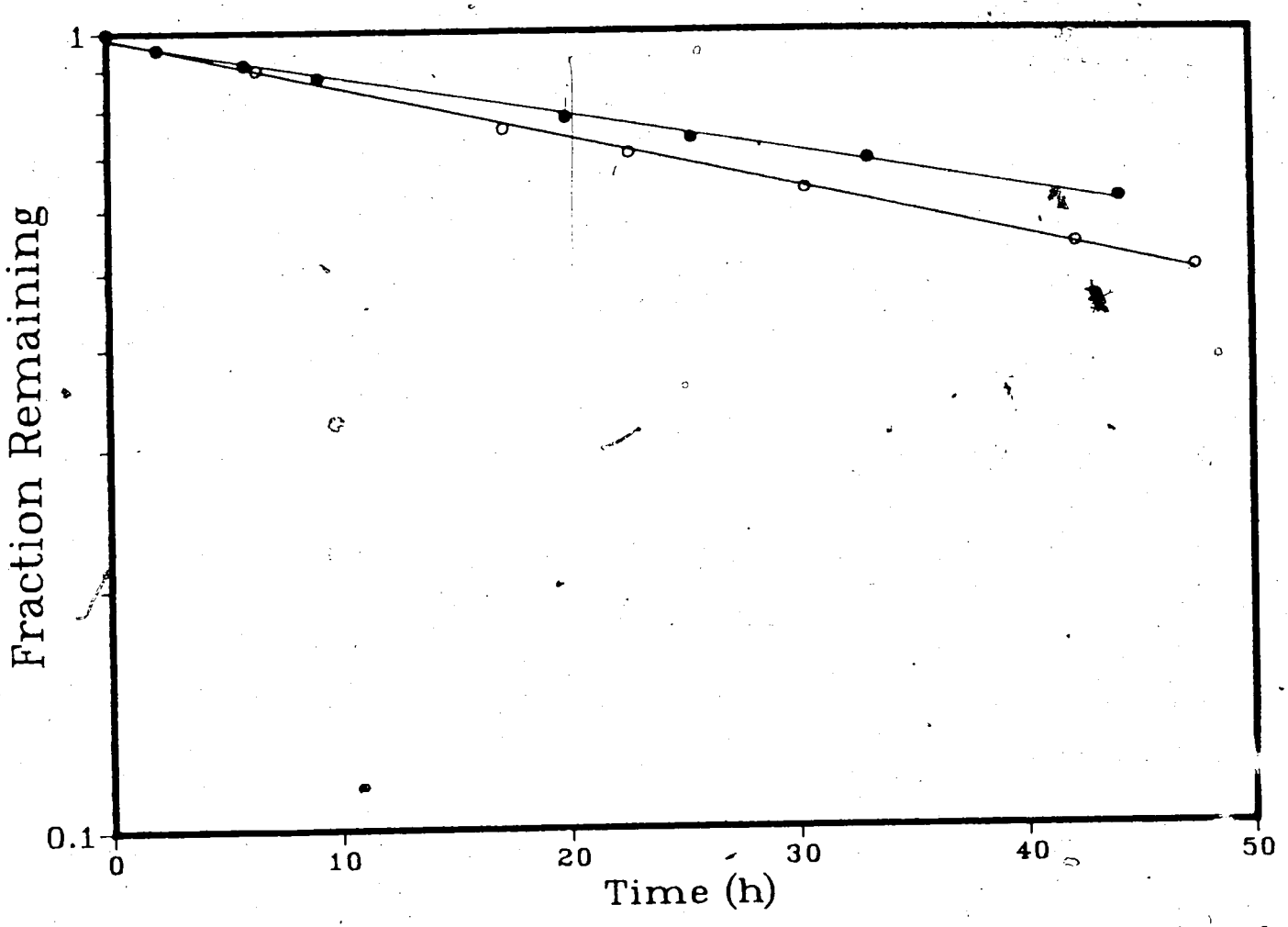


Fig. 15. Kinetics of Hydrolysis of ASA at pH 1.0 and 30°C in : (-o-), KCl-HCl Buffer Solution; (-•-), DMPC Liposomes in which the Unentrapped ASA was Removed by Centrifugation and then Resuspended in the Buffer Solution.

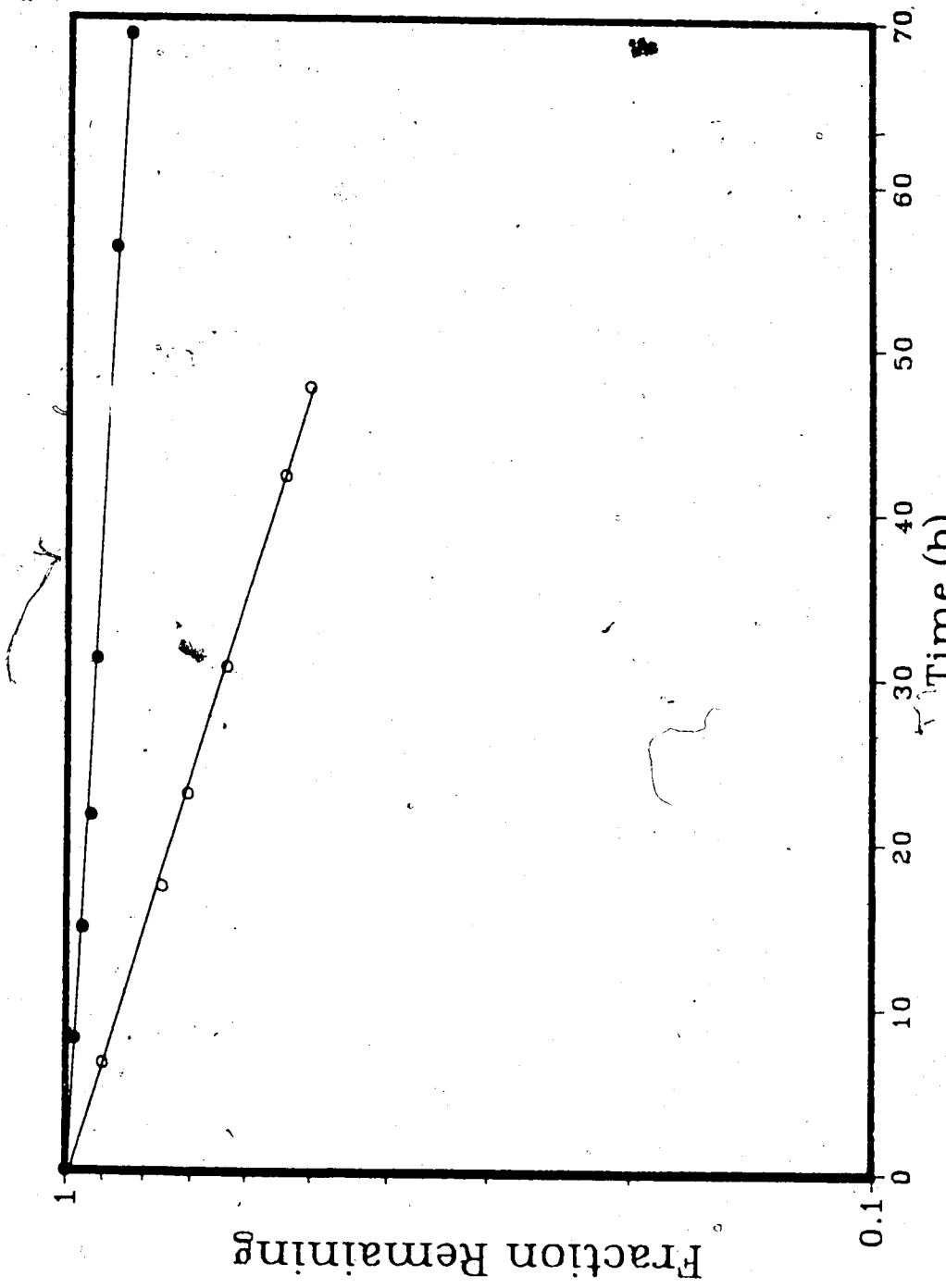


Fig. 16. Kinetics of Hydrolysis of ASA at pH 4.0 and 30°C in: (-o-), Acetic Acid-Sodium Acetate Buffer Solution; (-•-), DMPC Liposomes in which the Unentrapped ASA was Removed by Centrifugation and then Resuspended in the Buffer Solution.

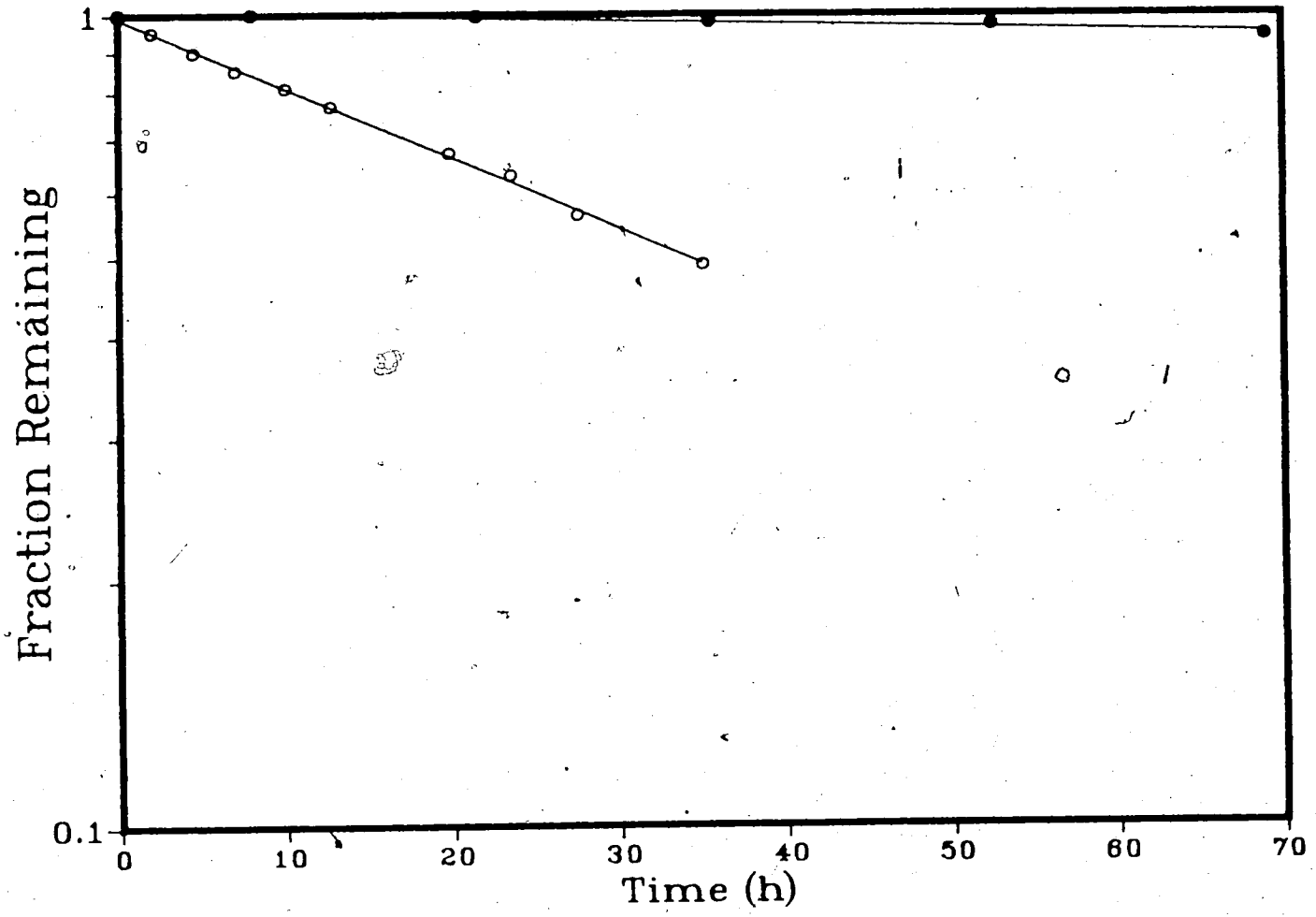


Fig. 17. Kinetics of Hydrolysis of ASA at pH 8.0 and 30°C in : (-o-), KH_2PO_4-Na_2HPO_4 Buffer Solution; (-•-), DMPC Liposomes in Which the Unentrapped ASA was Removed by Centrifugation and Then Resuspended in the Buffer Solution.

the pH but k_{obs} and k_B both decreased then increased over the range of pH corresponding to Figure 7 (see Table 3). The stabilization of ASA was maximal at pH 8.0 followed by at pH 4.0 but the lowest stabilization was obtained at pH 1.0. The comparison of k_L/k_B at various pH values can be obtained from Table 13. As can be seen it is lowest at pH 8.0 and highest at pH 1.0. Table 14 provides evidence that the determination of the percent increase in stability is unchanged at around 80 percent using either 14.4mM or 28.8mM as the initial concentration of DMPC.

4.2 THE LOCAL ANESTHETICS

4.2.1 Ultra-violet Spectrophotometric Analysis

Linear calibration curves which obeyed Beers law were obtained for each of the six local anesthetic drugs and are shown in Figures 18, 19 and 20. The slopes, intercepts and correlation coefficients obtained from regression analysis are shown in Table 15. The average values of ϵ (molar extinction coefficient, Eq.19) are shown in Table 2. The variation of ϵ values obtained from calibration curves determined at different times was ±1.2%. In addition, linear calibration curves of the degradation products p-aminobenzoic acid and p-butylamino benzoic acid are shown in Figure 21 and their ϵ values are also shown in Table 2.

TABLE 14

Hydrolysis of ASA in the Lipid Phase of Liposome as a Function of DMPC Concentration at pH 4.0 and 30°C.

DMPC (mM)	k_L (hr^{-1}) ($x10^2$)	k_B (hr^{-1}) ($x10^2$)	% Increase in Stability
14.4	0.304($8x10^{-5}$)	1.39($2x10^{-4}$)	78.1
28.8	0.291($8x10^{-5}$)	1.39($2x10^{-4}$)	79.1

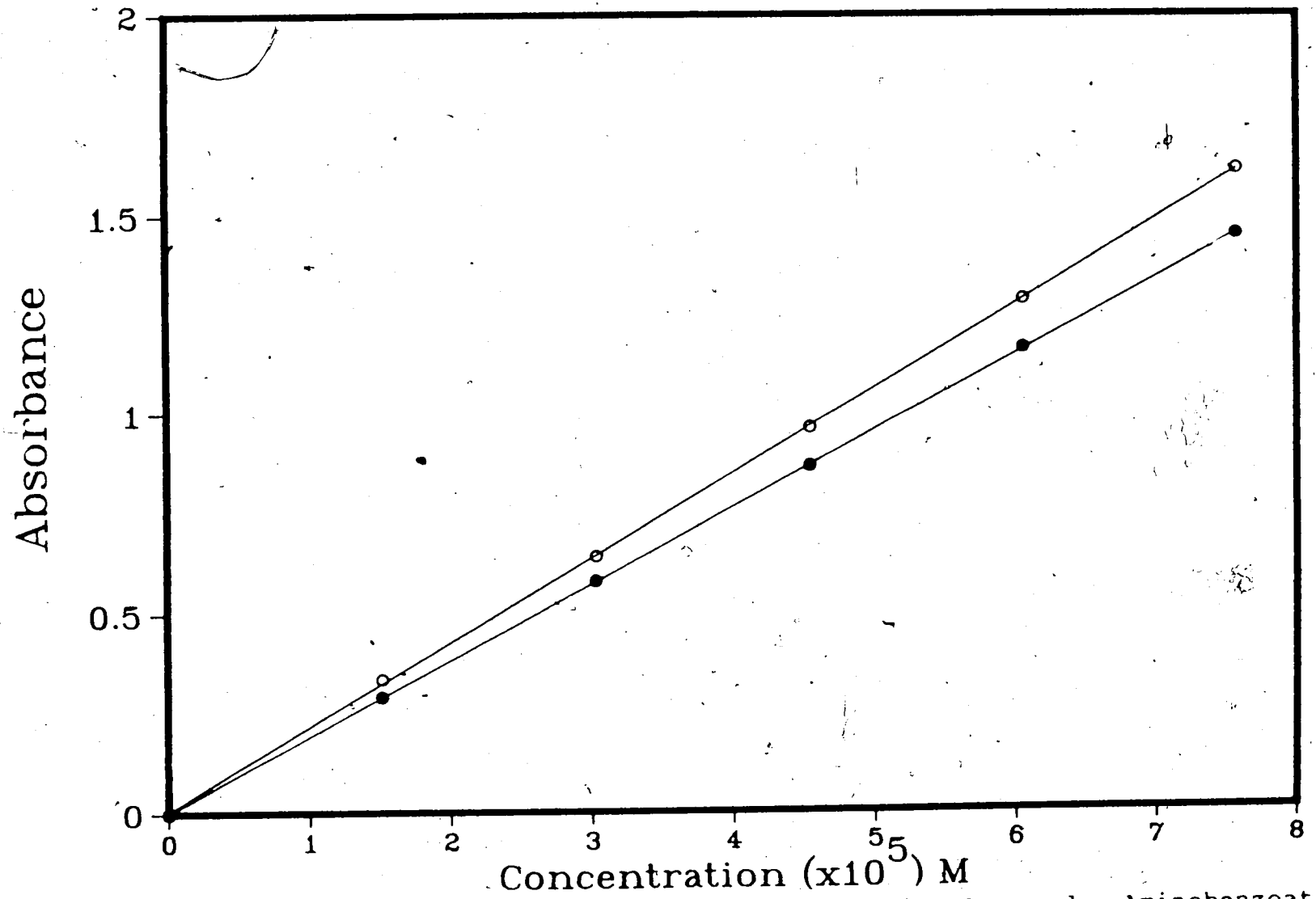


Fig. 18. Beers Plot of Benzocaine (-○-) (λ=291 nm) and Propyl p-Aminobenzoate (-●-)(λ=294 nm) in the Solution for Analysis (see text p-51).

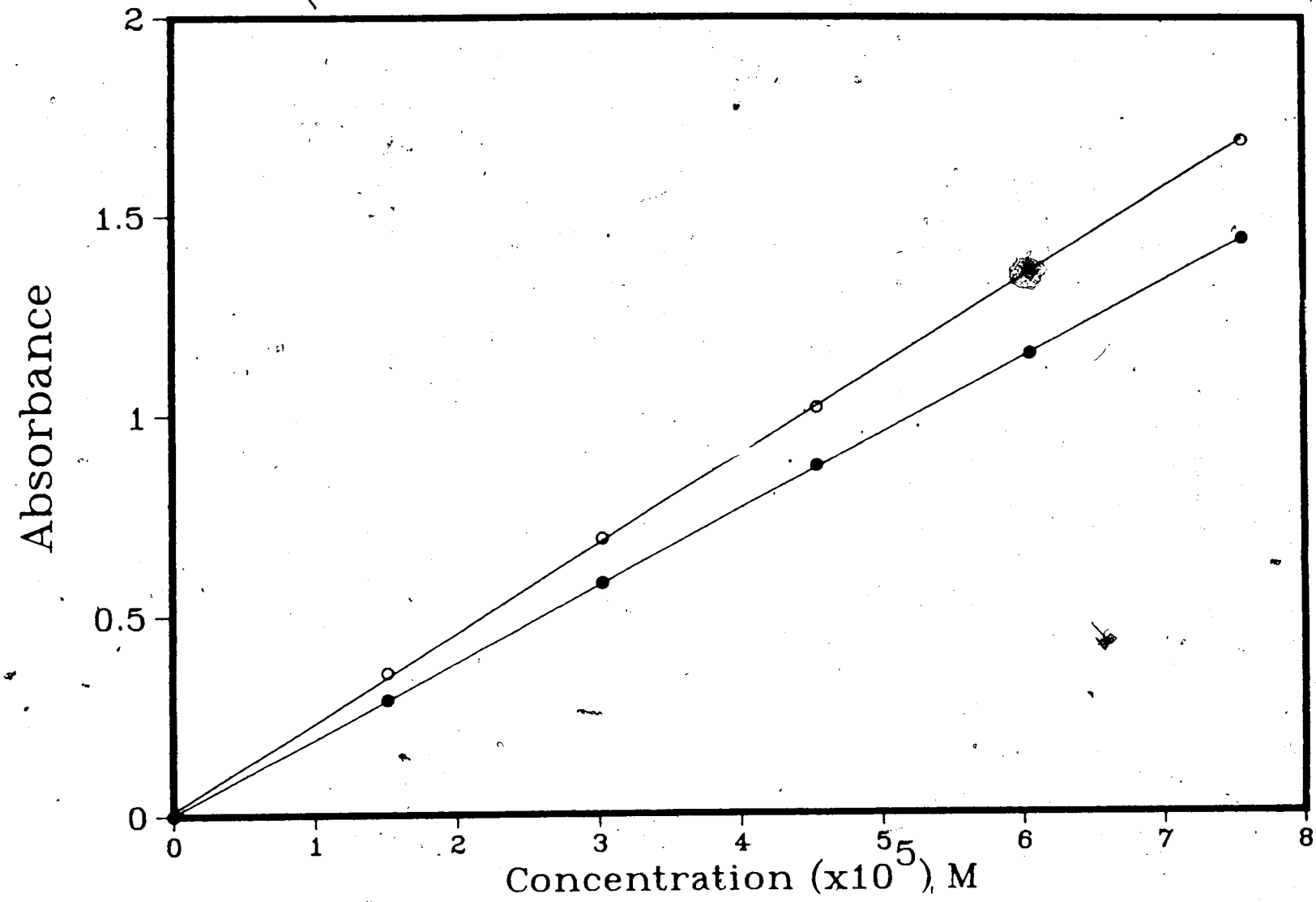


Fig. 19. Beers Plot of Procaine (-o-) (λ=293 nm) and Butyl p-Aminobenzoate (-•-) (λ=294 nm) in the Solution for Analysis (see text p-51).

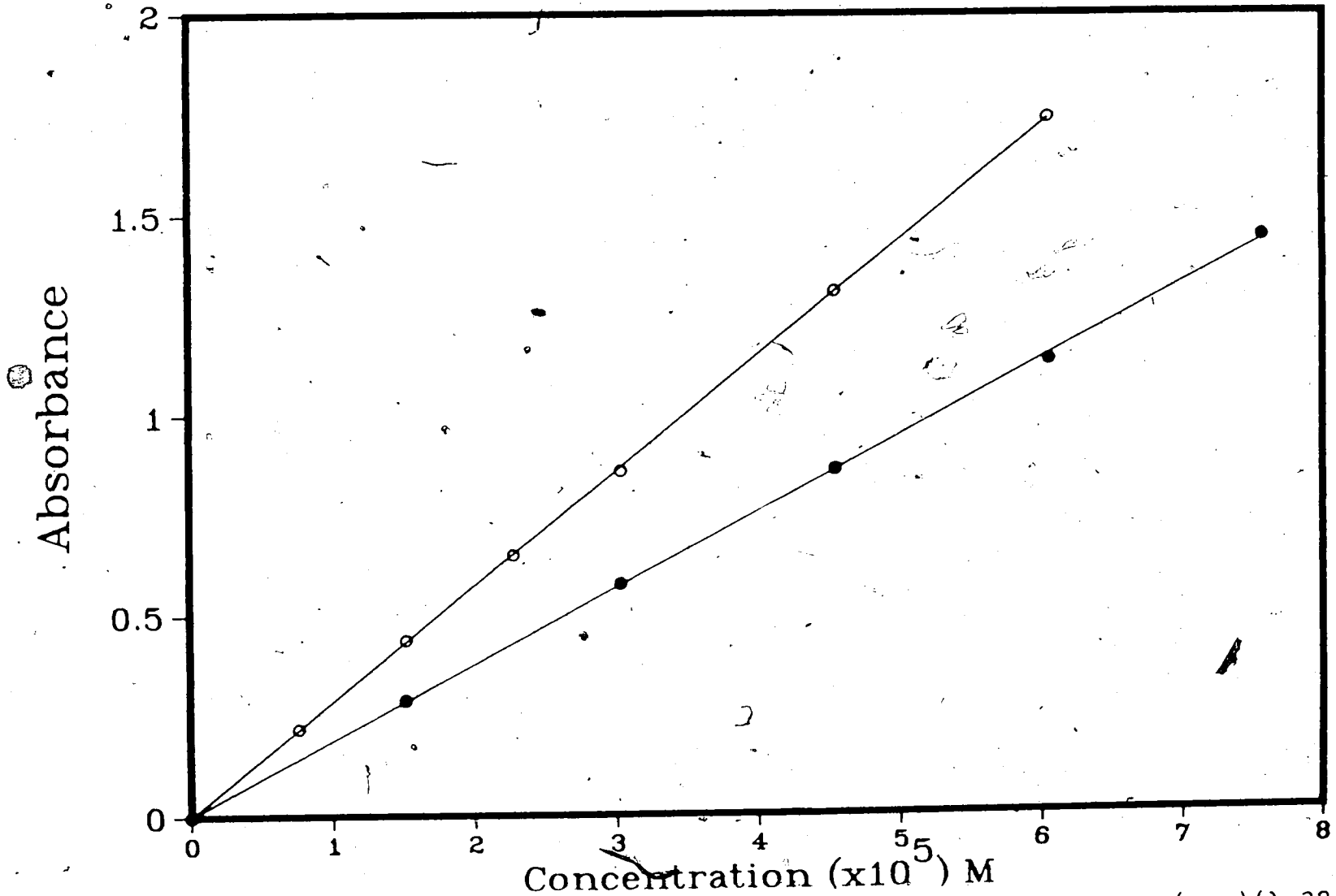


Fig. 20 Beers Plot of Tetracaine (-○-)(λ=309 nm) and Methyl p-Aminobenzoate (-•-)(λ=294 nm) in the Solution for Analysis (see text p-51).

TABLE 15

Regression Analysis of the Calibration Curves of Six Local Anesthetic Drugs

Drug	Slope	Intercept	Correlation Coefficient, r
Benzocaine	21119.9	0.0051	0.9999
Procaine	22230.9	0.0114	0.9999
Tetracaine	28694.2	0.0002	0.9999
Methyl PAB[a]	18880.9	0.0012	0.9999
Propyl PAB[b]	19075.3	0.0010	0.9999
Butyl PAB[c]	19025.1	0.0027	0.9999

[DMPC] = 14.4 mM.
Standard deviations (n=3) shown in brackets.
[a] Methyl p-amino benzoate
[b] Propyl p-amino benzoate
[c] Butyl p-amino benzoate

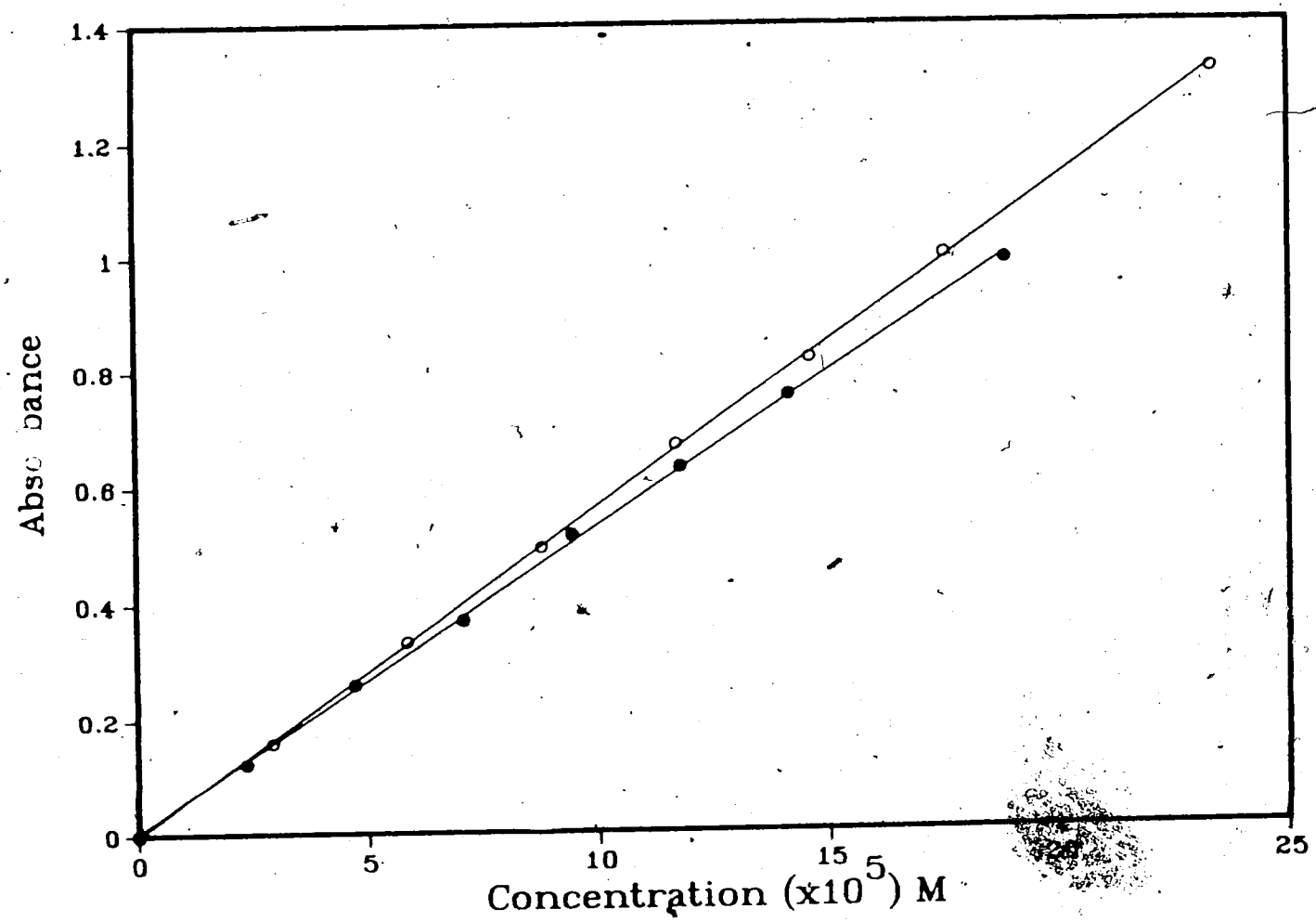


Fig. 21. Beers Plot of p-Aminobenzoic Acid (-o-)(λ=291 nm) and Butyl p-Aminobenzoic Acid (-•-)(λ=309 nm) in the Solution for Analysis (see text p-51).

4.2.2 Apparent First-Order Hydrolysis

The hydrolysis of all the local anesthetic drugs in aqueous buffer solutions or in liposomal preparations were found to follow apparent first-order hydrolysis for at least three half-lives as shown in Figures 22, 23 and 24 and is given by equation 22 :

$$C/C_o = e^{-kt} \qquad (22)$$

where C_o and C are the initial molar concentrations and at time t, respectively, and k is the pseudo first-order hydrolysis rate constant. For hydrolysis in aqueous buffer solution $k=k_B$ and in liposome systems $k=k_{obs}$. Hydrolysis in the lipid phase (k_L) and in the external aqueous phase of liposomes (k_b) was taken to obey Eq.22 and that $k_b=k_B$ (40). Thus,

$$k_{obs} = k_L f_L + k_B f_B \qquad (23)$$

where f_L and f_B are the fraction of drug in the lipid and aqueous phases, respectively. Linear least squares regression analysis was used to calculate all the rate constants and reproducibility was within 1 percent.

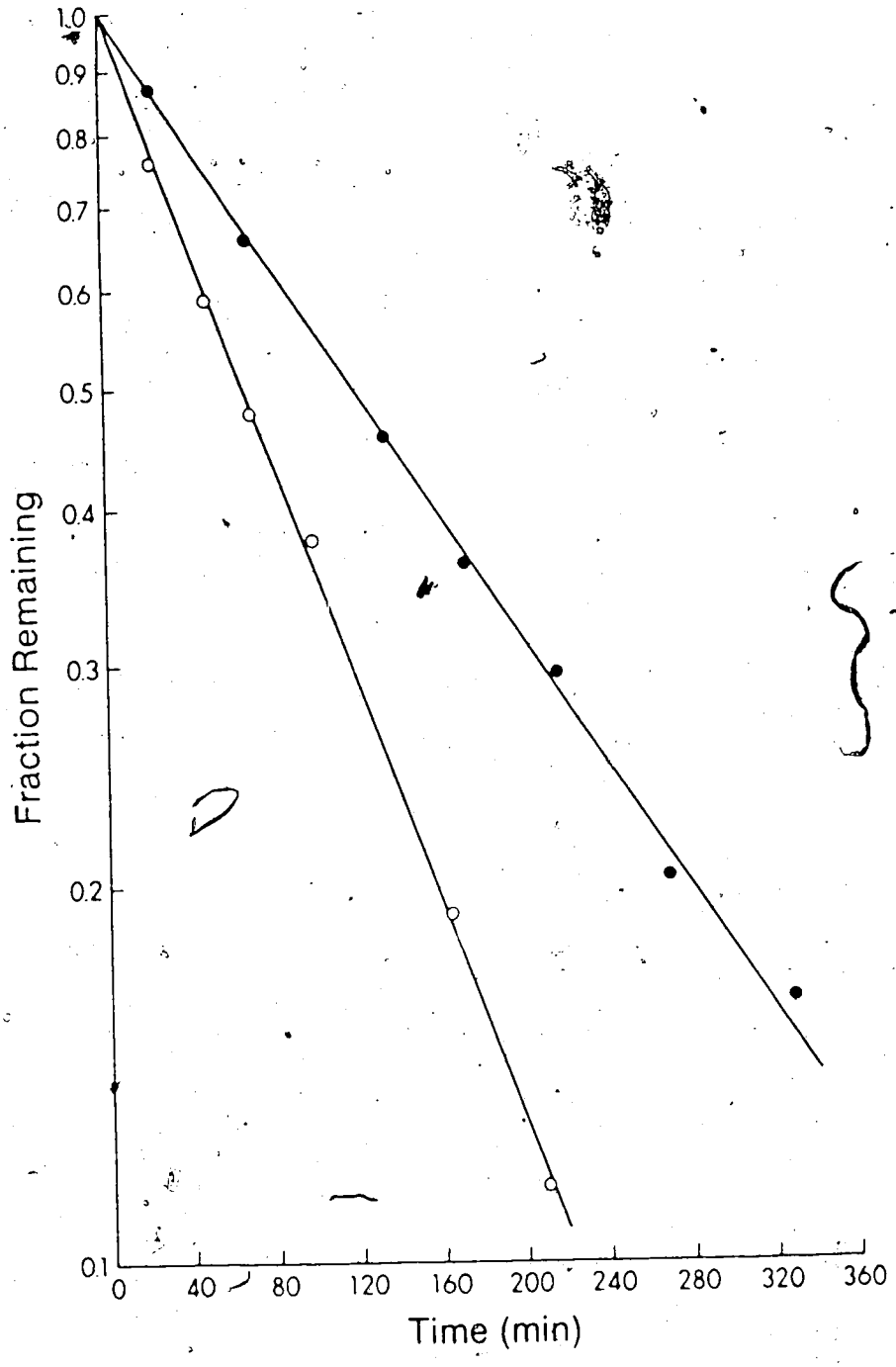


Fig. 22. First-Order Hydrolysis Kinetics of Benzocaine in Aqueous Buffer Solution, (-o-); and in Liposomes, (-•-); at pH 12.2 and 30°C. The Initial Benzocaine Concentration was 0.76 mM and the Phospholipid (DMPC) Concentration was 5.8 mM.

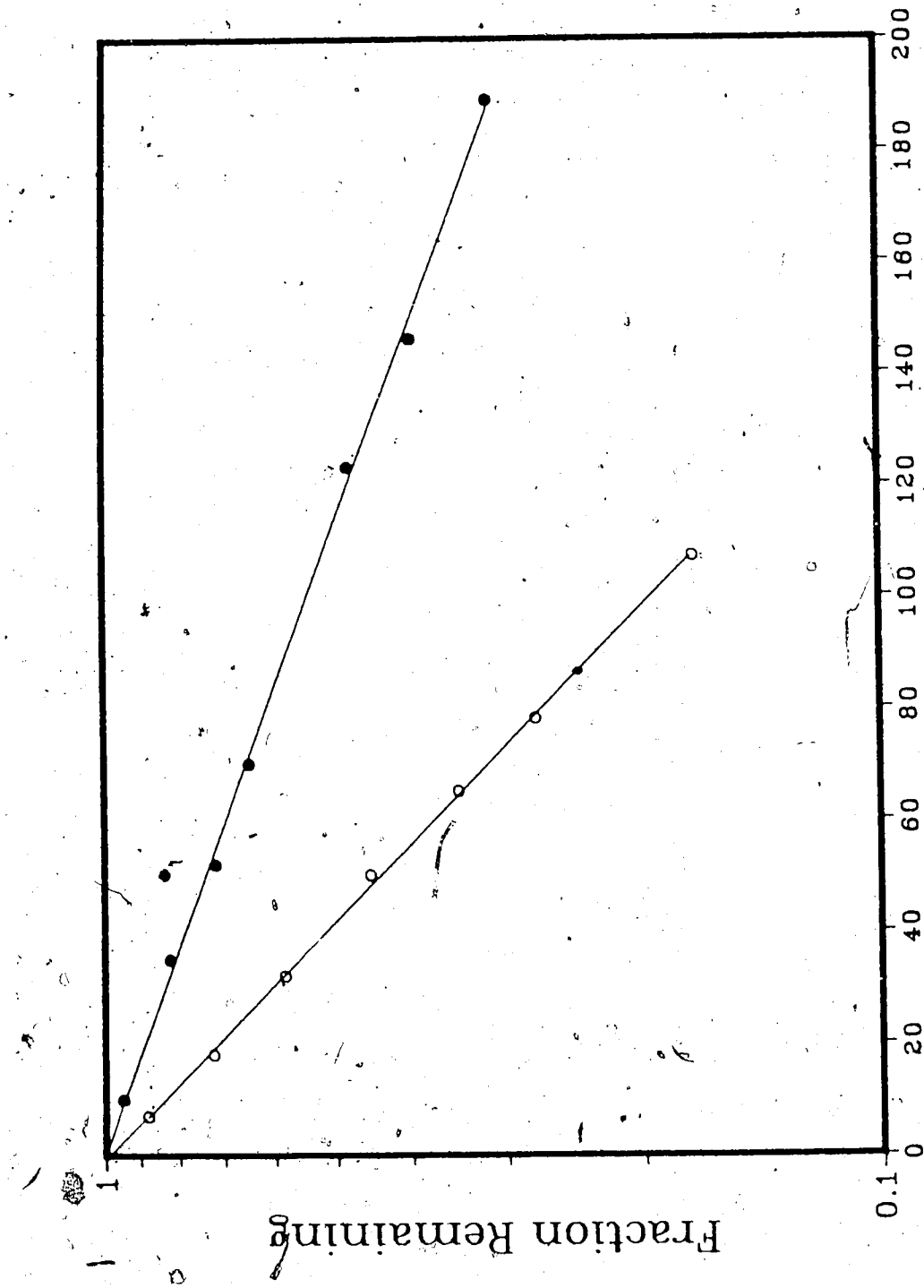


Fig. 23. First-Order Hydrolysis Kinetics of Procaine in Aqueous Buffer Solution, (-o-) and in Liposomes, (-•-) at pH 12.2 and 30°C. The initial Procaine Concentration was 0.76 mM and the Phospholipid (DMPC) Concentration was 5.8 mM.

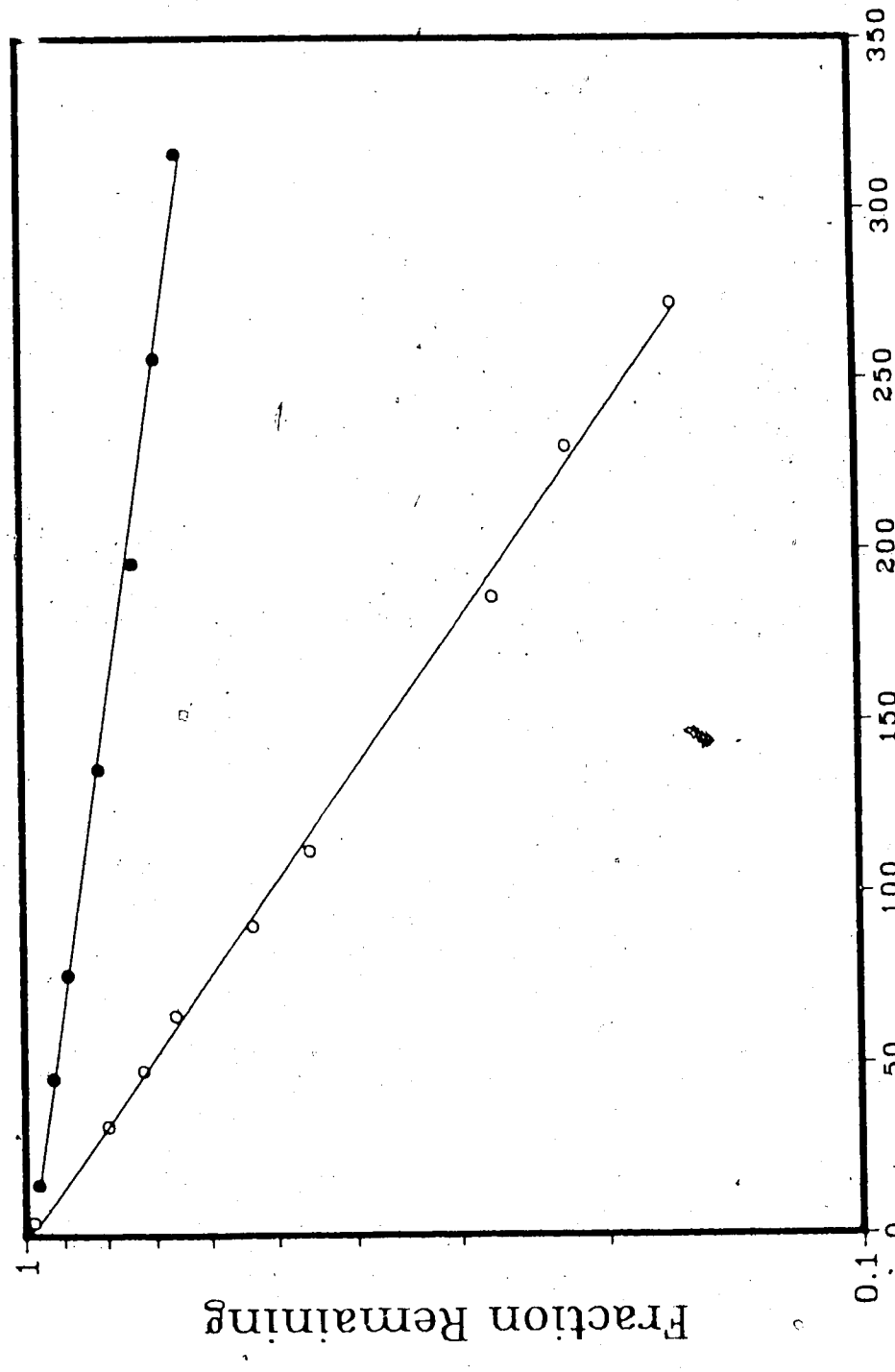


Fig. 24. First-Order Hydrolysis Kinetics of Tetracaine in Aqueous Buffer Solution, (-o-) and in Liposomes, (-•-) at pH 12.2 and 30°C. The Initial Tetracaine Concentration was 0.76mM and the Phospholipid (DMPC) Concentration was 5.8 mM.

4.2.3 Comparative Stabilities of Local Anesthetics in Liposomes

4.2.3.1 Effect of Liposome Composition

Table 16 shows the stabilization of benzocaine in liposomes of various compositions at pH 12.2 and 30°C. It indicates a significant variation in the stabilization of benzocaine (2-4 fold change) by selecting different phospholipids or phospholipid:cholesterol combinations. Neutral liposomes of phosphatidyl cholines, which exist in a fluid liquid crystalline state (e.g.EPC,DMPC) at the temperature of the experiment (30°C), protects benzocaine against hydrolysis to the extent of 35-40%. In contrast, DPPC is in a more rigid gel state at this temperature and the stability was increased by only 23%. SPHING liposomes, although in a fluid state, allow benzocaine to be stabilized only to the extent of 22%. On the other hand, negatively-charged liposomes of PS yielded the greatest protection against hydrolysis under the present conditions. The addition of CHOL to DMPC liposomes causes k_{obs} to increase and at a 1:1 mole ratio only a 12% increase in the stability of benzocaine was obtained. Thus, the addition of 50% CHOL to the liposome composition results in about a 3-fold decrease in the effectiveness of the phospholipid (DMPC) liposome to stabilize benzocaine.

TABLE 16

The Stabilization of Benzocaine in Liposomes of Various Compositions at pH 12.2 and 30°C.[a]

Liposome Composition[b]	% Increase in Stability[c]	T_c[d] (°C)
PS	47	6-8
EPC	39	0
DMPC	34	23
DPPC	23	41
SPHING	22	25-40
DMPC:CHOL (3:1)	25	-
DMPC:CHOL (1:1)	12	-

[a] the initial benzocaine concentration was 0.76 mM; the total lipid concentration was 2.9 mM.

[b] mole ratios are shown in brackets.

[c] using Eq. 10.

[d] Ref. 45

4.2.3.2 Effect of Phospholipid Concentration

The variation of k_{obs} of benzocaine, procaine and tetracaine in DMPC or DPPC liposomes as a function of the phospholipid concentration over the range 1.4-28.7 mM is illustrated in Figure 25. Each curve is characterized by an initial rapid decrease in k_{obs} followed by a more gradual lowering of k_{obs} as the phospholipid was further increased. The steepness of the slopes in the initial stages corresponding to DMPC liposomes is in the order benzocaine < procaine < tetracaine which is also the same order as their partition coefficients whereas the latter segments of the curves are approximately parallel. In contrast, the curve corresponding to benzocaine in DPPC liposomes exhibited a more gradual change indicating that k_{obs} is much less influenced by the amount of lipid when the liposomes exist in the gel state. In Table 17 a relationship has been shown between f_L and the relative stabilization of the local anesthetics in liposomes as a function of phospholipid concentration. It is significant to observe that in the fluid DMPC liposomes the relative increase in the stabilization of benzocaine or procaine parallels the increase in f_L whereas approximately a 4-fold increase in the stabilization of tetracaine was obtained by a 40% increase in the f_L at the higher DMPC concentration.

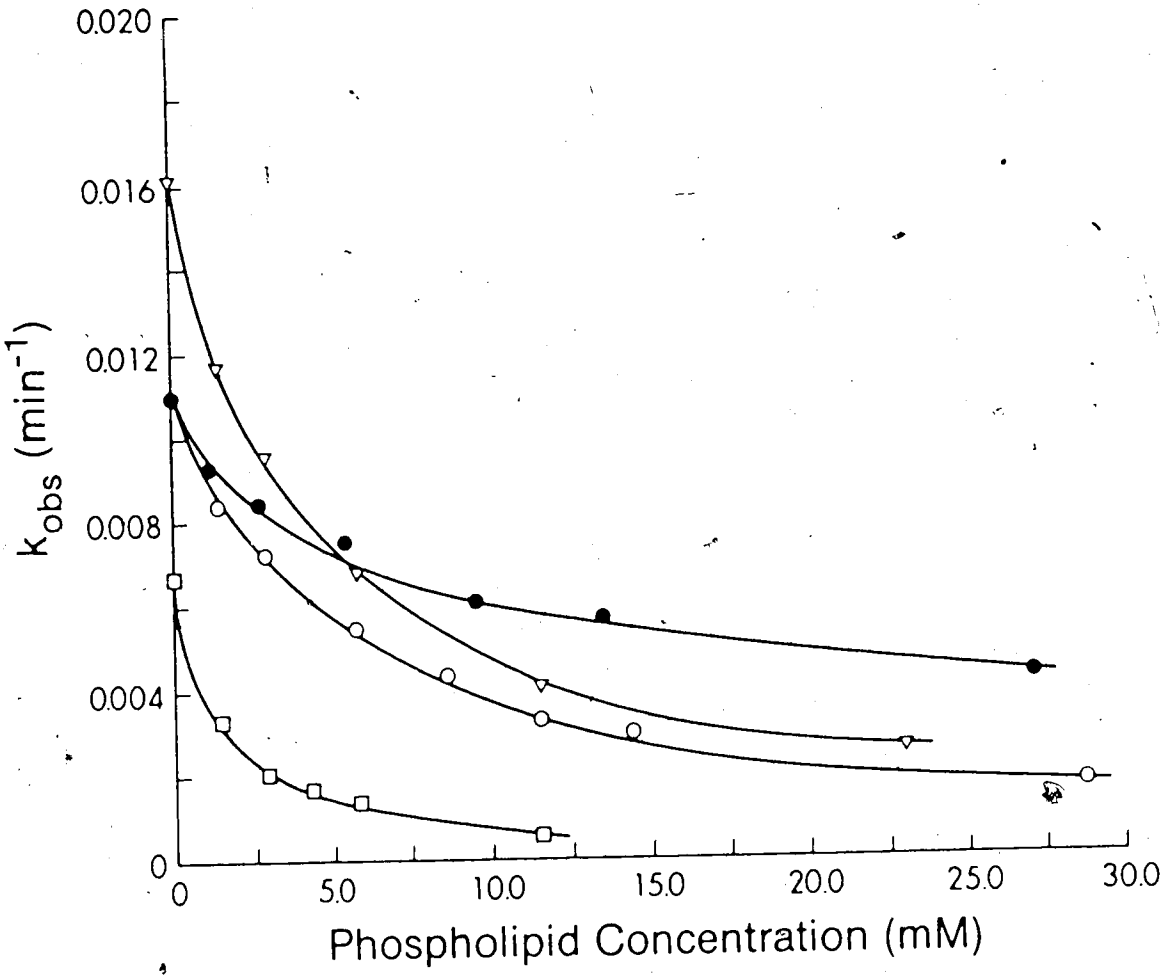


Fig. 25. Effect of Phospholipid Concentration on the Hydrolysis Rate Constant(k_{obs}) of Local Anesthetics in Liposomes at pH 12.2 and 30°C. The Initial Drug Concentration was 0.76mM. Benzocaine (DMPC), ○; (DPPC),●; Procaine (DMPC),▽; Tetracaine (DMPC),□.

TABLE 17

Relationship Between the Initial Fraction in the Lipid Phase (f_L) and the Relative Stability (k_{obs}/k_B) of Local Anesthetics in Liposomes as a Function of the Phospholipid (DMPC) Concentration.[a]

Local Anesthetic[b]	DMPC Conc. (mM)	f_L	k_{obs}/k_B[c]
Benzocaine	2.9	0.28	0.66
	11.5	0.63	0.31
Procaine	2.9	0.26	.60
	11.5	0.64	0.25
Tetracaine	2.9	0.63	0.31
	11.5	0.88	0.08

[a] experiments were conducted at pH 12.2 and 30°C.

[b] initial concentrations were 0.76 mM.

[c] values of k_B were as follows: benzocaine - $1.1 \times 10^{-2} min^{-1}$; procaine - $1.6 \times 10^{-2} min^{-1}$; tetracaine - $6.7 \times 10^{-3} min^{-1}$

4.2.3.3 Effect of pH

Incorporation of local anesthetic drugs into liposomes for the purpose of stabilization appears also to be pH dependent. Thus, the procaine stability data in Table 18 indicates that only undissociated procaine is able to accumulate in the lipid phase when introduced via the aqueous phase because at pH 7.4, at which procaine was 96% ionized (pKa=8.8), only 4% increase in stability was obtained. However, most of the increase in the stability of procaine at pH 12.2 was recoverable at pH 7.4 when the drug was incorporated in liposomes via the organic phase. At pH 12.2 the stability remained constant whether the drug was incorporated via the aqueous phase or via the organic phase.

4.2.3.4 Effect of Benzocaine Concentration and the Ionic Strength of the Medium

Studies were conducted to determine whether variation of the content of benzocaine in liposomes or the osmolarity of the aqueous medium produced any significant changes in the kinetics of degradation. The results in Figure 26 show a parallel decrease in k_{obs} with increasing benzocaine concentration in liposomes of DMRC or DPPC. However, a 4-fold increase in benzocaine concentration resulted in only a 15% decrease in k_{obs}. Similarly, ionic strengths of the aqueous medium ranging from 0.05 to 0.25 had little effect on k_{obs} (Table 19)

TABLE 18

Effect of the Method of Incorporation of Procaine in Liposomes on its Stability at 30°C.[a]

Incorporation of Procaine	% Increase in Stability	
	pH 12.2	pH 7.4
via aqueous phase	41.0	4.0
via organic phase	43.0	30.0

[a]DMPC concentration = 2.9 mM; procaine conc. =0.76 mM.

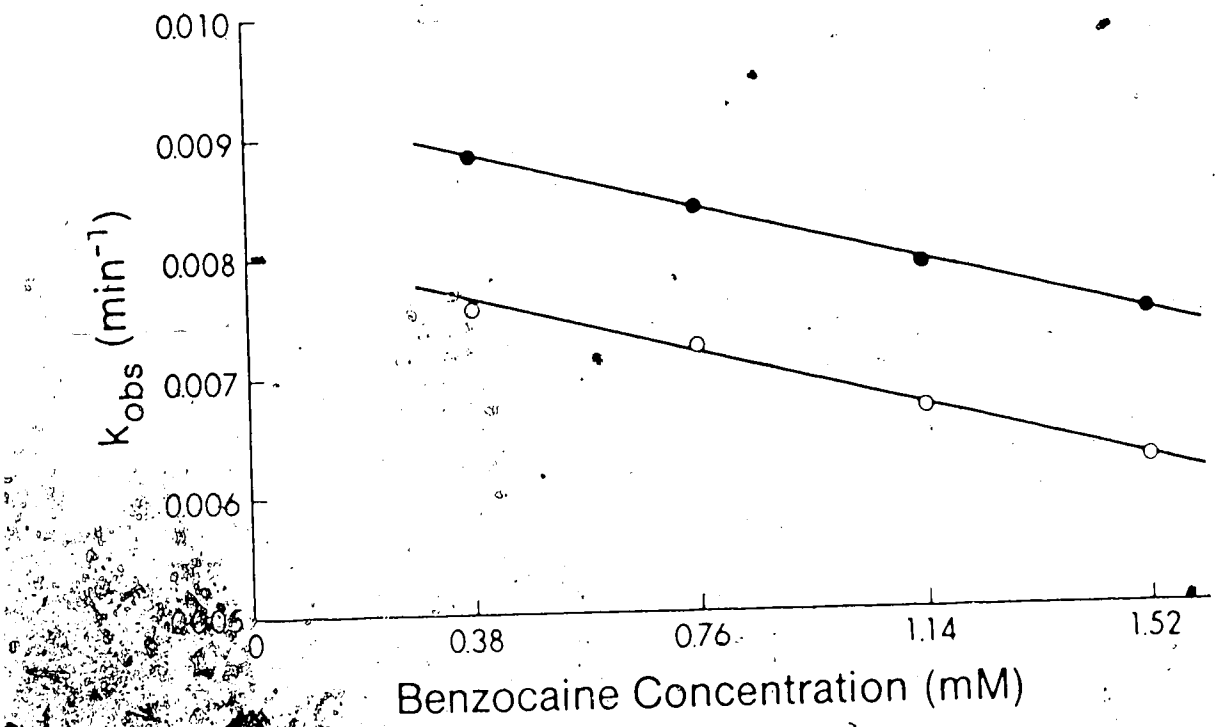


Fig. [illegible] Effect of Benzocaine Concentration on the Hydrolysis Rate Constant (k_{obs}) in Liposomes at pH 12.2 and 30°C. DMPC, ○; DPPC, ●.

TABLE 19

Hydrolysis Rate Constants of Benzocaine in DMPC Liposomes (k_{obs}) as a Function of the Ionic Strength of the Medium at pH 12.2 and 30°C.[a]

Ionic Strength	$10^3 k_{obs}$ (SD) (min^{-1})
0.046	7.3 (0.20)
0.060	7.2 (0.19)
0.100	6.9 (0.13)
0.154 (isotonic)	6.8 (0.09)
0.250	6.8 (0.11)

[a] the initial benzocaine concentration was 0.76 mM; the DMPC concentration was 2.9 mM.

even though the liposomes exist in various swelling states under these conditions (129).

4.2.4 Partition Coefficients of Local Anesthetics in the n-Octanol-Buffer and the Liposome-Buffer System

Partition coefficients of local anesthetics in the n-octanol-buffer (K_W^O) and in liposome-buffer (K_W^L) systems are given in Table 20. It can be seen that the partition coefficient values increase appreciably with an increase in the hydrophobicity or decrease in the water solubility of these compounds. Partition coefficients in the liposome-buffer system were considerably greater than those in the n-octanol-buffer system. A correlation between log K_W^L and log K_W^O is shown in Figure 27. Correlations of this type have also been found with other solutes (170,172,173). In Figure 28 a linear relationship between K_W^L and percent increase in stability is shown with r=0.996 and strongly supports the argument that the stability of drugs in liposomes is highly dependent on the partition coefficients of solutes. It even suggests that the percent increase in stability of a drug in liposomes can be predicted from its K_W^L. A test of this was demonstrated with indomethacin where its predicted stabilization in liposomes from its K_W^L determined experimentally strongly agreed with its reported stabilization in liposomes (40). The standard curve of indomethacin is shown in Figure 29.

TABLE 20

Partition Coefficient of Local Anesthetics in Octanol-Water (k_W^O) and DMPC Liposome-Water (K_W^L) System

Drug	$\log k_W^L$	$\log k_W^O$
MPAB[a]	1.844	1.365
Benzocaine	2.228	1.933
Procaine	2.255	1.869
PPAB[b]	2.823	2.313
Tetracaine	2.941	2.585
BPAB[c]	3.290	2.981

[a]MPAB - Methyl p-amino benzoate

[b]PPAB - Propyl p-amino benzoate

[c]BPAB - Butyl p-amino benzoate

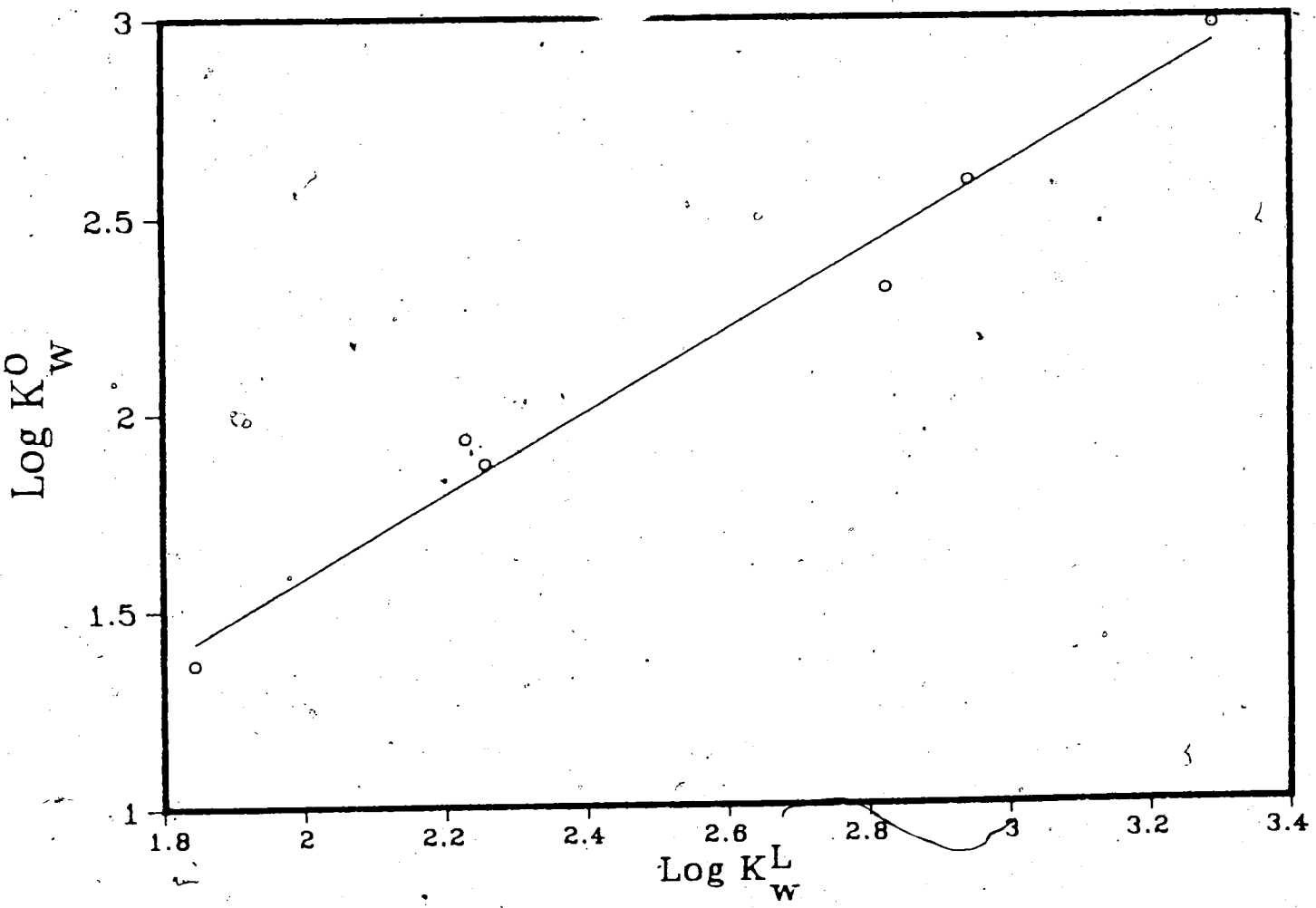


Fig. 27. Correlation of the Partition Coefficients of Local Anesthetics in n-Octanol-Buffer(K_w) and Liposome-Buffer (K_w) system at 30°C. (r=.99)

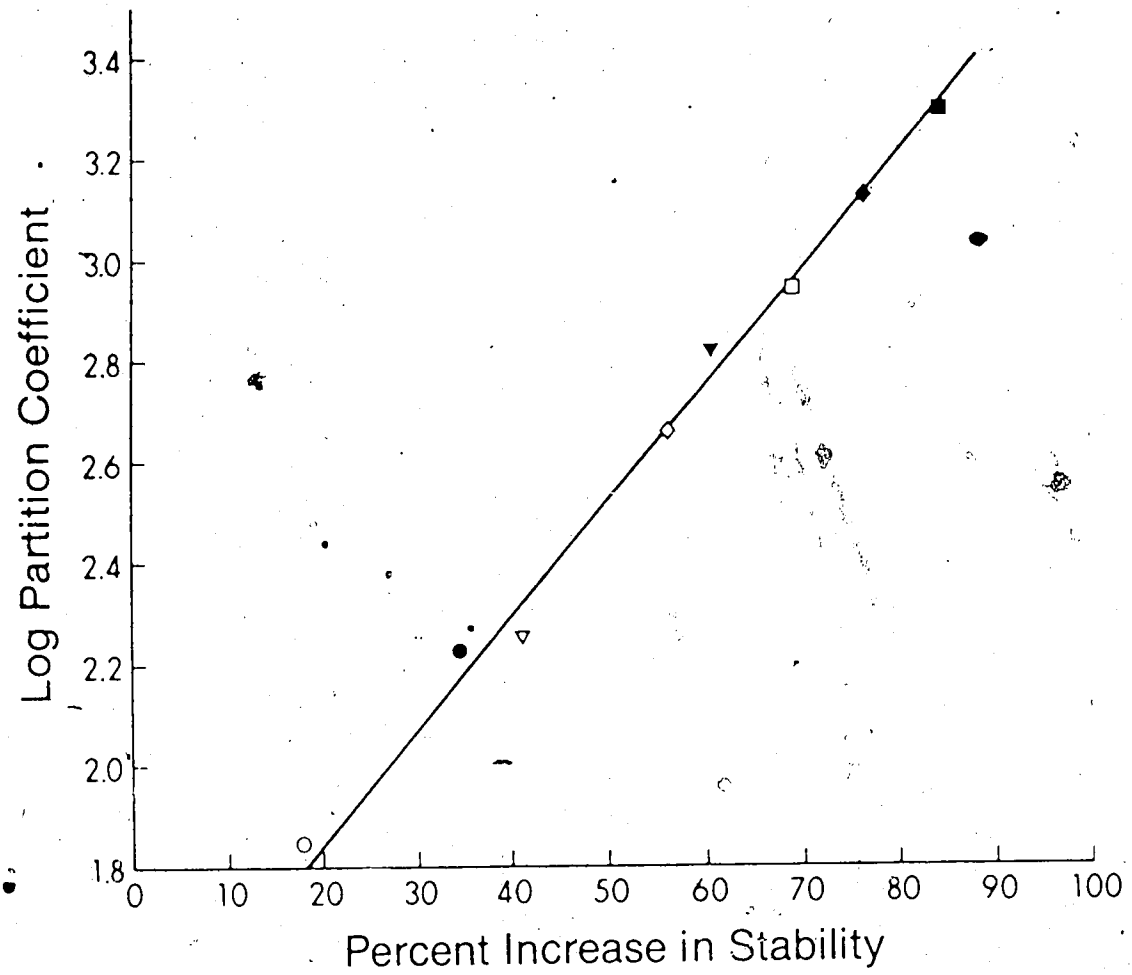


Fig. 28. Correlation of the Partition Coefficient (K_w^L and the Observed Percent Increase in Stability of Various Local Anesthetics and Other Solutes in DMPC Liposomes at pH 12.2 and 30°C. The Curve was Determined Using Linear Regression Analysis (r=0.996). Methyl p-Aminobenzoate, ○; Benzocaine, ●; Procaine, ▽; n-Propyl p-Aminobenzoate, ▼; Tetracaine, □; n-Butyl p-Aminobenzoate, ■; 2-Diethylaminoethyl p-Nitrobenzoate (ref.38), ◇; Indomethacin(ref.40), ◆.

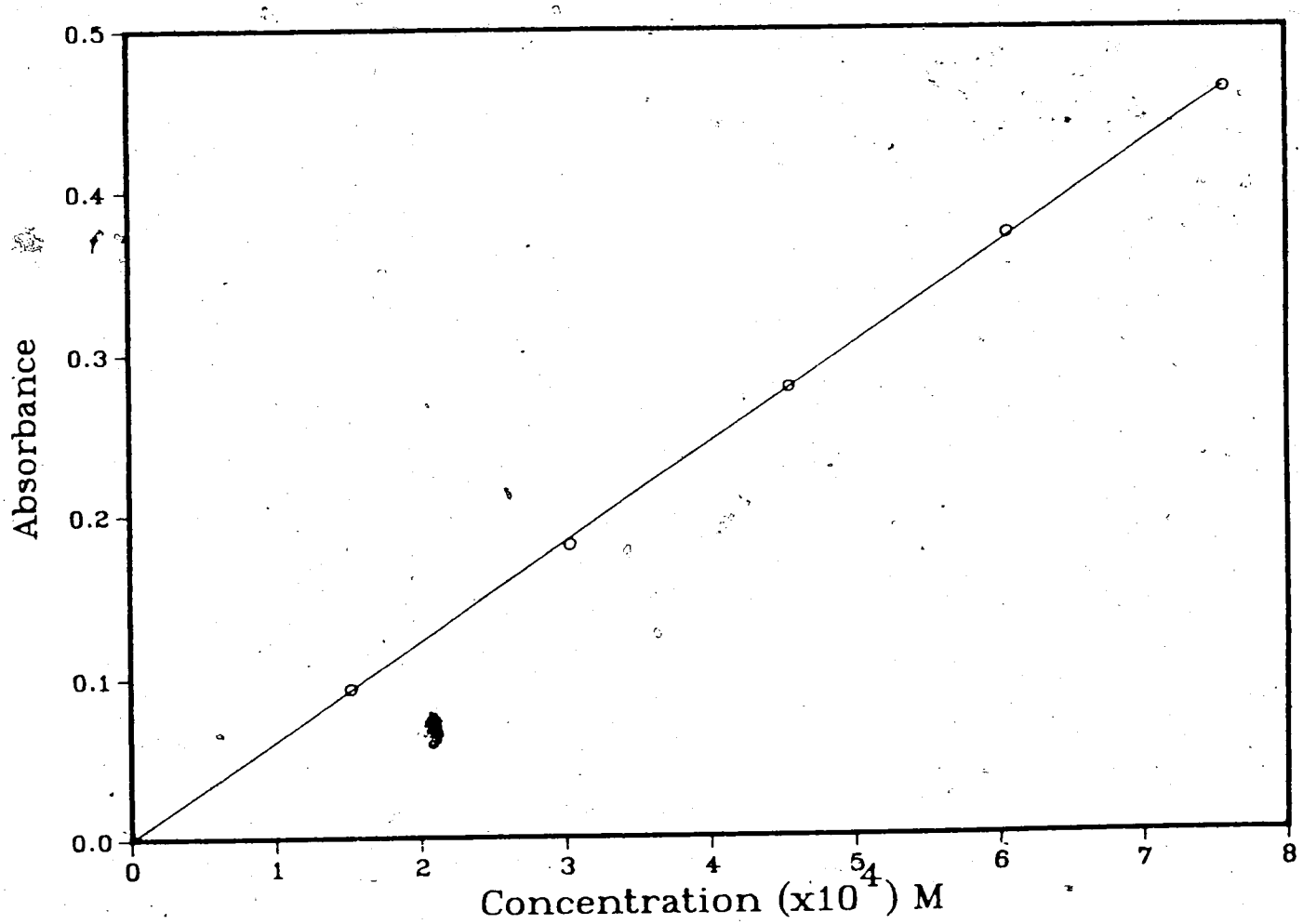


Fig. 29. Beers Plot of Indomethacin in KH_2PO_4-Na_2HPO_4 Buffer Solution at pH 6.0 (λ_{max}= 330 nm).

Similar results of K_W^L were obtained by incorporating the local anesthetics initially in the lipid film or in the aqueous phase during liposome preparation.

4.3 CEFOTAXIME-SODIUM

4.3.1 High Pressure Liquid Chromatographic Analysis

The ratios of the peak areas of CFX to internal standard for the two CFX concentrations using the two-point calibration curve method are shown in Table 21. The coefficient of variation of the peak area ratios were 0.20% and 0.10% respectively. These ratios were converted to a response factor by the integrator and later used in CFX determination. Further verification of the calibration curve was made in Table 22. Regression analysis of peak area versus CFX concentration (triplicates) over the range 0.25-3μg/20μl yielded r=0.9999 which constitutes a calibration curve obeying beer's law. Typical chromatograms resulting from analyses of control and liposomal CFX are shown in Figures 30 and 31. The retention time of cefotaxime was found to be 3.5 minutes and that of internal standard 4.6 minutes. In Figures 32 and 33, typical chromatograms are shown after approximately 21 hr and 24 hr of incubation of CFX after some of the drug has been degraded. A peak at 5.4 min in these chromatograms corresponds to degradation products of CFX. Although

TABLE 21

Precision of a Two-Point Calibration Curve for HPLC Determination of CFX in Liposome Samples.

Injection #	Peak Area Ratios 1st point[a]	2nd point[b]
1	0.4840	0.9757
2	0.4861	0.9740
3	0.4860	0.9736
Mean ±SD	0.4854±9.6x10^{-4}	0.9744±9.1x10^{-4}

[a] 1 µg/20 µl CFX, 6 µg/20µl ORM

[b] 2 µg/20µl CFX, 6 µg/20µl ORM

TABLE 22

Verification of the Two-Point Calibration Curve for HPLC Determination of CFX in Liposome Samples.

Theoretical Cefotaxime Concentration (μg/20μl)	Observed Cefotaxime Concentration (μg/20μl)	Mean ±SD Observed Concentration (μg/20μl)	Percent Deviation
3.00	2.956		
3.00	2.971	2.9797	-0.68
3.00	3.012	±.024	
2.00	2.009		
2.00	1.995	1.992	-0.40
2.00	1.972	±.003	
1.00	1.005		
1.00	1.002	1.001	+0.1
1.00	0.997	±.003	
0.50	0.5005		
0.50	0.4992	0.4985	
0.50	0.4959	±.002	-0.3
0.25	0.2515		
0.25	0.2486	.2507	+0.28
0.25	0.2521	±.002	

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Fig. 30. HPLC Chromatogram Obtained Following the Injection of a Solution of CFX at pH 1.0. Peak 1- CFX; Peak 2- Internal Standard. 20 µl were Injected on a 5 µ, C_{18} Novapak Column. The Flow Rate of the Mobile Phase (10% Acetonitrile in Acetate Buffer, pH 5.0) was 1.5 ml/min.

Fig. 31. HPLC Chromatogram Obtained Following the Injection of a 40% Methanol-in-Water Solution of a Liposomal Suspension of CFX at pH 1.0. Peak 1- CFX; Peak 2- Internal Standard. Other Conditions are as Described in Fig. 30.

Fig. 32. HPLC Chromatogram of CFX After Storing for 21 Hours at 30°C. Other Conditions are as Described in Fig.30. Peak 1- CFX; Peak 2- Internal Standard; Peak 3- Degradation Products of CFX.

Fig. 33. HPLC Chromatogram of CFX After Storing Liposomes for 24 Hours at 30°C. Other Conditions are as Described in Fig.30. Peak 1- CFX; Peak 2- Internal Standard; Peak 3- Degradation Products of CFX.

satisfactory separation of the degradation product (peak3) from the internal standard (peak2) was obtained the exact composition of peak3 was not determined. Furthermore, when aged samples were analyzed under conditions of varying the mobile phase (6,7,8, and 9% acetonitrile in acetate buffer), no additional peaks appeared in the chromatograms (not shown).

4.3.2 The Stability of Cefotaxime Sodium in Buffer Solution and in Liposomes

The hydrolysis of CFX in control buffer solution or in liposomal suspension followed pseudo-first-order kinetics at either acidic or alkaline pH. Figures 34 and 35 show typical first order plots for CFX at pH 1.0 and pH 9.0 respectively. Comparison of the rates of hydrolysis of CFX in the presence or absence of liposomes can be made from the results in Table 23. No significant difference was observed in k_{obs} and k_B and f_L was also negligible. it is apparent that the ratio k_{obs}/k_B would be near 1(see Table 23).

At pH 1.0, f_L could not be determined as solubilization of the liposomes occurred. Table 24 shows the results of the hydrolysis of CFX in DMPC:CHOL liposomes. Although solubilization of liposomes did not occur with these compositions, the stability of CFX was still not improved.

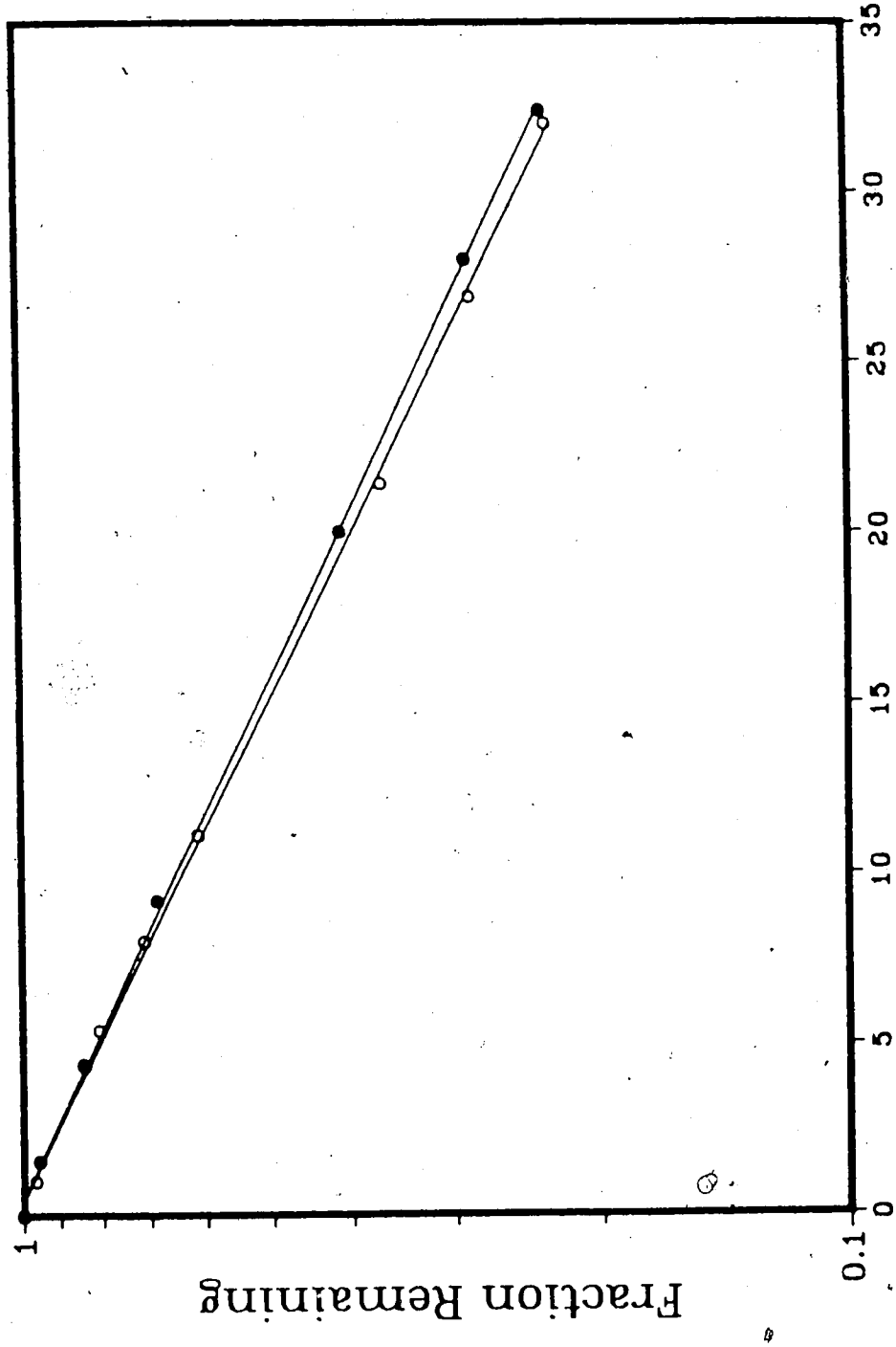


Fig. 34. First-Order Hydrolysis Kinetics of CFX at pH 1.0 and 30°C in: (-o-), HCl-KCl Buffer Solution and (-•-), DMPC Liposomes. DMPC Concentration was 14.4 mM; Initial CFX Concentration was 7.5 mM.

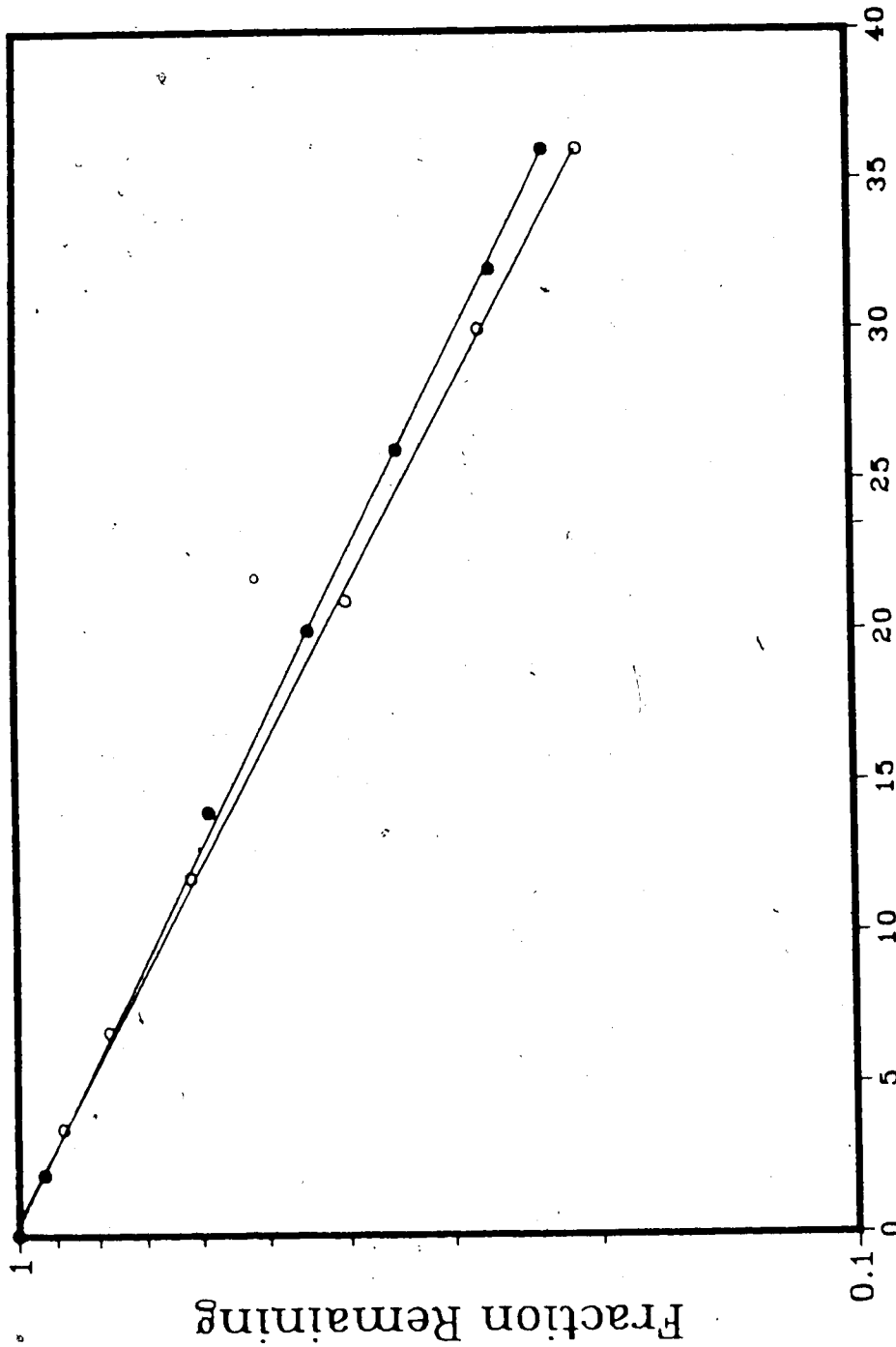


Fig. 35. First-Order Hydrolysis Kinetics of CFX at pH 9.0 and 30°C in: (-o-), Boric Acid-Sodium Borate Buffer Solution and (-•-), DMPC Liposomes. DMPC Concentration was 14.4 mM; Initial CFX Concentration was 7.5 mM.

TABLE 23

Comparison of the First Order Hydrolysis Rate Constants of CFX in DMPC Liposomes (k_{obs}), and Aqueous Buffer (k_B) and the Fraction of CFX Associated with the Lipid Phase (f_L).

pH	k_{obs} (hr^{-1}) ($x10^2$)	k_B (hr^{-1}) ($x10^2$)	f_L	k_{obs}/k_B
1.0	4.58 ($\pm5.4x10^{-4}$)	4.80 ($\pm4x10^{-4}$)	-	0.95
9.0	4.19 ($\pm1.7x10^{-4}$)	4.55 ($\pm3.8x10^{-4}$)	0.07	0.92

[DMPC] = 14.4 mM

[CFX] = 7.5 mM; 30°C

Standard deviations (n=3) shown in brackets.

TABLE 24

Apparent First-Order Hydrolysis of CFX in DMPC: CHOL Liposomes at pH 1.0, 30°C.

Mole Ratio of DMPC:CHOL	k_{obs}[a] (hr^{-1}) ($\times 10^{2}$)	k_{obs}/k_B
:1	4.77 ($\pm 2\times 10^{-4}$)	0.99
5:1	4.53 ($\pm 1\times 10^{-3}$)	0.94
10:1	4.42 ($\pm 9\times 10^{-4}$)	0.92
20:1	4.52 ($\pm 1\times 10^{-3}$)	0.94

[a] standard deviations in brackets (n=3).

4.3.3 Solubilization of Liposomes

Liposomes of synthetic phospholipid (DMPC) were found to undergo solubilization in the presence of CFX at pHs at which the drug was completely unionized. The rate of solubilization as determined from measurements of the turbidity of the liposomes at λ=520nm is shown graphically in Figure 36. A strong pH dependence is clearly indicated. At pH 1.0 and 1.5 (CFX is 93% and 80% unionized, respectively), the rate of solubilization was rapid but at pH 2.0 solubilization was retarded considerably (CFX is only 56% unionized). Hence, a slight change in pH exerted a profound effect on the solubilization of liposomes. Solubilization of liposome was not observed at any higher pHs. Figure 37 describes the dependency on pH of the time to attain certain levels of turbidity (i.e., degrees of solubilization). This dependency is more acute between pH 1.5-2.0 than between 1.0-1.5 (pK_a=2.1). Figure 38 shows the effect of temperature on the solubilization of liposomes by CFX at pH 1.0 and indicates that solubilization was much slower at $<T_c$ and sensitive to temperature than $>T_c$ (DMPC=23°) which was insensitive to the temperature.

After complete solubilization of liposomes at pH 1.0, it was observed that the clear solution gradually became turbid again. This is illustrated in Figures 36 and 38 by a gradual increase in turbidity after approximtely 20 hours. Figure 39 shows photomicrographs of control liposomes and

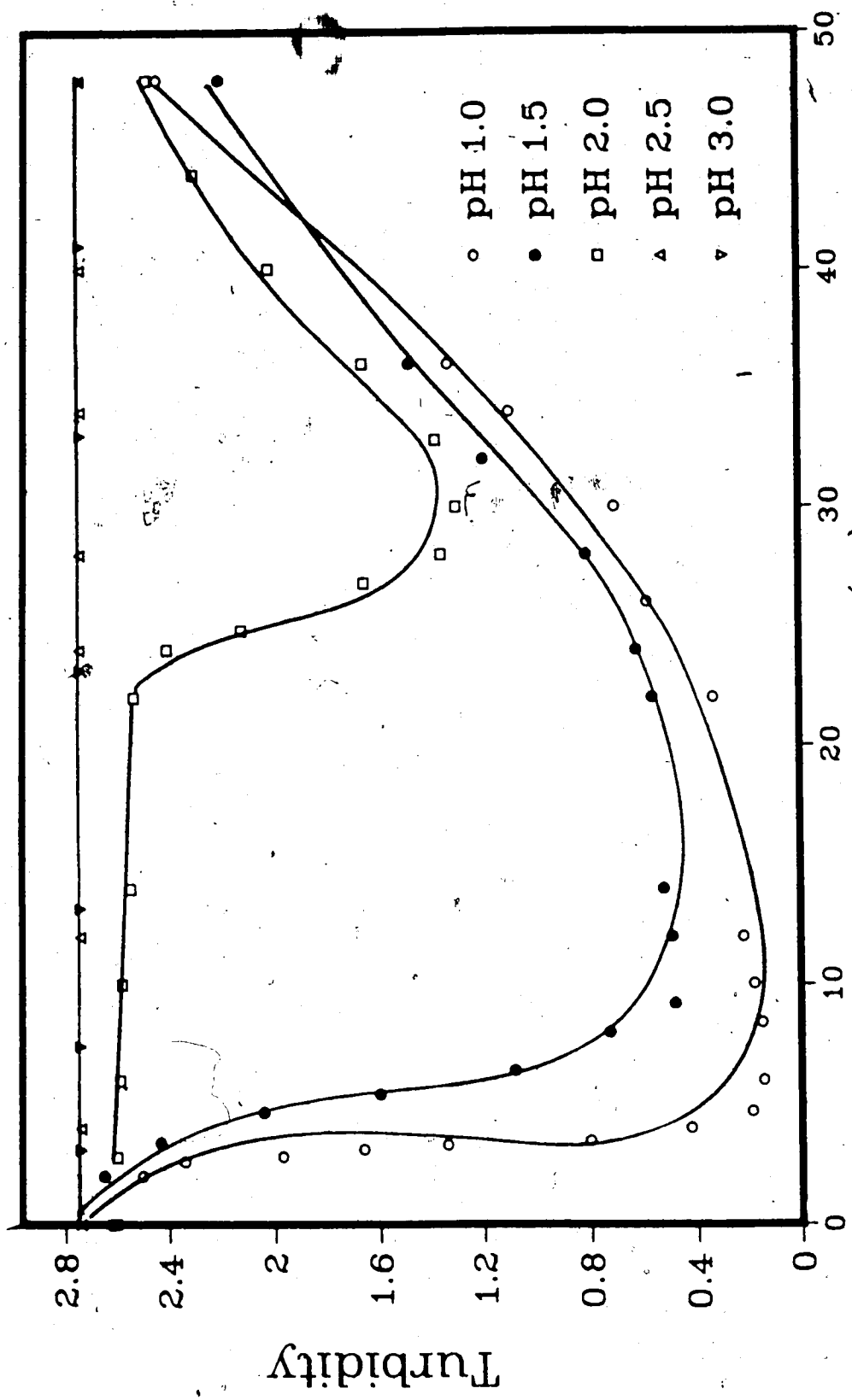


Fig. 36. Effect of pH on the Solubilization of Liposomes by CFX at 40°C. Phospholipid(DMPC) Concentration= 14.4 mM; CFX Concentration= 7.5mM.

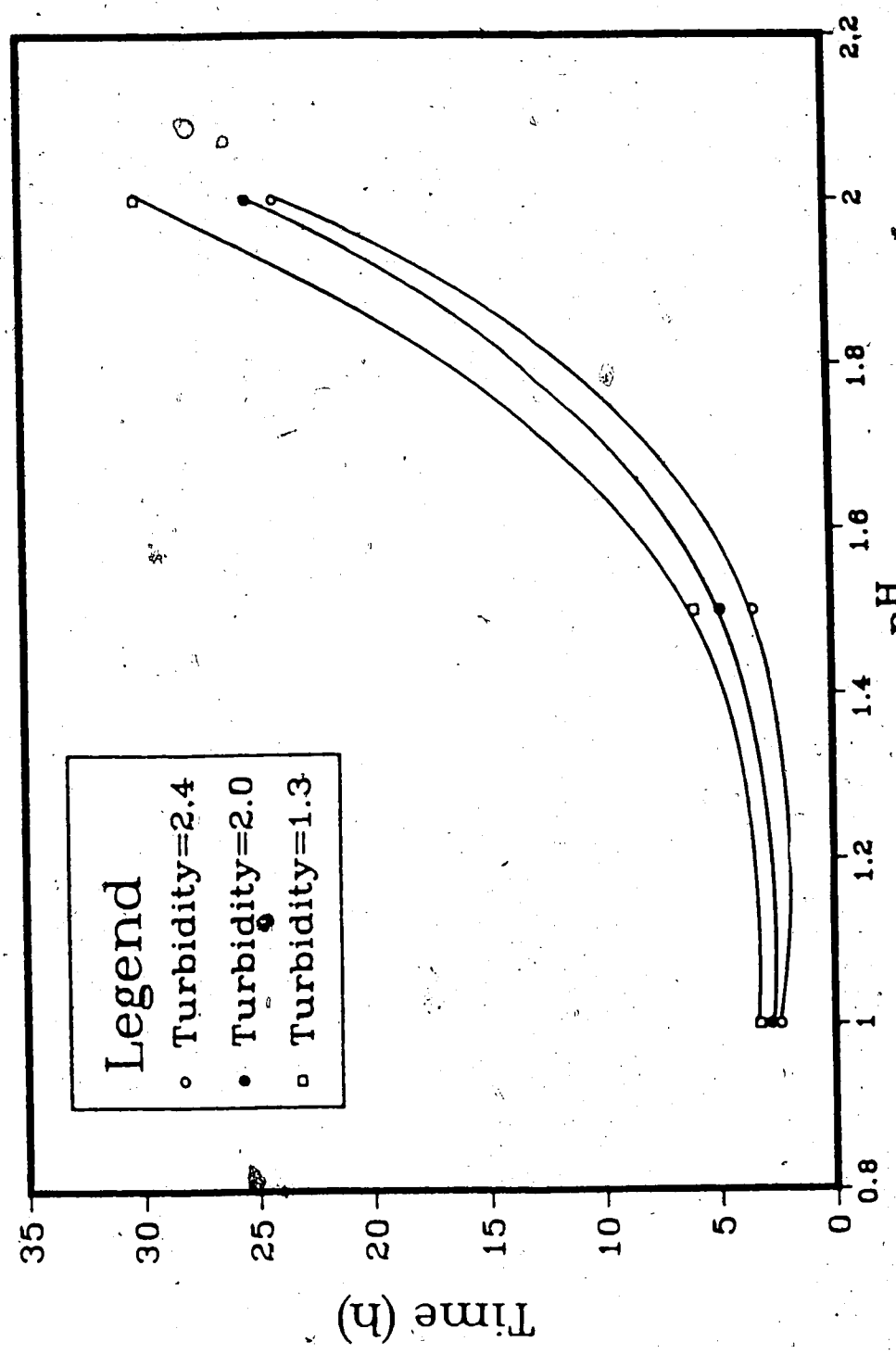


Fig. 37. Dependency of the Time for Solubilization of Liposomes by CFX on pH at Various Turbidity at 40°C. Phospholipid(DMPC) Concentration= 14.4 mM; CFX Concentration= 7.5 mM.

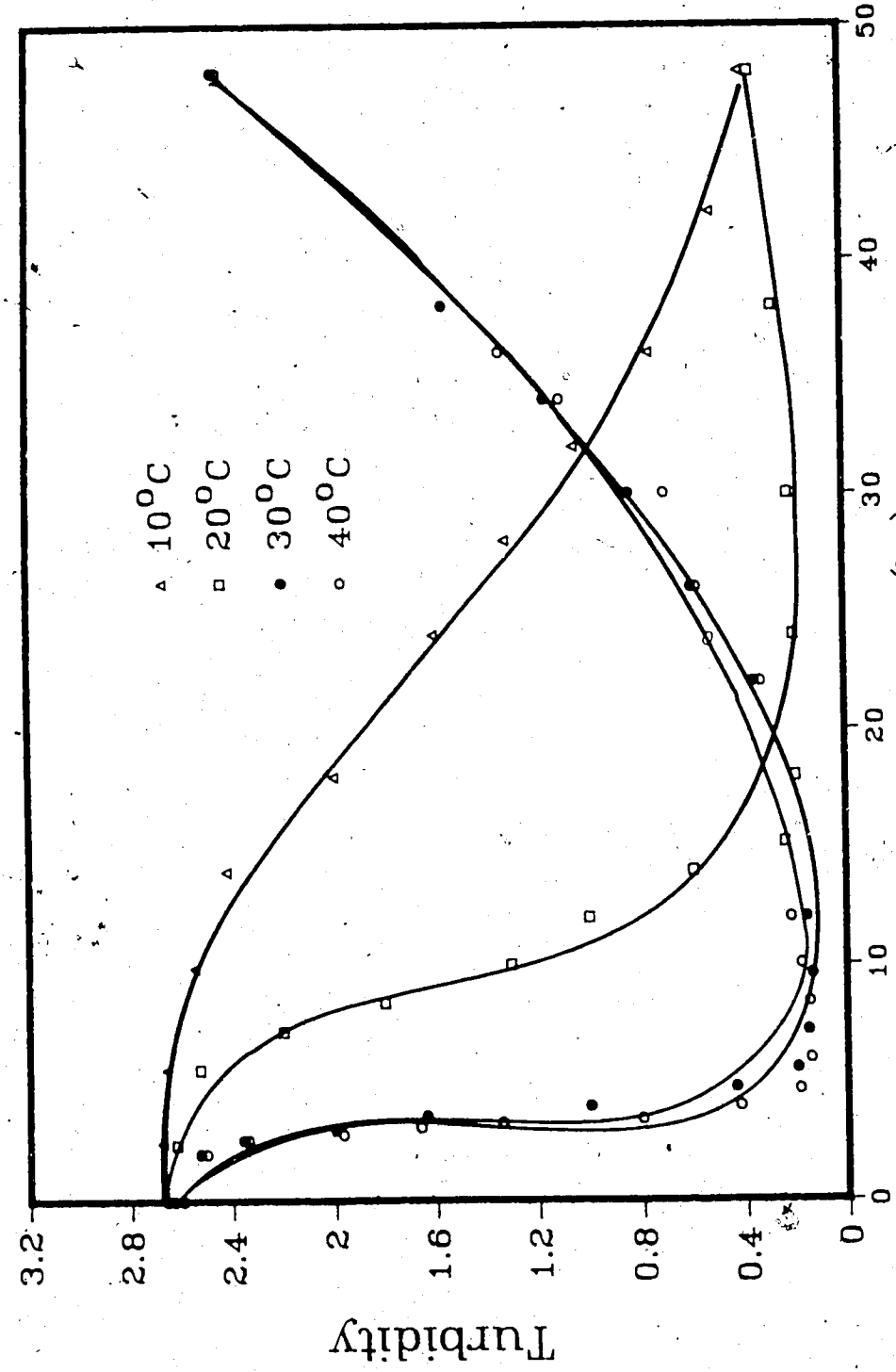


Fig. 38. Effect of Temperature on the Solubilization of Liposomes by CFX at pH 1.0. Phospholipid(DMPC) Concentration= 14.4 mM; CFX Concentration= 7.5 mM.

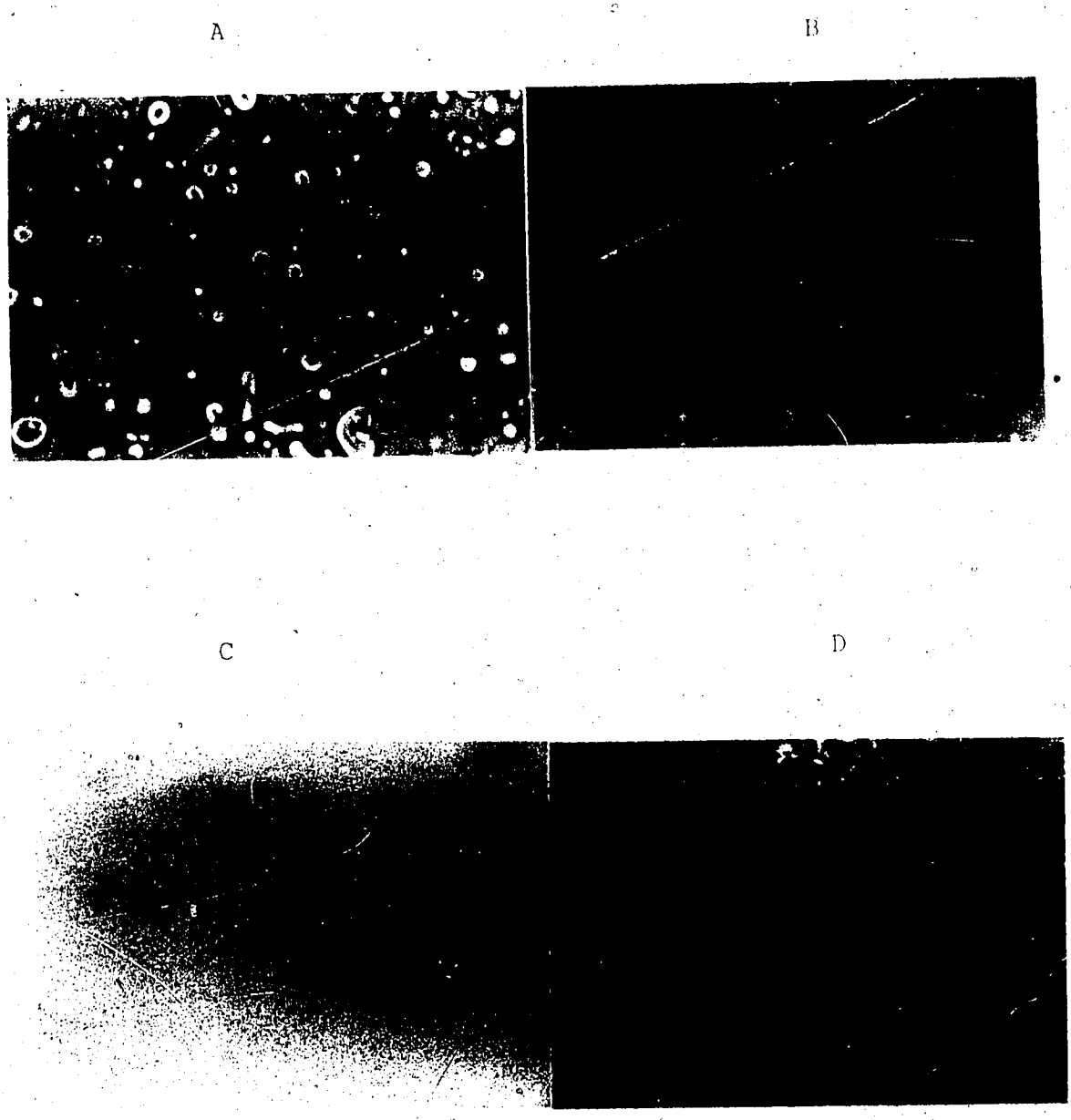


Fig. 39. Photomicrographs of DMPC Liposomes (14.4mM) at pH 1.0 and 40°C: A. in the Absence of CFX; B. with CFX after 10 min; C. with CFX after 20 hr; D. with CFX after 60 hr. Magnification: X500.

also liposomes at various stages of aging with CFX at pH 1.0. At early times (10 min) liposomes can be clearly identified. After 20 hr no liposomes were visible and the preparation appeared to be clear. In contrast, aging of control liposomes under the same conditions resulted in no significant changes in the liposomes (not shown). Figure 39 also displays photomicrographs of the preparation after 60 hr aging time when it had again become turbid. It is clear that large rectangular crystals had formed.

4.3.4 Surface Tension of Cefotaxime Sodium

The surface tension of the different concentrations of cefotaxime solutions were measured using a drop volume method. Results of surface tension measurements of CFX solutions are plotted according to the Gibbs adsorption isotherm (Figure 40). The curve is typical of that obtained for a surface-active agent although its surface activity is not great. A CMC of 0.006M was determined, which compares with 0.01M for sodium lauryl sulfate (146) but is below the concentration of CFX used in the present studies (0.0075M). The density versus concentration of cefotaxime curve which was used for the calculation of surface tension is shown in Figure 41.

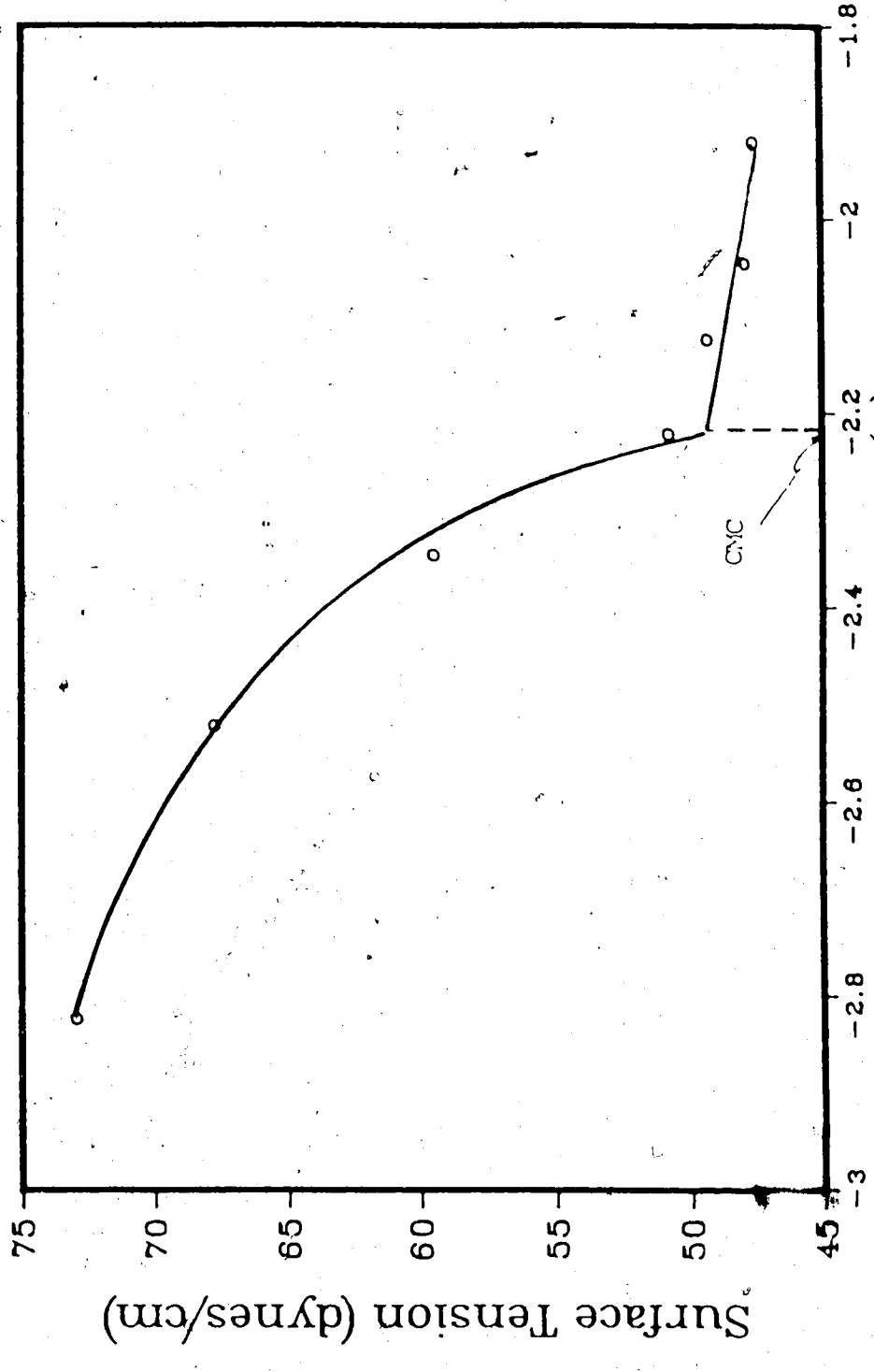


Fig. 40. A Gibbs Adsorption Isotherm Plot of CFX at pH 3.0 and 24°C.

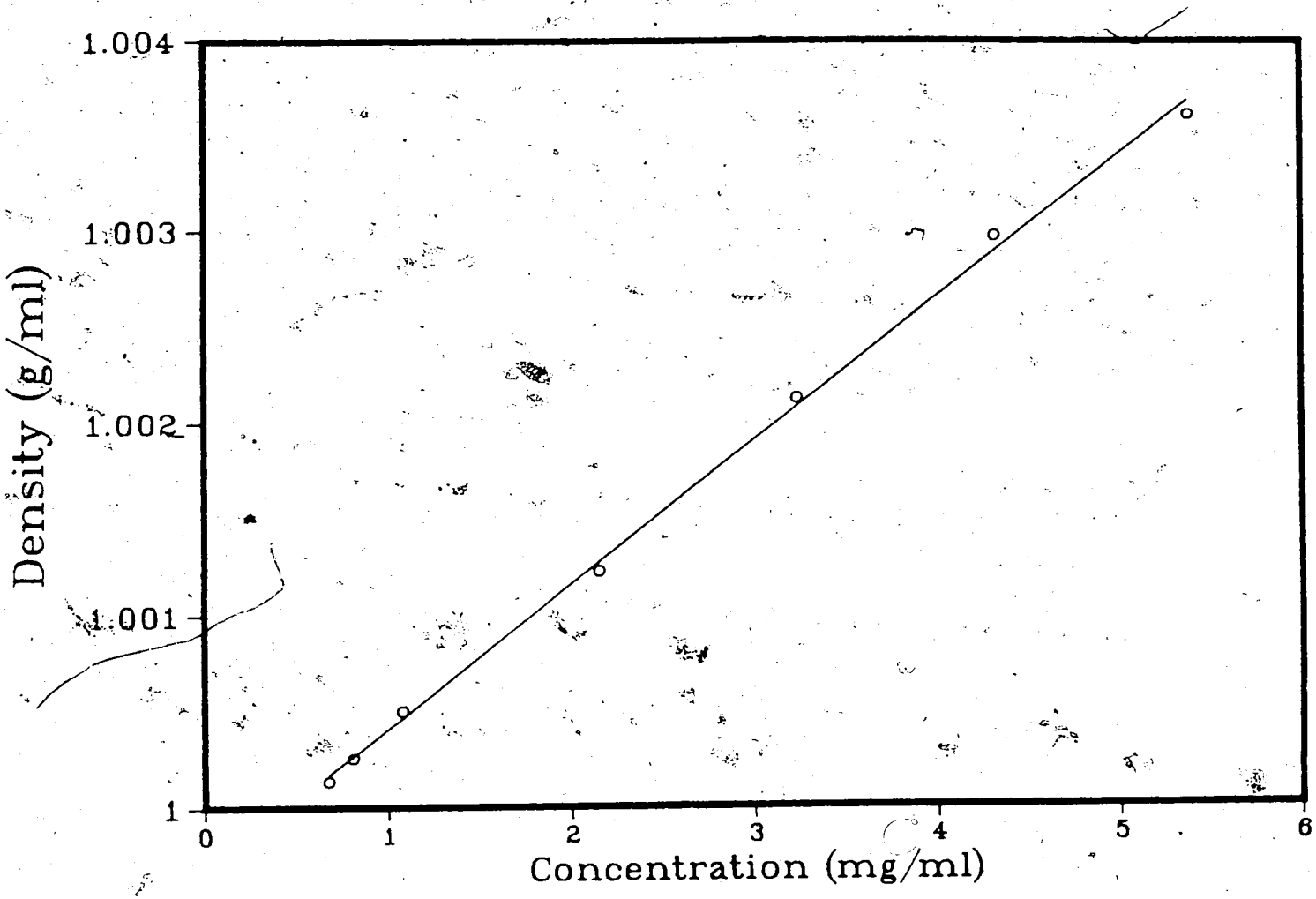


Fig. 41. Densities of CFX Solutions at 24°C (25ml pycnometer).

5. DISCUSSION

The stabilization of drugs against catalytic hydrolysis in liposomes has been previously shown to be related to the extent of association with or partition into the lipid phase (37,38,40,140) . In addition, the nature of the association and the location of the drug molecules within the bilayers have been found to be important determinants in the ultimate stability obtained (40,140). Thus, the effect of liposomes on the stabilization of drugs appears to be a function of both the hydrolysis rate constant in a structured phospholipid environment and the extracted fraction of the total initial concentration of drugs.

In this regard, it would be reasonable to expect that for solutes which partition into the bilayers of liposomes, their stabilities would be greater the greater their K_W^Ls. Although an indication of this has been found for some trimethylammonium bromides (140), others have not examined this property in detail. Furthermore, the association of solutes with liposomes has not been analyzed from the perspective of the manner of incorporation of the solute in liposomes, until now. In some instances, this can produce dramatically different results, such as occurred with ASA, whereas for others, such as CFX, it has very little importance. The stabilization of compounds in colloidal particles such as liposomes can also be altered by surface charge density. Thus, the interaction with and penetration

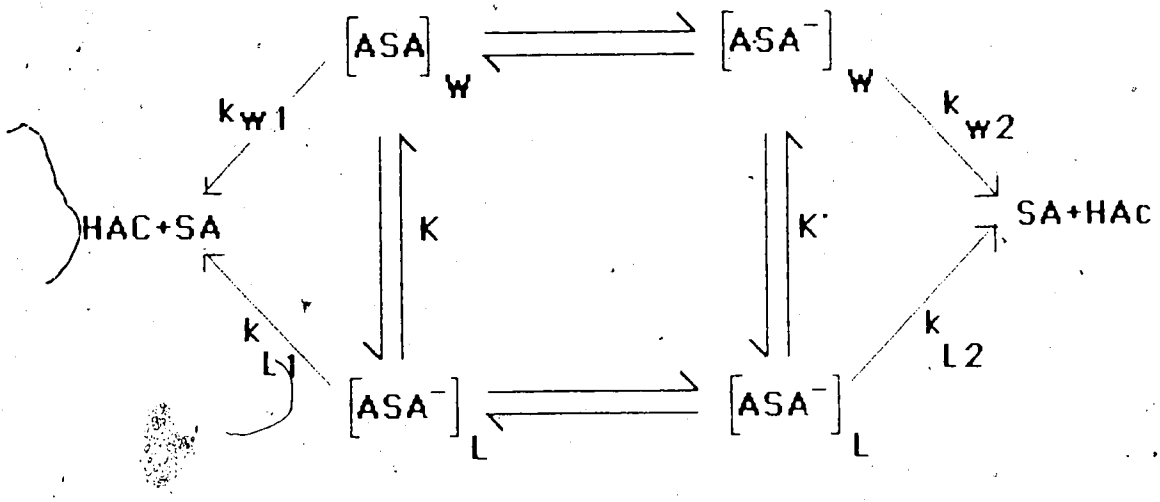
of H^+ or OH^- in liposomes will be dependent on the nature of the polar headgroups, their degree of hydration, and their net charge, i.e., positive or negative. Solutes embedded in the hydrophobic regions of the bilayer can be protected against catalytic attack of this type according to established principles (174). However, the extent of these influences on the hydrolysis degradation rates of solutes would be dependent on the reaction mechanism and the species involved.

5.1 ACETYLSALICYLIC ACID

ASA has a relatively low K_W^O (177) and its f_L in liposomes was low (Table 13). As expected, f_L at pH 1.0 was greater than at pH 4.0 because of the larger fraction of ASA in unionized form at pH 1.0 but k_{obs}/k_W was greater than at pH 4.0 or pH 8.0. Therefore, anionic ASA^- is associated in some fashion with the phospholipid, possibly binding to the choline regions of the polar groups, to form a complex having a favorable structure and orientation to protect ASA against catalytic hydrolysis. The fact that stabilization of ASA^- is significant only when it is incorporated in liposomes via the organic phase supports the argument since this method brings the PL and ASA in close, intimate contact before and during hydration. Otherwise, ASA^- must overcome surface oriented, hydrated, polar head groups of PL before being able to penetrate and complex and this does not appear

to be possible. Indeed, when ASA^- is introduced via the aqueous phase at pH 8.0, k_{obs} is greater than k_B suggesting that ASA^- adsorbs at the liposome surfaces in an orientation which increases exposure of its reactive center to OH^- attack. Similarly, the hydrolysis of 2-diethylaminoethyl p-nitrobenzoate was retarded in its free base form whereas it was enhanced in its protonated form (38).

ASA undergoes hydrolysis in both the aqueous buffer, and the liposome bilayers, and depending on the pH, hydrolysis of unionized ASA and/or anionic ASA^- is also occurring. Thus, scheme V, illustrates the kinetic and partitioning possibilities in liposome systems :



SCHEME V

k_{W1} and k_{L1} are the first order hydrolysis rate constants of the unionized species in the aqueous and phospholipid phases, respectively, k_{W2} and k_{L2} are the corresponding

first-order rate constants of the ionized species, K and K' are the partition coefficients between the phospholipid bilayer and aqueous phases of the unionized and ionized ASA, respectively.

Mathematical treatment may be applied to scheme V in order to theoretically derive the kinetic parameters and partition coefficients (37,38). Thus, the change in total concentration of ASA in the system with time is given by

$$-\frac{d[ASA]_T}{dt} = (1-V)(k_{W1}[ASA]_W + k_{W2}[ASA^-]_W) + V(k_{L1}[ASA]_L + k_{L2}[ASA^-]_L) \quad (24)$$

where V is the volume fraction of phospholipid. Using the following definitions

$$K_a = \frac{[ASA^-]_W[H^+]}{[ASA]_W}$$

$$K = \frac{[ASA]_L}{[ASA]_W}$$

$$K' = \frac{[ASA^-]_L}{[ASA^-]_W}$$

Eq.24 was modified to yield Eq.25:

$$-\frac{d[ASA]_T}{dt} = \frac{k_{W1}[H^+] + k_{W2}k_a + q(k_{L1}K[H^+] + k_{L2}K'K_a)}{[H^+] + K_a + qK[H^+] + qK'K_a}[ASA]_T \quad (25)$$

where q = V/(1-V). The volume fraction of the lipid phase was calculated from the density (=1.02 (37)) and the molecular weight of DMPC(= 696). Eq. 25 can be simplified to Eq.26 by introducing (37)

$$k_W = \frac{k_{W^1}[H^+] + k_{W^2}k_a}{([H^+] + k_a)}$$

giving

$$-\frac{d[ASA]_T}{dt} = \frac{k_W[H^+]+K_a+q(k_{L^1}K[H^+]+k_{L^2}K'K_a)}{[H^+]+K_a+qK[H^+]+qK'K_a}\,[ASA]_T \quad (26)$$

At pH 1.0,(ASA completely unionized) Eq.26 may be simplified yielding k_{obs}.Thus,

$$k_{obs} = \frac{k_{W^1} + qk_{L^1}K}{1 + qK} \quad (27)$$

Equation 27 is rearranged to give

$$k_{W^1} - k_{obs} = \frac{k_{obs} - k_{W^1}}{q}\cdot\frac{1}{K} + (k_{W^1} - k_{L^1}) \quad (28)$$

from which a plot of $(k_{W^1}-k_{obs})$ versus $\frac{k_{obs}-k_W}{q}$ is linear with a slope of 1/K and intercept of $(k_{W^1}-k_{L^1})$. A plot of the data for ASA at pH 1.0 is shown in Figure 42. Regression analysis yielded slope = 0.035, intercept = 0.0053, and r = 0.984. Calculated values of K and k_{L^1} are shown in Table 25. Since ASA is completely unionized at pH

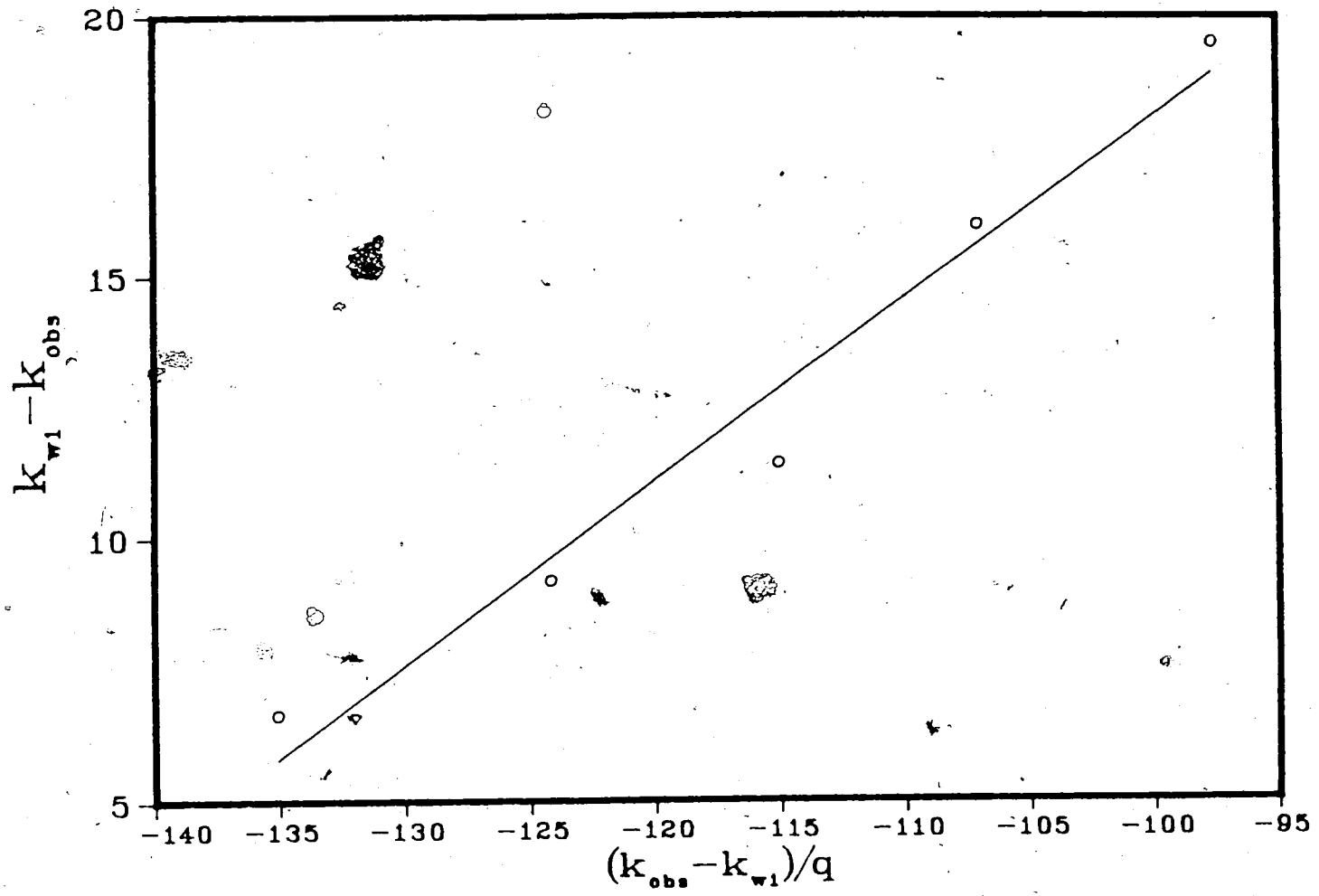


Fig. 42. A Plot of $(k_{obs} - k_{w1})/q$ versus $(k_{w1} - k_{obs})$ for ASA Based on Eq. 28

TABLE 25

Hydrolysis Rate Constant and Partition Coefficients of ASA from Kinetic Model at 30°C.

pH 1.0		pH 8.0	
k_{W^1}	$1.46 \times 10^{-2} hr^{-1}$	k_{W^2}	$2.04 \times 10^{-2} hr^{-1}$
k_{L^1}	$0.93 \times 10^{-2} hr^{-1}$	k_{L^2}	$\simeq 0$
K	29	K'	22

1.0, k_{W1} is the degradation rate constant of all ASA in the aqueous buffer solution.

At pH 8.0 essentially only ionized ASA is present in solution. Under these conditions and where k_{W2} is the overall rate constant in the aqueous buffer solution, mathematical treatment of Eq.26 yields Eq.29:

$$k_{obs} = \frac{k_{W2} + qK'k_{L2}}{1+qK'} \qquad (29)$$

Equation 29 is then rearranged to

$$k_{W2} - k_{obs} = \frac{k_{obs} - k_{W2}}{q} \cdot \frac{1}{K'} + (k_{W2} - k_{L2}) \qquad (30)$$

The results obtained at pH 8.0 were also plotted according to Eq.30 (Figure 43) yielding slope = 0.045, intercept = 0.0262, and r = 0.998. The generated hydrolysis rate constant, k_{W2} and K' are also given in Table 25. As expected, $k_{L2} << k_{L1}$ but K' is larger than would be expected for partitioning of the polar anionic ASA^-. However, a binding rather than a simple partitioning mechanism could account for this. The protection of ASA against hydrolysis appears to be independent of the concentration of OH^- ion since similar levels of stabilization were obtained at pH 4.0 and pH 8.0.

On the assumption that electrostatic binding of anionic ASA^- to PL appears to be the main mechanism contributing to

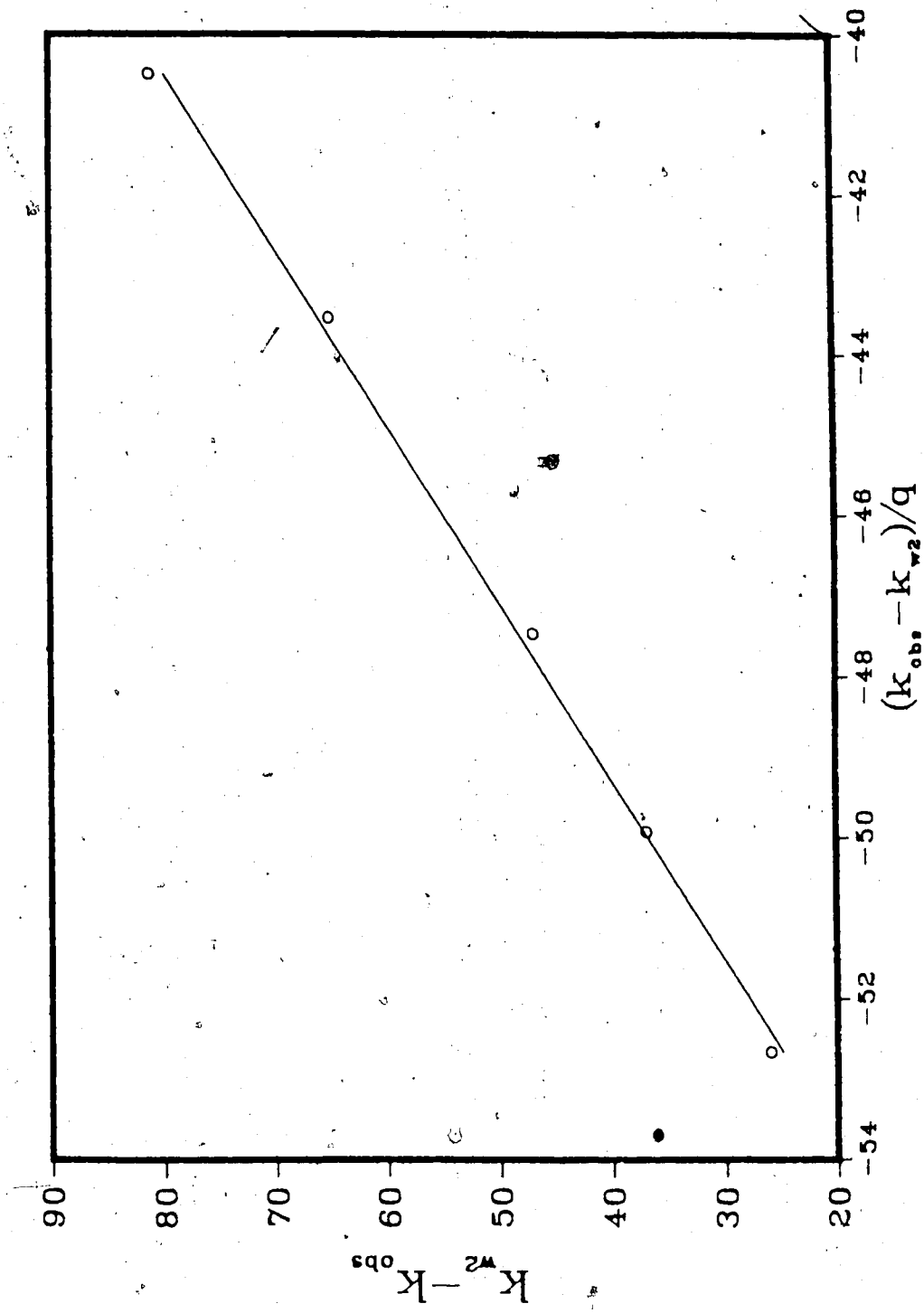


Fig. 43. A Plot of $(k_{obs} - k_{w2})/q$ versus $(k_{w2} - k_{obs})$ for ASA Based on Eq.30

its stabilization, it is expected that the bound ASA would remain in the bilayer following centrifugation, washing and resuspension in fresh buffer solution. Values of k_{L2} can, therefore, be verified experimentally by following the kinetics of hydrolysis of bound ASA only. Obtained values of k_L are shown in table 13. Comparison of the experimental k_L (Table 13) and calculated k_L values (Table 25) reveals close agreement at pH 8.0 and the percent increase in stability in the lipid phase is close to 100%. On the other hand, variation in partitioning of ASA at pH 1.0 during degradation in the external buffer resulted in less agreement between calculated and experimental values because the liposomes are continuously undergoing re-equilibration with the external environment.

The properties of liposomes are often closely related to their exact composition, the temperature, and the particle size distribution. In this work, MLVs were consistently employed and the reproducibility of many replicates of DMPC liposomes gave a strong indication that the techniques used produced consistent populations of liposomes. Furthermore, a linearity of first-order plots over several half-lives suggests that any changes in particle size which may be occurring during the kinetic studies have no impact on the rate constants obtained.

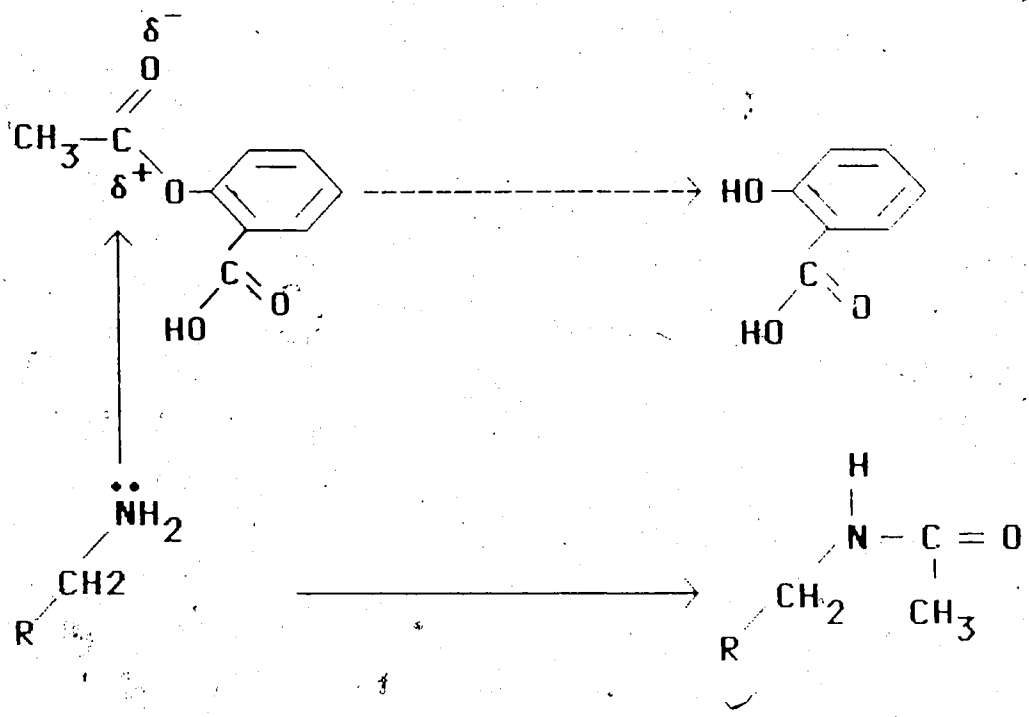
The uptake, partitioning or association of solutes with liposomes has repeatedly been shown to be a function of the

fluidity or structural order of the bilayers (175,176). This property of liposomes is determined mainly by composition and temperature. Thus, $<T_c$ of the PL, a gel-state of the bilayers requires greater energies of solute molecules in order to partition whereas $>T_c$ the liquid crystalline state of the bilayers facilitates uptake of solutes. Likewise, the ordered state of bilayers $>T_c$ may be increased by inclusion of certain molecules (e.g. CHOL) or decreased $<T_c$. Furthermore, charged additives to liposomes exert a more direct influence on the uptake or association of charged solute molecules or ions. It is not surprising, therefore, that k_{obs} of ASA in liposomes $<T_c$ (e.g. DPPC) or containing CHOL was higher than in DMPC liposomes since k_{obs} is a function of f_L. In other words, the same factors that determine partitioning in liposomes also determine the extent of binding of ASA^- to the PL in the bilayers. The displacement of chlorpromazine from hydrophobic binding sites in liposomes (179,181) due to addition of CHOL, or reduced liposome partitioning of mequitazine (180) or enhanced catalytic hydrolysis of p-nitrophenyl N-dodecyl-D(L)-phenylalaninate in artificial membranes (182) are all probably due to the same reasons that reduced the stabilization of ASA^- in DMPC liposomes.

The particular liposome composition of DMPC:STEAR produced some initially unexpected results which required some detailed analysis. The initial hydrolysis of ASA in

the chloroform solution of DMPC, ASA, and STEAR was due to nucleophilic attack of ASA by STEAR resulting in acetylation of the amine and formation of SA (see Figures 8,9,10). Illustration of the probable reaction mechanism is shown in Figure 44. A similar transformation of ASA in the body under the influence of proteins is believed to occur (158). The reactions of ASA with weakly basic amines exhibit hydrolysis rate acceleration of up to 150-fold which are ascribed to an intermolecular general acid-base reaction. The extent of this reaction is dependent on such factors as the relative concentrations of ASA and nucleophilic reagent (STEAR in this case). The exponential degradation as seen in Figure 8 arises because of acetylation of STEAR by ASA. A requirement of this reaction is a lone pair of electrons on the nitrogen atom of the STEAR molecule. It can be seen in Figures 9 and 10 that when ASA was incorporated via the aqueous phase at pH 4.0, there was no biexponential degradation of ASA indicating the absence of sufficient unprotonated amine to act as the nucleophile. However, at pH 8.0 because the amine possesses a lone pair of electrons the reaction occurred forming N-stearylacetamide and salicylic acid and a biexponential degradation of ASA was observed. The evidence shown in Figures 11 and 12 support this argument.

In a related study, it was found that p-nitophenyl acetate reacted with STEAR containing liposomes. But

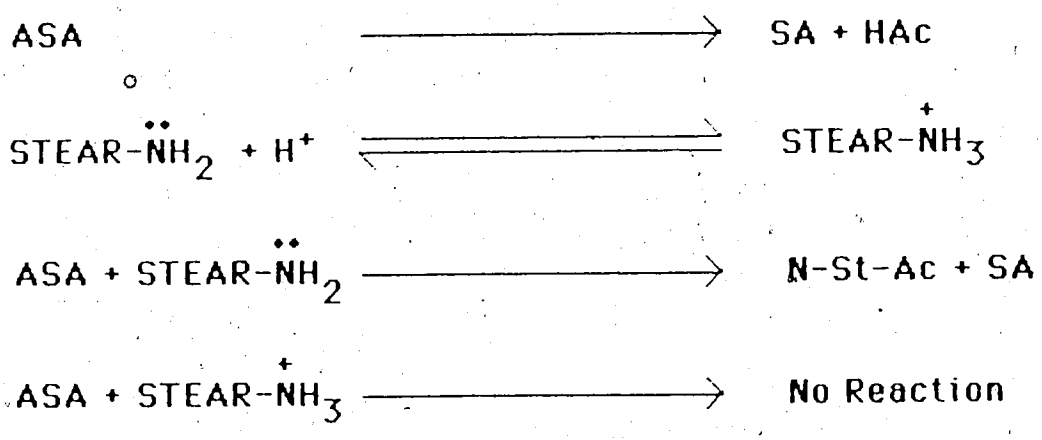


SCHEME VIII

Schematic representation of nucleophilic attack of ASA by STEAR resulting in its acetylation accompanied by the liberation of SA.

initially it was proposed that the increased loss of the ester in the presence of STEAR containing liposomes was due to the positively charged surface of the liposomes favoring formation of the negatively charged transition state during nucleophilic hydroxide ion attack (40). But later this theory was disproved and showed that the liposomal STEAR reacts with p-nitrophenyl acetate to form N-stearylacetamide, thus increasing the loss of the ester (141).

Loss of ester in the presence of STEAR containing liposomes involves the processes described in scheme VI.



SCHEME VI

Thus, at the appropriate pH or in chloroform STEAR behaves as a nucleophile which hydrolyzes ASA and forms N-stearylacetamide (N-st-ac) and SA.

A better understanding of the mechanisms involved is obtainable from inspection of the results of Table 8. After

the initial hydrolysis of ASA in the presence of STEAR or p-chloroaniline (which also has a free pair of electrons) the remaining ASA associated with the liposomes is stabilized. This is not due to the presence of N-stearylacetamide which was formed, since its inclusion in DMPC liposomes did not change the kinetics but likely due to the binding of ASA^- to the additional sites of positive charge introduced by the amines. However, in contrast, the addition of SA during the formation of liposomes resulted in a decreased stabilization of ASA because SA competed with ASA for the available binding sites, thereby reducing the fraction of total ASA associated with the liposomes (Table 26).

Using Eq. 21 and k_L obtained in DMPC liposomes k_{obs} was calculated for each liposome composition given in Table 26. When compared to the experimental k_{obs} agreement was within 6% except for DMPC:STEAR(2:1) liposomes suggesting that in these other liposome compositions k_{obs} is affected only through f_L but in STEAR-containing liposomes, both k_L and f_L contribute to k_{obs}. It is apparent from Table 26, column 5, that the phospholipid phase contribution to k_{obs} is greatest in DMPC:STEAR liposomes and least in liposomes containing either CHOL or SA. It would seem appropriate at this point to emphasize that k_L is only 5% of k_B at pH 8.0 (i.e., a small contribution to the overall ASA reactivity by k_L) whereas k_L is 75% of k_B at pH 1.0 underscoring the fact that

TABLE 26

Comparison of k_{obs} (calc'd) and k_{obs} (expt'l) and Contribution of the Lipid Phase to the Hydrolysis Reactivity of ASA in Liposome Formulations at pH 4.0 and 30°C.

Liposome Formulation[a]	k_{obs}(calc'd) (hr^{-1}) ($x10^2$)	k_{obs}(expt.) (hr^{-1}) ($x10^2$)	f_L	$k_L f_L / k_{obs}$
DMPC	1.11	1.05	0.258	0.074
DMPC:CHOL (3:1)	1.26	1.21	0.117	0.029
DMPC + 2.7mM SA	1.22	1.26	0.153	0.036
DMPC:STEAR (2:1)	1.01	0.80	0.349	0.131

Total Lipid Concentration = 14.4 mM (mole ratios shown in brackets)

$[ASA]_o$ = 7.5 mM.

anionic ASA^- is the form of ASA stabilized in liposomes.

The stabilization of drugs associated with liposomes under various conditions will depend on the integrity and stability of the liposome itself. Any changes which may occur in the structure of the liposome may influence the extent of partitioning of a solute and, therefore, its rate of degradation through the f_L term. The liposome systems in this study did not undergo any obvious visual changes when subjected to variations in pH, temperature or ionic strength and at pHs at which ASA is ionized its stability was little affected by such variations in the conditions of the system.

The stabilization of ASA in DMPC liposomes is compared to its stabilization in various surfactant solutions in Table 27. The hydrolysis of unionized ASA was suppressed in anionic, cationic and nonionic surfactant micelles but ionized ASA was suppressed only by cationic micelles (13). However, on a mole per mole basis a 16:1 ratio of sodium lauryl sulfate : ASA is required to provide a level of stability of ASA at pH 4.0 equivalent to a 4:1 ratio of DMPC:ASA. Also, DMPC liposomes are approximately 2.5 times more efficient than cetomacrogol micelles in stabilizing ASA (15). In contrast, at pH 1.0 the stabilization of ASA is 49% in 70mM cetomacrogol solution but only about 15% in 29mM DMPC liposomes indicating that for a semipolar solute such as ASA, the partitioning environment of a nonionic micelle is more favorable than that of a neutral liposome for

TABLE 27

Comparison of the Micellar and Liposomal Stabilization of ASA

Surfactant	pH	%increase in Stability	
		Micelles(Ref)	Liposomes[b]
Emulsgen 120 (10%)	4.03	30.6 (13)	41
CDAB (5%)	3.75	45 (13)	41
Bz.cl (10%)	3.85	20.6 (13)	41
SLS (5%)	3.58	77.1(13)	41
Cetomacrogol	4.6	15.0 (15)	41
(48.7%)	1.11	48.7 (15)	15[c]
Polysorbate 80	1.8	52.2 (15)	15[c]
(4%)			

CDAB = Cetylethyl dimethylammoniumbromide

Bz.cl = Benzalkonium Chloride

SLS = Sodium lauryl sulphate

[b]DMPC Conentration = 2.0%, pH 4.0

[c]DMPC Concentration = 2.0%, pH 1.0

protecting against hydrolytic degradation of unionized ASA by H^+.

5.2 LOCAL ANESTHETIC DRUGS

The stabilities of local anesthetics in liposomes were examined primarily to determine the importance of the partition coefficient on the stabilization of solutes by liposomes. Benzocaine in particular was selected as a model compound to examine in detail the influence of composition, concentration, and ionic strength and because its kinetics of degradation in micellar systems had been previously reported (151).

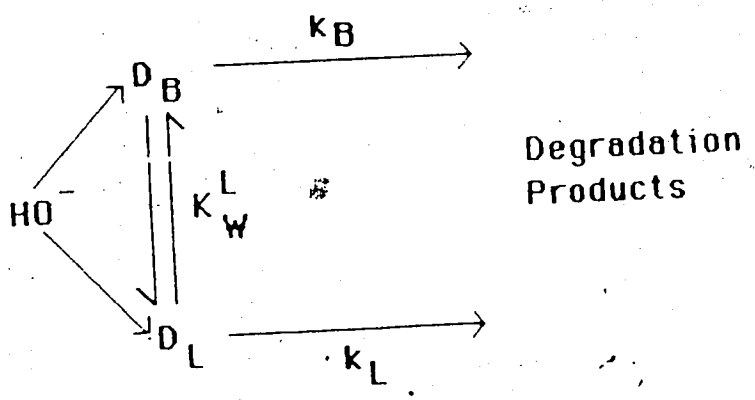
The kinetics of benzocaine hydrolysis of various compositions differed significantly as shown in Table 16. The association of the drug with the lipid phase appears to be responsible for a lower k_{obs} than k_B. A possible reason for the reduced hydrolysis in liposomes is the reduced reactivity in the lipid phase of that fraction of drug associated with it, in particular that which is partitioned deep into the bilayers. Thus, solutes which have a low k_L and a substantial f_L may be predicted to yield the greatest decrease in k_{obs}. Liposomal compositions which augment either of these parameters should provide stability improvement. The influence of f_L on benzocaine stability is well illustrated from the evidence of k_{obs} under the influence of varying concentrations of benzocaine (Figure

26), DMPC (Figure 25 and Table 17) and ionic strength (Table 19). However, the respective contributions of k_L and f_L to k_{obs} are not as obvious in liposomes of different compositions. For example, in liposomes of decreased bilayer fluidity less drug is able to be accommodated but the magnitude of k_L is not necessarily significantly different than in more fluid liposomes. The occurrence of a phase transition from a fluid liquid crystalline state to a more rigid gel-like state is a characteristic of phospholipids and for DMPC (45) T_c is 23° whereas it is 41° for DPPC (45) and 25-40° for SPHING (183). The partition coefficient of solutes in liposomes often exhibits a remarkable decrease upon cooling liposomes below the T_c (170,172,173,179,180) which is believed to be due to the higher energy requirement of accommodation of solute molecules in the rigid gel state of the bilayers (170). Thus, the increase in stability of benzocaine in DPPC liposomes is lower because less benzocaine associates with these bilayers. In a similar fashion reduced benzocaine accommodation in DMPC:CHOL liposomes, due to the decreased fluidity (184), contributes to its lower observed stability. Reduced binding, partitioning and stability of solutes in fluid liposomal membranes to which CHOL has been added has been previously reported (179-182). Although there is no direct evidence of the reactivity in PS or SPHING liposomes, differences in permeability to OH^- may be a major

contributing factor. In the case of PS liposomes, strong negatively-charged sites at the liposome surfaces repel OH^- and, therefore, catalytic attack of the reactive centers of benzocaine molecules partitioned in the bilayers is reduced. In contrast, the permeability of SPHING bilayers, like other phospholipids, is greater in the region of its T_c (129) and the result is increased OH^- penetration and hydrolytic attack of benzocaine molecules in the bilayers.

The reduction in the rate of hydrolysis of tetracaine in buffer as compared to hydrolysis of benzocaine or procaine can be attributed to the replacement of the hydrogen atom by the butyl group, which possesses greater electron-releasing properties (162,185), thus hindering the nucleophilic attack of the hydroxide ion at the acyl carbon atom. Similar reduction of hydrolysis was also observed in liposomal systems.

The degradation of the local anesthetic drugs in liposomes may be described by a simple kinetic model as depicted in scheme VII .



SCHEME VII

where D_B and D_L correspond to drug in the aqueous buffer solution and the lipid phase, respectively. The overall rate of hydrolysis is expressed accordingly :

$$-(V_B+V_L)\frac{dC}{dt} = k_B V_B C_B + k_L V_L C_L \qquad (31)$$

where V_B and V_L are the volumes of the aqueous, and the lipid phases, respectively, C_B and C_L are the concentration of drug in each phase, and C refers to the total concentration of drug in the system which is equal to $(V_B C_B + V_L C_L)/(V_B + V_C)$. V_L was estimated from the density of 1.02 of hydrated lipid (37).

Since the partition coefficient of the esters is given by :

$$K_W^L = \frac{C_L}{C_B}$$

then equation 31 may be expressed as

$$-\frac{d\ln C}{dt} = \frac{k_B - k_L}{1+K_W^L(V_L/V_B)} + k_L = k_{obs} \qquad (32)$$

and following re-arrangement gives

$$k_{obs} + \frac{k_{obs}}{K_W^L(V_L/V_B)} = \frac{k_B}{K_W^L(V_L/V_B)} + k_L \qquad (33)$$

A plot of this linear relationship yields k_B and k_L from the

slope and intercept, respectively, and are shown in Figure 45 for benzocaine, procaine and tetracaine.

Table 28 compares k_B determined from Eq.33 with k_B obtained experimentally. The excellent agreement found supports the validity of scheme VII in describing the events occurring during the degradation of these three local anesthetics. Values of k_L determined from Eq.33 were very low (in the order of 10^{-4} -10^{-5} min^{-1}) and were only about 0.5-1.0% of k_B indicating a strong protective capability of liposomes against hydrolysis of local anesthetics. It can be seen from Table 28 that the k_L/k_B are considerably smaller for tetracaine suggesting that the liposomal environment has a greater inhibiting effect on the rate of hydrolysis when the nonpolar portion of the compound is increased. A comparison of the reactivity in the lipid phase to that in the total liposomal suspension (last column,table 28) not only emphasizes the impact of a low k_L but also the significance of a higher f_L of tetracaine with respect to the observed increase in its stability.

The effect of the two constants, k_L and K_W^L, on the observed rate constant is particularly important for esters with a large partition coefficient and in solution where the ratio V_L/V_B is high since, under these conditions, nearly all of the ester is situated within the lipid phase of the liposome and the observed rate constant approaches the value of k_L. Table 29 illustrates this trend for each of the

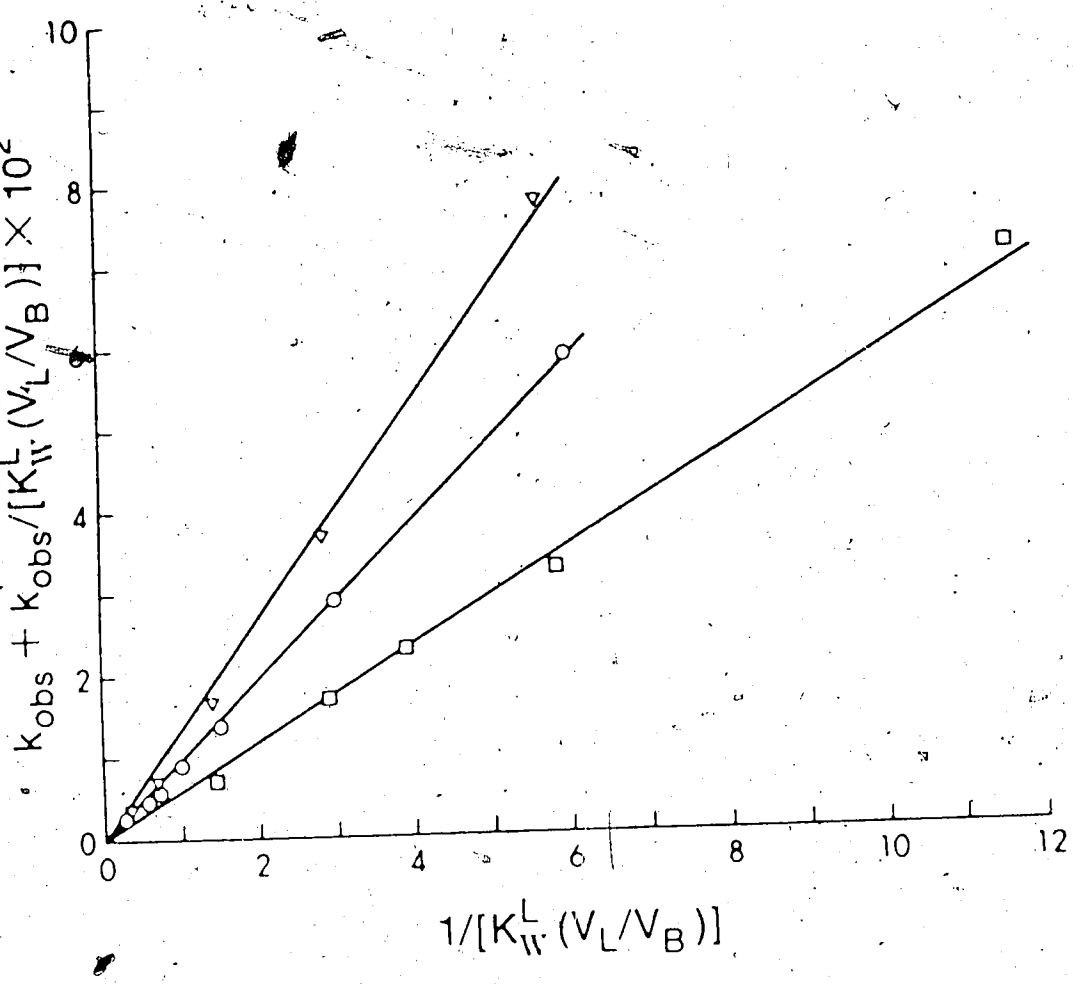


Fig. 45. Plots of Eq.33 for Benzocaine,○; Procaine,▽; and Tetracaine,□. A multiplication factor of 10 was applied to both axes for Tetracaine.

TABLE 28

Comparison of Hydrolysis in Aqueous Buffer Solution (k_B), Hydrolysis of Associated Drug Relative to Free (k_L/k_B) and Contribution of the Phospholipid Phase to Hydrolysis in the Liposomal Suspension ($k_L f_L/k_{obs}$)

Local Anesthetic	k_B(min^{-1}) (calc'd)[a] (x10^2)	k_B(min^{-1}) (expt'l) (x10^2)	10^2 k_L/k_B[b]	10^3 $k_L f_L/k_{obs}$
Benzocaine	1.0	1.1	1.2	4.5
Procaine	1.4	1.6	1.0	4.3
Tetracaine	0.6	0.7	0.4	8.4

[a] Eq. (33)

[b] k_L: benzocaine - 1.3×10^{-4} min^{-1}; procaine - 1.6×10^{-4} min^{-1}; tetracaine - 0.288×10^{-4} min^{-1}

TABLE 29

Comparison Between V_L/V_B and the Observed Rate Constant (k_{obs}) for Benzocaine, Procaine, and Tetracaine at pH 12.2 and 30°C.

Local Anesthetics	V_L/V_B ($\times 10^3$)	k_{obs} ($\times 10^3$)	k_L[a] ($\times 10^4$)
Benzocaine	0.981	8.40	1.3
	1.964	7.25	"
	3.935	5.49	"
	5.92	4.47	"
	7.905	3.34	"
	9.901	3.02	"
	20.00	1.77	"
Procaine	0.981	11.67	1.6
	1.964	9.6	"
	3.935	6.8	"
	7.905	4.1	"
	15.924	2.6	"
Tetracaine	0.981	3.05	0.288
	1.964	2.15	"
	2.95	1.65	"
	3.935	1.35	"
	7.905	0.62	"

[a]determined using Eq. (3.3).

local anesthetics.

The stabilization of undissociated drugs such as the local anesthetics under the present conditions would appear to be strongly influenced by K_W^L. For example, values of K_W^L for benzocaine, procaine and tetracaine of 169, 180 and 873 correspond to a 34%, 41%, and 69% increase in stability respectively. A test of such a correlation is demonstrated in figure 28 which also includes data points for other local anesthetics and solutes whose stabilities in liposomes have been previously reported (38,40). An excellent correlation (r=0.996) was obtained suggesting the possibility of predicting the decree of stabilization of a drug in a liposome formulation from its liposome K_W^L. However, the drugs which are stabilized by simple partitioning can only be predicted by this correlation curve.

Similarities between micellar and liposome systems have been referred to previously (40). however, phospholipid bilayers also have properties characteristic of lyotropic smectic mesophases (186,187). An indication of which physical state is most predominant in stabilizing drugs is given by a comparison of the stabilization of local anesthetics in liposomes and in surfactant micelles. This is presented in Table 30. On a mole per mole basis stabilization in liposomes on average is approximately 50% greater than in micellar systems and this would suggest that

TABLE 30

Comparison of the Micellar and Liposomal Stabilization of Local Anesthetics

Local Anesthetic	Surfactant[a]	%increase in Stability	
		Micelles(Ref.)	Liposomes[b]
Benzocaine	POE 24 monocetyl ether(≃4.5mM)	39.4(16)	34.0
	C-30 (≃12mM)	58.0 (16)	34.0
	SLS (9.3mM)	35.6 (16)	34.0
Procaine	PLE (8.3mM)	50.5 (15)	41.0
	SLS (10 mM)	65.3 (15)	34.0
	CTAB (4 mM)	39.1 (15)	41.0
	N-dodecyl betaine (2 mM)	18.3 (15)	41.0
n-Butyl-p-amino benzoate	POE 24 monocetyl ether (≃4.5 mM)	55.7 (14)	84.5

[a]C-30 = Cetyl alcohol polyoxyethylene ethers
SLS = Sodium lauryl sulfate
PLE = polyoxyethylene lauryl ether
CTAB = Cetyltrimethylammonium bromide

[b]DMPC Concentration = 0.2% (= 2.9 mM)

the liposomal bilayers in the liquid crystalline state may have some characteristics of a lyotropic smectic mesophase. For example, the hydrolysis reaction rate of procaine hydrochloride in lyotropic liquid crystalline phases of polyoxyethylene tridecyl ether was found to be approximately 300-fold slower than in aqueous media (186).

It appears that ionized drugs may also be stabilized in liposomes but only if they have hydrophobic moieties in their structures large enough to outweigh the surface polar group interactions and partition in the hydrocarbon regions of the bilayers, such as occurs with indomethacin (40). Otherwise, drugs such as procaine (37) or ASA are stabilized only if they are first incorporated directly into the lipid phase prior to liposome formation (Table 18). In this case, the decreased reactivity in the lipid phase is probably due to a more favorable orientation of the drug molecule deeper in the bilayers which are anchored by electrostatic association of the polar groups of the drug with those of the phospholipid molecules in the surface regions of the liposomes.

5.3 CEFOTAXIME SODIUM

The hydrolysis of CFX was not significantly affected by the presence of liposomes as evidenced by the similaritiy between the values of k_B and k_{obs} and a negligible value of f_L (Table 23). Thus, CFX remains in the bulk aqueous phase

of liposomal suspensions where it hydrolyzes to degradation products.

CFX is a highly water-soluble compound. Consequently, the lack of association between CFX and the liposomes can be attributed to its high hydrophilicity. At higher pHs CFX exists as a negatively-charged ion. However, unlike ASA no change in the stabilization of CFX was observed when it was added via the organic phase. Thus, CFX does not complex with PL and it does not partition sufficiently in the bilayers to be protected from OH^- catalytic hydrolysis.

At pH 1.0 determination of the stability of CFX in liposomes is impossible because CFX rapidly solubilizes the PL. Although the surface activity of CFX is not high (CMC at 48 dynes/cm) nevertheless it transforms the PL lamellar structures into mixed micelles yielding a transparent solution. The solubilization of lipid bilayers by surfactants has been previously studied (188, 189). A CMC of CFX of 6mM (Figure 40) is below the CFX concentrations used in the stability studies and, therefore, is able to solubilize membranes (189). The reappearance of turbidity at latter times following solubilization was shown to be due to precipitation of crystals. The complexation of DMPC and degradation products of CFX, which are gradually forming is a possible explanation for the appearance of these crystals (i.e., CFX degradation product becomes solvated by DMPC) since they do not appear in degraded CFX solutions in the

absence of DMPC.

5.4 GENERAL COMMENTS ON THE STABILIZATION OF DRUGS IN LIPOSOMES

The present study has demonstrated that the potential of liposomes to stabilize drugs in solution is variable depending on the nature of the interaction between the drug and liposome. The stabilization of ASA was achieved only through ionization. It was preferentially stabilized in its anionic form, although a larger fraction of ASA was associated with the lipid phase of the liposome at low pH. By increasing the rigidity of the liposomal bilayers a lowering of f_L resulted and consequently an increase in k_{obs}. However, ionic strength, temperature or varying the concentration product only minor changes. Stabilization of ASA was only observed if the drug was incorporated into liposomes via the organic phase. It is concluded that the contribution of the lipid phase to the hydrolysis reactivity of ASA is due to binding of anionic ASA to positively-charged sites on the phospholipid head groups. The fraction of ASA associated is approximately proportional to the number of binding sites available to it and is reduced by a process of competitive inhibition by SA, a degradation product of ASA.

The study of ASA has demonstrated that mechanisms other than simple partitioning can play an important role in

stabilizing certain drugs in liposomes. By appropriate choice of liposome composition and method of preparation it should be possible to formulate stabilized liquid preparations of a variety of electrolyte and ionic drug molecules.

On the other hand, unionized drugs are stabilized by simple partitioning into the lipid bilayer of liposomes. The important factors involved in this type of stabilization in liposomes are the depth of the reactive center of the solute molecule in the phospholipid bilayer and the fraction of the total drug associated with or partitioned into the liposomes. An excellent correlation of the percent increase in stability of local anesthetic drugs in liposome and the log K_W^L enabling the possible prediction of the degree of stabilization of a nonionic drug from its liposome partition coefficient. Simply changing the composition or concentration of liposomes with a concomittant change in bilayer fluidity, the partitioning or association of drug can be favorably modified to optimize stabilization.

When attempting to stabilize a drug in liposomes, the possibility of limited or complete solubilization of the PL should be considered. This would apply to molecules having inherent surface active properties or to other molecules which unsuspectingly become more surface active due to a pH change or, perhaps, a slight modification in chemical structure. If solubilization does occur, it can be a fast

or slow process but in any case, any benefits derived from liposome encapsulation are lost.

SUMMARY AND CONCLUSIONS

Acetylsalicylic acid, local anesthetics and cefotaxime sodium are all known to be subject to acid- or base-catalyzed hydrolysis in aqueous medium. Stabilization of these drugs in aqueous solution would, therefore, be clinically beneficial. Hence, the effect of phospholipid vesicles in stabilizing drugs have been investigated using these model compounds.

Multilamellar liposomes were prepared by the conventional method. The drugs were incorporated and the kinetics of their hydrolysis were investigated under various conditions of preparation, composition, concentration, ionic strength, pH and temperature and compared to that in the control buffer solution. The data were evaluated using different kinetic models and the contribution of the lipid phase of liposomes to the overall stabilization of drugs was calculated. Based on these studies, the following conclusions are made:

A. Acetylsalicylic Acid

1. Liposome incorporation of ASA has improved its stability by approximately 25% at 30°C and at pH values ranging from 4.0 to 8.0.
2. Anionic ASA is the predominant species to interact with the phospholipid bilayer to attain this level of stability, although a larger fraction of ASA was

associated with the lipid phase of the liposomes at low pH.

3. Interaction of anionic ASA with the bilayer likely binds electrostatically to the positively-charged sites of the polar groups of the phospholipid molecules.
4. Significant stabilization was only observed if ASA was incorporated into the liposomes via the organic phase.
5. Enhanced degradation of ASA occurred at pH 8.0 when incorporated into liposomes via the aqueous buffer phase. This suggests that anionic ASA adsorbed at the liposome surface is more susceptibile to OH^- attack.
6. Increasing the rigidity of the bilayers by the addition of CHOL or at temperatures $<T_c$ of the PL caused a lowering of f_L and an increase in k_{obs} indicating that some phosphorylcholine groups may not be available for binding of ASA.
7. Liposome concentrations of 14.4mM and 28.8mM yielded 25% and 42% increase in stability of ASA, respectively, at pH 4.0 over that in aqueous buffer solution.
8. Degradation was enhanced initially in the organic solvent during the preparation of DMPC:STEAR liposomes but ASA stability was enhanced once liposomes were fully prepared because STEAR contributes additional binding sites to increase f_L.
9. The stabilization of ASA in DMPC liposomes is as good as or better than its stabilization in various surfactant

solutions.

B. **Local Anesthetic Drugs**

1. Local anesthetic esters are stabilized to various extent in liposomes at pH 12.2 and 30°C depending on the liposome composition, phospholipid concentration and the liposome partition coefficient.
2. Incorporating the esters in the aqueous or lipid phase initially during liposome preparation yielded identical levels of stabilization.
3. An excellent correlation was obtained between % increase in stability and log K_W^L of the esters enabling possible prediction of the degree of stabilization of drugs in liposomes from their liposome K_W^L.
4. The magnitude of k_L is controlled by K_W^L whereas f_L is controlled by the fluidity of the bilayers and both determine the magnitude of k_{obs}.
5. PS provided the maximum stabilization of benzocaine probably as a result of electrostatic binding and repulsion of OH^- by the negatively-charged sites of PS.
6. Increasing either DMPC or benzocaine concentration increased benzocaine stabilization because of an increase in f_L
7. At pH 7.4 ionized procaine was stabilized in liposomes only when incorporated via the organic phase of lipid, similar to that observed for ASA.
8. On a mole per mole basis stabilization of local

anesthetics in liposomes is approximately 50% greater than in micellar systems possibly due to lyotropic smectic mesophase characteristics of the bilayers.

C. **Cefotaxime-Sodium**

1. CFX is a polar hydrophilic compound and as such incorporation of it to liposomes did not improve its stability compared to control buffer solution.
2. Liposomes have been found to undergo solubilization in the presence of CFX at pHs where the drug was unionized. However, as the ratio of ionized to unionized drug increases, the rate of solubilization decreases until at or near the pK_a of the drug, solubilization becomes insignificant.
3. Decreasing the temperature decreases the rate of solubilization but the effect is more pronounced above the T_c of the phospholipid than below.
4. At low temperature and increased ionization of the drug, a lag time before solubilization began suggesting that the structure of the liposome must be broken down before solubilization can commence.
5. Solubilization of DMPC:CHOL liposomes did not occur.
6. After about 20 hours of incubation at 30°, solubilized liposomes again became turbid. Photomicrographic studies revealed the existence of crystals of different sizes and shapes at these stages. It is concluded from the kinetic analysis of CFX that failure to hold the

solubilized system was due to the degraded products of CFX gradually formed an insoluble complex with DMPC which precipitate as large crystals.

7. The mechanism of solubilization process is postulated as being due to mixed micelle formation since the concentration of the drug used was above the C.M.C. of the drug.
8. The incidence of solubilization of pure phospholipid liposomes by unionized weak electrolyte drugs may limit the usefulness of liposomes in a variety of applications, including as drug delivery systems. However, liposomes having more highly ordered structures (e.g., addition of CHOL) resist solubilizat on.

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