MEASUREMENT OF SOME PHYSICAL PROPERTIES OF CERTAIN SIMPLE AND MIXED PHOSPHOLIPID FILMS

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Under the Supervision of Professor August P. Lemberger

Although soybean phosphatide mixtures have been employed for a number of years as a stabilizing agent for intravenous fat emulsions no systematic attempt has been made to correlate the physical and chemical properties of these compounds with their function in emulsion systems. This study was intended as the initial phase of an investigation of the properties of phospholipid films at the air/water interface. The scope of the study included the isolation in high purity of soybean lecithin and lysolecithin, the development of a new recording film balance and the preliminary study of lecithin films layered on various subsolutions.

A number of methods of isolating purified lecithin from a commercial soybean phosphatide mixture were investigated. These included the classic cadmium chloride method, ion exchange, DEAE chromatography and adsorption chromatography on silicic acid. The lecithin used in the study was prepared by ion exchange treatment with Dowex 1X4 resin followed by adsorption chromatography

on silicic acid. Purified soybean lysolecithin was prepared by treatment of lecithin with Vipera russellii venom.

The identities of the purified lecithin and lysolecithin were confirmed by comparison of their infrared spectra with those cited in the literature. The purity of the compounds was estimated by thin-layer chromatography on Silica Gel G.

The new recording film balance developed for use in this study was a modification of the Wilhelmy or vertical type film balance. The principal modification involved the use of a sensitive transducer element as the sensing device. The transducer used predominently in the apparatus was a Statham model G7A-0.15-350 unbonded displacement transducer. In this film balance, the immersion plate was suspended from the tip of a small aluminum extension arm attached to the probe of the transducer. The transducer was connected by a Wheatstone bridge circuit to a power supply and a d.c. amplifier. The apparatus was calibrated by suspending small weights from the extension arm of the probe and observing the meter deflection produced. The error due to meter drift over a 2 hour period was estimated to be approximately $\pm$0.03 dyne/cm. for an immersion plate with a perimeter of 3.0 cm. The instantaneous error due to meter noise was less than one-half this value.

A film trough was constructed for use in conjunction with the transducer sensing device. The trough was equipped with a moving barrier which could be manipulated externally while the trough was enclosed in a cover constructed of stainless steel and Plexiglas. A hollow chamber was built into the bottom of the trough for temperature control by circulation of water from a constant temperature bath.

The apparatus was used to determine the surface tensions of water and benzene at 23.0° C. Experimental values were found to agree within 0.01 dyne/cm. with literature values. The adsorption isotherm obtained by compression of a palmitic acid monolayer also was found to agree with those found in the literature.

It was concluded that the film balance which was developed in this study was a sensitive instrument capable of a precision well within the requirements of such an apparatus. The major advantage of the apparatus was the fact that a recorder could be conveniently operated off the d.c. amplifier. As a result, permanent records of all compression isotherms for the monolayers studied were obtained. The recorder was also useful for monitoring the equilibration rate of a film while being compressed. It was found that long period of time were required for complete equilibration of palmitic acid films during compression of the film. Lecithin films were found to equilibrate much more rapidly.

A preliminary study of the behavior of purified soybean lecithin films on various subsolutions was conducted using the film balance. It was found that purified lecithin formed liquid type films on deionized water. The limiting area per molecule of these films was approximately 65 $\mathring{A}^2$ per molecule. The lecithin films were found to be highly dependent on the age of the lecithin sample solutions. Aging of the sample solutions for several days resulted in a marked contraction of the film. Lecithin films layered on Pluronic F68 and lysolecithin subsolutions were found to exhibit reduced film pressures at equivalent areas per molecule. In general, the behavior could be related to the surface tensions of the subsolutions, lower film pressures being observed as the surface tension of the subsolution was successively reduced. Films obtained from commercial soybean phosphatide mixtures were compared with purified soybean lecithin films. The crude soybean phosphatides were observed to produce films that were contracted to a much greater extent than the pure lecithin films.

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MEASUREMENT OF SOME PHYSICAL PROPERTIES OF CERTAIN SIMPLE AND MIXED PHOSPHOLIPID FILMS

BY

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A thesis Submitted in Partial Fulfillment
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1963

DEDICATION

This thesis is affectionately dedicated to my wife, Gloria, and to my sons, Tom and Bart.

ACKNOWLEDGEMENT

The author wishes to sincerely thank Professor August P. Lemberger for serving both as counselor and friend during the course of this study.

Appreciation is also expressed to Dr. Stuart Eriksen for his help and suggestions.

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TABLE OF CONTENTS

Page

INTRODUCTION---------- 1

THE USE OF PHOSPHOLIPIDS IN INTRAVENOUS EMULSIONS---- 3

PLAN OF STUDY---------- 9

PART I. ISOLATION OF LECITHIN AND LYSOLECITHIN------ 10

PAST WORK---------- 10

Separation of Phospholipids---------- 10
Chemical Properties of the Phospholipids--- 16
Physical Properties of the Phospholipids--- 23

EXPERIMENTAL---------- 27

Source of Lecithin and Lysolecithin-------- 27
Other Materials and Equipment---------- 27
Isolation of Lecithin by Ion Exchange------ 29
Preparation of Lysolecithin---------- 31
Results and Discussion---------- 33

PART II. DEVELOPMENT OF A RECORDING FILM BALANCE---- 38

THEORY AND PAST WORK---------- 38

Surface Films---------- 38
Spreading and Formation of Films---------- 39
Film Pressure---------- 42

THE MEASUREMENT OF FILM PRESSURE---------- 51

The Horizontal Film Balance---------- 51
The Vertical Film Balance---------- 52

DESIGN OF APPARATUS---------- 55

Construction of Sensing Device---------- 55
Construction of Auxiliary Equipment-------- 62
Calibration of the Apparatus---------- 66

EXPERIMENTAL---------- 71

Materials and Equipment---------- 71
Experimental Methods---------- 71
Films of Palmitic Acid---------- 76

Page

RESULTS AND DISCUSSION---------- 81

PART III. A PRELIMINARY STUDY OF THE SURFACE PROPERTIES OF SOYBEAN LECITHIN AND LYSOLECITHIN---------- 85

PAST WORK---------- 85

EXPERIMENTAL---------- 88

Materials and Equipment---------- 88
Lecithin Films---------- 88

RESULTS AND DISCUSSION---------- 90

SUMMARY AND CONCLUSIONS----------103

APPENDIX----------107

A STUDY OF METHODS OF ISOLATION OF LECITHIN FROM SOYBEAN PHOSPHATIDE MIXTURES----------108

BIBLIOGRAPHY----------121

INTRODUCTION

Phospholipids comprise a class of compounds which apparently are present in varying amounts in all cells, plant and animal. The phospholipids have been the subject of an intensive amount of research in recent years. This is the result of the implication of certain phospholipids in various diseases and the increased industrial use of soybean phospholipids.

The exact function of the many phospholipids found in biological systems still is largely a matter of conjecture. It is believed that phospholipids are involved in blood clotting, fat and phosphate storage, fat transport and transport and ultilization of sodium and potassium ions(1). It has also been suggested that the phospholipids constitute the central structure of the cell wall(2). Recent studies indicate phospholipids may be involved in a number of diseases including Gaucher's disease, Niemann-Picks' disease, arteriosclerosis and cancer(3)(4)(5).

The principal commercial use of phospholipids is in the manufacture of edible products(6).

The phospholipids are also used as emulsifying or stabilizing agents in cosmetics and pharmaceuticals. It is the use of purified soybean lecithin in intravenous fat emulsions which stimulated this study. In view of the importance of intravenous fat emulsions and the extensive use of phospholipids in these preparations, a

systematic study of phospholipid-stabilized emulsion systems has been undertaken. As the study of the behavior at the air/water interface is the most direct and convenient approach yet developed to evaluate the properties of surface active agents, this method will be employed in the investigation. This phase of the study is concerned with the development of an apparatus for the measurement of film pressure and the preliminary investigation of lecithin films at the air/water interface. It is expected that this apparatus will be useful in the further study of the behavior of simple and mixed phospholipid systems.

THE USE OF PHOSPHOLIPIDS IN INTRAVENOUS EMULSIONS

It has been recognized for many years that the intravenous administration of fat emulsions is a potentially superior means of providing nutrition to those patients unable to ingest food by the oral route. The advantage of fat emulsions is that a high caloric content relative to the other commonly employed intravenous preparations can be achieved. Glucose, fructose and invert sugar are limited as parenteral sources of nutrition by osmotic pressure effects. One liter of 5 per cent glucose is isotonic, but contains only 200 calories. Higher concentrations are employed but are irritating to the veins and must be administered slowly to prevent urinary spillover. Ethyl alcohol infusions are also used but the depressing and intoxicating effects of alcohol limit its use.

Fat can be infused as a 15 per cent emulsion in glucose solution and provides approximately 9 calories per gram. The caloric content of a liter unit of such an emulsion would be approximately 1550 calories, several times that of isotonic sugar solutions. The preparation of therapeutic fat emulsions poses a number of specific problems in addition to the usual requirements of non-toxicity and stability. The emulsion must be sterile and hence stable to autoclaving. In addition, the oil globules must be sufficiently small so that there is no danger of embolism when the emulsion is injected

intravenously.

The history of intravenous fat emulsions is a long and troubled one. During the period 1873 to 1883, the intravenous administration of milk was employed with spectacular results in extremely malnourished patients. Serious reactions and infections were frequent, however, and the method was discontinued. During the 1920's and early 1930's, the Japanese investigator Yamakawa and his associates(7) were among the first to administer artificially prepared fat emulsions intravenously. The numerous side reactions associated with these fat emulsions, which employed egg lecithin as the emulsifying agent, led to their discontinuance after a short period of trial in the United States.

In 1943, Stare and his group at Harvard(8) introduced the use of soybean phosphatides as an emulsifier in intravenous fat emulsions. While the most suitable of a large number of emulsifiers tested by Stare(9), it soon became evident that soybean phosphatides were capable of causing a number of adverse physiological reactions in laboratory dogs. These included hemolysis, urticaria, anemia, and granulomatous lesions in the lung and liver(10). Further purification of the commercial soybean phosphatides (Asolectin*) by solvent extraction resulted in the elimination of these effects in dogs(11),

*Associated Concentrates, Inc., Elmhurst, Long Island, New York.

but later it was found that the phosphatide-stabilized fat emulsions produced a marked decrease in arterial blood pressure when administered to cats or humans(12).

Up to this time the emulsions prepared by Stare and his co-workers had contained approximately 3 per cent soybean phosphatide, 15 to 30 per cent refined vegetable oil as the internal phase and isotonic dextrose solution as the external phase. The emulsions were prepared by high pressure homogenization. Reduction of the phosphatide content of the emulsions to approximately 1 per cent resulted in nearly complete elimination of the vasopressor response to the fat emulsions but also yielded an emulsion which was physically unstable(13).

In 1955 Stare(14) found that the addition of 0.2 to 0.5 per cent Pluronic F68* as a co-emulsifier allowed the reduction of the soybean phosphatide content to 1 to 2 per cent. The use of the polyoxyethyleneoxypropylene polymer, Pluronic F68, also was found to reduce the number of particles larger than 1 micron in diameter when phosphatides were also present.

While the emulsions investigated by Stare were not entirely satisfactory for routine administration to malnourished patients his preliminary investigations probably were in part responsible for the revival of interest in intravenous fat emulsion.

*Wyandotte Chemicals Co., Wyandotte, Michigan

A number of intravenous fat emulsions employing phosphatides as the emulsifying agent have been described in the patent literature(15)(16)(17)(18)(19). At least one formulation, Lipomul IV*, has undergone extensive clinical trial(20) and has been on the market for several years. This fat emulsion contains 15 per cent cottonseed oil, 1.2 per cent purified soybean lecithin, 0.3 per cent Pluronic F68, and 4 per cent dextrose. While purification of the soya lecithin apparently has resulted in products considerably less toxic than earlier preparations it has been recommended that the use of these fat emulsions be restricted to carefully selected patients with severe nutritional deficiencies.

The individual roles of the various components of a soybean phosphatide mixture with respect to possible undesirable physiological responses have not been adequately studied. There is some indication that the more acidic phospholipid components, such as the phosphatidyl ethanolamines and phosphoinositides, are primarily responsible for the observed side effects. This is largely an empirical observation based on the fact that removal of these components substantially reduces undesirable responses such as thermogenic reactions and vasopressor effects(17).

*The Upjohn Company, Kalamazoo, Michigan

While fractionation of a soybean phosphatide mixture to yield principally lecithin has resulted in improved patient tolerance to phosphatide-stabilized fat emulsions, there is some evidence that such highly purified preparations may also be more subject to decomposition. Lambert, miller and Frost(21) found that aqueous solutions of pure synthetic lecithin became considerably more toxic over a period of time than either purified egg lecithin or crude soybean phosphatides. The soybean phosphatide mixture apparently was the least subject to decomposition.

There is, moreover, some question of the physical function of the various phospholipid components of a soya phosphatide mixture when employed as emulsifiers. A study conducted by Yeadon, Goldblatt, and Altschul(22) on the emulsifying properties of lecithin in oil-in-water emulsions clearly indicated that highly purified lecithin was an inefficient emulsifier producing emulsions particularly unstable to autoclaving, a procedure required in the preparation of intravenous emulsions. Addition of various materials to the purified lecithin including fatty acids and Pluronic F68 improved the characteristics of the emulsions but none of the mixtures employing purified lecithin were as effective as commercial soybean phosphatides. It should be pointed out that the results of these workers may have been compromised to a certain extent due to the fact that the purification of the lecithin was carried out by chromatography on alumina. These

lecithin samples were possibly contaminated by appreciable amounts of lysolecithin formed during the separation(23). It should also be noted that purified egg lecithin was used in this study. Due to the greater degree of saturation of egg lecithin, as compared to soybean lecithin, it is possible some minor differences in their emulsifying properties might exist.

There would seem to be a need for a systematic investigation of the components of soybean phosphatides with the object of relating their chemical and physical properties to their function in emulsion systems. Until a better understanding of the surface chemistry of the phospholipids singly, and in combination with other phospholipids or surfactants is achieved, little progress can be made in correlating the properties of phosphatide-stabilized emulsions with their composition.

PLAN OF STUDY

This study consisted of three distinct areas of effort. Consequently, as an aid to clarity, this presentation is divided into three separate parts. The first part describes the methods used to isolate purified lecithin and lysolecithin and the chemical and physical properties of these and other soybean phospholipids. The second part includes the theory, design, construction and evaluation of a new recording film balance. The third part is concerned with the use of this apparatus for the study of the surface properties of lecithin and lysolecithin.

PART I

ISOLATION OF LECITHIN AND LYSOLECITHIN

PAST WORK

Separation of Phospholipids: The separation and identification of the components present in a phospholipid mixture is an exceedingly difficult problem. Their strong tendency to associate with each other tends to reduce the effectiveness of separation methods which exploit solubility differences. As a result a large number of fractionation techniques have been reported in the literature for the isolation of the various phospholipids from natural sources. These have included simple solvent extraction, fractional precipitation, complex formation, counter current extraction and chromatography.

Some of the earliest separations of purified lecithin involved the formation of a lecithin-cadmium chloride complex. Strecker(24) in 1868 observed that cadmium chloride precipitated phospholipids from an alcoholic solution. It was found that the lecithin complex with cadmium was considerably less soluble in alcohol than the complexes formed with the other phospholipids. Levene and Rolf(25) ultilized this property to prepare purified lecithin from egg yolk and liver tissue in 1927. Their method involved repeated extractions and washings of the

complex with various organic solvents. The cadmium was removed from the complex by addition of a solution of ammonia in methanol to a chloroform solution of the complex. Pangborn(26)(27) later simplified their procedure and obtained purified lecithin from egg yolk and beef heart. The principal difference between the method used by Pangborn and those used by previous investigators was the means employed to break the cadmium chloride complex with lecithin. While Levene and Rolf(25) used ammonia for this purpose, Pangborn(27) found the removal of cadmium could be accomplished more simply and conveniently by repeated extractions of a chloroform solution of the complex with 30 per cent ethanol. The complex dissociated under those conditions and the cadmium chloride was washed out in the dilute alcohol.

The cadmium chloride method has a number of disadvantages. These include the great number of operations which must be performed during an isolation, low yields, and an incomplete removal of cadmium from the lecithin.

A number of methods involving column chromatography have been reported in the literature for the separation of lecithin and other phospholipids. In general, it may be stated that adsorption chromatography has come to be recognized as the most effective method for the separation of complex mixtures of phospholipids. In 1944, Taurog, Entenman, Fries and Chaikoff(44) separated liver

phospholipids into a choline-containing fraction and a non-choline-containing fraction by adsorption chromatography on magnesia. In 1951, Hanahan, Turner and Jayko(28) obtained purified lecithin from egg yolk by adsorption chromatography on alumina columns. Lea and Rhodes(29) later found that sphingomyelins are also eluted along with egg lecithin separated from egg yolk by adsorption chromatography on alumina.

In 1955, Lea, Rhodes, and Stoll(30) found that the phosphatides of egg yolk could be fractionated by elution from silicic acid columns with a solvent mixture consisting of 20 per cent methanol in chloroform. They also demonstrated that purified lecithin obtained by chromatography on alumina contained large amounts of lysolecithin. This was confirmed by Renhonen(23), who found that lecithin undergoes considerable hydrolysis on alumina columns at room temperature.

Hanahan et al(31) employed column chromatography on silicic acid for the separation of the more complex mixtures of phospholipids found in rat liver, beef liver and yeast. The solvent systems employed by Hanahan in order of addition to the silicic acid columns were chloroform-methanol 4:1, 3:2, and 1:4, v/v. The order of elution of the phospholipids from the columns was first phosphatidyl ethanolamine, followed by the phosphoinositides, lecithin, lysolecithin, and sphingomyelin. While reasonable separations were

obtained with low load levels of less than 1 mg. of the phospholipid mixture per gram of silicic acid, some overlapping of the fractions did occur. This was particularly true of the phosphoinositides which were found to be contaminated with nitrogenous impurities.

Silicic acid chromatography also has been used for the fractionation of phospholipids derived from cabbage(32) and human serum(33).

It has been recognized for some time that purification of lecithin should be possible by treatment with a suitable ion exchange agent because most of the phospholipid contaminants are of an acidic nature(30). The introduction of ion exchangers with a low degree of cross-linkage improved the feasibility of such a separation of the relatively high molecular weight phospholipids. It was discovered that although it was possible to remove the acidic phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl inositol compounds by ion exchange, the lecithin was almost completely converted to the lyso-compound in the process. However, in 1960, Saunders and Perrin(34) found the use of the strong anion exchange resin Dowex $1x^4$ in the bicarbonate form permitted the removal of the acidic components of a methanol solution of egg phospholipids with a reduced amount of lecithin hydrolysis.

In 1961, Rouser, Bauman, and Kritchevsky(35) reported the use of diethylaminoethyl cellulose (DEAE) for

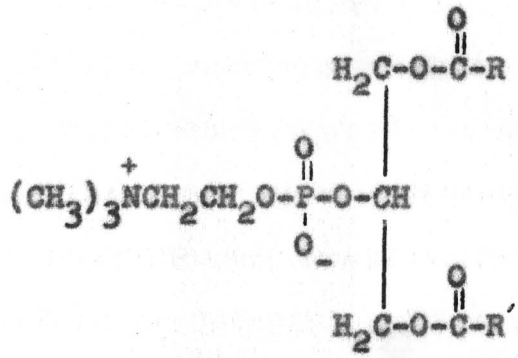
phospholipid separations. DEAE is an anion exchange agent prepared by treatment of cellulose to yield a material having diethylaminoethyl residues affixed by ether linkages to the alcohol groups of cellulose. The relatively low density of ionic sites on the cellulose fibers results in rather loose binding of high molecular weight compounds such as proteins. As the cellulose also acts as a stationary phase in chromatography the material has a dual nature which has proved to be effective for the separation of many high molecular weight substances under very mild conditions. Rouser and co-workers found that lecithin and phosphatidyl ethanolamine could be separated from soybean phosphatides by chromatography on DEAE. The lecithin was eluted first from the column with chloroform-methanol 7:1 and the phosphatidyl ethanolamine with chloroform-methanol 3:2. It was found some oxidation products of phosphatidyl ethanolamine also were eluted with the parent compound.

Since lysolecithin is a hydrolysis product of lecithin and occurs only in trace amounts in natural phospholipid mixtures, this substance is ordinarily prepared by synthesis or derived from the parent compound lecithin.

Lysolecithin may be prepared conveniently from lecithin by treatment of an ether solution of lecithin with snake venom Lecithinase A. This enzyme specifically catalyzes the release of the fatty acid constituent in the

β-ester position to yield α'-(acyl) lysolecithin. The lysolecithin formed during the reaction precipitates out of the ether solution after a short period of time. The method was introduced by Hanahan, Rodbell and Turner(36) in 1954, who employed Naia naia and Crotalus admanteus snake venoms as sources of Lecithinase A. Later, Saunders(37) modified their method slightly and also substituted Russell viper venom as the enzyme source.

existence of natural β-lecithin is unlikely. The isolation of β-lecithin from natural sources apparently was due to the formation of the β-form by phosphate migration during the isolation procedure(45)(46).



(III)

Even highly purified lecithin from a natural source is a highly complex mixture. This is due to the variation of the fatty acid residues of the molecule.

The fatty acid composition of the lecithin varies with the source. Thornton and co-workers(47) found that soybean lecithin purified by the cadmium chloride complex method had the following fatty acid composition:

palmitic	15.77%
stearic	6.30%
oleic	12.98%
linoleic	62.92%
linolenic	2.02%

Lecithin isolated from hen's eggs is considerably more saturated(2) with stearic and palmitic acids making up about 36 per cent of the total fatty acids.

In 1950, Baer and Kates(48) reported the first successful synthesis of fully saturated lecithins. Baer

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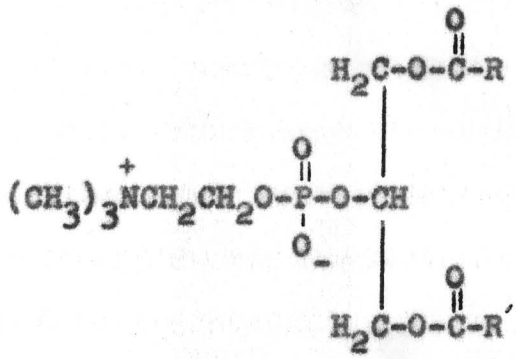

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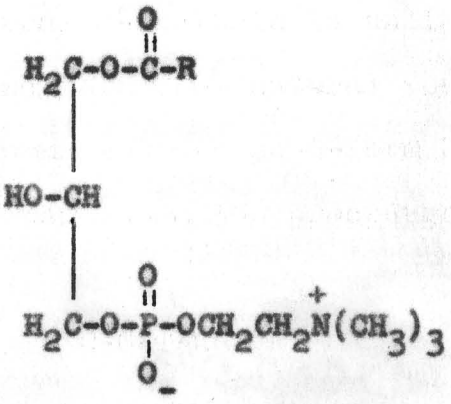
and Maurukas(49) subsequently simplified the synthesis in 1952.

Later in 1956, Baer and co-workers(50) were able to synthesize a fully unsaturated α-lecithin, L-α-dioleyl-lecithin. By comparison of the natural lecithins with synthetic lecithin of known configuration, Baer has shown that the configuration of the naturally occurring lecithins may be designated as belonging to the L-α-series relative to the stereochemical reference compounds D- and L-glyceraldehyde.

Since Baer's first synthesis of lecithin, methods have been worked out for the synthesis of almost all phospholipids of known structure and configuration(1)(6)(51). An interesting and important synthesis is the preparation of lecithins of known, mixed fatty acid composition. The synthesis of mixed-acid L-α-lecithins was achieved by the Dutch scientists de Haas and van Deenen(52)(53) in 1960. Later, these investigators devised a simpler, partial synthesis by ultilizing snake venom phospholipase to remove one fatty acid from the β-position of lecithin(54). The lyso-compound obtained was then re-esterified with the desired fatty acid at the β-position.

Lysolecithin: Lysolecithin (IV) is the partial hydrolysis product of lecithin formed by the loss of the fatty acid at the β-position.

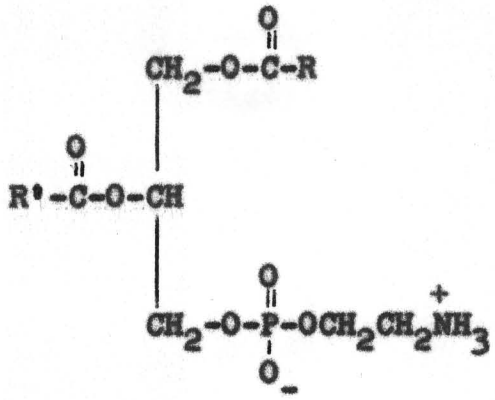


(IV)

The loss of one fatty acid residue from the β-position of lecithin to form the α'-(acyl)lysolecithin is catalyzed by a component of cobra venom known as lecithinase A. It has been conclusively demonstrated that the lecithinase A splits off only the fatty acids attached to the β-position of lecithin(55)(56)(57)(58).

While traces of lysolecithin are found in most phospholipid extracts, the use of snake venom is the most convenient method for the preparation of lysolecithin. In 1954, Hanahan, Rodbell, and Turner(36) found that the enzyme-catalyzed hydrolysis of lecithin to form lysolecithin proceeds in ether solution on addition of a small volume of an aqueous solution of the snake venom. According to their procedure, the mixture is shaken thoroughly and the lysolecithin, being insoluble in ether, precipitates in good yield after a short interval of time. As the enzyme will attack other phospholipids such as phosphatidyl ethanolamine, the lyso-compounds of other phospholipids present in the reaction mixture will also be formed.

Phosphatidyl Ethanolamine: At one time it was believed that phosphatidyl ethanolamine constituted the alcohol-insoluble portion of a phospholipid mixture. The alcohol-insoluble fraction was called cephalin and was believed to differ in structure from lecithin by containing ethanolamine as the base rather than choline. Accordingly, cephalin was assigned the structure (V) corresponding to the compound phosphatidyl ethanolamine.



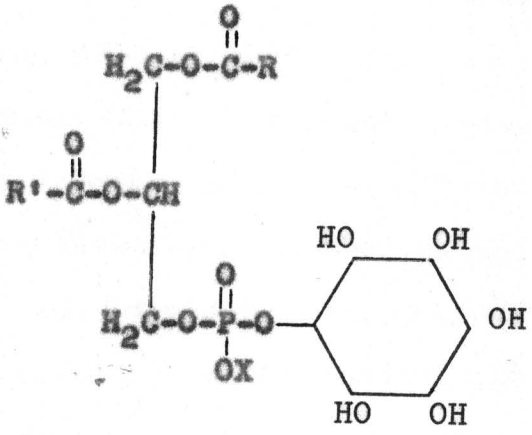
(V)

Eventually it was discovered that although the structure (V) suggested for phosphatidyl ethanolamine is correct for a major constituent of soybean and brain phospholipids, the compound itself is quite soluble in the warm ethanol used as a basis for the separation of the two major fractions. Thus the alcohol-soluble fraction consists of phosphatidyl choline, phosphatidyl ethanolamine and other minor contaminants. The alcohol-insoluble fraction still is occasionally referred to as the cephalin fraction but it is incorrect to designate any specific phospholipid as a single compound, cephalin.

Phosphoinositides: The alcohol-insoluble fraction still has not been adequately characterized. It consists predominently of a group of compounds known as the inositol phospholipids, phosphoinositides, or lipositols.

While the existence of the phosphoinositide fraction has been recognized for some time(59)(60), it is only in the past few years that appreciable progress has been made in its characterization(36). Apparently this group consists of simple monophosphoinositides and other more complex phosphoinositides.

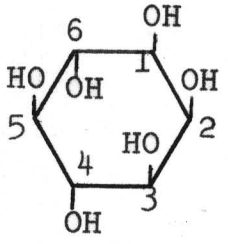
The monophosphoinositides are diacyl esters of glycerylphosphorylinositol (VI).



(VI)

X = Na, Mg, K, or Ca

The hexahydroxycyclohexane, inositol, apparently is present to a large extent as myo-inositol(VII).

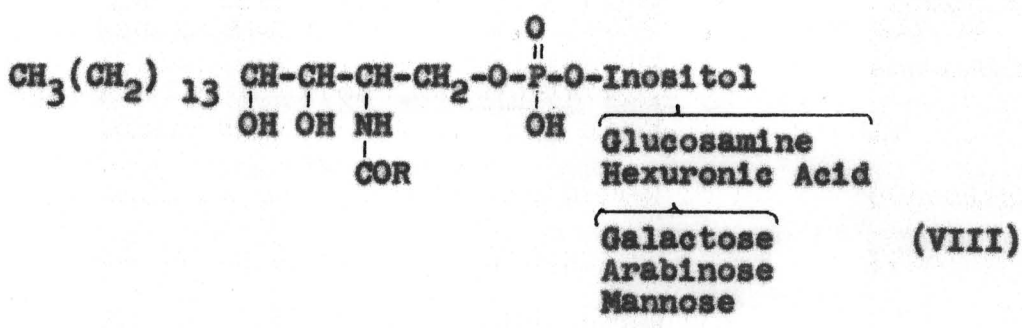


(VII)

While some investigators previously had speculated that the most likely site of attachment would be the number 2 position of inositol(61)(62), Ballou and co-workers(63)(64)(65) have established that the phosphatidyl group is attached to the number 1 position of myo-inositol in simple phosphoinositides isolated from soybean. They suggest that this compound be designated 1-phosphatidyl-L-myo-inositol.

Folch(66)(67) reported the existence of diphosphoinositides in beef brain in 1949. Subsequently, in 1961, Ballou et al(63)(68) concluded that the myo-inositol components present in beef brain actually are triphosphoinositides. The structures of these compounds are still uncertain at this time.

Other complex phosphoinositides may exist in soybean phosphatides. Carter and co-workers(69)(70) have reported the existence of a complex inositol-phytosphingosine containing lipid in plant seed phospholipids which they termed phytoglycolipide. A partial structure (VIII) was proposed.



(VIII)

Physical Properties of the Phospholipids

Lecithin: Pure lecithin is a white, waxy solid which is extremely hygroscopic. Natural lecithin containing unsaturated fatty acids quickly darkens in color and develops a disagreeable odor and taste. Once isolated in a pure state, great care must be taken to prevent its decomposition by protection from heat, light, water and atmospheric oxygen. Pure lecithin is usually preserved by dissolving it in anhydrous, absolute alcohol and storing the solution under nitrogen at low temperatures.

Although natural lecithin has no definite melting point, Baer and Maurakas(49) observed sintering points of 90° C., 120° C., and 120° C. for L-α-(dimyristoyl)-lecithin, L-α-(dipalmitoyl)lecithin and L-α-(distearoyl)-lecithin, respectively. They reported that the meniscus points were much more reproducible and found values of 226-227° C., 225-226° C., and 222-223° C., respectively, for these same compounds.

The L-α-lecithins possess an asymmetric carbon and are dextrorotatory(49).

Lecithin is readily soluble in most of the common fat solvents including ether, chloroform, carbon tetrachloride and petroleum ether. It is also soluble in pyridine, ethanol, methanol and acetic acid. It is insoluble in acetone. The fully saturated lecithins, or hydrolecithins,

have somewhat different solubility properties, being insoluble in ether, acetone and cold ethanol.

Saunders and Elworthy(71) have reported that purified natural lecithins can be obtained in crystalline form by precipitation from acetone-methylethyl ketone mixtures.

The lecithins from natural sources form cloudy, colloidal solutions with water which are quite stable(72). Synthetic hydrolecithins also form sols with water but these settle out rapidly.

Lecithin sols may be prepared by a number of methods, including simple shaking of the solid with water. It has been found that these sols are easily precipitated by small concentrations of divalent metal ions but are stabilized by higher concentrations(71). Recently, Saunders, Perrin and Gammack(73) have prepared clear lecithin sols by ultrasonic irradiation of turbid sols of both egg and unsaturated synthetic lecithins. These sols were found to contain micelles of molecular weight about 10^7. The sols were stable to electrolytes.

Lysolecithin: While lysolecithin resembles lecithin in some of its physical properties, it is strikingly different in others. When pure, lysolecithin is a white, hygroscopic solid. As with lecithin, precautions must be taken to retard the degradation of lysolecithin. Lysolecithin, due to an unexplained natural tendency for distribution of predominently saturated fatty acids at the α'-position,

is considerably more saturated than lecithin and therefore is less subject to oxidation.

Lysolecithin may be obtained as needlelike crystals by crystallization from pyridine(74) or from ethanol(37). Like lecithin, lysolecithin has no definite melting point. Lysolecithin apparently is dextrorotatory in pyridine, glacial acetic acid and ethanol solutions but is levorotatory in chloroform solutions(75).

Lysolecithin is soluble in chloroform, methanol, ethanol, pyridine and acetic acid. It is insoluble in ether, petroleum ether and acetone. Lysolecithin dissolves in water to give optically clear solutions. Saunders and Robinson(76) found that thick fluid solutions were formed at concentrations of 50% by weight. They found that lysolecithin is highly surface active, concentrations of less than 0.001 per cent w/v lysolecithin reducing the surface tension of water to 45.35 dyne/cm. Increasing the concentration of lysolecithin to 1 per cent produced a further lowering only to 37.6 dyne/cm. The critical micelle concentration of lysolecithin in water was estimated to be less than 0.01 per cent(77). It was also found that no appreciable lowering of surface tension occurred in chloroform and ethanol solutions of lysolecithin in the concentration range 0.001 to 0.1 per cent w/v.

Other Phospholipids: Pure phosphatidyl ethanolamine is a white, waxy solid which is extremely sensitive to

atmospheric oxidation and light(78). The solubility properties of phosphatidyl ethanolamine are similar to those of lecithin.

Little information is available on the physical properties of pure phosphoinositides. In contrast to most of the other phospholipids, they are known to be relatively insoluble in alcohol. Hanahan and Olley(62) found that monophosphoinositides prepared from liver were stable, solid compounds that could be stored for long periods of time.

EXPERIMENTAL

Source of Lecithin and Lysolecithin: Since the group of compounds known as the phospholipids are found in varying concentrations in all living cells, a large number of materials from natural sources may be used as a source of lecithin. Eggs, brain and liver tissue and soybean oil are excellent sources. As the lecithin used in the pharmaceutical, food and cosmetic industries usually is derived from soybean oil, a soybean phosphatide mixture was chosen as the starting material. Soybean oil contains from 1.6 to 3.0 per cent phosphatides. Commercial lecithin derived from soybean oil ordinarily is a yellow, granular substance. Asolectin*, the soybean phosphatide mixture used in this study, contains roughly equal amounts of lecithin, phosphatidyl ethanolamine and phosphoinositides amounting to approximately 95 per cent of the total. The remaining 5 per cent consists mainly of oil, waxes and sterols.

Other Materials and Equipment: U.S.P. grade diethyl ether and ethanol were used for preliminary solvent extractions or precipitations. Prior to use, the diethyl ether was passed through an alumina column to remove the peroxides(79). Reagent methanol was shaken with calcium

*Obtained from Associated Concentrates, Inc., Elmhurst, Long Island, N.Y.

sulfate and redistilled prior to use. The reagent grade chloroform was also redistilled before using. The deionized water was prepared by passing distilled water through a mixed bed resin (Bio-Rad AG 501-X8 (D)) followed by redistillation.

The silicic acid (Mallinckrodt, 100 mesh) and alumina (aluminum oxide, reagent, Merck) were activated by heating overnight at 110° C. The Dowex 1X4 resin (50-100 mesh) was obtained in the chloride form. It was converted to the bicarbonate form by placing the resin in a large chromatography column and washing it first with 2 liters of 5 per cent sodium hydroxide solution. The resin was then washed with distilled water until the wash was neutral to litmus. This was followed by 5 liters of 5 per cent sodium bicarbonate solution and the resin again rinsed with deionized water until neutral to litmus. About 2 liters of absolute methanol were then allowed to drain slowly through the column in order to remove most of the water from the resin.

The applicator used for preparation of the silica gel plates for thin-layer chromatography was of the type designed by Mutter and Hofstetter(82). The Silica Gel G (prepared according to Stahl) was applied on 20 x 20 cm. plates to a thickness of approximately 250 microns. The plates were activated before use by heating at 110° C. for 60 minutes.

The developing solvent mixtures for the thin-layer chromatography were chloroform-methanol-glacial acetic acid-water 65:25:8:4, v/v, and chloroform-methanol-water 65:25:4, v/v. The solutions were prepared with redistilled, reagent grade methanol and chloroform. The glacial acid, reagent grade, was used without redistilling.

The ninhydrin spray was prepared by dissolving 0.2 per cent ninhydrin in butanol saturated with water. The ammonium molybdate-perchloric acid spray was prepared by dissolving 3 g. of ammonium molybdate in 50 ml. of water, 5 ml. 6N HCl and 13 ml. of 70 per cent perchloric acid.

Isolation of Lecithin by Ion Exchange: The method used for isolation of lecithin was essentially the same as that used by Saunders and Perrin(80)(81) for the preparation of pure lecithin from egg yolk.

Two hundred g. of Asolectin were extracted with 1 liter of 95 per cent ethanol by shaking on a mechanical shaker for 6 hours. The solution was decanted off and the residue extracted again with an equal volume of fresh solvent. The two extracts were pooled and evaporated to dryness at room temperature with a Rinco rotary evaporator. About 50 g. of residue was obtained. This was dissolved in a minimum of ether and the crude lecithin precipitated by pouring the ether solution into a volume of acetone 4 to 5 times greater than that of

the ether solution. The solvent was decanted off and the procedure repeated 3 times.

The residue was redissolved in 1 liter of absolute methanol and shaken for no longer than 5 minutes with 100 g. of Dowex 1X4 resin (50-100 mesh) in the bicarbonate form. After the lecithin solution had been rapidly decanted from the Dowex 1X4 resin it was evaporated to dryness with the rotary evaporator at room temperature. About 20 g. of residue were obtained. A spot test with 0.2 per cent ninhydrin in methyl cellosolve was performed on a few mg. of the residue. The test was negative indicating essentially complete removal of phosphatidyl ethanolamine.

Two hundred g. of Mallinckrodt silicic acid (100 mesh) were slurried with chloroform-methanol, 4:1 by volume, and packed into a 2.1 inch diameter column. The lecithin was dissolved in a minimum volume of chloroform and added to the column. After the solution had soaked into the silicic acid packing, the inner walls of the column were carefully rinsed with a small volume of chloroform which was also allowed to soak into the silicic acid. This was repeated once and then a sufficient volume of the chloroform-methanol 4:1 mixture was added to the column to provide enough head for a reasonable flow rate. In some of the columns about 20 per cent Celite was added to the silicic acid to improve the flow rate of the column.

The first material to move down the column was observed as a sharp yellow band. Saunders and Perrin(81) observed a similar band in the chromatographic separation of egg lecithin and have suggested it may consist of highly colored pigments. This fraction was disgarded and the bulk of the lecithin fraction was collected in the next 1000 to 1500 ml. of solvent. The lysolecithin was retained by the column but could easily be eluted by changing the eluting solvent to pure methanol. Fig. 1 shows the silicic acid chromatography of a sample of the residue obtained by treatment of soybean phospholipid with Dowex 1X4. The lecithin was eluted easily with chloroform-methanol 4:1, v/v, and the lysolecithin with anhydrous methanol.

The lecithin fraction was evaporated to dryness at room temperature with the rotary evaporator and the residue dissolved in ether and precipitated in acetone. This was repeated 3 times.

The purified lecithin obtained by this procedure was a nearly-white, waxy solid. It was dissolved in anhydrous, absolute ethanol and stored under nitrogen in the freezing compartment of a refrigerator. The yield from such an isolation varied considerably but was usually 6 to 8 g.

Preparation of Lysolecithin: Lysolecithin was prepared by Saunders modification(37) of Hanahan's method(36). The

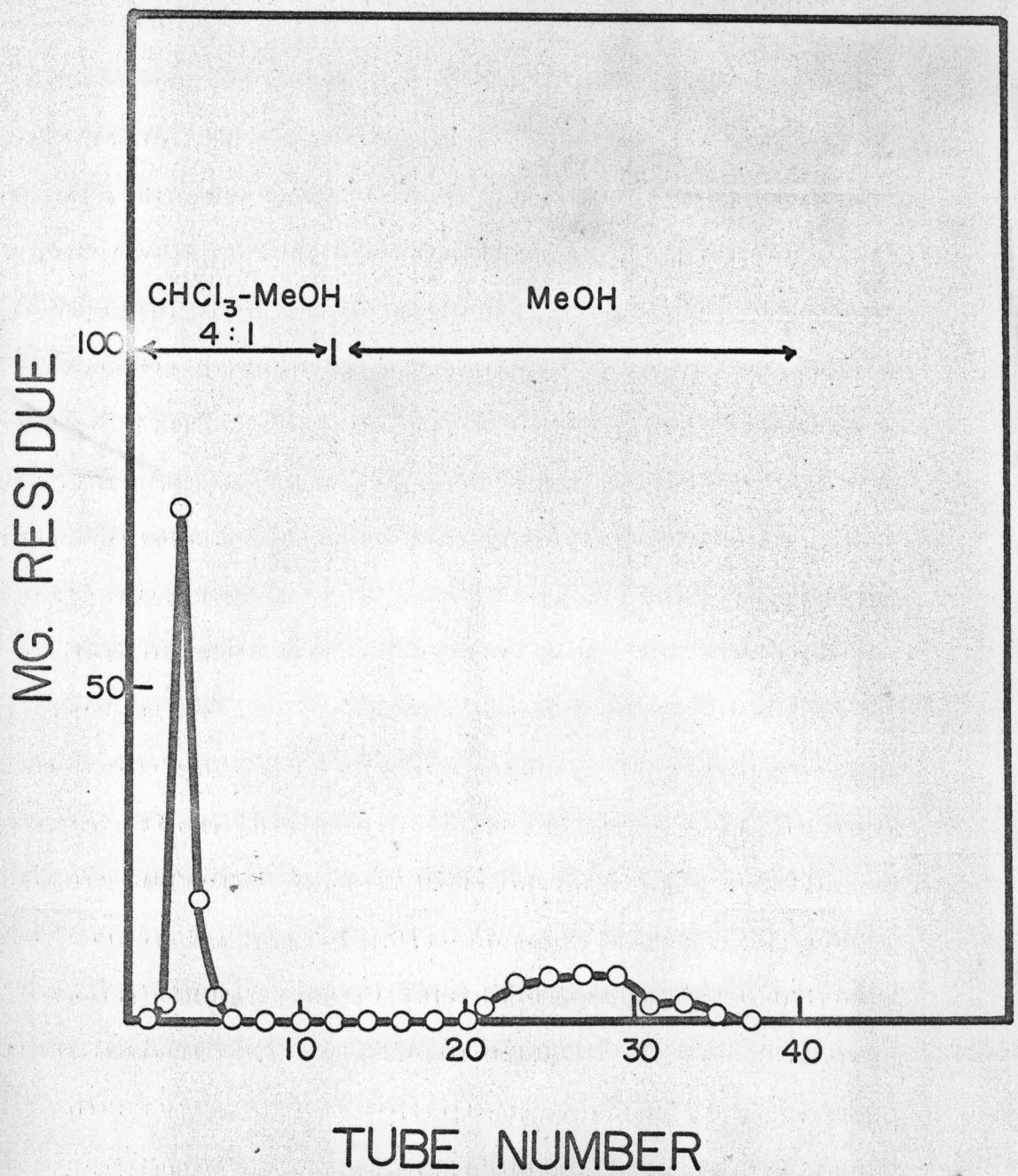


Fig. 1. **Silicic acid chromatography of soybean phosphatides treated with Dowex 1X4 ion exchange resin.**

procedure was the same as for the preparation of purified lecithin up to the point of the separation of lecithin from lysolecithin by adsorption chromatography on silicic acid. At this point, the residue obtained from the treatment with the anion exchange resin was dissolved in 1 liter of ether. Ten mg. of lyophilized Vipera russellii venom* dissolved in 10 ml. of water were added and the flask shaken for a few minutes. The lysolecithin precipitated out overnight and the solvent was decanted off. The lysolecithin was washed with ether and then with acetone. The lysolecithin then was dissolved in a minimum amount of chloroform and precipitated by pouring it into a 6 times greater volume of ether. This was repeated 4 times. The lysolecithin was dissolved in 200 ml. of warm absolute ethanol and centrifuged to remove traces of ethanol insoluble material. The alcoholic solution was decanted off, evaporated to dryness in a rotary evaporator and recrystallized from warm ethanol. The yield was low, amounting to 2 to 3 g. for an alcoholic extract of 100 g. of Asolectin. The purified lysolecithin was dissolved in anhydrous, absolute ethanol and stored under nitrogen in a refrigerator.

Results and Discussion: The identity of the purified lecithin and lysolecithin was confirmed by their solubility

*Obtained from Miami Serpentarium, Miami 56, Florida

properties and infrared spectra. The purified lecithin was found to be soluble in ether, chloroform, methanol, and ethanol, and was insoluble in acetone. The lysolecithin was soluble in chloroform, methanol and ethanol, and was insoluble in ether and acetone. The infrared spectrum of the lecithin was essentially the same as the infrared tracing obtained for a sample of L-α-(dimyristoyl)-lecithin generously provided by Dr. Erich Baer of the University of Toronto. The infrared spectrum of the purified lysolecithin was nearly identical to the literature spectrum for lysolecithin(36).

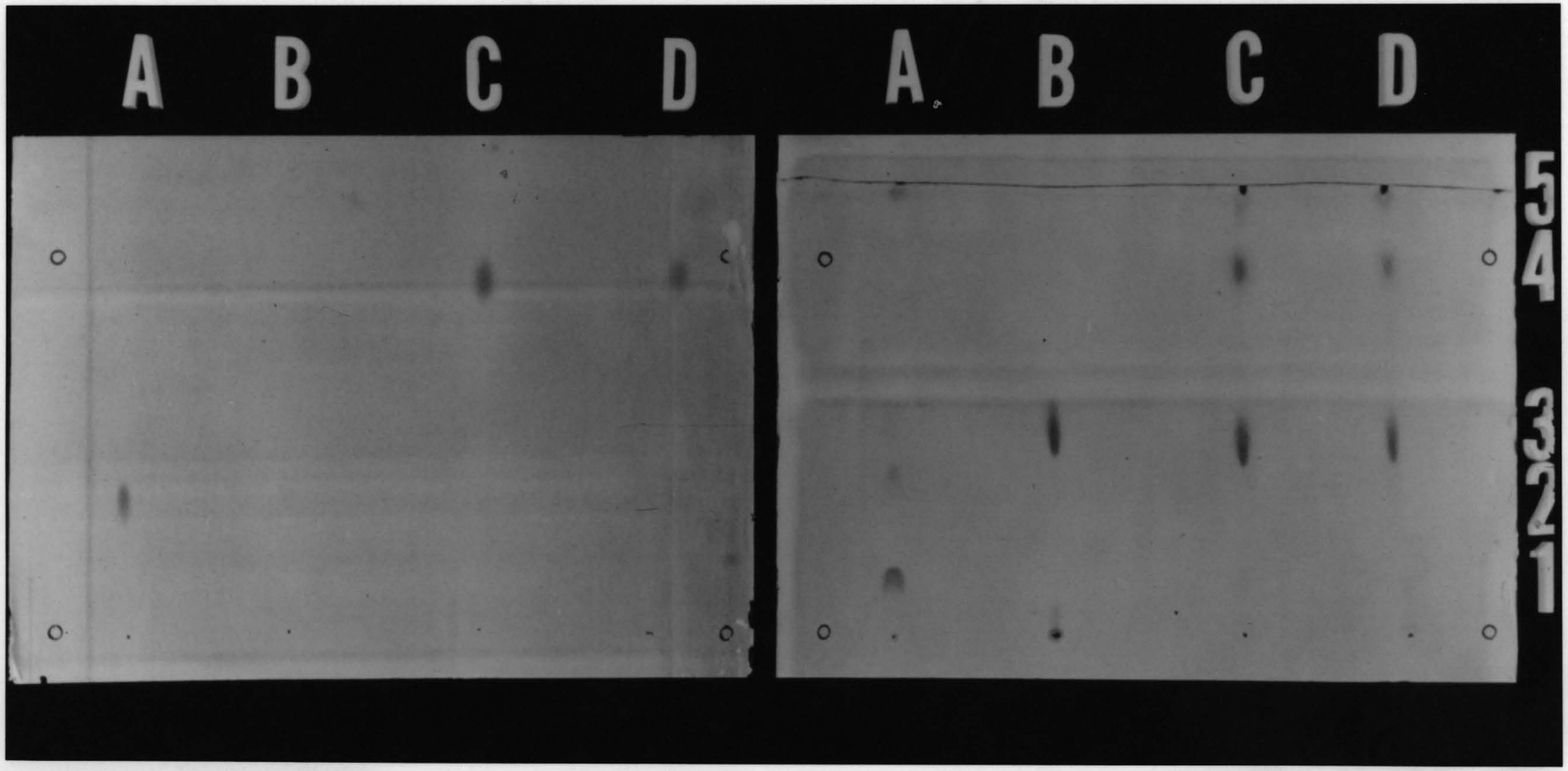
The purity of the purified lecithin and lysolecithin was estimated by thin-layer chromatography on Silica Gel G. The chromatograms for purified lysolecithin, purified lecithin, Asolectin and Armour sterile lecithin are shown in Fig. 2 and 3. Two different developing solvent mixtures which have been found effective for the thin-layer chromatography of phospholipids were used(82)(83)(84). The chromatograms, Fig. 2, were obtained using chloroform-methanol-glacial acetic acid-water 65:25:8:4, v/v. The chromatograms, Fig. 3, were obtained with chloroform-methanol-water 65:25:4, v/v, as the developing solvent. The phospholipid samples were applied to the silica gel plate in amounts of approximately 500 micrograms from 2 per cent chloroform solutions. The chromatogram on the left in each figure was developed with ninhydrin spray and the chromatogram on the right with ammonium

molybdate-perchloric acid spray. The ninhydrin spray detected phospholipids with free amino groups while the ammonium molybdate-perchloric acid spray was used as a universal indicator.

The chromatograms were compared with those given in the literature for mixtures of synthetic phospholipids(82)(83)(84). The purified lecithin was detected as a single spot with the molybdate-perchloric acid spray in both Fig. 2 and 3. The ninhydrin plates did not reveal the presence of contaminating amino compounds. The lysolecithin was stained with difficulty with the molybdate-perchloric acid and appeared only as a faint spot in both figures. The chromatograms showed that the purified lysolecithin contained traces of contaminates, principally a ninhydrin-reactive material suspected to be lyso-phosphatidyl ethanolamine.

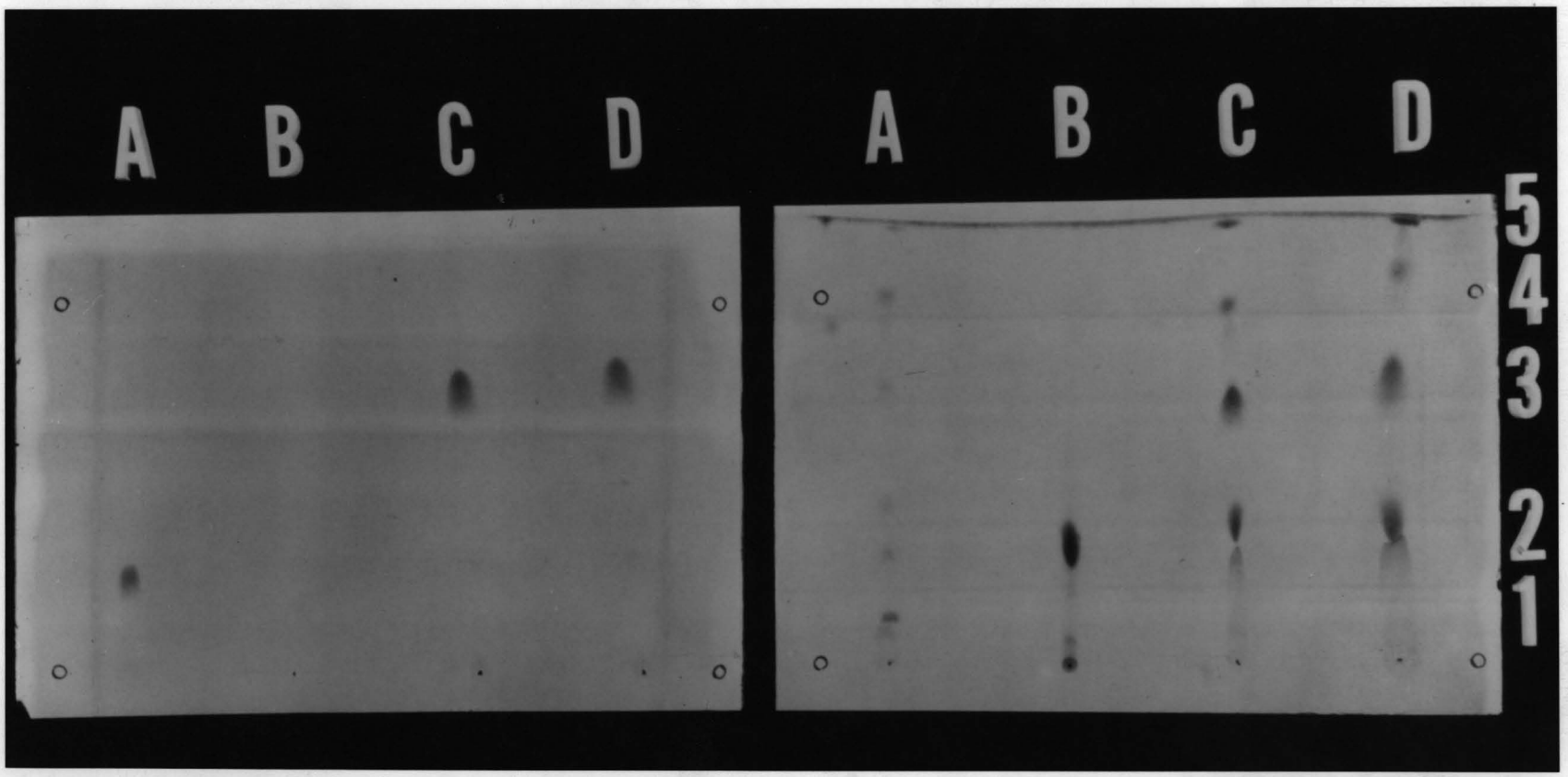
The thin-layer chromatograms indicated the soybean lecithin was obtained in a high state of purity. The lysolecithin apparently contained traces of other compounds. This suggests that, at least with soybean lysolecithin, it may be desirable to prepare the lysolecithin by direct treatment of previously purified lecithin.

Fig. 2. Thin-layer chromatograms obtained with chloroform-methanol-glacial acetic acid-water 65:25:8:4 as the developing solvent. Detection methods: left plate, ninhydrin spray; right plate, ammonium molybdate-perchloric acid spray. Samples: (A) purified soybean lysolecithin, (B) purified soybean lecithin, (C) Asolectin,



and (D) Armour Sterile Lecithin. Spots: (A-1) right plate, lysolecithin; (A-2) left plate, ninhydrin-reactive contaminant of lysolecithin; (B-3)(C-3)(D-3) right plate, purified lecithin and lecithin as a component of Asolectin and Armour Sterile Lecithin; (C-4)(D-4) both plates, ninhydrin-reactive components of Asolectin and Armour Sterile Lecithin; and (5) both plates, solvent front.

Fig. 3 . Thin-layer chromatograms obtained with chloroform-methanol-water 65:25:4 as the developing solvent. Detection methods: left plate, ninhydrin spray; right plate, ammonium molybdate-perchloric acid spray. Samples: (A) purified soybean lysolecithin, (B) purified soybean lecithin, (C) Asolectin, and (D) Armour Sterile Lecithin. Spots: (A-1) right plate, lysolecithin; (A-2) left plate,



ninhydrin-reactive contaminant of lysolecithin; (B-2)(C-2)(D-2) right plate, purified lecithin and lecithin as a component of Asolectin and Armour Sterile Lecithin; (C-3)(D-3) both plates, ninhydrin-reactive components of Asolectin and Armour Sterile Lecithin; (C-4)(D-4) right plate, unidentified component of Asolectin and Armour Sterile Lecithin; and (5) both plates, solvent front.

PART II. DEVELOPMENT OF A RECORDING FILM BALANCE

THEORY AND PAST WORK

Surface Films: Probably any discussion of surface films must begin with J. Willard Gibbs, who in 1878 derived the exact relationship between adsorption and surface tension. According to thermodynamics, processes which occur spontaneously are accompanied by a decrease in free energy. If a solute lowers the free surface energy of a solution there must be a greater proportion of solute to solvent at the interface than in the bulk of the solution. The Gibbs equation for binary systems is

$$\Gamma = -\frac{(a)}{(RT)}\frac{(d\gamma)}{(da)} \qquad (1)$$

where Γ is the excess surface concentration of one component, a is the activity of the solute, R is the gas constant, T the absolute temperature and γ is the surface or interfacial tension. Γ is called the surface concentration of solute per unit area of interface. It is not strictly a concentration term as it represents a number of moles divided by an area.

If a solute causes a decrease in surface tension, $(d\gamma/da)$ is negative and the surface concentration of the solute is greater than the concentration in the bulk solution. If the solute causes an increase in surface tension, $(d\gamma/da)$ is positive and the surface concentration

of the solute is less than the concentration in the bulk solution. This phenomenon is exhibited by some electrolytes and is called negative adsorption.

While very large decreases in surface tensions as a result of accumulation of a surface active agent at an interface are possible because the surface tension can approach that of the solute, negative adsorption will result in only small changes in the surface or interfacial tension. This is because it represents a situation where solute is being rejected from the surface. Thus the surface tension of the solution differs but slightly from the surface tension of the pure solvent.

The experimental verification of the Gibbs equation by direct measurement of the amount of substance adsorbed at the surface of solutions has been attempted by various methods. Initially, gross discrepancies between theory and experimental results were frequently found. More recent experiments, particularly those which have ultilized tracer techniques(85)(86), have shown closer agreement between experimental values obtained for surface adsorption and those values predicted by the Gibbs equation.

Spreading and Formation of Films: The spreading of liquids on other liquids or solids was not well understood prior to 1922. In that year, Harkins and Feldman(87) clearly explained the spreading process in terms of thermodynamics. They proposed that any material could spread on any

substance providing the spreading process was accompanied by a decrease in free energy. According to this theory, a substance will spread only if the work of surface adhesion, W_A, between substance and subsolution is greater than the work of cohesion, W_C, of the substance to be layered. The difference between these terms is called the spreading coefficient. The spreading coefficient, $S_{b/a}$, of substance b on liquid a is defined as:

$$S_{b/a} = W_A - W_C \qquad (2)$$

The work of adhesion, W_A, is the work necessary to pull apart 1 cm.^{2} of the interface ab and is given by the equation of Dupre':

$$W_A = \gamma_a + \gamma_b - \gamma_{ab} \qquad (3)$$

where γ_a is the surface tension of the subsolution a, γ_b is the surface tension of substance b, and γ_{ab} is the interfacial tension of the interface ab. In terms of surface free energy this equation represents the disappearance of unit surfaces a and b and the appearance of unit interface ab. The work of cohesion, W_C, is defined as

$$W_C = 2\gamma_b \qquad (4)$$

where γ_b is equal to the free surface energy of 1 cm.^{2} of substance b. More specifically, it is the work required to break apart a bar of liquid 1 cm.^{2} in area to yield two

surfaces which have a combined area of 2 cm.^{2}. Thus equation (1) may also be represented as:

$$S_{b/a} = \gamma_a - (\gamma_b + \gamma_{ab}) \qquad (5)$$

If spreading is accompanied by a decrease in free energy, i.e., the spreading coefficient is positive, then spreading is spontaneous. The equation for the spreading coefficient predicts a substance will spread on a liquid of higher surface tension. For this reason almost all organic liquids will spread on a clean water surface. By the same token, virtually any liquid should spread on a clean mercury surface.

Equation (5) applies for pure liquids and is called the initial spreading coefficient. As the thin spreading liquid b becomes saturated quickly with a, a semi-initial spreading coefficient is defined

$$S_{b'/a} = \gamma_a - (\gamma_{b'} + \gamma_{ab'}) \qquad (6)$$

where the prime indicates b is saturated with a. If the semi-initial spreading coefficient is also positive, this indicates b will spread on a even when b is saturated with a.

If the two liquids become mutually saturated then a final spreading coefficient is defined:

$$S_{b'/a'} = \gamma_{a'} - (\gamma_{b'} + \gamma_{a'b'}) \qquad (7)$$

If this value becomes negative, as is often the case, then b will not spread on a. If spreading had occurred initially before mutual saturation, then the liquid b

will retract to form a lens. In this instance, the surface will have a tension corresponding to a Gibbs monolayer for a saturated solution of b in a.

Film Pressure: If the surface tension of a liquid is lowered by the addition of a solute, then, as predicted by the Gibbs equation, the solute must be adsorbed at the surface or interface. This adsorption may be sufficient to produce a molecular layer or film of solute molecules on the surface. The monolayer may be considered to exert a film pressure, π, which may be defined as

$$\pi = \gamma_{solvent} - \gamma_{solution} \qquad (8)$$

where $\gamma_{solvent}$ is the surface tension of the solvent and $\gamma_{solution}$ is the surface tension of the film covered solution. One way to view the film pressure is to consider it as being a force which has the tendency to expand the surface, as opposed to surface tension which tends to contract the surface. The surface pressure has also been likened to a two-dimensional pressure. Surface pressure has the units of dyne/cm. and can be measured directly by a number of means.

Harkins(88) has said that a film exists wherever a layer which has a different composition from the body of the liquid or solid is present at the boundary surface. The area and shape of the film are independent of the gravitational forces which are present. If the area and shape of the layer depend on both surface and gravitational

forces, a lens is said to exist.

In general, films may be formed at the gas-liquid interface by:

(1) adsorption from a vapor

(2) adsorption from the solution

(3) spreading of the substance by placing the solute itself, usually dissolved in a volatile solvent, directly on the surface.

Films may be classified as monolayers where the film is the thickness of one molecule, and as duplex films where the film is thick enough that the two interfaces are independent and possess their own characteristic surface tensions.

When a monolayer of an insoluble substance such as a fatty acid is layered on the surface of water and the available surface area slowly reduced, the surface pressure will increase to a maximum and the film will collapse. If the film pressure is plotted versus the area per molecule at constant temperature, an isotherm is obtained which is analogous to the familiar pressure-volume isotherms of gases.

Monomolecular films have been found to exhibit 6 different surface phases in contrast to three-dimensional systems which possess only 3 states of matter(89)(90). These 6 phases have been designated as: (1) gas phase, (2) liquid expanded phase, (3) intermediate liquid

phase, (4) liquid condensed phase, (5) superliquid phase and (6) solid phase. The phase relations of monlayers have been discussed in detail by Harkins(91), Dervichian(92) and Adam(93).

There are a number of factors which may influence the isotherm for a monolayer of a particular substance. The type of isotherm obtained is largely a function of the temperature. Increasing the temperature results in expansion of the isotherm. The subsolution also may have a considerable influence on the behavior of the monomolecular film. In general, this may be related to the affinity of the polar group of the film-forming substance for the water of the subsolution. It appears that the greater the solubility of this polar group in the subsolution, the higher is the surface pressure a fluid monolayer can resist before collapse occurs(92). In agreement with this, it has been found that films of triglycerides or esters in the fluid state collapse at pressures below 20 dyne/cm. and fatty acids withstand pressures up to 30 dyne/cm. Lecithin, which possesses a highly polar group, forms monolayers which can be compressed to pressures well above 40 dyne/cm.

As would be expected, the pH and ionic strength of the subsolution are important factors. pH has been observed to strongly influence the behavior of fatty acid monolayers. At low pH values the films are contracted

due to suppression of the ionization of the carboxyl groups of the fatty acid. Expansion of the film occurs at high pH values.

Harrap(94) has shown that the surface pressure of insulin monolayers is at a minimum at the isoelectric point. Changes in the ionic strength of the subsolution exerted the least effect at pH values near the isoelectric point.

Harkins and Myers(95) have studied the effect of calcium ion on stearic acid films. The effect of the calcium ion was to convert the liquid film into a solid film. This was considered to be purely a chemical effect resulting from formation of calcium stearate.

When chemical effects are absent, the effect of a neutral salt is to increase the area of the monolayer. Some substances such as phenanthrene, which do not give a film on water, yield films on concentrated salt solutions. The short chain fatty acids such as myristic acid also have been studied on concentrated salt solutions.

MacRitchie and Alexander(96) found that the addition of dextrose to protein films appreciably increased the stability of these films at higher pressures. The isotherms were also displaced to slightly higher areas per molecule. The explanation for this was thought to be the effect of sucrose on hydrogen bonding of the

protein film with the subsolution.

As might be expected, there is no general rule which may be ultilized to predict the behavior of mixed films. The properties of mixed films apparently range from near ideal behavior, as seen with two similar long chain fatty acids, to the extreme of what appears to be complex formation.

A general observation is that solutions of two liquid type films ordinarily yield a mixed film of the same type. Addition of a solid type film to a liquid type may condense the liquid film. In such a system the molar ratio determines whether contraction or expansion occurs.

Schulman(97) has found that mixed monolayers in which one of the components is a crooked molecule such as oleic acid are unstable at pressures above the collapse pressure of the oleic acid. At this point, the oleic acid appeared to be forced out of the film. The squeezed-out oleic acid formed a visible lens. It was then possible to compress the film to the collapse point of the second film component.

The question of whether actual complex formation occurs in mixed monolayers has been a subject of controversy for nearly 30 years. The question may be in part a matter of terminology. If the term complex is restricted to include only molecular associations with

definite dissociation constants and equilibrium pressures, then apparently some of the conclusions drawn by certain investigators over the past 20 or 30 years may be questionable.

Dervichian(98) has shown that in some cases the observed expansions or contractions of mixed films should probably be explained simply in terms of changes in physical state rather than by complex formation. This has been shown to be true with mixtures of fatty acids. Dervichian has also questioned the practice of assuming complex formation merely because of the existence of definite breaks in the plots obtained by plotting molecular area per molecule versus the mole fractions of the components at a fixed film pressure. The breaks in the plots often correspond to definite molecular ratios of the two film species. Such a plot of mixed lecithin and cholesterol films shows breaks corresponding to two different molecular ratios: cholesterol 3, lecithin 1 and cholesterol 1, lecithin 3. Dervichian pointed out that certain preferred orientations of long chain components which make up such a film might logically be expected and that the breaks in the cholesterol-lecithin plot could well represent ratios which are preferred by reasons of symmetry alone rather than as a result of complex formation.

In 1935 Schulman and Hughes(99) observed that large changes in film pressure and surface potential could be obtained by the injection of soluble surface active materials beneath an insoluble monolayer. This phenomenon was termed film penetration. Schulman has considered film penetration may result in the formation of definite compounds or complexes. The fact that films which have been penetrated may be more stable to surface pressure than either of the two film components has been cited as evidence for the existence of complexes.

Schulman and Cockbain(100) have proposed that in order for stoichiometric complexes to be formed by film penetration, strong polar and strong or moderately strong van der Waal's interactions must be possible between the two film components. Schulman and co-workers have studied a number of systems in which film penetration was believed to have resulted in complex formation. These systems include cetyl alcohol and sodium cetyl sulfate(101), octadecylamine and soluble organic acids such as benzoic acid(100) and cholesterol and saponin(102).

At least two investigators, Harkins(103) and Dervichian(92)(98) have questioned whether Schulman and his co-workers have actually demonstrated the existence of stoichiometric complexes in penetrated films. Harkins found that equilibration during the compression of a cetyl alcohol film penetrated by sodium cetyl sulfate

was attained very slowly. He observed that if the compression was carried out very slowly over a period of 12 hours, the kink in the compression isotherm which schulman had attributed to complex formation did not appear. Harkins did find, however, that these films could be compressed to pressures as high as 60 dyne/cm. before collapse occurred. Harkins concluded that while complexes may exist in mixed films, Schulman had not proved that they do exist. Jolly(104) has since observed that Harkins failure to reproduce Schulman's data may have been due to the fact insufficient time was allowed for the sodium cetyl sulfate to penetrate the insoluble cetyl alcohol film before the compression was begun.

Dervichian has criticized the idea of complex formation by film penetration for much the same reason he opposed the concept of complex formation in mixed insoluble films. It is Dervichian's belief that Schulman and his collaborators have failed to consider that the rate changes in the penetration or ejection of a soluble surfactant from an insoluble monolayer may be due simply to changes in the cohesive forces in connection with the passage from one regular molecular arrangement in the monolayer to another.

The situation at this time is that there appears to be no conclusive evidence that chemical complexes exist in

mixed monomolecular films. This does not, however, alter the fact that some special interaction, dependent on the ratios of the species making up the monolayer, does occur in some systems.

THE MEASUREMENT OF FILM PRESSURE

Theoretically any method for the measurement of surface tension allows the calculation of the film pressure. However, some of the common methods, particularly those involving capillarity, are not well suited for measurement of film pressure.

Agnes Pockels(105), in 1891, was the first to employ a long rectangular trough, filled to overflowing with water, for the study of surface films. This basic apparatus, with which a film is layered on the surface of a clean liquid and then compressed or expanded by means of movable barriers has since been used and modified by many investigators. The principal refinements have been made by Langmuir(106), Adam and Jessop(107), and Harkins and Nutting(108). This classical apparatus is frequently referred to as a horizontal film balance in order to distinguish it from the vertical-type film balance developed by Wilhelmy(109) in 1863.

The Horizontal Film Balance: A modern horizontal film balance consists of a rectangular trough with a moving barrier that compresses the surface film against a fixed floating barrier. The floating barrier usually consists of a strip of mica attached to the walls of the trough with paraffined strips of platinum or gold foil. The mica strip is attached to a torsion devise so that a

lateral displacement of the float causes a twist in the torsion wire. A mirror arrangement is commonly employed to follow the displacement of the float. A calibration arm upon which calibration weights may be placed is attached to the torsion wire. The apparatus may then be calibrated by noting the displacement produced by addition of a known weight.

The horizontal film balance actually measures the difference between two surface tensions, which meet the float at two different contact angles. Harkins and Anderson(110) have derived the mathermatical relations involved and have concluded that the horizontal force on the float is simply its length times the difference in surface tensions and is independent of the tilt of the float or the way the liquid surface wets its surface.

The Vertical Film Balance: The Wilhelmy or vertical pull type of film balance has several advantages over the horizontal type. Perhaps the most important advantage is that it is far less expensive to construct a high precision vertical film balance than a horizontal type. It is also a much more versatile instrument than the horizontal type and can be used for ordinary surface tension measurements, interfacial tension measurements, and the study of soluble films as well as insoluble surface films.

Basically the Wilhelmy balance consists of an immersion plate, usually glass or platinum, which is attached to the arm of a balance. The plate is partially immersed in the film-covered subsolution. When the plate is partially immersed in a liquid, there is a downward pull on the plate which is directly proportional to the surface tension. If a film is layered on the liquid, the surface tension is reduced and the plate will rise until buoyancy compensation is reached.

The theory involved in the Wilhelmy method is quite simple(110). For a plate of perimeter p partially immersed in a liquid, the downward pull on the plate, F_d, is equal to

$$p\gamma\cos\theta \qquad (9)$$

where γ is the surface tension of the liquid and θ is the contact angle it makes with the plate. If the contact angle is zero then:

$$F_d = p\gamma \qquad (10)$$

The upward force, F_u, on the plate due to buoyancy is

$$gdhtw$$

where g is the acceleration due to gravity, d the density of the liquid, h the depth the plate is immersed, t the thickness of the plate, and w the width of the dry plate. If the surface tension is reduced by film formation, the plate will rise until the reduction in downward pull

($-F_d$) is balanced by the decrease in buoyancy ($-F_u$). Thus we have the relation:

$$\Delta F_d = \Delta F_u \qquad (11)$$

or

$$p \Delta \gamma = gdtw \Delta h \qquad (12)$$

The change in h may be measured by a cathetometer. Usually, however, a galvanometer mirror is mounted with its center in the axis of rotatation of the beam, and the deflection of the beam observed. In this case, Δh is proportional to the change in the scale reading, ΔS, and

$$- p \Delta \gamma = k \Delta S \qquad (13)$$

The constant of proportionality, k, is obtained by determining the change in scale reading, ΔS, caused by placing calibration weights, M, on the balance pan with the immersion plate in the subsolution. Thus

$$k = -g \Delta M / \Delta S \qquad (14)$$

Thus the surface pressure, which is equal to the reduction in surface tension, is

$$\pi = - \Delta \gamma = k \Delta S / p \qquad (15)$$

DESIGN OF APPARATUS

Construction of Sensing Device: The apparatus constructed for use in this study was a modification of the Wilhelmy or vertical type film balance. The modification involved the use of a displacement type transducer element as a sensing device instead of the customary analytical balance. The photograph, page 56, depicts the general appearance of the transducer element and other apparatus components as they are employed in the film balance.

Transducers, or strain gages, rely on changes in resistance induced in certain strain-sensitive filaments as the means whereby very small displacements may be detected. The two transducers which were evaluated in this study were model G7A unbonded, displacement transducers manufactured by the Statham Instrument Company*.

The transducer element consists of a small frame which supports a movable armature by 2 thin cantilever plates. Four sets of strain sensitive filaments are strung under initial tension between the frame and armature. A probe is attached to the armature and extends through a small opening in the stainless steel case which houses the transducer element. When the armature is displaced longitudinally due to force applied to the

*12401 West Olympic Blvd., Los Angeles 64, California

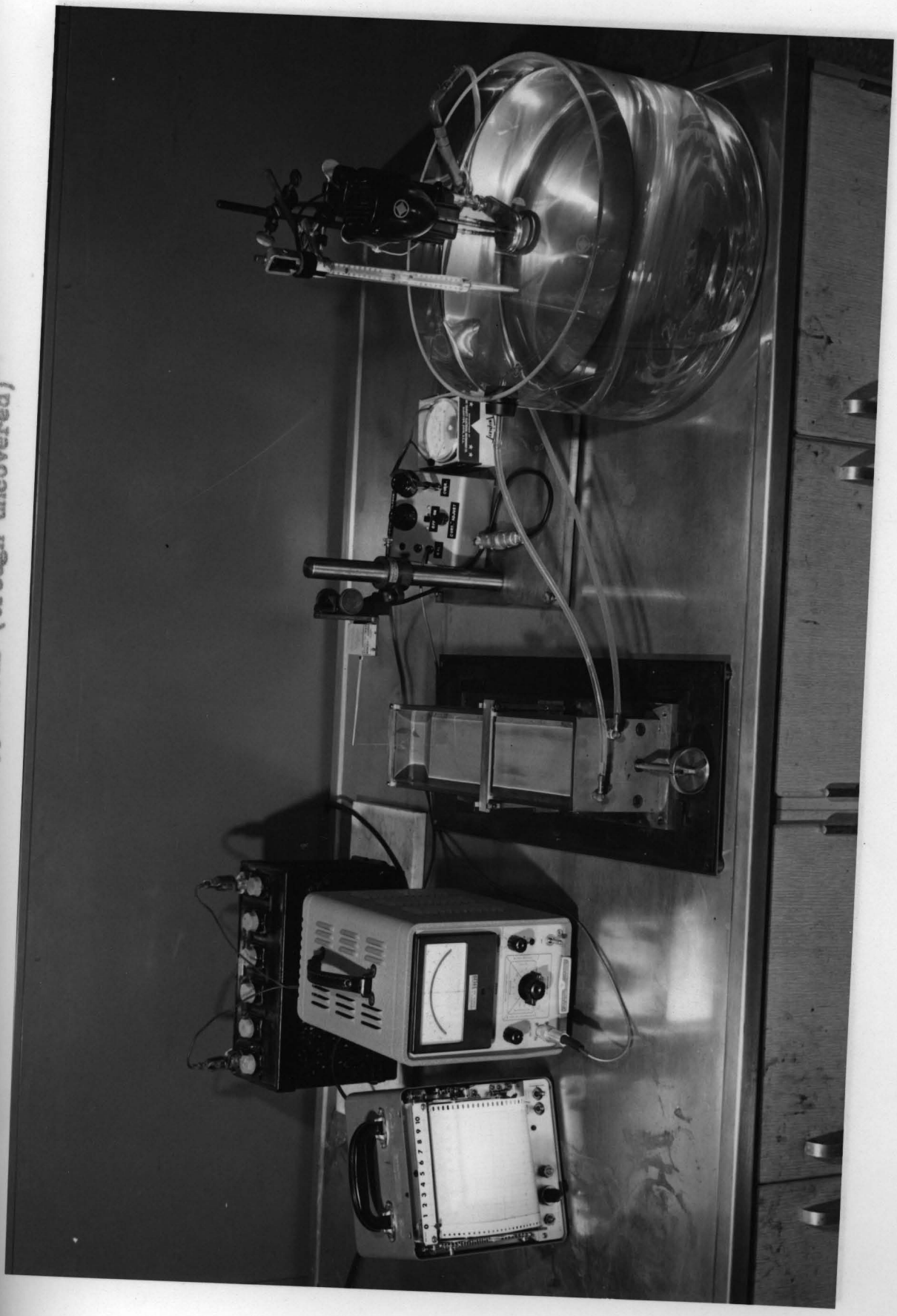
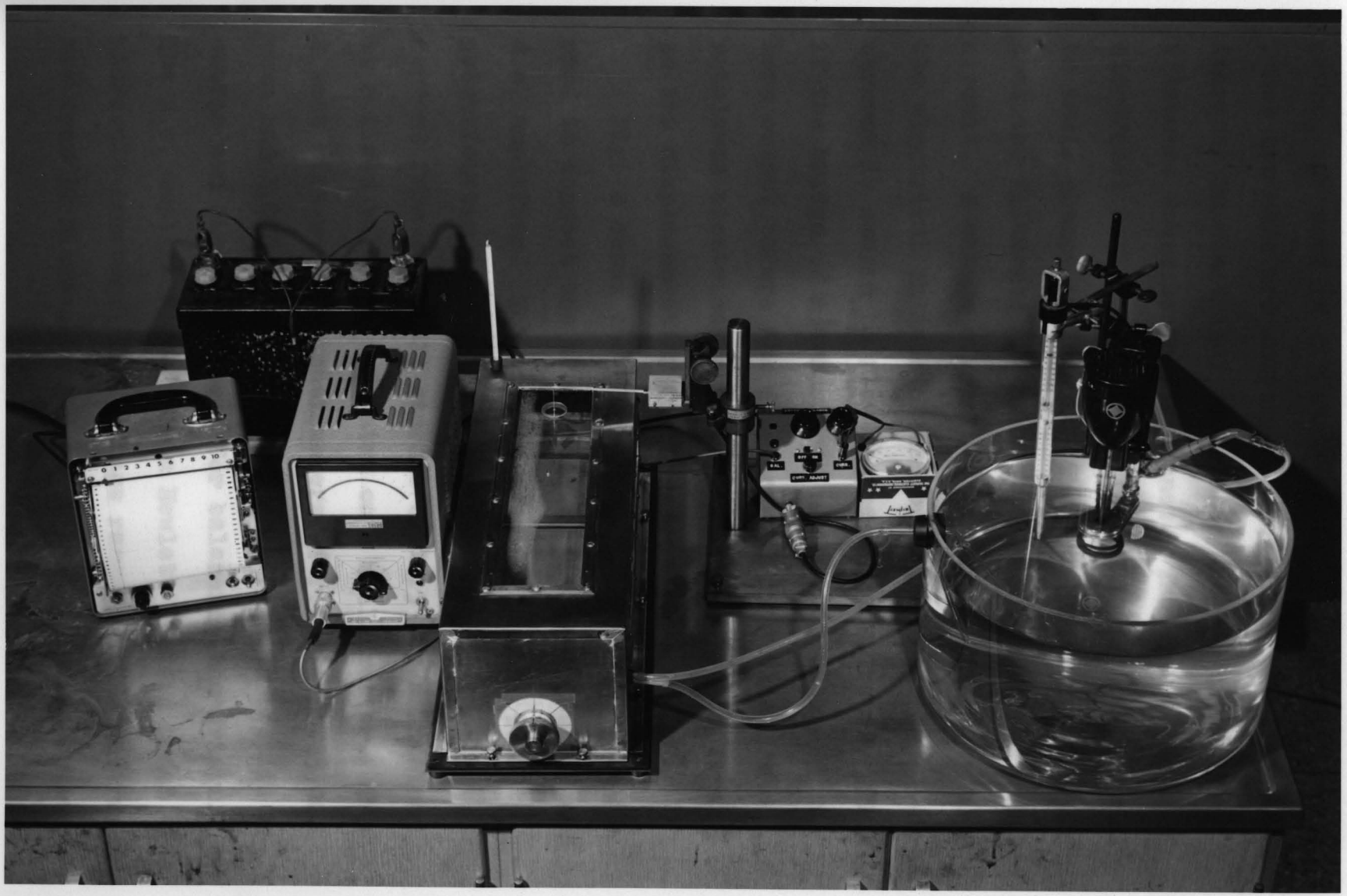


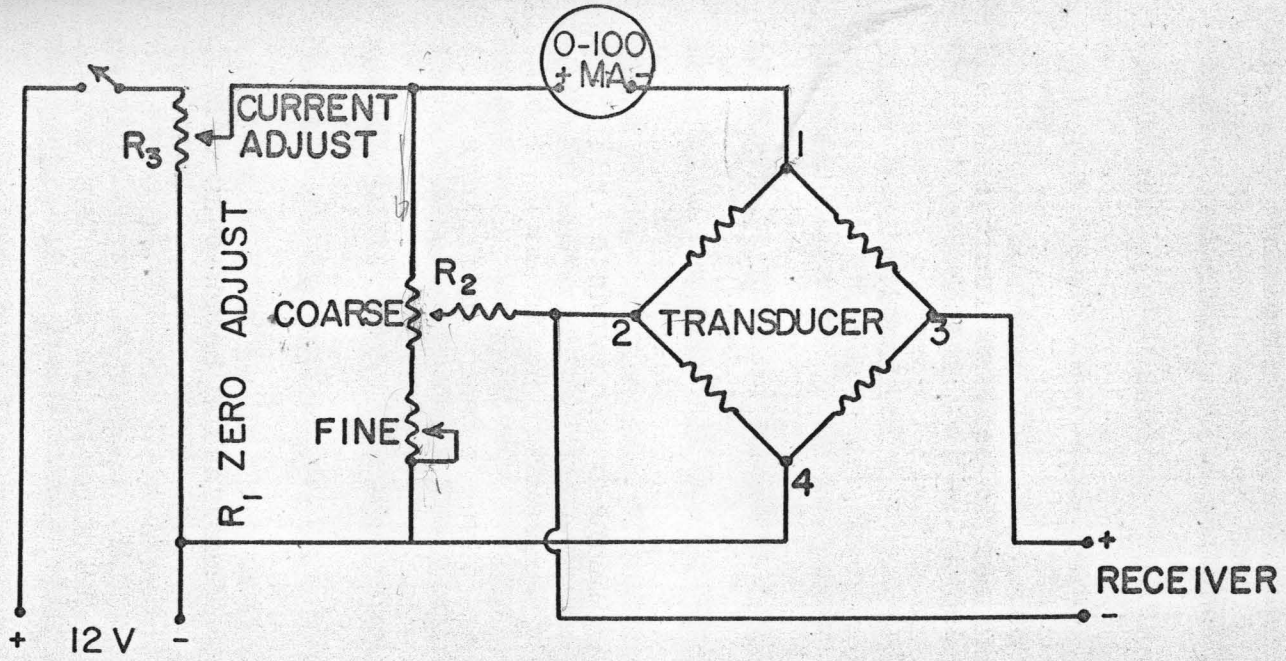
Fig. 4. Photograph of apparatus (trough uncovered)

Fig. 5. Photograph of apparatus (trough covered)



probe, two sets of filaments are elongated, while the other two sets are shortened. The elongated filaments increase in electrical resistance and the shortened filaments decrease in resistance. The change in the electrical resistance of the filaments is proportional to their change in length. The transducer is wired so that the filaments are connected in a Wheatstone bridge circuit. A change of resistance in the filaments alters the electrical balance of the bridge so that an electric current is caused to flow in the output circuit. Thus, if the bridge of a transducer element is balanced at zero output before the displacement is applied, the unbalance electrical output is directly proportional to the displacement of the transducer probe.

Fig. 6 shows the diagram of the external circuit used to connect the transducer to the power supply and d.c. amplifier. The circuit used a 100 ohm, 5 watt potentiometer as a voltage divider whereby the input current from a 12 volt storage battery was maintained at a fixed value as indicated on a milliammeter. Two 2 watt wire-wound potentiometers of resistance 25,000 ohms and 1,000 ohms were wired in series to provide a coarse and fine adjustment for balancing the bridge. The d.c. amplifier used was a Hewlett-Packard Model 425A Microvolt-Ammeter. The voltage range on this instrument was from 10 microvolts end scale to 1 volt end scale.



Transducer terminals 1 and 4 = Power input

Transducer terminals 2 and 3 = output to receiving instrument

R_1 - 1,000 and 25,000 ohm wire-wound potentiometers

R_2 - 25,000 ohm wire-wound ~~potentiometers~~ resistor

R_3 - 100 ohm, 5 watt wire-wound potentiometer

Fig. 6. External circuit diagram

The output of the transducer element could be read directly from the meter of the amplifier or obtained from the tracing of the recorder which was operated off the amplifier. The recorder was a model G-11A Varian Graphic Recorder.

The transducers used were Statham models G7A-0.15-350 and G7A-0.30-350. The model G7A-0.15-350 transducer was the more sensitive of the two, undergoing a maximum probe displacement of ± 0.015 inch full scale with an applied force of ±0.15 oz. A force of ±0.30 oz. was required to produce maximum displacement of ±0.015 inch with the model G7A-0.30-350. Both transducers had a bridge resistance of approximately 350 ohms.

In order to increase the sensitivity of the transducer, an aluminum extension arm was attached to the transducer probe. The length of the extension arm was 11.5 cm. The immersion plate was suspended from the tip of the extension by a length of small diameter aluminum tubing to which a small hook and ring had been attached at opposite ends. The transducer itself was mounted on an adjustable rack which in turn was supported by a heavy brass base equipped with leveling screws.

The usual vertical type film balance relies on the buoyancy effect in order to measure changes in film pressure, i.e., as the film pressure increases the

partially immersed plate rises out of the liquid a distance proportional to the reduction in surface tension. If the buoyancy effect were to cause appreciable displacement of the immersion plate attached to a transducer, then the entire electrical output of the transducer could not be ascribed solely to changes in the film pressure. If such were the case it would be necessary to position the immersion plate so that the bottom edge just comes in contact with the surface of the water. While this is an alternative, it is a definite advantage when working with surface films to have the plate partially submerged so that successive determinations can be made on the same film simply by raising the plate to a new higher position and exposing a fresh, uncontaminated plate perimeter for the next compression.

An analysis of the force-displacement characteristics of the model G7A transducer allows an estimation of the effect of buoyancy changes during a typical compression of a surface film.

The probe of the G7A-0.30-350 transducer will undergo maximum displacement of 0.015 inch or 0.0381 cm., if a weight of 9.5 grams is applied normal to the base. The maximum film pressure likely to be encountered is on the order of 50 dyne/cm. If the dimensions of the immersion plate are 1.50 by 0.0145 cm., or approximately 3 cm. in perimeter, this constitutes a force of roughly 150 dynes.

The displacement a force of 150 dynes would produce then is equal to:

$$\frac{(150 \text{ dyne})(0.0381 \text{ cm.})}{(9.5 \text{ g.})(980 \text{ cm./sec.}^2)}$$

or approximately 6.14×10^{-4} cm. The effect due to buoyancy would be equal to the mass of the water no longer displaced multiplied by the acceleration due to gravity, or: (1.5 cm.)(0.0145 cm.)(0.000614 cm.) (1 g./cm.^{3})(980 cm./sec.^{2}) or 1.35×10^{-2} dyne. As the total force involved is 150 dynes, the error due to buoyancy effects in terms of per cent would be approximately

$$\frac{0.0135}{150} \times 100$$

or 0.009%. This would be the approximate error produced by buoyancy if the plate was suspended directly from the transducer probe. The use of an aluminum extension arm increases the buoyancy effect to a degree directly proportional to its length. This has been calculated as increasing the error due to buoyancy approximately four-fold. As the extension also increases the sensitivity by four-fold its use was probably justified.

Construction of Auxiliary Equipment: The aluminum extension arm for the transducer probe was hand-shaped from 1/8 inch aluminum rod. The arm was flattened on two sides tapering gradually to the tip. The bottom edge was also tapered

to a lesser extent and a small notch was made on the upper edge of the tip for the immersion plate hook assembly to ride on. The butt of the extension arm was flattened slightly on both sides normal to the taper for a distance of approximately 3/16 inch. A hole 2/56 inch in diameter was tapped through the center for attachment to the probe of the transducer. A nylon screw was used to make the attachment.

The immersion plate was attached to the end of the aluminum arm by a 6 cm. length of aluminum tubing (outer diameter 1 mm.) to which a small platinum hook and ring were affixed at opposite ends with epoxy resin cement.

The weight of the entire arm assembly attached to the transducer probe was 0.828 g. The weight of the aluminum arm itself, including the nylon screw, was 0.793 g.

The immersion plate consisted of special precision thickness Corning microscope cover glasses. The dimensions of these cover glasses were 15 X 50 mm. The thickness was 0.14 to 0.15 mm. The glass plate was carefully perforated in the center of one of the 15 mm. sides about 3 mm. from the edge. The diameter of the hole was about 1 mm. in diameter. A sand blaster equipped with a fine abrasive and a small tip was used to perforate the cover glass. It was found the results appeared to be

more reproductible if the surface of the cover glass was also entirely depolished by the abrasive. The perimeter of each glass immersion plate was individually measured with a vernier gauge and stored prior to use in individual, labelled containers. The perimeters of 24 immersion plates prepared in this manner ranged from 3.00 to 3.03 cm.

An immersion plate was also prepared from platinum foil 0.0005 inch in thickness. This immersion plate was also perforated near one edge and its surface depolished by sand blasting with fine abrasive. The dimensions were measured by a cathetometer and found to be 5.10 X 2.04 cm. The weight was 0.282 g.

The platinum immersion plate was found to have no particular advantage over the depolished glass immersion plates. The metal plate of course was not subject to breakage whereas the glass plates were extremely fragile. On the other hand, the glass plates were inexpensive, easy to prepare, and could be cleaned more easily. The platinum foil was difficult to depolish, tending to curl up at the corners. As the total weight which could be attached to the probe of the transducers was limited, the lightness of the glass plates was also an advantage.

A film trough was constructed to be used in conjunction with the transducer elements previously described. The general features of the trough are shown

in Fig. 4

The film trough was constructed of 0.25 inch stainless steel plates which were welded together and then milled so the upper surface was perfectly flat. The upper inner walls of the trough were also carefully milled to provide a rectangular enclosure of known dimensions. The inner dimensions were found by measurement to be 9.99 by 39.32 cm. The trough was provided with a hollow chamber beneath the upper reservoir through which water from a constant temperature bath was circulated. One end of the trough had a well designed to accomodate the immersion plate of the apparatus. The threaded rod which moved the moving barrier of the trough was made to extend beyond the cover for the trough thus providing for external manipulation of the trough when covered. The threaded drive rod was carefully machined from brass. The barrier yoke was found to traverse a distance of 0.508 cm. per complete revolution of the drive rod. The fixed barrier of the trough was adjustable and was aligned parallel to the moving barrier used to compress the film.

A cover constructed of 24 gauge stainless steel and Plexiglas was constructed to fit closely over the top of the trough. The general features of the cover are shown in Fig. 5. The drive rod extended through a Teflon seal in one end which allowed the drive rod to turn

freely but was also reasonably air-tight. A circular hole was made in the Plexiglas top of the cover at one end to allow passage of the immersion plate. A disk of Plexiglas with an opening approximately 4 mm. in diameter in its center was used to seal the larger opening. The length of aluminum tubing used to attach the immersion plate to the extension arm of the probe passed through the small opening in the disk.

Additional openings in the cover were provided for sample introduction, the placing of a thermometer, and hoses for circulation of water through the hollow chamber in the trough. The sample introduction opening was covered during the course of a determination.

The cover itself bolted down on a Neoprene rubber covered steel base to give a tight fit. While the cover did not provide an absolutely air-tight enclosure nor a good thermal barrier, it did allow control of the temperature and humidity at temperatures near room temperature. Water at 25.0° C. from a constant temperature bath was circulated through the hollow chamber beneath the trough.

Calibration of the Apparatus: The first step in the use of the apparatus was the calibration of the transducer output and confirmation that the response was linear. This was done by suspending small platinum weights

ranging from 5.18 mg. to 123.65 mg. from the end of the aluminum extension arm.

Before use the calibration weights were washed thoroughly first with ether and then with acetone and dried to constant weight in an oven. The calibration weights were weighed on a microbalance and stored in a dust-proof container in a dessicator between calibrations.

The following are typical data from the calibration of the two transducer units.

TABLE I

Calibration Wt. in Mg.	Output, Microvolts G7A-0.15-350	Output, Microvolts G7A-0.30-350
5.18	123	68
11.18	265	148
23.49	552	313
40.27	949	540
63.04	1490	843
89.96	2120	1200
123.65	2940	1650

A comparison of the relative sensitivities of the two transducer units may be obtained by inspection of the plot shown in Fig. 7. The G7A-0.15-350 transducer was approximately 1.8 times as sensitive as the G7A-0.30-350 transducer, and was employed for the majority of the experiments. The plot also shows that the electrical

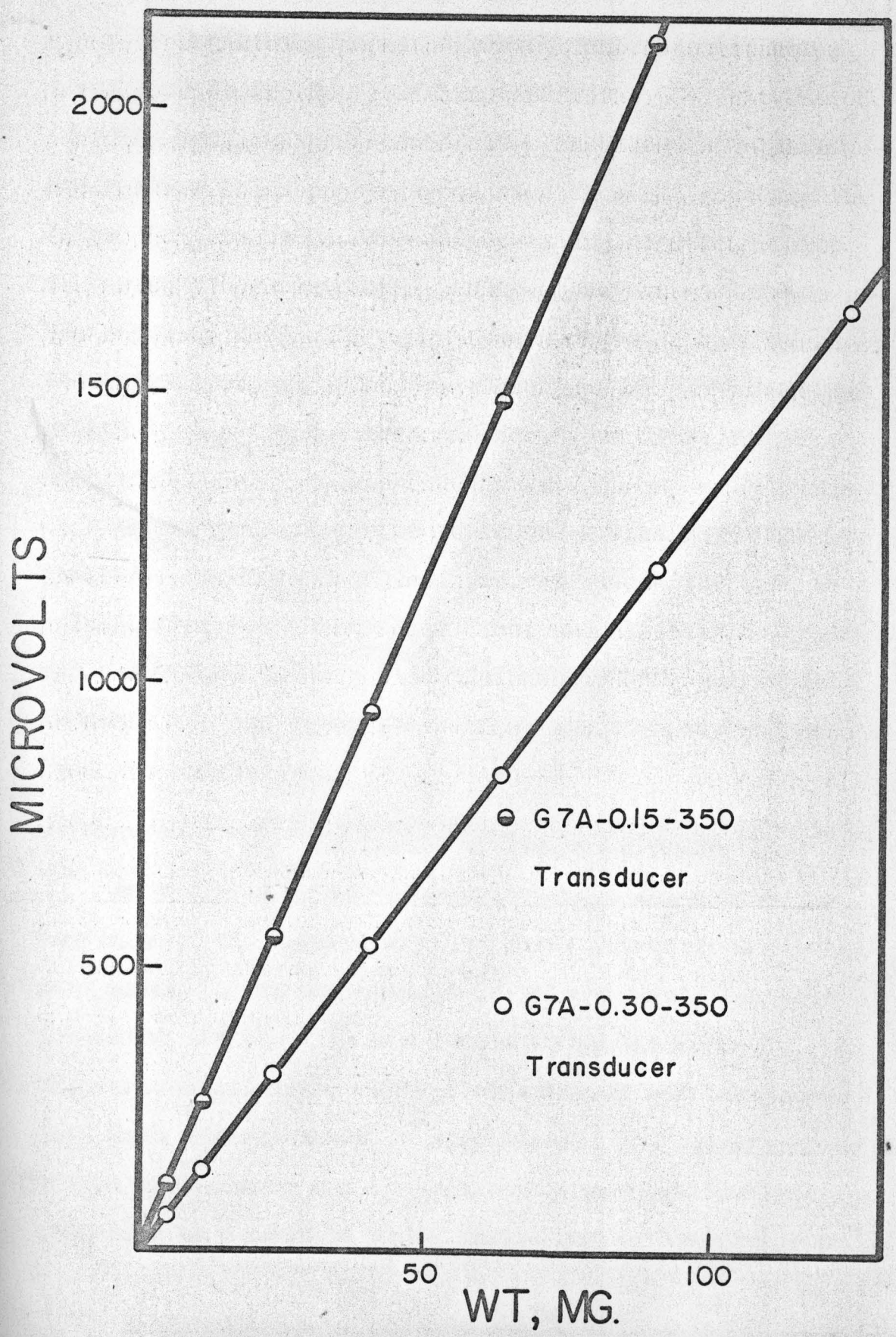


Fig. 7. Calibration plots for Statham model G7A-0.15-350 and G7A-0.30-350 transducers.

ouput of the apparatus was directly proportional to the force applied at the end of the probe.

The relationship between the transducer output and the surface pressure was calculated from the calibration data. This was done by multiplying the applied weight by the acceleration due to gravity, thereby obtaining the force in dynes which produced a certain observed electrical output. Dividing this force by the electrical output in microvolts, gave the number of dynes per microvolt.

For example, from the calibration data given for the G7A-0.15-350 transducer, the mean value for the relationship between applied force and electrical output was calculated to be 0.0412 dyne/microvolt. The surface pressure for any given measurement was then calculated from the relationship:

$$\pi \text{ (dyne/cm.)} = \frac{\text{(0.0412 dyne/microvolt)(output, microvolts)}}{\text{(perimeter of immersion plate, cm.)}}$$

An analysis of the possibility that changes in the buoyancy of the immersion plate might introduce a significant error in the measurement of film pressure has been presented earlier. It was concluded on the basis of the force-displacement characteristics of the transducer that such effects would be very small. The negligible effect of buoyancy was also checked experimentally by attaching an immersion plate to the end of the aluminum

arm, partially immersing the plate in water and then adding the calibration weights to the arm. The calibration plot remained linear and had the same slope as previous calibration plots.

The sensitivity of the film balance was limited by the stability of the signal obtained from the d.c. amplifier. The amplifier noise was estimated to be approximately 1 microvolt. The drift was found to be a maximum of 2.4 microvolts over a 2 hour period. The error due to drift during this time period can be calculated in terms of its effect on the film pressure using the calibration data for the G7A-0.15-350 transducer:

$$\pm \pi = \frac{(0.0412 \text{ dyne/microvolt})(2.4 \text{ microvolt})}{(\text{perimeter of immersion plate, cm.})}$$

$$\pm \pi = \frac{0.10 \text{ dyne}}{\text{perimeter of immersion plate, cm.}}$$

Thus, the error would be $\pm$ 0.03 dyne/cm. for an immersion plate with a perimeter of 3.0 cm.

EXPERIMENTAL

Materials and Equipment: Triple-distilled reagent grade benzene was used as the spreading solvent for the sample solutions. The water used in the subsolutions was prepared by passing distilled water through a mixed bed ion exchange column and then redistilling the deionized water. The paraffin (m.p. 65-70° C.) used to coat the film trough was melted and extracted several times with hot ethanol.

The sample solutions were delivered to the surfaces of the subsolutions in volumes ranging from 0.010 to 0.100 ml. Calibrated micro-pipettes were normally used to deliver the solutions for the compression experiments with the film trough although a 0.100 ml. capacity Hamilton microliter syringe with a Chaney Adaption was also used with equal success in some of the experiments. In the titration experiments the volume increments were conveniently added by means of 0.010 and 0.025 ml. capacity gas chromatography syringes.

Experimental Methods: Two different methods were used for the evaluation of the apparatus and the preliminary investigation of the properties of lecithin and lysolecithin at the air/water interface. The first of these involved the conventional technique of compressing the monolayer with the moving barrier of the film trough. In the

second method, successive volume increments of the sample solution were added to a surface of known, constant area.

In a typical compression experiment the film trough was steamed and carefully washed with Alconox solution. The trough then was rinsed thoroughly with distilled water and with methanol.

The trough was dried in an air stream and placed in a large drying oven. After the trough was sufficiently hot a thin film of paraffin was melted onto the edges of the trough. After the trough had been wax-coated it was allowed to cool slowly to room temperature and then rinsed with deionized water and finally filled so the surface of the water was slightly above the edge of the trough. The surface of the water was swept clean with strips of Teflon sheet to remove foreign particles floating on the surface. The moving bar of the film balance was then placed in a position directly against the fixed barrier. The trough was covered with the stainless steel and Plexiglas cover which bolted down to the Neoprene-covered base upon which the trough rested. The drive rod for the moving barrier extended through the Teflon seal in one end of the cover. Two shallow polyethylene containers filled with water-saturated filter paper were enclosed inside the cover with the trough. Hoses from the trough were attached to the constant temperature bath.

The pointer dial was attached to the drive rod and the moving barrier moved to the end of the trough opposite the fixed barrier. This provided a final sweep of the surface prior to layering of the film. The position of the moving barrier during the compression of the film was determined by reference to a circular scale fastened flush to the end of the trough cover.

The transducer element was positioned so the tip of the extension arm was directly above the opening in the Plexiglas top of the cover. A depolished glass immersion plate was attached to the probe extension assembly. The aluminum connection tubing passed through a small hole in a Plexiglas disk which in turn fit tightly into the circular opening in the Plexiglas top of the trough cover. The transducer platform was then lowered so the immersion plate was partially submerged in the water and the bridge balanced so there was zero output. Then an appropriate amount of the film-forming substance dissolved in benzene was layered on the clean water surface. It was essential that the solution be added slowly or the benzene film spread to the edges of the trough and dissolved the wax coating. The appropriate amount of the sample was calculated from the substance's molecular weight and a knowledge of the area occupied per molecule at the surface. The quantity of material added was less than that required to form a compact monolayer

over the entire surface available. The system was flushed with nitrogen to aid in removal of the volatile solvent and compression begun as soon as the tracing on the recording indicated equilibrium had been attained. The compression was carried out by moving the barrier a fixed distance at specified time intervals. The unbalance electrical output of the transducer was read directly off the amplifier meter or obtained from the recorder tracing.

The second method used for obtaining the compression isotherm for a monolayer was less precise but was judged to offer a substantial advantage in convenience. In this method the immersion plate was partially submerged in the subsolution contained in a shallow, circular polyethylene tray. The dimensions of this tray were 155 by 8.5 mm. The isotherms for the films were obtained by adding small volume increments of the sample solution. Since the surface area available remained constant as increasing amounts of solvent were added to the interface the effect was essentially the same as the standard compression of a film, i.e., the area available per molecule was gradually reduced.

The procedure used in this method was as follows: The polyethylene tray was washed with Alconox solution, carefully rinsed with deionized water and dried. The tray was placed in the bottom of a large crystallizing

dish which was positioned on a steel base equipped with leveling screws. Since rapid evaporation of the volatile solvent used to layer the film was desired the top of the crystallizing dish was only partially covered with transparent plastic film. The plastic film provided some protection from air currents and air-borne contaminants.

The sample solution was added to the surface of the subsolution in discreet volume increments. The volume increments were added either at regular time intervals or after the film had reached equilibrium as indicated by the recorder. The total volume added was usually about 0.20 ml.

The film balance was also used to obtain approximate surface tension values for certain of the subsolutions. The bridge was balanced with the immersion plate suspended above the surface of the subsolution. Then the plate was lowered until the lower edge touched the surface. The plate was then detached from the surface by slowly raising the plate. The maximum output of the transducer at detachment from the surface was directly proportional to the surface tension. The surface tension was calculated from the relation:

$$\gamma(\text{dyne/cm.}) = \frac{(0.0412 \text{ dyne/microvolt})(\text{output, microvolts})}{(\text{perimeter of immersion plate, cm.})}$$

As a rough check of the accuracy of the method the surface tensions of distilled water and reagent benzene were

determined at room temperature (23.0° C.). The values obtained were 72.28 dyne/cm. for water and 28.47 for benzene.

Films of Palmitic Acid: The isotherms obtained by compression with the film trough and by the titration method for monolayers of high purity palmitic acid are shown in Fig. 8 and 9. These isotherms were obtained at room temperature in an air conditioned laboratory. In no instance did the room temperature vary by more than 0.2° C. during the course of a single experiment.

The compression isotherm for palmitic acid at 23.5° C. was obtained by rapid compression of the film at low π values and then slow compression at 2 minute time intervals during the steep portion of the isotherm. The tracing of the compression isotherm obtained with the recorder clearly indicated the π values observed during the steep portion of the isotherm were not equilibrium values. The compression was carried out over a total time of 1.5 hours. The subsolution was freshly redistilled deionized water. The limiting area for the monolayer, obtained by extrapolation, was approximately 21 $Å^2$ per molecule.

The titration isotherm at 22.0° C. was obtained by adding sample volume increments of 0.025 ml. at low π values and 0.010 ml. volumes at high π values. The total volume of sample solution added was 0.250 ml. The

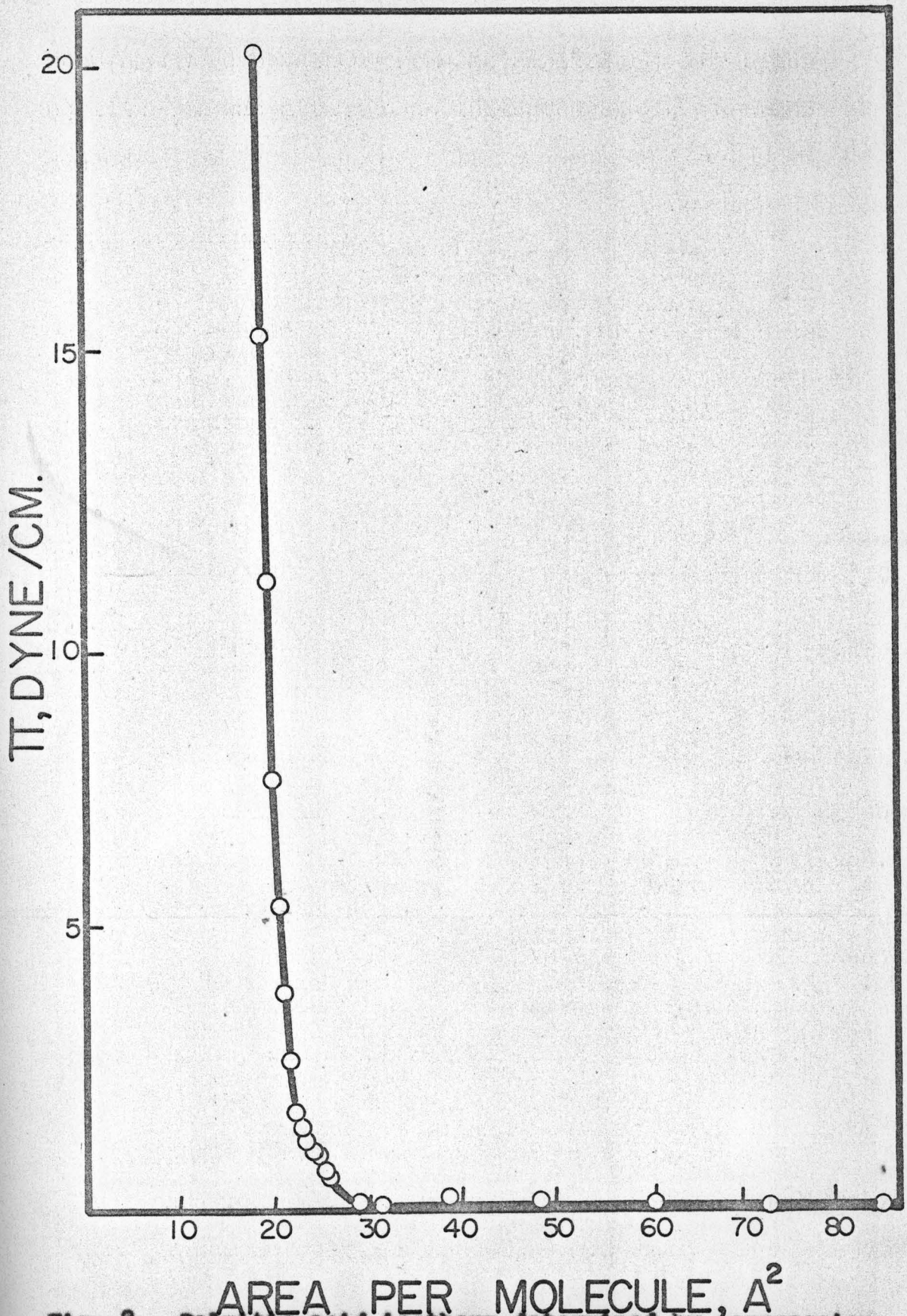


Fig. 8. Palmitic acid isotherm determined by compression with film trough (23.5° C.)

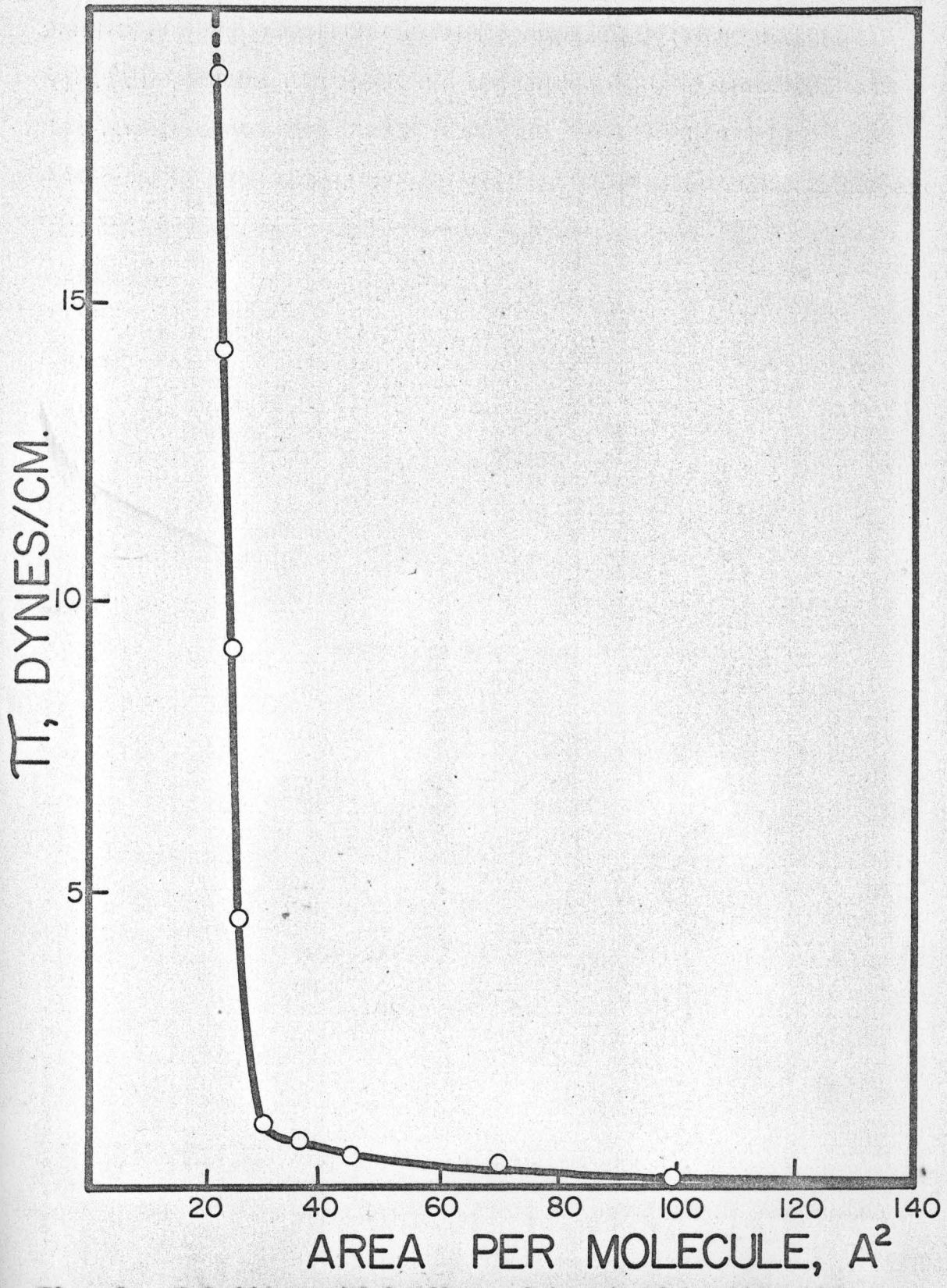


Fig. 9. Palmitic acid isotherm determined by titration method (22° C.)

readings were obtained from the amplifier meter after equilibrium was attained as indicated by the recorder. The subsolution was 0.005 N HCl in deionized water. The total time required for the experiment was 3 hours. The limiting area per molecule was approximately 26 $\mathring{A}^2$ per molecule.

RESULTS AND DISCUSSION

The experimental surface tension values obtained at 23.0° C. of 72.28 dyne/cm. for water and 28.47 dyne/cm. for benzene by the detachment method compared favorably with those given in the International Critical Tables (111) of 72.28 ± 0.05 dyne/cm. for water at 23° C. and 28.88 ± 0.03 dyne/cm. for benzene at 20° C.*

The isotherms obtained for palmitic acid were very similar to those reported by Harkins(112) for palmitic acid at comparable temperatures. The isotherm obtained by compression was somewhat more contracted than those given by Harkins. This may have been due to the presence of metallic contaminants leeched out from the metal of the trough by the subsolution. The isotherm obtained by the titration method was also similar to the literature isotherm. The curve is slightly expanded at low π values, presumably due to the influence of the benzene solvent, but the extrapolation value for the limiting area is comparable to the value obtained from Harkin's data.

The recordings obtained for a number of compression isotherms for palmitic acid indicated that equilibrium was approached very slowly at high film pressures for this substance.

*Calculated value for benzene at 23° C.: $\gamma(\text{air}) = 31.58 - 0.137t + 0.00001\ t^2 \pm 0.2 = 28.48 \pm 0.2$ dyne/cm.

Palmitic acid was observed to exhibit sharp decreases in film pressure following a small reduction in surface area per molecule. This initial rapid fall in film pressure was of short duration, ordinarily less than 2 minutes, and was followed by a slow equilibration of much longer duration. As the time required for complete equilibration films would have been prohibitively long, the films were compressed at regular time intervals after the equilibration effect became less significant, as indicated by the recorder. Titration isotherms were obtained in a similar fashion although the time intervals required between addition of the sample increments were much longer.

While the isotherms obtained by the titration method are less accurate and less informative than those obtained by compression with the film trough, the method has the advantage of great simplicity. The use of the small polyethylene trays also avoids the necessity of wax-coating the edges and walls of the container. Several of these polyethylene trays were obtained and a series of isotherms for a given substance could be obtained in the course of a few hours. Another important advantage of the method was that only small volumes of the subsolution were required. The volume of subsolution employed in the experiments was only 50 ml. The capacity

of the conventional film trough was 1140 ml. The small volumes required was a particular advantage in subsequent experiments with lysolecithin subsolutions. Lysolecithin was obtained by a complicated separation procedure and conservation of material was an important factor. A disadvantage of the method was the tendency for the benzene used as the spreading solvent to produce a slight expansion in the adsorption isotherms. In addition, as the trays were only partially filled with the subsolution, the surface of the subsolution could not be swept clean before the sample was added.

The analysis of the force-displacement characteristics of the Statham G7A transducers and the calibration data obtained for these elements indicated that a suitable sensing device for a recording film balance could be assembled from these components. The results of surface tension measurements and the reproduction of standard isotherms for palmitic acid strongly supported the view that the data obtained with the apparatus was comparable with that obtained with other conventional film balances.

While the use of a transducer element as the sensing device for a film balance is not a radical departure from a conventional Wilhelmy type apparatus, it does offer certain substantial advantages. The principal advantage of the apparatus is that a recorder may be conveniently operated with the film balance. Other

investigators have previously devised film balances with some sort of recording device. These recording devices have included complicated photographic techniques(113) or mechanical contrivances such as the use of a kymograph(114). Apart from the fact that the use of a recorder provides a convenient and permanent record of each compression isotherm, there are other advantages which dictate the use of a recorder if possible. The use of a recorder is particularly advantageous for surface tension measurements by the detachment method. The visual determination of the maximum displacement obtained at the instant of detachment of the liquid surface from the immersion plate is less reliable and reproducible than obtaining the maximum value conveniently from a tracing of the detachment process.

The use of a recorder also allows the operator of the film balance to estimate the rapidity with which equilibrium is reached during the compression of the film. Another advantage is that the present apparatus could be easily modified to provide automatic recording of the compression isotherm as the film is compressed slowly over a period of hours by means of a motor connected to the drive rod of the film trough.

While the error due to meter drift over a 2 hour period was estimated to be approximately $\pm$ 0.03 dyne/cm. for an immersion plate of perimeter 3.0 cm., the

instantaneous error due to meter noise in the apparatus would be less than one-half this value. While this represents a satisfactory degree of sensitivity for most film pressure measurements, the sensitivity could be increased appreciably by the use of larger immersion plates. Recently, extremely sensitive semiconductor strain gages have been developed and are commercially available(115). It is likely that it would be possible to design a film balance with many times the sensitivity of the present apparatus using a semiconductor strain gage as the sensing device. The use of a more sensitive detection device would necessarily have to be accompanied by similar improvements in temperature control of the apparatus.

PART III. A PRELIMINARY STUDY OF THE SURFACE PROPERTIES OF SOYBEAN LECITHIN AND LYSOLECITHIN

PAST WORK

While monolayers formed by lecithin have been studied extensively for a number of years, most of these investigations have been conducted with crude phosphatide mixtures or with lecithin samples which very likely were contaminated by other lipid components. It is only recently that developments both in isolation techniques and synthetic methods have made the phospholipids readily available in a state of high purity.

Anderson and Pethica(116), in 1956, were among the first to study monolayers of synthetic lecithin. They found that distearoyl lecithin formed liquid films at 20° C. The effects of changes in the pH and ionic strength of the subsolution were studied. It was found that for pH values less than 7, and sodium chloride concentrations less than 1.0 molar, the curves for the synthetic lecithin were all identical. The films expanded at higher pH values and dissolved at very high pH values. Very high sodium chloride concentrations were also found to expand the film. The closest stable packing of the films was found to be about 39 $Å^2$ per molecule.

In 1962, the Dutch workers, van Deenan, de Haas,

Houtsmuller, and Mulder(117) reported an extensive study of the behavior of synthetic phospholipid films, both simple and mixed. These workers found that the effect of increasing the chain length of the fatty acid residues in synthetic lecithins was similar to that observed with fatty acids, i.e., the long-chain compounds L-α-(distearoyl)-lecithin and L-α-(ditetracosanyl) lecithin produced liquid condensed films, whereas the shorter chain lecithins, L-α-(dipentadecanoyl)lecithin and L-α-(didecanoyl)-lecithin gave liquid expanded films. L-α-(diheptanoyl)-lecithin and L-α-(dibutyryl)lecithin were water soluble and failed to form stable films.

It was found the degree of saturation also affected the compression isotherms. The films of L-α-(dioleoyl)-lecithin were found to be considerably more expanded than films of L-α-(distearoyl)lecithin or (α'-oleoyl-β-stearoyl)-L-α-lecithin.

The mixed films of cholesterol and synthetic lecithin showed that with equimolar mixtures the effect of cholesterol was to condense the lecithin film. This effect was less pronounced with the saturated lecithins.

Lysolecithin is highly water soluble and for this reason would not be expected to form surface films capable of being compressed. Hughes(118) claimed to have obtained liquid expanded type monomolecular films of lysolecithin on water which could be compressed to

65.5 $\mathring{A}^2$ per molecule before collapsing. Robinson(75) has suggested that the lysolecithin used by Hughes may have been contaminated by a considerable amount of lecithin.

EXPERIMENTAL

Materials and Equipment: The recording film balance and auxiliary equipment used in this study have been previously described. The purified lecithin and lyso-lecithin were isolated from a commercial soybean phosphatide mixture (Asolectin) by methods which also have been previously described.

The benzene used as the spreading solvent for the lecithin sample solutions was triple-distilled reagent grade benzene. The deionized water was prepared by ion exchange treatment of distilled water followed by redistillation.

The analytical grade sodium chloride, Pluronic F68, and the Asolectin and Armour Sterile Lecithin* were all used without further treatment or purification.

Lecithin Films: The recording film balance was used to study the behavior of purified soybean lecithin films on various subsolutions. The adsorption isotherms were obtained by compression with the film trough and also by spreading small volume increments of lecithin solutions on a surface of fixed area until film collapse occurred. For

*Generously provided by the Armour Pharmaceutical Co., Kanakee, Illinois

the sake of convenience, the latter method has been referred to as the titration method. The surface pressure determinations for a single film was obtained over a period of time ranging from 1.5 to 3 hours. As with palmitic acid films, a rapid, initial decrease in film pressure following each compression was observed with lecithin films. This was followed by a slow equilibration of longer duration. This effect was much less pronounced with the lecithin films and the π-area isotherms were obtained under essentially equilibrium conditions.

Isotherms for lecithin on deionized water were obtained by both compression and titration. The effect of the degradation with time of lecithin in benzene solution on the adsorption isotherms of lecithin was studied by the titration method.

In addition, the effects of Pluronic F68 and lyso-lecithin subsolutions on lecithin films were studied by the titration method. Surface tension measurements were obtained for these subsolutions by the detachment method.

Surface films were also obtained for two different commercial soybean phosphatide mixtures and their behavior compared with purified lecithin.

RESULTS AND DISCUSSION

Fig. 10 shows the π versus area isotherms obtained for purified soybean lecithin layered on deionized water by the titration method and by compression with the film trough. In order to plot the adsorption isotherms in terms of film pressure versus area per molecule, an average molecular weight of approximately 794 for pure soybean lecithin was calculated from the data of Thornton, Johnson and Ewan(47) for the fatty acid composition of soybean lecithin. The concentration of lecithin in the benzene solutions used for sample spreading was approximately 10^{-7} molar. The compression isotherm for lecithin in Fig. 10 is slightly more expanded than the titration isotherm. This can be attributed in part to the fact that the titration isotherm was obtained at a room temperature of 21.0° C. whereas the compression was carried out at 25.0° C. by circulation of water from a constant temperature bath through the film trough.

A considerable variability in the lecithin isotherms was observed dependent on the relative age of the lecithin sample solution. Fig. 11 shows the titration isotherms at 23° C. obtained for lecithin at times of 24 hours, 48 hours and 33 days after the sample solution had been prepared. The sample solutions were stored in

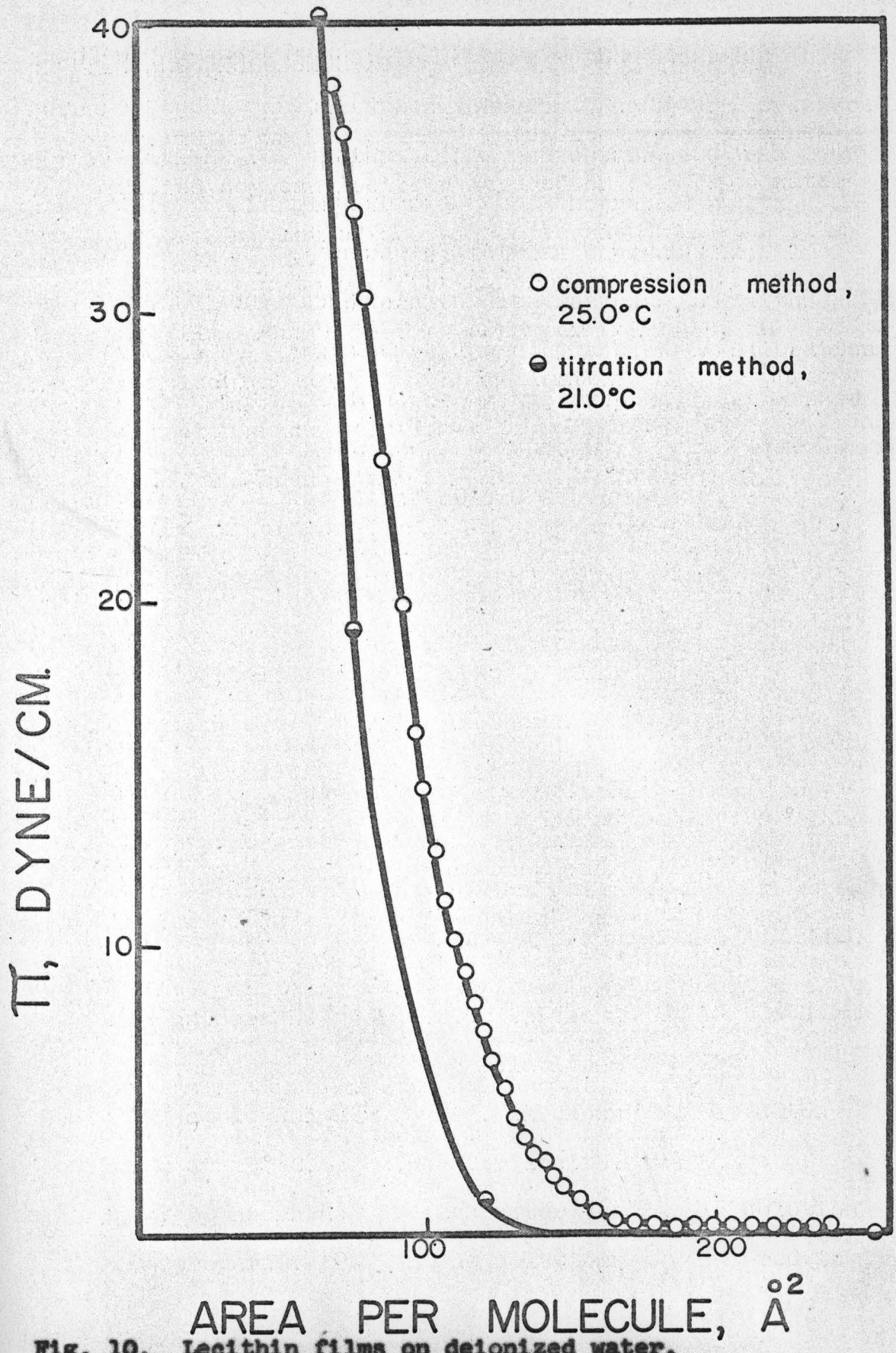


Fig. 10. Lecithin films on deionized water.

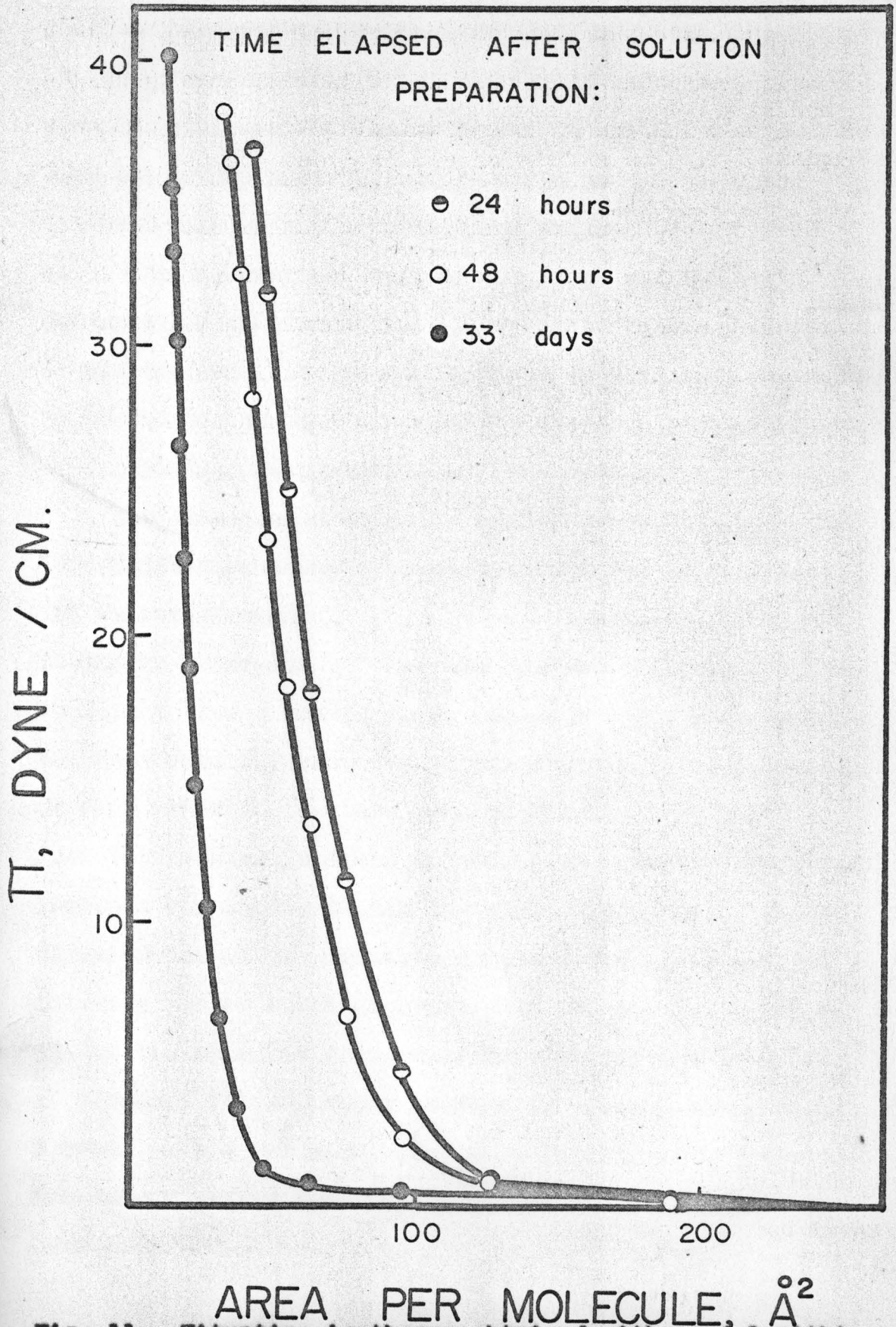


Fig. 11. Titration isotherms obtained with aged lecithin solutions.

the dark at room temperature (21-24° C.). The effect of aging the lecithin solutions was to produce a marked reduction in the π values obtained at any particular area per molecule. This contraction of the lecithin isotherm was particularly evident after a period of 33 days. No attempt was made to ascertain whether the contraction was a result of physical or chemical change of the lecithin. Although lecithin is extremely sensitive to hydrolysis in aqueous media it seems unlikely the rate of degradation would be rapid in benzene solutions.

The behavior of lecithin monolayers on Pluronic F68 subsolutions of varying concentrations was studied by the titration method. Fig. 12 and 13 show adsorption isotherms obtained for lecithin spread from sample solutions aged 6 and 21 days, respectively. Although the isotherms obtained for the lecithin solution prepared 21 days prior to use were more contracted than those from the fresher lecithin solution, the overall effect of Pluronic F68 appeared much the same. In general, the π values obtained for lecithin at specified areas per molecule can be related directly to the surface tensions of the Pluronic F68 subsolutions. The surface tensions of Pluronic F68 solutions ranging in concentration from 0.00001 to 1.0 per cent, w/v, were determined by the detachment method described previously. Depolished glass immersion plates were used for the surface tension

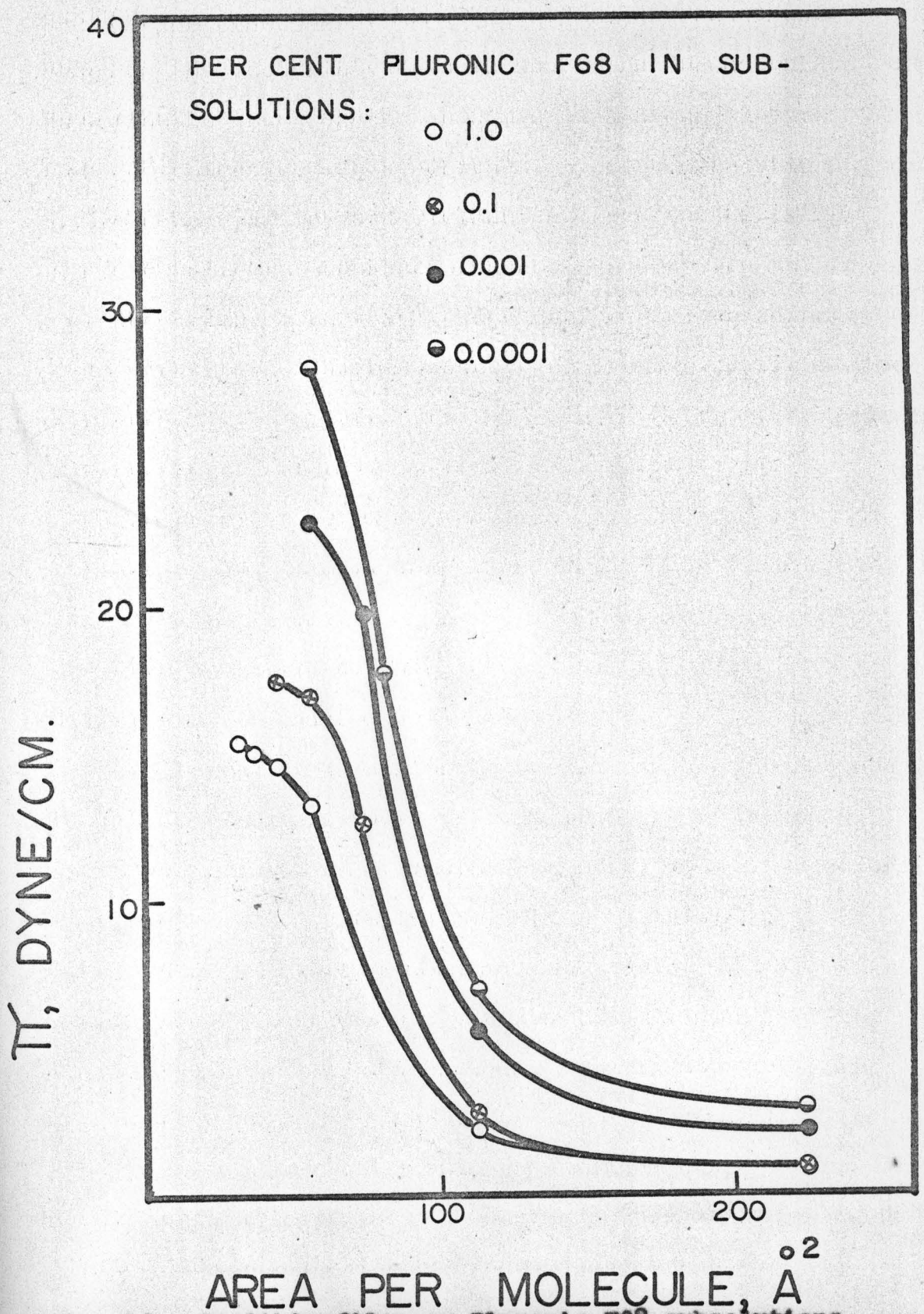


Fig. 12. Lecithin films on Pluronic F68 subsolutions. (sample solution age: 6 days)

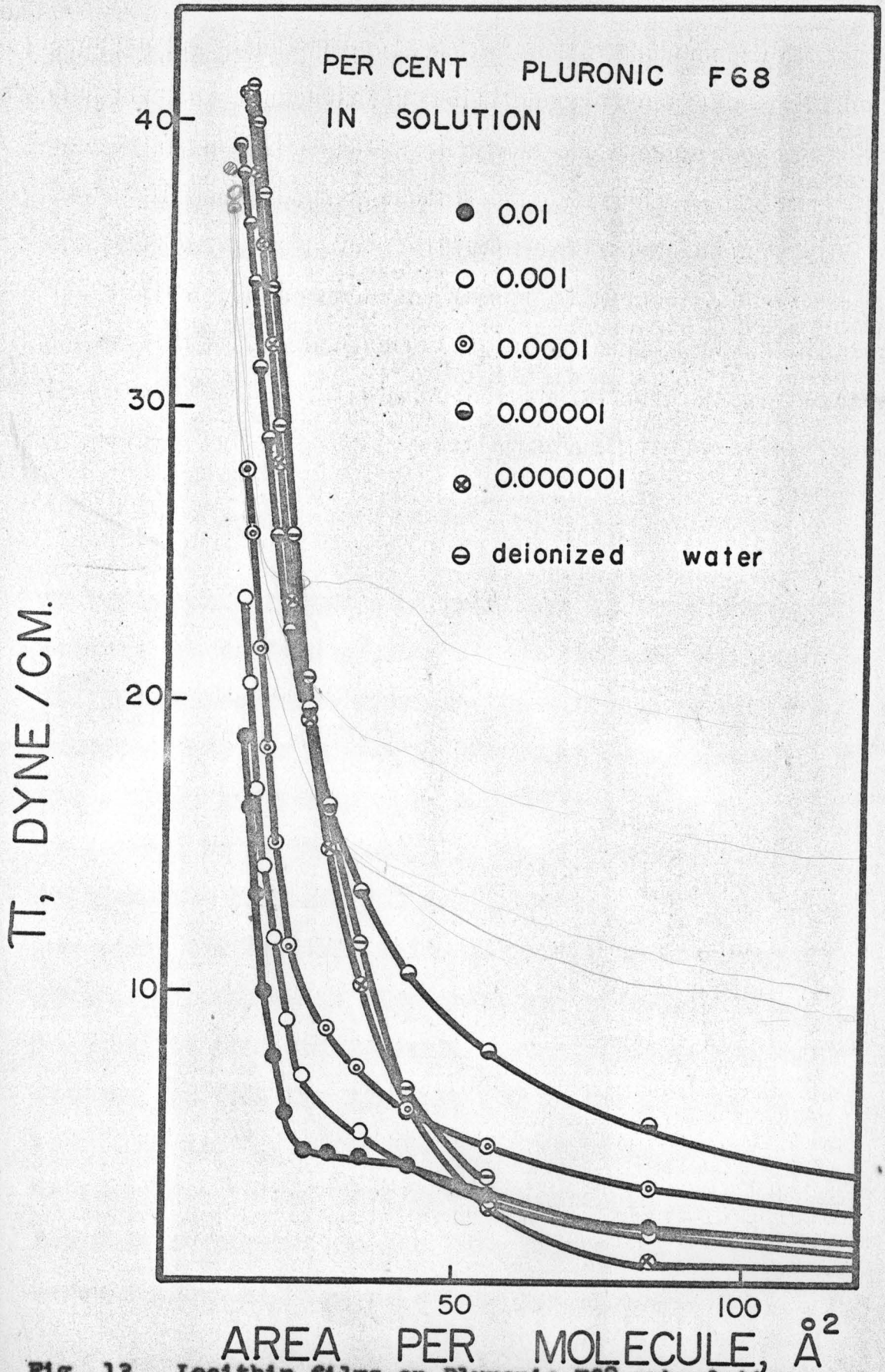


Fig. 13. Lecithin films on Pluronic F68 subsolutions (sample solution age: 21 days)

measurements. The temperature of the solutions was 20.0° C. The experimental values are tabulated below.

TABLE II
Surface Tensions of Pluronic F68 Solutions

per cent, w/v	surface tension, dyne/cm.
1	45.57
1×10^{-1}	49.29
1×10^{-2}	51.55
1×10^{-3}	56.35
1×10^{-4}	63.95
1×10^{-5}	72.52

As the surface pressure in this case is the difference between the surface tension of the Pluronic F68 solution and the film-covered Pluronic F68 solution it could be expected that the film pressure would be proportional to the surface tension of the subsolution. This was found in general to be the case. It is of interest that at intermediate Pluronic F68 concentrations (10^{-4} to 10^{-2} per cent) the lecithin films exhibited proportionately greater expansions at high areas per molecule. The possibility that sufficiently strong interactions occur between lecithin and Pluronic F68 at the surface to result in actual solvolysis of lecithin is deemed unlikely due to the fact that the collapse point for the lecithin monolayers was the same regardless of the concentration of Pluronic F68 in the subsolution.

The effect of lysolecithin subsolutions on lecithin films was also investigated. The titration isotherms were obtained at room temperature by spreading the lecithin solution (age: 3 days) on lysolecithin subsolutions ranging in concentration from 12×10^{-5} to 12×10^{-2} per cent, w/v, in deionized water. The surface tensions for the lysolecithin solutions are tabulated below.

TABLE III

Surface Tensions of Lysolecithin Solutions

per cent, w/v	temperature, °C.	surface tensions, dyne/cm.
1.2×10^{-2}	21.2	36.85
1.2×10^{-3}	21.2	40.85
1.2×10^{-4}	21.3	69.80
1.2×10^{-5}	21.6	71.69

The effect on the lecithin films of adding lysolecithin to the subsolutions is shown in Fig. 14. In general the effect appears analogous to that observed on Pluronic F68 subsolutions. It was observed that the lecithin film layered on a subsolution containing 1.2×10^{-4} per cent lysolecithin was greatly expanded.

Purified lecithin films were compared with films produced by Asolectin and Armour Sterile Lecithin. Fig. 15 shows the adsorption isotherms of purified soybean lecithin, Asolectin, and Armour Sterile Lecithin determined

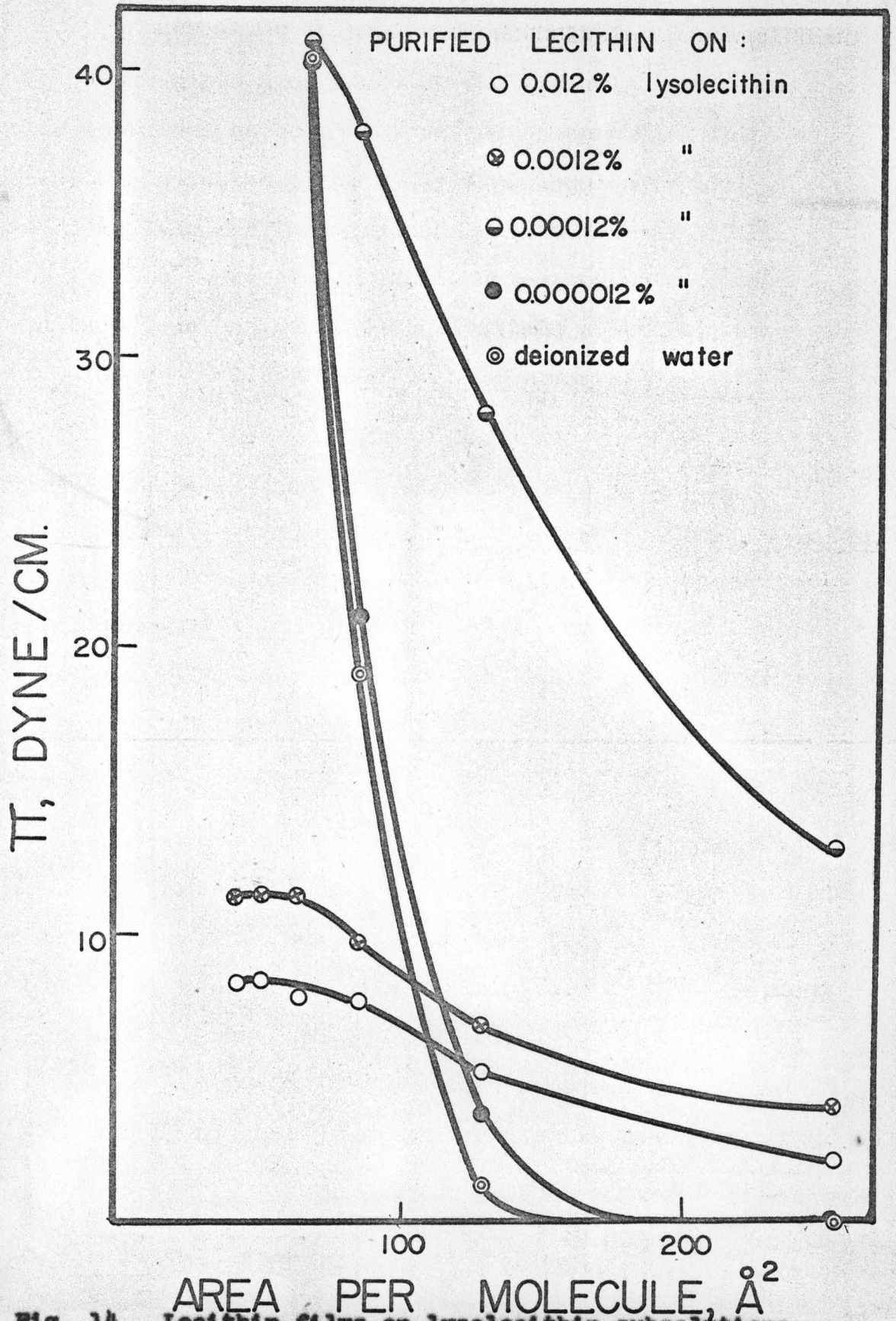


Fig. 14. Lecithin films on lysolecithin subsolutions.

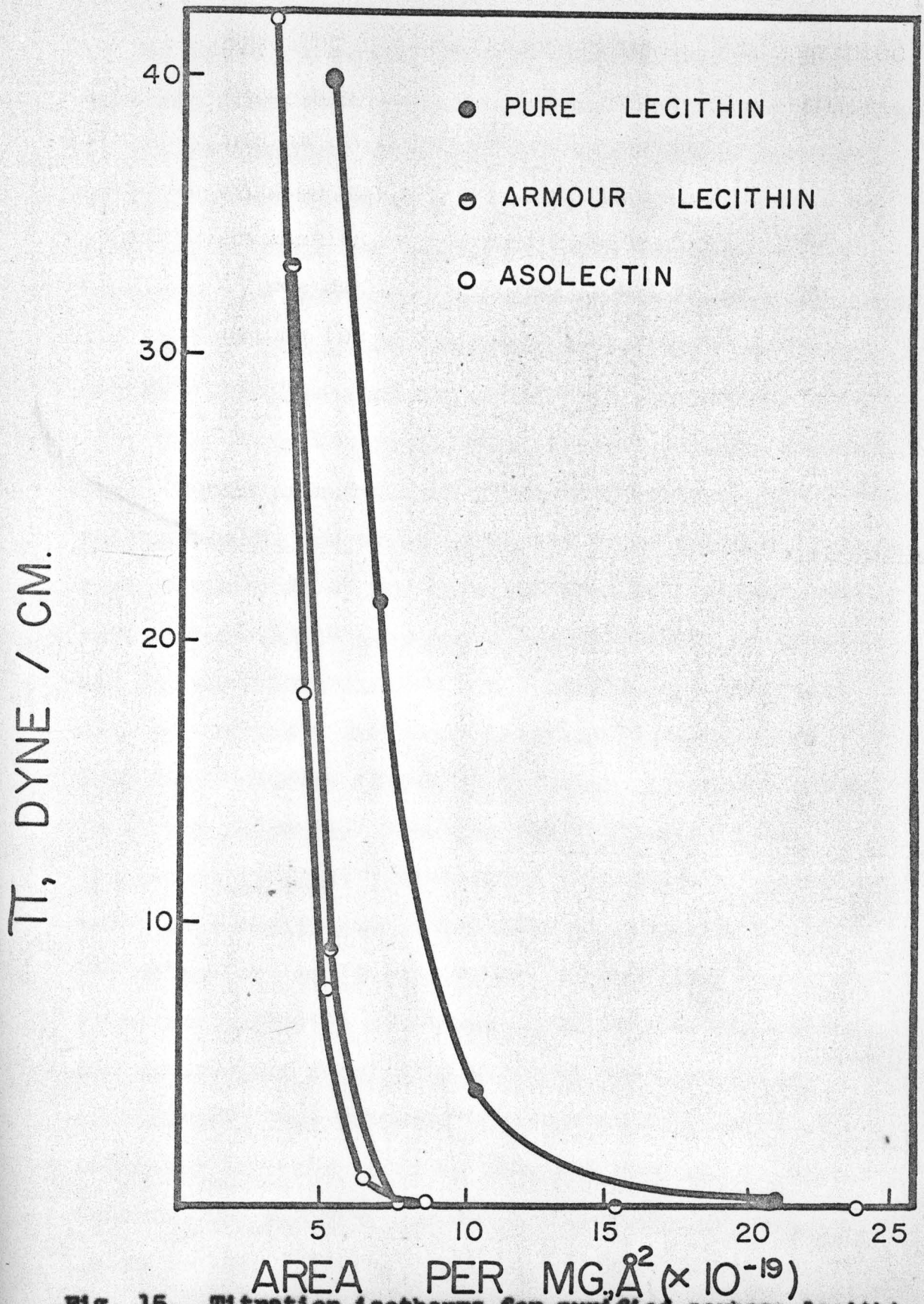


Fig. 15. Titration isotherms for purified soybean lecithin and two commercial soybean phosphatide mixtures.

by the titration method at room temperature. The lecithin films were spread from sample solutions prepared 5 days previously. Fig. 16 shows compression isotherms at 25.0° C. obtained with 1 day old sample solutions of purified lecithin and Asolectin. For comparative purposes, the film pressures were plotted versus the areas per mg. In both plots the purified lecithin isotherms are more expanded than the commercial phosphatide mixtures.

This study indicated that purified soybean lecithin forms liquid expanded type films on deionized water. The collapse point for these films was found to be approximately 65 $\mathring{A}^2$ per molecule when the lecithin was spread from sample solutions prepared within 48 hours of the actual determinations. The theoretical limiting area per molecule for lecithin molecules with their long axes oriented normal to the water surface is about 40 $\mathring{A}^2$ per molecule. Anderson and Pethica(116) and van Deenan et al(117) have found that films of synthetic L-α-(distearoyl)lecithin collapse at approximately 40 $\mathring{A}^2$ per molecule. van Deenan and co-workers(117) found that films for synthetic lecithins containing either one or two unsaturated fatty acid residues produced films considerably more expanded than those obtained for the saturated lecithins. It is believed that the isotherms obtained for purified soybean lecithin in this study are in accordance with the unsaturated nature of soybean

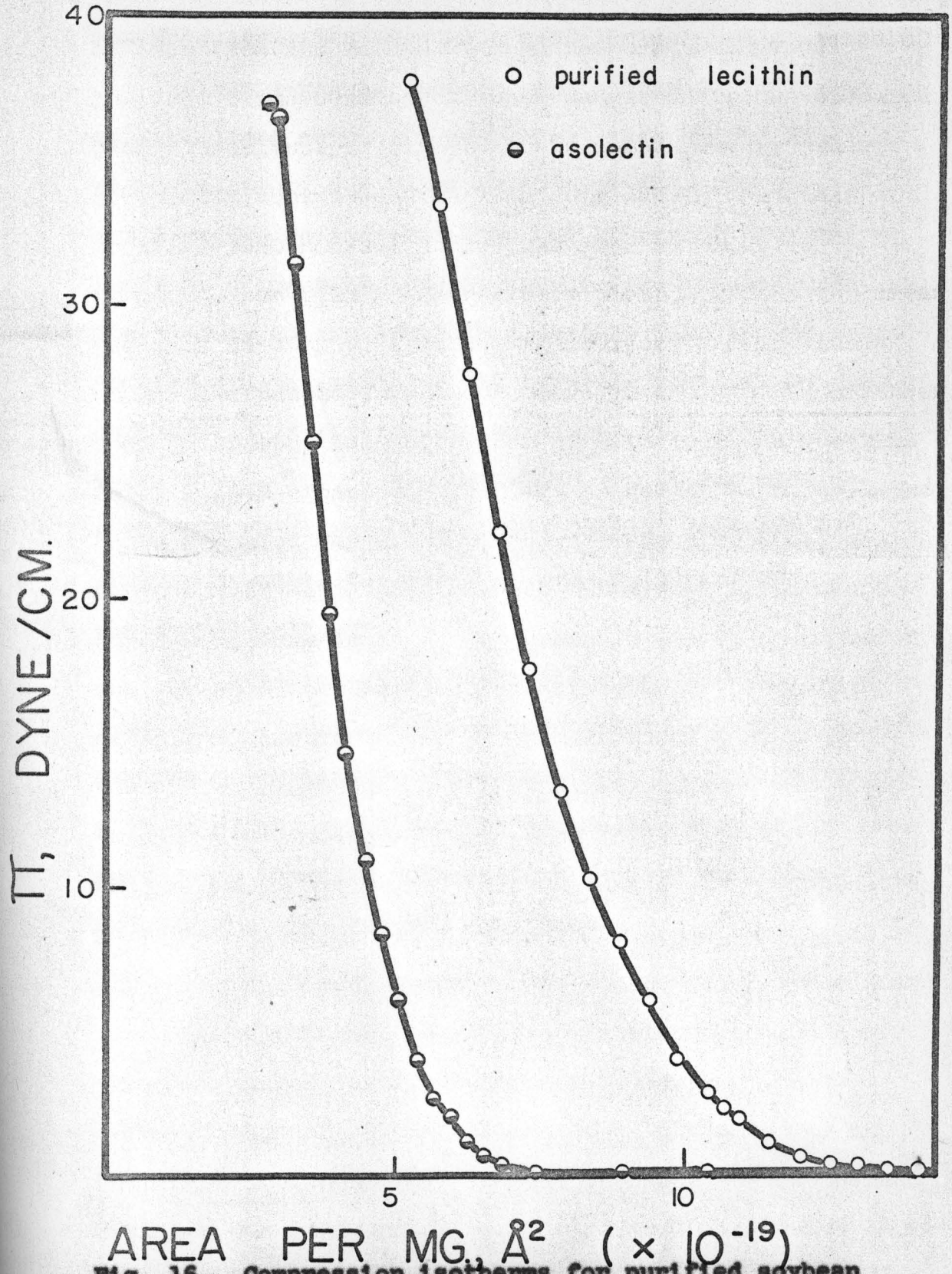


Fig. 16. Compression isotherms for purified soybean lecithin and Asolectin.

lecithin.

It was observed that the age of the lecithin solution from which the monolayers were spread modified the behavior of the films to a surprising degree. This would appear to indicate that any appraisal of purified lecithin films must also include a careful evaluation of the history of the lecithin solutions used in the study. An interesting aspect of the apparent physical or chemical degradation of the benzene solutions of lecithin is the gross similarity of the adsorption isotherms obtained with aged solutions of purified soybean lecithin and freshly prepared solutions of commercial soybean phosphatide mixtures.

The behavior of lecithin films on Pluronic F68 and lysolecithin monolayers appeared to be similar in nature. Further investigation under carefully defined conditions will be necessary before any generalizations can be made concerning specific interactions between the subsolution solutes and the lecithin monolayers.

SUMMARY AND CONCLUSIONS

After experimental evaluation of several methods it was found that the most convenient separation of purified lecithin from a commercial soybean phosphatide mixture was obtained by the treatment of an alcoholic phosphatide solution with Dowex 1X4 to remove the acidic phospholipids. As lecithin was partially hydrolyzed to lysolecithin by ion exchange treatment further purification by adsorption chromatography on silicic acid was necessary.

Lysolecithin was prepared from soybean lecithin previously treated with Dowex 1X4 resin but which had not been chromatographed on silicic acid. The lecithin was selectively hydrolyzed to lysolecithin by the action of Lecithinase A.

The infrared spectra of soybean lecithin and lysolecithin confirmed their identity. Thin-layer chromatography on Silica Gel G indicated that the lecithin was obtained in an extremely pure state but that the lysolecithin contained traces of contaminates.

The film balance developed for the study of phospholipid films was a modification of the classic Wilhelmy film balance. The Wilhelmy film balance was modified by substitution of a Statham model G7A unbonded transducer for the analytical balance used as a sensing device in conventional film balances of this type. The

output of the transducer was read directly off the meter of a d.c. amplifier connected to the transducer by a Wheatstone bridge circuit or alternatively from the recorder operated off the amplifier. The sensitivity of the transducer element was increased by attachment of an aluminum extension arm to the probe of the transducer. The accuracy of a given measurement was limited by the stability of the d.c. amplifier. The error due to meter noise for a single reading was estimated to be approximately $\pm$0.02 dyne/cm. for an immersion plate with a perimeter of 3 cm. Surface tension measurements on water and benzene using the apparatus and a detachment method seemed to indicate a somewhat higher degree of accuracy. The error due to meter drift was estimated to be $\pm$0.03 dyne/cm. over a two hour period.

Surface pressure-area isotherms of high purity palmitic acid films were obtained by compression of the film on a film trough constructed for use with the transducer sensing device. Surface pressure-area isotherms were also obtained by a simple alternative method. This method involved addition of small volume increments of a benzene solution of the film forming substance to the surface of the subsolution contained in a small polyethylene tray. While this method was less precise than compression of a film with the film trough it had the advantage of simplicity, required a volume of subsolution

less than one-tenth that of the film trough and avoided the possibility of contamination of the film by wax or other trough impurities.

The surface tension studies with water and benzene and the reproduction of π-area isotherms for palmitic acid comparable to those found in the literature for this substance indicated that the sensitivity and precision of the recording film balance was well within the requirements of a film balance. The convenient recording of the compression isotherm for surface films was considered a substantial improvement over conventional film balances which can be modified only with difficulty to provide for recording of the compression process.

The preliminary investigation of lecithin films on deionized water indicated that purified soybean lecithin formed liquid expanded films. These films produced π-area isotherms comparable to those found by other investigators for films of synthetic, unsaturated lecithins. The compression isotherms also indicated lecithin in benzene solutions are highly susceptible to aging effects. The effect of aging the lecithin solution was to produce a marked contraction in the compression isotherms. Lecithin films layered on Pluronic F68 and lysolecithin solutions of varying concentrations were found to undergo reductions in the film pressure which could be roughly correlated with the surface tensions

of the subsolutions. Comparison of purified soybean lecithin with two different commercial soybean phosphatide mixtures showed the purified lecithin produced films which are considerably more expanded than films containing a mixture of phospholipids.

APPENDIX

A STUDY OF METHODS OF ISOLATION OF LECITHIN FROM SOYBEAN PHOSPHATIDE MIXTURES

Several of the methods which have proven suitable for the isolation of lecithin primarily from sources other than soybeans, were investigated during the course of this study with the objective of determining which would be the most convenient for obtaining relatively large amounts of highly pure lecithin. The lecithin eventually used in the study was prepared by the ion exchange method of Saunders and Perrin(80)(81) and has been described previously. The other methods are described below.

EXPERIMENTAL

Reagents and Materials: Asolectin, a commercial soybean phosphatide mixture, was the source of lecithin in this study. The petroleum ether (Skellysolve B, b.p. 67-68° C.) used in the chromatography experiments and the U.S.P. ether used in the initial extractions of Asolectin were treated to remove peroxides by passing through an alumina column(79). All other reagents and chemicals employed in the isolation procedures were of reagent or analytical grade unless otherwise specified. The solvents used in the column chromatography procedures were redistilled in pyrex prior to use.

Isolation of Lecithin by the Cadmium Chloride Complex Method: The method of Pangborn(27) was followed with only slight modification. The procedure was as follows:

One hundred g. of Asolectin were dissolved in a minimum volume of diethyl ether and the phospholipids precipitated by pouring the ether solution into a volume of acetone 4 times that of the ether solution. This was repeated twice. The precipitate was extracted with 1500 ml. of absolute alcohol by shaking the mixture overnight on a mechanical shaker. The solvent was filtered off and the phospholipids precipitated by addition of a slight excess of a saturated solution of $CdCl_2$. The mixture was refrigerated for 1 hour and filtered by suction. The precipitate was washed several times with acetone on the filter. The precipitate was dissolved in 100 ml. of chloroform and the solution poured with constant mixing into 700 ml. of alcohol to which 10 ml. of 50 per cent aqueous $CdCl_2$ solution had been added. The mixture was allowed to stand for 10 minutes at room temperature with frequent shaking and then filtered by suction. This step was repeated twice.

The precipitate was suspended in approximately 150 ml. of petroleum ether and 500 ml. of 80 per cent alcohol saturated with petroleum ether and containing 0.1 per cent $CdCl_2$ was added. The suspension was shaken vigorously in a separatory funnel until solution was

complete. The alcoholic layer was drawn off and the amount of material left in the petroleum ether layer estimated by evaporation of a small aliquot.

The petroleum ether was extracted with the 80 per cent alcoholic mixture described above. One hundred ml. of the mixture was used for each gram of material estimated to be in the petroleum ether. The extractions were combined and concentrated to approximately two-thirds the original volume by evaporation under reduced pressure. The concentrated mixture was refrigerated overnight at about -5° C.

The lecithin - $CdCl_2$ complex was removed from the chilled 80 per cent alcoholic mixture by suction filtration and dissolved in 100 ml. of chloroform. This was extracted for 5 minutes with an equal volume of 30 per cent alcohol. The $CdCl_2$ was removed in the alcoholic wash. This required about 4 extractions. Completeness of removal was verified by testing the alcoholic wash for chloride ion with silver nitrate solution. When emulsification occurred during the extractions the mixture was separated by centrifugation.

The cadmium-free chloroform solution was vacuum distilled to remove the chloroform and the residue washed with a small quantity of acetone. The residue was then dissolved in 100 ml. of anhydrous ether and 20 ml. of acetone added. The lecithin was precipitated

out of solution by refrigerating overnight. The solvents were removed by filtration and the lecithin dried in a vacuum dessicator. The purified lecithin was dissolved in anhydrous, absolute ethanol and stored in the freezing compartment of a refrigerator. The lecithin was a faintly-yellow, waxy material. The yield was about 10 g.

Isolation of Lecithin by Adsorption Chromatography on Silicic Acid: The most successful fractionation of Asolectin by adsorption chromatography on silicic acid was obtained by elution of the soya phosphatides from Mallinckrodt silicic acid, 100 mesh, with 10 per cent by volume ethanol in petroleum ether, 20 per cent methanol by volume in chloroform, and methanol. The elution pattern obtained from a 60 gram silicic acid column is shown in Fig. 17.

The silicic acid was activated prior to use by heating overnight at 110° C. Sixty grams of the silicic acid were slurried with 10 per cent ethanol in petroleum ether to form a thick suspension. The suspension of silicic acid was poured into a 1.25 inch diameter column. The column was equipped with a coarsely sintered glass plate support which was covered by a circular disk of ether-extracted filter paper. The stopcock at the bottom of the column had a polished barrel fitted with a Teflon plug. The top of the column consisted of a

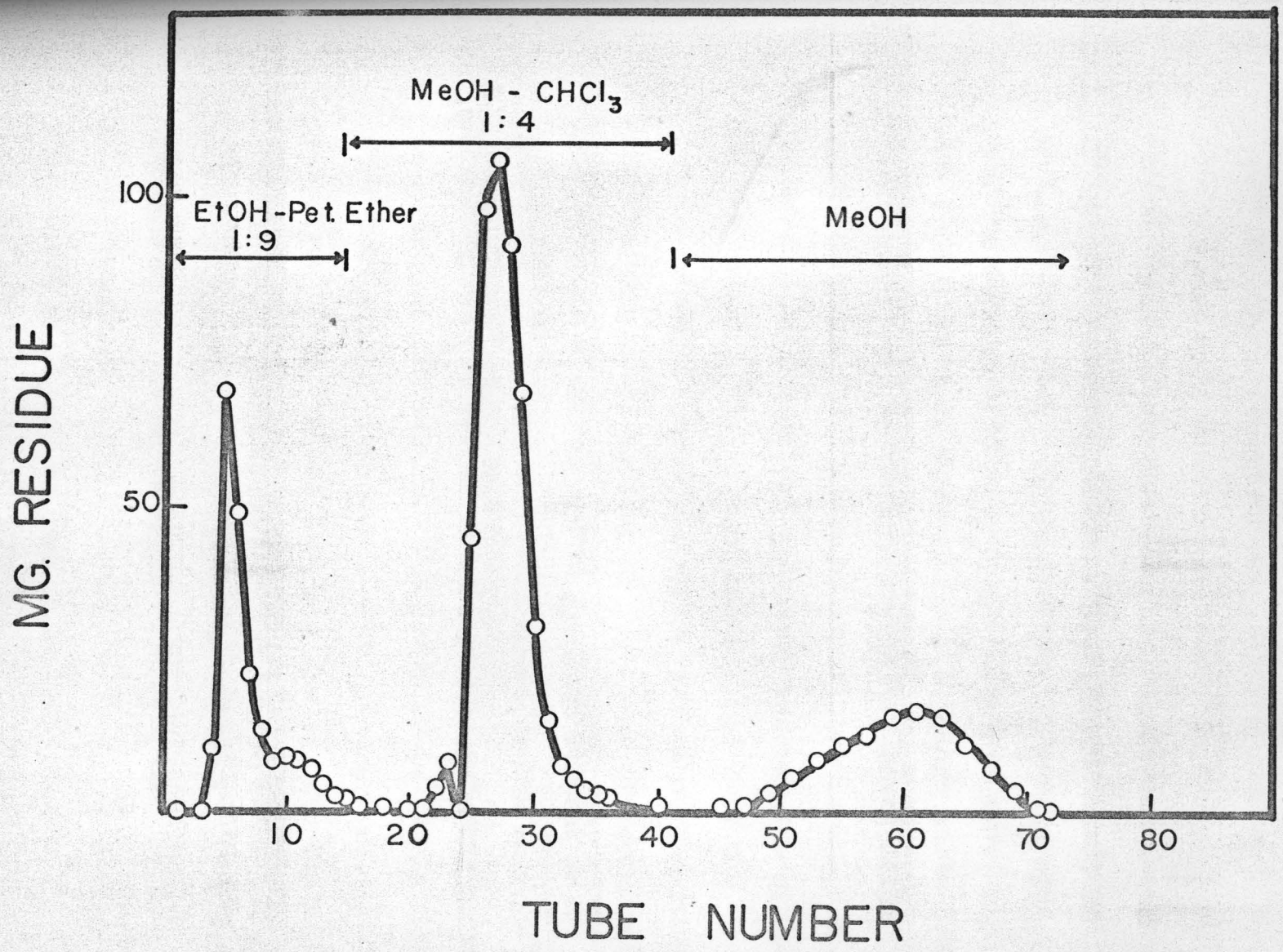


Fig. 17. Adsorption chromatography of Asolectin on silicic acid.

1,000 ml. capacity reservoir. After addition of the slurry of silicic acid the sides of the column were tapped until the upper surface of the silicic acid packing was flat and even. The surface was covered with another ether-extracted disk of filter paper. Ignited ether-extracted sea sand was then carefully added to a depth of approximately 0.5 inch above the silicic acid and covered with a filter paper disk. The sand prevented the surface of the silicic acid from being disturbed when the sample or solvent was added to the column. The solvent was drained from the column to the level of the upper surface of the sand and the sample, consisting of 1.00 gram of Asolectin dissolved in a few ml. of 10 per cent ethanol in petroleum ether, was carefully added to the column. The stopcock was opened and the sample solution allowed to soak into the column. The inner walls of the column were then washed with 10 ml. of 10 per cent ethanol in petroleum ether. After this had soaked into the packing, 150 ml. of the same solvent mixture were added to the column. Fractions were collected every 30 minutes with the aid of an automatic fraction collector. The volume of the fractions was approximately 10 ml. initially but diminished gradually to less than 5 ml. toward the latter part of the separation. 250 ml. of the second solvent, consisting of 20 per cent methanol in chloroform, was

added to the column after the first solvent mixture had passed through the column. The third solvent added was 250 ml. of anhydrous methanol. The appearance of the peak obtained by elution with methanol was flattened slightly due to the greatly reduced flow rate following addition of this solvent.

The plot shown in Fig. 17 was obtained by evaporation of the fractions obtained at 30 minutes time intervals in tared aluminum pans. The pans were weighed and the weight of the residue found in each tube plotted versus the tube number.

Isolation of Lecithin by DEAE Chromatography: The DEAE cellulose (Selectacel*, type 40) used in this study had a theoretical exchange capacity of 0.9 $\pm$ 0.1 meq./g. and was supplied in the chloride form.

Fig. 18 shows the elution pattern obtained from chromatography of 1.00 g. of Asolectin on a 20 g. DEAE(Cl^-) column. The DEAE was slurried with chloroform-methanol 7:1, v/v, and packed with the aid of a glass plunger into a column with inner diameter 2.5 cm. The DEAE was then washed with the chloroform-methanol mixture. Initially a copious amount of fine white powder was washed from the DEAE. The washing was continued

*Carl Schleicher and Schuell Co., Keene, N.H.

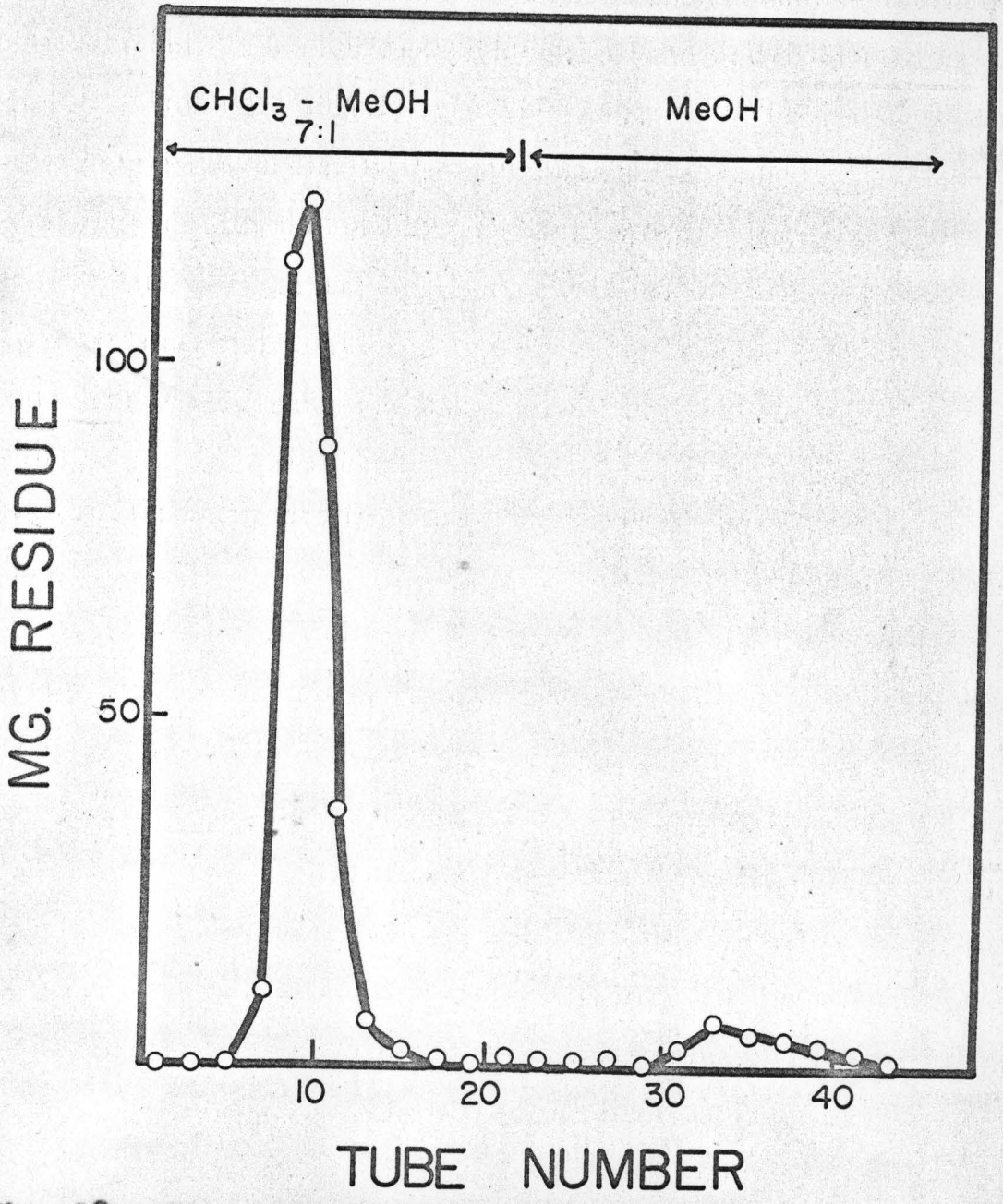


Fig. 18. DEAE chromatography of Asolectin.

until no residue was found on evaporation of a small volume of the wash. This required 200 to 300 ml. The flow rate was extremely fast for a column operation and was regulated with the column stopcock to a rate of approximately 15 ml. per 5 minutes. The 1.00 g. sample of Asolectin dissolved in 10 ml. of chloroform-methanol 7:0 was allowed to soak into the column and the inner walls washed with 10 ml. of additional solvent mixture. Three hundred ml. of chloroform-methanol 7:0 were then added to the column and fractions collected every five minutes with the aid of an automatic fraction collector. After the first solvent mixture had passed through the column, 300 ml. of chloroform-methanol 3:2 were added and fractions collected with this solvent as the eluting agent. The fractions were evaporated to dryness in tared aluminum pans and the residue weighed. The residue obtained from evaporation of each of the fractions was tested with ninhydrin reagent in order to detect the appearance of phosphatidyl ethanolamine. A positive test was obtained for all fractions collected after tube number 31. The estimated recovery from the column was small, amounting to only about 50 per cent of the total material added to the column.

Hydroalcoholic solutions of 5 per cent KOH were used for regeneration of the DEAE columns without any obvious change in the column structure. An attempt to

use aqueous solution of base resulted in the swelling of the DEAE and formation of a gel which completely blocked the passage of the regenerating solution through the column. Following regeneration, the column was washed with large volumes of anhydrous methanol until no residue appeared in the wash. The column was then washed with 200 ml. of chloroform-methanol 7:1 immediately before use. Conversion of the ion exchange sites to the OH^- form did not appear to alter the elution pattern.

As exposure of lecithin to other ion exchange resins has been observed to result in hydrolysis of lecithin to lysolecithin, material obtained from the first peak of DEAE chromatography of soybean phosphatides was rechromatographed on silicic acid. A 250 mg. sample of this material was placed on a 40 g. silicic acid column and eluted first with chloroform-methanol 3:2 and then with 51 per cent methanol in chloroform. As Fig. 19 shows, two distinct peaks were obtained. Material from the first peak was ether-soluble and acetone-insoluble and probably was lecithin. The material from the second peak was insoluble in ether and in acetone and likely consisted of lysolecithin.

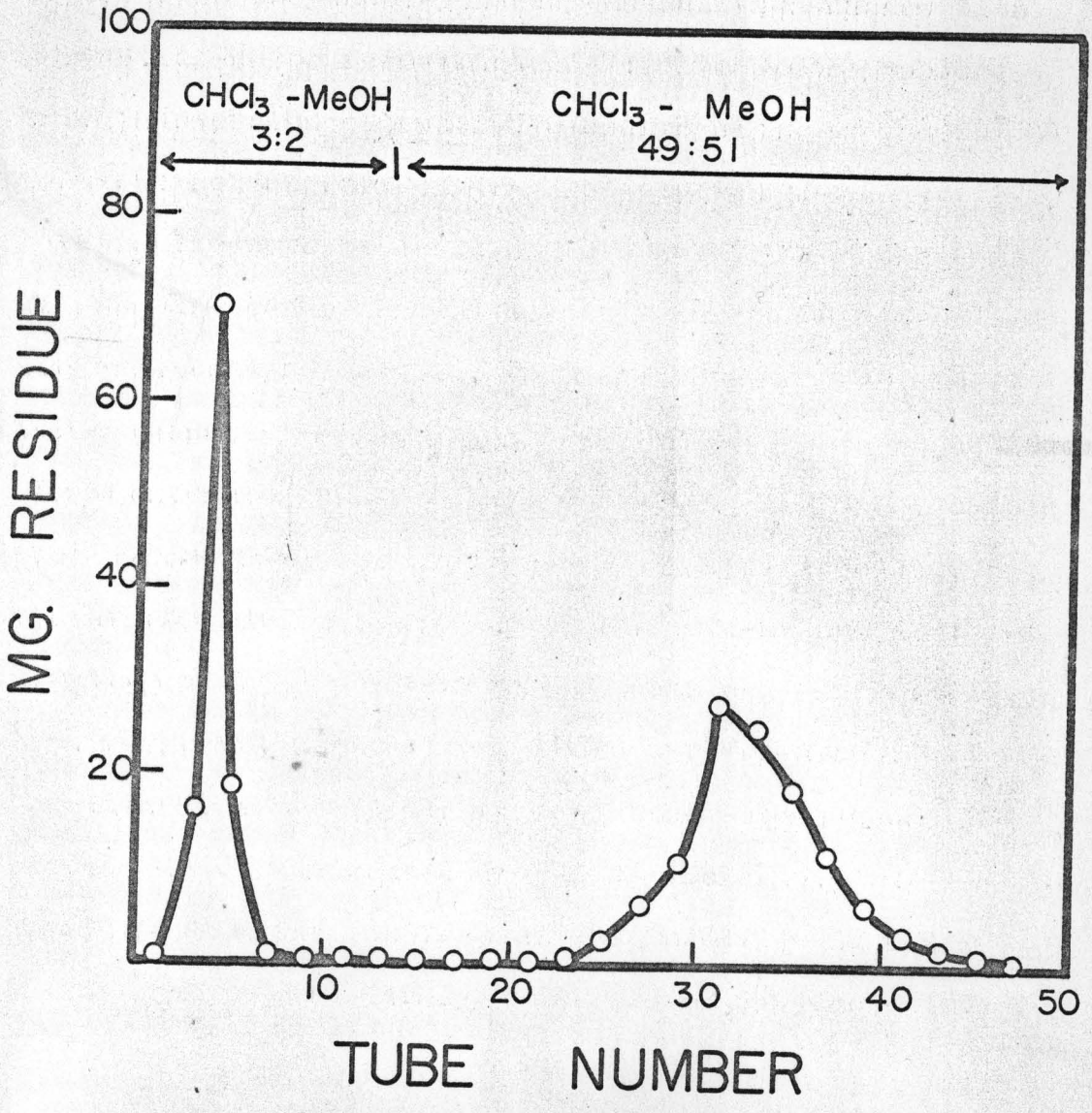


Fig. 19. Silicic acid chromatography of first fraction obtained from DEAE chromatography of Asolectin.

RESULTS AND DISCUSSION

The major disadvantage of preparation of lecithin by ion exchange treatment with Dowex 1X4 is the method is limited to the isolation of lecithin. The cadmium chloride complex method is similarly limited to the preparation of lecithin. Purified lecithin prepared from soybean phosphatides by the cadmium chloride method was rechromatographed on silicic acid. The results strongly suggested appreciable contamination of the lecithin. In addition, Saunders and Elworthy(72) have found that lecithin prepared by the cadmium chloride complex method retains traces of cadmium which influence the surface properties of the lecithin. For these reasons, it was decided that the lecithin prepared by this method was unsuitable for studies with lecithin films.

Asolectin was successfully separated into 3 major fractions by chromatography on silicic acid. The third fraction eluted from the column was identified as lecithin by its infrared spectrum. The first fraction gave a strong ninhydrin reaction and apparently consisted predominently of phosphatidyl ethanolamine. The second fraction, which was eluted with 20 per cent methanol in chloroform gave a faint ninhydrin reaction and probably was the phosphoinositide fraction.

A disadvantage of separation of lecithin by adsorption chromatography on silicic acid is the necessity for very careful monitoring of the columns to prevent overlapping of the fractions. In order to assure that the lecithin fraction was cleanly separated, rechromatography of the fraction would be necessary. The major advantage of the method is that other phospholipid components are also separated.

As lecithin is eluted first from DEAE columns it appeared DEAE chromatography might be a convenient method for obtaining purified lecithin. However, rechromatography on silicic acid of the lecithin fraction from a DEAE column indicated the lecithin was a mixture apparently consisting of lecithin and lysolecithin.

While isolation of purified soybean lecithin by chromatography on DEAE or silicic acid was found to be less convenient than the ion exchange method of Saunders and Perrin the procedures may prove to be of some value for the isolation of other phospholipid components of commercial soybean phosphatide mixtures.

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