SOLUBILISATION PROPERTIES OF PHOSPHATIDYLCHOLINE

by

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ABSTRACT

The solubilisation of glucose and related sugars by thoroughly-purified phosphatidylcholine (lecithin) in benzene solution has been studied. The extent of solubilisation of the sugar has been found to depend on the concentration of lecithin, the structure of the sugar and the presence of cholesterol as a co-solute. The limiting solubility of glucose was achieved at low lecithin concentrations and corresponds to a 1:1 molar ratio of glucose to lecithin. Osmometric evidence indicated that, at higher concentrations, glucose was carried within the lecithin micelle. For other sugars, solubilisation was found to depend on the size of the sugar molecule relative to that of the lecithin micelle and on the number and acidity of its free hydroxyl groups. Enthalpy and entropy of solubilisation were determined for glucose and the results are consistent with an interaction mechanism involving hydrogen bonding via the free hydroxyl groups. Cholesterol, when present as a co-solute, competed for available hydrogen bonding sites on the lecithin molecule and thus decreased the amount of glucose solubilised, but in benzene solutions lecithin exhibited a 5:1 preference for glucose hydroxyl over cholesterol hydroxyl. In aqueous-ethanolic solutions inversion of the lecithin micelle occured and accordingly cholesterol could be solubilised. As the dielectric constant of the medium was decreased by the addition of ethanol, the micellar weight decreased and the uptake of cholesterol increased. In 90% ethanol the limiting solubility of cholesterol corresponded to a 1:1 cholesterol/lecithin ratio. In 70% ethanol the uptake of cholesterol corresponded to a 1:9 cholesterol/lecithin ratio, and this ratio was independent of lecithin concentration below 7.0 mg/ml. In media of higher dielectric constant lecithin formed an opalescent sol rather than a solution and the uptake of cholesterol decreased further, but the cholesterol/lecithin molar ratio continued to be independent of the lecithin concentration of the sol. In none of the aqueous solutions was glucose found to have any effect on the uptake of cholesterol.

It is possible that these results may have biological significance.

ACKNOWLEDGEMENT

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CHAPTER 1

INTRODUCTION

A molecule having a polar 'head' grouping and a non-polar 'tail', is capable, under certain circumstances, of aggregation into micelles. In polar solvents, for example, the polar groupings are oriented facing outwards, while the non-polar groupings form a non-polar core into which other non-polar substances may be physically incorporated. In this way aqueous solutions of soap are able to solubilise oils[1].

In biological systems, one encounters chemically similar molecules which are glycerol esters. At least one of the three glycerol hydroxyl groups is esterified with a non-polar long chain fatty acid and one other hydroxyl group is esterified with a polar phosphate 'head' group. Such molecules are known generally as phospholipids and constitute a large proportion of such natural products as egg yolk.

It has been shown that at least two of these phospholipids (lecithin and lysolecithin) are able to form micelles in both polar and non-polar solvents[2]. It has also been shown that water[3] and dibasic fatty acids[4] may each be solubilised in benzene by phospholipid and that these solubilisates are transported within

the micelle. On the basis of this and other evidence, it has been suggested[4] that lecithin is concerned in the transport of substances involved in metabolism between oil and water phases in living tissue. In particular, it has been reported[5] that the addition of glucose accelerated the precipitation of cholesterol from supersaturated cholesterol/triglyceride solutions containing lecithin and it has been suggested that this result has possible biological significance,in terms of ischaemic heart disease.

Although the ability of phospholipids to form chloroform-soluble 'complexes' with sugars[6] is well known, no quantitative results have been reported which indicate the extent to which this solubilisation occurs, the nature of the cohesive forces responsible, or the effect of a co-solute such as cholesterol. It is the purpose of this thesis to provide answers to these questions in terms of physical chemistry.

Since no meaningful equilibrium study can be attempted when using a mixture of phospholipids, the first stage of the work was concerned with the selection, purification and characterisation of a single phospholipid. Also, since phospholipids may form micelles in either polar or non-polar solvents, it was desirable to conduct

solubilisation studies in both types of media. The selection of an appropriate phospholipid and of appropriate media, was simplified by the fact that micelle formation by phosphatidylcholine in both benzene and aqueous ethanol has already been accurately and quantitatively studied[7,8].

In attempting to interpret the solubilisation process, it was necessary to study such related phenomena as, for example, the effects of the dielectric constant of the medium and various structural features of the solubilisate. It was also necessary to determine the enthalpy and entropy changes accompanying the solubilisation process.

Since the entire approach to the problem has been from the viewpoint of physical chemistry, the conclusions can be discussed only in chemical terms and to assign a biological significance to the results at the present stage would be presumptuous.

CHAPTER 2

HISTORICAL REVIEW

2.1 Solubilisation.

Solubilisation refers to a particular mode of bringing into solution substances which are otherwise insoluble. Solubilisation involves the previous presence of a solution whose particles enhance the solubility of a material of limited solubility. This phenomenan involves the disappearance of the original substance of limited solubility into the particles of the solution itself.

Examples of solubilisation are to be found in the literature as far back as the late 19th century when it was noted[9] that soap solutions have the power to increase the solubility of a variety of substances. In 1868 Kuehne[10] reported that cholesterol is soluble in solutions of soaps and bile salts but almost insoluble in water or aqueous acid and alkali solutions. One of the first attempts to explain this phenomena was published by Verzar[11]. He showed that bile salt solutions will dissolve not only fatty acids but also various organic and inorganic substances. He suggested that the solvent action might be attributed to the formation of a protective ring of bile salt molecules around the

fatty acid or other molecules, with the outer group of the bile salt directed towards the solvent.

In 1918 McBain and Bolam[12] suggested that substances could be solubilised by adsorption on aggregates called micelles. McBain[13] suggested a form of micelle which consists of two layers of soap molecules or ion pairs partially dissociated and arranged side by side with the two hydrocarbon layers inside. X-ray evidence[14] for the existence of the McBain micelle was reported in 1946.

In their discussion on solubilisation McBain and Hutchinson[15] state that there are a number of mechanisms which may be involved in the process such as incorporation of the solubilised material between the layers of the solubiliser or adsorption on the micelle. They also discuss the possibility that all solubilisation phenomena begin with truly dissolved molecules of the solubilised material which then associate with molecules of the solubiliser to form small, loose colloidal particles which grow into larger and more complex structures as the extent of solubilisation increases.

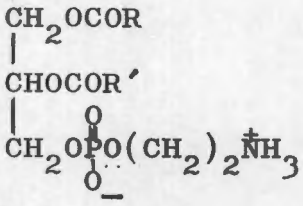
2.2 Phospholipids.

2.2.1 General Discussion.

Phospholipids are naturally occuring compounds found

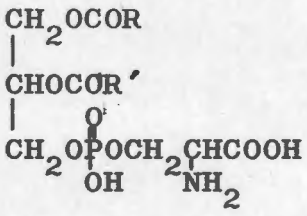
in small amounts in all animal and plant cells, abundantly in brain and nerve tissue, bile, the envelope of red blood cells, egg yolk and seeds. Their general structure is that of a mono or diglyceride with a phosphate group or derivative thereof esterified with the remaining hydroxyl group. The major phospholipids obtained from egg yolk are the following:

I



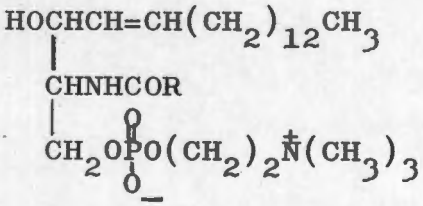
Phosphatidylethanolamine

II



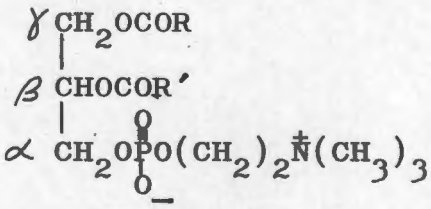
Phosphatidylserine

III



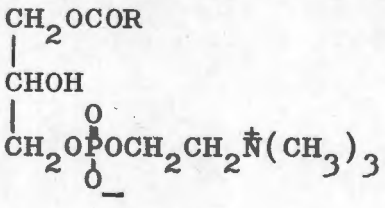
Sphingomyelin

IV



Phosphatidylcholine
(Lecithin)

V



Lysophosphatidylcholine

where R and R′ represent different hydrocarbon chains which may contain one or more double bonds.

2.2.2.Lecithin (Phosphatidylcholine).

The chemical structure of lecithin was first proposed in 1868[16] from hydrolysis experiments on egg yolk lecithin. As can be seen from structure IV, the molecule has a soap-like structure with water-insoluble hydrocarbon chains joined to a complex water-soluble polar group.

Since the phosphate group is attached to the α-carbon atom and as the central or β-carbon atom of the molecule is asymmetric, optical isomers are possible. Natural lecithins are optically active[17] with $[\alpha]_D^{25} = +6.62^o$ and have been shown[18] to have the L-α-conformation.

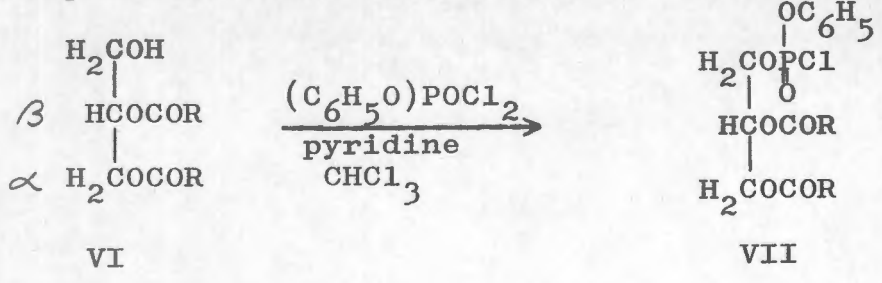
Natural lecithin incorporates a mixture of fatty acids which have been identified from hydrolysis of lecithin. Levene and Rolf[19,20] found the only saturated acids to be palmitic (C_{16}) and stearic (C_{18}) while unsaturated acids included oleic (C_{18} with one double bond), linoleic (C_{18} with two double bonds) and arachidonic (C_{20} with four double bonds). The nature of the unsaturation has been found to vary with the diet of the hens[21].

In 1934 Jukes[22] proposed a zwitterionic structure (IV) for the phosphate-choline moiety of the lecithin molecule which is supported by potentiometric titration evidence[22,23].

2.2.3 Preparation of Lecithin.

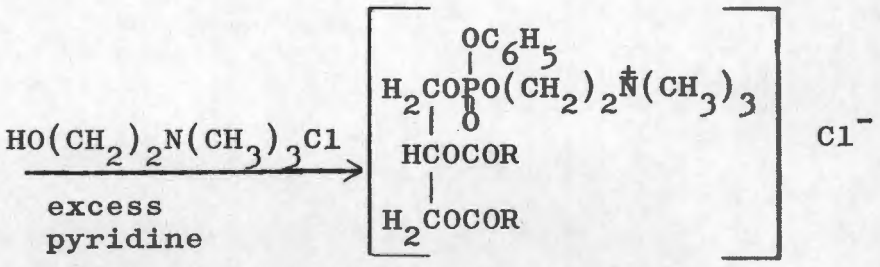
2.2.3.1 Synthesis of Lecithin.

Syntheses of lecithins containing fully saturated fatty acid chains have been carried out by Baer and Kates[24]. These workers used a D-α,β-diglyceride (structure VI) as the starting material, which was treated with monophenylphosphoryldichloride in the presence of pyridine. The reaction product,(VII), was treated with choline chloride in the presence of a large excess of pyridine and the material,(VIII), isolated as a reineckate (by stirring with alcoholic ammonium reineckate). The corresponding sulphate was hydrogenated to remove the phenyl group. The sulphate ion was removed with barium carbonate.



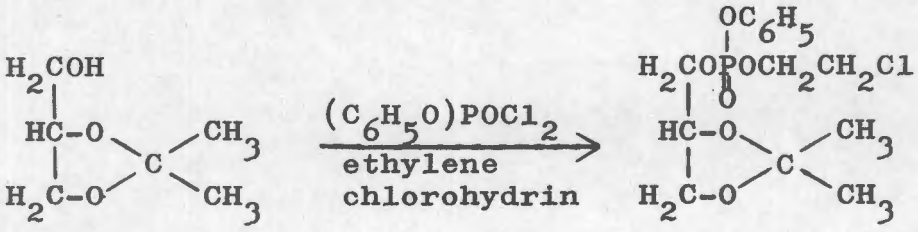
VI VII

Structure VII

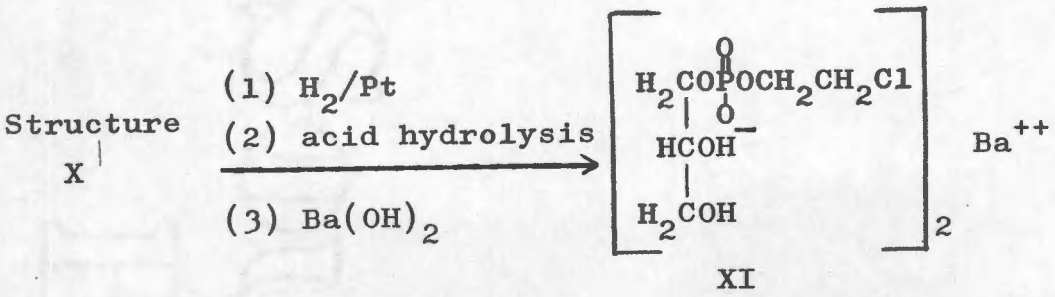


VIII

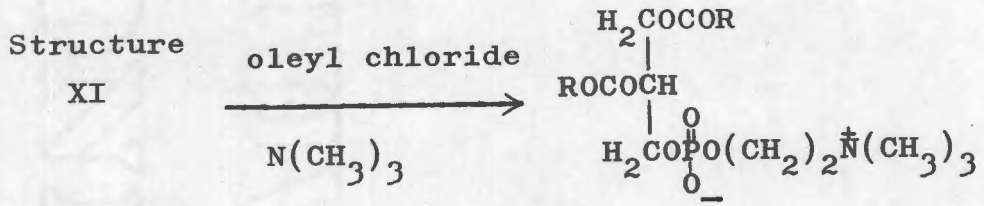
Baer et al[25,6] also successfully synthesised unsaturated L-α-(dioleyl)-lecithin. D-acetone glycerol (IX) was phosphorylated with monophenylphosphoryldichloride and the product esterified with ethylene chlorohydrin. The resulting structure (X) was hydrogenated, subjected to acid hydrolysis and isolated as the barium salt (XI). Compound XI was treated with oleyl chloride and heated with trimethylamine to give a mixture of L-α-(dioleyl)-lecithin (XII) and the corresponding lysolecithin. The pure L-α-(dioleyl)-lecithin was isolated by column chromatography on silicic acid.



IX X



XI



XII

R= $-(CH_2)_7CH=CH(CH_2)_7CH_3$

2.2.3.2 Purification of Natural Lecithin.

Much work has been published on the purification of lecithin from egg yolk and the methods have been extensively reviewed by Lea and Rhodes[26,27]. The phosphatide fraction is removed from the egg yolk by extraction with acetone, which removes lipids such as cholesterol and leaves the phosphatide residue. This is further extracted to destroy any lipo-protein complexes, and also remove the fully saturated lecithins and accompanying lipid contaminants. Until fairly recently treatment of the phosphatide mixture to yield lecithin

was carried out by a method devised by Bergell[28] and developed and modified by Levene and Rolf[29] and Pangborn[30]. In this method, a saturated cadmium chloride solution is added to an ethanolic solution containing mixed phosphatides. The complex formed between the cadmium chloride and lecithin precipitates from the ethanolic solution. The complex is destroyed by further extraction or partition between immiscible solvents[29,30] or by ion exchange resins[31]. The disadvantage of this method is that the lecithin is always contaminated with lysolecithin and it is impossible to remove all traces of this contaminant and of the cadmium ions. Since phosphatides tend to associate with one another in solution, a method based on fractional precipitation is not completely efficient. The most widely applied purification procedures for lecithin are therefore based on column chromatography. Adsorption chromatography was first applied to the separation of mixed lipids by Trappe[32] who demonstrated that lipids are more strongly adsorbed as their polarity increases. In 1951 Hanahan et al[33] effected separation of phospholipids on alumina columns but the lecithin isolated also contained small amounts of lysolecithin. Lea and

Rhodes[27] separated cephalins, lecithin and lysolecithin on silicic acid/celite columns with chloroform/methanol solvents for elution. This method was the first to separate completely lysolecithin from lecithin. In 1957 the same workers[26], using both silicic acid columns and improved alumina columns, quantitatively separated the main components of egg phospholipids, as determined by analytical techniques. Hanahan et al[34] have also separated the various classes of phospholipids using only silicic acid columns and chloroform/methanol eluants.

2.3 Physical Chemistry of Lecithin Solutions.

2.3.1 Non-polar Media

Boiling-point elevation measurements[35] and dialysis[36] experiments in non-polar solvents such as benzene confirm the fact that lecithin is present in the form of small micelles. Micelle formation by lecithin in benzene has been studied in detail by Elworthy[7,37]. The critical micelle concentration of lecithin in benzene at 25°C has been reported by Blei and Lee[38] to be 33×10^{-5}%. Above this concentration small micelles containing four[7] monomer units are present. By an osmometric study of benzene solutions of lecithin, Elworthy found a second association limit at 0.073%, at which

the small micelles aggregate into large ones. Below this concentration the micellar weight was found to be 3180 at 25°C and 1830 at 40°C. The molecular weight of monomer is approximately 780. The large micelles were found to have molecular weights of 57,000 at 25°C and 43,000 at 40°C which would correspond to 73 monomers at 25°C and 55 at 40°C. Enthalpy and entropy values were found to be fairly constant in the concentration region 2-10 g/l where mainly large micelles are present. At 25°C the values are the following:

$$\Delta H_m \cong 1.6 \text{ kcal/mole}$$

$$\Delta S_m \cong 6.0 \text{ cal/deg,mole}$$

where the subscript m denotes micellisation.

Elworthy[37] also determined the diffusion constants of lecithin micelles in benzene solutions. By means of a moving boundary technique the diffusion of lecithin solutions into pure benzene was studied. The measurements yielded data indicative of two-component diffusion from which the molecular weight of the small micelles was determined. Semi-differential diffusion experiments involving diffusion from concentrated to dilute lecithin solutions gave results corresponding to single solute diffusion and the molecular weight of the large micelles

was determined. The molecular weights of both large and small micelles at 25°C agreed closely with those obtained from osmometry. Viscosity measurements were used to study the shape of the micelles in solution. The shape factor, ν, was obtained by dividing the specific viscosity of the solution, η_{sp}, by ϕ, the the volume fraction of solute. By plotting η_{sp}/ϕ versus concentration the intercept at zero concentration (equal to 2.5 for spherical particles[39,40]) gave a value of 2.8, indicative of asymmetry.

From molecular models, the dimensions of the lecithin molecule are determined to be 55Å^2 for the phosphoryl-choline head group area and 35Å for the length of the molecule[37]. For micelle structure in benzene Elworthy[37] allowed a gap of 2Å between polar groups in the micelle such that the overall length of the micelle is 72Å. From the agreement of the shape factor (from viscometry) with the micellar model, Elworthy concluded that the large micelles had a laminar or sandwich-type structure with the polar head groups in the interior of the micelle and the hydrocarbon chains directed outward towards the solvent.

2.3.2 Polar Media

In polar non-aqueous solvents such as ethanol, lecithin exists in the monomeric form, as confirmed by boiling-point elevation measurements[35] and dialysis[36]. Robinson[41] found lecithin micelles in water to have a molecular weight (by light-scattering techniques) of 20×10^6 and an orientation such that the polar head groups faced the solvent while the hydrocarbon chains formed the interior of the micelle. Since in benzene the micellar structure was reversed, Elworthy[8,42] made a study of lecithin micelles in solvents of varying intermediate dielectric constants to find out at what point this inversion of micellar structure occured. Light-scattering, viscosity and diffusion techniques were used to study lecithin in solvents of dielectric constant 2.3 to 42.8. Results indicated that as the dielectric constant of the solvent medium increased from that of benzene ($\mathcal{E} = 2.3$) the micelle size decreased until at $\mathcal{E} = 30$, monomers were present. Further increase in dielectric constant towards that of water increased the micellar size promoting the formation of micelles in which the hydrocarbon chains were placed in the interior and the polar groups formed the micelle exterior. Similar results to that in benzene were found[8,41] for the shape of the

lecithin micelles in aqueous solvents except that the structure of the bimolecular leaflets was inverted.

2.3.3 Lecithin Sols.

Natural lecithin forms colloidal sols with water which are quite stable in the absence of inorganic salts, but which are easily precipitated by small concentrations of divalent metal salts[2]. Much work has been carried out on the interface of lecithin sols and water in attempts to simulate a cell membrane environment. Elworthy[2] stated that the simple cell membrane is a film built on a fatty structure which contains lecithin and cholesterol and which separates two aqueous fluids. Saunders[43,44] studied the problem of forming stable films between two aqueous liquids by examining the sharp boundaries formed when the sol and water were allowed to flow through a common orifice. Using optical techniques he found that interfaces were formed which were stable over a matter of days. Elworthy and Saunders[45,46] studied the surface forces at the sol/water boundary by measuring the force required to draw a platinum ring up through the interface under various conditions. No surface force was observed for natural lecithin if all small ions were removed by ion exchange columns. The effect of added electrolytes

was studied and it was concluded that the surface force appeared to be due to a film of insoluble lecithin-salt complex formed at the boundary between the sol and water. Elworthy[2] also mentioned that surface force differed from interfacial tension in that there were no unbalanced molecular attractive forces at the sol/water interface. Aqueous solutions of hydrocarbon films stabilised by lecithin have also been investigated[47] in attempts to make and examine artificial bimolecular leaflets separating two aqueous layers.

In general the systems studied are attempts at simulating biological systems but it may be realised that the complexity of the actual biological system makes it extremely difficult to approach identical conditions experimentally.

2.4 Solubilisation by Lecithin.

2.4.1 Non-polar Media.

2.4.1.1 Qualitative Evidence for Solubilisation.

As early as 1909, Porges and Nebauer[48] reported that ethereal solutions of lecithin "dissolved" many substances, such as glucose, which were normally insoluble in ether. Baer[6] found that glucose and sucrose could be solubilised by lecithin in solutions of ether (anhydrous and moist) and chloroform to as

high as 6% (w/w) for glucose in moist ether. In 1961 Billimoria[49] found that a reducing sugar eluted from a silicic acid column with the lecithin fraction, and suggested a "chemical combination" between the two, as separation attempts between water and chloroform gave an aqueous layer free of sugar. LeFevre et al[50] studied the solubility of various sugars in hexane solutions of phospholipids and lecithin. They reported that the sugar remained chemically unmodified and was held by the phospholipid in a "weak association". Little attempt was made by these workers to investigate the nature or extent of the interaction between the sugars and phospholipid.

2.4.1.2 Quantitative Evidence for Solubilisation.

Evidence for the solubilisation of various substances by lecithin micelles in benzene solution has been reported by several workers. Elworthy[51] studied the solubilisation of twelve dibasic fatty acids by lecithin micelles in benzene and found that the apparent volume solubilised was too large to be accounted for by accomodating the entire acid within the micelle. It was therefore necessary to suggest a structure in which only one of the polar carboxyl groups need be accomodated within the micelle, while the remainder of the molecule

may protrude into the benzene. The protruding section was therefore analogous to a monobasic fatty acid and such substances are known to be freely soluble in benzene. Elworthy showed that the required amounts of solubilisate could be accounted for by arranging the acids with their long axes parallel either to the major or minor axes of the rectangular polar 'sheet' of the micelle head. Acids with an even number of carbon atoms were solubilised to a somewhat greater extent than acids with odd numbers of carbon atoms.

Elworthy also studied the adsorption of water vapour by lecithin[4,52] and the interaction of water with lecithin micelles in benzene[3]. Viscosity measurements indicated that the micelles resembled ellipsoids and the addition of water initially increased their asymmetry. Above 0.055 g water/g lecithin the micelles tended towards a more spherical shape. Nearly all the water solubilised was found to be associated with the lecithin. Elworthy suggested that in order to prevent contact between the hydrated polar heads and benzene, the micelle shape became spherical (as confirmed by light scattering) as the volume of water in the micelle centre increased. Sphericity was achieved at 33% water which agreed closely with the value reported by Demchenko[53] who found this

value to be independent of the solvent used (benzene, toluene and xylene). Blei and Lee[38] reported the solubilisation of potassium and sodium dye salts by lecithin micelles in benzene. The critical micelle concentration (c.m.c.) between monomer and small micelles was detected at 33 x 10^{-5}% lecithin at 25°C. A second aggregation, of small micelles to large, was observed close to 0.08% which was the c.m.c. value reported by Elworthy[7].

2.4.2 Polar Media.

The solubilisation of cholesterol by lecithin micelles in aqueous solution has been reported by Fleischer et al[54] and Saunders[55]. The former group treated lecithin in the presence of cholesterol with "the reagents for inducing micelle formation (butanol/water/cholate mixture followed by dialysis)". Micelles of lecithin were formed containing 20% cholesterol by weight. They also reported that the presence of cholesterol did not affect the opalescence of the solution, or the amount of cholate retained after dialysis. Saunders[55] irradiated lecithin sols ultrasonically and found the large micelles (20 x 10^6 molecular weight) to be reduced to 5 x 10^6. The resulting sols were clear and showed no increase in turbidity on standing. These

sols were capable of solubilising 10% cholesterol by weight in a 20% lecithin solution or one molecule of cholesterol per molecule of lecithin,however these sols were unstable and gelled on standing.

2.5 Biological Significance.

Much work has been published suggesting that phospholipids are involved in the structure or function of the cell membrane[2,47,56,57].

Since the early 1900's, cholesterol has been studied as a possible factor in ischaemic heart disease. In 1957, Friedman et al[58] induced atheroma in rabbits by feeding them cholesterol and successfully reversed the atheroma by lecithin infusions. In recent years, Yudkin[58,60], from statistical analysis of nutrition studies, has suggested a relation between sugar intake and ischaemic heart disease by studying the correlation of diet and the incidence of the disease in several major countries. Cohen[61] also reported the intake of sucrose to be a dietary factor in the development of ischaemic heart disease among the new immigrants to Israel. It has also been reported[5] that glucose addition accelerates the crystallisation of cholesterol from supersaturated cholesterol/triglyceride solutions containing lecithin.

It is possible that glucose is involved in the deposition of cholesterol in atheroma formation. A study of lecithin, cholesterol and glucose was necessary to confirm if previous evidence[5] was due to rate or equilibrium phenomena. Quantitative evidence was also lacking and no investigation had been made as to the chemical nature of the interactions between these three components. For these reasons, the present study was undertaken.

CHAPTER 3

EXPERIMENTAL PROCEDURE

3.1 Purification of Lecithin.

The method followed was largely that of Lea, Rhodes and Stoll[27], and Hanahan et al[34], which involved column chromatography using silicic acid as the adsorbent.

Materials.

The silicic acid used was Mallinckrodt's analytical reagent, 100 mesh. All solvents were either analytical reagent or re-distilled. Egg lecithin was extracted from hen's egg yolks by repeated extractions with acetone and chloroform-methanol (1:1 v/v) following the method of Rhodes and Lea[26].

Apparatus.

A large glass column approximately 4" in diameter by 20" in length was used. This was fitted with a sintered glass disc above a teflon stopcock. The top was connected to a ground glass joint containing inlet and exit tubes such that a stream of nitrogen could be fed simultaneously into the column and, by means of polyethylene tubing, into the receiver.

A Büchi rotoevaporator (Switzerland) was used to concentrate to dryness the fractions obtained from

the column.

Method.

The silicic acid was first sieved through a 200 mesh screen to remove fine particles which might clog (or wash through) the sintered glass disc. It was then suspended in acetone to remove traces of moisture, filtered on a Büchner funnel and dried at 120°C overnight. The silicic acid was slurried in acetone for column packing and the column washed first with one litre of acetone and then three litres of chloroform. The standard amount of silicic acid used in this column was 500 g.

When the solvent had drained nearly to the surface of the silicic acid, a sample of 5 g of crude lecithin (mixed phosphatides)dissolved in 50 ml of chloroform was introduced and allowed to flow slowly down the inside wall of the glass column so as not to disturb the silicic acid surface. All the solvents subsequently used were added in this manner. A flow rate of approximately 2 ml/min was maintained which ultimately allowed the pure lecithin to be collected in 50 hours running time. The elution solvents used were as follows: 500 ml of chloroform to wash the sample completely on to the column; one litre of 35% methanol-chloroform (v/v) to remove neutral lipids,cephalin (phosphatidylserine ,

phosphatidylethanolamine) and phosphoinositides. The lecithin was removed by 1-2 litres of 45% methanol-chloroform (v/v) and if it was desired to remove the sphingolipids and lysolecithin, one litre each of 65% and 85% methanol-chloroform (v/v) were added. As no automatic fraction collector was available, the initial fraction taken was as large as possible (1.5 l) since it was not necessary to separate one cephalin from another. Then fractions of approximately 250 ml were collected regularly (under nitrogen), each fraction was evaporated to dryness on a rotary evaporator and dissolved in methanol, small samples being taken for thin-layer chromatography (T.L.C.). All samples were kept under nitrogen in methanol at -16°C. The column was monitored by means of T.L.C. and the lecithin could easily be obtained quite pure by selectively discarding the initial and final lecithin fractions where contamination by any other phospholipids might occur. The plates were sprayed either with a fluorescein solution, an ammoniacal silver nitrate solution, or with dilute ethanolic sulphuric acid. The R_F of lecithin was found to be ca. 0.6. The details of the T.L.C. method and sprays are given below. The yield obtained on a 500 g silicic acid

column was approximately 2 g of pure lecithin from 5 g of mixed phosphatides.

A total of seventeen 500 g silicic acid columns were run, during which time various parameters were studied. An attempt at separating lecithin on an alumina column[26] was found to be not as efficient a separation as on silicic acid. From all the various columns run, several conclusions were drawn.

1) If fresh silicic acid was not available, then regenerated material was used after vigorous washing with 3N HCl and distilled water to a neutral pH. Columns with twice regenerated silicic acid were not satisfactory, however, as determined by T.L.C.

2) When both the column and sample were kept under nitrogen, the lecithin was colorless, whereas the product from a column exposed to the air was pale yellow, perhaps owing to slight oxidation.

3) The optimum phospholipid load for the conditions given above was found to be approximately 1 mg total phosphorus per 2 g silicic acid corresponding to 5 g of phospholipid per 500 g of silicic acid. If this load was exceeded, poor separation was found to result.

3.2 Thin-layer Chromatography.

Materials.

Silica gel G (Keiselgel G, "according to Stahl", Merck) and analytical reagent grade or re-distilled solvents were used. Standard lipids and phospholipids used for reference were obtained mainly from the following sources: Light Co., United Kingdom; Nutritional Biochemicals, Chagrin Falls, Ohio, U.S.A.; and Pfansteihl Chemical Co., Waukegan, Illinois, U.S.A..

Method.

The method was mainly that of Vogel et al[62], in which glass plates were coated with silica gel G (30 g mixed with 60 ml distilled water to a smooth fluid) using an appropriate applicator. The plates were dried at 120°C for 2 hours and stored over anhydrous calcium chloride.

The sample was applied by means of a microsyringe either as a band or a single spot. The plates were developed in 85:15:1.5 benzene/hexane/glacial acetic acid for neutral lipids and 80:25:3.25 chloroform/methanol/water for polar lipids (phospholipids). Standard samples were used for reference and identification.

The stains[63] used on the plates were the following:

1) Reducing substances were detected by ammoniacal silver nitrate. Colour: dark brown on white background.

2) Lipids were detected using a 0.05% 2',7'-dichlorofluorescein solution in ethanol. Colour: pinkish-white on an orange background under ultraviolet light.

3) Organic materials were detected by spraying with 5% sulphuric acid in ethanol and heating in an oven or over a hot plate. Colour: black spots on a white background.

4) Amino phosphatides were detected with ninhydrin. Colour: purple on a white background.

5) Choline groups were detected with the Dragendorf reagent. Colour: orange spots on a white background.

6) Phosphatides were detected by molybdic acid spray. Colour: blue spots on a white background.

By means of the above techniques neutral lipids (triglyceride, diglyceride, cholesterol, glycerol) and phospholipids (phosphatidylserine, phosphatidylethanolamine, sphingomyelin, lysolecithin) were found to be contaminants in the crude lecithin but were found to be entirely absent in the purified product.

3.3 Phosphorus Analysis.

The phosphorus content of lecithin was determined by the Bartlett[64] modification of the Fiske-Subbarow[65] method. The phosphorus was oxidised by peroxide and allowed to react with ammonium molybdate and the Fiske-Subbarow colour reagent (aminonaphtholsulphonic acid). The resulting blue colour absorbs at 830 mμ and obeys Beer's law in the concentration range studied, from 0.005 to 0.05 mg of phosphorus. A Unicam SP600 spectrophotometer was used for the determinations.

3.4 Determination of Iodine Value.

The iodine value of the pure lecithin was determined using the micro-analytical method of Trappe[66] based on the method of Kaufmann[67]. The sample was brominated using a solution of bromine in methanol saturated with sodium bromide. The mixture was treated with excess potassium iodide and the resulting iodine was titrated with a standard sodium thiosulphate solution. The results are discussed in section 4.1.3.

3.5 Infra-red Spectrophotometry.

A double beam Unicam SP100 high-resolution recording spectrophotometer was employed for deter-

mining the infra-red spectrum of the purified lecithin.

3.6 Viscometry.

All viscometric measurements were carried out using two Ostwald capillary viscometers which were standardised and calibrated as described in section 4.1.7.

3.7 Vapour Pressure Osmometry.

The vapour pressure osmometer used was a Mechrolab V/P/O, Series 300 (Mechrolab, Inc.). Results are discussed in sections 4.1.6 and 4.2.1.

3.8 Spectrophotometry.

All spectrophotometric determinations were carried out on a Unicam SP600 spectrophotometer (Unicam Instruments, Ltd.).

3.9 Sugar Analysis.

Since the amounts of sugars solubilised by lecithin in various solutions had to be determined, a method of sugar analysis was needed. The requirements for a satisfactory method of analysis are that it be general for a variety of sugars, and sensitive in the microgram range. For these reasons, the Smith[68] method was chosen. This method depends on the reaction of the carbohydrate with phenol and sulphuric acid to give a red-orange product of unknown structure. The intensity of the colour produced was measured by spectrophotometry.

Materials.

All sugars were analytical reagent grade, obtained in anhydrous form. Melting points determined on a Fisher-Johns melting point apparatus (Fisher Scientific Co.) gave values within 0.5°C of literature. All other reagents (concentrated sulphuric acid and phenol) and solvents used were also analytical reagent grade.

Apparatus.

A Unicam SP600 spectrophotometer (Unicam Instruments, Ltd.) was used for the colorimetric determinations.

Method.

The method employed in the analysis of the sugars is based on that of Smith[68] with several modifications. Since the solutions to be analysed contained sugar, lecithin, and in some cases, cholesterol, a method had to be devised to separate the sugar from the lecithin and cholesterol as these two substances interfered appreciably in the colorimetric determination. Various extraction techniques were attempted before the final successful method was developed. Since lint (or any cellulose matter) is automatically degraded to a reducing sugar by the sulphuric acid reagent and thereby biases the result, all glassware had to be scrupulously cleaned with chromic acid and all tubes were capped

with aluminium foil to prevent the introduction of lint. All determinations were carried out in triplicate. Benzene/lecithin solutions saturated with glucose were prepared according to the procedure described in section 3.12. The solutions (or aliquots) were filtered then triplicate samples containing 0.005 to 0.070 mg of sugar removed by means of a pipette, and introduced into a test tube. The samples were evaporated down to dryness on a steam bath. Three empty test tubes were heated alongside the samples for use as reference on the spectrophotometer. To the dried residues were added 2.0 ml distilled water followed by 1.0 ml ether. The tubes were shaken with a definite rotary motion until all the solid material had dissolved (approximately 40-60 seconds). The lecithin and cholesterol were dissolved in the benzene-ether layer, while the glucose (or other sugar) remained in the water layer. The water layer (lower) in each case was removed to a second clean test tube by means of fine glass capillary pipettes. These solutions were heated for 10 min in a steam bath to remove any traces of ether and benzene which might cause opalescence in the final solution. The solutions were cooled to room temperature and 1.0 ml samples were removed to

a third test tube. The required amount of phenol (5% aqueous solution) was added by means of a pipette, and 5.0 ml of concentrated sulphuric acid was added rapidly from a 5 ml glass syringe, such that the stream of acid was directed towards the liquid surface for thorough mixing. The tubes were allowed to stand for 10 min, they were shaken thoroughly (again using a rotary motion) and placed in a water bath at 25-30°C for 10-20 minutes. The colour formed was found to be stable over a period of several hours.

Standard solutions were also run for each sugar to ensure that Beer's law was obeyed over the concentration range studied. The absorbance was measured at 480-490 mμ depending on the absorbance maximum for the particular sugar being determined. The sugars studied, the conditions employed and the optical density values of their respective Beer's law plots are presented in Table I and illustrated in Figure 1.

The extraction procedure was tested by determining the amounts of glucose in standard solutions containing known amounts of lecithin and cholesterol and the results were found to be experimentally indistinguishable from results for the standard glucose solutions themselves.

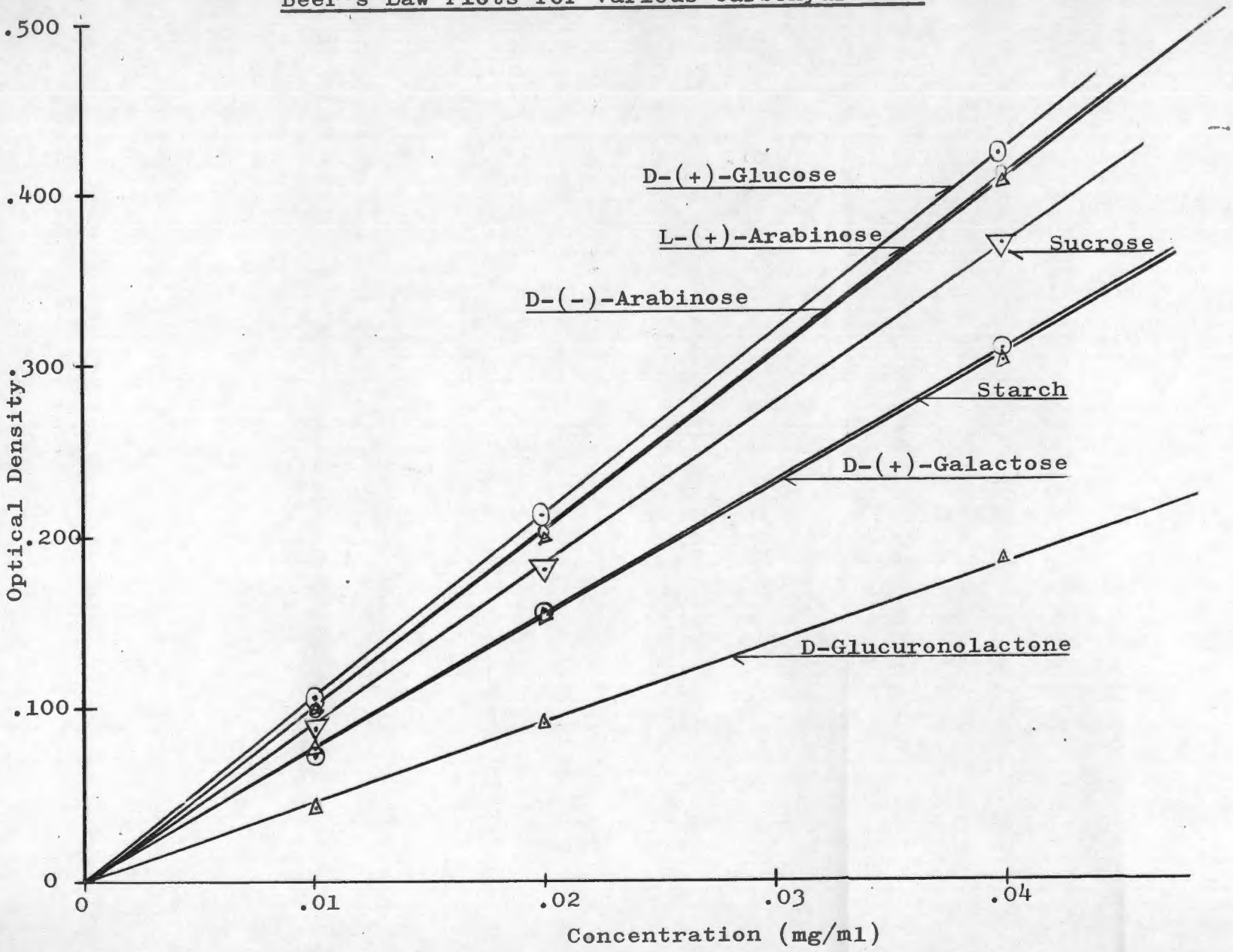
TABLE I

Analytical Conditions and Results for Various Carbohydrates.

Carbohydrate	Wavelength (mμ)	Amount of Phenol (mg)	Concentration (mg/ml)	Optical Density (1.0cm cuvette)
D-(+)-Glucose	485	50	0.010	0.107
			0.020	0.213
			0.040	0.417
			0.060	0.640
D-(+)-Galactose	487	40	0.010	0.078
			0.020	0.153
			0.040	0.307
L-(+)-Arabinose	480	40	0.010	0.103
			0.020	0.205
			0.040	0.413
D-(-)-Arabinose	480	40	0.010	0.100
			0.020	0.201
			0.040	0.407
D-Glucuronolactone	480	40	0.010	0.047
			0.020	0.093
			0.040	0.188
Sucrose	490	100	0.010	0.087
			0.020	0.182
			0.040	0.370
Starch	488	100	0.010	0.070
			0.020	0.157
			0.040	0.310

FIGURE 1

Beer's Law Plots for Various Carbohydrates.



The order of solvent addition in the extraction procedure was found to be important: if the benzene were added first, rather than the water, very low results were obtained, as the lecithin micelles continued to hold some of the glucose in the benzene-ether layer.

The precision of the measurement was tested by experimental determination of the concentrations of a set of standard glucose solutions of known concentrations. The deviation of any individual determination from the experimental mean was in no case greater than ±6% and the deviation of the experimental mean from the known concentration was in each case usually less than ±1% for a set of triplicate determinations.

3.10 Cholesterol Analysis.

Since the amount of cholesterol solubilised by lecithin in various solutions had to be determined, a method of cholesterol analysis was needed. Cholesterol was determined by means of the methods of Zak et al[69] and Abell et al[70] adapted to a micro scale by Wilkens and de Wit[71].

Materials.

The sulphuric acid, glacial acetic acid, petroleum ether (60-80°C) and absolute ethanol were all analytical reagent grade. The cholesterol used was recrystallised

three times from absolute ethanol and dried to a constant weight. Melting point: 148-8.5°C.

The colour reagent stock solution consisted of 5.0 g anhydrous ferric chloride and 5.0 ml distilled water, made up to 100 ml with glacial acetic acid. A 33% aqueous potassium hydroxide solution was prepared for the saponification procedure.

Method.

An aliquot of the solution to be tested was either filtered (if the supernatant was clear) or centrifuged (if the supernatant was turbid). Triplicate samples of the aliquot were then pipetted into 10.5 by 1.5 cm pyrex test tubes fitted with B14 ground glass joints and stoppers. The samples were heated to dryness on a boiling water bath (usually under a stream of nitrogen). Potassium hydroxide,(0.5 ml of a solution made from 2.0 ml of 33% KOH and 28 ml absolute ethanol), was added and the stoppered tubes were placed in a water bath at 45°C for 1.5 hours.for saponification. The tubes were cooled and 0.5 ml of distilled water and 3.0 ml of petroleum ether were added to each tube. The stoppers were replaced and the tubes were shaken thoroughly for 1.5 minutes. Two-thirds of the petroleum ether layer (2.0 ml) was removed in each case to second test tubes

and evaporated to dryness over a steam bath. Glacial acetic acid (1.5 ml) was then added from a glass syringe and if necessary the tubes were heated slightly until all the material had dissolved. Finally 1.0 ml of colour reagent (1.0 ml of ferric chloride stock solution diluted to 100 ml with concentrated sulphuric acid) was added from a glass syringe to each tube and the tubes were shaken by hand to ensure proper mixing. The tubes were kept in a water bath at 30°C for 20 min and then the absorbance of the solutions was measured spectrophotometrically at 560 mμ. The violet colour produced was stable over a period of several hours. Values for the absorbance of standard solutions of cholesterol treated in the same manner are listed in Table II and illustrated in Figure 2.

The precision of the measurement was tested by experimental determination of the concentration of a set of standard cholesterol solutions of known concentrations. The deviation of any individual determination from the experimental mean was in no case greater than ± 2% and the deviation of the experimental mean from the known concentration was in each case usually less than ± 2% for a set of triplicate determinations.

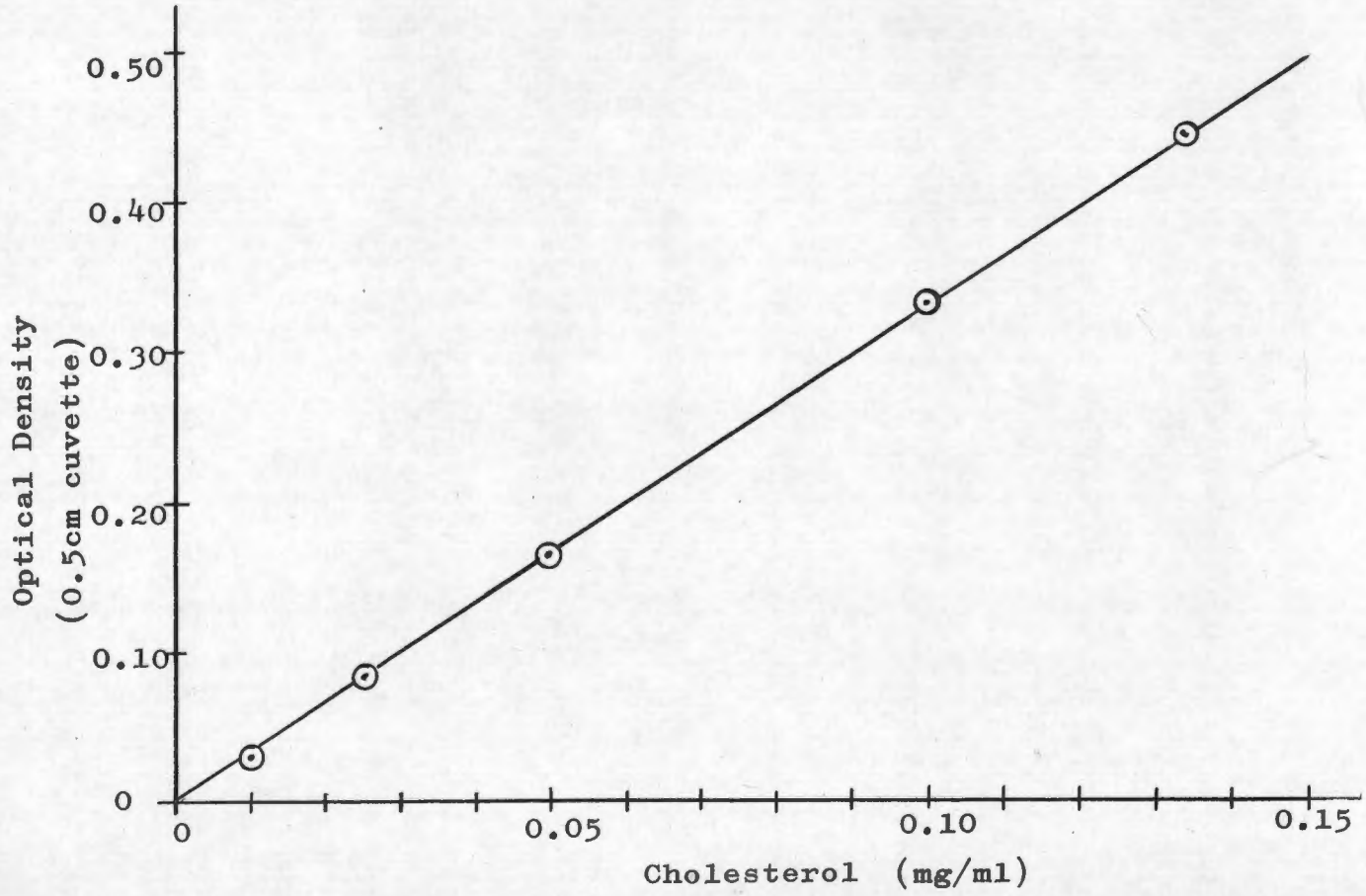
TABLE II

Absorbance of Standard Cholesterol Solutions at 560 mμ.

Concentration (mg/ml)	Optical Density (in 0.5 cm cuvette)
0.010	0.030
0.025	0.085
0.050	0.165
0.100	0.333
0.134	0.445

FIGURE 2

Beer's Law Calibration for Cholesterol Analysis at 560 mμ.



3.11 Sorbitol and Inositol Analyses.

Neither of these sugars responded to the Smith[68] analysis method, therefore alternative procedures had to be found. After extraction into aqueous solution sorbitol was determined by periodate oxidation, followed by spectrophotometric determination using chromotropic acid[72]. Inositol was determined by oxidation with bromine water at 100°C, followed by the use of the Smith[68] method on the oxidation product.

3.11.1 Sorbitol Analysis.

The solution was extracted by means of the method described in section 3.9. The method of Speck[72] was modified by using more concentrated reagents. This method involved periodate oxidation of the sugar, and the formaldehyde formed was reacted with chromotropic acid. The intensity of the resulting purple solution was measured spectrophotometrically.

Materials.

Chromotropic acid,(4,5-dihydroxy-2,7-naphthalene-disulfonic acid), analytical reagent grade, was used. The colour reagent was made up as follows: chromotropic acid,0.5 g, was dissolved in 50 ml of water and filtered. The solution was diluted to 250 ml with 2:1 concentrated sulphuric acid/water.

All other reagents, including the formaldehyde, were analytical reagents.

Method.

To each of three triplicate samples of sorbitol solution containing 0.02 to 0.10 mg was added 0.4 ml of 1.5 M periodic acid. The tubes were shaken and 2.0 ml of 1M sodium bicarbonate was added. The tubes were stored in the dark at 25°C for 2 hours. Sulphuric acid (3.0 ml,2.5M) and sodium arsenite(5.0 ml,1M) were added. The tubes were shaken until the reduction of the iodine which formedon addition of the sodium arsenite solutionwas complete. To 1.0 ml of each solution was added 10.0 ml of the chromotropic acid reagent. The tubes were heated over a steam bath for 30 min and stoppered when hot. On cooling it was found necessary to centrifuge the samples to remove residual iodine.

The formaldehyde content of standard solutions was determined by measuring the absorbance after reaction with chromotropic acid under identical conditions. The results are presented in Table III. Each determination was carried out in triplicate and reagent blanks were run for reference purposes. The absorbance of the purple colour was measured spectrophotometrically at 570 mμ..

TABLE III

Absorbance of Standard Formaldehyde Solutions

at 570 mμ.

Formaldehyde (μ moles/ml)	Optical Density (1.0 cm cuvette)
0.091	0.145
0.227	0.359
0.590	0.906

3.11.2 Inositol Analysis.

As periodate oxidation of inositol does not yield formaldehyde, the previous method (section 3.11.1) was unsuitable. Inositol was therefore determined by preliminary oxidation with bromine water.

Method.

Inositol was extracted into aqueous solution as described in section 3.9. To each of three triplicate samples containing 0.01 to 0.15 mg of inositol was added 1.0 ml of saturated bromine water. Standard inositol solutions and reagent blanks were determined along with the inositol samples. The tubes were placed in a boiling water bath for one hour until the solutions were colourless. They were heated for 5 hours longer and evaporated to dryness under nitrogen. A modification of the Smith[68] method was used, by adding to each dried residue 1.0 ml of 5% aqueous phenol solution. The tubes were shaken until all solid material was dissolved and 5.0 ml of concentrated sulphuric acid was added. The absorbance of the resulting colour was measured spectrophotometrically at 480 mμ. Table IV gives the absorbance of the oxidised inositol standards determined by this method.

TABLE IV

Absorbance of Standard Inositol Solutions

at 480 mμ.

Inositol Concentration (mg/ml)	Optical Density (1.0 cm cuvette)
0.036	0.039
0.072	0.077
0.144	0.155

3.12 General Procedure for the Determination of Solubilisation.

The pure lecithin was made up to a known concentration with methanol (analytical reagent grade) and kept under nitrogen at a maximum temperature of -16°C. This solvent was found by Hanahan[73] to be the most suitable as solvents such as chloroform develop over a period of time acids which would cause significant Hydrolysis of lecithin. All glassware was chromic acid washed and thoroughly rinsed with distilled water prior to use. For all experiments a measured aliquot of the lecithin solution was evaporated to constant weight in a tared 25 ml erlenmeyer flask fitted with ground glass joints. The resulting weight of lecithin was thus determined directly and confirmed by the known weight per unit volume of solution. To prevent the lecithin from absorbing water, all flasks were kept in a dessicator until immediately before use. Additional reagents were added in solid form (glucose or cholesterol, etc.) and the solvent was pipetted into the flask. All flasks were purged with nitrogen and tightly stoppered. They were put into a thermostatted water bath with continuous mechanical agitation. The bath was used without a cover as water tended to condense on the cover and drop

onto the glass flasks. The shaking action was controlled to one full cycle per second.

If filtration of the solution was necessary, the filter paper was pre-dried in an oven at 120°C to remove as much moisture as possible and kept in a dessicator until used. This was mainly to prevent the dry benzene solutions from becoming contaminated with water. During all manipulations the solutions were kept under a nitrogen blanket, and stored at -16°C when not in use.

Sampling techniques varied depending on the particular experiment and the material (i.e. glucose, cholesterol,etc.) being determined and are dealt with specifically in the appropriate sections.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterisation of Lecithin.

Before the solubilisation study could be commenced, it was necessary to characterise the lecithin using the chemical and physical techniques described in this section.

4.1.1 Thin-layer Chromatography.

During the course of the column chromatographic separation of the pure lecithin from its contaminants, the fractions were each analysed using the technique of thin-layer chromatography. For details, refer to section 3.2.

Plates were stained with 0.05% 2',7'-dichloro-fluorescein in ethanol and examined by ultraviolet light. The phospholipid fluoresces under these conditions and appears as a pinkish-white spot against an orange background.

Typical results for pure lecithin are illustrated in the photograph designated as Figure 3. No tail, halo, or other evidence of impurity is visible. The R_F value (approximately equal to 0.6) is identical to that of a reference sample of pure lecithin kindly supplied by Professor D.J.Hanahan, of the University of Washington.

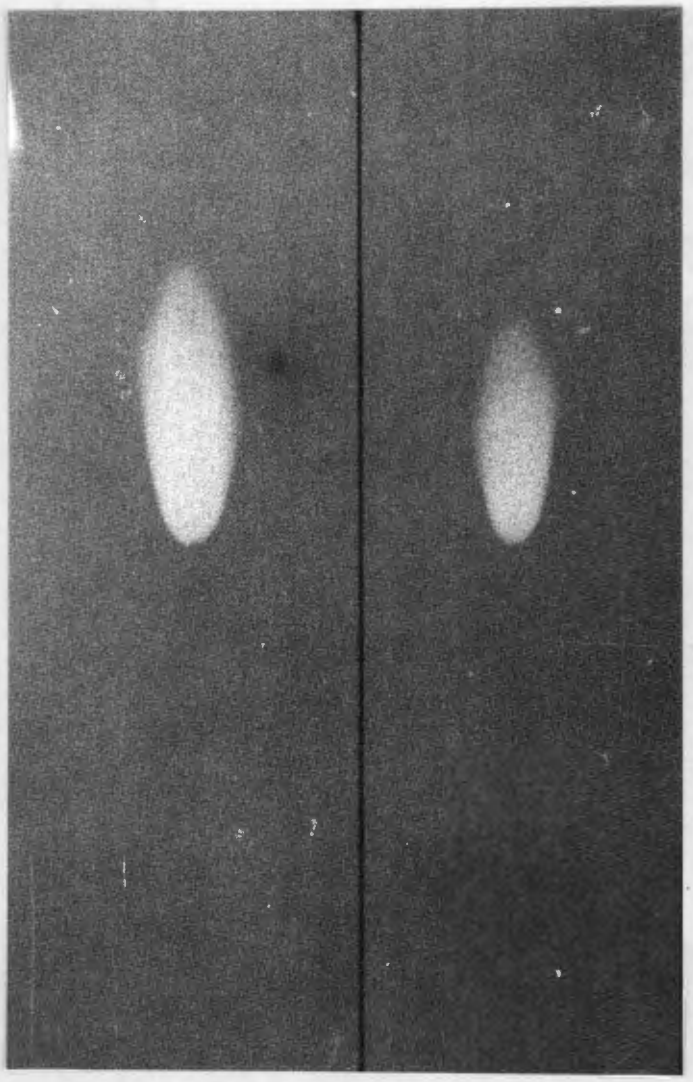
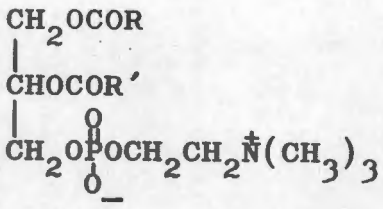


FIGURE 3

Fluorescein-sprayed T.L.C. Plate of Lecithin Photographed Under Ultra-Violet Light.

4.1.2 Fatty Acid Content.

Lecithin has been shown[16,22] to have the structure



where R and R′may represent the alkyl components of various saturated or unsaturated fatty acids. A sample (3 mg) of purified lecithin was methanolysed by refluxing with 4 ml of 5% methanolic sulphuric acid for 2 hours, followed by extraction of the resulting methyl esters with petroleum ether (40-60°). The excess solvent was evaporated off under a stream of dry nitrogen and the residual esters subjected to gas-liquid chromatography, using ethylene glycol glutarate supported on Chromosorb W (80/100 mesh) as a stationary phase at a temperature of 176°C. Argon was used as carrier gas at an inlet pressure of 20 lbs/in^2. Elution of components was monitored using a flame ionisation detector and peaks were identified using reference mixtures of esters of known fatty acids.

The acids found are reported in Table V. Retention

TABLE V

Analysis of Lecithin Fatty Acids by G.L.C.

Number of Carbon Atoms in Acid	Number of Double Bonds	Relative Retention Time	Relative Peak Area
16	0	1.00	1.000
16	1	1.14	0.075
18	0	1.90	0.450
18	1	2.11	0.907
18	2	2.54	0.333
20	4	5.82	0.112

Retention Times and Peak Areas are expressed relative to those of the methyl ester of palmitic acid.

times and peak areas of the methyl esters are reported relative to $C_{15}H_{31}COOCH_3$.

This distribution of fatty acids is similar to that found by Levene and Rolf[20].

The fatty acid analysis is in accord with the experimentally determined iodine value (section 4.1.3), infra-red spectrogram (section 4.1.4) and micro-analysis (section 4.1.5).

4.1.3 Iodine Value.

The method for determination of the iodine value was presented in detail in section 3.4.

The iodine value found was 63.8 (mean of three results: average deviation = $\pm$ 0.45). This value is in agreement with those of Elworthy[42,74,75] (60,62,66.7) and can be shown to be in reasonable accord with the unsaturation indicated by the G.L.C. analysis, since each molecule of lecithin has an average of 1.463 double bonds, according to the results shown in section 4.1.2. The detailed calculation is shown in the Appendix, sections 6.1-6.3.

4.1.4 Infra-red Spectrophotometry.

The infra-red spectrum of a 1% solution of lecithin in spectral quality carbon tetrachloride was run on a double-beam Unicam SP100 high-resolution recording

spectrophotometer against a reference cell containing solvent. The path length was 1 mm, and the range scanned was 650-3650 cm^{-1}. The spectrogram of the pure lecithin is illustrated in Figure 4, and the assignment of peaks found in both pure and crude lecithin is presented in Table VI.

Spectrograms of lecithins[17,25] have appeared in the literature and peaks shown here are in perfect accordance with the generally accepted structure of this compound. Baer[6], in his work on synthetic lecithins has reported a peak at 970 cm^{-1}, also found in this spectrogram, which is attributed to absorbance by the covalent phosphate group.

4.1.5 Micro-analysis.

Micro-analysis of a sample of pure lecithin gave the following result:

C = 65.07% H = 10.96% N = 1.81% P = 3.92%.

Theoretical:

C = 65.14% H = 10.85% N = 1.78% P = 3.94%.

Calculations of the expected percentages of carbon, hydrogen, nitrogen and phosphorus were carried out using the fatty acid analysis reported in section 4.1.2. The detailed calculation is presented in the Appendix, section 6.4.

FIGURE 4

Infra-red Spectrogram of Lecithin.

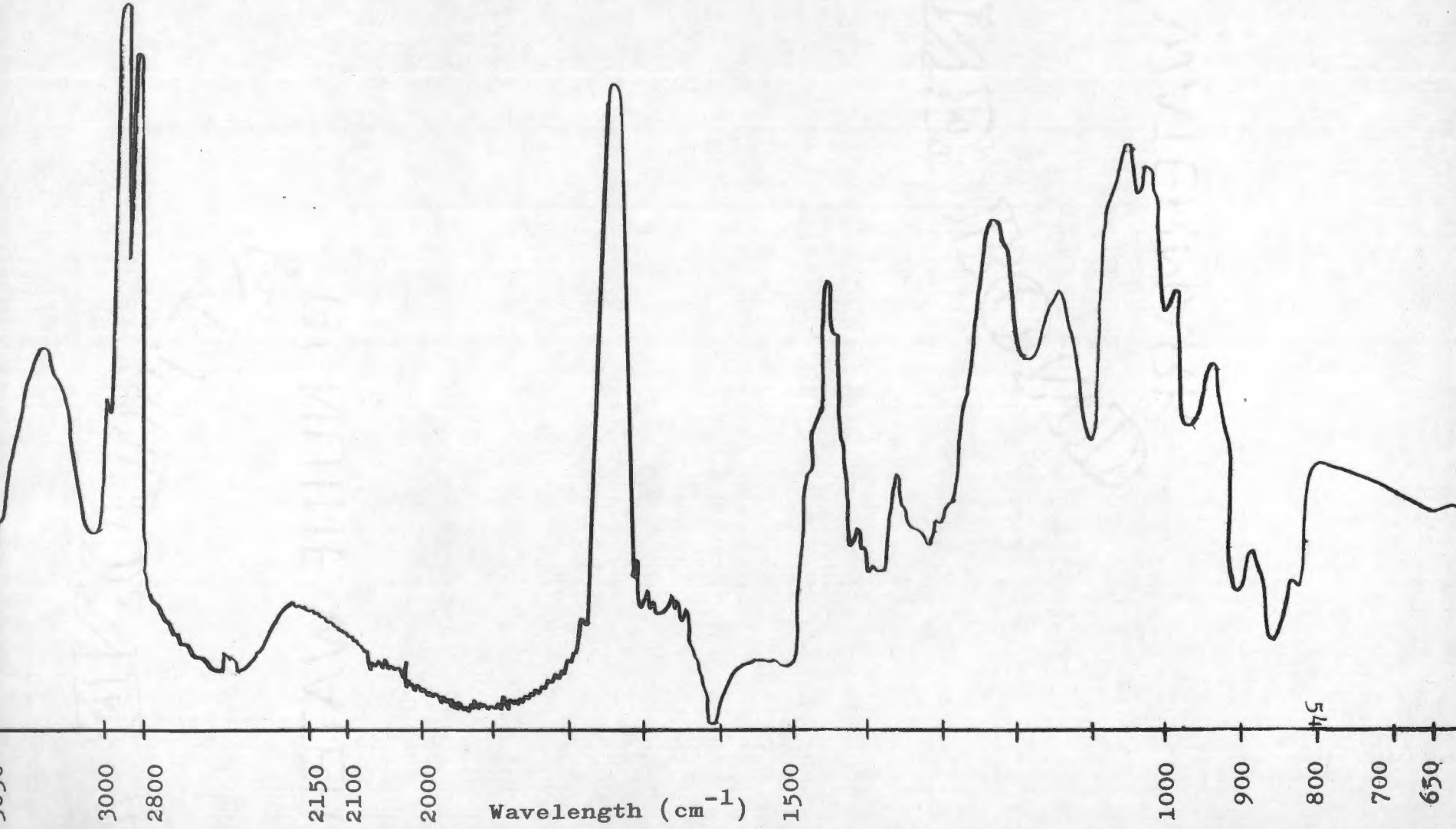


TABLE VI

Infra-red Spectrograms of Lecithin: Peak Assignments.

Wavelength (cm^{-1})	Peak Intensity		Assignment
	Pure	Crude	
3400	M	S	-OH
3010	W	/	$\rangle C=CH-$
2930	S	S	$-C-CH_3$
2880	S	S	$-CH_2-$
2360	/	W	
1740	S	M	$R\overset{O}{\overset{\|}{C}}OR$
1710	/	S	
1660	W	/	$\rangle C=CH-$
1590	/	M	
1475	M	M	$-CH_2-$
1430	/	M	
1385	W	/	$-\overset{H}{\overset{\|}{C}}=\overset{H}{\overset{\|}{C}}-$
1265	M	merged	$-CH_2OPO, P=O, -C\equiv N$ [76]
1170	M	merged	ester(butyrates and up)
1110	S	broad shoulder	-C-N, -C-O
1075	S	broad shoulder	-P-O-C-
1060	/	S	
1030	M	merged	n-propyl or i-propyl
975	M	merged	$-CH_2-O-P\rightarrow O$
930	W	merged	$-CH_2CH_2CH_3$
850	M	S	$\rangle C=CH-$
675	M	S	CCl_4 (solvent)

S = strong ; M = medium ; W = weak

4.1.6 Micellar Weight of Lecithin.

Elworthy has already proved the existence of micellar aggregates in benzene/lecithin solutions using a wide variety of methods[7,37]. The presence of micelles in benzene solutions of the lecithin used in this study and a determination of their micellar weight as a function of concentration, was shown using the diffusion method of Polson[77]. Using this technique, a solution of lecithin is allowed to diffuse freely into pure solvent for a known time, after which the experiment is terminated and the lecithin content of the solvent compartment is determined using the phosphorus analysis method[64,65] described in section 3.3.

The diffusion constant, D, was determined from the equation:

$$D = \left[\frac{C_t}{C_o} H \right]^2 \frac{\pi}{t}$$

where C_t = concentration of final solution

C_o = concentration of original solution

H = height of column containing diffusate

t = duration of experiment in seconds.

The molecular weight of the diffusing particle is then given by the equation[78]

$$[\eta] = \frac{k}{M\ D'^3}$$

In this equation

$[\eta]$ = intrinsic viscosity = $\left[\dfrac{\text{specific viscosity } (\eta_{sp})}{\text{concentration (C g/ml)}}\right]_{C\to 0}$

$$\left[\frac{\eta_{sp}}{C}\right]_{C\to 0} = \left[\frac{\left(\dfrac{\text{viscosity of solution}}{\text{viscosity of solvent}}\right) - 1}{\text{concentration (g/ml)}}\right]_{C\to 0}$$

where k = a constant of value 6.16×10^{-14} (C=g/ml) based on the Stokes-Einstein equation[79]. In recent years Broesma[80] has modified the Einstein equation for large molecular weight particles, giving a value $k=7.39 \times 10^{-14}$ (C=g/ml).

M = molecular weight

$$D' = D\left[\frac{\text{viscosity of benzene (poise)}}{\text{viscosity of water (poise)}}\right] = 0.588D.$$

Intrinsic viscosity measurements are presented in detail in section 4.1.7.

Molecular weights for lecithin/benzene solutions are presented in Table VII. Both values of the constant k have been used in the calculations.

TABLE VII

Micellar Weights of Lecithin/Benzene Solutions.

$T = 21.1^{\circ}C$ $[\eta] = 3.61$ ml/g

Concentration (g/ml)	D x 10^6 (cm^2/sec)	Molecular Weight	
		$k=6.16 \times 10^{-14}$	$k=7.39 \times 10^{-14}$
0.0006	4.90	710	860
0.0012	4.94	700	840
0.0060	3.88	1,400	1,700
0.0120	1.69	17,000	21,000
0.0240	1.29	39,000	47,000

It is interesting to note that Elworthy[7] reported the critical micelle concentration for benzene/lecithin solution as 0.00073 g/ml, and that at comparable concentrations the molecular weight reported in this study corresponds to that of monomer molecules. This need not mean that no micelles exist at these concentrations, but it does mean that the weight fraction of monomers is sufficiently large that the diffusion process occurs predominantly through the mechanism of monomer migration.

At higher concentrations of lecithin, the molecular weight increases, attaining a value of 47,000 at a concentration of 24 mg/ml. This is in good agreement with Elworthy's[7] value of 57,000 under comparable conditions: the difference between the two numbers being easily attributed to the fact that Elworthy's measurement involved osmometry (and thus yielded a number-average molecular weight) whereas the diffusion measurement yields a weight-average molecular weight.

Further evidence for the existence of micelles in lecithin/benzene solutions was obtained by vapour pressure osmometry reported in detail in section 4.2.1. Molecular weights in the region of 700 were found for solutions of concentration 0.001 g/ml, increasing to 6,950 at concentration 0.02 g/ml. The molecular weight

found from vapour pressure osmometry is consistently lower than that found by membrane osmometry: this is undoubtedly due to the well-known fact that membranes are permeable to low molecular weight particles and consequently the monomer units diffuse out of the membrane osmometer without contributing their full effect to lowering the number average molecular weight.

Further evidence for the existence of micelles in the lecithin/benzene system is presented in section 4.1.7.

4.1.7 Determination of Viscosity.

Viscosity measurements were carried out for use together with the diffusion data in molecular weight determination (section 4.1.6). Additional viscosity measurements were then run for the determination of ν ($= \eta_{sp}/\phi$) since the numerical value of this parameter yields an indication of the shape of particles in solution.

All measurements were run at 21.1°C (since this was the temperature used for the diffusion study) utilising two Ostwald capillary viscometers. The viscometers were standardised and the kinetic energy corrections were determined using standard liquids.

The detailed calculation of the kinetic energy correction is given in the Appendix, section 6.5. Flow times were determined using an Omega stopwatch.

The factor ν is obtained by dividing the specific viscosity, η_{sp}, by ϕ, the volume fraction of solute. This was determined using the known density[37] (1.015 g/ml) of lecithin.

Experimental results are presented in Table VIII, and a plot of ν versus concentration is illustrated in Figure 5.

A system obeying the postulates on which Einstein's equation[39,40] is based would give $\nu = 2.5$ when $C = 0$. Broesma[80] has shown that for colloidal particles this value should be 3.0 rather than 2.5. In either case, however, higher values are attributed either to asymmetry or to solvation of the particles.

Viscosity data collected by Elworthy[37] show a similar trend of ν values, from which the author deduces that a laminar arrangement of monomer molecules in the micelle is the most probable. The results of this study are in agreement with Elworthy's data.

The lowest concentration used in the Elworthy study is 0.73 g/l, at which he reports that the observed value of ν is approximately 6. The results of the present

TABLE VIII

Viscosity of Benzene/Lecithin Solutions.

T = 21.1°C

Viscometer No. 0 — **Flow time of pure solvent= 1231.2sec**

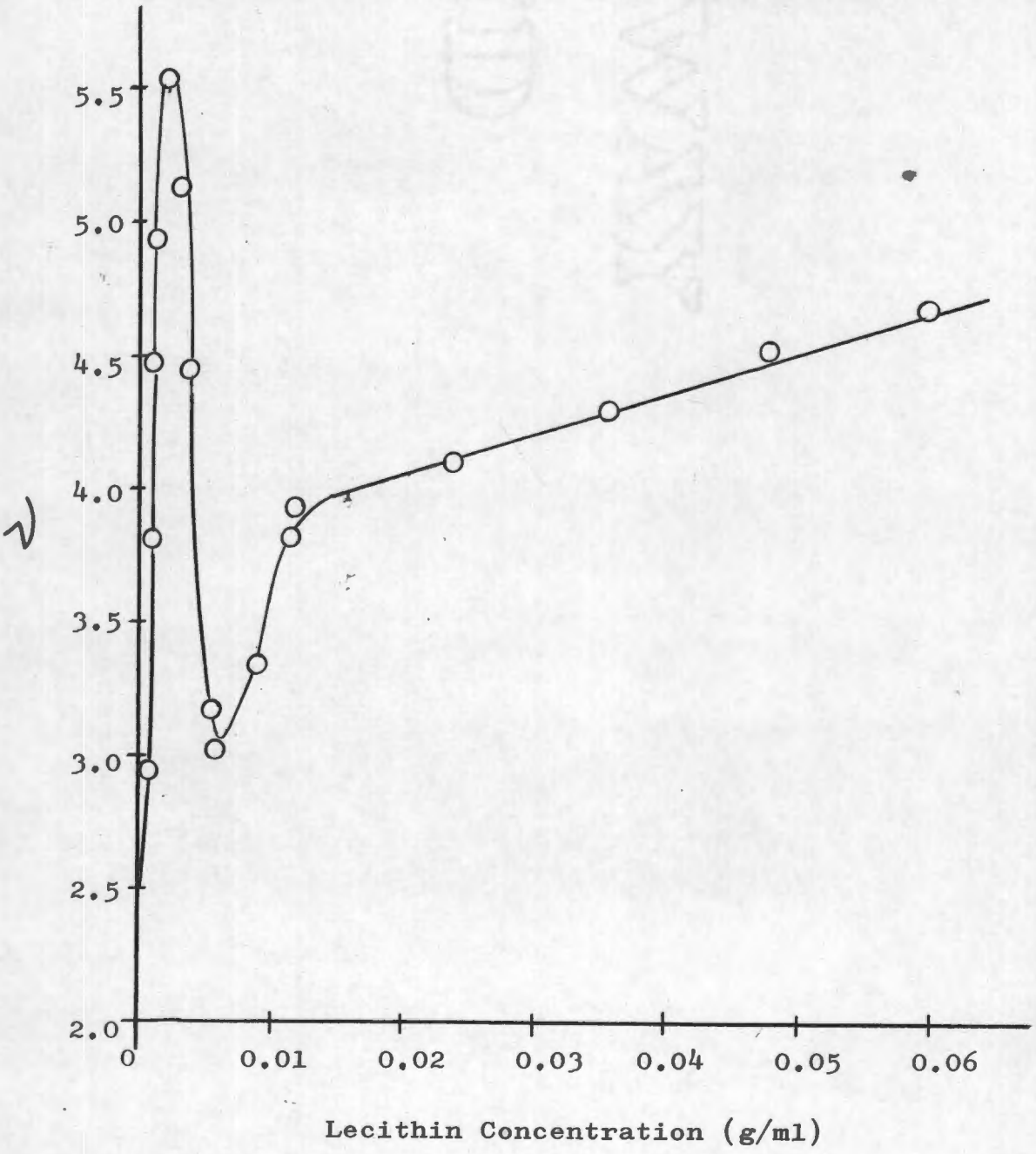
Concentration (g/ml)	Flow Time of Solution (seconds)	η_{sp}	ν
0.00073	1233.8	0.0021	2.95
0.00110	1236.2	0.0041	3.81
0.00146	1239.1	0.0064	4.47
0.00195	1242.8	0.0095	4.94
0.00234	1246.9	0.0129	5.59
0.00292	1249.2	0.0147	5.11
0.00389	1252.1	0.0171	4.46
0.00584	1253.5	0.0182	3.17
0.00605	1253.2	0.0180	3.02
0.00912	1268.3	0.0303	3.37
0.01170	1285.0	0.0440	3.81

Viscometer BT/L — **Flow time of pure solvent= 55.8sec**

Concentration (g/ml)	Flow Time of Solution (seconds)	η_{sp}	ν
0.012	58.2	0.0465	3.93
0.024	60.8	0.0969	4.10
0.036	63.7	0.1530	4.31
0.048	66.9	0.2140	4.53
0.060	70.2	0.2780	4.70

FIGURE 5

Viscosity of Lecithin/Benzene Solutions at 21.1°C.



study show that there is indeed a dramatic change in the value of ν between the concentrations of 0.73 g/l and 6 g/l. It is not possible to interpret this result in terms of specific changes in the shape of the dissolved particle but it is clear that at concentrations just higher than the critical micelle concentration reported by Elworthy, the shape of the particle becomes far less symmetrical.

Since viscosity studies[37] have shown that lecithin forms a disc-like micelle in benzene, it is natural to assume that the observed increase in ν merely reflects the change of shape due to the micellar aggregation.

4.1.8 Summary of Section 4.1.

Lecithin has been characterised by thin-layer chromatography, G.L.C. analysis of the esters of its fatty acids, iodine value determination, infra-red spectrophotometry, and micro-analysis. The material is free from contaminating lipids and the identities of its alkyl side-chains are known. Benzene solutions of lecithin have been studied by osmometric, viscometric and diffusion techniques. The results are in accord with previously published work, and clearly indicate the presence of micelles in solution..

4.2 Solubilisation of Sugars in Benzene.

4.2.1. Solubilisation of Glucose.

In order to determine the extent to which glucose is solubilised by lecithin, a series of lecithin solutions, in sodium-dried analytical reagent benzene, were saturated under nitrogen with finely-powdered anhydrous glucose at a temperature of 25°C with continuous mechanical agitation. Samples of the supernatant liquid were withdrawn, filtered and analysed for glucose using the method described in detail in section 3.9. By sampling at a series of intervals it was found that equilibrium was established after 200 hours, since no further uptake of glucose could be detected at subsequent times. In order to correct for the small solubility of glucose in pure benzene, a blank determination was run in which the solvent itself was saturated with glucose for a similar time under identical conditions.

The experimental results are listed in Table IX in which the apparent solubility of glucose is already corrected for solubility of glucose in the solvent itself. The same results are illustrated in Figure 6, in which the uptake is plotted on a weight basis, and also in Figure 7, in which the uptake is plotted on a molar basis.

TABLE IX

Solubilisation of Glucose in Lecithin/Benzene Solution.

T = 25°C

Solubility of glucose in pure benzene = 0.015 mg/ml.

Lecithin Concentration (mg/ml)	Apparent Solubility of Glucose (mg/ml)	Wt.Glucose / Wt.Lecithin	M of Glucose / M of Lecithin
0.48	0.105	0.219	0.935
2.20	0.381	0.173	0.739
4.45	0.545	0.123	0.522
7.58	0.635	0.084	0.358
9.30	0.716	0.077	0.328

Wt. = Weight ; M = Moles

FIGURE 6

Solubilisation of Glucose by Lecithin at 25°C.

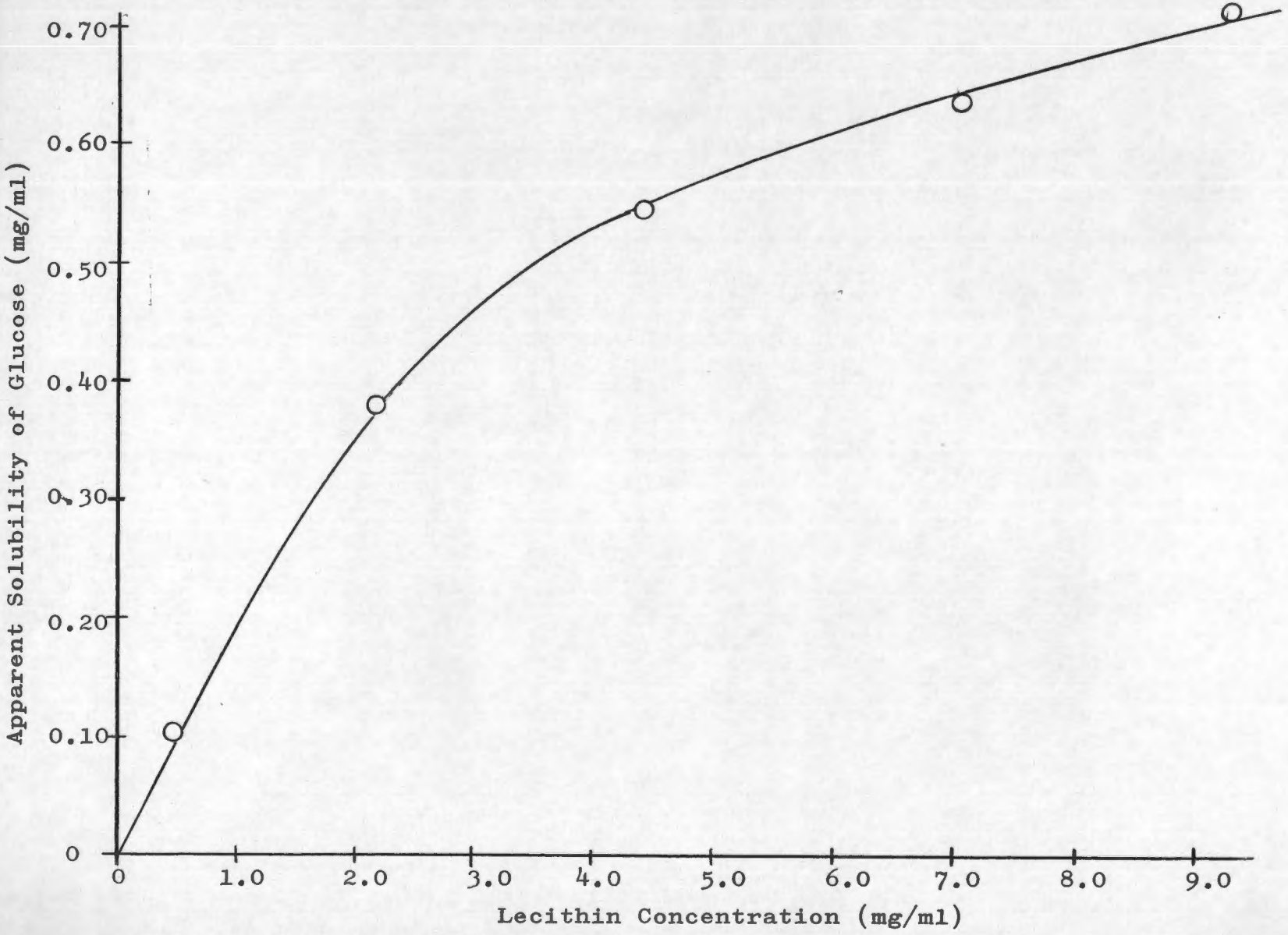
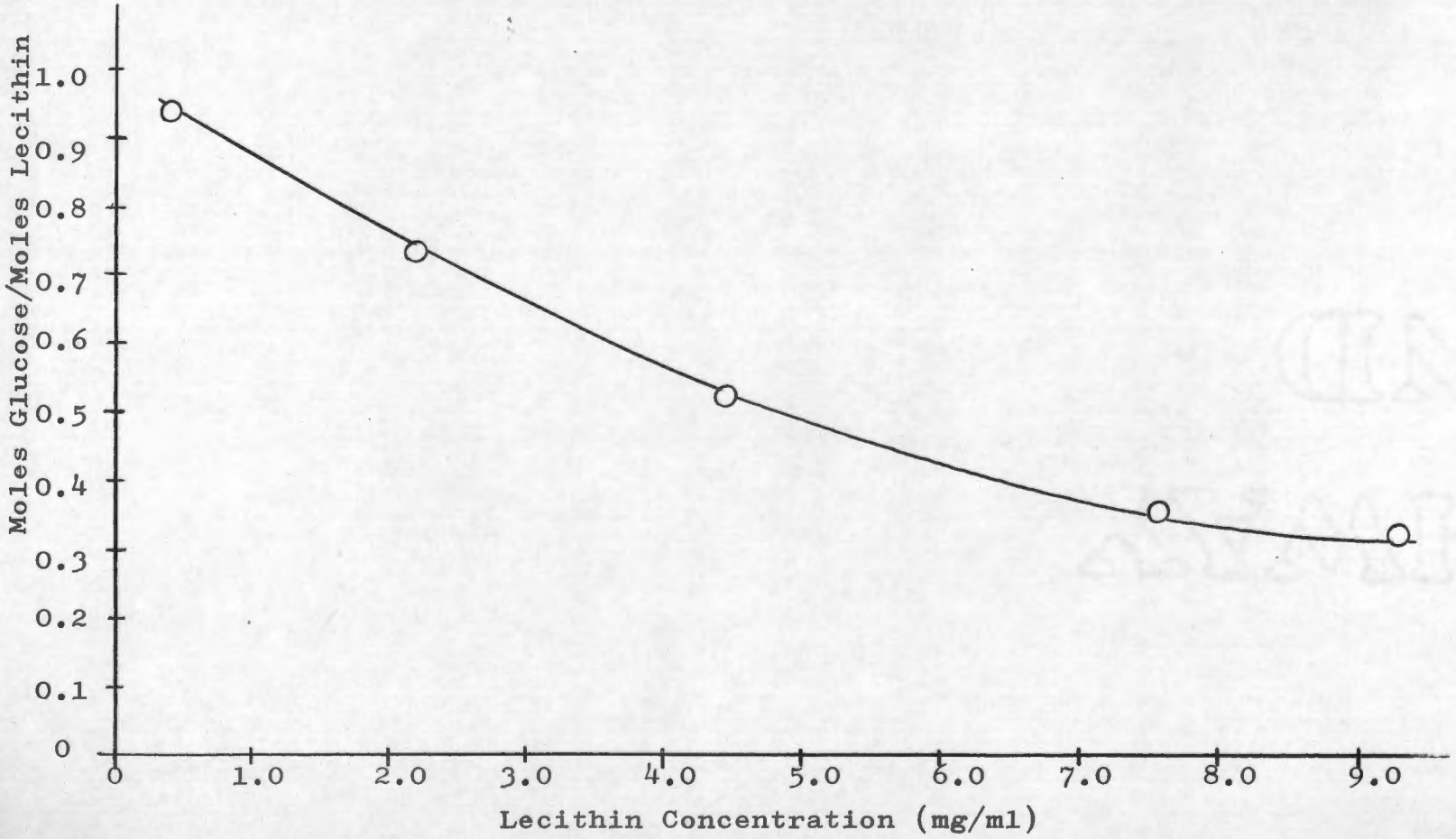


FIGURE 7

Limiting Uptake of Glucose by Lecithin at 25°C.



It is seen that the uptake of glucose is dramatically enhanced by the presence of lecithin.

This is in agreement with the semiquantitative data of Baer[6] and LeFevre et al[50], who reported 'complexes' between lecithin and various sugars, which were soluble in moist ether, chloroform, and hexane. Blei and Lee[38] have reported the solubilisation of potassium and sodium salts of the dye m-(p-anilino)-phenylazobenzenesulphonic acid by lecithin in benzene solutions. Elworthy has also studied the solubilisation of fatty acids[57] and water[3,4,52] by lecithin. In the former case he observed an approximately 1:1 molar ratio between solubilised fatty acid and lecithin, while in the latter case much larger molar amounts of water were adsorbed, namely up to one third of the weight of lecithin. The present study has yielded similar values, both on the basis of weight and on the basis of molar ratio.

It is also interesting to note that the relative uptake of glucose increases as the lecithin concentration decreases and that the limiting solubility is achieved at a concentration lower than Elworthy's[7] critical micelle concentration, the point at which small micelles aggregate into large micelles. It is also significant that the limiting solubility corresponds

to a 1:1 molar ratio of glucose to lecithin.

Although it is not possible to specify the nature of the association between glucose and lecithin at concentrations below the critical micelle concentration (0.73 g/l) it seems reasonable to suggest that hydrogen bonding via the glucose hydroxyl groups might well provide the mechanism for solubilisation.

As regards the actual positioning of the solubilisate, Elworthy[51] has suggested that in the case of dibasic fatty acids the solubilisate lies between polar sheets formed by strips of suitably aligned lecithin molecules, but his work was confined to a range of concentrations higher than the work reported here. The problem is discussed further in section 4.2.3.

At higher concentrations of lecithin, micelles are known to exist and in order to determine whether or not the glucose was being transported within the micelle a comparison was made (using the technique of vapour pressure osmometry) between the micellar weights of lecithin/benzene solutions with and without glucose. The vapour pressure osmometer (described in section 3.7) was standardised using a reference benzil/benzene solution and the micellar weights of a series of lecithin/benzene solutions were compared with the

micellar weights of the same solutions after being saturated with glucose. The concentration of lecithin was varied over the range 1.0 mg/ml to 40.0 mg/ml. Over this range, the low sensitivity of the instrument resulted in poor precision, but the average of the glucose-saturated micellar weights was 13% higher than that of the solutions without glucose. Since the vapour pressure osmometer yields a number-average molecular weight it would be very sensitive to the presence of monomeric particles (such as glucose molecules) which would depress the observed micellar weight. Since this is not the case, one must conclude that over this concentration range, the glucose is carried within the micelle, and that this accounts for the 13% increase in micellar weight.

This result is in accordance with the work of Elworthy[3] who found that when water was carried by lecithin micelles, the weight of the micelle increased by a similar amount, and light-scattering measurements indicated that micellar asymmetry developed in that case.

4.2.2 Thermodynamic Functions of Glucose Solubilisation.

By determining the limiting solubility of glucose by lecithin at two different temperatures and applying the van't Hoff isochore to the two equilibrium concentrations thus obtained, one may find the enthalpy and entropy changes associated with the solubilisation of glucose. It is, of course, necessary to correct for the difference in solubility of glucose in the solvent itself as the temperature is increased. It is also necessary to correct for the enthalpy and entropy changes associated with micellisation: these changes have been determined by Elworthy[7] and their magnitude is small.

The experimental results are presented in Table X. From Table X it can be seen that the apparent solubility of glucose by lecithin is:

$298.2^{o}K$: 0.716 mg/ml

$308.2^{o}K$: 1.092 mg/ml.

Using the van't Hoff isochore:

$$\log \frac{1.092}{0.716} = \frac{\Delta H}{2.303(1.987)} \left(\frac{308.2 - 298.2}{(308.2)(298.2)} \right)$$

$$\Delta H = 7.68 \text{ kcals/mole}$$

TABLE X

Enthalpy and Entropy Changes Associated with Glucose Solubilisation.

Concentration of lecithin = 9.3 mg/ml.

Temperature (^{o}K)	Solubility of Glucose in Pure Benzene (mg/ml)	Solubility of Glucose in the Presence of Lecithin (mg/ml)
298.2	0.015	0.731
308.2	0.018	1.110

It follows that:

$$\Delta S = \frac{7,680 \text{ cals}}{298.2^{\circ}K}$$

$$\Delta S = 25.8 \text{ cals/deg.mole}$$

For micellisation at 298.2°K Elworthy[7] gives the following:

$$\Delta H_m = 1.61 \text{ kcal/mole}$$

$$\Delta S_m = 6.2 \text{ cal/deg.mole}$$

where the subscript m denotes micellisation.

For the solubilisation process therefore:

$$\Delta H_s = 6.07 \text{ kcal/mole}$$

$$\Delta S_s = 19.6 \text{ cal/deg.mole}$$

where the subscript s denotes solubilisation.

It is observed that the solubilisation is accompanied by entropy and enthalpy changes far greater in magnitude than those associated with the micellisation itself. The sign and magnitude of the enthalpy change would be consistent with the view that hydrogen bonding was responsible for the association of glucose with lecithin (the typical value for hydrogen bond energy is 6 kcal[81]), while the positive entropy change presumably reflects the increase in the mobility of the glucose molecule on being transferred from a rigid crystalline lattice to a mobile micelle.

4.2.3 Solubilisation of Sugars Other Than Glucose.

Having determined the extent of solubilisation of glucose it was of interest to check whether or not other similar sugars could be solubilised in analogous fashion. The sugars examined and the results obtained are listed in Table XI and illustrated in Figure 8. For all sugars other than sorbitol and inositol the analytical method of Smith[68] was used, modified as described in section 3.9. For each sugar, individual Beer's law plots had to be done (see Figure 1) and in each case the solubility of the sugar in the solvent also had to be determined. Sorbitol was determined by periodate oxidation[72] followed by colorimetric determination of the resultant formaldehyde, as described in section 3.11.1, and inositol was determined by bromine oxidation, followed by colorimetric determination of the oxidation products by the Smith[68] method, modified as described in section 3.11.2. The apparent solubilities listed in Table XI are already corrected for the solubility of the material in the pure solvent, and represent the equilibrium amounts of various sugars solubilised by lecithin in benzene solution.

TABLE XI

Solubilisation of Carbohydrate Materials.

T = 25°C

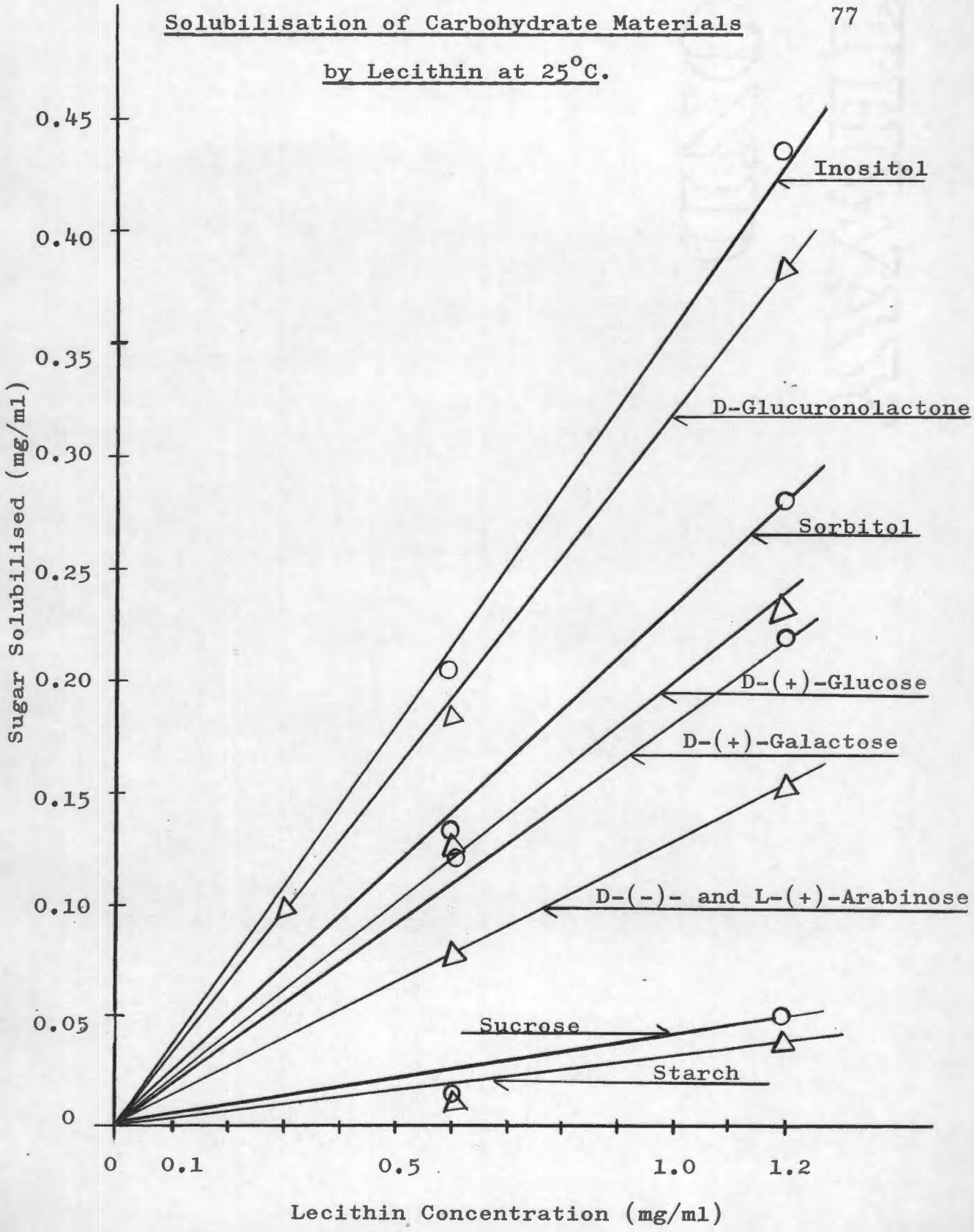
Sugar	Lecithin Concentration (mg/ml)	Sugar Uptake (mg/ml)	$\frac{M_{sugar}}{M_{lecithin}}$	$\left[\frac{M_s}{M_l}\right] / n_{OH}$
D-(+)-Glucose	0.6	0.129	0.917	0.18
	1.2	0.234	0.830	0.17
D-(+)-Galactose	0.6	0.129	0.917	0.18
	1.2	0.219	0.780	0.16
D-(-)-Arabinose	0.6	0.077	0.661	0.17
	1.2	0.159	0.649	0.16
L-(+)-Arabinose	0.6	0.079	0.674	0.17
	1.2	0.159	0.649	0.16
Sorbitol	0.6	0.130	0.915	0.15
	1.2	0.282	0.994	0.17
Inositol	0.6	0.205	1.460	0.24
	1.2	0.436	1.550	0.26
D-Glucuronolactone	0.3	0.100	1.460	0.49
	0.6	0.184	1.330	0.45
	1.2	0.389	1.420	0.47
Sucrose	0.6	0.011	0.042	
	1.2	0.051	0.096	
Starch	0.6	0.018		
	1.2	0.042		

M_s = Moles of sugar; M_1 = Moles of lecithin

n = Number of free hydroxyl groups on the sugar.

FIGURE 8

Solubilisation of Carbohydrate Materials by Lecithin at 25°C.



It is observed that neither starch nor sucrose is solubilised to any appreciable extent, but that all of the other sugars are solubilised to the extent of 0.6 to 1.5 moles of sugar per mole of lecithin. Elworthy has determined[37] that the area of the polar head of the lecithin molecule is approximately 50 $Å^2$, whereas the surface area of the glucose molecule (determined from known van der Waals' radii, Pauling's interatomic distances and known bond angles[82]) would be somewhat smaller than this (38 $Å^2$). Thus a 1:1 molar ratio of hexose to lecithin could be accounted for whether the hexose were being solubilised by either the tetrameric micelle, or whether it were being solubilised by monomeric lecithin.

The failure of sucrose to be appreciably solubilised cannot be attributed entirely to its molecular weight, since Elworthy[51] successfully solubilised approximately equimolar ratios of dibasic fatty acids of up to sixteen carbon atoms in benzene/lecithin solutions. In the latter case however, the area of solubilisate associated with lecithin could be accounted for only by assuming that one of the polar head groups resided inside the micelle, whereas the rest of the molecule, which was consequently akin to

a monocarboxylic acid, protruded into the solvent. The view is consistent with the fact that monocarboxylic acids are freely soluble in benzene. However, in applying the same reasoning in the present case, one would conclude that the sucrose molecule would need to be accomodated entirely within the micelle for solubilisation to occur. Using Elworthy's[37] data for the size and shape of the small (tetrameric) micelles, and determining the size and shape of the sucrose molecule (from known van der Waals' radii, Pauling's interatomic distances and known bond angles[82]), it is apparent that sucrose cannot easily be accomodated. Elworthy[7] has also shown, however, that it is possible to find, from a mass-action equation, the amounts of lecithin present in the form of small (n=4) and of large (n=18) micelles. From Elworthy's treatment it may be shown that for a lecithin concentration of 1.2 g/l, the weight fraction of large micelles (n=18) is ca. 0.3 and hence the number fraction of large micelles is close to 0.1. If sucrose could be solubilised only within the large micelles one might expect the molar uptake of sucrose to be about one-tenth of that of glucose. Experimentally it is found to be 0.12. At lower lecithin concentrations the relative proportion

of large micelles decreases even more and the sucrose/glucose ratio falls yet farther (0.045) at 0.6 g/l of lecithin.

Among the other sugars, it is remarkable to find that the amount of sugar solubilised is directly proportional to the number of free hydroxyl groups with the exception of D-glucuronolactone and, to a much lesser extent, inositol. This is consistent with the view that hydrogen bonding, via the free hydroxyl groups, supplies the mechanism by which the intermolecular interaction occurs. Glucuronolactone would be expected to exhibit an enhanced hydrogen bonding activity on account of the acidity due to the carbonyl group. It is not as simple to see why inositol should be favoured, unless possibly its all-equatorial configuration should make it easier to accomodate.

As might be expected, changes in the configuration of a single hydroxyl group do not significantly affect the solubilisation behaviour; both D-(-)-and L-(+)-arabinose are solubilised to an identical extent and the difference between D-(+)-glucose and D-(+)-galactose is within the experimental deviation of the analytical method.

4.2.4 Effect of Cholesterol on Glucose Uptake.

As explained in Chapter 1, it was of interest to determine whether or not cholesterol modified the ability of the phospholipid to solubilise glucose and vice versa. This was examined by adding known amounts of cholesterol to benzene solutions of lecithin and then allowing the solutions to equilibrate with an excess of anhydrous glucose. In each case solvent blanks were run to determine whether the cholesterol affected the solubility of glucose in the solvent itself, but no such effect was detected. As before, a period of 200 hours with continuous mechanical agitation was found to be necessary for equilibration. The solutions were sampled, the samples filtered and analysed for glucose at time intervals of 158 hours, 230 hours and 276 hours.

Experimental results are presented in Table XII. It is observed that the addition of cholesterol depresses the uptake of glucose. In accordance with previous suggestions it is quite conceivable that the cholesterol hydroxyl group competes with the glucose hydroxyls for available hydrogen bonding sites on the lecithin. If there were no discrimination between these two types of competing hydroxyls then one would expect

TABLE XII

Effect of Cholesterol on Glucose Uptake.

$T = 25^{\circ}C$

Concentration of Lecithin = 0.60 mg/ml.

Sample Time (hours)	Cholesterol Concentration (mg/ml)	Moles Cholesterol / Moles Lecithin	Glucose Uptake (mg/ml)
158	0.00	0.00	0.111
	0.30	1.03	0.104
	0.60	2.09	0.100
	1.18	4.14	0.091
	4.76	16.70	0.033
230	0.00	0.00	0.148
	0.30	1.03	0.126
	0.60	2.09	0.114
	1.18	4.14	0.105
	4.76	16.70	0.040
276	0.00	0.00	0.149
	0.30	1.03	0.135
	0.60	2.09	0.125
	1.18	4.14	0.118
	4.76	16.70	0.050

a relationship of the form:

$$5\ (\text{glucose}) + 1\ (\text{cholesterol}) = \text{constant}.$$

The data in Table XII do not conform to such a relationship.

If, however, there exists a systematic preference for an aromatic rather than an aliphatic hydroxyl, then the relationship will have the form:

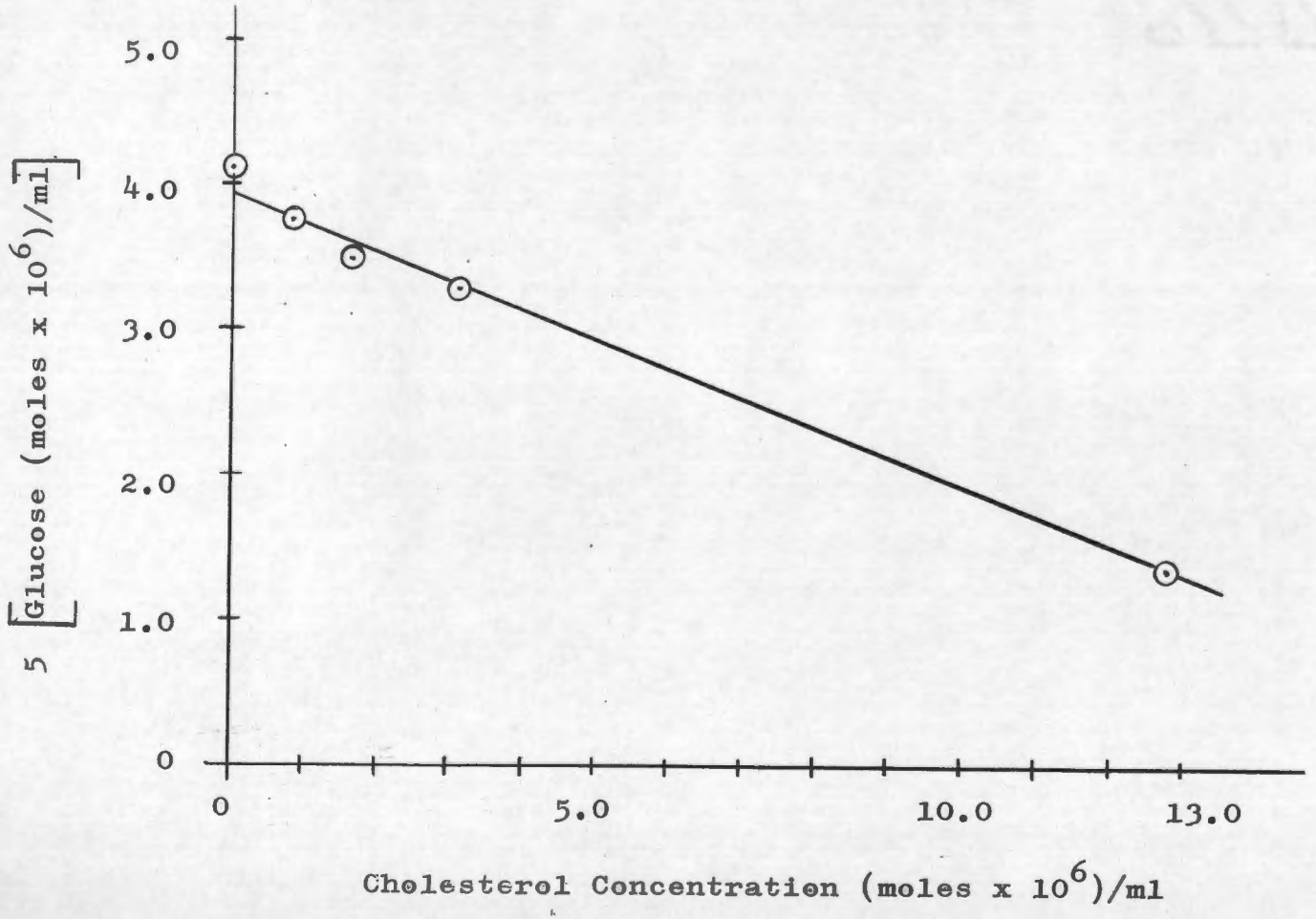
$$5\ (\text{glucose}) + \lambda\ (\text{cholesterol}) = \text{constant}$$

A plot of 5(glucose in moles) versus cholesterol (moles) is presented in Figure 9 and the value of the preference factor, λ , is found to be ca. 0.20 from the slope of the line. The physical significance of this is that lecithin will associate with a glucose hydroxyl five times as readily as with a cholesterol hydroxyl. When this preference is considered together with the statistical abundance of glucose hydroxyls (5 per molecule) relative to cholesterol hydroxyls, it follows that the overall preference of the phospholipid for glucose over cholesterol involves a factor of 25 in benzene solvent systems.

Similar effects would be expected to hold for other compounds capable of competitive hydrogen bonding.

FIGURE 9

Effect of Cholesterol on Glucose Solubilisation by Lecithin at 25°C.



4.2.5 Summary of Section 4.2.

It has been shown that lecithin/benzene solutions are capable of solubilising glucose and related substances. Criteria determining whether or not a sugar may be solubilised appear to include the size of the molecule relative to that of the lecithin micelle, the number of free hydroxyl groups and the acidity of the hydroxyl groups. The uptake of glucose is most pronounced at low concentrations where the quantity solubilised corresponds to a 1:1 molar ratio of glucose to lecithin. At higher concentrations the uptake is somewhat lower. Vapour pressure osmometric data indicate that the glucose is carried within the micelle. Enthalpy and entropy changes associated with solubilisation are consistent with the possibility that hydrogen bonding via one of the glucose hydroxyl groups provides the mechanism for solubilisation. Addition of cholesterol to the solution depresses the uptake of glucose in a quantitative manner, possibly by competitive hydrogen bonding by the cholesterol hydroxyls for available sites on the phospholipid molecule. It is observed that lecithin exhibits a consistent preference for glucose over cholesterol in this system.

4.3 Solubilisation of Cholesterol in Aqueous Ethanolic Media.

It has been shown[37] that lecithin micelles in benzene have structures such that the polar sections of the molecules are surrounded by the non-polar hydrocarbon chains. In polar media, however, an inversion of the micelle occurs and the polar portions of the molecule face outwards surrounding the hydrocarbon material.[41] Elworthy[8] has studied the micellar weights of lecithin in a series of ethanol/water and methanol/water solvent media. In pure ethanol, the lecithin exists in the form of monomer molecules, but as the dielectric constant of the medium increases the micellar weight increases rapidly[8]. When the volume fraction of water increases above 0.3, the solutions begin to exhibit the opalescence characteristic of colloidal sols.

It was of interest to determine whether the inverted lecithin micelle could solubilise non-polar materials and cholesterol was selected for study on account of its association with lecithin in cell wall structures[2].

The experimental procedure was parallel to that described in section 4.2 for the determination of glucose solubilisation. A solution containing a

known concentration of lecithin in the particular solvent of interest was equilibrated with excess cholesterol at 25°C with continuous mechanical agitation until equilibrium was achieved (usually 90-150 hours). For those cases in which the volume fraction of water exceeded 0.3 a preliminary period (1-2 hours) of vigorous mechanical agitation was necessary to ensure proper dispersion of the lecithin. After equilibration, samples of the mixture were filtered or centrifuged to remove excess undissolved cholesterol and the supernatant liquid or filtrate was analysed for cholesterol using the method described in section 3.10. Since lecithin itself interferes in the analysis, a preliminary saponification was carried out followed by an extraction to remove the cholesterol from the mixture. The experimental details of the method are also found in section 3.10.

For each solvent system, the solubility of cholesterol in the absence of lecithin had to be determined separately.

The extent of solubilisation of cholesterol by lecithin in a series of water/ethanol solvent media was thus determined and the experimentally observed results are presented in Table XIII. The results are

TABLE XIII

Solubilisation of Cholesterol by Lecithin in Aqueous Ethanol Solvent Systems.

T = 25°C

Lecithin Concentration = 5.5 mg/ml.

Volume Fraction Ethanol	ε^{83} (Debye units)	Micellar Weight	Reference	Solubility of Cholesterol: Solvent	Solvent plus Lecithin	Net Uptake Chol. (mg/ml)	Molar Ratio (Cholesterol)/(Lecithin)
0.90	29.8	800	8	4.89	7.080	2.190	0.817
0.80	34.1	2,600	8	1.87	2.520	0.650	0.242
0.70	38.7	20,300	8	0.54	0.799	0.259	0.096
0.60	43.9	>68,000	8	0.12	0.186	0.066	0.025
0.50	49.4	$>10^6$	84	0.03	0.049	0.019	0.007

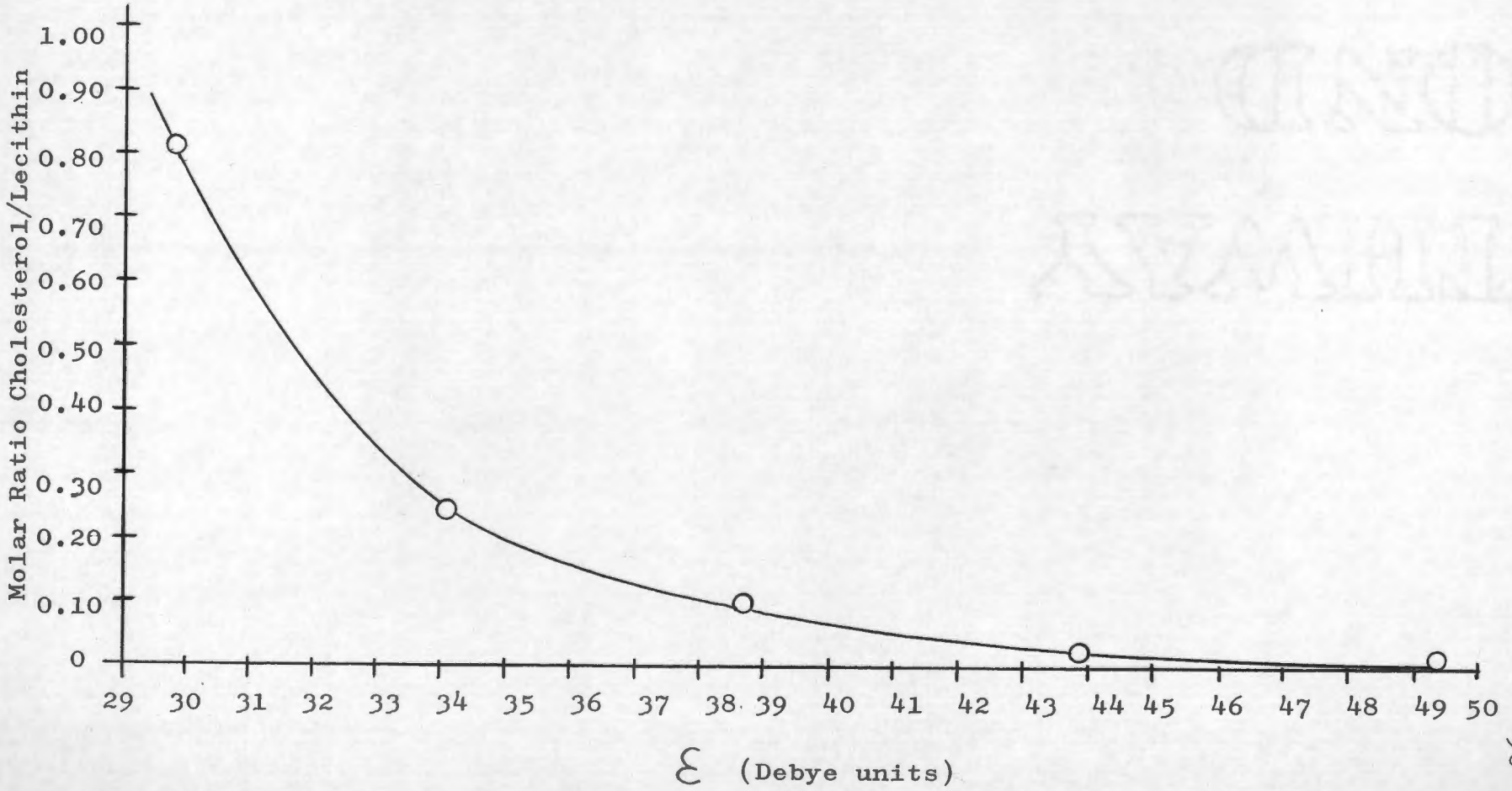
illustrated by a plot of the molar ratio of cholesterol to lecithin as a function of dielectric constant in Figure 10.

It is observed that the uptake of cholesterol is strongly dependent on the dielectric constant of the medium and that the limiting solubility in the aqueous ethanolic systems tested occurs in 90% ethanol, in which solvent the lecithin exists in monomeric form. A similar result was found for the uptake of glucose by lecithin in benzene solution. In that case the limiting solubility was found at a concentration below Elworthy's critical micelle concentration[7] and and the limiting amount solubilised corresponded to a 1:1 molar ratio of glucose to lecithin, similar to the present case.

It is remarkable that the solubilisation of cholesterol continued to be exhibited even when the dielectric constant of the medium was so high that the lecithin existed as a colloidal dispersion rather than as a solution. Under these conditions equilibrium was established by 150 hours.

FIGURE 10

Molar Ratio of Solubilised Cholesterol/Lecithin as a Function of Dielectric Constant at 25°C.



4.3.1 Solubilisation of Cholesterol by Lecithin in Homogeneous Solution.

The uptake of cholesterol as a function of lecithin concentration was investigated in a solvent system consisting of 70% by volume of ethanol and 30% by volume of water. In this medium, lecithin existed as a completely clear homogeneous solution over the range of concentrations used. Equilibration was achieved by mild agitation in a 25°C thermostatted water bath and was found to have been achieved after 90-150 hours. The range of lecithin concentrations and the corresponding uptake of cholesterol for each, is presented in Table XIV, and illustrated in Figure 11.

The uptake of cholesterol per mole of lecithin is not affected to any measurable extent by a change in the concentration of lecithin up to a concentration of 7.0 mg/ml, above which concentration a slight decrease in cholesterol uptake is observed. This occurs close to the critical micelle concentration (c.m.c.) reported by Elworthy[8] (8.8 mg/ml) and demonstrates that the micelle is less capable of solubilising cholesterol than the monomer, since as Table XIV shows, the amount of cholesterol solubilised below the c.m.c. is greater than

TABLE XIV

Cholesterol Uptake as a Function of Lecithin Concentration.

T = 25°C

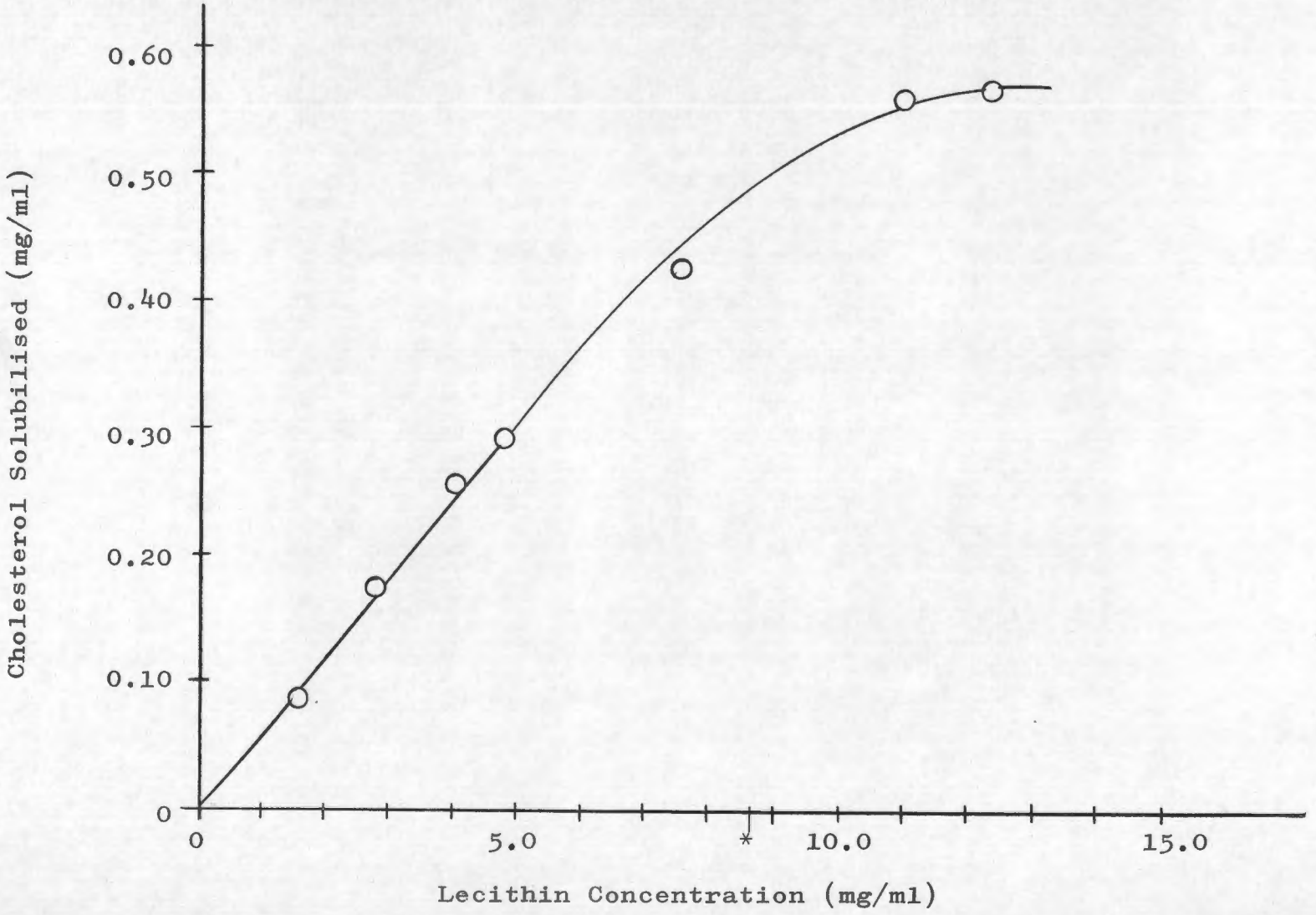
Solvent = 70% Ethanol/Water

Lecithin Concentration (mg/ml)	Solubilised Cholesterol (mg/ml)	Molar Ratio (Cholesterol)/(Lecithin)
1.60	0.088	0.114
2.80	0.177	0.131
4.04	0.258	0.133
4.80	0.291	0.125
7.60	0.426	0.116
*		
11.00	0.561	0.105
12.40	0.567	0.095

* c.m.c. point in 70% ethanol/water is 8.8 mg/ml.

FIGURE 11

Cholesterol Uptake (mg/ml) as a Function of Lecithin Concentration (mg/ml) in 70% Ethanol/Water at 25°C.



* critical micelle concentration for lecithin micelles in 70% ethanol/water.

that solubilised when micelles are present.

4.3.1.1 Enthalpy and Entropy of Solubilisation.

The equilibrium amount of cholesterol solubilised by lecithin in 70% aqueous ethanol was determined at two different temperatures for the same concentration of lecithin (9.4 mg/ml). The following results were obtained:

a) At 25°C cholesterol uptake was found to be 0.56 mg/ml at equilibrium and corrected for solvent blank.

b) At 35°C cholesterol uptake was found to be 0.642 mg/ml at equilibrium and corrected for solvent blank.

From these results, it is possible to calculate ΔH_s, the apparent enthalpy of solubilisation, and ΔS_s the apparent entropy of solubilisation. The values found were the following:

$$\Delta H_s = 2.55 \text{ kcals/mole.}$$

$$\Delta S_s = 8.57 \text{ cal/deg.mole}$$

The experimental value of ΔH is significantly lower than the corresponding value for glucose solubilisation (see section 4.2.2), possibly indicating van der Waals' forces rather than hydrogen bonding are operative in this case. However, since the enthalpy and entropy changes associated with the actual process of

micellisation itself have not been evaluated for cholesterol, these values cannot be accepted unreservedly.

4.3.2 Solubilisation of Cholesterol by Lecithin in Colloidal Sols.

The uptake of cholesterol as a function of lecithin concentration was investigated in a solvent system containing 50% by volume of ethanol and 50% by volume of water. In this particular solvent system the resulting sols were opalescent to milky, depending on the lecithin concentration. Excess cholesterol was added and the dispersion of the lecithin was ensured by a preliminary period (1-2 hours) of vigorous mechanical agitation. This was followed by gentle agitation in a 25°C thermostatted water bath until equilibration was achieved. Samples of the mixture were withdrawn and centrifuged for 30 minutes to precipitate undissolved material. An aliquot of the slightly hazy liquid was then subjected to hydrolysis and cholesterol analysis. The range of lecithin concentrations and the cholesterol uptake corresponding to each, are presented in Table XV, already corrected for solvent blank (i.e. cholesterol solubility in 50% ethanol/water). Figure 12 illustrates the cholesterol uptake as a function

TABLE XV

Cholesterol Uptake as a Function of Lecithin Concentration.

$T = 25^{o}C$

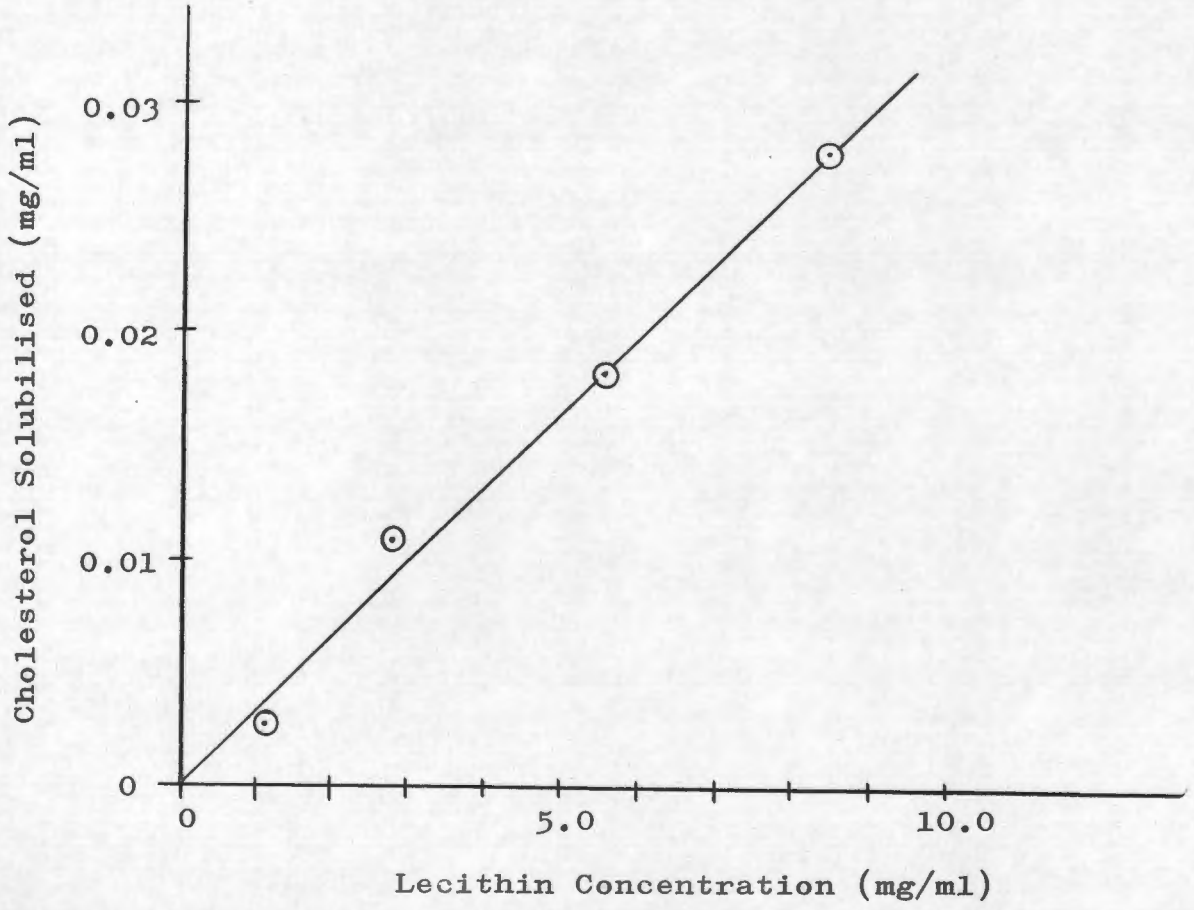
Solvent = 50% Ethanol/Water

Lecithin Concentration (mg/ml)	Solubilised Cholesterol (mg/ml)	Molar Ratio $\frac{\text{(Cholesterol)}}{\text{(Lecithin)}}$
1.15	0.0027	0.00483
2.80	0.0111	0.00821
5.55	0.0181	0.00673
8.42	0.0279	0.00682

FIGURE 12

Cholesterol Uptake (mg/ml) as a Function of

Lecithin Concentration (mg/ml) in 50% Ethanol/Water at 25°C.



of lecithin concentration in 50% ethanol/water.

Although the lecithin is incompletely dissolved in the solvent medium, the uptake of cholesterol is still clearly dependent on the concentration of lecithin. The solubilisation of the cholesterol still occurs, but the magnitude of the effect is far smaller than in the 70% ethanol/water solvent medium.

4.3.3 Effect of Glucose on the Solubilisation of Cholesterol.

A series of tests was carried out to determine whether or not the addition of glucose affected the cholesterol uptake in either the 70% or the 50% aqueous ethanol media. A wide range of molar ratios (210/1 to 30/1 and 5,000/1 to 2,000/1) of glucose to cholesterol were used. In no case, however, was any effect observed on the cholesterol solubilisation, although the addition of larger amounts of glucose did affect the solubility of cholesterol in the 50% aqueous ethanol itself, the solubility first decreasing slightly and then increasing appreciably.

By analogy to the glucose solubilisation discussed in section 4.2.4 one would not expect glucose to affect the cholesterol uptake, since the mechanism involving competitive hydrogen bonding would not be applicable

in aqueous ethanolic media.

4.3.4 Summary of Section 4.3.

The solubilisation of cholesterol by glucose in aqueous ethanolic solvent systems has been investigated, and the uptake of cholesterol has been found to depend on the dielectric constant of the solvent. In 90% ethanol, in which the lecithin exists as monomer, the limiting solubility is found corresponding to a 1:1 molar ratio of cholesterol to lecithin. In more polar solvent systems, the uptake decreases, but even when the dielectric constant of the medium increases to the point where the lecithin exists as a colloidal sol, the solubilisation of cholesterol still occurs. In both 70% ethanol (clear solution) and 50% ethanol (milky sol) the concentration of cholesterol solubilised is linearly proportional to the lecithin concentration except at concentrations above 7.0 mg/ml in 70% ethanol, at which concentration micelles begin to form[8]. This indicates that the micelle is less capable of solubilising cholesterol than is the monomer. In neither case is it affected by the addition, even of large amounts, of glucose. Values have been calculated for the enthalpy and entropy changes associated with solubilisation, but the lack of the

corresponding data for the micellisation process itself makes it difficult to assign a physical interpretation to these results.

CHAPTER 5

GENERAL DISCUSSION

In Chapter 1 it was proposed that a study of the solubilisation of materials such as glucose and cholesterol by lecithin in both polar and non-polar media would be of interest in terms of physical chemistry and possibly biologically significant. For this study to be meaningful, the lecithin would have to be thoroughly purified and characterised. This was accomplished as discussed in section 4.1. Thin-layer chromatography, infra-red spectrophotometry, micro analysis and G.L.C. analysis of the esters of its fatty acids enabled one to determine the purity and the average molecular weight of the lecithin used.

In section 4.1 it was also shown by osmometric, viscometric and diffusion techniques that lecithin formed micelles in benzene solution. These micelles were shown (in section 4.2) to be capable of solubilising glucose and other sugars in benzene media and the factors determining whether or not a sugar might be solubilised were found to include the size of the sugar molecule relative to the lecithin micelle, the number of free hydroxyl groups and the acidity of the hydroxyl groups. The uptake of glucose was most

pronounced at low lecithin concentrations, where the quantity solubilised corresponded to a 1:1 molar ratio of glucose to lecithin. At higher concentrations the uptake was found to be somewhat lower. Vapour pressure osmometric data indicated that glucose was carried within the micelle. Enthalpy and entropy changes associated with solubilisation were found to be consistent with the possibility that hydrogen bonding via one of the glucose hydroxyl groups provided the mechanism for solubilisation. Cholesterol was found to depress the uptake of glucose in a quantitative manner, possibly by competitive hydrogen bonding by the cholesterol hydroxyls for available sites on the phospholipid molecule. It was observed that lecithin exhibited a consistent preference for glucose over cholesterol in benzene media.

The solubilisation of cholesterol by lecithin in aqueous ethanolic media was investigated as described in section 4.3 and the uptake of cholesterol was found to depend on the dielectric constant of the medium. In 90% ethanol, in which the lecithin existed as monomer, the limiting solubility was found to correspond to a 1:1 molar ratio of cholesterol to lecithin. In more polar solvent systems, the uptake decreased, but

solubilisation still occurred even when the dielectric constant of the medium increased to the point where the lecithin existed as a colloidal sol. In both 70% and 50% ethanol the concentration of cholesterol solubilised was found to be linearly proportional to the lecithin concentration except at concentrations above 7.0 mg/ml of lecithin in 70% ethanol, at which concentration micelles began to form. This indicated that in this solvent the micelle was less capable of solubilising cholesterol than was the monomer. In neither case was the uptake of cholesterol affected by the addition of glucose. Values were determined for the enthalpy and entropy changes associated with solubilisation but the lack of the corresponding data for the micellisation process itself made it difficult to assign a physical interpretation to these results.

CHAPTER 6

APPENDIX

6.1 Calculation of the Average Molecular Weight of the Fatty Acid Chains of Lecithin.

As discussed in section 4.1.3, the average molecular weight of the fatty acid chain of the lecithin molecule, the number of double bonds per fatty acid chain and thus the theoretical iodine value can be determined using only the G.L.C. results of the esters of the fatty acids. The calculated iodine value can then be compared with the value found experimentally.

To determine the average molecular weight of the fatty acid chains, the weight of each fragment is multiplied by its abundance as determined by G.L.C. analysis, and the product sum is divided by the sum of the proportions.

Fragment	Fragment Weight	Proportion(%)	Product
$C_{15}H_{31}$	211.4	34.90	7385
$C_{15}H_{29}$	209.4	2.62	547
$C_{17}H_{35}$	239.4	15.65	3740
$C_{17}H_{33}$	237.4	31.75	7550
$C_{17}H_{31}$	235.4	11.60	2730
$C_{19}H_{31}$	259.4	3.89	1009
		100.41	22961

22961/100.41 = 228.7

Therefore the average molecular weight is 228.7.

6.2 Calculation of the Average Number of Double Bonds in Each Fatty Acid Fragment.

To determine the average number of double bonds per acid fragment, the total product of the double bonds in each fragment and its proportion in the lecithin molecule is divided by the sum total of the proportions.

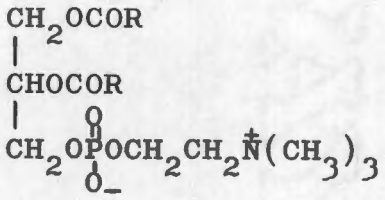
Fragment	Number of Double Bonds	Proportion (%)	Product
$C_{15}H_{31}$	----	34.90	------
$C_{15}H_{29}$	1	2.62	2.62
$C_{17}H_{35}$	----	15.65	------
$C_{17}H_{33}$	1	31.75	31.75
$C_{17}H_{31}$	2	11.60	23.20
$C_{19}H_{31}$	4	3.89	15.56
		100.41	73.13

$$73.13/100.41 = 0.73$$

Therefore the average acid chain on the lecithin molecule has 0.73 double bonds.

6.3 Calculation of the Iodine Value of Lecithin.

As discussed in section 2.2.1, the structure of the lecithin molecule has been shown to be the following:



where R represents the average fatty acid chain. From section 6.1 the molecular weight of R was calculated to be 228.7. Therefore the molecular weight of the lecithin molecule would equal 2(228.7) + the sum of the atomic weights of the remainder of the molecule.

Atom	Total Number	Atomic Weight	Product
C	10	12.01	120.1
O	8	16.00	128.0
P	1	31.00	31.0
N	1	14.00	14.0
H	18	1.00	18.0
			311.1

The molecular weight is thus equal to :

$$311.1 + 2(228.7) = 768.5.$$

From section 6.2, the total number of double bonds per molecule of lecithin is 2(0.73) or 1.46.

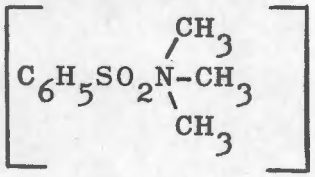
Each double bond will consume 2(127) grams of iodine. Thus 768.5 g of lecithin will consume 1.46 x 2 x 127 g of iodine.

$$\text{Iodine Value} = \frac{1.46 \times 2 \times 127 \times 100}{768.5}$$

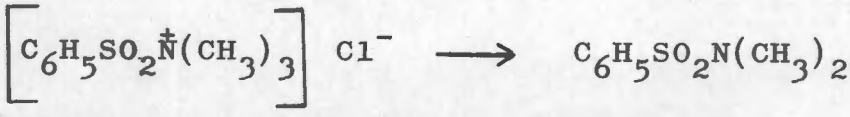
$$= 49.4$$

The experimental iodine value determined was 63.8 $\pm$0.45.

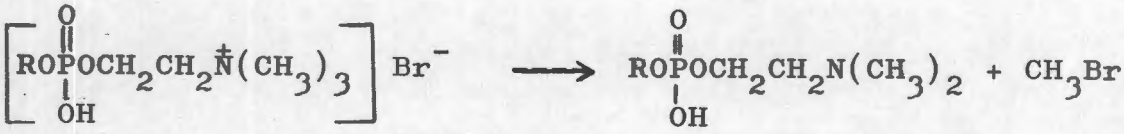
A possible explanation for this discrepancy might be the fact that quaternary ammonium compounds such as



are known to react in the following manner[85]:



Lecithin may react in analogous fashion:



The method used for the iodine value determination involved Br_2 addition and the oxidation of iodide ion to iodine by the excess bromine in solution. Therefore if the above degradation did occur, the amount of iodine consumed would be:

$$= \frac{2(127)(1.46) + (127)}{768.5} = \underline{65.}$$

6.4 Calculation of the Theoretical Percentages of C,H,N and P in Lecithin.

To calculate the theoretical percentages, it is first necessary to find the average percent of carbon and of hydrogen in each fatty acid chain.

Fragment	Total Weight	Weight C	Weight H	%C	%H	%C x Prop.	%H x Prop.
$C_{15}H_{31}$	211.4	180.15	31.25	85.2	14.8	2975	517.0
$C_{15}H_{29}$	209.4	180.15	29.23	86.1	13.9	226	36.5
$C_{17}H_{35}$	239.4	204.17	35.28	85.2	14.8	1339	232.0
$C_{17}H_{33}$	237.4	204.17	33.26	86.0	14.0	2730	445.0
$C_{17}H_{31}$	235.4	204.17	31.25	86.8	13.2	1008	153.0
$C_{19}H_{31}$	259.4	228.19	31.25	88.4	12.6	345	46.9
						8623	1430.4

(Prop. represents the proportion as found by G.L.C.analysis)

$$8623/100.41 = 86.1$$

$$1430.4/100.41 = 14.29$$

Therefore: %C = 86.1 and %H = 14.29.

To find the ratio of carbon to hydrogen, the percentage is divided by the atomic weight.

C: $86.1/12.01 = C_{7.16}$

H: $14.29/1.008 = H_{14.17}$

Dividing each by 7.16 we obtain $C_{1.0}$ and $H_{1.98}$.

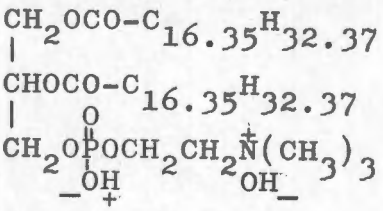
The number of carbon atoms multiplied by the proportion found by G.L.C. analysis, gives the total number of carbon atoms per fatty acid fragment, and therfore,from the ratio, one can determine the number of hydrogen atoms per fatty acid fragment.

Fragment	Number of Carbon Atoms	Proportion (%)	Product
$C_{15}H_{31}$	15	34.9	525.0
$C_{15}H_{29}$	15	2.62	39.4
$C_{17}H_{35}$	17	15.65	266.5
$C_{17}H_{33}$	17	31.75	540.0
$C_{17}H_{31}$	17	11.60	197.5
$C_{19}H_{31}$	19	3.89	73.9
			1642.3

$$1642.3/100.41 = 16.35$$

Hence fragment is composed of $-C_{16.35}H_{32.37}$.

Assuming that under analysis conditions the lecithin molecule exists in the following form:



the molecular weight equals the following sum.

	Number	x	Atomic Weight	=	Product
C: 10 + 2(16.35)	= 42.7		12.01		512.80
H: 18 + 2(32.37)	= 84.74		1.01		85.40
N:	= 1		14.00		14.00
P:	= 1		30.98		30.98
O:	= 8		16.00		144.00
					787.18

Therefore:

$$\%C = \frac{512.8}{787.2} = 65.14$$

$$\%H = \frac{85.4}{787.2} = 10.85$$

$$\%N = \frac{14.0}{787.2} = 1.78$$

$$\%P = \frac{30.98}{787.2} = 3.94$$

6.5 Calculation of the Kinetic Energy Correction for Ostwald Viscometer Used.

The relationship between viscosity and flow time is given by an equation of the form:

$$\eta = A\rho t - \frac{B\rho}{t}$$

where η = viscosity (poise)

ρ = density t = flow time in seconds

A,B = constants characteristic of viscometer, evaluated by plotting $\eta/\rho t$ versus $1/t^2$.

The flow times of standard liquids were determined in both the Ostwald O and BT/L viscometers. The viscosity and density of each liquid were found from the International Critical Tables and for each viscometer a plot of η/ρ t versus $1/t^2$ was made from which A and B, the intercept and slope, could be determined. The data and results are presented in Table XVI.

TABLE XVI

Data and Results of Kinetic Energy Corrections.

T = 21.1°C

Viscometer	Solvent	η (poise) x 10^4	ρ (g/ml)	t(sec)	$\eta/\rho t \cdot (10^6)$	$1/t^2$ x 10^6	A x 10^6	B (slope)
Ostwald "0"	acetone	30.75	0.78530	683.1	5.735	2.17		
	ether	22.40	0.70768	561.5	5.638	3.18	5.95	0.0984
	dioxane	120.30	1.0279	1970.0	5.950	0.26		

Viscometer	Solvent	η (poise) x 10^5	ρ (g/ml)	t(sec)	$\eta/\rho t \cdot (10^3)$	$1/t^2$ x 10^3	A x 10^4	B (slope)
Ostwald	acetone	307.5	0.78530	32.4	1.208	0.96		
"BT/L"	ether	224.0	0.70768	27.8	1.140	1.30	1.37	0.0172
	dioxane	1203.0	1.0279	87.2	1.345	0.13		

A = intercept of plot η/ρ t versus $1/t^2$; B = slope of same plot

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