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THE SYNTHESIS AND BIOLOGICAL PROPERTIES OF LECITHIN ANALOGS

by

YISRAEL A. ISAACSON

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1978

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Chairman of Examining Committee

9/5/78
date

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Abstract

THE SYNTHESIS AND BIOLOGICAL PROPERTIES
OF
LECITHIN ANALOGS

by

Yisrael A. Isaacson

Adviser: Professor Robert Bittman

The chemical synthesis of racemic diacyloxypropylphosphonylcholines is described. The route involved reaction of the appropriate diacyloxypropyliodide with tris(trimethylsilyl)phosphite to yield the corresponding bis(trimethylsilyl)phosphonate. Removal of the trimethylsilyl groups by neutral aqueous hydrolysis gave the free diacylpropylphosphonic acids, which, when treated with choline toluenesulfonate, yielded the desired choline derivatives. The isosteric compound 3,4-dimyristoyloxybutylphosphonylcholine was synthesized in a similar manner starting from the 3,4-dimyristyloxybutyl-1-bromide. Also described is the synthesis of 2-octadecyleicosylphosphorylcholine and a series of its analogs containing modified headgroups. The synthesis of tritiated dipalmitoyllecithin is presented.

The compounds synthesized were tested to determine their efficacy in reactivating β-hydroxybutyrate

dehydrogenase, a lecithin requiring enzyme. Both the D and L isomers of dipalmitoyllecithin reactivated the enzyme to identical extents and efficiencies. Similarly both the dimyristoylpropyl compound and the dimyristoylbutyl compound reactivated the enzyme to high levels with high efficiencies. The 2-octadecyleicosylphosphorylcholine was also a powerful activator of the enzyme. These results indicated that the requirements for activation are a hydrophobic group coupled to a phosphorylcholine moiety. The specificity for a choline group on the activating lipid was also investigated. Extending the distance between the phosphate group and quaternary ammonium group by the insertion of one or two methylene groups did not alter the lecithin's ability to reactivate the enzyme. The substitution of an ethyl group for one of the quaternary ammonium methyl groups yielded a reactivating lecithin. On the other hand, N,N-dimethylphosphatidylethanolamine, 2-octadecyleicosylphosphoryl-l-methylcholine, 2-octadecyleicosylphosphoryl-N,N,N-triethylethanolamine and sphingomyelin could not reactivate the enzyme. The enzyme does not appear to bind to non-reactivating lecithins. The rate of the enzyme-lecithin interaction is governed by the charge in the lipid mixture, negatively charged lipid lowering the rate of interaction.

The interaction of cholesterol with the 2-octadecyleicosylphosphorylcholines was studied by measuring the efflux of glucose from liposomes. Cholesterol inhibited the efflux of glucose from liposomes prepared from 2-octadecyleicosylphosphorylcholine and its analogs. This indicates that an interaction has taken place and that carbonyl groups are not necessary for such an interaction.

The work reported in this thesis has been published and/or submitted for publication in the following journals:

1. Tetrahedron Letters 12, 977 (1975)
2. Chemistry and Physics of Lipids 16, 60 (1976)
3. Biophysical Journal 17, 72A (1977)
   Abstract No. W-PM-G 12
4. Journal of Biological Chemistry - submitted for publication
5. Portions of Chapter III - manuscript in preparation

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Limitations of space make it impossible for me to enumerate the scores of individuals who, over the years, either directly or indirectly ignited my interest in science and who were most generous in imparting the fruits of their wisdom and knowledge. Their numbers are legion. Needless to say, I am profoundly grateful to all of them.

There are, however, certain teachers, colleagues, friends who loom large on the horizons of my mind by dint of their recent, intimate association during the years of my studies at the Graduate School of CUNY.

First and foremost, I thank my teacher, mentor, and adviser, Dr. Robert Bittman whose sage counsel, advice and concern were a source of encouragement to me and whose infinite patience, though justly strained and tried at times, never faltered nor wavered.

I am indebted to Dr. Arthur F. Rosenthal, of the Long Island Jewish-Hillside Medical Center, whose numerous insights aided me in hurdling what seemed at the time to be impassable obstacles. He guided me through a maze of dead-ends to the broad, expansive avenues of clarity and progress.

I was most fortunate in being the recipient of the gracious hospitality and assistance of Dr. Sidney Fleischer of Vanderbilt University who was most lavish in his encouragement and helpful suggestions. The members of his laboratory were most cooperative and extended an inordinate measure of courtesies and kindnesses for which I am very appreciative.

In reviewing retrospectively the years while I was engaged in study and research I freely acknowledge with profound appreciation my greatest source of inspiration--that team of highly motivated graduate students with whom I was privileged to work in the laboratories of Queens College (CUNY). I shall fondly remember these colleagues and will always cherish their friendship. Particularly, I cite Messrs. Zenowij Majuk and Tze Chien Wun.

These acknowledgments would not be complete without a special gesture of gratitude to my dear friend and colleague, Dr. Paul Deroo. I consider myself to have been signally blessed to have enjoyed his friendship, his help, and the unfailing kindnesses which he rendered so generously.

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SYNTHESIS OF PHOSPHATIDYLCHOLINE ANALOGS

Introduction

Within the past decade there has developed significant interest in the preparation and investigation of phosphonic acids and their derivatives which might be considered analogs of naturally occurring phosphates.[1] For the most part, this interest was generated by the recognition that phosphonic acids and their esters, used as analogs of naturally occurring phosphates, possessed intriguing possibilities for metabolic regulation or perturbation. This was dependent on the fact that the carbon-phosphorus bond of these compounds was incapable of being hydrolyzed by ordinary phosphatases.

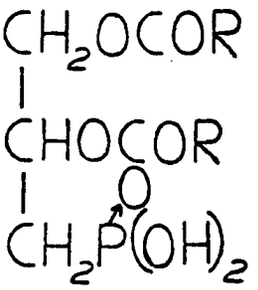
An additional factor in the development of this interest was the isolation of naturally occurring phosphonic acids.[2,3] They have been found mostly in invertebrates such as Metridium dianthus (sea anemone)[4] and unicellular organisms such as Tetrahymena pyriformis.[3] Within these organisms the compounds that have been found to contain a C-P bond include 2-aminoethylphosphonic

acid, the N-methyl, N,N-dimethyl, N,N,N-trimethyl derivatives of 2-aminoethylphosphonic acid, and 2-amino-3-phosphonopropionic acid.[5] In addition these C-P compounds have been found incorporated in both lipids[6] and proteins.[4] For example, the lipids isolated from Tetrahymena cilia have been demonstrated to consist predominately of phosphonolipid.[3] Theories as to the function of these compounds are at present speculative. It is believed that the resistance of the C-P bond to hydrolysis may be relevant to their function in nature. Higher organisms contain phosphonates in both brain[7a] and atheroslerotic plaques[7b] in trace quantities, but it is not clear whether they represent compounds synthesized by the organism or whether their presence is due to the chance ingestion of substances containing C-P bonds.

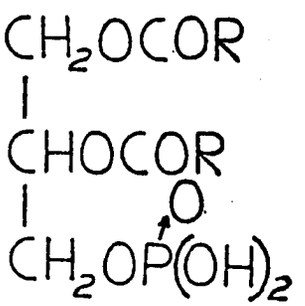
A third direction from whence interest in these compounds has been generated is their potential use as analogs of phospholipids in physicochemical and model biological systems. Before utilizing any of these compounds in a biological situation one must consider the significant decrease in acidity of the phosphorus-containing acid function attendant upon the introduction of an electron-donating alkyl group. This could result in the existence of a different state of dissociation for

the analog compared to the natural compound at physiological pHs. In comparing a phosphonic acid with a simple phosphate, i.e., a monoester of phosphoric acid, it is the second pK_a which is of significance. As for both compounds the first pK_a represents a relatively strong acid. However, when a primary alkyl group is attached to the phosphorus, the second pK_a for a series of phosphonic acids is found to be in the range of 7.7 - 8.2[a] as compared with second pK_a values of ca. 7.0 for the corresponding monoalkyl phosphates.[9] Thus under physiological conditions (pH 7.4) this class of compounds should be considered to be only dissociated to a monoionic state.

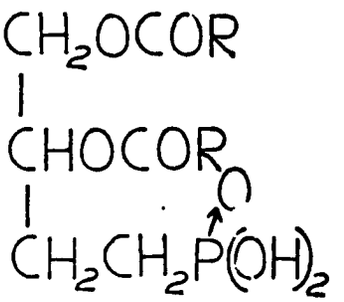
A second factor which must be considered when utilizing these analogs is that of physical size and shape. Comparing structure I to its "parent compound" Ia, one can see that the former is obviously contracted in overall size and, more specifically, the distances between the phosphoryl oxygen and other possible binding sites or sites of interaction are significantly changed unless extremely strained and highly unlikely conformations are invoked. This difference could result in great variation of biochemical or physiological activity unrelated to the mere substitution of a carbon-phosphorus linkage for that of a phosphate ester.



I



IA



IB

A more suitable analog of Ia would be Ib where the ester oxygen is replaced by a methylene group. Such an analog is known as an isosteric analog. Though it is not identical in size and shape to the parent compound, bond angles and length are similar enough that the term isostere may be applied.[1] This is not to say that subtle differences in size and shape cannot cause profound influences in the biological properties of these compounds.

Compounds containing a carbon-phosphorus bond have been used extensively in biological investigations. Already a vast literature has accumulated detailing the numerous studies utilizing phosphonates in biochemical systems. An extensive review of this subject is impossible at this point but the interested reader is referred to a review by Engel[1] and references therein for a complete treatment of the subject. A number of examples where phosphonates have been used in biochemical studies are presented here.

Methylenediphosphonic acid has been shown to be an inhibitor of the polynucleotide phosphorylase[10] but to be ineffective in inhibiting pyrophosphatases from yeast,[11] rat liver microsomes[12] and bacteria.[12] Furthermore, this compound has been shown to have considerable clinical potential as a stable, non-toxic Ca^{2+} chelating agent that can be used to reduce pathological calcium deposition in hypercalcemic states.[13,14]

Until now, the phosphonates that have provided the most information about the role of phosphate groups in biological processes are phosphonic acid analogs of nucleotides. The most extensively used compound has been the analog of GTP in which the β,γ-pyrophosphate oxygen is replaced by a methylene group (GMPPCP). This compound has been used to elucidate the mechanism of

protein synthesis, a process which requires GTP. In this case it was demonstrated that the formation of the peptide bond[15] and the translocation step of protein synthesis[16] cannot proceed with GMPPCP. However, in the formation of an initiation complex[17] and in the binding of aminoacyl t-RNA to the ribosome[18] which are also GTP-requiring processes, GMPPCP can substitute for GTP. GMPPCP has also been used to define the absolute role of GTP in the specific binding of glucagon with plasma membranes.[19]

Obviously, the wide range of biochemical processes in which ATP plays a role makes the analogs of ATP where either of the two diester oxygens are replaced with methylene groups versatile biochemical tools. For example, the α,β analog has been found to be capable of replacing ATP in the enzymic reactions catalyzed by RNA polymerase[20] and adenylate deaminase.[21] With other enzymes this analog is a potent inhibitor, e.g., PEP synthetase,[22] adenylate cyclase,[23] adenosine kinase,[24] formylglycinamide ribonucleotide amidotransferase[25] and carrot juice ATPase.[26]

In the area of phosphonate analogs of glycolysis intermediates a number of investigations have shown that some of these compounds, namely 3,4-dihydroxybutyl-1-phosphonic acid,[27] 3-hydroxy-4-oxobutyl-1-phosphonic

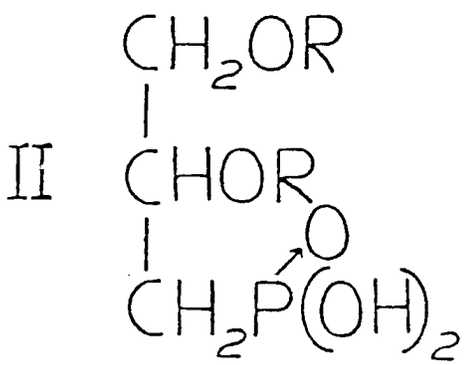
acid,[28] and 4-hydroxy-3-oxobutyl-1-phosphonic acid[1] demonstrate bacteriocidal and/or bacteriostatic activity in certain bacterial strains.

Phospholipid analogs have been investigated for their ability to replace natural phospholipids in a number of systems. For example, 2-hexadecoxy-3-octadecoxypropylphosphonic acid is a powerful inhibitor of pig kidney phosphatidate phosphohydrolase.[29] The use of isosteric and nonisosteric analogs of lecithin has shown that the length of the glycerol backbone is an important determinant in the interaction of sterols with phospholipids; only the isosteric compounds interacted with sterol.[30]

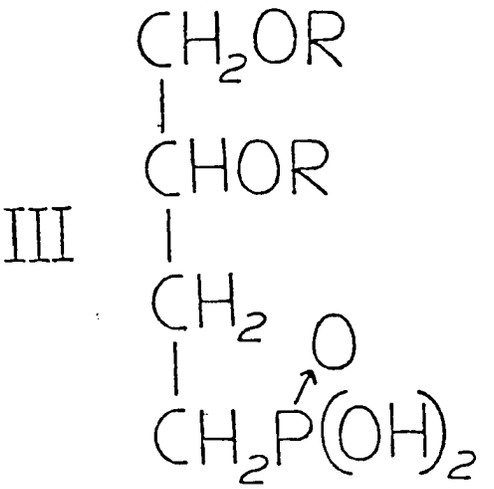
It should also be noted that all analogs are potential candidates for use as antibacterial, antiparasitic and antineoplastic agents.

Phosphatidic acid analogs containing the phosphonic acid moiety in place of phosphoric acid (I) have been termed "phosphonic acids" by Baer and Basu[31] who recently synthesized the L-distearoyl and L-dipalmitoyl compounds. Actually synthetic diether analogs of this class, represented by both nonisosteric (dialkoxypropyl, II) and isosteric (dialkoxybutyl, III) forms, have been known for a longer time.[32,33] The corresponding diether choline and ethanolamine esters are also

known,[34,35,36] constituting a class of lecithin and phosphatidylethanolamine analogs.



II



III

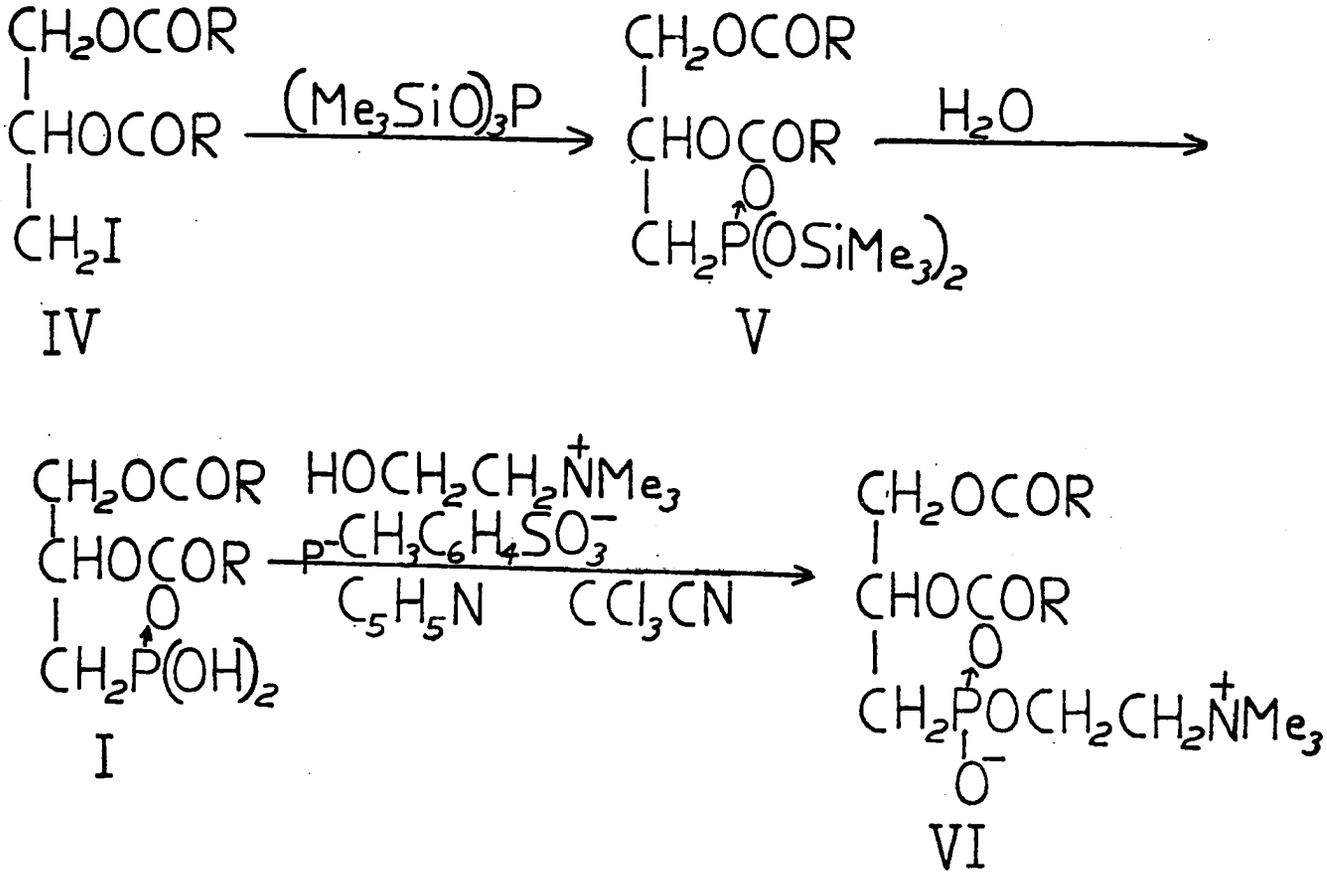
Only one representative of a diesterphosphonate lecithin of this type, the dioctanoyl homolog, appears to have been reported until very recently. The synthesis of this compound by Bonsen and co-workers[37] was accomplished by esterification of 2,3-dihydroxypropyloxyphosphonate with the carboxylic acid anhydride (the method used by Baer and Basu), followed by condensation of the phosphotidic acid with choline. In my experience, acylations of 2,3-dihydroxypropyloxyphosphonic acid did not always proceed satisfactorily. A method was therefore sought employing the well-known and easily prepared glycerol halohydrin 2,3-diacylates.

Obviously a route employing conventional Arbuzov reagents, e.g., triethyl phosphite, with the halohydrin

diesters, could not proceed beyond the diacylpropylphosphonate diester, since the phosphorus ester functions are hydrolyzed with more difficulty than the carboxylic esters. The interesting silyl exchange reaction of phosphonate diesters reported by Rabinowitz[38] usually fails, in my experience, with lipid phosphonates, but it was at least suggestive of a possible alternative.

Orlov and co-workers[39] recently described the preparation of tris(trimethylsilyl)phosphite, and showed that it would react with n-butyl bromide to give a normal Arbuzov product.[40] The great advantage of this reagent is the extreme ease with which the resulting phosphonate diesters are hydrolyzed by water alone, thus leaving intact any potentially hydrolyzable moieties, e.g., carboxylic esters, that may be present. In this dissertation, the synthesis of lecithins derived from the phosphotidic acids (I) (R = myristoyl, oleoyl, stearoyl, and octanoyl) will be reported in detail.

The synthetic route is given in Scheme I, all the compounds reported being the DL forms. The iodohydrin diacylates, prepared in the usual manner by acylation of glycerol α-iodohydrin, were treated with an excess of tris(trimethylsilyl)phosphite. Treatment of the crude product with aqueous solvents at room temperature liberated the free phosphonic acids without damage to the



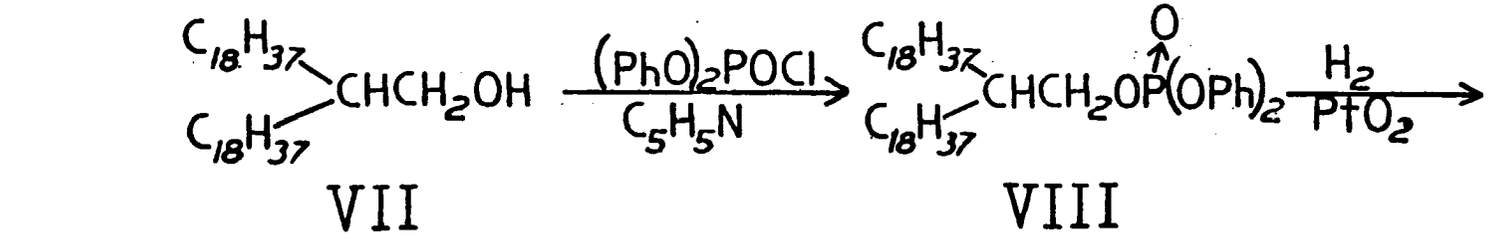
SCHEME I

carboxylic esters. The phosphotidic acids in pyridine were reacted with the toluenesulfonate salt of choline in the presence of trichloroacetonitrile to yield the corresponding lecithin analogs.[34] The isolation procedure for all intermediates varied with each particular acyl group. The lecithin analogs thus prepared differ from the natural in only one structural detail, that of the "glycerol" to phosphorus bond.

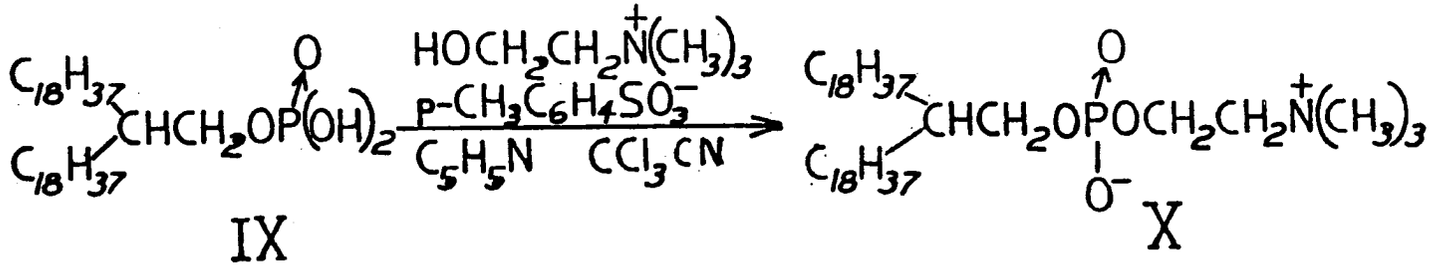
Also reported is the synthesis of dimyristoylbutylphosphonocholine, a representative of an isosteric phosphonate analog of lecithin having a methylene group in place of the oxygen between the glycerol and phosphorus.

Also synthesized was the lecithin analog, 2-octadecyleicosylphosphorylcholine (X), which differs in structure from natural lecithin in quite a different respect: the carboxylic esters are replaced completely by hydrocarbon chains. The synthesis follows a standard route (Scheme II). 2-Octadecyleicosanol was phosphorylated by diphenylphosphonylchloride, the phosphatidic acid analog was liberated by hydrogenolysis of the protecting phenyl group, and the choline ester was formed as with the above analogs.

During the course of the research reported in this thesis it became desirable to have lecithin analogs



VII VIII



IX X

SCHEME II

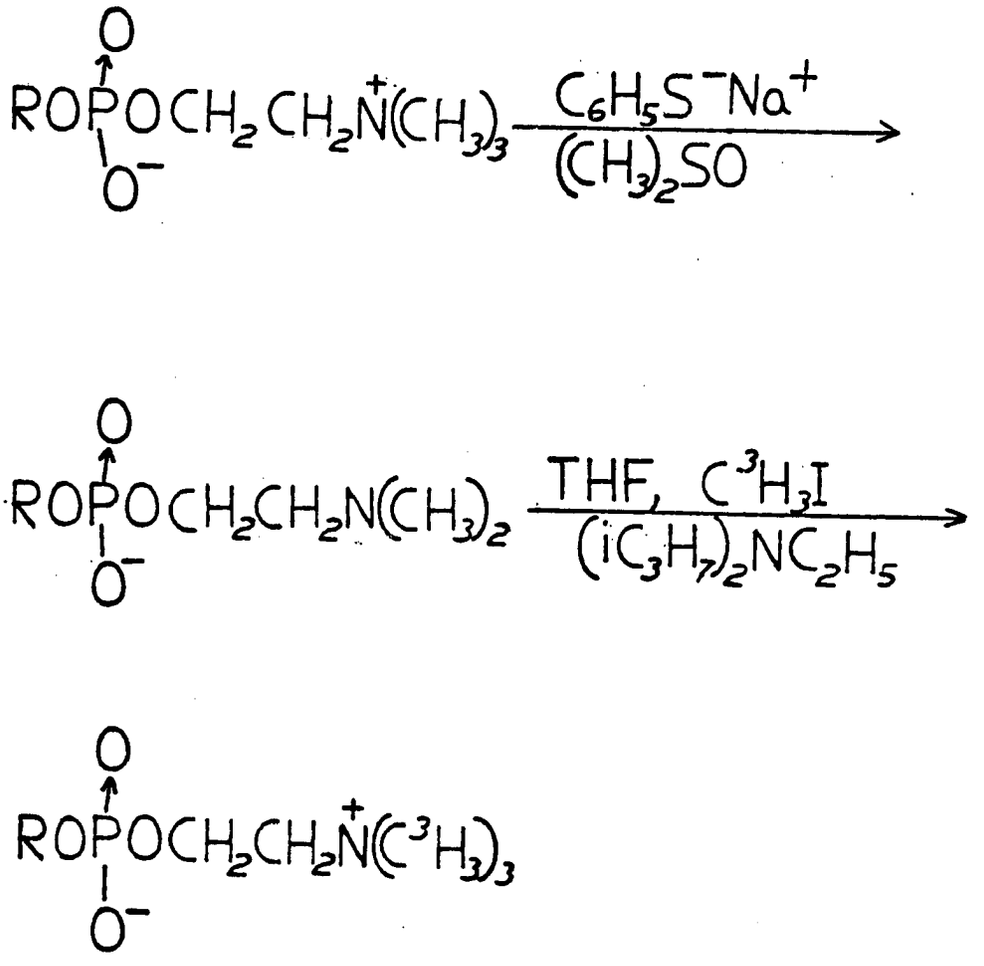
with modified head groups. This need was met by coupling choline analogs to the above-mentioned 2-octadecyleicosylphosphatidic acid using procedures exactly analogous to the coupling of choline to this compound.

The preparation of radioactive lecithins was accomplished by demethylating the choline head group with sodium benzenethiolate followed by remethylation with tritium-labeled methyl iodide (Scheme III).[41] The procedure described for dipalmitoylphosphatidylcholine is representative of the general method.

Experimental Procedure

a. Materials

DL-Glyceroliodohydrin (2,3-dihydroxyiodopropane) was prepared from glycerolchlorohydrin (Sigma Chemical Co., St. Louis, Mo.) by the procedure of Rosenthal and Geyer.[42] Methyl p-toluenesulfonate, ethyl p-toluenesulfonate, N,N-dimethylaminoethanol, N,N-diethylaminoethanol, N,N-dimethylamino-1-propanol, N,N-dimethylamino-1-butanol, and N,N-dimethylamino-2-propanol were purchased from the Aldrich Chemical Company. All solvents were analytical grade. Tetrahydrofuran (THF) was distilled immediately before use. Analyses were performed by Schwarzkopf Microanalytic Laboratories. Choline p-toluenesulfonate was prepared by mixing equimolecular quantities of N,N-dimethylethanolamine and



SCHEME III

methyl-p-toluenesulfonate in THF solution. After the reaction was allowed to proceed for 48 hours, the mixture was quickly filtered. The solid product was dried in a vacuum dessicator over P_2O_5. Yields for this reaction were typically 90%.

b. Tris(trimethylsilyl)phosphite

To a cold (8°C) solution of phosphorus acid (82 g; 1.0 mol) in 300 ml of freshly dried and distilled 1,2-dimethyoxyethane was added 475 ml of trimethychlorsilane. Triethylamine (417 ml, 3.0 mol) was then added over a period of thirty minutes while the reaction mixture was vigorously stirred and maintained at 8°C. After all the triethylamine had been added, the mixture was refluxed for 4 hours with constant stirring and was then left at room temperature overnight. After the reaction mixture was diluted with one liter of freshly dried ligroin, the precipitated triethylamine hydrochloride was removed by filtration and was washed with 3 liters of ligroin. The filtrate and washings were combined and the solvents removed by distillation at atmospheric pressure (temperature not over 90°C). The remaining liquid was vacuum distilled and the fraction distilling at 84°C/5 mm was collected. The yield of tris(trimethylsilyl)phosphite was 233 g, 78%.

c. DL-2,3-Diacyloxyiodopropane (IV, RCOO- = oleoyl)

All solvents used in the synthesis of oleoyl compounds were preserved by addition of a trace of t-butylhydroxytoluene (BHT). To a solution of 3.36 g (17 mmol) of DL-glycerol iodohydrin in 10 ml of alcohol-free, anhydrous chloroform and 2.7 ml (33 mmol) of anhydrous pyridine at 0°C was added, dropwise, over a period of 1 hour, a solution of 10 g (33 mmol) of oleoyl chloride (99% pure, Sigma Chemical Co.) in 10 ml of anhydrous alcohol-free chloroform. After the addition of the oleoyl chloride, the reaction mixture was allowed to warm to room temperature and was kept in the dark for 24 hours. The solution was diluted with 75 ml of diethyl ether and was washed twice with 20 ml of cold 0.5 N sulfuric acid, and then with water until neutral. The ether solution was then washed with 10% aqueous sodium thiosulfate solution and finally with water. The solution was dried over anhydrous sodium sulfate, filtered, and the solvent was removed under reduced pressure. The colorless oil was dissolved in hexane and applied to a column of silicic acid. It was necessary to perform the chromatography in semi-darkness because of the light sensitivity of the iodo compound. The column was first eluted with 750 ml of hexane and then with 1 liter of 25% ethyl acetate in hexane to remove the

dioleoyliodopropane. Removal of the solvent under reduced pressure left a chromatographically pure colorless oil. Yield: 10.7 g: 86%.

Analytical data for dioleoyliodlpropane and its homologs are given in Table 1.

d. 2,3-Dioleoyloxypropylphosphonic acid (I, RCOO = oleoyl)

To 3.65 g (5mmol) dioleoyloxyiodopropane was added 17 ml (10-fold excess) of tris(trimethylsilyl)-phosphite plus a trace of BHT. The reaction mixture was heated to 125°C with stirring under a static nitrogen atmosphere for 16 hours. Trimethylsily iodide and excess tris(trimethylsilyl)phosphite were removed by high vacuum distillation (both at 100°C), leaving a colorless oil which was immediately used for the next step.

The product (V) of the previous reaction was dissolved in 50 ml of THF-water (9:1, v/v) and left to hydrolyze in the dark for 12 hours at room temperature. The solvent was removed under reduced pressure and the residue was dried by repeated azeotropic distillation with 2-propanol at reduced pressure. The residual oil was dissolved in chloroform and applied to a column of silicic acid. The column was eluted first with 500 ml of chloroform and then with 500 ml of chloroform-methanol

Table 1
Physical and analytical data for 2,3-diacyloxyiodopropanes.

Acyl	R_f	% Yield	% C		% H		% I	
			Calc'd	Found	Calc'd	Found	Calc'd	Found
Dioctanoyl	0.54	64	50.22	50.29	7.76	7.84	27.93	28.06
Dioleoyl	0.61	86	64.09	64.11	9.79	9.83	17.36	17.44

The dimyristoyl and distearoyl homologs are known compounds [43] and are therefore not included. R_f's on silica gel G in hexane–ether–acetic acid, 80 : 10 : 1 (v/v/v). The dioctanoyl and dioleyl compounds are oils.

Infrared spectra of these compounds were in accordance with the structures given, all the homologs showing a strong ester carbonyl at about 1725 cm.$^{-1}$.

(9:1, v/v) to remove the product. Removal of the solvent left a viscous oil which was dissolved in chloroform and passed through a Gelman Metricel™ Alpha-6 (0.45μ) filter to remove suspended silicic acid. Removal of the solvent under reduced pressure gave a chromatographically pure viscous oil. Yield (from V): 2.8 g: 81%.

Analytical and physical data for the 2,3-diacyloxypropylphosphonic acids are given in Table 2.

e. 2,3-Dioleoyloxypropylphosphonylcholine (VI, RCOO = oleoyl)

A solution containing dioleoyloxypropylphosphonic acid (685 mg: 1 mmol), choline toluenesulfonate (2.75 g: 10 mmol), trichloroacetonitrile (20 ml) and anhydrous pyridine was stirred at 50°C for 48 hours, during which time the reaction mixture turned dark brown. The solvents were removed under reduced pressure. The residue was dissolved in 10 ml of THF-water (9:1, v/v) and applied to a column of Amberlite MB-3, a mixed-bed ion-exchange resin, which had been pre-washed with the same solvent mixture. The column was eluted with 250 ml of THF-water (9:1, v/v) at a flow rate of 2 ml/min. The colorless eluate was concentrated and dried by repeated azeotropic distillation with 2-propanol at reduced pressure. The oily residue was dissolved in chloroform and applied to a column of silicic acid. The

Table 2
Physical and analytical data for 2,3-diacyloxypropylphosphonic acids.

Acyl	R_f	% Yield	m.p.	% C		% H		% P	
				Calc'd	Found	Calc'd	Found	Calc'd	Found
Dioctanoyl	0.10	71	–	55.87	56.10	9.13	9.24	7.58	7.31
Dimyristoyl	0.21	79	–	64.55	64.81	10.66	10.69	5.37	5.06
Dioleoyl	0.25	81	–	68.39	68.83	10.74	10.90	4.52	4.48
Distearoyl	0.45 ★	89	73–76°C	–	–	–	–	–	–

★ In chloroform–methanol–formic acid 85 : 8 : 8 (v/v/v).

The R_f values are for silica gel G, in chloroform–methanol–formic acid (97%), 90 : 5 : 5 (v/v/v), except for the distearoyl homolog.

The distearoyl analog was obtained in almost homogeneous form directly from the hydrolysis mixture but was not purified further for analysis.

Yields are given from the 2,3-diacyloxyiodopropanes.

Infrared spectra of these compounds showed the expected absorbances, including strong ester carbonyl at 1720 cm^{-1} and a phosphoryl band peaking at about 1260 cm^{-1}.

column was eluted first with chloroform-methanol (9:1, v/v) to remove unreacted dioleoylpropylphosphonic and then with chloroform-methanol (6:4, v/v) to remove the desired compound. After removal of the solvent, the residue was redissolved in chloroform and passed through a Metricel™ filter as described above. Removal of the solvent yielded a chromatographically pure white solid. Yield: 570 mg: 74%.

Analytical and physical data for the 2,3-diacyloxypropylphosphonylcholines are given in Table 3.

f. Other 2,3-diacyloxypropylphosphonylcholine homologs

The synthesis of dioleoyloxypropylphosphonyl choline described above is representative of the synthesis of the dioctanoyl, dimyristoyl, and distearoyl homologs, with the exception that treatment of solvents with BHT was not employed with the saturated homologs. Also to be noted is the fact that the distearoyl and dimyristoyl compounds could be precipitated when in chloroform solution by the addition of cold acetonitrile. This aided in the separation of lipid components from non-lipid impurities.

Table 3
Physical and analytical data for 2,3-diacyloxypropylphosphonylcholines.

Acyl	R_f	% Yield	m.p.	H_2O of hydration	% C		% H		% P		% N	
					Calc'd	Found	Calc'd	Found	Calc'd	Found	Calc'd	Found
Dioctanoyl	0.49	62	--	1.5	55.37	55.30	9.87	9.61	5.95	5.45	2.69	2.64
Dimyristoyl	0.52	71	--	1.5	62.76	62.48	10.97	10.67	4.50	4.12	2.03	2.47
Dioleoyl	0.60	74	--	0	68.63	68.29	10.99	11.08	4.02	3.88	1.82	2.34
Distearoyl	0.54	79	204 - 206°C dec	0.5	67.48	67.48	11.39	11.52	3.95	4.02	1.79	2.12

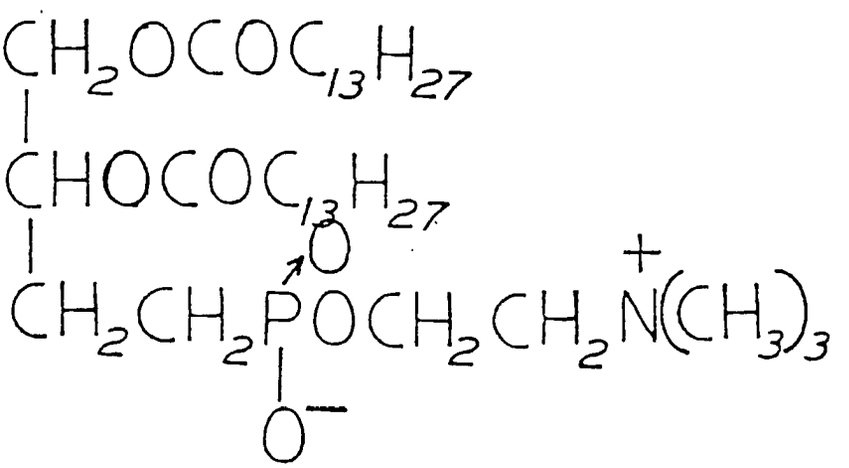
The R_f values are for silica gel G in chloroform--methanol--water, 65 : 25 : 4 (v/v/v).

Yields are given from the 2,3-diacyloxypropylphosphonic acids.

The first three lecithin analogs listed are gums, melting points of which were difficult to determine accurately.

The lecithin analogs showed the expected infrared absorbances including the following: P → O, 1220 cm^{-1} (m); P-O C, 1050 cm^{-1} (m) and possibly 970 cm^{-1} (m); and C=O, 1720 cm^{-1} (s). 60 MHz ^1H nmr showed the quaternary ammonium methyl as a singlet at 3.4 ppm.

g. 3,4-Dimyristoyloxybutylphosphonylcholine



The preparation of this compound followed the above procedure with the exception that 3,4-dihydroxybromobutane (kindly supplied by Dr. Robert Engel) was used in place of the glycerol iodohydrin. The substitution of bromine for iodine did not seem to adversely affect the yield of the Arbuzov reaction. Physical and analytical data for the intermediates in this synthesis are given in Table 4.

This compound resembles a natural lecithin more closely than the above compounds since it is isosteric with the natural compound having a methylene group substituted for the oxygen atom.

h. Diphenyl 2-octadecyleicosylphosphate (VIII)

To a solution of 550 mg (1.00 mmol) of 2-octadecyleicosanol (provided by Dr. A. Rosenthal) in 10 ml

TABLE 4

Physical and analytical data for intermediates in the synthesis of 3,4-dimyristoyloxybutylphosphonocholine

Compound	R_f	% Yield	%C		%H		%P		%N	
			Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found
3,4-Dimyristoyloxy-butylbromide	0.60[a]	73	64.89	64.99	10.35	10.42	---	---	---	---
3,4-Dimyristoyloxy-butylphos-phonic acid	0.23[b]	82	65.30	65.24	10.68	10.62	5.26	5.44	---	---
3,4-Dimyristoyloxy-phosphono-choline	0.51[c]	70	65.78	65.69	10.95	10.98	4.59	4.71	2.07	1.83

The R_f values are for Silica Gel G in the solvents indicated.

Yields are from the previous compounds in the reaction scheme.

a) Hexane-ether-acetic acid, 80:10:1 (v/v/v)

b) Chloroform-methanol-formic acid (97%), 90:5:5 (v/v/v)

c) Chloroform-methanol-water, 65:25:4 (v/v/v)

of anhydrous pyridine-chloroform (4:1, v/v) was added diphenylchlorophosphate (296 mg, 10% excess). The solution was stirred at room temperature for 24 hours.

Thin-layer chromatography in chloroform showed that the 2-octadecyleicosanol (R_f 0.78) had been completely converted to a faster moving compound (R_f 0.85). The solvents were removed at reduced pressure leaving a colorless oil which crystallized to a waxy solid on standing at room temperature overnight. Yield: 764 mg: 98%: m.p. 33-34°C.

Analysis calculated for $C_{50}H_{87}O_4P$ (783.22):

C, 76.68: H, 11.20: P, 3.95

Found: C, 76.41: H, 11.23: P, 4.17

The infrared spectrum (KBr) showed a free P⟶O at 1295 cm^{-1} and aryl absorbances (e.g., 1520 cm^{-1} (s), aromatic skeletal), while the 60 MHz ^1H nmr showed phenyl groups (10 H) at δ7.30 ppm.

i. 2-Octadecyleicosylphosphoric acid (IX)

To a solution of diphenyl 2-octadecyleicosylphosphate (VIII) (700 mg 0.89 mmol) in cyclohexane (5 ml) was added 25 ml of glacial acetic acid. Platinum catalyst was prepared by suspending 50 mg of platinum oxide (Adams' catalyst, Matheson, Coleman and Bell) in 25 mg of glacial acetic acid and reducing with hydrogen at atmospheric pressure. The solution containing the

diphenyl ester was added to the rapidly stirred suspension of platinum by injection through a rubber septum. When hydrogen was no longer taken up, the platinum was removed by filtration through a sintered glass filter (positive N_2 pressure) and was washed with a small volume of chloroform. The filtrate and washings were combined and the solvent was completely removed under reduced pressure, whereupon 2-octadecyleicosylphosphoric acid (IX) crystallized. The compound was dissolved in chloroform and applied to a column of silicic acid. The column was eluted first with chloroform (200 ml) to remove nonpolar material and was subsequently eluted with 500 ml of chloroform-methanol (3:1, v/v) to remove the desired product. After removal of the solvent under reduced pressure, the compound was redissolved in chloroform and passed through an alpha Metricel^{TM} filter to remove suspended silicic acid. Removal of the solvent left a chromatographically pure (R_f 0.46, in chloroform-methanol-97% formic acid, 90:5:5, v/v/v) white solid. Yield: 446 mg: 79%: m.p. 51-52°C.

Analysis calculated for $C_{38}H_{79}O_4P$ (631.02):

C, 72.33: H, 12.62: P, 4.91

Found: C, 72.16: H, 12.73: P, 4.79.

The infrared spectrum showed the expected absorbances, the P-O-C falling at 1030 cm^{-1} (s).

j. 2-Octadecyleicosylphosphorylcholine (X)

To a solution of 315 mg (0.5 mmol) of 2-octadecyleicosylphosphoric acid (IX) in a mixture of pyridine (25 mg) and trichloroacetonitrile (5 ml) was added 1.4 g (10-fold excess) choline toluenesulfonate. The reaction mixture was stirred at 50°C for 48 hours. Approximately two-thirds of the solvent was removed under reduced pressure and the desired product was precipitated by adding 40 ml of cold acetonitrile. After filtration the beige-colored solid was dissolved in THF-water (9:1, v/v) and applied to a column of Amberlite MB-3 resin previously equilibrated with the same solvent. The column was eluted with 250 ml of THF-water (9:1, v/v) at a flow rate of 2 ml/min. The colorless eluate was concentrated under reduced pressure and dried by repeated azeotropic distillation with 2-propanol. The waxy white product was dissolved in chloroform and applied to a column of silicic acid. Unreacted 2-octadecyleicosylphosphonic acid was eluted with 250 ml of chloroform-methanol (3:1, v/v). The required compound (X) was then eluted with 500 ml of chloroform-methanol (1:4, v/v). The solvent was removed under reduced pressure and the compound was redissolved in chloroform and filtered through a Metricel^{TM} filter to remove silicic acid. The solvent was removed leaving a chromatographically pure (R_f 0.57,

in chloroform-methanol-water, 65:25:4, v/v/v) white solid. Yield: 263 mg, 71% m.p. 217-219°C (decomposition).

Analysis calculated for $C_{43}H_{90}O_4PN$ (943.193):

C, 69.49: H, 12.61: P, 4.17: N, 1.88

Found: C, 69.62: H, 12.81: P, 4.26: N, 2.17.

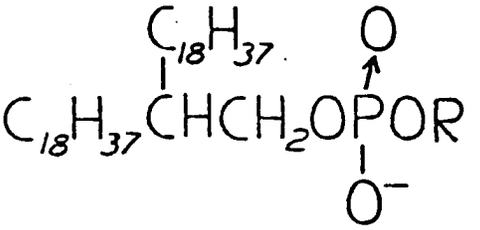
The infrared spectrum (KBr) showed the expected bands; e.g., P⟶O, 1240 cm^{-1}, P-O-C (?), 960 cm^{-1} (s); $P-O^-$ (s), 1090 cm^{-1}.

k. 2-Octadecyleicosylphosphorylcholine analogs with modified head groups

Compounds were also synthesized which differed from the 2-octadecyleicosylphosphorylcholine in having a modified head group in place of the choline moiety (Figure 1). These include 2-octadecyleicosylphosphoryl-N,N,N-trimethylamino-1-propanol (XII), a compound which has an extra methylene group between the phosphate and choline, thus increasing the distance between the charged centers of the molecule; 2-octadecyleicosylphosphoryl-N,N,N-trimethylamino-1-butanol (XIII), a compound with an increased separation of the phosphate and quaternary ammonium groups; 2-octadecyleicosylphosphoryl-N-ethyl-N,N-dimethylaminoethanol (XIV), a compound possessing an ethyl group substituted for an N-methyl group on choline; 2-octadecyleicosylphosphoryl-N,N,N-triethylaminoethanol (XV), a compound with the three N-methyl groups of

FIGURE 1

STRUCTURES OF 2-OCTADECYLEICOSYLPHOSPHORYLCHOLINE ANALOGS



X $R=CH_2CH_2\overset{+}{N}(CH_3)_3$

XII $R=CH_2CH_2CH_2\overset{+}{N}(CH_3)_3$

XIII $R=CH_2CH_2CH_2CH_2\overset{+}{N}(CH_3)_3$

XIV $R=CH_2CH_2\overset{+}{N}(C_2H_5)(CH_3)_2$

XV $R=CH_2CH_2\overset{+}{N}(C_2H_5)_3$

XVI $R=CH(CH_3)CH_2\overset{+}{N}(CH_3)_3$

choline substituted by ethyl groups; 2-octadecyleicosyl-phosphoryl-1-methylcholine (XVI), a compound having a methyl group substituted for a hydrogen atom on the carbon alpha to the phosphate group. The synthesis of these compounds was accomplished by coupling the respective choline analog to 2-octadecyleicosylphosphoric acid using the procedure described above for the synthesis of 2-octadecyleicosylphosphorylcholine. The tosylate salts of these choline analogs were readily prepared by reacting a suitable tertiary amine with either methyl-p-toluenesulfonate or ethyl-p-toluenesulfonate, using conditions similar to those described above for the choline tosylate. All of the salts crystallized as white solids from THF except the N,N,N-triethylaminoethanoltosylate which separated as an oil. After separation of excess solvent the product was solidified by triturating with ice-cold acetone. Yields were typically in the vicinity of 85% (range 81-92%). All the tosylate salts were extremely hydroscopic and required great care in storage. Analytical and physical data for the above-mentioned lecithin analogs are given in Table 5.

1. N,N-Dimethyl-3,4-dipalmitoylbutylphosphonylethanolamine

Sodium benzenethiolate was prepared according to the following procedure.[44] Equivalent amounts of sodium

TABLE 5

Physical and analytical data for octadecyleicosylphosphorylcholine analogs with modified head groups

Compound	R_f	% Yield	m.p.	%C		%H		%P		%N	
				Calc.*	Found	Calc.*	Found	Calc.*	Found	Calc.*	Found
XII	0.62	69	222-224	72.38	70.01	12.70	12.79	1.92	1.58	4.24	4.11
XIII	0.59	58		72.68	70.94	12.70	12.52	4.06	4.13	1.84	1.69
XIV	0.68	63	185-187	72.38	69.27	12.70	12.89	1.92	1.72	4.24	3.94
XV	0.73	59		72.87	70.94	12.76	12.81	1.85	1.76	4.09	3.91
XVI	0.61	64	206-207	72.38	70.69	12.70	12.83	1.92	1.81	4.24	4.07

R_f values are for silica gel G in chloroform-methanol-water, 65:25:4 (v/v/v).

Yields are given from 2-octadecyleicosylphosphoric acid.

Compound XV was a soft wax, the melting point of which was difficult to determine.

*Calculated for the anhydrous compound.

(17 g, 0.75 mol) and thiophenol (81 g, 0.75 mol) were refluxed in 100 ml of absolute methanol for one hour, after which time 200 ml of toluene was added. Upon cooling to 10°C, a solid crystalline product precipitated which was taken to be sodium benzenethiolate; nmr (D_2O) showed the sole presence of aromatic protons (multiplet, 7.0 ppm) and the complete absence of aliphatic protons.

DMSO was dried by refluxing over BaO for 2 hours followed by distillation. Twenty-five ml of the fraction boiling at 189°C was distilled directly into a reaction vessel containing 160 mg (0.22 mmol) of dipalmitoylphosphatidylcholine and 132 mg (1 mmol) of sodium benzenethiolate. The mixture was heated under anhydrous conditions at 100°C for 24 hours after which time another 132 mg of sodium benzenethiolate was added. The reaction was allowed to proceed for another 24 hours. At the end of this time, the starting material had disappeared; tlc of the reaction mixture (chloroform: methanol: water, 65:25:4, v/v/v) showed the concomitant appearance of a less polar phosphorus-containing spot (R_f = 0.83). The reaction mixture was allowed to cool and was poured into 25 ml of ice-cold 0.5 N sulfuric acid. The solution was extracted three times with chloroform. The combined chloroform extracts (75 ml)

were dried over sodium sulfate. Removal of the chloroform under reduced pressure left a heterogeneous mixture which consisted of a colorless liquid intermixed with a white solid. Since tlc showed the presence of only one major spot, it was inferred that the solid was the desired product and the liquid was DMSO that was extracted into the chloroform. The heterogeneous mixture was dissolved in a minimal amount of chloroform (5 ml) and the desired lipid product was precipitated with 40 ml of ice-cold acetonitrile. The crude product was purified by silicic acid chromatography. The column was eluted first with chloroform: methanol (9:1, v/v) to remove the desired product. Yield: 110 mg: 66%; tlc in chloroform: methanol: water (65:25:4, v/v/v) gave a single spot, R_f = 0.83.

m. Tritiated dipalmitoylphosphatidylcholine[41]

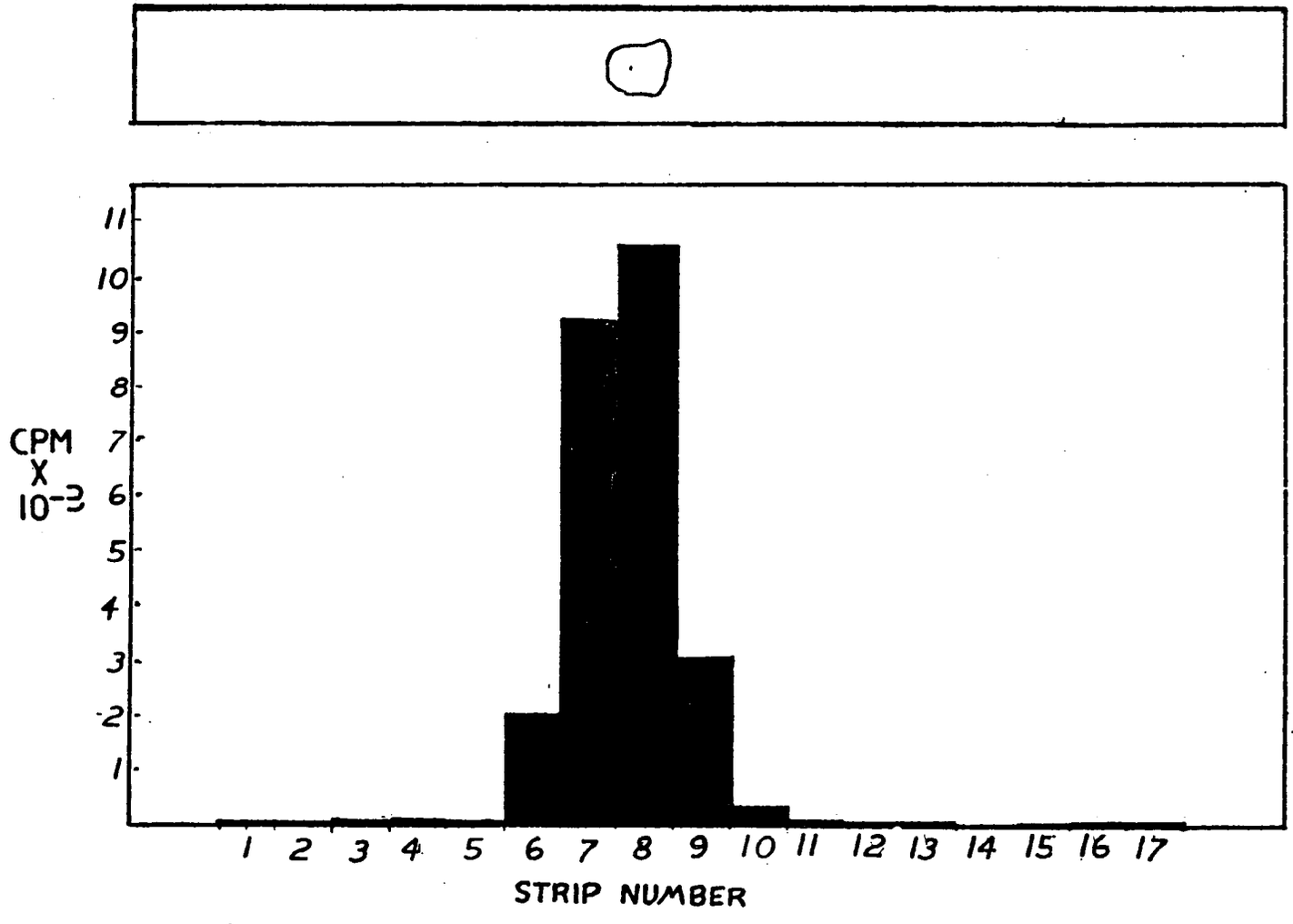
Dipalmitoyl-N,N-dimethylphosphatidylethanolamine (100 mg, 0.14 mmol) was dissolved in 20 ml of THF. To this was added 30 μl of N,N-diisopropylethylamine (0.23 mmol) and 100 μl (0.7 mmol) ^{3}H- methyl iodide (Amersham Searle). The sealed reaction mixture was maintained in the dark at room temperature for 24 hours, during which time it turned light yellow. Thin-layer chromatography of the reaction mixture showed an almost complete disappearance of the starting material (R_f = 0.83) with the

appearance of a more polar spot that co-chromatographed with dipalmitoylphosphatidylcholine (R_f = 0.45). Then 25 ml of ether was added and the ether solution was extracted twice with an aqueous solution of 5% sodium thiosulfate, followed by two extractions with cold 2N hydrochloric acid and one extraction with water. The combined aqueous phases were re-extracted twice with 10 ml of chloroform, and the combined ether-chloroform extracts were dried over sodium sulfate. Evaporation of the solvents left a white solid residue. Purification on silicic acid gave a chromatographically pure compound which appeared to be identical to the starting lecithin using chromatographic (tlc, R_f = 0.47), spectroscopic (ir, P$\longrightarrow$O 1260 cm^{-1}), and physical (mp 231-233) criteria. Yield: 70 mg. A radiogram was constructed by cutting strips (0.125") from a commercially available plastic-backed silica gel G tlc plate (Eastman Kodak) and counting the strips in a liquid scintillation counter. A major radioactive spot appeared which co-chromatographed with unlabeled lecithin and represented greater than 99.5% of the radioactivity, along with a smaller spot corresponding to a more polar compound which represented less than 0.5% of the radioactivity spotted (Figure 2). The latter is probably lysolecithin. Thus, the compound is greater than 99.5% pure. The specific activity of the

FIGURE 2

Radiogram of synthetic ^{3}H dipalmitoylphosphorylcholine constructed by cutting 0.125" strips of plastic backed tlc plate and counting them.

Upper strip is tlc of unlabeled lecithin visualized with the Dittmer-Lester spray.



product was 10 millicuries/mmol.

In a similar manner the radioactive analogs of 2-octadecyleicosylphosphorylcholine and 3,4-dimyristoylbutylphosphonylcholine were synthesized. The reactions proceeded smoothly and the yields obtained are shown in Table 6. Although in principle the above procedure could be applied to any lecithin, those that cannot be precipitated with acetonitrile; e.g., dioleoyllecithin or natural lecithins, could not be readily freed from excess DMSO which, in turn, may interfere with the purification of the N,N-dimethyl compounds with silicic acid. Care must also be taken in the storage of the product as a preliminary observation indicates that the label may be labile.

TABLE 6

Yields and specific activities of radioactive lecithin analogs synthesized

Compound	% Yield	Millicuries/mmol
Dipalmitoyllecithin	40	10
2-Octadecyleicosylphosphorylcholine	15	10
3,4-Dimyristoylbutylphonylcholine	35	10

% Yield is from the unlabeled compound.

CHAPTER II

REACTIVATION OF β-HYDROXYBUTYRATE DEHYDROGENASE WITH LECITHIN ANALOGS

Introduction

Definition of the precise role of phospholipid in enzymic activity is basic to an understanding of the structure and function of biologic membranes. The study of lipid-protein interactions in the cell membrane has been hampered by the difficulty of obtaining purified membrane proteins that can be reconstituted into functionally active complexes resembling the native system. Methods used to dissociate membrane components, i.e., detergents, sonication, high or low salt concentrations, often result in an irreversible loss of activity. It is extremely difficult to completely rid the protein of contaminating detergents or lipids.[45]

The reversible oxidation of free D-β-hydroxybutyrate to acetoacetate in animal tissues was first observed many years ago.[46] The properties of the enzymic reaction

were initially studied by Green et al.[47] who demonstrated that the reaction in pig heart resided in respiratory particles, was stereospecific for the D isomer of β-hydroxybutyrate and required NAD^+. The next in-depth study of the enzyme was that of Lehninger et al.[48] who described the assay, kinetic properties of the enzyme, its occurrence in various tissues and subcellular fractions and the role of mitochondrial structure in the activity of the enzyme. Specifically they showed that the enzyme as it occurs in particulate fractions obtained from mitochondrial fragments is specific for the D-stereoisomer ($K_m = 4.7 \times 10^{-4}M$) and for NAD^+ ($K_m = 2.5 \times 10^{-4}M$) confirming the earlier work of Green. Furthermore the pH optimum for enzymatic activity was 8.0 to 8.5. The enzyme was ubiquitously distributed among rat tissue but was found only in the mitochondria in tightly bound form. Freshly prepared mitochondria show essentially no BDH activity when tested with added NAD; activity is yielded only after disruption of mitochondria by relatively drastic means, the most effective being ultrasonic irradiation. This effect was ascribed to the inaccessibility of the NAD+ to the mitochondrial interior.

Interest in this enzyme was stimulated by the observation that the enzyme could be solubilized with

cholate and that the solubilized enzyme had a specific requirement for lecithin,[49] other phospholipids (DPG, phosphatidic acid, phosphatidyl inositol, PE and sphingomyelin) being incapable of restoring activity.[50] Cholesterol inhibits the reactivation by lecithin.[51] These findings indicated that the enzyme has considerable potential as a model for membrane-bound enzymes and for the study of protein-lipid interactions.

Over the years there have been continued attempts to purify BDH.[52,53,54] The most successful approach involved the use of cholate to release the enzyme from the membrane, followed by the formation of a lecithin-enzyme complex which was not sedimentable in 50% saturated ammonium sulfate.[55,56] However, the purified enzyme prepared by this method is complexed to lipid, and therefore cannot be used as such to study interaction with lipids. Attempts to remove lipid with solvents lead to denaturation of the protein.[55,56]

In this regard a breakthrough has been achieved in the laboratory of Dr. Sidney Fleischer where the enzyme has been purified to homogeneity and to a lipid-free state (< 1 mole Pi/mole enzyme) without adding detergent.[57] The enzyme which is firmly bound to the inner mitochondrial membrane is initially released by controlled digestion of freeze-thawed beef heart

mitochondria with phospholipase A. Optimal release and recovery of activity occur when 25% of the phospholipids have been degraded. After an ammonium sulfate precipitation step, the released enzyme is then purified by adsorption to glass beads and subsequent elution with LiBr solutions. The homogeneous apodehydrogenase thus isolated has a subunit molecular weight of 31,500. Gel exclusion chromatography of the soluble enzyme showed components equivalent to the monomer and dimer. The enzyme has been purified 260 fold and comprises 0.4% of the total bovine heart mitochondrial protein complement. Amino acid analyses of the purified protein shows its composition to be similar to that of other soluble proteins, e.g., bovine serum albumin.[57]

The interaction of the soluble BDH with lipids has been studied. Mitochondrial phospholipid reactivates with the highest specific activity (approximately 100 μmol NAD reduced/min/mg) and with the greatest efficiency--2.5 to 4.0 mol of lecithin/mol of enzyme subunit (efficiency is defined as the mole PL to mole enzyme ratio that gives 50% of maximal reactivation). Each of the lecithins of varying chain length and unsaturation reactivated the enzyme, albeit to differing extents and efficiencies. In general, lecithins containing unsaturated fatty acid moieties reactivated better

than those containing the comparable saturated lipid. For reasons that are unclear, optimal reactivation can be obtained for the various lecithins when they are codispersed with PE (PC:PE 1.0:0.8). Also, when lecithins are codispersed with PE and DPG (PC:PE:DPG 1:0.8:0.4) maximal efficiency is obtained. The reactivation of the enzyme with dioctanoyl or dihexanoyl lecithins demonstrate that a phospholipid bilayer is not necessary to reactivate the enzyme. These results with the lipid-free dehydrogenase mirror those obtained for the lecithin complexed enzyme of earlier workers.[58]

The same energies of activation are obtained from Arrhenius plots for the membrane-bound enzyme and for the purified soluble enzyme whether it is reactivated with MPL or with saturated lecithins below their transition temperatures or with unsaturated lecithins above their transition temperatures.[58] This, coupled with the observation that the membrane-bound enzyme and the enzyme released by phospholipase A treatment show identical kinetics,[59] indicates that the enzyme has not been adversely affected by the isolation procedure and that lipid phase transitions do not affect the enzyme's activity.

It has recently been demonstrated that the enzyme complexed with lecithin or with a phospholipid mixture

containing lecithin binds NADH while the apoenzyme phospholipid alone or complexes formed with non-reactivating phospholipids do not bind NADH.[60] Thus it would appear that at least one role the lipid plays in reactivating this enzyme is to allow the binding of the cofactor.

The subunit structure of the active enzyme is presently unknown. Attempts to elucidate the subunit structure of the active lecithin-enzyme complex using crosslinking reagents yielded equivocal results.[61] Presently an attempt is being made to utilize the sedimentation properties of the soluble but active dioctanoyl-lecithin-enzyme complexes to answer this question.[62]

Despite the advances made in the isolation and physical properties of this enzyme, its physiological significance remains obscure. The acetoacetate formed is not further metabolized in isolated mitochondria.[63] It was suggested that the enzyme may function in a transport system for the passage of reducing equivalents across the mitochondrial membrane.[64] Others have hypothesized that the enzyme might function in a mechanism for coordinating the oxidation-reduction levels in the mitochondria from one tissue to another via the ratio of β-hydroxybutyrate to acetoacetate in the circulating blood.[65] The finding that the NAD^+:NADH ratio affects the inherent activity of the enzyme suggests the

additional possibility that the enzyme might function as a biological buffer system for NAD^+ and NADH. As more NADH is formed the enzyme becomes less active and thereby slows the generation of NADH.[53]

In the study presented in this thesis the reconstitution of BDH with a number of lecithin analogs whose synthesis was described in the first part of the thesis is reported with a view toward further delineating the structural requirements necessary for the functional interaction between BDH with phospholipid.

Experimental Procedure

a. Materials

L-N,N-Dimethylphosphatidylethanolamine (dipalmitoyl), L-palmitoyllysolecithin, DL-dipalmitoyllecithin, and bovine brain sphingomyelin were purchased from the Sigma Chemical Company. A second source of sphingomyelin was Supelco. L-Dipalmitoyllecithin was also synthesized following the procedure of Cubero-Robles and Van den Berg.[66] Mitochondrial phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) were purified from beef heart mitochondrial lipid extracts using silicic acid chromatography. Each lipid was shown to be homogeneous by tlc on silica gel plates. Detection was by spraying with Dittmer-Lester spray[67] (specific for phosphorus-containing compounds) followed by charring.

b. Preparation of D-dipalmitoyllecithin

Approximately 250 mg of DL-dipalmitoyllecithin was sonicated in 15 ml of distilled water until no further decrease in turbidity could be visually detected. To the sonicate was added 2.5 ml of 0.025 M glyclglycine buffer, pH 7.4, 4 ml of Naja Naja Siamensis snake venom (1 mg/ml), 0.75 μl of 1 M $CaCl_2$ and 3.5 ml of H_2O. The suspension was allowed to stand for 48 hours with occasional shaking at 37°C, after which time it was extracted three times with equal volumes of chloroform: methanol (2:1, v/v). The chloroform phases were combined, the organic solvents removed in vacuo, and the residue dissolved in a small volume of chloroform. Thin-layer chromatography showed the presence of two phosphorus-containing compounds; one co-chromatographed with the starting material and the other was more polar, possibly lysolecithin. The individual spots were scraped off the plate. A phosphate analysis of the two spots was performed as previously described except that the silica was removed just prior to determining the absorbance. Nearly equivalent amounts of phosphorus in both spots indicated a greater than 90% digestion of the L isomer. The D-lecithin was purified to homogeneity on a silicic acid column by eluting with 40% MeOH in chloroform. Yield: 70 mg, 56%; $[\alpha]D = -6.7$.

c. Preparation of Aqueous Dispersons of Phospholipids

Dispersions having a final concentration of 200 μg of phosphorus/ml in a total volume of 3 ml of Tris-EDTA, pH 8.1, were prepared. In experiments with mixtures of phosphatidylcholine and phosphatidylethanolamine, a final concentration of 100 μg of PC phosphorus per ml and 80 μg of PE phosphorus per ml was used. The lipids were sonicated under a stream of nitrogen using a Branson model S 110 sonifier set at 6 amps. With the exception of dioleoyloxypropylphosphonylcholine, which was immersed in an ice-water bath during the sonication, no provision was made for the maintenance of low temperatures. During the sonication the liquid suspension heated to approximately 50°C. The suspensions were sonicated for 15-second bursts with one-minute intervals between bursts to allow for cooling. Sonication was continued until no further decrease in turbidity could be visually noted. The suspensions were then centrifuged in a Beckman Ti50 rotor at 30,000 rpm for 30 minutes. The supernatants obtained were used in the reactivation studies. In order to determine the total and relative amounts of lipid in the suspensions, aliquots of the suspension were chromatographed, scraped from the plates, and quantitatively assayed for phosphorus. From the ratio of phosphorus in the various spots and from the

total phosphorus, the amounts of lecithin and phosphatidylethanolamine in the dispersion were calculated. Lipid phosphorus was measured using a modification of the procedure of Chen et al.[68] For the phosphonolipids, digestion with perchloric acid was allowed to proceed for 16 hours. Control studies showed that digestion for this length of time gave a linear relationship between the absorbance at 800 nm and the amount of phosphonolecithin present.

The standard assay mixture for D-β-hydroxybutyrate dehydrogenase activity contained 10 mM potassium phosphate buffer, pH 7.35, 2 mM NAD^+, 0.5 mM EDTA, 0.3 M dithiothreitol, 1.3% ethanol, 400 μg/ml bovine serum albumin, 1.2 μg enzyme, and varying amounts of lipid. The above assay mixture (0.9 ml) was preincubated for 15 minutes at 37°C. The reaction was initiated by the addition of 0.1 ml of 0.2 M DL-β-hydroxybutyrate. The reduction of NAD^+ was followed at 340 nm using a Gilford model 2000 spectrophotometer. Tracings were typically linear, thus showing saturation of the enzyme under these conditions. Controls were run using saturating amounts of a suspension of mixed mitochondrial phospholipids (MPL).

The extent of reactivation is expressed as the percent of activity obtained with a specific lecithin

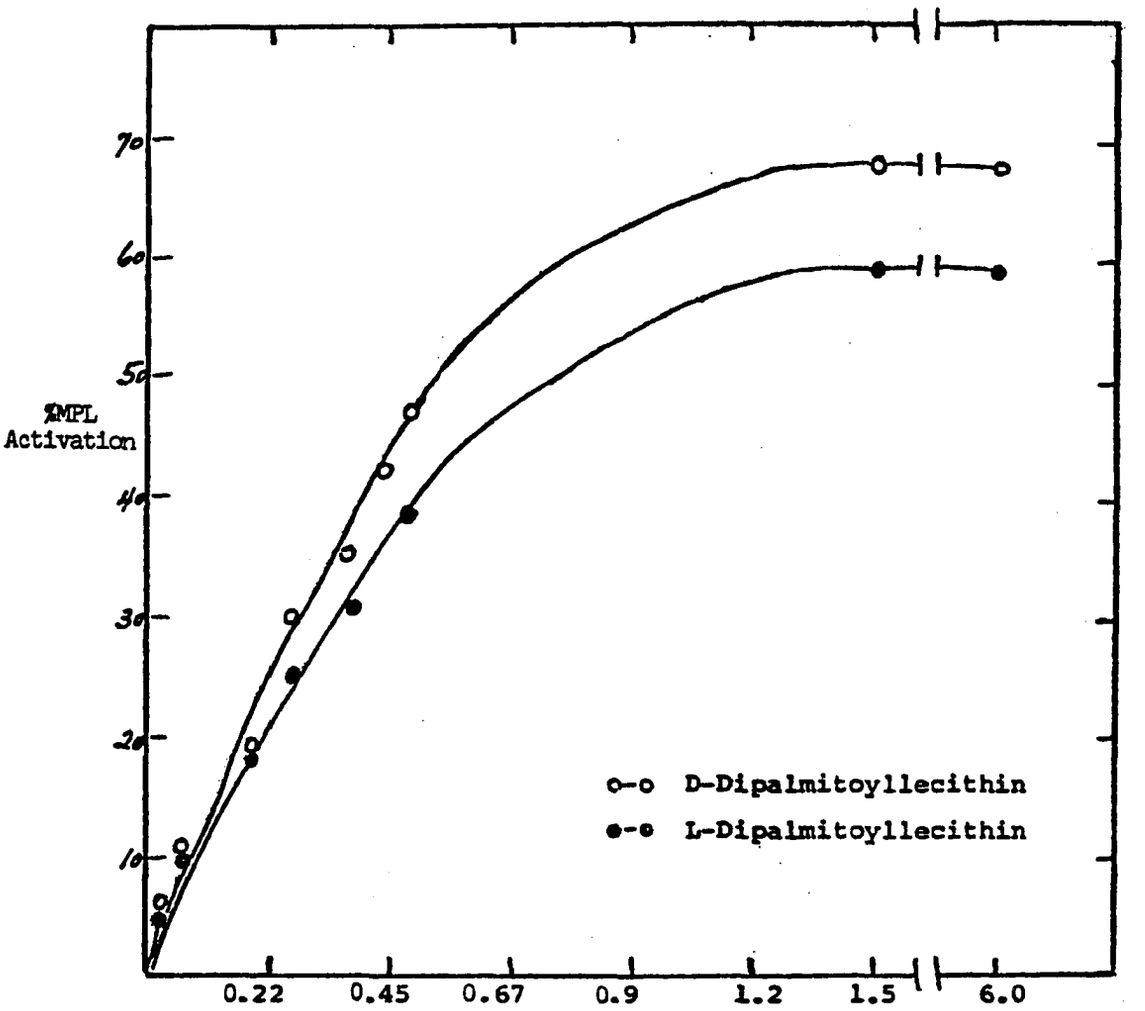
relative to that obtained with a mixed mitochondrial phospholipid (MPL) dispersion, which is taken to be 100%. To determine the efficiency of reactivation, titration curves were constructed by plotting the activity of the enzyme versus the amount of lipid added to the cuvet. The molar ratio of lecithin to enzyme which gives 50% maximal activity is reported.

Results

a. Effect of modifications on the glycerol side of the phosphate

Since the phosphono compounds synthesized were racemic mixtures, it was necessary to establish whether the configuration around the 2-carbon atom of the activating lecithin was critical to its ability to reactivate the enzyme. Figure 3 shows the titration curves of the D and L forms of dipalmitoyllecithin in the presence of PE. Both lecithins showed nearly identical extents of reactivation and efficiency. D-Dipalmitoyllecithin activated to 68% mitochondrial phospholipid (MPL) activity when dispersed with PE while the L form activated to 58% MPL under the same conditions. In the presence of diphosphatidylglycerol (DPG), the efficiency of both lecithins was approximately 4.2 moles of lecithin per mole of enzyme. Thus the enzyme showed a nearly identical preference toward the D or L forms

FIGURE 3



Titration curves for D and L isomers of dipalmitoyllecithin in the presence of PE (PC:PE, 1:0.8*). For experimental details see text.

*Unless otherwise indicated, all ratios are in terms of moles phosphorus.

of the activating lecithin.

Phosphono compounds that lack the ester oxygen were tested in order to examine the role of this portion of the molecule on enzyme reactivation. These included distearoyl, dioleoyl, and dimyristoyloxypropylphosphonylcholines, as well as dimyristoyloxybutylphosphonylcholine. The latter compound differed from the dimyristoylpropyl compound in that it is an isosteric analog in which the oxygen atom of the ester was replaced with a methylene group. All of the phosphonylcholines activated the enzyme albeit to different extents and efficiencies (Table 7). The low level of activation demonstrated by the distearoyl compound probably reflected a difficulty in maintaining this lipid in a dispersed state. The dimyristoylcholines were effective activators, activating to the extent of about 75%. The titration curves for the dimyristoyloxybutyl and dimyristoyloxypropylphosphonylcholines were practically superimposable (Figure 4). This indicated that steric considerations governing the spacing between the acyl groups and the polar head groups were unimportant within these limits in determining the activating efficacy of a given lecithin.

To gain a further insight into the structural requirements of the glycerol side of the molecule, we attempted to reactivate the enzyme with 2-octadecyleicosyl-

TABLE 7

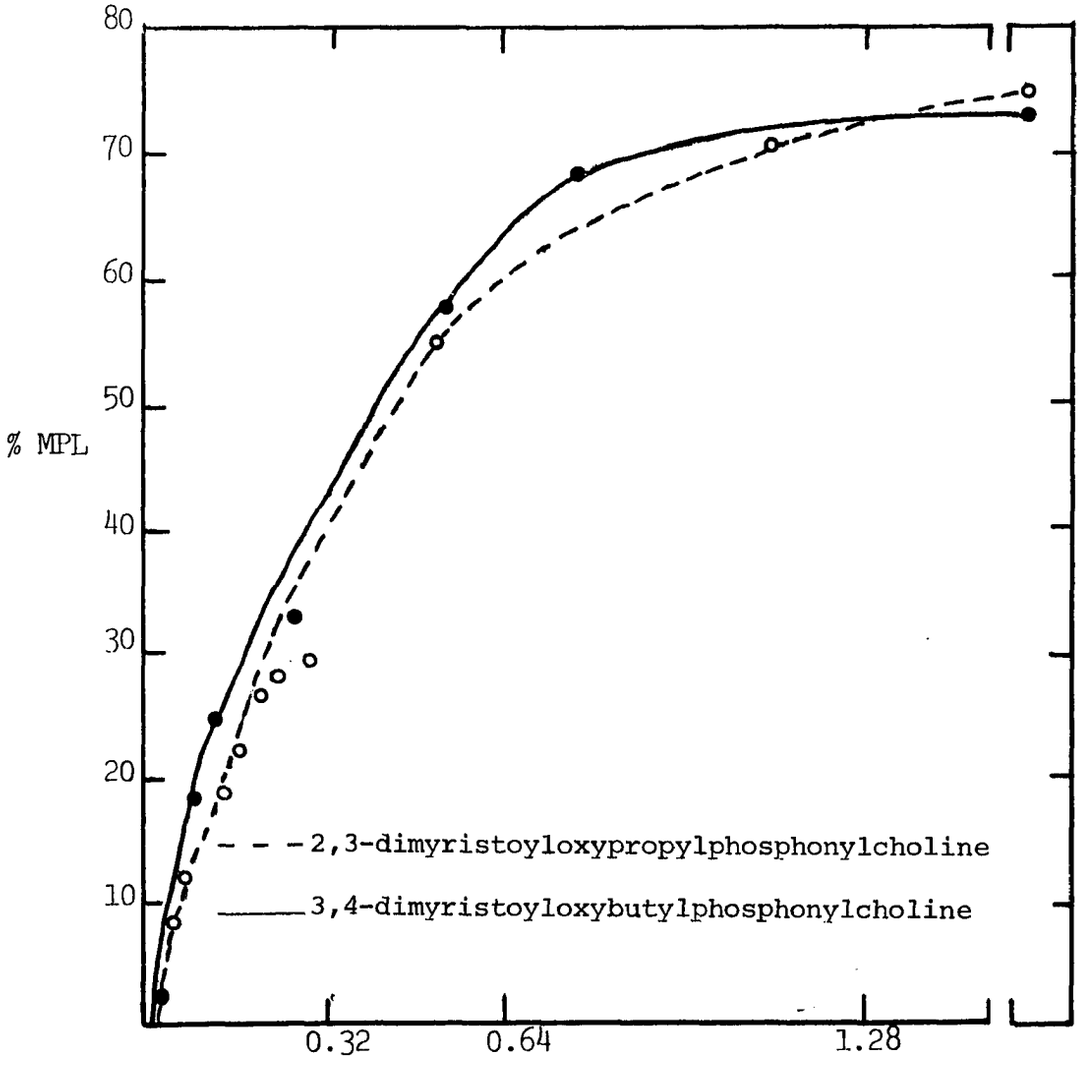
Reactivation of BDH with lecithin analogs having modifications on the glycerol side of the phosphate

Compound	Compound Alone		Compound + PE (1.0 : 0.8)		Compound + PE + DPG (1.0 : 0.8 : 0.4)	
	Extent of reactivation	Lipid/protein mole ratio	Extent of reactivation	Lipid/protein mole ratio	Extent of reactivation	Lipid/protein mole ratio
Distearoyloxypropylphosphonylcholine	0	-	8.3	-	3.0	-
Dioleoyloxypropylphosphonylcholine	29	120	50	12	5*	5*
Dimyristoyloxypropylphosphonylcholine	4.7	-	73	85	42	3.8
Dimyristoyloxybutylphosphonylcholine	4.2	-	75	81	34	3.7
Sphingomyelin	0	-	0	-	0	-
L-Palmitoyllysolecithin	0	-	15	-		
D,L-Dipalmitoyllecithin	-	-	65	88	2.7	4.3
2-Octadecyleicosylphosphorylcholine	26	275	105	93	72	5.2

Extent reactivation and mole ratios were determined as explained in methods. Mole ratios were not determined for activations of less than 10%.

*Done at a phosphorus ratio of 1.0 : 0.8 : 0.8.

FIGURE 4



Titration curves of the reactivation of BDH with 2,3-dimyristoyloxypropylphosphonocholine and 3,4-dimyristoyloxybutylphosphonocholine. Both reactivation studies were run in the presence of PE (PC:PE, 1:0.8). For experimental details, see text.

phosphorylcholine, a compound which lacks the 1,2-diester groups of lecithin and bears straight hydrocarbon chains in these positions instead. Table 7 shows that this compound is an excellent activator, activating to high levels of activity (105%) with a low efficiency of reactivation (5.2).

Sphingomyelin, whether alone or in the presence of PE or PE and DPG, was completely ineffective in reactivating the enzyme (Table 7). Lysolecithin was partially active, activating to the extent of 15% (Table 7).

b. 2-Octadecyleicosylphosphorylcholine analogs with modified head groups

Since 2-octadecyleicosylphosphorylcholine reactivated the enzyme to high levels of activity with high efficiency, it was used to test the effect of head group modifications on the ability of a lecithin to reactivate the enzyme. Table 8 shows the results obtained with a series of head-group analogs. Extension of the distance between the charged centers of the head-group, i.e., between the phosphate and quaternary ammonium groups, by one or two methylene groups had little effect on the ability of the lipid to reactivate the enzyme (propyl compound and butyl compound). Substitution of one N-ethyl group for an N-methyl group also did not affect

TABLE 8

Reactivation of BDH with 2-octadecyleicosylphosphorylcholine analogs

Compound	Compound Alone		Compound + PE (1.0 : 0.8)		Compound + PE + DPG (1.0 : 0.8 : 0.4)	
	Extent of reactivation	Lipid/Protein mole ratio	Extent of reactivation	Lipid/Protein mole ratio	Extent of reactivation	Lipid/Protein mole ratio
Octadecyleisosyl-phosphorylcholine	26	275	105	93	72	5.2
Propyl Compound[a]	12.5	295	101	82	70	4.8
N-Ethyl, N,N-dimethyl Compound[b]	4	-	81	70	45	4.0
Isopropyl Compound[c]	0	-	0	-	0	-
Butyl Compound[d]	27	-	93	78	64	3.9
N,N,N-Triethyl Compound[e]	0	-	3	-	0	-

The extent of reactivation and lipid to protein mole ratios were determined as described in the Experimental Section. Mole ratios were not determined for reactivations less than 10%.

a. Compound XII, p. 29

b. Compound XIV, p. 29.

c. Compound XVI, p. 29.

d. Compound XIII, p. 29.

e. Compound XV, p. 29.

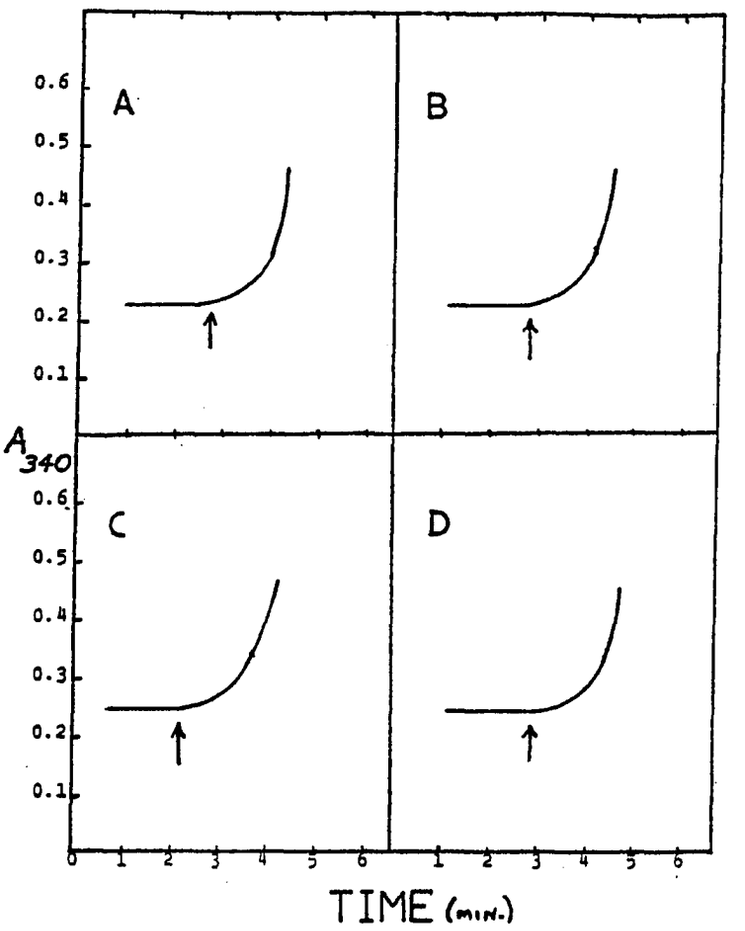
the ability of the phospholipid to form an active complex with BDH (N-ethyl, N,N-dimethyl compound). However, the placing of a methyl group on the methylene group adjacent to the phosphate (isopropyl compound), which changed the compound from an ethanolamine derivative to an isopropylamine derivative, completely blocked the lipid's ability to reactivate the enzyme. The N,N,N-triethylamine derivative (N,N,N-triethyl compound) also was unable to reactivate BDH.

Dipalmitoyl-N,N-dimethylphosphatidylethanolamine was totally unable to reactivate the enzyme.

c. Binding and kinetics of reactivation

Experiments shown in Figure 5 suggest that the enzyme does not bind to those lipids that are non-activators. The addition of MPL (enough MPL to just give maximal stimulation) dispersions to cuvets containing BDH pre-incubated with a non-activating lecithin (curves B,C,D) resulted in a rapid rise in activity which was nearly identical to that obtained when no lipid was present in the pre-incubation mixture. This indicated that in the presence of these lipids the enzyme was available for interaction with MPL and not firmly bound to the inactive lipid. This assumes that the exchange reaction would have affected the rate of lipid binding

FIGURE 5



Interaction of MPL with BDH in the presence of lipids which do not activate with enzyme.

A--No lipid
B--Isopropyl compound + PE (1.0:0.8) (1.2 μg lipid P)
C--Dipalmitoyl-N,N-dimethylphosphatidylethanolamine + PE (1.0:0.8) (1.2 μg lipid P)
D--Sphingomyelin + PE (1.0:0.8) (1.2 μg lipid P)

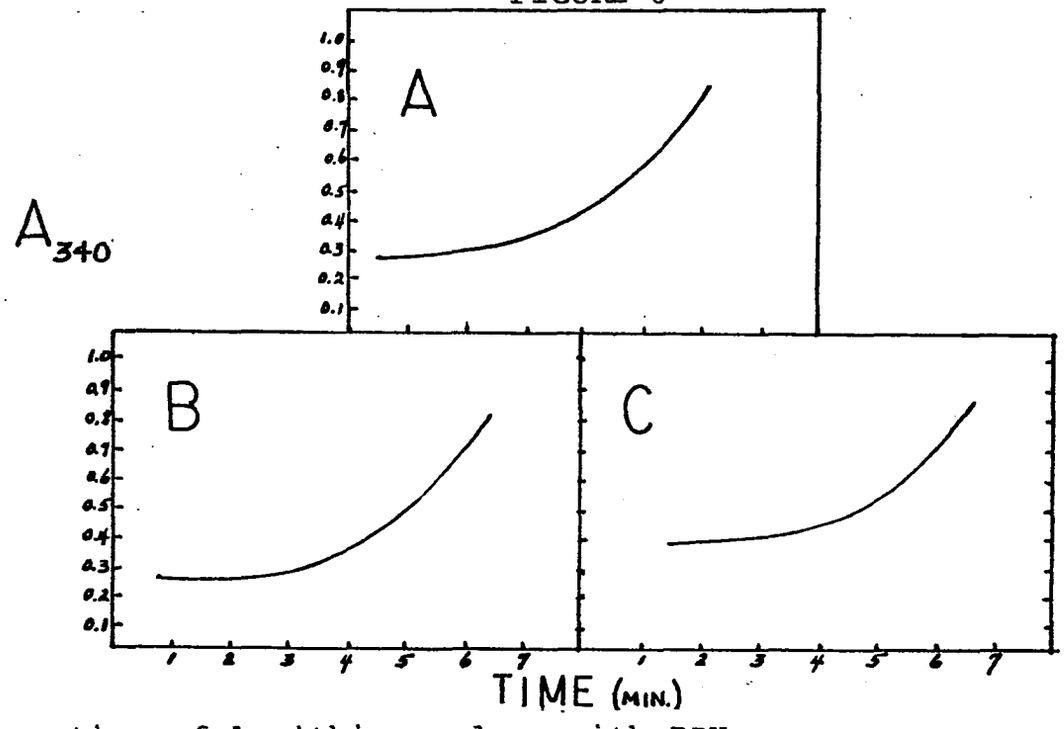
At the time indicated by the arrow, MPL (2 μg lipid P) was added to a complete reaction mixture containing the lipids mentioned above. Monitoring of the absorbance at 340 nm indicates the extent of the reaction. For further details see text.

which is the rate-limiting step.[59]

While one might expect that the rate at which a given lecithin would bind to the enzyme to form a functionally active complex may vary for the different phospholipids, such did not appear to be the case. The addition of the enzyme to an otherwise complete reaction mixture yielded a non-linear A_{340} of increasing slope, the rate of increase reflecting the rate of active complex formation. The rate of increase was similar in the presence of octadecyleicosylphosphorylcholine, propyl compound, and N-ethyl, N,N-dimethyl compounds (Figure 6), indicating that the rate at which they reacted with the enzyme was the same for all three compounds. Studies of this nature showed that the rate of interaction of the enzyme with phospholipid dispersions containing PC, DPG, and PE was considerably faster than that with dispersions prepared with PC and PE alone (Figure 7).

If the ability of DPG to sharply increase the efficiency of a reactivating lecithin results from its imparting a negative charge to the vesicle, then one may expect that other lipophilic anions would have a similar effect. Table 9 shows a comparison of the effects of DPG and dicetyl phosphate on the ability of D,L-dipalmitoyllecithin to activate BDH. While both acidic lipids depressed the activity of the enzyme to similarly low values,

FIGURE 6



Rate of reaction of lecithin analogs with BDH

A--Octadecyleisosylphosphorylcholine + PE (1.0:0.8) (2.1 μg P)

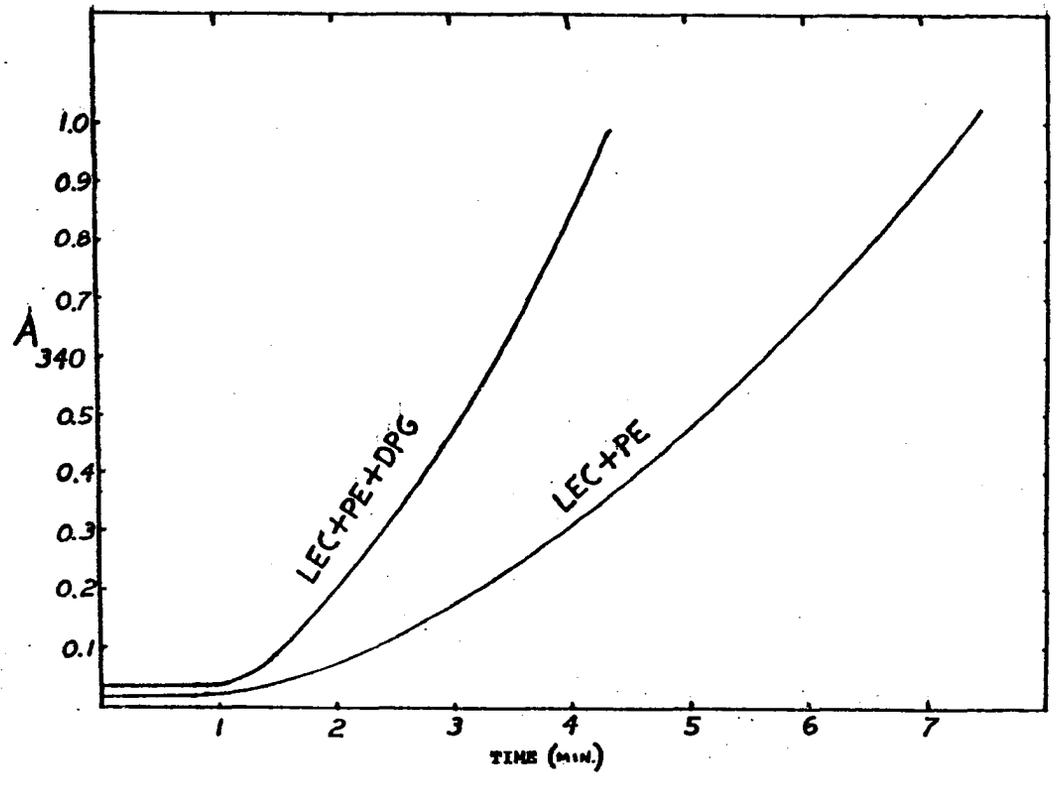
B--Propyl compound + PE (1.0:0.8) (2.1 μg P)

C--N-Ethyl, N,N-dimethyl compound + PE (1.0:0.8) (2.1 μg P)

At the time of the beginning of the tracing the lipids were added to an otherwise complete reaction mixture.

The reaction is measured by monitoring the absorbance at 340 nm.

FIGURE 7



Effect of DPG incorporation on the rate of lecithin BDH interaction. To an otherwise complete reaction mixture containing either 2-octadecyleicosylphosphorylcholine and PE (1.0:0.8) (0.9 μg lipid P) or 2-octadecyleicosylphosphorylcholine + PE + DPG (1.0:0.8:0.4) (0.08 μg lipid P) was added 1.0 μg of BDH at time T = 1 min. The figure represents the Gilford tracing of the reaction.

TABLE 9

Reactivation and efficiency of activation by dipalmitoyllecithin in the presence of acidic phospholipids

Lipids	Extent of Reactivation	Efficiency (mol lecithin per mol BDH)
DL-Dipalmitoyl-lecithin + PE (1.0:0.8)	65	90
DL-Dipalmitoyl-lecithin + PE + DPG (1.0:0.8:0.5)	35	4.5
DL-Dipalmitoyl-lecithin + PE + dicetyl phosphate (1.0:0.8:0.5)	32	17

dicetyl phosphate was less effective than DPG in reducing the number of moles of DL-dipalmitoyllecithin per mole of BDH required for 50% of maximal activation.

Discussion

Over the past several years a growing number of membrane-bound enzyme systems have been shown to require lipid or a membrane structure for activity. These include cytochrome oxidase,[69] the Ca^{2+}, Mg^{2+} ATPase of sarcoplasmic reticulum,[70] UDP galactose : lipopolysaccharide α, 3-galactosyltransferase of Salmonella typhimurium,[71] rat liver glucose-6-phosphatase,[72] succinate-cytochrome c reductase,[73] Na^+, K^+ ATPase,[74] enzyme II of the E. coli phosphotransferase system,[75] C_{55}-isoprenoid alcohol phosphokinase,[76] and the oxidative phosphorylation "coupling device"[77] among others.

The role of lipid in these systems is highly variable. Thus in certain instances, e.g., C_{55}-isoprenoid alcohol phosphokinase, the large molar excess of lipid to enzyme ($>10^3:1$)[76] and the ability of various detergents to effectively reconstitute activity[78] indicated that the lipid is providing a hydrophobic medium in which the enzyme must operate. In other cases, e.g., the glucosyl and galactosyl transferases of Salmonella typhimurium, it has been shown that the requirement for

lipid is attendant upon its interaction with the substrate of the enzymic reaction, in this instance the lipopolysaccharide isolated from mutants of Salmonella typhimurium.[79] In those situations where the enzyme serves a role in the transport of certain substances, e.g., Na^+, K^+ATPase, it is obvious that an enclosed membranous structure is required in order to measure the transport process.[80] Also to be considered is the fact that should the chemiosmotic theory of oxidative phosphorylation hold up, the reconstitution of oxidative phosphorylation would require the membranous separation of two spaces in order that the appropriate proton gradients be generated.

The nature of the lipids which are necessary in order to reactivate varies from system to system. Thus, as already mentioned, the C_{55}-isoprenoid alcohol phosphokinase has a lipid requirement which can be met by a broad range of amphipathic molecules including common phospholipids and detergents.[76,78] Other systems generally show requirements of varying specificity. Often one class of phospholipid will give maximal reactivations while other classes give reduced levels of reactivation. The Na^+, K^+ ATPase has been claimed by some workers to have an absolute requirement for phosphatidylserine.[74] Other workers, however, have not been able to demonstrate

such a requirement.[82] The glycosyltransferases discussed above have been shown to work optimally with phosphatidylethanolamine having unsaturated fatty acid chains.[79] Although the major phospholipid of sarcoplasmic reticulum is phosphatidylcholine, the reconstitution of the Ca^{2+}, Mg^{2+} ATPase and the Ca^{2+} uptake properties of this organelle is said to be optimal with phosphatidylethanolamine.[83] In the above two cases it should be noted that the molar ratio of lipid to enzyme was on the order of 10^3:1.

The situation with BDH differs considerably from those of other membrane bound enzymes in at least two ways. First, this enzyme has a specific requirement for lecithin and no other naturally occurring phospholipid or detergent can substitute to give even reduced reactivation. Secondly, the molar ratio of lipid to enzyme is on the order of four to one.[58]

Various aspects of the phospholipid-BDH interaction have been investigated previously.[84] BDH preparations in the previous studies were never fully freed of residual phospholipid.[85] The studies reported in this thesis were done with a homogeneous protein devoid of lipid ($<$1 mole P/mole enzyme). Thus the results can confidently be interpreted to reflect the actual enzyme-phospholipid interaction.

In concurrence with earlier studies, the results reported in this paper indicate that the mole ratio of lecithins to enzyme needed to yield 50% maximal activity are on the order of four to one. This indicates that (1) the activation is due to lecithin and not due to trace contaminants that may be present and (2) the lecithins act in a cofactor-type role and are not required to form a bulk phase as is found with other enzymes.[76] This implies that the enzyme has a lecithin binding site of at least moderate specificity. Binding of lecithin or a lecithin-like molecule to this site is a necessary prerequisite for the activation of this enzyme.

The studies outlined in this paper show that the structural requirements of a lipid necessary to reactivate BDH are a hydrophobic group (of little specificity) coupled to a phosphoryl quaternary ammonium unit. This conclusion is based on studies with the phospholipids and 2-octadecyleicosylphosphorylcholine. The requirement for a hydrophobic moiety has been shown by Gazzotti et al.[58] who showed that decreases in length of the hydrophobic chains of lecithin gave progressively poorer activation of the enzyme, minimum activity being found with the dihexanoyl lecithin. The finding that lysolecithin, when dispersed with PE, is also partially

effective in reactivating the enzyme is consistent with the above hypothesis. (PE is necessary to minimize the detergent properties of the lysolecithin.)

The fact that the configuration of the lecithin does not influence the extent or the efficiency of the reactivation indicates a further lack of specificity in the hydrophobic portion of the molecule.

In terms of the extents of activation and mole ratios, the results obtained in this study with the dimyristoyllecithin analog are comparable to those obtained in a previous study for the parent compound (Table 10). There is, however, an unexplained discrepancy between the 3-carbon dioleoyllecithinphosphonate and dioleoyllecithin with reference to mole ratios.

The inability of sphingomyelin to activate the enzyme is surprising since sphingomyelin has a hydrophobic side chain as well as a head group identical to that of lecithin. Recent reports indicate, however, that the conformation of the sphingomyelin head group differs from that of phosphatidylcholine.[86] NMR studies of PC-PE mixed vesicles indicate that the predominant orientation of the PC head group must be parallel to the bilayer with the N-methyl protons interacting closely with the phosphates of neighboring PE molecules. The situation with sphingomyelin, on the other hand, was

TABLE 10

Comparison of ractivation and mole ratios of lecithin analogs with parent compounds[58]

Compound	Lecithin		Lecithin + PE (PC:PE, 1.0:0.8)		Lecithin + PE + DPG (PC:PE:DPG,1.0:0.8:0.4)	
	Percent reactivation	mole ratio	Percent reactivation	mole ratio	Percent reactivation	mole ratio
PC 14:0	6	-	50	40	35	3.5
Dimyristoyloxypropyl-phosphonylcholine	4.7	-	73	85	42	3.8
Dimyristoyloxybutyl-phosphonylcholine	4.2	-	75	81	34	3.7
PC 18:1 cis $\Delta 9$	70	8.0	72	5.1	-	-
Dioleoyloxypropyl-phosphonylcholine	29	120	50	12		

found to be somewhat different. Here the N-methyl protons were not interacting closely with the PE phosphate group, indicating that despite their structural similarity the head group conformation of sphingomyelin and PC differ. Thus, the activation process may be sensitive to the orientation of the head group relative to the phospholipid fatty acyl chains.

While the requirements for activation on the glycerol side of the phosphate can be met by a hydrophobic group, the requirements for activation on the choline side of the phosphate are more stringent. A methyl group substituted for one of the two hydrogen atoms on the carbon nearest the phosphorus abolishes activity. This is probably a steric effect. The ability to reactivate is also dependent on the structure of the quaternary ammonium group. The removal of one methyl group destroys the lipid's ability to reactivate the enzyme. While the N,N-dimethylphosphatidylethanolamine is zwitterionic at the pH at which the reaction is run (7.4), it should be realized that the lecithin cannot lose its positive charge while the N,N-dimethyl compound can. While the addition of one N-ethyl group in place of an N-methyl group has little effect on the extent of interaction, the substitution of a N,N,N-triethyl group results in a severe inhibition of activity. This

could arise from a steric effect where the N,N,N-triethyl group cannot be accommodated in the binding site or an electronic effect where the triethyl group effectively shields the positive charge. The above results demonstrate the importance of the trimethylammonium group in the activation of the enzyme.

It was thus surprising to find that when the distance between the phosphate and the positive center is varied by the linear addition of one or two methylene groups, the ability of the enzyme to activate was not diminished. This may reflect a conformational mobility in the lipid which allows its quaternary ammonium moiety to assume an approximately equivalent position on the enzyme as does the ammonium group of the choline compound.

If the quaternary ammonium group must assume a conformation that allows it to appear as an ethanolamine derivative to the enzyme, one might expect that the rate at which the different lecithins reactivate the enzyme would differ, i.e., before reactivation could occur, the lecithin would have to assume an energetically unfavorable conformation and reactivation could not occur immediately. This, however, may not be the case. All lecithins react with equal rates, indicating that if a change in conformation does have to occur, it occurs sufficiently rapidly not to affect the overall rate of

interaction between lipid and enzyme. The enzyme does not bind to sphingomyelin-PE mixtures, N,N-dimethyphosphatidylethanolamine, PE mixtures or isopropyl compound-PE mixtures, as judged by the increase in A_{340} upon addition of MPL dispersion. Although one cannot rule out the possibility that the rate-limiting step is not the exchange of one lipid for the other, this is in contrast to that which is found with mixtures of DPG-PE, PS-PE and PI-PE, to which the enzyme binds, forming enzymatically inactive complexes which can be disrupted by high salt concentrations, whereas the enzyme-PC interaction is not disrupted by a high ionic strength.[62] The above facts indicate that the enzyme binds to negatively charged phospholipid vesicles in a manner where electrostatic forces predominate.

The finding that the incorporation of DPG into a lipid mixture significantly increases the rate of interaction between lipid and protein can have a number of explanations. One explanation would be that the binding of the enzyme by the DPG-containing vesicle increases the concentration of enzyme near the surface of the bilayer, thus increasing the rate of interaction by a simple mass action principle. Alternatively, the binding of the enzyme with the DPG-containing vesicle may align the enzyme in the proper spatial relationship

relative to the vesicle surface to allow the proper interaction to take place. Also to be considered is the possibility that incorporation of the acidic phospholipid into the bilayer, which imparts a negative charge to the bilayer, acts to inhibit low levels of aggregation by electrostatic repulsion. This has the effect of increasing the available surface area of the vesicle and increases the rate of the reaction. Also to be noted is that this explanation may partially explain the increased efficiency of reactivation shown by DPG-containing vesicles. Yet it is clear that the negative charge imparted to the bilayer is not sufficient to explain all the facts, such as the difference in the efficiency of a given phospholipid in the presence of equivalent amounts of dicetylphosphate and DPG. Thus other factors must be invoked to explain the effect of DPG. These might include the spatial arrangement of the DPG negative charges and the unsaturated fatty acyl side chain of the DPG versus the saturated fatty acyl side chain of dicetyl phosphate.

CHAPTER III

THE INTERACTION OF 2-OCTADECYLEICOSYLPHOSPHORYLCHOLINE ANALOGS WITH CHOLESTEROL

Introduction

Cholesterol is a major component of many biological membranes and obviously fulfills an important function in them. This function is as yet not clearly understood. The sterol appears to interact with phospholipids in the liquid-crystalline state, as judged by a number of physical techniques. These include x-ray[87] and monolayer studies,[88] which demonstrate a reduction in the mean molecular area occupied by a phospholipid upon interaction with cholesterol, and esr[89] and nmr[90] studies which show a reduction in the mobility of the fatty acid chains due to increased hydrophobic interactions. These effects influence the simple diffusion of solutes through the lipid bilayer. Cholesterol suppresses the permeability of phospholipid membranes for water,[91,30] cations, glycerol, and glucose,[92] and

the penetrability of monolayers by proteins.[93]

With phospholipids in the crystalline state, cholesterol reduces the chain interactions. This so-called liquefying effect can be demonstrated using differential scanning calorimetry[94] and nmr[90] with model membrane systems below their lipid phase transition temperatures. Growth experiments with Acholeplasma laidlawii show that varying the cholesterol content of the membranes by manipulation of cell growth conditions causes a change in the fatty acid composition of the cell membrane.[95] This indicates that the liquefying effect can be very essential for proper membrane function.

The structural requirements of the sterol necessary for the sterol to interact with the phospholipid have been extensively studied. The conclusions drawn from these studies are that a planar ring system, an intact side chain at C_{17} and a 3β-hydroxyl group are essential for both the liquefying and condensation effect of the sterol.[96] While it is clear that the phospholipid-sterol interaction is highly hydrophobic in nature, the requirement for a 3β-hydroxyl group indicates that polar region interactions may also be important. A number of formulations have been put forward to explain the mode by which the 3β-hydroxyl group influences the interaction between lipid and sterol. Based on infrared studies of

hydrated phospholipid bilayers, it was concluded that the 3β-hydroxyl group interacts with the phosphate moiety of a phospholipid.[97] This conclusion was supported by water permeability measurements which show (a) that only isosteric butylphosphonates show an increased permeability to water upon interaction with cholesterol, the propylphosphonates showing a sharply reduced response, and, (b) diether phosphonates can interact with cholesterol even though they lack carbonyl groups.[30] The above conclusion has been claimed to be irrelevant by Brockerhoff.[98] Using both model and thermodynamic arguments, he came to the conclusion that the β-carbonyl group of lecithins is bound to the 3β-hydroxyl group, and that cholesterol and proteins compete for bonding to the ester carbonyl oxygen atom of phospholipids. The nmr work of Yeagle et al.[98a] supports hydration of phospholipid C=0 groups both in the absence and presence of cholesterol. In a recent publication, Huang proposed a model having the β-carbonyl oxygen atom hydrogen bonded to the cholesterol hydroxyl group; in this model only a β-hydroxyl group can hydrogen bond to the phospholipid, a 3α-hydroxyl group being incapable of forming a strong hydrogen bond.[99,100]

2-Octadecyleicosylphosphorylcholine and the head group analogs whose syntheses were described earlier (pp. 28-30) lack not only the carbonyl oxygen, but also

the acyl oxygen atoms. Thus they represent a series of compounds with desirable properties to study the interaction of sterol with lipid. Glucose permeability was studied in order to try to establish the requirement for the carbonyl or carboxyl oxygen atoms in the phospholipid-sterol interaction as well as the effect of head group modifications on such an interaction.

Materials and Methods

^{14}C-Labelled glucose was purchased from New England Nuclear (specific activity 48.2 Ci/mole). Cholesterol and visking dialysis tubing were purchased from Sigma Chemical Company. Epicholesterol was purchased from Schwarz-Mann BioResearch. The sterols were recrystallized from acetone and the purity was checked by thin-layer chromatography on silica gel plates using solvent systems consisting (by volumes) of benzene-methanol (92:8) and chloroform-acetone (98:3.5). The melting points of the sterols were cholesterol, 147.5-148°C, and epicholesterol, 141.5°C.

Prior to use the dialysis tubing was boiled in distilled water three times, followed by boiling in 0.075 M KCl - 0.075 M NaCl twice.

Preparation of Liposomes Containing Trapped Radioactive Glucose

Negative-staining electron microscopy (kindly provided by Dr. O. R. Anderson) revealed that multilayered liposomes are formed when 2-octadecyleicosylphosphoryl-choline is dispersed in water.

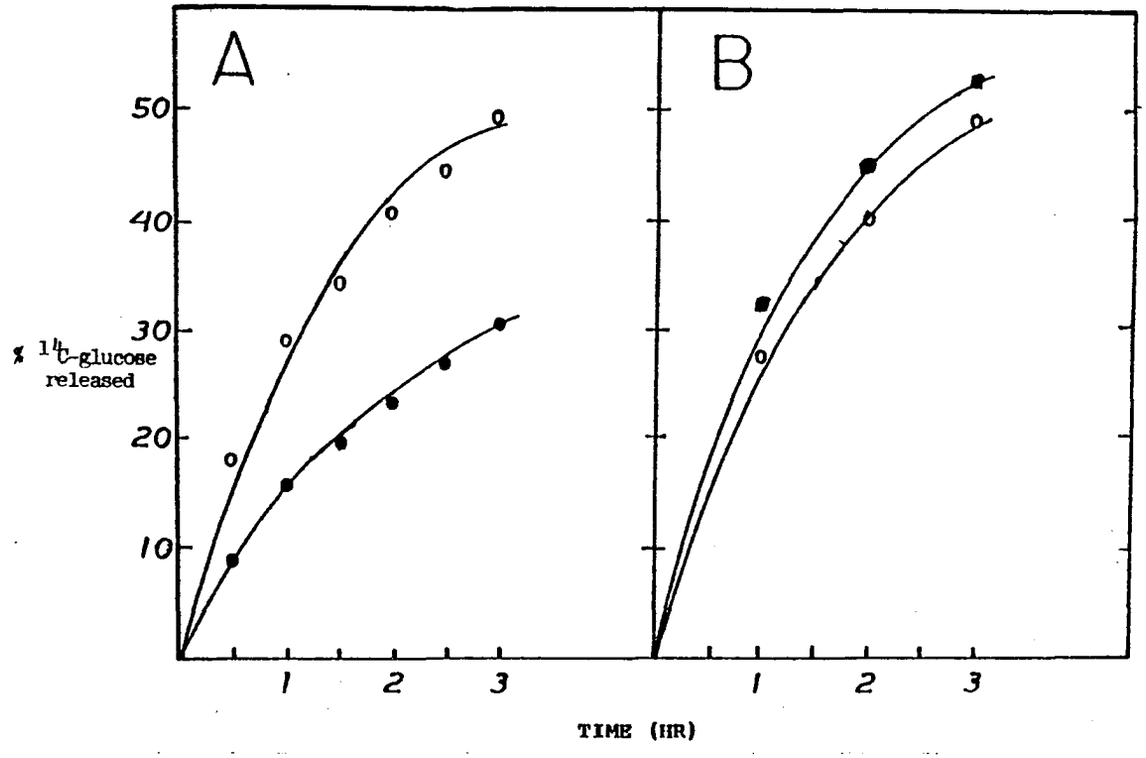
Approximately 8 mg of lecithin analog in a chloroform solution was placed in a small conical centrifuge tube and appropriate quantities of other lipids (dicetyl phosphate [DCP 10 mol %] or sterols) were added to obtain the desired mole ratio. The solvent was removed by blowing nitrogen over the solution followed by vacuum dessication for at least 15 minutes. To the dried lipid film was added 0.5 ml of 0.075 M KCl - 0.075 M NaCl and a trace of radioactive glucose (2×10^7 cpm). The conical tube was immersed in a hot water bath ($> 60°C$) for a few seconds and the suspension was then agitated with a glass bead by placing it on a Vortex mixer for ten seconds. The heating-agitation cycle was repeated three times. The lipid suspension was then transferred to a dialysis bag and dialyzed at room temperature extensively (5 hours) against large volumes (1 L) of 0.75 M KCl - 0.075 M NaCl with frequent changes of dialysate in order to remove untrapped glucose. After this time, the dialysis bags were transferred to small test tubes containing 3 ml of 0.075 M NaCl - 0.085 M KCl

placed in a water bath at 55°C, and manually shaken. At half-hour intervals, 100 μl aliquots were taken from the test tube and counted in a liquid scintillation counter. After 3 hours, aliquots were taken from the dialysis bag in order to measure the amount of trapped glucose.

Results

Preliminary trials indicated that, at temperatures less than 40°C, dialyses for periods as long as 48 hours resulted in a negligible release of labeled glucose from liposomes prepared from 2-octadecyleicosylphosphorylcholine. However, at 55°C, glucose was released at a rate which readily allowed its measurements. This fact allowed the removal of untrapped glucose from the phospholipid suspension by extensive dialysis at room temperature without appreciable loss of internal glucose. Figure 8A shows the time course of the release of glucose from liposomes prepared with octadecyleicosylphosphorylcholine and 10 mole percent DCP both in the presence and absence of 33 mole percent cholesterol. Cholesterol caused a striking decrease in the amount and rate of release of trapped glucose. The incorporation of epicholesterol into the phospholipid dispersion, Figure 8B, had no such effect, indicating that the decrease in the rate of glucose permeability requires the presence of the 3β-hydroxyl group of cholesterol.

FIGURE 8



Effect of sterols on glucose permeability of liposomes. Liposomes composed of 2-octadecyleicosylphosphorylcholine (8.0 mg) containing trapped ^{14}C-glucose were prepared either in the presence or absence of sterol (33 mol%) as described in methods. The efflux of radioactive glucose was then measured as described in the text.

A. o-o in the absence of cholesterol
   ●-● in the presence of 33 mol% cholesterol

B. o-o in the absence of epicholesterol
   ●-● in the presence of 33 mol% epicholesterol

Varying the head group of the phospholipid does not qualitatively influence the interaction between phospholipid and sterol. This is shown in Figure 9 where glucose release experiments were done with 2-octadecyleicosylphosphoryl-N,N,N-trimethyl-3-aminopropanol, 2-octadecyleicosylphosphoryl-1-methylcholine, and 2-octadecyleicosylphosphoryl-N-ethyl-N,N-dimethylethanolamine, respectively. Also run was dihexadecyloxydietherlecithin which interacted with cholesterol.

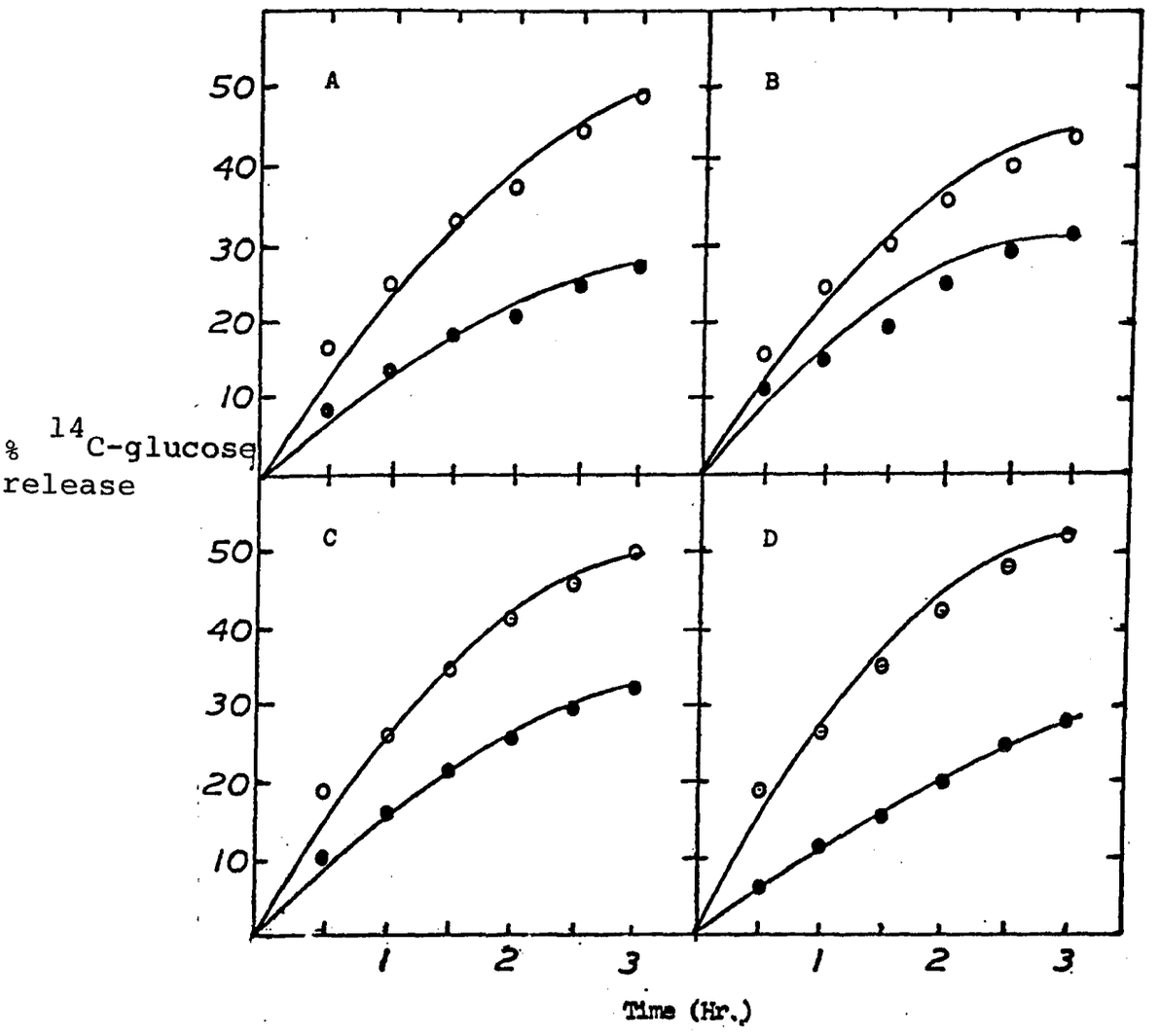
Discussion

It is clear that cholesterol does not require a carbonyl oxygen in order to interact with lecithin. The study reported above is in agreement with nmr and differential scanning calorimetry studies with octadecyleicosylphosphorylcholine which confirm that cholesterol does interact with this phospholipid in a manner analogous to its interaction with natural and artificial lecithins.[101]

It may be of importance to note that the permeability studies were done at temperatures higher than the transition temperature of the lecithin (46°C). Thus the objection about the relevance of studies below the transition temperature is negated.

The octadecyleicosylphosphorylcholines which lack the two ester groups present a relatively high hydrophobic barrier to the hydrophilic permeant glucose.

FIGURE 9



Release of glucose from liposomes prepared from lecithin analogs. Liposomes containing ^{14}C-glucose were prepared from octadecyleicosylphosphorylcholine (A), isopropyl compound (B), N,ethyl, N,N-dimethyl compound (C), and dihexadecyldietherlecithinphosphate (D) in the absence of added sterol (o-o) and in the presence of added sterol (●-●) (33 mol%). The release of ^{14}C-glucose from the liposomes was measured as described in the text.

$^{45}Ca^{2+}$ efflux from liposomes formed from 2-octadecyl-eicosylphosphorylcholine was negligible even at 60°C.

Changes in the head group did not appreciably change the rate of glucose permeability or the extent of interaction betwee lipid and cholesterol.

The requirement of a β-hydroxyl group for the cholesterol-lecithin interaction indicates that an electrostatic interaction between this hydroxyl group and a portion of the lecithin molecule is important in stabilizing the interaction. With the elimination of the ester bonds as possible sites for interaction, one is left with the phosphate group of lecithin as being the portion of the molecule most likely to participate in this bonding. However, this conclusion has also been questioned as recent work has shown that the ^{31}P nmr is unaffected by cholesterol.[102] Thus the dynamics of the phospholipid-cholesterol interaction remain unsolved.

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