ANALOGUES OF CHOLINE

AND RELATED COMPOUNDS

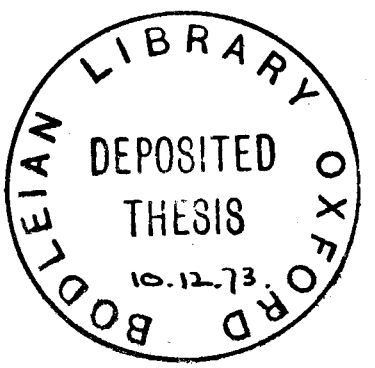
A thesis submitted in part

requirement for the Degree of

Doctor of Philosophy

by

R.G.Edwards, B.Sc., M.A.



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Department of Biochemistry

Oxford

Oriel College

Oxford

IN MEMORIAM

IDRIS ROWLANDS PAULL-JAMES

28.VIII.1896 - 13.IV.1970

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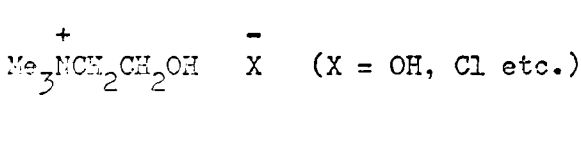
ABBREVIATIONS

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
CDP	cytidine-5'-diphosphate
CMP	cytidine-5'-monophosphate
CTP	cytidine-5'-triphosphate
cpm	counts per minute
DCC	dicyclohexylcarbodiimide
GLC	gas-liquid chromatography
IR	infrared
MS	mass spectrum
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
ppm	parts per million
PMR	proton magnetic resonance
^{31}P-NMR	phosphorus-31 nuclear magnetic resonance
rpm	revolutions per minute
TLC	thin-layer chromatography

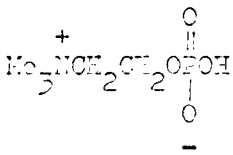
INTRODUCTION

THE BIOLOGICAL ROLE OF CHOLINE

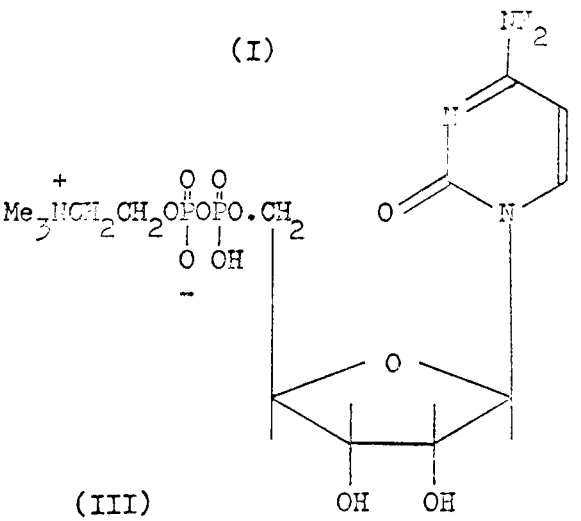
Choline (I) is an accessory dietary factor required by all forms of animal life, and is sometimes classed as a vitamin,[1] although the term vitagen has been proposed[2] for such an intermediary. Its metabolic role is at least threefold; firstly, it is a precursor of phosphorylcholine (II), cytidine-5'-diphosphate choline (CDP-choline) (III) and thence such phospholipids as phosphatidylcholine (lecithin) (IV), sphingomyelin (V) and choline plasmalogen (VI). Secondly, it is a precursor of betaine (VII), a donor of methyl groups, and thirdly, it is a precursor of acetylcholine (VIII), the neurotransmitter of the autonomic nervous system.



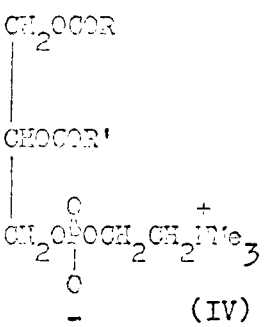
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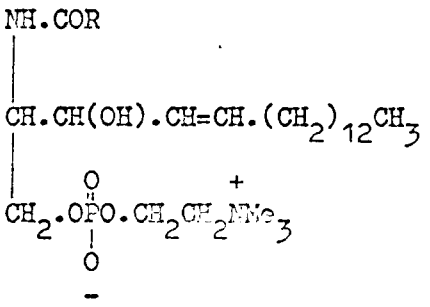
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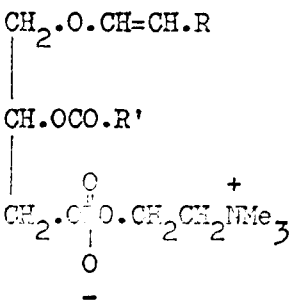
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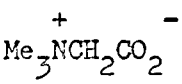
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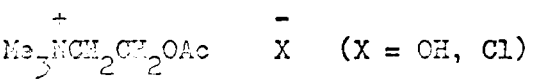
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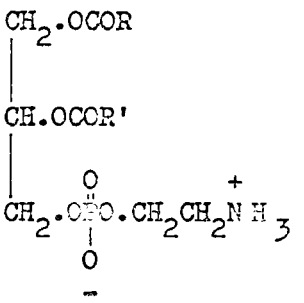
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BIOSYNTHESIS OF CHOLINE PHOSPHOLIPIDS

(i) Cytidine pathway.

Phosphatidylcholine or lecithin (IV) is the predominant phospholipid in most animal cells, and the details of its biosynthesis have been more thoroughly investigated than those of any other common lipid. It can be synthesized by two major routes, namely by the utilization of exogenous choline from dietary sources or salvage pathways, or by sequential

methylation of phosphatidylethanolamine (IX). The initial stage in the utilization of free choline is its phosphorylation to phosphorylcholine (II), involving the transfer of a phosphate group from ATP to choline by the



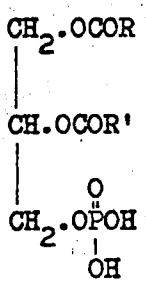
(IX)

enzyme choline phosphokinase; the latter was first isolated from yeast by Wittenberg and Kornberg[3] in 1953, and requires magnesium ions and sulphydryl groups for activation.

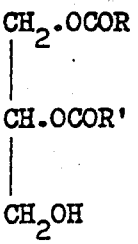
ATP + CHOLINE ⟶ PHOSPHORYLCHOLINE + ADP

Kennedy and Weiss[4] showed that there was no reaction between phosphorylcholine (II) and phosphatidic acid (X), and found that the necessary intermediate was CDP-choline (III), formed from CTP and phosphorylcholine in the presence of the enzyme phosphorylcholine-cytidyltransferase.

CTP + PHOSPHORYLCHOLINE ⟶ CDP-CHOLINE + PP_i



(X)



(XI)

Crude preparations of this enzyme, extracted from rat liver, were investigated by Borkenhagen and Kennedy[5] and by Schneider *et al.*,[6-10] and it was later purified and separated from phosphorylethanolamine-cytidyltransferase by Chojnacki *et al.*[11-12] Moreover, it was shown[5] that this enzyme could readily bring about the reverse reaction.

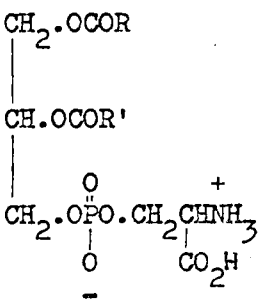
Kennedy and Weiss[4] also isolated from rat liver a second enzyme, phosphorylcholine-diglyceridetransferase, which brings about the reaction between CDP-choline (III) and 1,2-L-diglyceride (XI) to form lecithin (IV):

CDP-CHOLINE + 1,2-L-DIGLYCERIDE $\rightleftharpoons$ LECITHIN + CMP

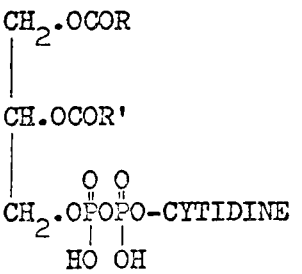
Further investigations were carried out by Weiss *et al.*[13] and Schneider *et al.*,[6-10] and it has been shown that this reaction is also freely reversible, both *in vitro*[13] and *in vivo*.[14] Very recently, Kanoh and Ohno,[15] in a study of the different molecular species of lecithin, found that the reverse reaction would degrade not only lecithins synthesized by this pathway, but also those produced by the methylation of phosphatidyl-

ethanolamine (IX). The enzymes involved in the biosynthesis of lecithin by this route are not confined to the liver, but have been found in the brain, seminal vesicle, intestinal mucosa and skeletal muscle.

The biogenesis of phosphatidylethanolamine (IX) follows a very similar pathway, although the final step is thought to be much less freely reversible than the corresponding one for lecithin.[14-15] The biogenesis of phosphatidylserine (XII) is less well understood, although it is thought to involve the reaction of L-serine and CDP-diglyceride (XIII).[16]

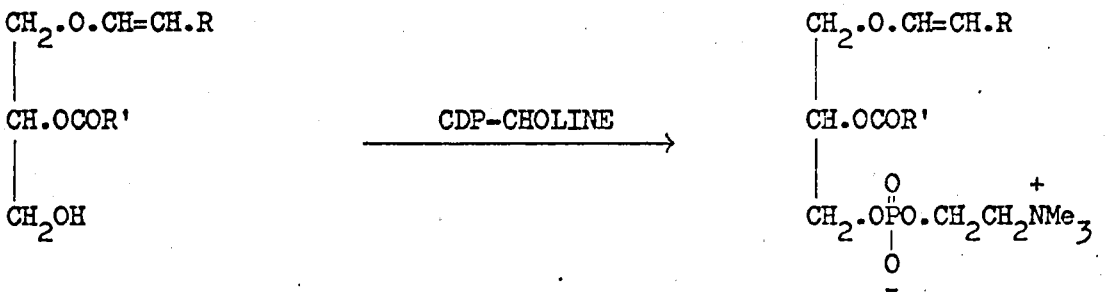


(XII)



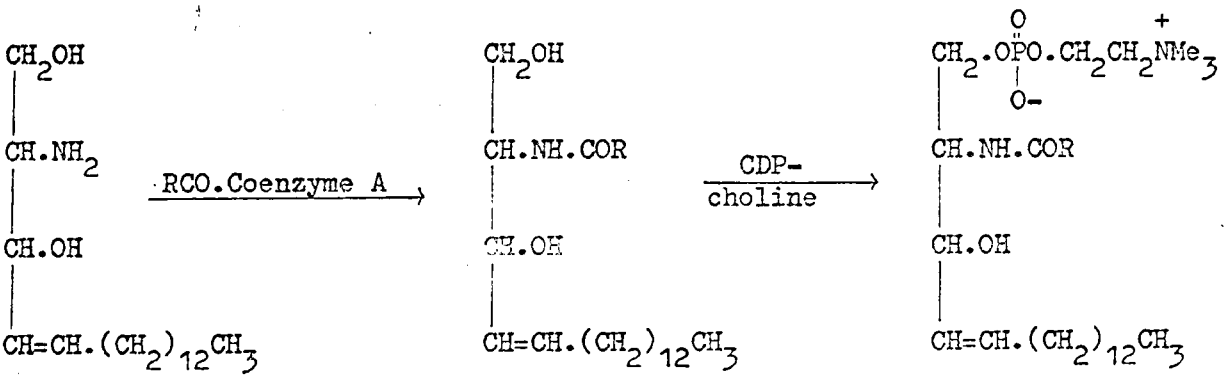
(XIII)

It has been assumed that the biosynthesis of plasmalogens and similar phospholipids resembles that of the diacyl analogues, and this view is supported by the finding of Kiyasu and Kennedy[17] that 1-alkenyl-2-acylglycerol (XIV) reacts with CDP-choline (III) to form choline plasmalogen (VI).



(XIV) (VI)

Sphingomyelin (V) has been shown, by Sribney and Kennedy,[18] to be formed from CDP-choline (III) and ceramide (XV) in the presence of the widely-distributed enzyme CDP-choline : ceramide cholinephosphotransferase. The ceramide is synthesized from sphingosine (XVI); ceramides formed from dihydrosphingosine will not serve as substrates.



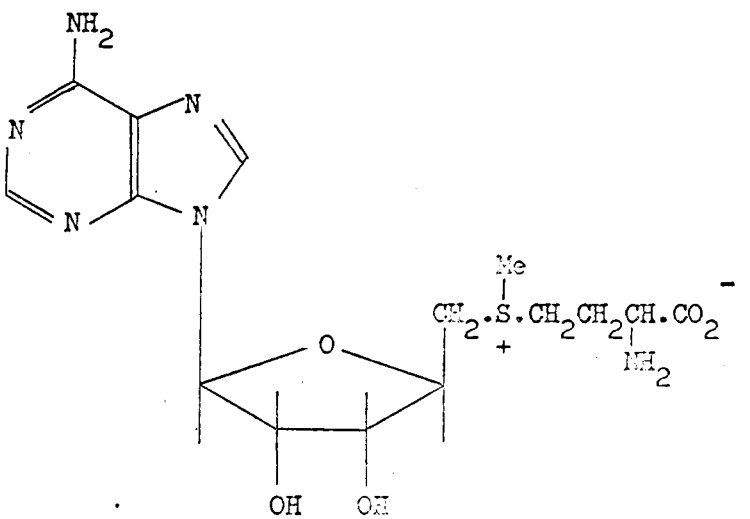
(XVI) (XV) (V)

An alternative pathway has been observed in young rat brain,[19] in which sphingosylphosphorylcholine couples with acyl-Coenzyme A. Recently,

Diringer et al.[20] have observed the synthesis of sphingomyelin in transformed mouse fibroblasts by the immediate transfer of phosphorylcholine to sphingosine or N-acylsphingosine.

(ii) Sequential methylation.

The synthesis of lecithin may also occur by successive transfer of three methyl groups from S-adenosylmethionine (XVI)[21] to phosphatidyl-ethanolamine (IX) which can in turn be synthesized either from CDP-ethanolamine or by decarboxylation of phosphatidylserine (XII).



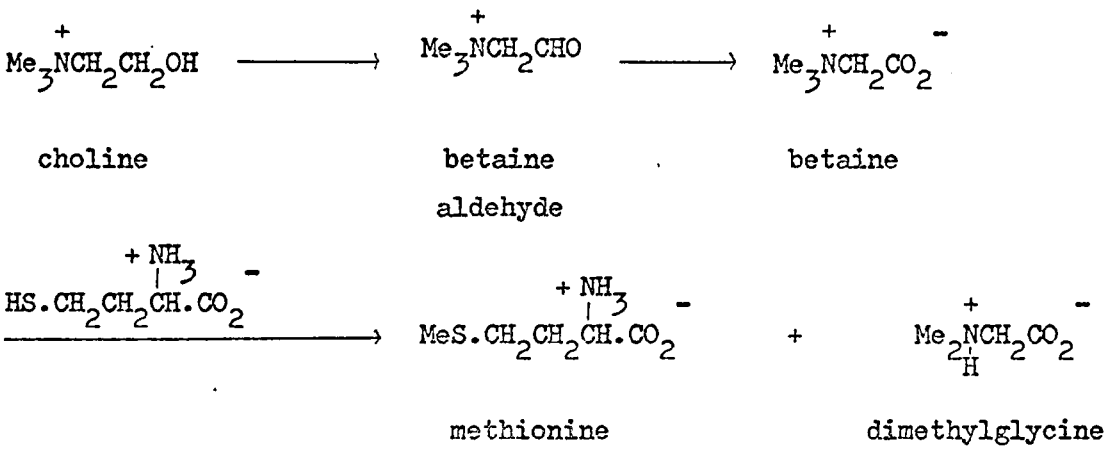
(XVI)

S-adenosylmethionine (XVI) is formed from methionine and ATP, in a unique

reaction producing both inorganic phosphate and pyrophosphate:

$$\text{METHIONINE} + \text{ATP} \longrightarrow \text{S-ADENOSYLMETHIONINE} + PP_i + P_i$$

The methionine may be dietary in origin, or formed from homocysteine by the transfer of a methyl group from betaine, an oxidation product of choline:



This phenomenon of transmethylation was first postulated by du Vigneaud *et al.*[22-3] in order to explain the lipotropic action of methionine and betaine in choline-deficient rats, and the concept was elaborated by Bremer, Greenberg and Figard,[24-7] and by Wilson *et al.*,[28] to include the decarboxylation of phosphatidylserine and the subsequent step-wise methylation of phosphatidylethanolamine. Such a mechanism had been suggested by the isolation of N-methyl- and N,N-dimethylethanolamine phosphatides from rat liver; labelling experiments proved that the methyl acceptor was the fully-formed phosphatide and not free ethanolamine,

phosphorylethanolamine or CDP-ethanolamine.[25] The enzymes required for this process appear to be present in the livers of all mammals, as well as the testes, heart, kidneys and other tissues;[27] it is thought that one enzyme catalyzes the first methylation and a second carries out the final two.[29] Bjørnstad and Bremer[14] postulated that lecithin biosynthesis by the methylation pathway was of significance only in the liver, and occurred in female rats to a greater extent than males.

Recently, Sinclair[30] has shown that not only labelled choline, but also ethanolamine and methyl-labelled methionine, are incorporated into the sphingomyelin of Ehrlich ascites tumour cells; preliminary work by Balint, Treble et al.[31-2] had shown that methyl-labelled methionine was incorporated into liver sphingomyelin. With other evidence, he has proposed the following sequence:

Ethanolamine ——————→ phosphatidylethanolamine (direct synthesis)

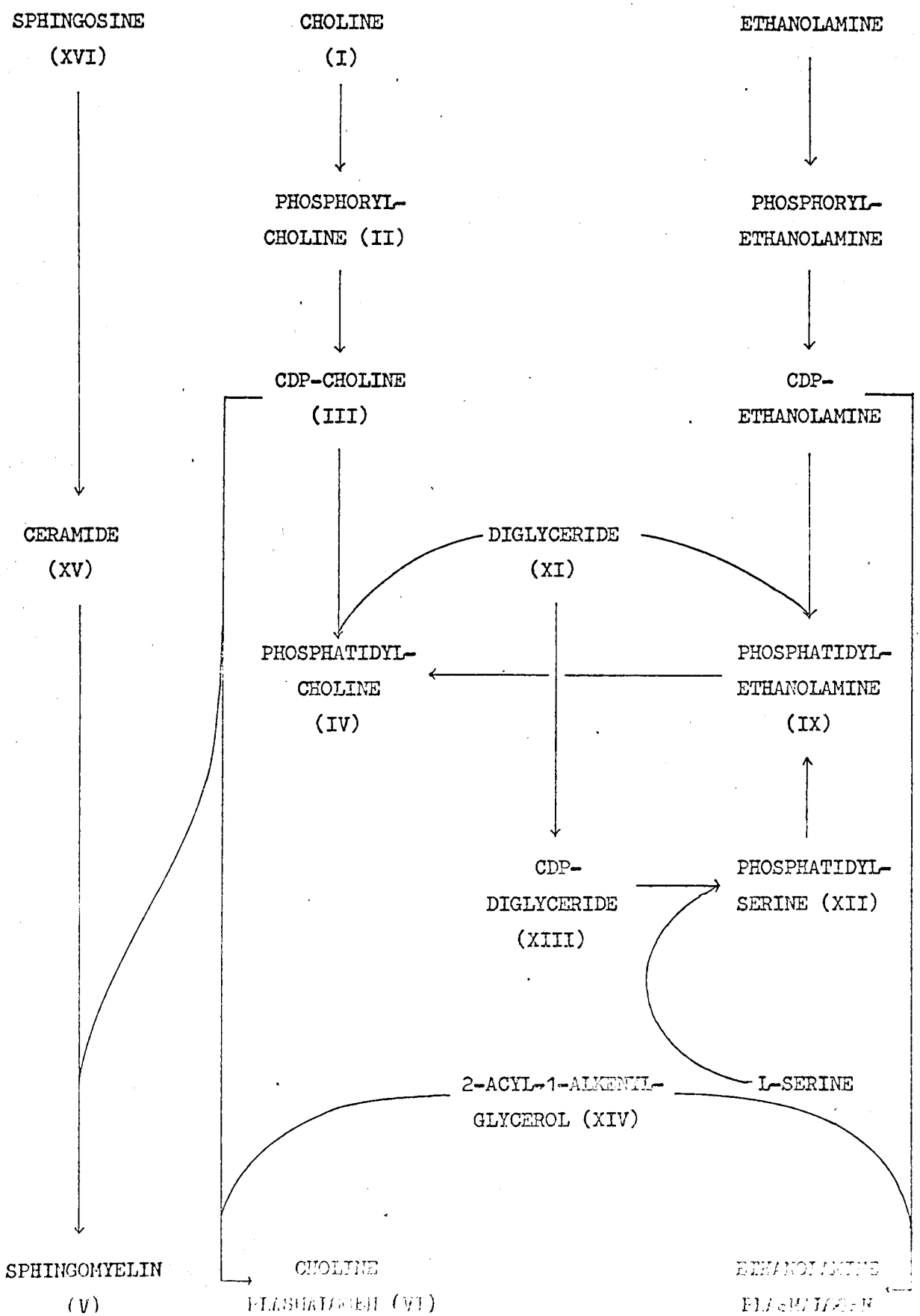
Phosphatidylethanolamine ——→ phosphatidylcholine (methylation pathway)

Phosphatidylcholine ————→ CDP-choline (reversal of synthesis)

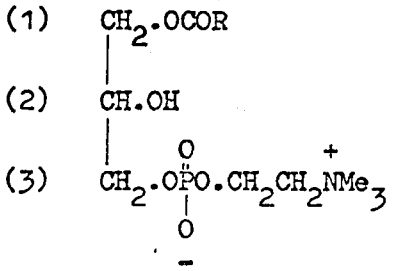
CDP-choline ——————→ sphingomyelin (direct synthesis)

(iii) Other routes.

Lands and Merkl[33] have shown that lecithin can be formed by acylation


SPHINGOSINE
(XVI)
CHOLINE
(I)
ETHANOLAMINE
PHOSPHORYL-
CHOLINE (II)
PHOSPHORYL-
ETHANOLAMINE
CDP-CHOLINE
(III)
CDP-
ETHANOLAMINE
CERAMIDE
(XV)
DIGLYCERIDE
(XI)
PHOSPHATIDYL-
CHOLINE
(IV)
PHOSPHATIDYL-
ETHANOLAMINE
(IX)
CDP-
DIGLYCERIDE
(XIII)
PHOSPHATIDYL-
SERINE (XII)
2-ACYL-1-ALKENYL-
GLYCEROL (XIV)
L-SERINE
SPHINGOMYELIN
(V)
CHOLINE
PLASMALOGEN (VI)
ETHANOLAMINE
PLASMALOGEN

of lysolecithin (XVII) by means of acyl-Coenzyme A. Since lysolecithin



(XVII)

may be formed by hydrolysis of lecithin, this pathway does not represent a de novo synthesis; however, the mechanism may be of considerable importance for the formation of a species of lecithin with specific fatty acids at positions 1 and 2. As either of the fatty acids of lecithin can be removed by phospholipases, to provide either 1- or 2-acylglycerophosphoryl-choline, the appropriate specific acyl-Coenzyme A : acylglycerophosphoryl-choline acyltransferase described by Lands and Hart[34] can then acylate this degradation product to form a new lecithin, thus accounting for asymmetric distribution of saturated and unsaturated fatty acids.[35]

Ehrbland and Marinetti[36] claim that the following reaction takes place in the supernatant of a rat liver homogenate:

2 LYSOLECITHIN ⟶ LECITHIN + GLYCEROPHOSPHORYLCHOLINE

Stoichiometry may be lost through acylation of some lysolecithin during the incubation. Ferber,[35] in a very recent review, cosidered this pathway to be of no biological significance.

In addition, it has been reported[37-8] that interconversion of phospholipids can occur by calcium-stimulated direct base exchange; thus free serine, choline and ethanolamine can exchange with the existing base of a phospholipid. In 1970 Treble et al.[32] proposed that the rate of choline entry into lecithin by such base exchange is several times higher, in vivo, than via the cytidine pathway; Porcellati and di Jeso[39] reported that this mechanism was probably the only way by which serine was incorporated into chick brain phospholipids. In 1971 Plagemann[40] decided that direct exchange did not play a significant role in the incorporation of choline into lecithin in vitro in Novikoff hepatoma cells, and in the following year Kanfer[41] studied calcium and pH dependence of the reaction in rat brain. Sundler et al.[42] found that the rate of lecithin synthesis by base exchange in rat liver, in vivo, was only 5 per cent of that by the cytidine pathway; a high proportion of the base-exchanged lecithin contained arachidonic acid, whereas lecithin formed by the cytidine pathway contained more oleic and linoleic acid. In 1973 Trewhella and Collins[43] suggested that, in rat liver, 1-stearoyl-2-arachidonyl lecithin is formed largely by the methylation pathway and the 1-palmitoyl-2-linoleoyl species by the cytidine pathway, while base exchange was of relatively minor importance.

Bjerve[44] measured conditions of optimal pH and calcium concentration *in vitro*, and also the mutual inhibition between choline, ethanolamine and L-serine. He found that both choline and L-serine were competitively inhibited by the other bases, whereas ethanolamine was not. He concluded that a single common phosphatidyl-enzyme complex was not involved; moreover, calcium-stimulated incorporation of phospholipid bases *in vitro* is of major importance in the biosynthesis of phosphatidylserine, but is negligible in the cases of phosphatidylethanolamine and lecithin. Very recently he has also found[44a] that while phosphatidylserine and lecithin are substrates for exchange with all three bases, phosphatidylethanolamine cannot exchange with choline, and he has postulated the existence of two pools of phosphatide in rat liver microsomes, a very small pool that is highly active in base exchange, and a much greater and more stable pool which is inactive.

LECITHIN CATABOLISM

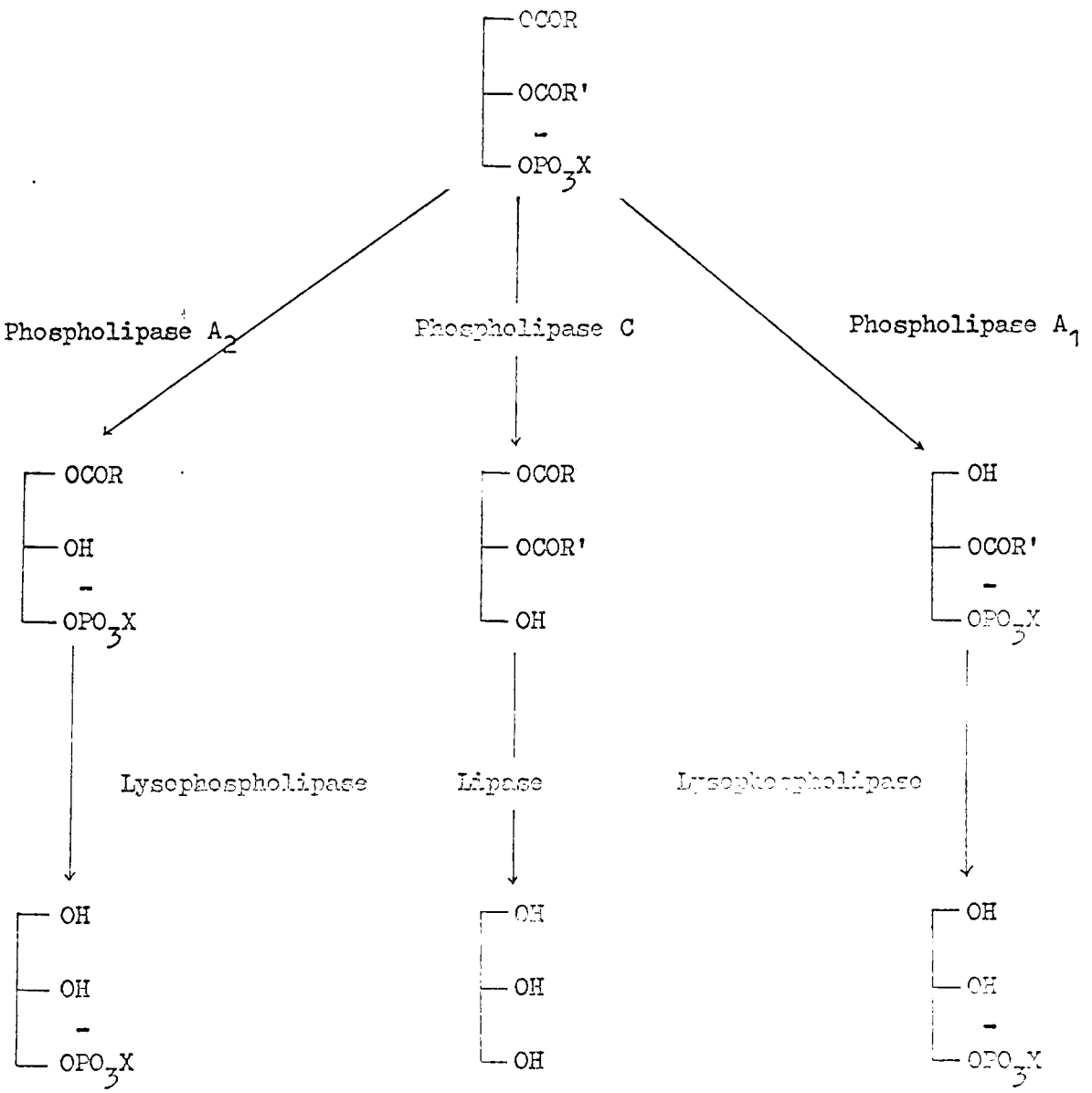
Phospholipase A_2 (phosphatidyl acyl-hydrolase) was first isolated from snake venom, and catalyzes hydrolytic cleavage at the 2-position to give lysolecithin (XVII) and fatty acid. A second enzyme, designated *Phospholipase A_1* and catalyzing hydrolysis at the 1-position, has been

discovered more recently. Waite and van Deenen[45] have separated the A_1 and A_2 enzymes from rat liver homogenate, and have found A_1 to be located mainly in the microsomal fraction and independent of divalent cations, while A_2 is located in the mitochondria and is dependent upon calcium ions; moreover, A_2 is less sensitive to heat than A_1.

Lysophospholipase (lysolecithin acyl-hydrolase), sometimes called phospholipase B, has been the subject of some confusion. By present definition,[35] it catalyzes the hydrolysis of the second acyl chain from either 1-acyl- or 2-acylglycerophosphorylcholine (lysolecithin or its 2-acyl isomer). It is far more sensitive to heat than phospholipase A_1 or A_2; activity is located in the supernatant fluid of rat liver homogenate after sedimentation of the microsomal fraction at 100,000 x g.[45] It is still uncertain whether this is really a single enzyme or a mixture of two, each specific for one or other of the two lysophosphatide isomers.

Phospholipase C (phosphatidylcholine cholinephosphohydrolase) has been found in the α-toxin of Chlostridium welchii and other bacteria, but is not well known in animal tissues. It catalyzes the hydrolytic cleavage of the phosphoryl- or phosphonyl-base moiety from lecithin, phosphatidyl-ethanolamine, sphingomyelin and their phosphonate analogues, and choline and ethanolamine plasmalogens. It is calcium-dependent.

Phospholipase D (phosphatidylcholine phosphatidohydrolase) catalyzes the hydrolysis of phosphatides to phosphatidic adid (X) and base, and has only been isolated from plant tissues.



(X = $CH_2CH_2\overset{+}{N}Me_3$, $CH_2CH_2\overset{+}{N}H_3$, or $CH_2CH(CO_2H)\overset{+}{N}H_3$)

DEGRADATION OF CHOLINE

Davies,[46] in 1936, reported that 14 to 43 per cent of choline or betaine consumed by cows was excreted in the urine as trimethylamine oxide: no unchanged betaine could be detected. Later, Castro-Mendoza et al.[47] reported a similar finding in the rat, and de la Huerga and Popper[48] found that the same applied to humans: within 24 hours, 60 to 67 per cent of orally-administered choline was excreted in the urine, regardless of dose size, as trimethylamine or its oxide. The oxide formed 95 to 97 per cent of this excretion, but free choline never amounted to more than 0.3 per cent. If, however, choline was also administered by intravenous injection, there was no increase in trimethylamine oxide secretion, but the urine now contained appreciable quantities of unchanged choline. A similar investigation with rats[49] led to the conclusion that the degradation of choline to trimethylamine and its oxide was caused by the bacteria of the intestinal tract.

PHOSPHOLIPIDS IN NATURAL AND ARTIFICIAL MEMBRANES

(i) Current views on cell membrane structure.

At present the most acceptable model for the structure of cell membranes

is based on the fluid mosaic model of Singer and Nicolson;[50-2] this makes the assumption that the most important non-covalent interactions in the cell membrane are hydrophilic. Thus in a phospholipid bilayer structure the non-polar fatty acid chains are directed away from contact with water, minimizing hydrophobic interactions, while the polar heads are in direct contact with the aqueous phase at the exterior surfaces of the bilayer, maximizing hydrophilic interactions; dipole-dipole interactions at the bilayer surface may also help to stabilize the structure. Differential calorimetry,[53-4] X-ray diffraction,[55-6] and spin-label studies,[57-9] in producing evidence that the majority of membrane phospholipids are in bilayer form, have shown that lipid structure in membranes and in aqueous dispersion are closely similar. However, a small portion of the lipid may be more intimately associated with the membrane protein.

Much of this protein probably plays an important part in determining membrane structure. Once again, in order to attain the lowest free energy for the membrane in an aqueous environment, the non-polar protein chains are directed away from the surface, while the ionic and polar groups are in contact with the aqueous phase. Thus the classical concept of Danielli and Davson,[60] of a lipid bilayer sandwiched between layers of protein, is made highly unlikely.

While peripheral proteins may be held to the membrane surface only by weak, non-covalent interactions, and are easily dissociated from the

membrane, freeze-cleaving experiments, in which the membrane is thought to be split open along the plane of the bilayer, indicate a random distribution of globular proteins which form an integral part of the membrane, requiring drastic treatment to remove them and often remaining associated with the lipid when isolated.

Moreover, there is some experimental evidence (now questioned) that certain integral proteins of the appropriate size and structure may span the entire thickness of the membrane and be exposed at both membrane surfaces.[61-2] More evidence[54-9] supports the view that the membrane consists of a matrix of phospholipid bilayer as a discontinuous fluid, in which orientated integral protein may undergo translational diffusion at rates determined by the effective viscosity of the lipid.

(ii) Physical studies of membranes and lipid dispersions.

In the last ten years, X-ray diffraction of both natural and artificial membranes has contributed an appreciable amount to the understanding of lipid bilayer formation.[63] Thus Phillips et al.[64] have shown that the polar group of lecithin has the zwitterion extended normal to the bilayer, so that rapid uptake of water is necessary to shield mutually repulsive dipoles. On the other hand, the polar groups of phosphatidylethanolamine are arranged with the zwitterions either approximately tangential to the plane of the membrane, or intertwined with opposing groups in a multilayer

system; hence such molecules are difficult to hydrate, and their aqueous dispersions are less stable than those of lecithin.

Nuclear magnetic resonance (NMR) has probably made the most significant contribution to the elucidation of lipid behaviour, but apart from a few studies [65-7] on the spectra of phospholipids in organic solvents, the technique is dependent upon satisfactory dispersion of lipids in aqueous media for the investigation of artificial membranes.[68-80] This is normally achieved by prolonged sonication, but the merits and demerits of this procedure, and the possibility of appreciable lipid decomposition, have been the subject of some controversy.[81-7] The use of paramagnetic ions such as ferricyanide, manganese^{II}, europium^{III}, and gadolinium^{III} has recently provided information on the distribution of different phospholipid species between the internal and external surfaces of artificial membranes.[88-90]

By taking advantage of the natural abundance of carbon-13, and by the incorporation of ^{13}C-enriched species into membranes, recent workers[91-3] have pioneered the use of pulsed Fourier transform ^{13}C-NMR in this field; greatly increased resolution is possible.

The similar use of phosphorus-31 NMR has been restricted to the investigation of the behaviour of the phosphate moiety of phospholipids in aqueous dispersion.[94-6]

The technique of spin-labelling phospholipids with nitroxide groups

has been adopted for electron-spin resonance studies of mobility at different regions along the hydrocarbon chains of bilayers.[97-9]

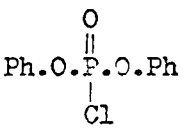
Thermal investigations of phospholipids, such as the determination of transition temperatures, have yielded important information about membrane fluidity. Phase transitions are dependent upon headgroup, hydrocarbon chain length, and degree and type of unsaturation present;[100] thus lipids with more unsaturated chains have lower transition temperatures than more saturated ones,[101] longer chains higher transition temperatures than shorter ones,[102] and cis-unsaturated chains lower transition temperatures than trans-unsaturated ones.[103] Chapman[102] has pointed out that, at 37°, the hydrocarbon chains of the unsaturated phospholipids present in natural tissues will be in a highly mobile condition unless there are interactions with other types of molecules; even the limiting transition temperature (41°) for the major lung phospholipid, dipalmitoyllecithin, is close to body temperature. Such factors, along with the possible influence of cholesterol, water, and changes in head-group, have to be taken into consideration in any theories of biological membrane structure.

CHEMICAL SYNTHESIS OF LECITHIN AND PRECURSORS

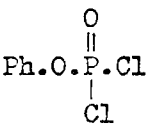
(i) Phosphorylcholine (II).

The first acceptable preparation was by Plimmer and Burch[104] in 1937,

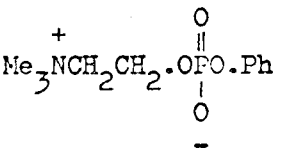
by heating choline under reflux in polyphosphoric acid. Baer[105-6] claimed a higher yield from a lengthy process involving the use of diphenylphosphorochloridate (XVIII) as a phosphorylating agent, followed by alkaline hydrolysis of the resulting phosphate triester. Bird,[107] in a more elegant synthesis, used phenylphosphorodichloridate (XIX) as a phosphorylating agent, isolated the zwitterionic product (XX) by ion exchange, and removed the phenyl group by hydrogenolysis.



(XVIII)



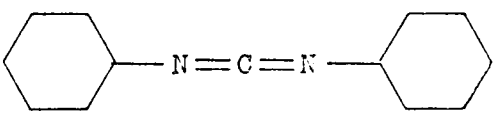
(XIX)



(XX)

(ii) CDP-choline (III).

This was first synthesized by Kennedy[108] from CMP and phosphorylcholine, using the dicyclohexylcarbodiimide (DCC) (XXI) technique of Khorana et al.[109-11]



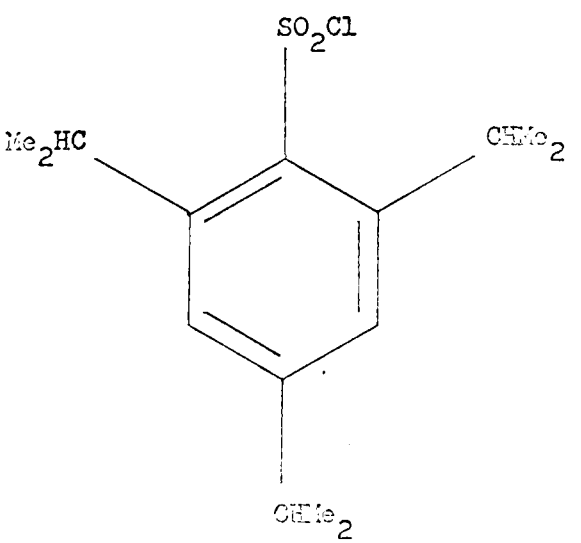
(XXI)

The product was separated from unchanged CMP and by-products by gradient elution from an anion-exchange column with dilute formic acid.

CMP + PHOSPHORYLCHOLINE $\xrightarrow[\text{aqueous pyridine}]{\text{excess DCC}}$ CDP-CHOLINE

(iii) Lecithin (IV) and other phospholipids.

Many synthetic routes have been described in the literature, and these have been thoroughly reviewed by van Deenen and de Haas[112] in 1964, and by Slotbloom and Bonsen[113] in 1970. A recent synthesis of lecithin by Aneja and Chadha[114] involved the direct esterification of phosphatidic acid (X) with choline in the presence of pyridine and triisopropylbenzene-sulphonyl chloride (TPS) (XXII) as the condensing agent.



(XXII)

ANALOGUES OF CHOLINE AND RELATED COMPOUNDS

(i) Early syntheses and pharmacological investigations.

Wurtz[115] first prepared the chloride of choline (I) by heating trimethylamine with excess ethylene chlorohydrin. However, analogues, homologues and derivatives of choline were not seriously investigated until the beginning of the century, when Hunt and Taveau [116] discovered such remarkable properties of acetylcholine (VIII) as its powerful hypotensive effect. These workers also examined the pharmacological properties of propionyl-, butyryl-, valeryl-, phenylacetyl-, cinnamyl-, and m- and p-nitrobenzoylcholine, all of which were found to be pharmacologically active, although the introduction of the phenyl group diminished the depressant action very markedly. The effect of moving the hydroxyl group in relation to the nitrogen atom was also noted:[117] thus if the hydroxyl group was left in the β-position, various changes could be made in the side chain without affecting the level of toxicity, but decreasing or increasing its proximity to the nitrogen atom resulted in a greater toxic effect.

Renshaw, who in 1910 devised a method for producing choline salts in almost quantitative yields by an improvement[118] of Wurtz's method,[115] later carried out a long series of investigations, with various co-workers, "on the basis for physiological activity of certain -onium compounds,"[119-135]

in which homologues, analogues and derivatives of choline (I), betaine (VII), acetylcholine (VIII) and formocholine (XXIII) were synthesized and tested

$$Me_3\overset{+}{N}CH_2OH \quad \overset{-}{X} \qquad (X = OH, Cl \text{ etc.})$$

(XXIII)

for pharmacological activity. Hunt and Renshaw[136] also collaborated in a thorough pharmacological investigation of some analogues of choline and quaternary ammonium salts in which the nitrogen atom was replaced by others from Group Vb or even Group VIb. It was known, chiefly from the work of Dale and Burn,[137-8] that a number of quaternary ammonium salts exhibited not only a curare-like action upon motor nerve endings but also muscarinic and nicotinic actions upon the autonomic nervous system; Hunt and Renshaw found[119,136] that the sulphonium, phosphonium, arsonium and stibonium analogues of tetramethylammonium iodide had a marked muscarinic action, less marked than that of the ammonium compound but, in the case of sulphur and phosphorus, still very pronounced. These two analogues also exhibited a marked (stimulating) nicotinic action, and a curare-like action about one tenth that of the nitrogen compound, while the arsenic and antimony compounds possessed neither. In mice, the nitrogen compound was the most toxic, followed by those of phosphorus, arsenic, sulphur and antimony, in order of decreasing toxicity. Of the possible analogues of choline itself, only

$$Me_3\overset{+}{P}CH_2CH_2OH \quad \overset{-}{Cl}$$

(XXIV)

phosphocholine chloride (XXIV), first prepared by Hofmann[139] in 1861, was investigated,[136,140] and was found to have a marked muscarinic action, much increased by acetylation , as in the case of choline. Hunt and Renshaw deduced[119,136] that, since the phosphorus compounds produced qualitatively the same physiological action as their nitrogen counterparts, the -onium group was not, per se, of primary significance in the physiological action of these compounds. It was therefore postulated that the -onium group merely determined an effective geometric structure which, in turn, made possible some specific type of adsorption or desorption process.

Later, Welch and Roepke[141-2] undertook similar work on the arsenic analogue, arsenocholine chloride (XXV), first prepared by various chemical

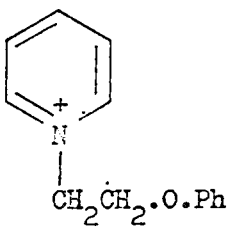
$$Me_3\overset{+}{As}CH_2CH_2OH \quad \overset{-}{Cl}$$

(XXV)

companies,[143-4] and also on its acetyl derivatives: they showed that, while the pharmacological actions of the phosphorus and arsenic analogues were qualitatively identical to those of acetylcholine, the arsenic analogue

had only one-eighth of the activity of its phosphonium counterpart, which in turn had 5 to 10 per cent of the activity of acetylcholine. A rough comparison of the action of the unacetylated analogues on guinea-pig and rabbit intestine indicated that phosphocholine had 77 per cent, and arsenocholine 3.5 per cent, of the activity of choline. Moreover, arsenocholine was, like choline, an inhibitor of choline esterase.[142]

Thereafter, Hunt[145-156] investigated the pharmacological activity of the many choline analogues prepared by Renshaw, but these were now confined to nitrogen and sulphur compounds including many homologues of choline, betaine, acetylcholine and formocholine, their ethers, and phenyl and heterocyclic derivatives such as (XXVI). The sulphur derivatives



(XXVI)

investigated included not only those compounds in which sulphur replaced nitrogen, such as (XXVII), but also those in which it replaced oxygen, such as (XXVIII) and (XXIX).

$Me_2\overset{+}{S}CH_2CH_2OH \quad \overset{-}{Cl}$ (XXVII)

$Me_3\overset{+}{N}CH_2CH_2SH \quad \overset{-}{Cl}$ (XXVIII)

$Me_3\overset{+}{N}CH_2SH \quad \overset{-}{Cl}$ (XXIX)

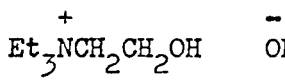
There have been few publications[157-9] on this subject in the last forty years, although Inouye et al.[158] have calculated what they term the relative delocalizability of the quaternary ammonium, phosphonium and arsonium heads, and have found a direct correlation with the muscarinic effect of the acetoxyethyl derivatives. Bartels[159] has published a study of the structure-activity relationship of acetylcholine and its arsenic and sulphur derivatives, using the isolated single electroplax preparation; replacement of nitrogen by sulphur reduced the potency of the blocking effect by a factor of 10, and arsenic by a factor of 25. However, substitution of the three methyl groups by ethyl reduced the potency by a factor of over 2000.

(ii) Choline deficiency: choline and analogues as lipotropic agents.

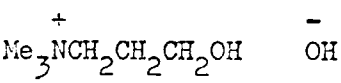
Choline is an essential factor for the normal growth and health of most organisms, but it was not until 1932 that Best, Hershley and Huntsman[160-2] discovered it to be a dietary factor protecting the liver from accumulation of fat: the term "lipotropic" is now used to describe this property.[163] In the same year, betaine was found to have a similar effect,[162] and in 1937 Tucker and Eckstein[164] realised that methionine was the principal lipotropic agent in protein. Two years later du Vigneaud et al.[22] discovered the process of transmethylation, thus linking betaine and methionine as precursors of choline. Diets deficient in these three compounds are termed hypolipotropic.

Apart from fatty livers, the administration of hypolipotropic diets has been found to give rise to haemorrhagic lesions of the kidneys[165-9] (especially in weanling rats)[170] and other organs, cirrhosis,[171-2] growth failure in most species including insects,[173] perosis in poultry,[174-5] impaired reproduction[176-7] and lactation[178] in rats, and muscle weakness or even paralysis.[178] Other symptoms have been recorded, but these are not necessarily alleviated by the provision of lipotropic agents in the diet.

It was not until 1936 that homologues, analogues and derivatives of betaine, methionine and choline were first examined for lipotropic properties Thus Channon et al.[179-80] prepared and tested 2-hydroxyethyltriethylammonium hydroxide (XXX) and 2-hydroxypropyltrimethylammonium hydroxide ("homocholine" (XXXI). Although they were slightly more toxic than choline, both exhibited



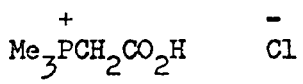
(XXX)



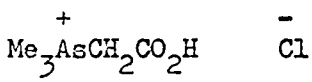
(XXXI)

an appreciable lipotropic effect, and McArthur and Lucas[181-3] later showed, by a lengthy chemical and microbiological assay, that the homologue (XXX) had been incorporated into the lecithin of rats whose choline-free diet had been supplemented by it. The proportion of phospholipid-bound choline replaced by the triethyl analogue ranged from 17 to 29 per cent over time intervals of ten to forty days.

Meanwhile Welch[104] had given arsenocholine (XXV) to rats fed on a high fat/low choline diet, had isolated their brain and liver lecithins as cadmium chloride complexes, and had found that the carbon-arc spectrograms of these extracts contained strong arsenic lines absent from the lecithin of control animals. He estimated that the lecithin of rats fed on this diet for six days contained the equivalent of 2 per cent "arsenolecithin".[185] Moreover, less intense but definite arsenic lines were observed in the spectrum of the mercuric chloride complex of sphingosinephosphorylcholine isolated from the kidneys. Welch and Welch[185] extended this investigation to phosphocholine chloride (XXIV), phosphobetaine hydrochloride (XXXII) and arsenobetaine hydrochloride (XXXIII) and found that phosphocholine,

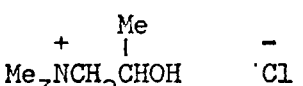


(XXXII)

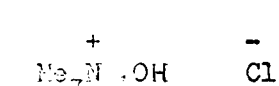


(XXXIII)

like arsenocholine, possessed lipotropic activity, while both betaine analogues were inactive. Activity was, however, exhibited by betaine itself, and also by phosphorylcholine (II), but not by β-methylcholine chloride (XXXIV) or trimethylamine oxide hydrochloride (XXXV). They



(XXXIV)



(XXXV)

concluded that the phosphorus and arsenic analogues of choline act *per se*, and not by coversion to the betaine derivatives. Welch[186] prepared dimethylthetin (XXXVI), the sulphur analogue of betaine, and found it

$$Me_2\overset{+}{S}CH_2CO_2^-$$

(XXXVI)

possessed strong lipotropic activity; it was also shown to have a biologically-labile methyl group, unlike sulphocholine (XXVII).[187]

Welch[186] included in his investigation the renal anti-haemorrhagic action of choline analogues, and reported that both phosphocholine and arsenocholine were active; his work was hardly more than qualitative, however, since the dose levels of phosphocholine used were inexplicably higher than those of the nitrogen and arsenic analogues, and the sample size was much smaller. He also noted that the arsenic and phosphorus analogues of betaine were as ineffective against kidney lesions as against fatty livers.

The hypothesis of Welch and Welch[185] that betaine was a precursor of choline was discounted by Jukes,[188] who suggested exactly the reverse. Thus betaine was found to have none of the antiperotic activity of choline when administered to poultry; arsenocholine, however, was an effective anti-perotic agent.[188-9]

Welch and Landau[190] later accepted this view, after du Vigneaud et al.[22] and Stetton[191] had shown that the action of betaine was an indirect one, as a donor of methyl groups. This also explained why arsenobetaine was inactive, since it did not serve as a methyl donor; the latter also applied to arsenocholine,[192-4] but this was acting per se, and a methyl-donating capacity would have been unnecessary for its activity.

These deductions were in keeping with the observations of Almquist and Grau,[195] who found that while arsenocholine was capable of assuming a large portion of the function of choline in the chick, the remainder could be supplied by betaine or methionine; thus the effects of arsenocholine and betaine (or methionine) were additive, and together formed a virtually complete substitute for choline.

Mann et al.[196] investigated the in vitro oxidation of arsenocholine to arsenobetaine, and found that the liver of the rat firstly oxidizes it, rapidly, to arsenobetaine aldehyde (XXXVII), which they could not isolate.

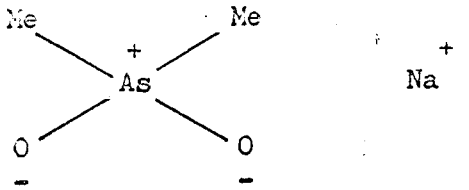
$$Me_3\overset{+}{A}sCH_2CHO \quad \overset{-}{Cl}$$

(XXXVII)

They did, however, isolate a 2,4-dinitrophenylhydrazine derivative during the oxidation, and also detected a secondary reaction, blocked by the addition of semi-carbazide, leading to the production of a substance with

a strong garlic-like odour suggestive of trimethylarsine and capable of reducing acid permanganate. No smell reminiscent of trimethylamine was detected in the case of choline, nor any compound capable of reducing permanganate. A similar effect was observed *in vivo*, since the exhalation and tissues of animals fed arsenocholine chloride or arsenobetaine chloride also had a marked garlic-like odour. The absence of toxic effects indicated that the product could not be arsine, nor its mono- or dimethyl derivatives; trimethylarsine, on the other hand, did not appear toxic at the concentrations used. Welch and Landau[190] re-reported the findings of Mann *et al.*,[196] and proposed that arsenocholine was more likely to lose its two-carbon side-chain than its methyl groups because of the greater strength of the (methyl) carbon - arsenic bonds; the reverse was true for choline.

Ciusa[197-8] later claimed to have disproved Maw and du Vigneaud's theories[199] on the ability of onium derivatives to donate methyl groups, by showing that both arsenocholine and sodium cacodylate (XXXVIII) were methyl donors after all, since the arsenic atom was in the $(sp^3)^+$ state.



(XXXVIII)

(iii) Choline analogues in metabolic studies.

In the last thirty years the number of publications concerning the metabolism of Group Vb analogues of choline has been negligible. In 1945 Horowitz et al.[200] investigated the response of two choline-requiring mutants of Neurospora crassa to choline and its derivatives and analogues. In liquid medium, the growth-promoting activity of arsenocholine was identical to that of choline, while in agar it was still 91 to 97 per cent. In 1959 Kiss et al.[201] used an arsenocholine diet to determine the rate of increase of phosphatide synthesis in previously fasted normal and adrenalectomized rats.

The study of the metabolism of nitrogen analogues of choline has proved more popular. Thus Artom and Crowder[202] found measurable quantities of N,N-dimethylethanolamine (XXXIX) incorporated into the phospholipids of rats 3 to 6 hours after the administration of a dose of the compound, although under normal circumstances mere traces of phosphatidyl-N,N-dimethylethanolamine can be found only occasionally; thus N,N-dimethylethanolamine can be incorporated into phospholipids before methylation to choline. Ansell and Spanner,[203] in a review of the biochemical role of dimethylethanolamine, later confirmed these findings.

$Me_2NCH_2CH_2OH$

(XXXIX)

$H_2N.C(CH_3)_2.CH_2OH$

(XL)

Longmore and Mulford[204] demonstated the *in vivo* conversion of labelled 2-amino-2-methylpropanol (XL) to the corresponding analogue of phosphatidyl-ethanolamine; the resulting phospholipid mixture was resolved by paper chromatography. Mersman and Strength,[205] and Bell,[206-7] have investigated the phospholipid incorporation of various N-ethyl derivatives of choline and ethanolamine in the rat, while Bieber *et al.*[208-9] have studied a similar incorporation by the larvae of the blowfly, *Phormia Regina*, of dimethyl-ethanolamine (XXXIX), dimethylaminoisopropanol (XLI) and carnitine (XLII);

$Me_2NCH_2\overset{\overset{Me}{|}}{C}HOH$ (XLI)

$Me_3\overset{+}{N}CH_2\overset{\overset{CH_2CO_2^-}{|}}{C}HOH$ (XLII)

all were found in the extracted lecithin, although carnitine had been decarboxylated to β-methylcholine (XXXIV). Bridges *et al.*[210] obtained similar results by incubating the larvae of the housefly, *Musca domestica*, with carnitine (XLII) or acetyl-β-methylcholine (XLIII) in place of choline.

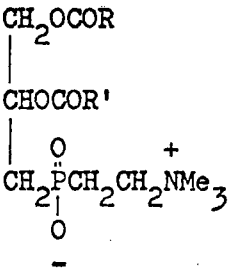
$Me_3\overset{+}{N}CH_2\overset{\overset{Me}{|}}{C}HOAc \quad Cl^-$

(XLIII)

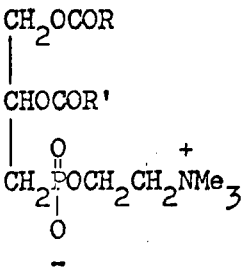
However, no phosphatidyl-β-methylcholine was found in adult flies fed on carnitine, demonstrating that the adults, unlike the larvae, cannot decarboxylate this compound. Ansell, Chojnacki and co-workers[211-9] have used several unnatural bases, mainly N-alkyl derivatives of ethanolamine, propanolamine, butanolamine and pentanolamine, to investigate the cytidine pathway to lecithin; this was achieved by the incubation of phosphorus-32-labelled CDP derivatives of these bases with rat liver and brain homogenates. Very recently Jezyk and Hughes[220] studied the effect on rates of acylation of the 2-position of lysolecithin(XVII) when different homologues of choline, ethanolamine and their intermediates form the polar end of the molecule; it was hoped that this might lead to a better understanding of the processes determining the asymmetrical distribution of fatty acids in the phosphatides of mammalian tissue.

(iv) Phosphono- and phosphino- analogues of phospholipids.

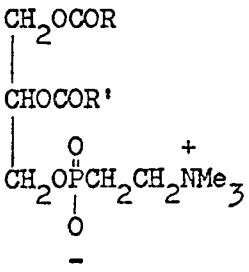
Analogues of phosphorylcholine (II) and phosphorylethanolamine, in which the phosphoryl group is replaced by phosphonyl or phosphinyl, have been the subject of recent investigation. Several phosphinate (e.g. (XLIV)) and phosphonate (e.g. (XLV), (XLVI)) analogues of phosphatides have now been chemically synthesized, as have the equivalent ethanolamine compounds.[221-230] Compounds such as (XLIV)are of interest because of the complete resistance of the polar chain to hydrolytic cleavage.



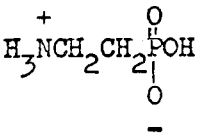
(XLIV)



(XLV)



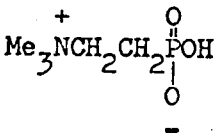
(XLVI)



(XLVII)

Hariguchi and Kandatsu[231] provided the first evidence for the natural occurrence of phosphonolipids with the discovery of 2-aminoethylphosphonic acid (XLVII) in the protozoa of sheep rumen, and since then the ceramide derivative has been found widely distributed among invertebrates and protozoa,[232] as have its N-methyl analogues.[233] Thompson[234] isolated the phosphonate analogue of phosphatidylethanolamine from Tetrahymena pyriformis, using an adaption of the novel thin-layer chromatographic method of Kapoulas,[235] who had realized that phosphonolipids were far more widely distributed than previously expected; it had merely been extremely difficult

to separate them from their phospholipid counterparts and so identify them. Berger and Hanahan[236] have since developed a column chromatographic separation method, and Bjerve[237] has studied lecithin biosynthesis in the rat by observing the incorporation of phosphonylcholine (XLVIII) into phospholipids



(XLVIII)

both *in vitro* and *in vivo*, with the conclusion that the normal cytidine pathway is followed.

RECENT STUDIES OF CHOLINE DEFICIENCY

Lombardi,[238] in a general investigation of the pathogenesis of fatty liver, considered the very short term effects of choline deficiency and noted a 2- to 3-fold increase in the level of rat liver triglyceride after 6 to 9 hours; this accumulation was completely halted by the oral administration of choline. He obtained conclusive evidence that dietary fat was the source of excess triglycerides by the inclusion of labelled palmitic acid in the diet, and deduced that in such animals there is impairment of the ability of

the liver to metabolize chylomicra triglycerides as a result of defective phospholipid synthesis, since the incorporation of the label into liver and plasma phospholipids was lower in choline-deficient rats than in controls. Hence the synthesis of the phospholipid moiety of very low-density serum lipoproteins would become progressively insufficient. With absorption of dietary fat, more and more of the chylomicra triglycerides taken up by the liver would remain there instead of being secreted into the plasma as a moiety of very low-density lipoprotein, this being one of the major pathways by which the liver disposes of fatty acids. Lombardi et al.[239] later produced further support for the hypothesis that fatty liver results from, rather than is a cause of, impaired release of hepatic triglyceride into the plasma, by experiments involving the simultaneous injection of labelled palmitic acid and Triton, a detergent inducing hyperlipemia (mainly hypertriglyceridemia).

Thompson et al.[240] have studied the effects of the early stages of choline deficiency on phosphorylcholine and lecithin levels in rat liver and plasma. Despite a 70 per cent reduction in phosphorylcholine levels, and a greatly decreased incorporation of labelled choline into this intermediate, lecithin levels were only slightly depressed, with increased incorporation of labelled choline and labelled methyl groups from methionine. It was postulated that choline deficiency induced a more rapid conversion of phosphorylcholine to lecithin, thus reducing its concentration in the liver. Since an accelerated turnover of the polar moiety of lecithin was also noticed, it was suggest-

ed that this reflected an increased metabolic instability of the membrane lipids . While increase incorporation of methionine methyl groups into lecithin indicated a higher utilization of the sequential methylation pathway, the increased incorporation of choline, not *via* phosphorylcholine, suggested possible base-exchange reactions at the phosphatide level.

Fallon *et al.*[241] have studied the effects of long-term (1 month to 1 year) choline deficiency and ethanol ingestion on hepatic lecithin metabolism in the rat, and have found that chronic ethanol ingestion results in a significant increase in total phospholipid content and a similar increase in utilization of the sequential methylation pathway, with no changes in the cytidine pathway or in triglyceride levels. Choline deficiency, as expected, resulted in a similar increase in lecithin formed by trans-methylation, with no change in the cytidine pathway but with increased triglyceride levels. Supplementation of the diet with 2 per cent choline completely prevented the changes in lecithin synthesis induced by ethanol ingestion.

Glenn and Austin[242] confirmed that methylation of phosphatidylethanol-amine to lecithin increased in choline-deficient rats, although the extent was somewhat sex-dependent. They also showed that there were dramatic changes in the relative proportions of microsomal lecithin subspecies, and that these were produced by methylation of phosphatidylethanolamine at different rates; thus relative proportionsof arachidonic acid increased

greatly, as did the rate of methylation of the species of phosphatidylethanolamine in which it occurred. In work complementary to this, Beare-Rogers[243-5] found lowered levels of arachidonic acid in hepatic phospholipids of rats fed a diet deficient in both choline and methionine; choline increased the content of this fatty acid in phosphatidylethanolamine but not lecithin, whereas methionine increased both the amount of lecithin and its content of arachidonic acid.

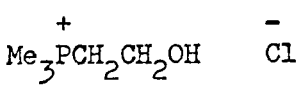
Wong and Thompson[246] studied the effect of choline deficiency on the initial stages of choline metabolism in liver, and found that free choline levels dropped by 50 per cent after 2 days, while free betaine levels were reduced to an unmeasurable quantity. Also, triglyceride levels increased 4-fold in 2 days, 20-fold in 6 days and 40-fold in 13 days.

Trewhella and Collins,[247] in an attempt to elucidate the effects of choline deficiency on fatty acid distribution, have very recently studied the consequences of essential fatty acid deficiency on liver lecithins, and find that the lecithin species with the slowest rate of turnover in normal rats is the 1-stearoyl-2-arachidonyl compound, while in deficient rats it is the 1-stearoyl-2-eicosatrienoyl compound.

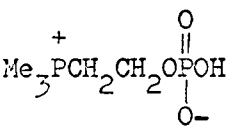
AIMS OF THE PRESENT WORK

Although phosphocholine (XXIV) is a known lipotropic agent,[185-6] there is no positive evidence that it is metabolized in the same way as choline. However, since its pharmacological and lipotropic properties bear a greater resemblance to those of choline than do those of the arsenic analogue,[141-2, 185-6] and because there is some reason to believe that the latter is incorporated into lecithin *in vivo*,[184-5] the circumstantial evidence for choline-like metabolism is strong. The aims of the present work are as follows.

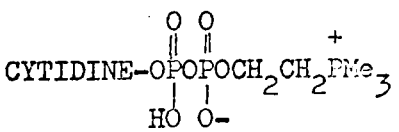
Firstly, it was intended to synthesize phosphocholine chloride (XXIV) and the other, as yet unknown, phosphorus analogues of the cytidine-pathway metabolites of choline, namely phosphorylphosphocholine (XLIX), CDP-phosphocholine (L) and phosphatidylphosphocholine ("phospholecithin") (LI).



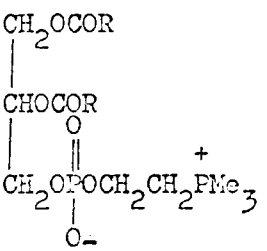
(XXIV)



(XLIX)



(L)



(LI)

Secondly, it was hoped to compare some of the properties of synthetic distearoylphosphatidylcholine and its phosphorus analogue (LI, R = $C_{17}H_{35}$), for example, susceptibility to hydrolytic cleavage by phospholipase C, ability to form bilayers in aqueous dispersion, and spectral and thermal properties.

The third stage of the work was to test the ability of these analogues to substitute for their nitrogen equivalents in each enzyme system of the cytidine pathway in vitro, using tritium-labelled choline and carbon-14 -labelled phosphocholine as precursors. It was hoped to isolate and identify the enzyme reaction products by comparison with their synthesized counterparts.

Fourthly, a comparison was to be made of the tendencies of choline and phosphocholine to be incorporated into membrane phospholipids in cell culture, again using labelled precursors.

Fifthly, it was hoped to investigate the metabolism and degradation of phosphocholine in vivo, using choline-deficient weanling rats. In 1942 Welch and Landau[190] had written: "Incontrovertible proof that the arsenic component (of a lecithin extract) is actually the arsenic analogue of choline requires the separation of that substance from choline, and the positive identification of the pure compound; however, the difficulties attending such separation appear to be great." The aim of this work was to provide such incontrovertible proof for the phosphonium analogue; in

contrast to arsenocholine, an investigation of its possible incorporation into lecithin has never even been attempted, presumably because of the insurmountable difficulties of positive identification facing the earlier workers. To this end, it was hoped to employ such physical methods as high resolution proton and phosphorus-31 magnetic resonance, isotopic labelling, and thin-layer, column and gas-liquid chromatographic techniques. It was thought that the use of ^{31}P-NMR would be especially useful, since it would enable any phosphocholine-derived phospholipids to be studied in lipid mixtures and in membranes, independently of the other phosphatides present. Finally it was intended to investigate any gross changes in the lecithin fatty acid distribution of rats fed on such a choline-free, phosphocholine-supplemented diet, and to search for any manifestations of choline deficiency.

RESULTS AND DISCUSSION

CHAPTER 1 : CHEMICAL SYNTHESES

(i) Phosphorus analogues of choline.

Phosphocholine (XXIV) was first prepared as the very deliquescent, crystalline chloride by the quaternization of trimethylphosphine with 2-chloroethanol at 100° in a sealed tube, after the method of Renshaw and Bishop.[140] Carbon-14 -labelled phosphocholine chloride was synthesized in a similar manner using (1,2-^{14}C)-2-bromoethanol (LII) diluted with

$$HO.{}^{14}CH_2.{}^{14}CH_2.Br$$

(LII)

chloroethanol; any bromide ion in the product was replaced by chloride using an ion-exchange method. When phosphocholine iodide (XXIVb) was required in a later preparation, it was obtained in high yield by heating the chloride (XXIVa) with sodium iodide in acetone solution.

$$PMe_3 + HOCH_2CH_2Cl \xrightarrow[100^\circ]{} Me_3\overset{+}{P}CH_2CH_2OH\ \overset{-}{Cl} \xrightarrow{NaI} Me_3\overset{+}{P}CH_2CH_2OH\ \overset{-}{I}$$

(XXIVa) (XXIVb)

Owing to the high cost and handling difficulties of trimethylphosphine, it was also decided to synthesize the tri-n-butylphosphonium analogue of choline, 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb) from tri-n-butyl phosphine, which was inexpensive and readily available, and to use this rather than phosphocholine in all exploratory synthetic work. Quaternization was achieved with iodoethanol, at atmospheric pressure, under

$$(n\text{-Bu})_3P + HOCH_2CH_2X \longrightarrow (n\text{-Bu})_3\overset{+}{P}CH_2CH_2OH \quad \overset{-}{X}$$

(LIII) a) X = Cl
b) X = I

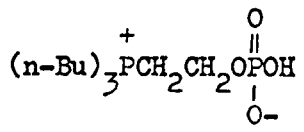
nitrogen, in very high yield. The iodide was the salt synthesized since it was crystalline, although very deliquescent; the chloride (LIIIa), when required as an enzyme substrate, was readily obtained as an oil by treating the iodide with (chloride) anion-exchange resin.

Witschard and Griffin[248] claim to have prepared these two salts, but the physical properties they attribute to the iodide are in total disagreement with those recorded here, and are completely uncharacteristic of this type of compound in general.

(ii) Phosphorus analogues of phosphorylcholine.

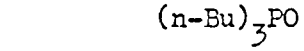
An initial attempt was made to phosphorylate 2-hydroxyethyltri-n-butyl-

phosphonium iodide (LIIIb) to 2-(tri-n-butylphosphonio)-ethyl phosphate (LIV)



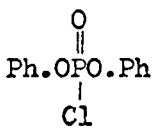
(LIV)

by the standard method of Plimmer and Burch,[104] which involved heating the compound under reflux in a mixture of orthophosphoric acid and phosphorus pentoxide for several hours. It was possible to simplify their extraction procedure through the availability of ion-exchange resins; after removal of excess phosphoric acid by precipitation with calcium hydroxide, the aqueous solution was passed through a mixed-bed (formate)/(proton) ion-exchange column to remove all charged species, while allowing free passage of uncharged species and inner salts such as (LIV). However, the only product was a colourless liquid identified by PMR, ^{31}P-NMR and infrared spectroscopy, not as the expected product (LIV), but as the hydrate of tri-n-butylphosphine oxide (LV); this was confirmed by independent synthesis of (LV) by the method of Waters,[249] involving oxidation of tri-n-butylphosphine with hydrogen peroxide. Thus the reaction mixture of Plimmer and Burch[104] had resulted in the oxidation of the choline analogue rather than its phosphorylation.

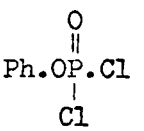


(LV)

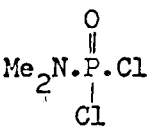
A number of well-documented phosphorylating agents were then prepared for possible use, including diphenylphosphorochloridate (XVIII) and phenylphosphorodichloridate (XIX) from phenol and phosphoryl chloride, dimethylphosphoramidic dichloride (LVI) and bis-(dimethylamido)-phosphoryl chloride (LVII) from dimethylamine and phosphoryl chloride, and o-phenylene-phosphorochloridate (LVIII) from catechol (LIX) and phosphorus pentachloride via catechyl phosphotrichloride(LX).



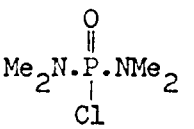
(XVIII)



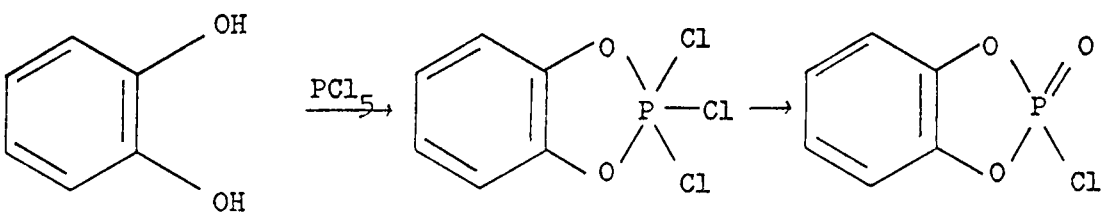
(XIX)



(LVI)

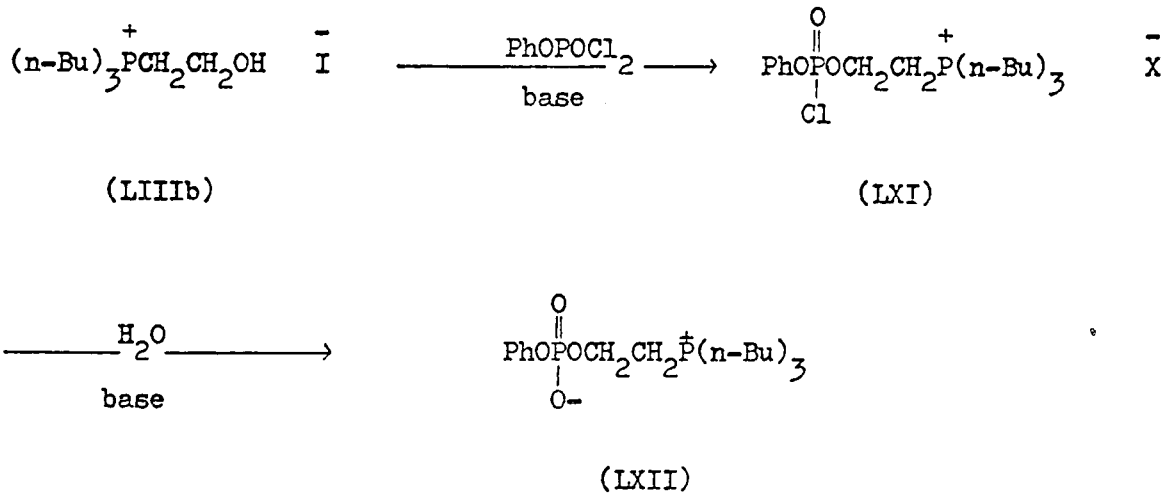


(LVII)



(LIX) (LX) (LVIII)

The next attempted phosphorylation involved the modern method of Bird[107] and the use of phenylphosphorodichloridate(XIX). Phosphorylation was carried out in the presence of excess anhydrous base, with acetonitrile as solvent, to give the intermediate (LXI), which was then hydrolysed to the phosphate diester (LXII). The latter was isolated by ion-exchange

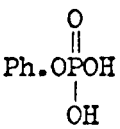


(LIIIb) (LXI)

(LXII)

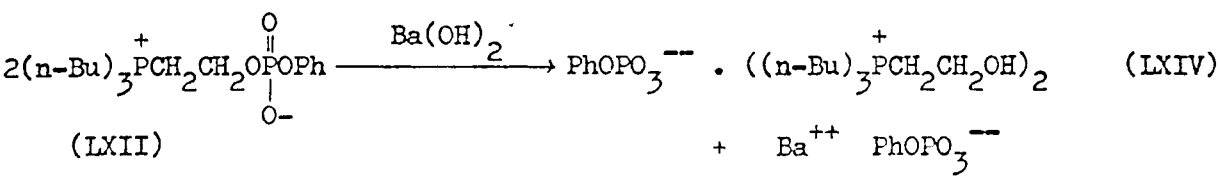
with monobed resin (H,OH) unsuitable for the preparation of monophosphate esters such as (LIV), but ideal for such a zwitterionic diester; the product, a hygroscopic wax obtained in 46 per cent yield, needed no further purification. However, the application of the second stage of Bird's method, removal of the phenyl group by hydrogenolysis, was unsuccessful. As feared, the formation of even minute amounts of free phosphine during the reaction was enough to poison the platinum dioxide catalyst, and even the presence of 5 per cent hydrogen chloride in the acetic acid solvent did not prevent this. It was therefore decided to attempt removal

of the phenyl group by hydrolysis in concentrated alkali. Baer, in a review[105] published in 1947, had criticized earlier methods of phosphorylcholine preparation for their complex reaction mixtures and low yields, and had advocated phosphorylation with diphenylphosphorochloridate (XVIII) followed by hydrolysis of the resulting diphenyl phosphate triester with boiling aqueous barium hydroxide. The monophenyl ester (LXII) was therefore subjected to this treatment, but addition of ethanol to the aqueous extract of the reaction mixture induced crystallization of the barium salt, not of 2-(tri-n-butylphosphonio)-ethyl phosphate (LIV) as expected, but of phenyl phosphoric acid (LXIII).



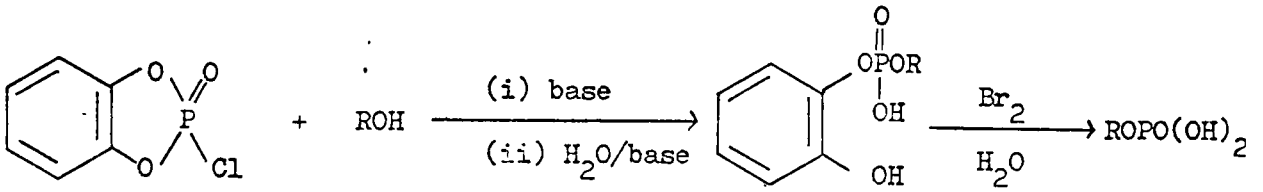
(LXIII)

The mother liquor was shown, by NMR and microanalysis, to contain the 2-hydroxyethyltri-n-butylphosphonium salt of phenylphosphoric acid, (LXIV); thus hydrolytic cleavage had occurred between phosphate and alkyl group , rather than between phosphate and phenyl group as was found with the choline derivative.[105]



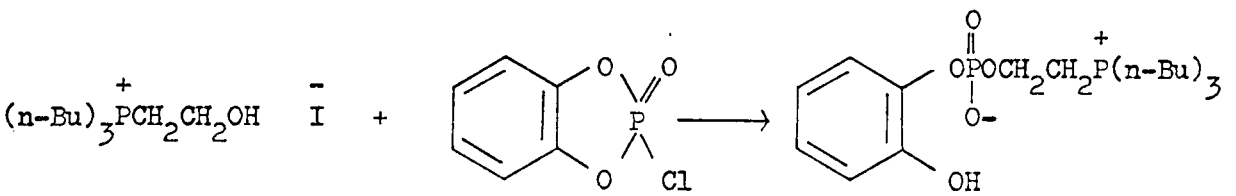
(LXII) (LXIV)

Since the removal of the phenyl group to give the desired inner salt had proved so difficult, attention was turned to a recent method devised by Khwaja et al.[250] involving phosphorylation with o-phenylenephosphorochloridate (LVIII), one of the most reactive phosphorylating agents known; in the final step the catechyl group is oxidatively cleaved from the phosphate diester with bromine water:



(LVIII)

The method had not previously been applied to the phosphorylation of choline or its analogues. The phosphorylation was performed in the same manner as before, and the o-hydroxyphenyl ester (LXV) was isolated, after ion-exchange, in37 per cent yield.

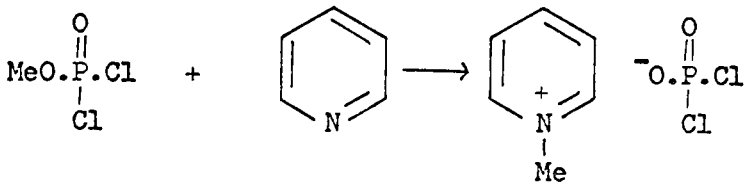


(LIIIb) (LVIII) (LXV)

However, attempts to remove the o-hydroxyphenyl group by oxidation with bromine water were a total failure; ion-exchange of the aqueous extract

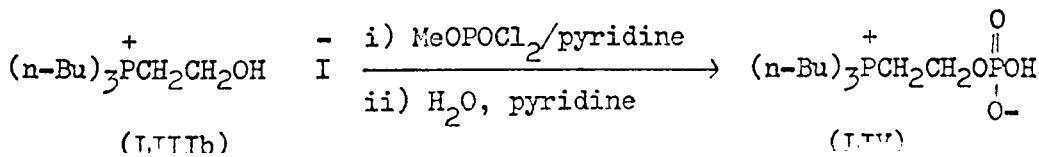
of the reaction mixture, using the (H)/(formate) resin system, yielded no inner salt whatsoever.

Smrt and Catlin[251] recently observed that phosphorylation with methylphosphorodichloridate (LXVI) in the presence of pyridine led to the formation of phosphate monoesters rather than the expected methyl phosphate diesters. They claimed that the phosphorylating agent and base formed a complex (LXVII) which could be obtained in crystalline form and which was the effective phosphorylating agent.



(LXVI) (LXVII)

Methylphosphodichloridate was therefore prepared from methanol and phosphorylchloride, and was used in the phosphorylation of the phosphonium salt (LIIIb) in the usual way, in the presence of excess pyridine. Water was added to hydrolyze the intermediate, and after treatment of the reaction mixture with (H)/(formate) mixed-bed ion-exchange resin the desired phosphorylphosphonium salt (LIV) was finally obtained in 40 per cent yield.



(LIIIb) (LIV)

Thus phosphorylation had been achieved in a single-step procedure, in a yield comparable to those obtained in the first stages of the other methods considered. Before this was adopted as the standard phosphorylation method, however, the use of dimethylphosphoramidic dichloride (LVI) was investigated. No attempt was made to isolate the phosphate diester (LXVIII) but the crude reaction mixture was boiled, after the removal of organic solvents, with an aqueous suspension of Amberlite IR-120 (H); this cation-exchange resin was sufficiently acidic to effect the acid hydrolysis of the dimethylamino group. After ion-exchange, the inner salt (LIV) was isolated as before, but in an appreciably lower yield, 16 per cent.

$$(n\text{-Bu})_3\overset{+}{P}CH_2CH_2OH \quad I^- \xrightarrow[\text{(ii) } H_2O,\ \text{pyridine}]{\text{(i) } Me_2NPOCl_2,\ \text{pyridine}}$$

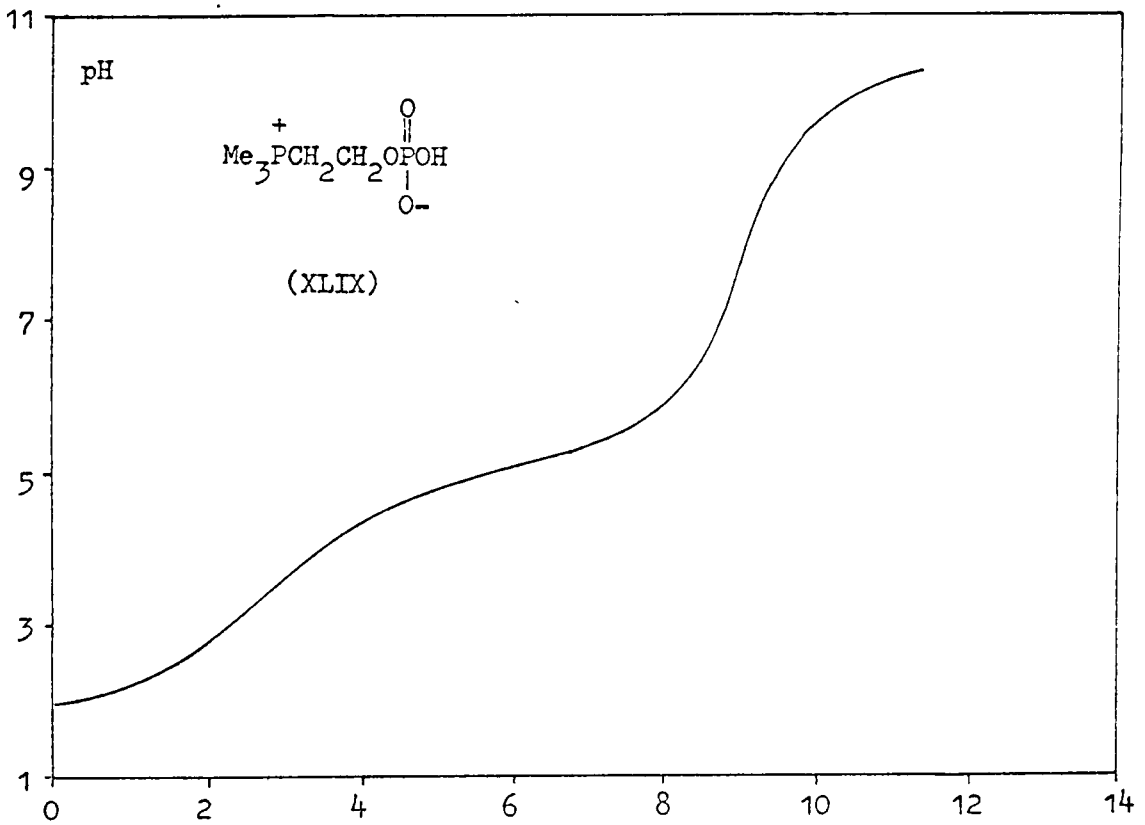
(LIIIb)

$$\left[(n\text{-Bu})_3\overset{+}{P}CH_2CH_2O\overset{O}{\overset{\|}{P}}NMe_2\ \ {}^{O-}\right] \xrightarrow{H^+} (n\text{-Bu})_3\overset{+}{P}CH_2CH_2O\overset{O}{\overset{\|}{P}}OH\ \ {}^{O-} + Me_2NH$$

(LXVIII) (LIV)

Methylphosphorodichloridate (LXVI) was therefore the phosphorylating agent of choice, and this was used in the phosphorylation of phosphocholine (XXIV) itself to phosphorylphosphocholine (XLIX); for this purpose phosphocholine iodide (XXIVb), being far less hygroscopic than the chloride

(XXIVa) and far more soluble in acetonitrile, was the salt used. The yield of inner salt (XLIX) was 31 per cent; initial attempts to obtain it as anything but a deliquescent gum failed, and conversion to a calcium chloride or barium salt was considered. However, it was found that residual water could be removed by azeotroping with absolute ethanol; the free inner salt (XLIX) was left as a white solid which analyzed satisfactorily, and which was not deliquescent. A pH titration curve was established, and compared with that published by Attias[252] for phosphorylcholine (II):



$Me_3\overset{+}{P}CH_2CH_2O\overset{O}{\overset{\|}{P}}OH$ (with O⁻ on P)

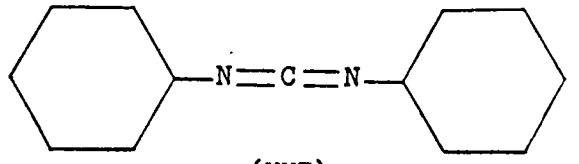
(XLIX)

Titration curve of 0.1M phosphorylphosphocholine (30 ml.)

	Phosphoryl-phosphocholine	Phosphorylcholine (from Attias)
	pH	pH
First acidity neutralized	2.75	3.50
Second pK	5.00	5.50
Second acidity neutralized	8.00	8.00

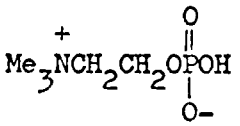
(iii) Phosphorus analogues of CDP-choline.

Kennedy[108] first synthesized CDP-choline (III) in 1956, using an adaption of the dicyclohexylcarbodiimide (DCC) (XXI) technique developed by Khorana et al.[109-11] for the preparation of nucleotides. Cytidine-5'-monophosphate (CMP) (LXIX) and phosphorylcholine (II) were condensed in the presence of DCC in pyridine; because of the insolubility of CMP in the solvent, the reaction was carried out in aqueous pyridine, which necessitated the use of a large excess (130-fold) of DCC. After 10 days, precipitated dicyclohexylurea (LXX) was removed by filtration, and the aqueous extract chromatographed on Dowex-1 (formate) anion-exchange resin with a gradient of 0 - 0.04N formic acid, after the method of Hurlbert et al.[253] The CDP-choline (III) was thus obtained free from starting materials and the by-product P_1, P_2-dicytidine-5'-pyrophosphate.



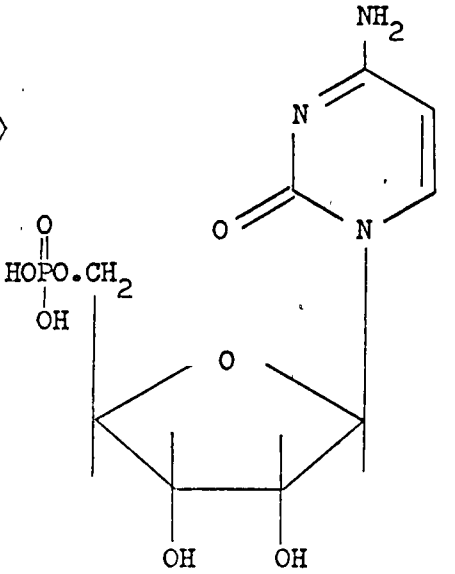
(XXI)

+

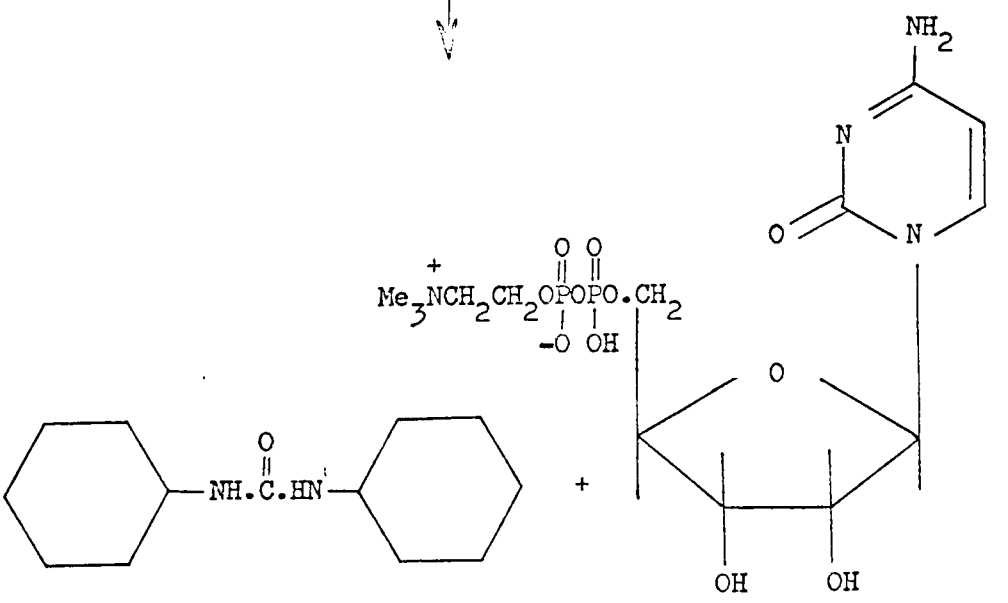


(II)

+



(LXIX)

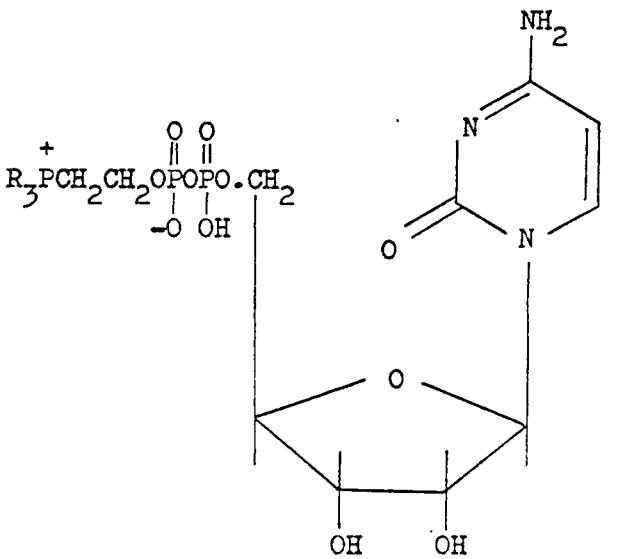


(LXX)

+

(III)

This method was applied successfully to the preparation of both CDP-phosphocholine (L) and its tri-n-butylphosphonium analogue (LXXI), the yield of the former being 24 per cent and of the latter only 1.3 per cent. Both structures were confirmed by proton magnetic resonance.



(L): R = Me

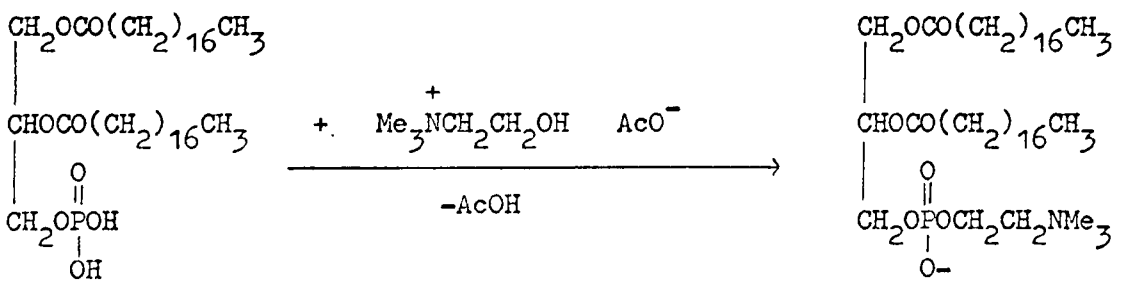
(LXXI): R = n-Bu

(iv) Phosphorus analogues of distearoylphosphatidylcholine.

Many routes for the synthesis of lecithins have been proposed and investigated; a large number have been reviewed by van Deenen and de Haas,[112] and more recently by Slotbloom and Bonsen.[113] For the preparation of

phosphatidylphosphocholine ("phospholecithin") (LI), however, the large majority were considered unsuitable, as final synthetic stages included the use of silver salts (which would complex with phosphonium derivatives), hydrogenolysis in the presence of platinum catalysts (which would be poisoned by traces of trialkylphosphine), or the formation of the onium head as the very last step (handling of trimethylphosphine is far less desirable than the handling of phosphocholine).

A timely publication by Aneja and Chadha,[114] describing the synthesis of distearoylphosphatidylcholine (LXXII) by the condensation of 1,2-distearoyl-glycero-3-phosphoric acid (distearoylphosphatidic acid) (LXXIII) and choline (I) in the presence of triisopropylbenzenesulphonyl chloride (TPS) (XXII), was therefore investigated and found to be eminently suitable. The problem of the solubility of the choline salt in pyridine was overcome, not by the use of the acetate as suggested by the authors,[114] but by the use of the iodide in acetonitrile. A second problem was the synthesis of

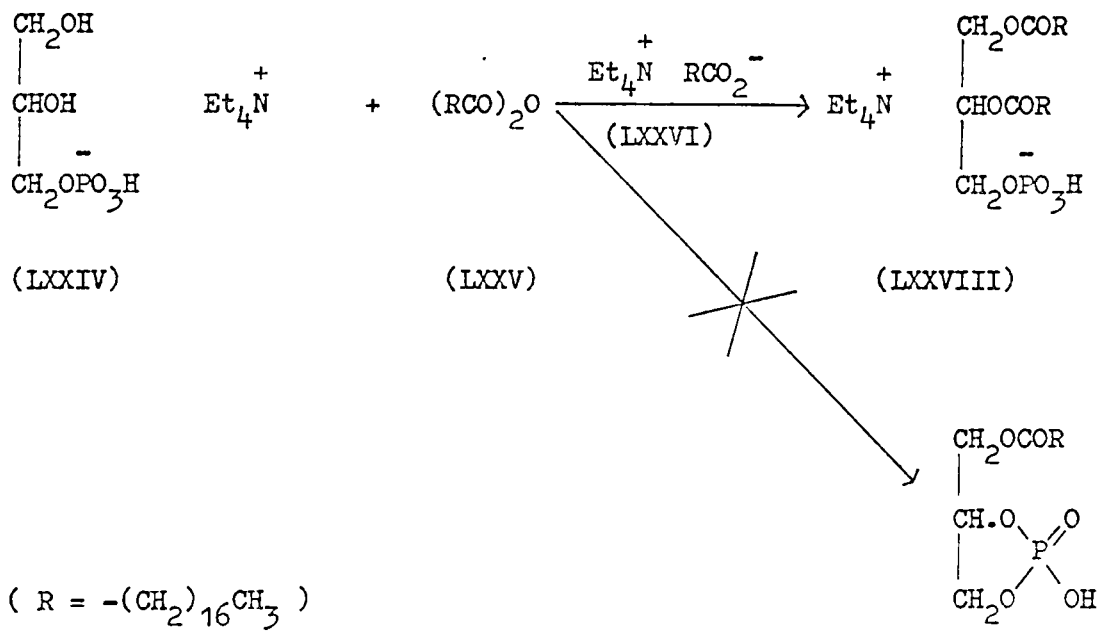


(LXXIII) (LXXII)

distearoylphosphatidic acid (LXXIII). A small amount was prepared by the method of Lapidot et al.,[254] involving the reaction of the tetraethylammonium salt of glycero-3-phosphoric acid, (LXXIV), with the fatty acid anhydride (LXXV), in the presence of the tetraethylammonium salt of the fatty acid (LXXVI) to prevent the formation of the cyclic phosphate (LXXVII). The fatty acid anhydride is prepared by the action of dicyclohexylcarbodiimide on the free acid:

(i) $RCO_2H \xrightarrow[CCl_4]{DCC} (RCO)_2O$

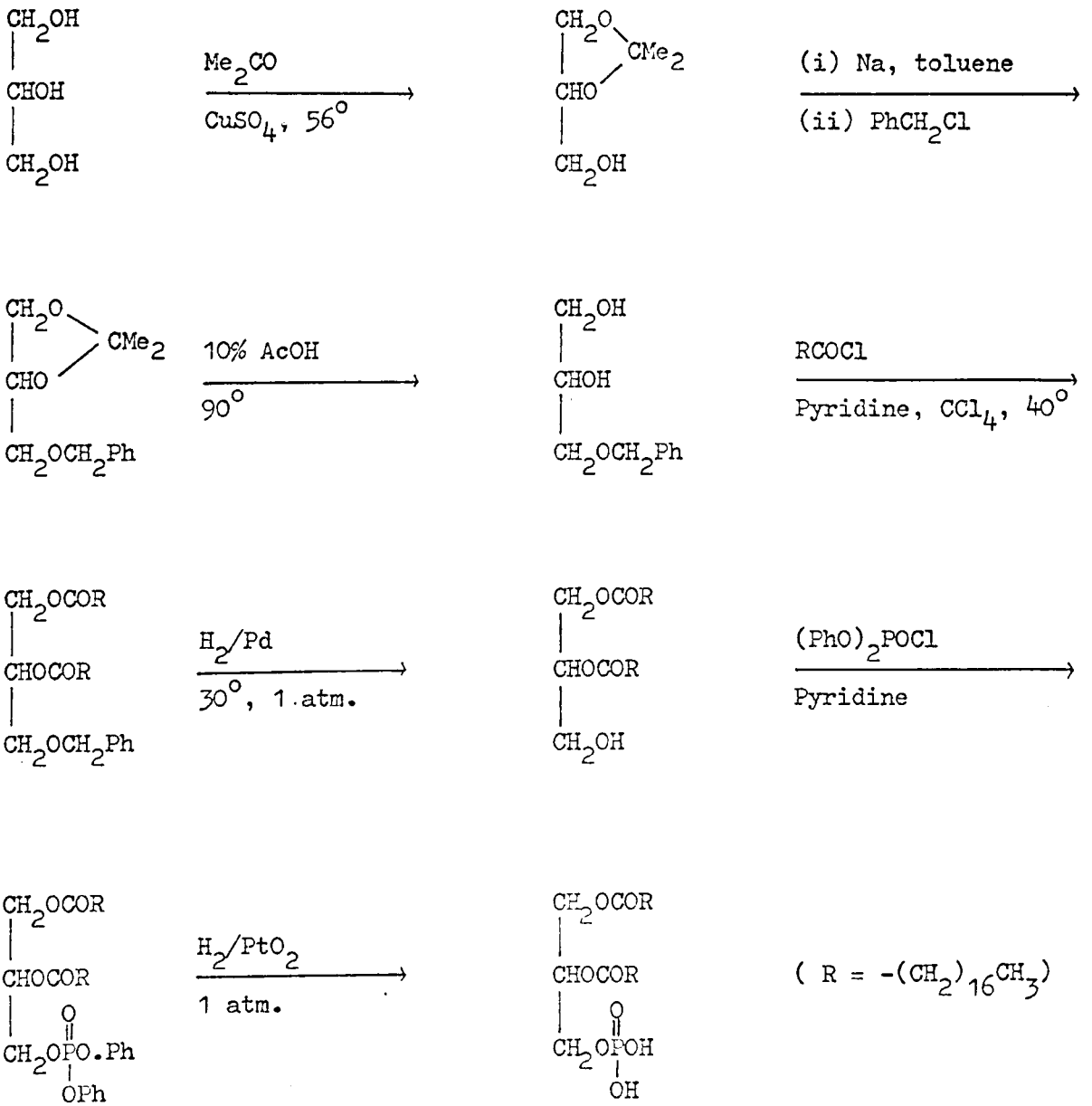
(ii)



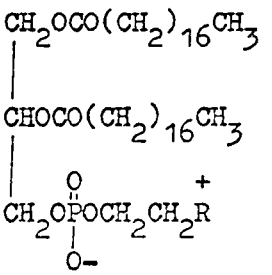
(R = $-(CH_2)_{16}CH_3$)

The tetraethylammonium phosphatidate (LXXVIII) was isolated by gel

filtration with Sephadex LH-120 lipophilic medium, but this was an expensive procedure for the amount of product obtained. In large-scale syntheses of the phosphorus analogues of lecithin, therefore, the distearoyl-phosphatidic acid used was that prepared in bulk in this laboratory by a well-established route from glycerol:[255-6]



Thus, using the method of Aneja and Chadha,[114] DL-distearoyl-phosphatidylcholine (LXXII), DL-distearoylphosphatidylphosphocholine (LI) and its tri-n-butylphosphonium analogue (LXXIX) were prepared in 40, 56 and 33 per cent yield respectively; the products were purified by silica column chromatography for large-scale preparations, and by preparative TLC for small-scale work.



(LXXII) : R = NMe_3

(LI) : R = PMe_3

(LXXIX) : R = $P(n\text{-}Bu)_3$

CHAPTER 2 : PHYSICAL AND OTHER PROPERTIES OF THE PHOSPHORUS ANALOGUES

(i) Thermal properties of lecithin and analogues.

Transition temperatures were observed using a Koffler heating stage, while melting points were measured in sealed capillary tubing:

	Transition temperature	Melting point
1,2-Distearoyl-DL-phosphatidylcholine	79-82°	233° (decomp.)
Trimethylphosphonium analogue	65-74°	135-7° (")
Tri-n-butylphosphonium analogue	58-65°	138-9° (")

At the transition temperature, which is an endothermic transition well below the true melting point, a change of state from crystalline to liquid-crystalline takes place, representing the melting of the hydrocarbon chains; at this point the structure is held together by the polar moieties.

Chapman *et al.*[102] have shown that the melting point of distearoyl-phosphatidylcholine coincides with the loss of choline from the molecule, leaving phosphatidic acid. Williams and Chapman[267] have observed that the melting points of phosphatidylcholines are virtually independent of

chain length, and deduced that, since an ionic network probably exists in the crystal structure, the stronger is the base, the higher is the melting point. This is indeed found; in order of decreasing basicity of head-group there is phosphatidylcholine (m.p. 233°), phosphatidylethanolamine (m.p. 196°) and phosphatidylserine (160°) in the distearoyl series. Thus the polar head-group is the major factor controlling melting points, and this would explain the remarkable difference in melting point between lecithin and its phosphonium analogues, since choline itself, a tetraalkyl ammonium hydroxide, is appreciably more basic than phosphocholine, a tetraalkyl phosphonium hydroxide.

(ii) Infrared absorption spectra of lecithin and its phosphonium analogues.

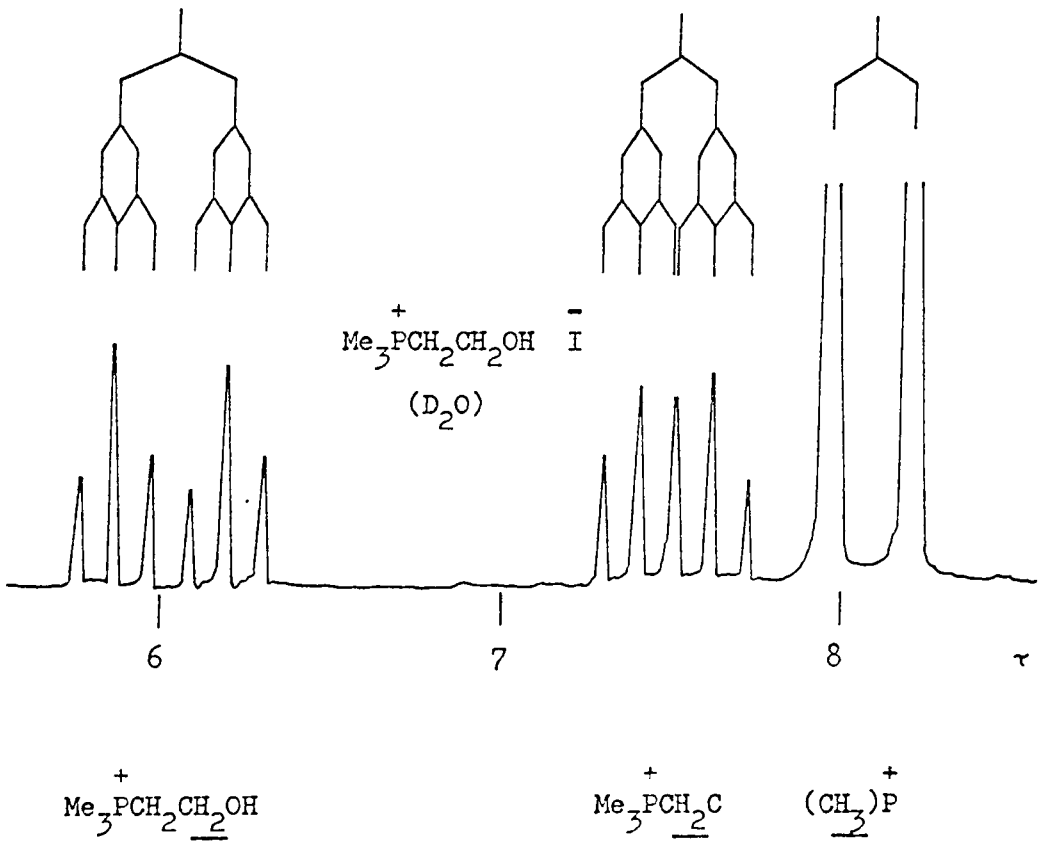
The mull spectra of the three synthetic lecithins prepared were very similar. Although it is accepted that there are few correlations for P - C absorptions,[267] some bands in the spectrum of phosphatidylphosphocholine were attributable to these: at 1302-1295 nm. (P - CH_3 antisymmetrical deformation)[268] and at 780 nm. (P - C stretch).[269]

INFRARED BANDS OF DISTEAROYLLECITHINS

ABSORPTION, nm.			ATTRIBUTION [102, 268-9]
Lecithin:			
$-NMe_3$	$-PMe_3$	$-P(n\text{-}Bu)_3$	(ν = stretch, δ = deformation, bend)
2923	2920	2924	ν C-H (antisym.) of $-(CH_2)_n-$
2860	2850	2858	ν C-H (sym.) of $-(CH_2)_n-$ and $-CH_3$
1744	1740	1746	ν >C=O
	1600		
	1560		
1470	1468	1470	δ $-(CH_2)_n-$; $-R-CH_2-$ scissors deformation
1420	1420	1418	δ C-H; N^+-CH_3 antisym. bend
1380	1378	1380	δ C-H; $R-CH_3$ sym. bend
	1302		δ $P-CH_3$ antisym.
	1295		
1254	1254	1259	
1235	1235	1240	$-(CH_2)_n-$ wag;
	1215	1220	ν P=O or ν PO_2^- (antisym.)
	1195	1198	
1170	1170	1172	ν C-O, C-O-C
1108	1100	1103	ν C-O-(P) (antisym. out of plane)
1070	1072	1070	ν PO_2^- (sym.)
1015	1010	1015	ν C-C of alkyl chain (sym.)
	990	1000	ν C-C of alkyl chain (antisym.)
972	970	962	ν C-C " " " "
	780		ν P-C (antisym.)
720	720	730	ν $-(CH_2)-$ rock
680	680		

(iii) Proton magnetic resonance.

Since the ^{31}P nucleus couples with the proton, the PMR spectra of phosphocholine and its derivatives are quite distinguishable from those of choline, with the characteristic splitting of the phosphonium methyl and α- and β-methylene protons. One interesting observation is that the coupling constant $J_{(\underline{P}.C.\underline{CH_2})}$ is greater than $J_{(\underline{P}.\underline{CH_2}.C)}$; thus for phosphocholine:



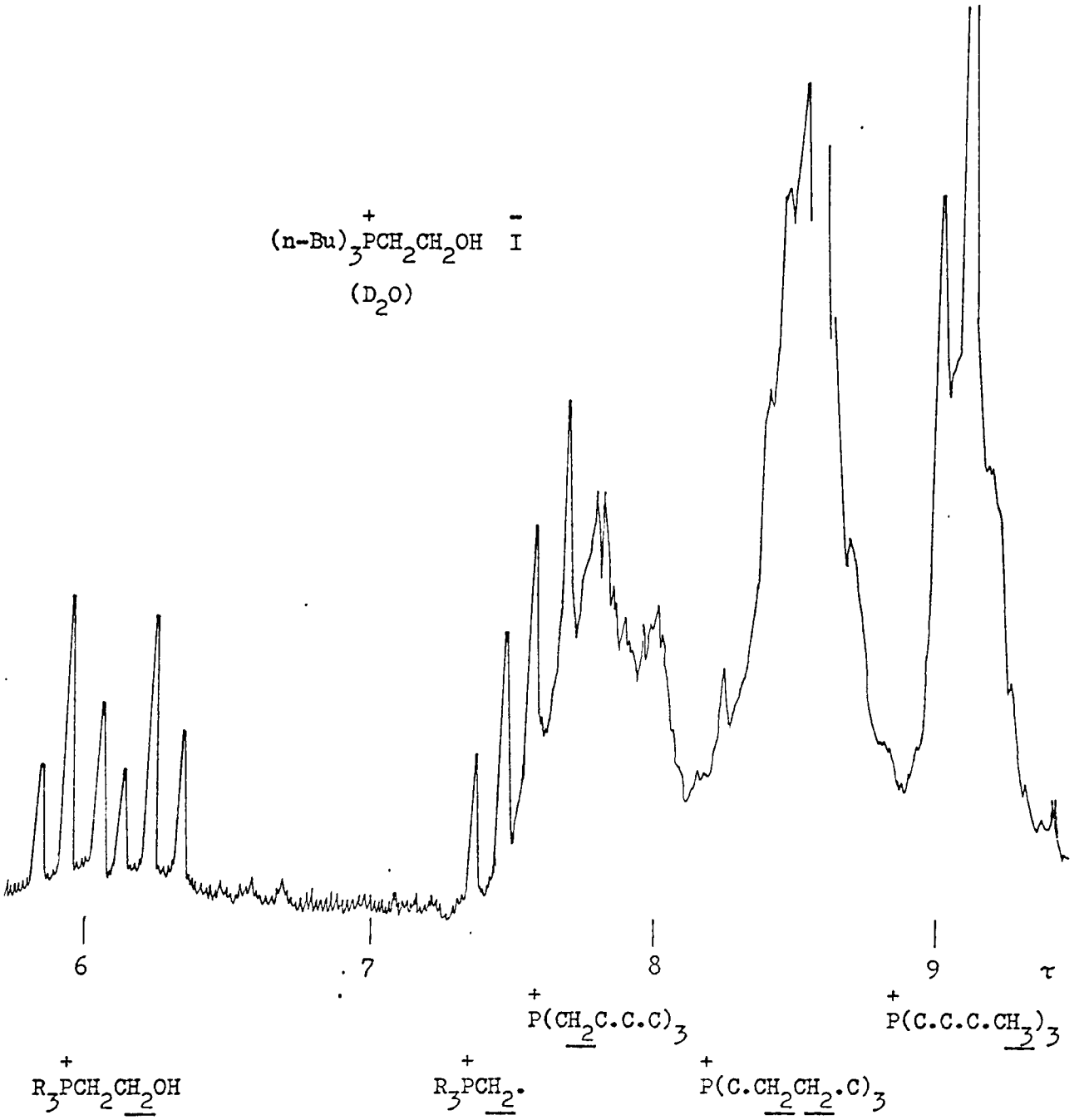
$Me_3\overset{+}{P}CH_2\underline{CH_2}OH$ $\qquad$ $Me_3\overset{+}{P}\underline{CH_2}C$ $\qquad$ $(\underline{CH_2})\overset{+}{P}$

The signals at 6τ and 7.5τ are pairs of triplets, although those at 7.5τ overlap. The coupling constants may be compared with those measured by

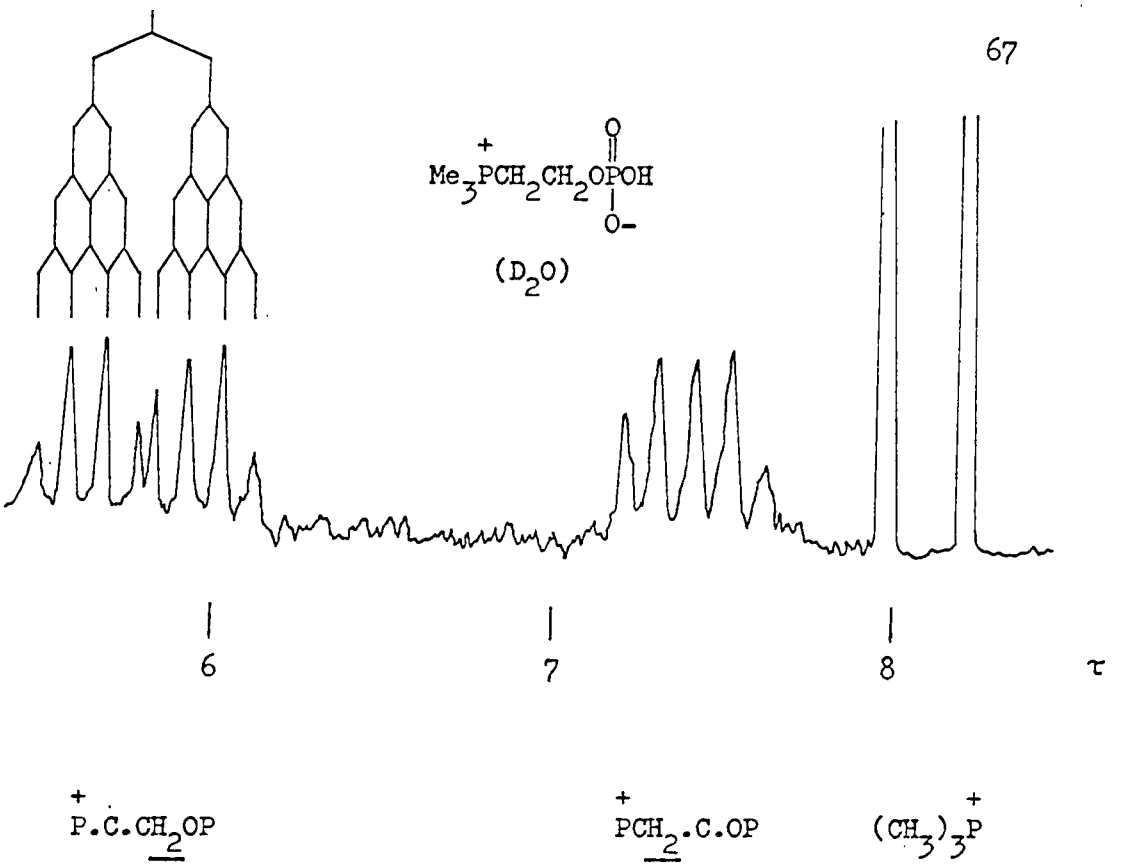
Massey *et al.*[270] for the $Me_3\overset{+}{P}Et$ ion:

	$Me_3\overset{+}{P}CH_2CH_2OH$	$Me_3\overset{+}{P}CH_2CH_3$ [270]
	Hz	Hz
$J_{(P.CH_3)}$	14.4	14.5
$J_{(P.CH_2.C)}$	12.9	14.0
$J_{(P.C.CH_2)}$	20.0	20.6
$J_{(H.H)}$	6.2	7.4

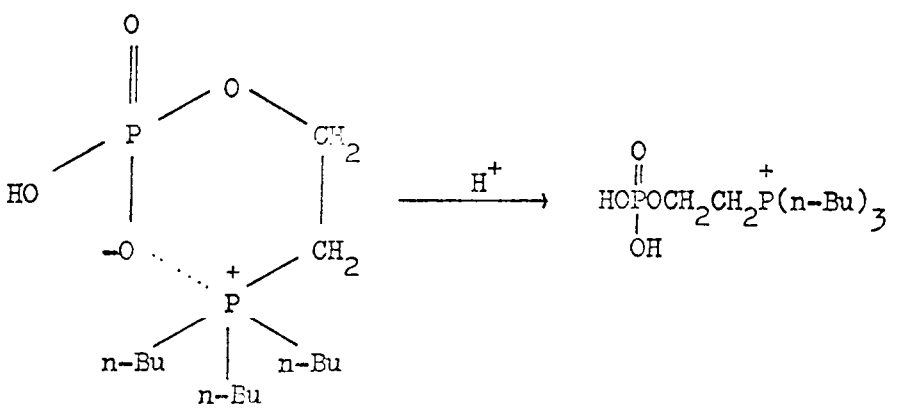
The spectrum of 2-hydroxyethyltri-n-butylphosphonium iodide is similar, although the α-methylene signals of the n-butyl and 2-hydroxyethyl groups overlap, and the spectrum is therefore more complex:

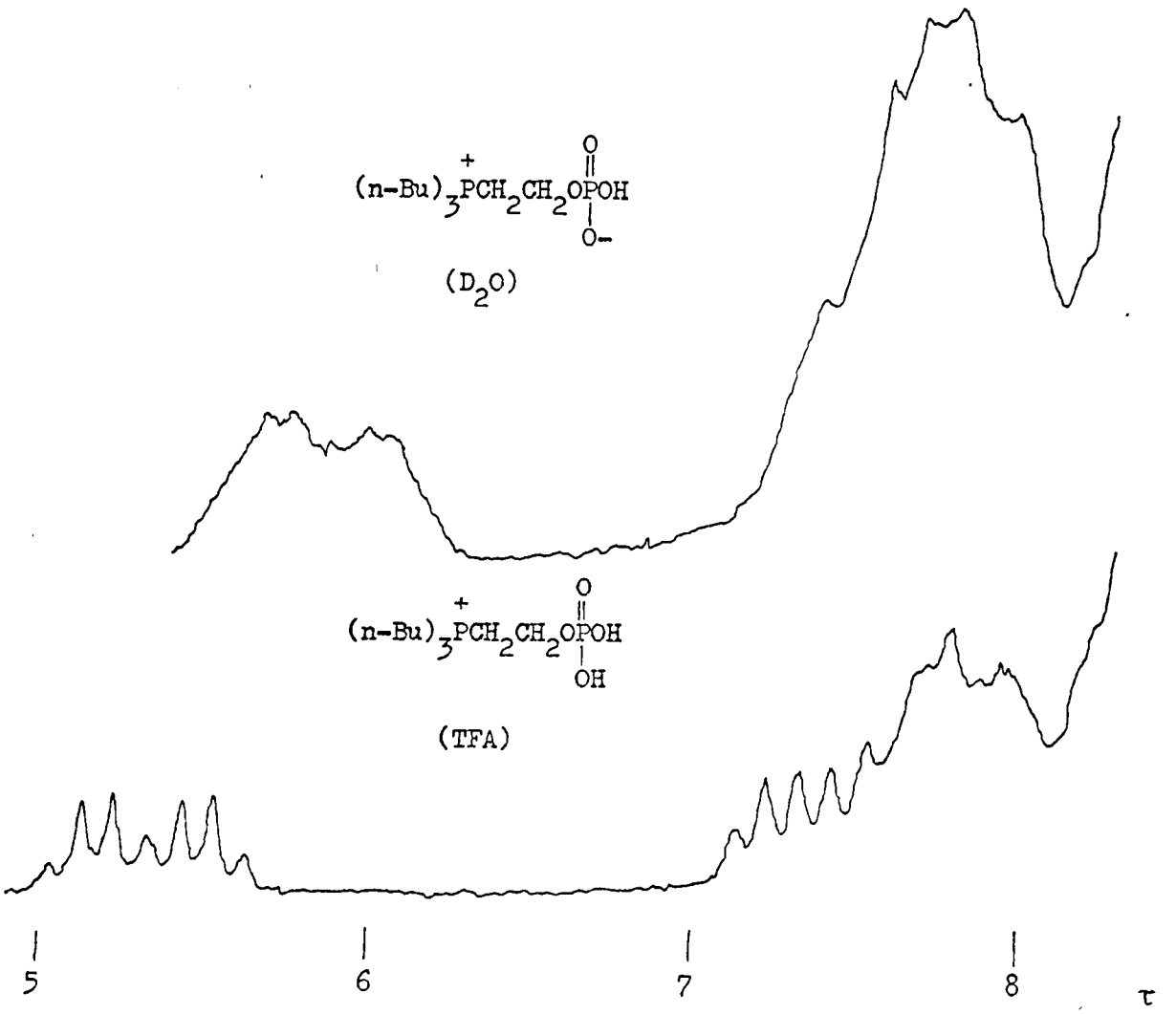


Introduction of a second phosphorus atom into the molecule, by phosphorylation of phosphocholine, causes further splitting of the β-methylene signal, to a pair of quartets:



With the tri-n-butylphosphonium derivative, the resolution is greatly increased when the solvent is changed from deuterium oxide to trifluoracetic acid. It is possible that in neutral solution the compound assumes a cyclic conformation, which is only disrupted by protonation in the acid:

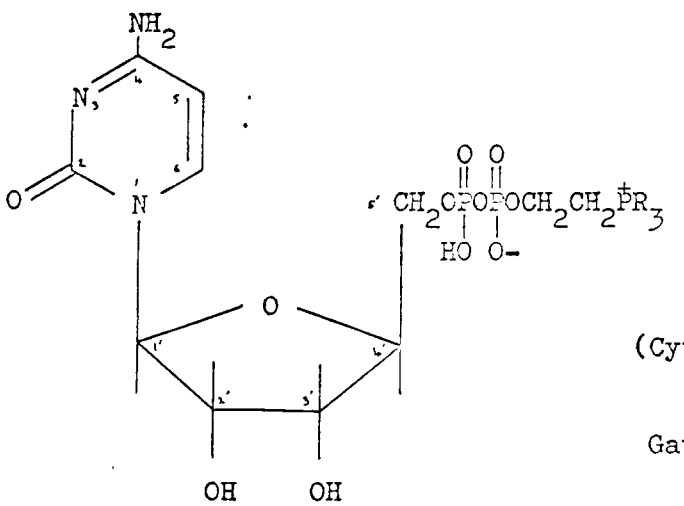




In the more complex spectra of the CDP-choline and lecithin analogues, the distinguishing feature is the doublet of the trimethylphosphonium protons, and this later provided useful corroboration when the phosphatidyl-phosphocholine structure was attributed to certain lipid extracts of rat organs.

PMR spectra of phosphonium analogues of CDP-choline (D_2O)

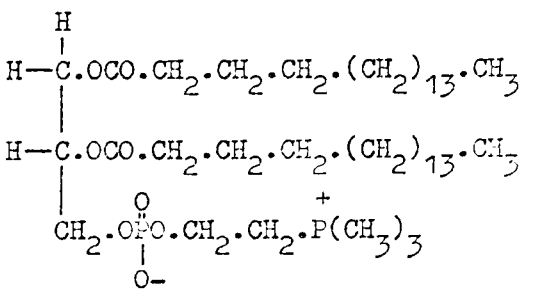
ASSIGNATION	DESCRIPTION	CHEMICAL SHIFT (τ)		J (Hz)
		$-\underline{P}Me_3$ (60 MHz)	$-\underline{P}(n\text{-}Bu)_3$ (90 MHz)	
H_6	Doublet	1.81 - 1.94	1.78 - 1.88	7.7
H_5	Doublet	3.81 - 3.93	3.78 - 3.88	3.0
$H_{1'}$	Broad singlet	4.10	4.08	—
$H_{2'}$, $H_{3'}$, $H_{4'}$, + P.C.$\underline{CH_2}$, $2H_{5'}$, +	Complex	5.53 - 6.05	5.50 - 6.00	—
P.$\underline{CH_2}$.C +	2 triplets	7.19 - 7.60	7.27 - 7.56	12.5
$P(\underline{CH_2})_3$ +	Doublet	7.97 - 8.22	————	14.4
$P(n\text{-}Bu)_3$	Complex	————	7.56 - 9.19	—



(Cytidine assignations from Gatlin and Davis.)[271]

270 MHz PMR spectrum of phosphatidylphosphocholine (LI)

ASSIGNATION	CHEMICAL SHIFT (τ)	
	Phosphatidyl-phosphocholine	Phosphatidyl-choline (220 MHz)[114]
CH.OCO	4.82	4.81
CH_2.OCO	5.68, 5.92	5.62, 5.90
N.C.CH_2OP	——	5.71
P.C.CH_2OP	5.78	——
glycerol.CH_2OP	6.08	6.07
NCH_2.C.OP	——	6.15
$N(CH_3)_3$	——	6.65
PCH_2.C.OP	7.30	——
2 CH_2CO_2	7.75	7.73
$P(CH_3)_3$	7.97 - 8.03 *	——
2 CH_2.C.CO_2	8.37	8.40
2 CH_2.C.C.CO_2	8.58	——
2 $-(CH_2)_n$	8.76	8.77
2 stearate $-CH_3$	9.13	9.12



(LI)

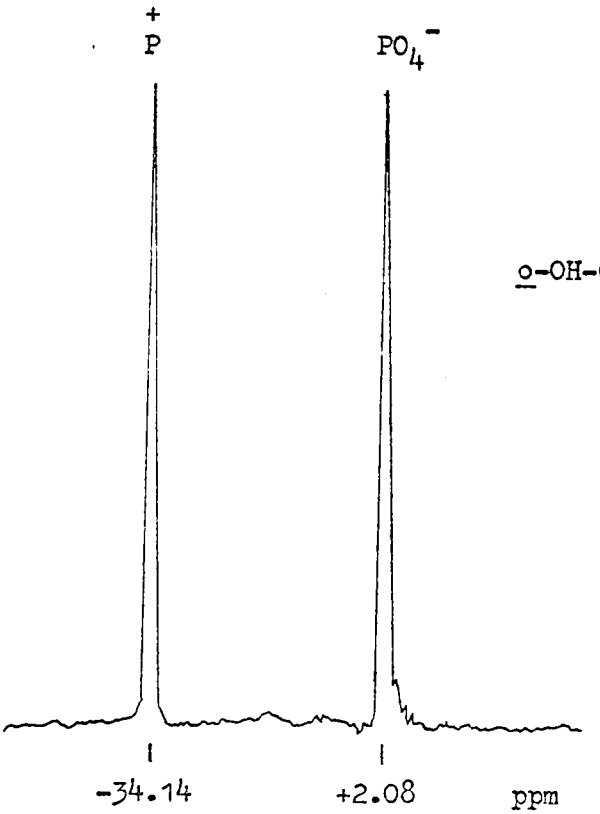
*J = 14.7 Hz

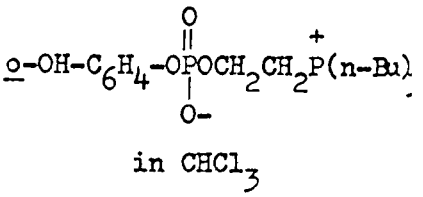
(iv) Phosphorus-31 nuclear magnetic resonance.

Phosphorus-31 is the only naturally-occurring isotope of phosphorus, and has a nuclear spin of $\frac{1}{2}$. Because of a smaller magnetic moment, the NMR sensitivity is only 6.64 per cent of that of the proton at constant magnetic field. Nevertheless, the ^{31}P nucleus is well suited for the application of high resolution techniques because it gives rise to sharp signals; since possible chemical shifts extend over a range of ca. 500 ppm for trivalent compounds, and ca. 100 ppm for pentavalent, conditions may be used which favour high sensitivity at the expense of resolution.

Spectra were obtained by the pulse Fourier transform technique, at an operating frequency of 84.5 MHz; chemical shifts were measured in parts per million (ppm) relative to an 87 per cent phosphoric acid external reference (84.570000 MHz).

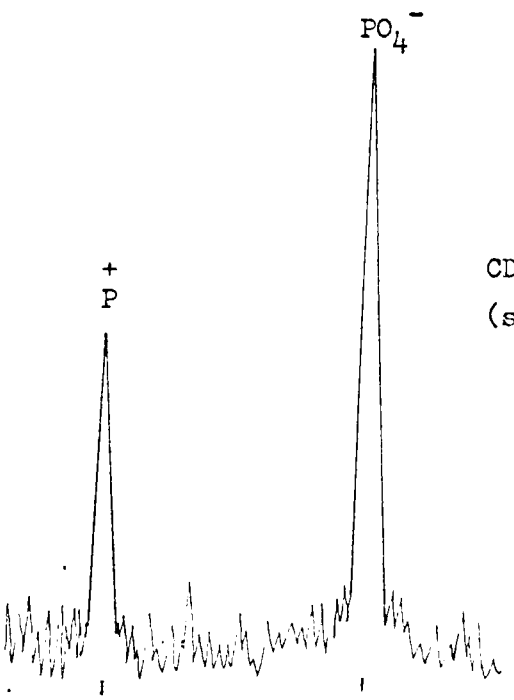
The spectra of all phosphonium compounds prepared in this work were measured; PMe_3^+ was found to absorb in the region -26 to -30 ppm, and $P(n\text{-}Bu)_3^+$ at -34 to -35 ppm. With solutions of phosphorylphosphonium derivatives, two sharp signals of equal area were obtained:





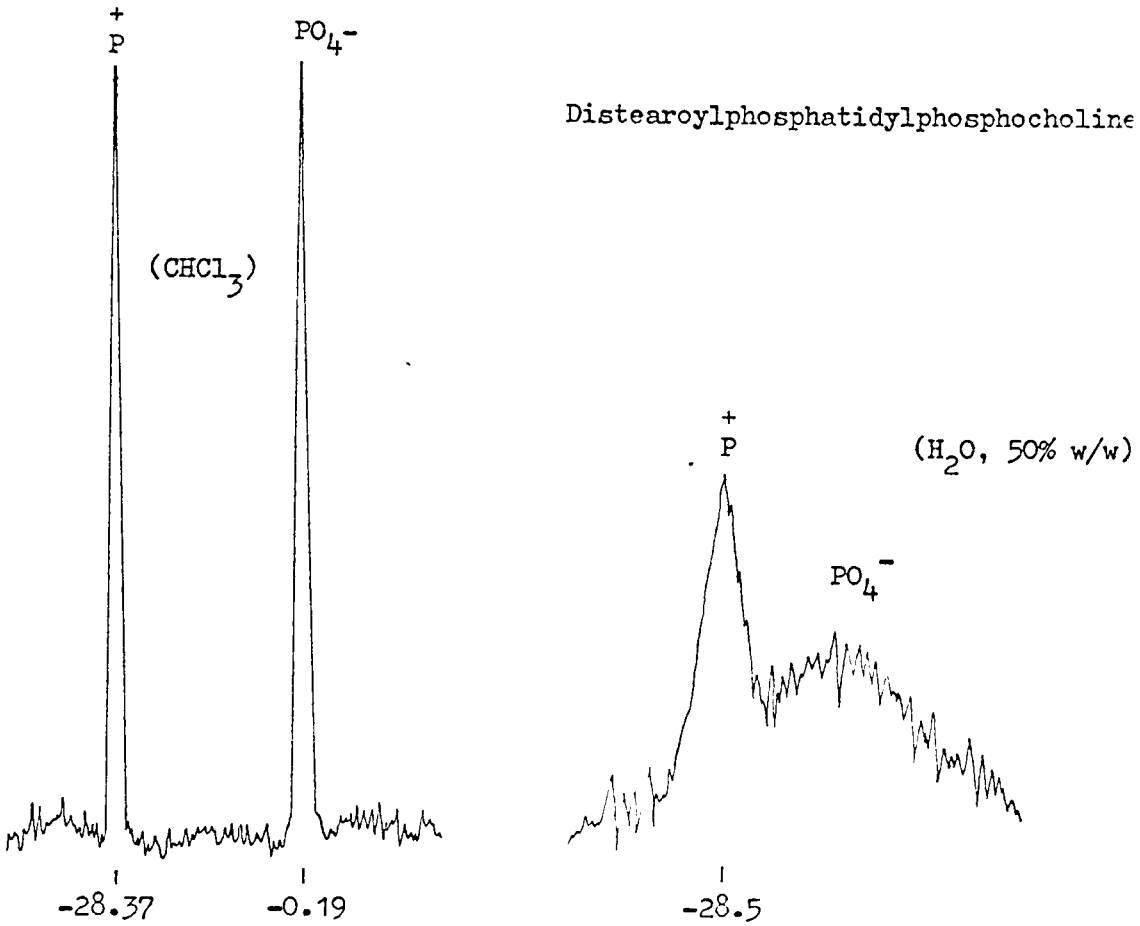
in $CHCl_3$

except for the CDP derivatives, in which the two phosphate groups were equivalent, giving rise to a signal twice the area of that from phosphonium:



CDP-phosphocholine (sodium salt) in H_2O

Spectra were recorded for phosphatidylphosphocholine both in chloroform solution and in aqueous dispersion (50% w/w):

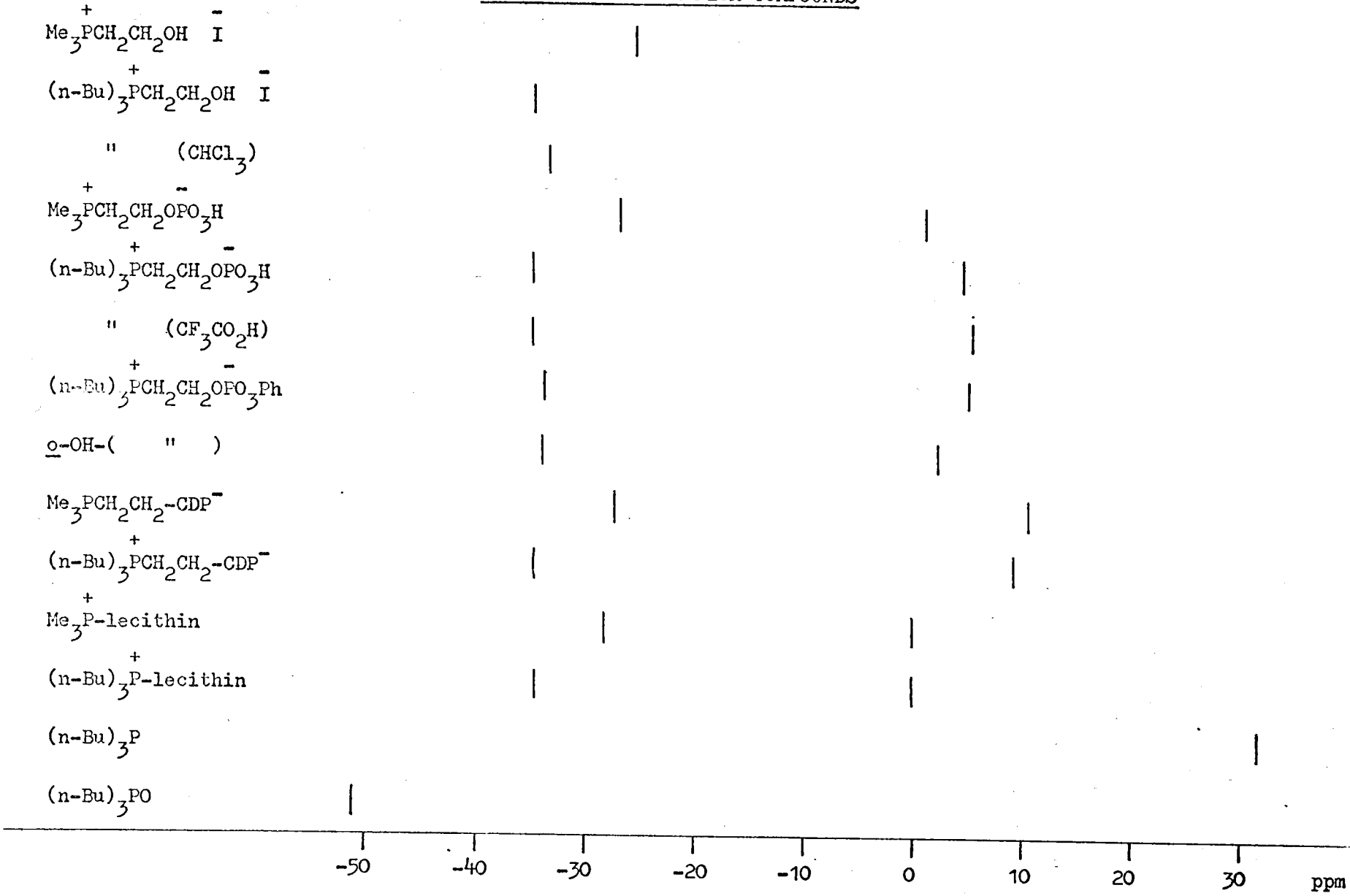


Thus in aqueous dispersion there is disproportionate line-broadening, far more for phosphate than for phosphonium; this would appear to corroborate the X-ray work of Phillips et al.[64] who have shown that in phosphatidylcholine the head-group is extended normal to the bilayer, rather than tangentially. If the latter were the case, the phosphonium signal would be broadened considerably more through spin-spin relaxation associated with the

^{31}P-NMR of phosphonium derivatives

COMPOUND	SOLVENT	CHEMICAL SHIFTS (ppm)		
		P^+	PO_4^-	$\Delta_{PO_4-P^+}$
$Me_3\overset{+}{P}CH_2CH_2OH\ \overset{-}{I}$	H_2O	-25.9		
$(n\text{-}Bu)_3\overset{+}{P}CH_2CH_2OH\ \overset{-}{I}$	H_2O	-35.0		
"	$CHCl_3$	-33.7		
$Me_3\overset{+}{P}CH_2CH_2O\overset{-}{P}O_3H$	H_2O	-27.2	-0.8	26.4
$(n\text{-}Bu)_3\overset{+}{P}CH_2CH_2O\overset{-}{P}O_3H$	$CHCl_3$	-35.0	+4.2	39.2
"	CF_3CO_2H	-35.0	+5.0	40.0
$(n\text{-}Bu)_3\overset{+}{P}CH_2CH_2O\overset{-}{P}O_3Ph$	$CHCl_3$	-34.0	+4.8	38.8
o-OH-(")	$CHCl_3$	-34.1	+2.1	36.2
$Me_3\overset{+}{P}CH_2CH_2$-CDP$^-$	H_2O	-27.4	+10.4	37.8
$(n\text{-}Bu)_3\overset{+}{P}CH_2CH_2$-CDP$^-$	H_2O	-34.9	+9.1	44.0
$Me_3\overset{+}{P}$-lecithin	$CHCl_3$	-28.4	-0.2	28.2
$(n\text{-}Bu)_3\overset{+}{P}$-lecithin	$CHCl_3$	-34.6	-0.2	34.4
$(n\text{-}Bu)_3P$	neat	+31.3		
$(n\text{-}Bu)_3PO$	$CHCl_3$	-51.8		

^{31}P-NMR OF PHOSPHONIUM COMPOUNDS



Grim et al.[272] have published tables correlating the chemical shifts of phosphonium salts with the nature of their substituents. Thus in dimethylsulphoxide solution the predicted contributions are:

GROUP	SHIFT CONTRIBUTION (ppm)
Me	-6.3
Et	-10.1
n-Bu	-8.5

giving total shifts of -35.6 ppm for $(n\text{-Bu})_3\overset{+}{P}Et$ and -28.9 ppm for $Me_3\overset{+}{P}Et$. Allowing for an overall factor of +2.2 ppm in changing to aqueous solvent:

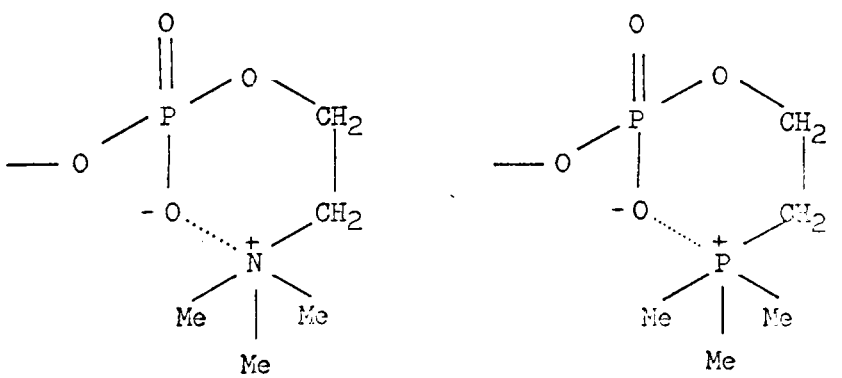
	PREDICTED SHIFT	OBSERVED SHIFT (ppm)
$(n\text{-Bu})_3\overset{+}{P}Et$	-33.4	
$(n\text{-Bu})_3PCH_2CH_2OH$		-35.0
$Me_3\overset{+}{P}Et$	-26.7	
$Me_3\overset{+}{P}CH_2CH_2OH$		-25.9

a fair agreement is reached with such empirically-derived correlations.

(v) Mass spectra of phosphatidylcholine and phosphatidylphosphocholine.

The mass spectra of distearoylphosphatidylcholine and its phosphorus analogue were very similar, corresponding essentially to the fragmentation patterns described by Klein.[273-5] With the spectrometer operating in the normal mode, the observed fragmentation was that of distearin, since loss of the phosphoryl-base moiety would have occurred well before the molecular ion reached the collector and possibly even before it left the source.

According to Klein,[275] the molecular ions are of such low intensity that they may only be recorded with the spectrometer operating in the defocused mode. Unfortunately this was not possible, although it was hoped that such an operation might yield information about the possible relative internal stabilization of the phosphorylcholine and phosphoryl-phosphocholine groups:



(Cf. chapter 2 (iii) and chepter 3 (iv).)

(vi) Electron microscopy of liposomes of distearoyllecithins.

The normal method of preparation of aqueous lecithin dispersions for physical studies is by prolonged sonication at high amplitude ; this procedure has been developed for natural lecithin, which contains a high proportion of unsaturated fatty acids, but dispersion becomes more difficult the longer and more saturated the fatty acid chains. Thus great difficulty was encountered in preparing suitable dispersions of unnatural, saturated distearoyllecithin and its phosphonium analogues. Initial attempts involved sonication under argon for periods of 30 to 60 minutes; undispersed lipid and titanium particles from the probe were then removed by centrifugation. Negatively-stained samples of these dispersions, when viewed in the electron microscope, revealed only very large lipid aggregates and no small vesicles.

Moreover, the chemical consequences of prolonged sonication remain controversial,[83-5] although Huang and Charlton[85] claimed that there was no detectable difference in the chemical state of lecithin molecules before and after treatment by ultrasonic irradiation followed by gel filtration.

A very recent paper by Batzri and Korn[276] described the preparation of single bilayer liposomes without sonication, by the injection of an ethanolic solution of phospholipid into water; egg-yolk lecithin liposomes

prepared in this way were apparently indistinguishable from those prepared by sonication and gel filtration. The method was therefore adopted for the preparation of synthetic lecithin liposomes, although with these distearoyl derivatives it was found necessary to use hot (60-70°) solutions and a pre-heated syringe. Samples were injected directly into solutions of negative stain (1% aqueous sodium phosphotungstate solution, pH 7.0) as suggested by Batzri and Korn, thus preparing them for electron microscopy in a single stage. However, the 10 per cent of stearylamine added to lipid samples by these workers was omitted, as it was thought that the resulting liposomes would not necessarily be truly representative.

Positively-stained samples were also prepared, by injecting ethanolic solutions into water, fixing with osmium, mounting in resin and thin-sectioning, and were compared with a sample of "natural" phosphatidyl-phosphocholine extracted from rat brain (Chapter 5 (vi)).

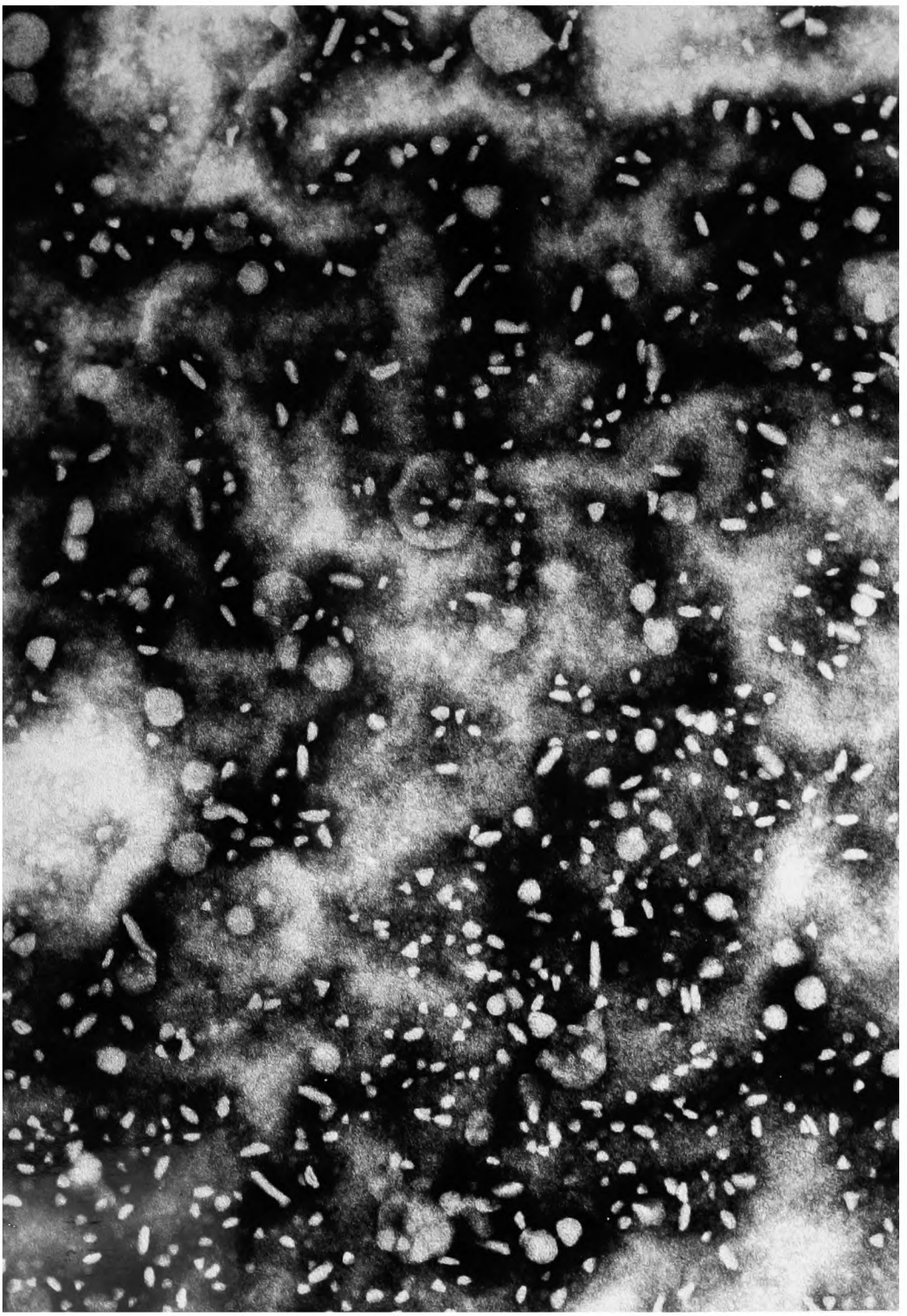
Electron micrographs are shown on the following pages. The negatively-stained samples of distearoylphosphatidylcholine and distearoylphosphatidyl-phosphocholine dispersions are very similar, consisting mainly of single-bilayer liposomes of circular and elliptical cross-section, with a predominance of the latter in the phosphonium sample. The liposomes of the tri-n-butylphosphonium analogue, however, were mainly long and thin in cross-section; although a single-bilayer structure was evident at higher magnification, the overall appearance was of collapsed vesicles.

The positively-stained thin-sectioned liposomes were mainly circular in cross-section, although the average diameter of those of the tri-n-butylphosphonium analogue was appreciably greater (850-1000Å) than the others, (250-320 Å). Liposomes of rat-brain phosphatidylphosphocholine were of intermediate diameter (550-600 Å).

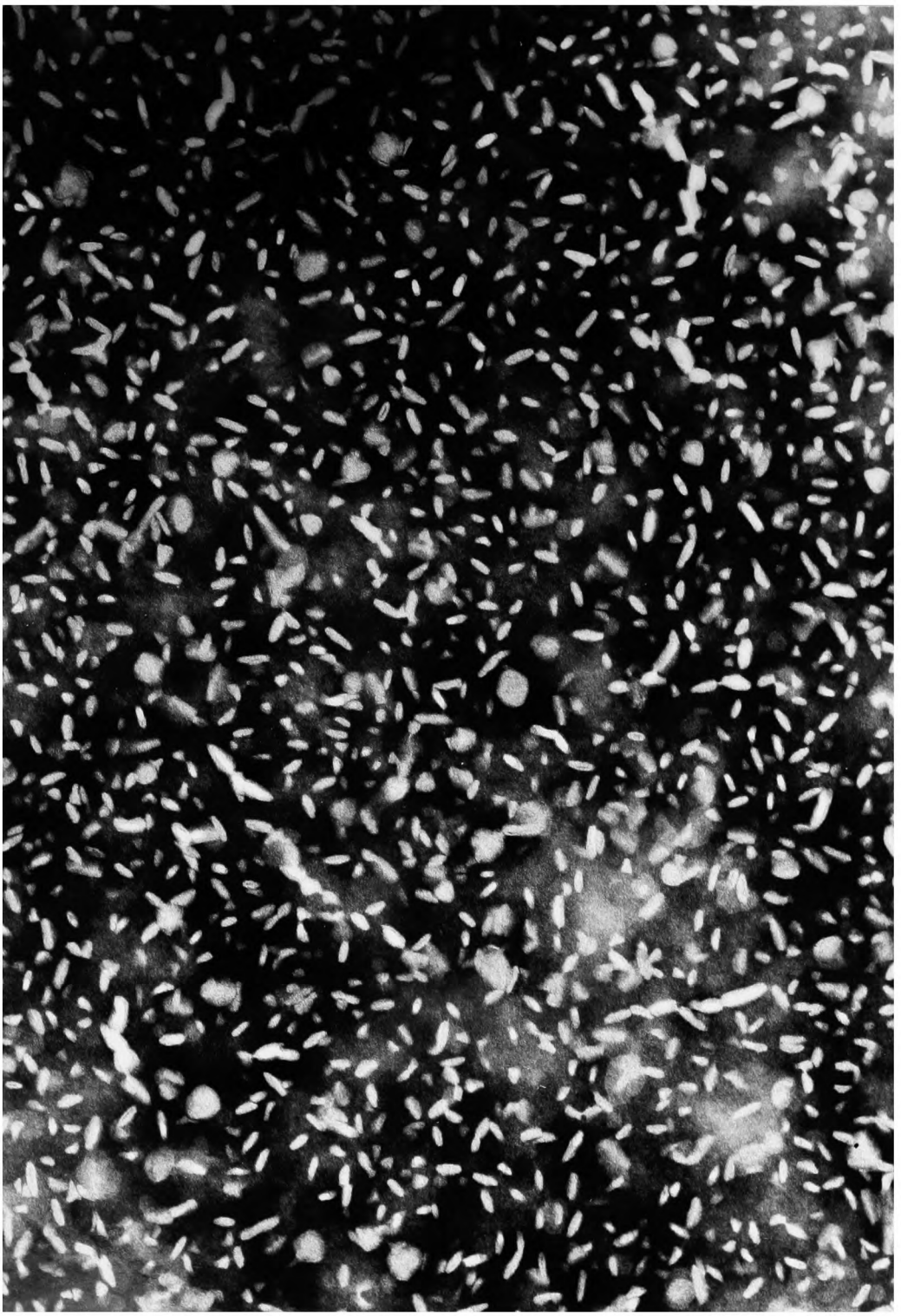
Thus even the tri-n-butylphosphonium analogue of lecithin, with its bulky head-group probably possessing a significant amount of hydrophobic character, will form single-bilayer liposomes, although these differ considerably, both in size and shape, from those of the trimethyl derivatives.

Key to electron micrographs:

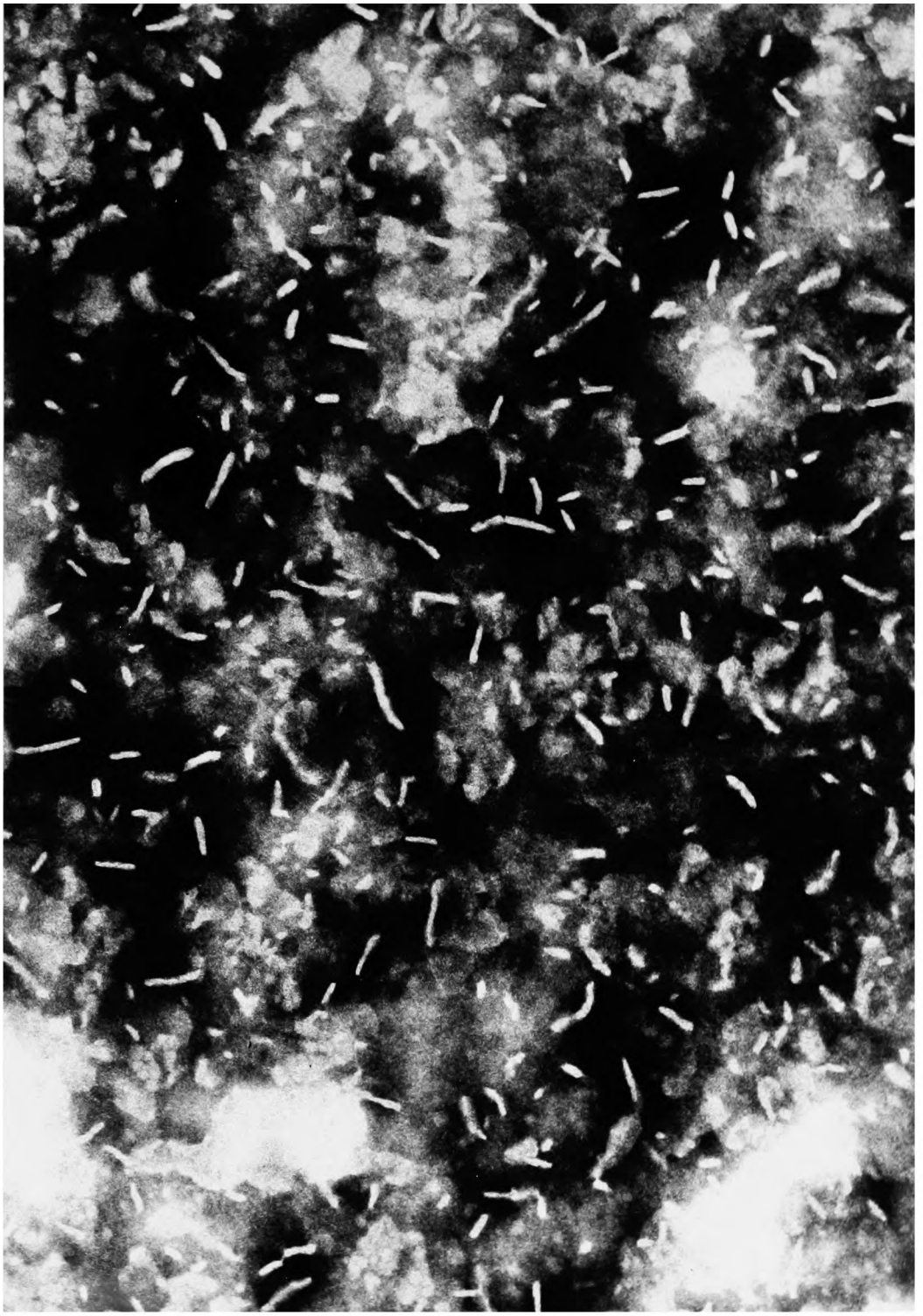
PHOTOGRAPH	LECITHIN		STAIN	MAGNIFICATION
A	Synthetic	$-\overset{+}{N}Me_3$	-	120000
B	"	$-\overset{+}{P}Me_3$	-	120000
C	"	$-\overset{+}{P}(n\text{-}Bu)_3$	-	120000
D	"	$-\overset{+}{N}Me_3$	+	20000
E	"	$-\overset{+}{P}Me_3$	+	20000
F	"	$-\overset{+}{P}(n\text{-}Bu)_3$	+	20000
G	Rat brain	$-\overset{+}{P}Me_3$	+	20000



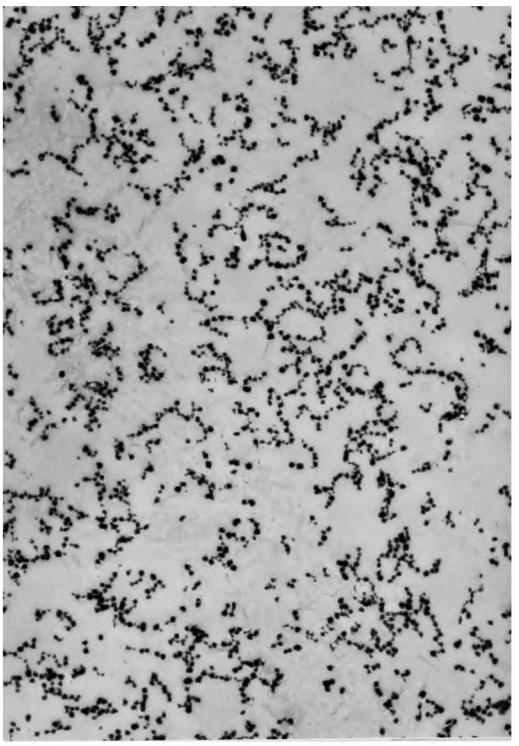
A

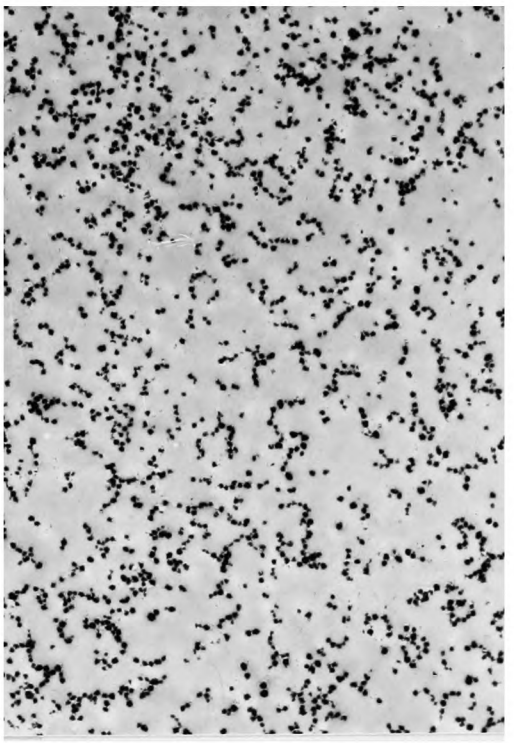


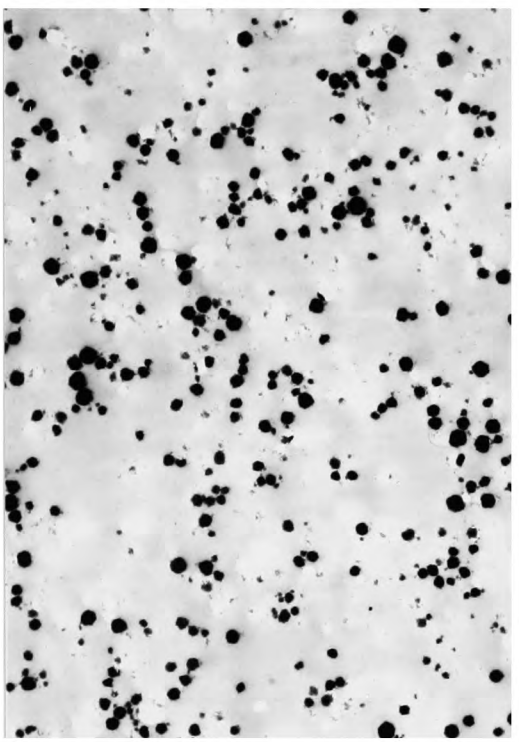
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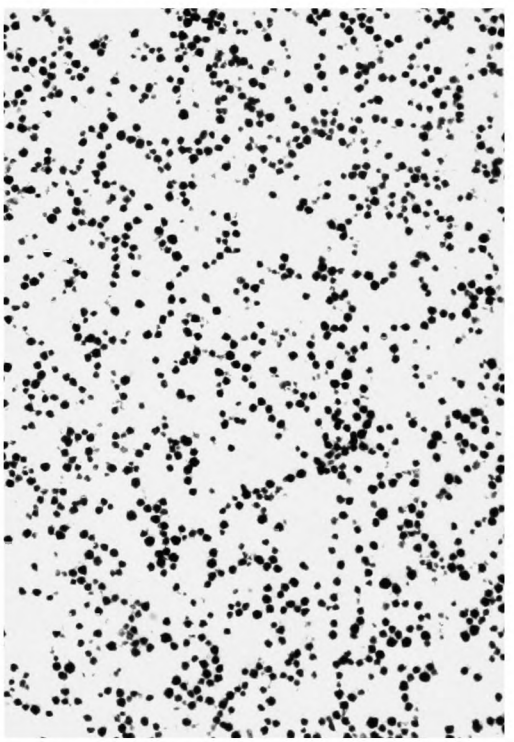


C









D

E

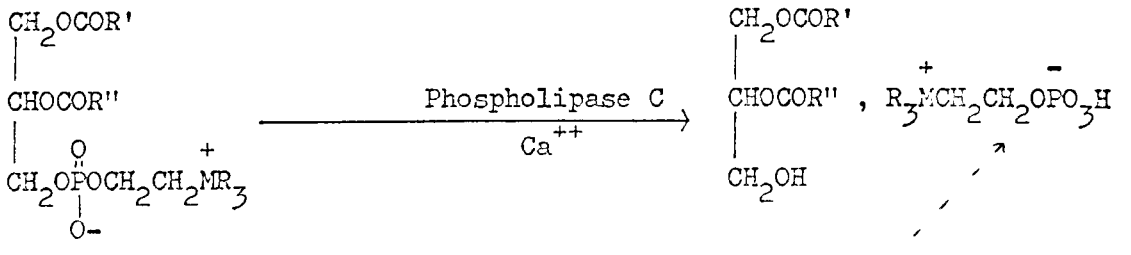
F

G

(vii) Hydrolysis of distearoyllecithin and phosphorus analogues by phospholipase C.

Several preliminary experiments were carried out to determine the optimum conditions for attack on the distearoyllecithins by phospholipase C; difficulties were initially encountered in producing satisfactory lipid dispersions, but these were finally overcome by the use of a detergent.

Chloroform solutions of egg-yolk lecithin, distearoyl-DL-phosphatidylcholine, and its trimethylphosphonium and tri-n-butylphosphonium analogues, each containing the detergent cetyltrimethylammonium bromide, were evaporated to dryness and dispersed by 10 seconds of sonication in aqueous tris buffer. Calcium chloride and enzyme solutions were added, and the reaction mixtures were sampled over a period of an hour. These samples were extracted with a chloroform-methanol-water system, and aqueous and organic phases were assayed for phosphate colorimetrically.



Organic phase

Aqueous phase

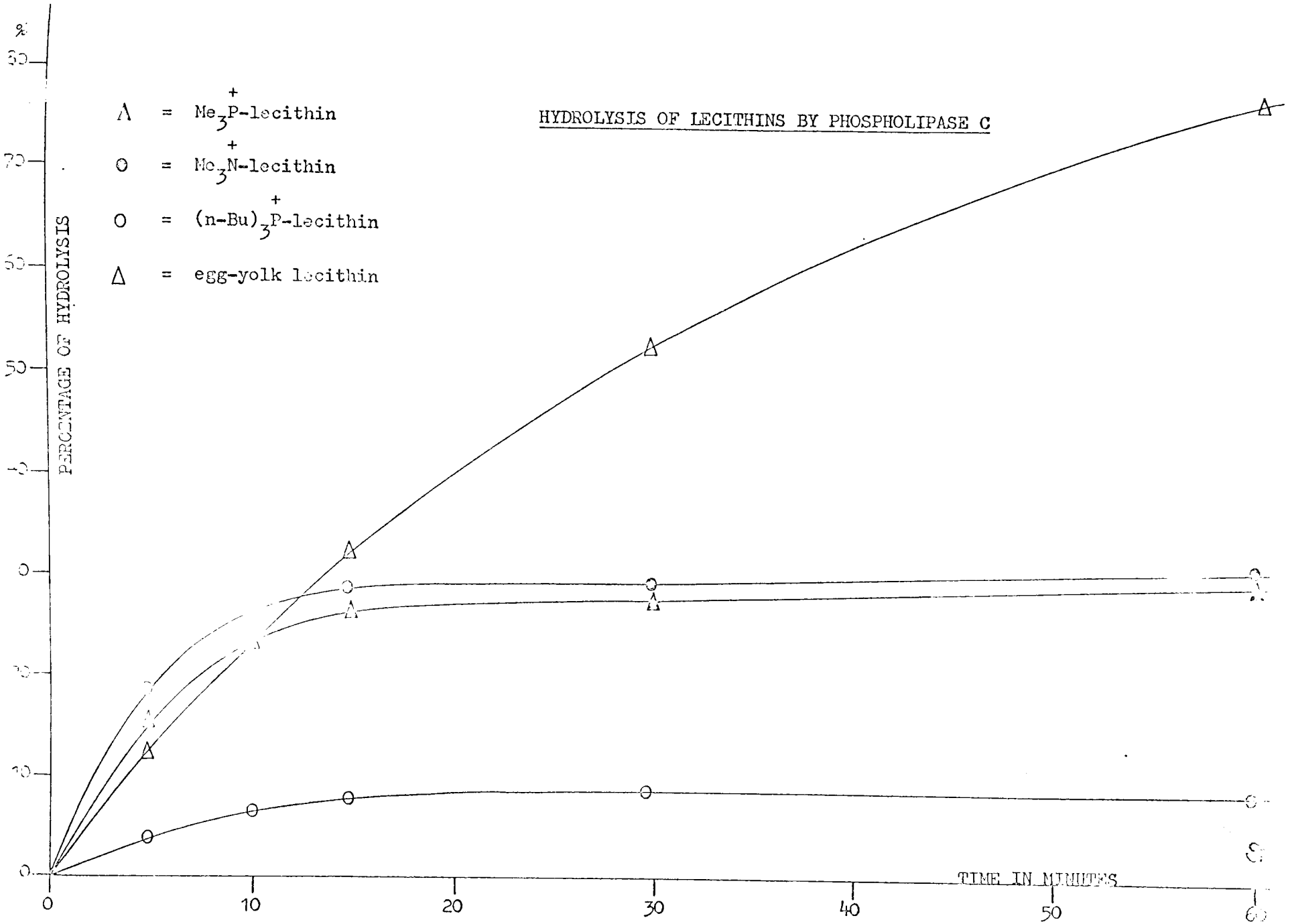
(M = N or P; R = Me or n-Bu)

Thus for each sample, the quantity of phosphate in the aqueous phase divided by total phosphate gave the degree of hydrolysis; the latter was plotted as a function of time.

The initial rates of hydrolysis of egg lecithin, distearoyllecithin and its trimethylphosphonium analogue were very similar, ca. 5 μ mole per minute per mg. enzyme, while that of the tri-n-butylphosphonium analogue was appreciably slower at ca. 1.2 μ mole per minute per mg. enzyme. However, after 1 hour the hydrolyses of all synthetic lecithins had reached a limiting value, whilst that of egg lecithin was still proceeding at 20 per cent of the original rate.

The major inference to be drawn from the experiment was that the action of phospholipase C on distearoyl-DL-phosphatidylcholine and -phosphatidylphosphocholine was almost identical, both in initial rate and final degree of hydrolysis, whereas the tri-n-butylphosphonium analogue is a far less acceptable substrate. Thus the change in the alkyl substitution of the quaternary group has a far greater effect in this case than the change from ammonium to phosphonium.

LECITHIN	INITIAL RATE OF HYDROLYSIS	PERCENTAGE OF SUBSTRATE HYDROLYZED AFTER 1 HR.
Egg-yolk	4.2 μmole. $min.^{-1}$ $mg.^{-1}$ enzyme	77.1%
Distearoyl, $-\overset{+}{N}Me_3$	5.7 " " "	31.1%
Distearoyl, $-\overset{+}{P}Me_3$	4.8 " " "	29.5%
Distearoyl, $-\overset{+}{P}(n\text{-}Bu)_3$	1.2 " " "	8.9%


HYDROLYSIS OF LECITHINS BY PHOSPHOLIPASE C
Λ = Me3P+-lecithin
O = Me3N+-lecithin
O = (n-Bu)3P+-lecithin
Δ = egg-yolk lecithin
PERCENTAGE OF HYDROLYSIS
%
TIME IN MINUTES
0
10
20
30
40
50

CHAPTER 3 : IN VITRO METABOLISM OF PHOSPHOCHOLINE BY THE ENZYMES OF THE CYTIDINE PATHWAY

(i) Choline phosphokinase.

The enzyme catalyzing the phosphorylation of choline in the presence of ATP:

$$\text{ATP} + \text{CHOLINE} \longrightarrow \text{PHOSPHORYLCHOLINE} + \text{ADP}$$

was first isolated from yeast by Wittenberg and Kornberg[3] in 1953, and a similar preparation was used in the following experiments. It was reported[3] that this partially purified yeast enzyme possessed a rather general capacity to phosphorylate a series of compounds chemically related to ethanolamine; based on the ability to phosphorylate choline, its affinity for dimethylaminoethanol was 70%, for monomethylaminoethanol 20%, and for ethanolamine 7%. It was inactive towards serine, ethanol and ethylene glycol.

It was therefore decided to investigate the action of the enzyme on phosphonium analogues of choline. Once an enzyme preparation had been obtained from Brewer's yeast, a preliminary experiment was performed

to standardize it against choline, and to ascertain its affinity for the phosphonium analogues. Four incubation mixtures were prepared, each containing choline phosphokinase, magnesium ion, L-cysteine, ATP and buffer (pH 8.5). To the first was added choline chloride (I), to the second phosphocholine chloride (XXIVa), to the third 2-hydroxyethyltri-n-butylphosphonium chloride (LIIIa), and the fourth was left as a control. The mixtures were incubated at room temperature for 2 hours, deproteinized and passed through a (H)/(formate) mixed-bed ion-exchange column to isolate any phosphate inner salts formed.

The first problem encountered was the choice of assay method. Wittenberg and Kornberg[3] had determined kinase activity by measuring the ability of the enzyme to convert choline to a form not determined by the iodine method of Appleton et al.,[277] involving the precipitation of an iodine complex of the unreacted choline in the incubation mixture. Since they found this method to have an error of up to 15%, it was hardly satisfactory. They also estimated the formation of phosphorylcholine by measuring the liberation of free choline from the final reaction mixture by the enzyme semen phosphatase. Since this assay involved the introduction of a second enzyme system, with an unknown affinity for analogues of phosphorylcholine, in order to measure the activity of the first enzyme system, it was deemed unsatisfactory, and new methods were sought.

An obvious solution seemed to be chemical hydrolysis of the reaction products, followed by routine phosphate estimation. However, other workers[252, 278-84] had found that phosphorylcholine was remarkably stable and resistant to hydrolysis; thus even in 2N hydrochloric acid in sealed tubes at 125° and optimum pH (ca. 3.5), hydrolysis is found to be only 80% complete after 12 hours.[284] Moreover, an oxidative method such as that of Dyatlovitskaya et al.,[285] using 1:1 concentrated sulphuric acid and 72% perchloric acid, might cause difficulties in the determination of phosphate from phosphorylphosphocholine, due to an indeterminate degree of oxidation of the phosphonium group.

The procedure eventually adopted was a modified version of the method of Hayashi et al.,[286] involving the precipitation of a phosphorylcholine-molybdenum blue complex in the presence of stannous chloride, followed by optical density measurements of a solution of the complex in acetone/hydrochloric acid. Preliminary work showed this to be equally effective for the estimation of phosphorylcholine and its trimethylphosphonium and tri-n-butylphosphonium analogues.

Application of this assay method to the enzyme reaction mixtures gave the following values for the degree of phosphorylation after 2 hours:

SUBSTRATE	PERCENTAGE PHOSPHORYLATED
Choline chloride	46%
Phosphocholine chloride	46%
2-hydroxyethyltri-n-butylphosphonium chloride	2.9%

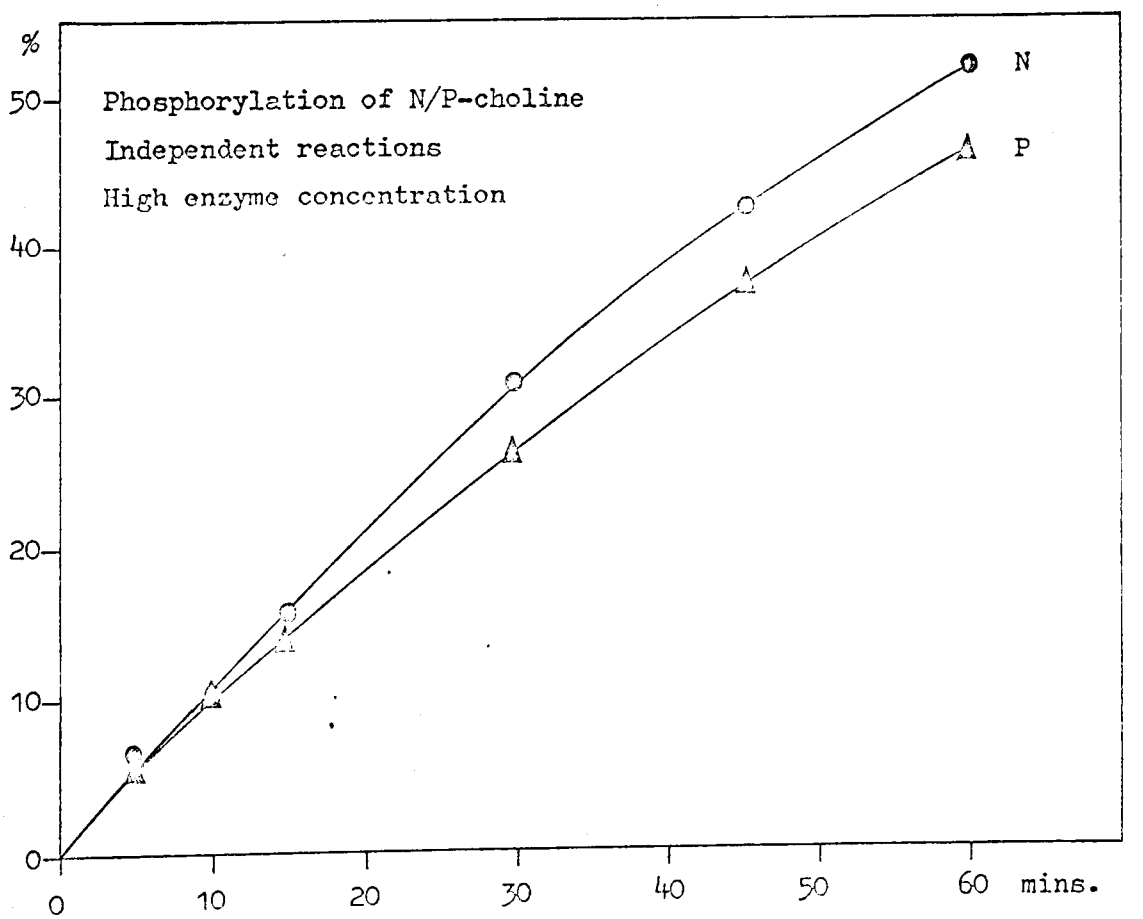
In addition, the PMR spectra of the reaction products were measured, and were found to be identical to those of the synthetic phosphorylated bases.

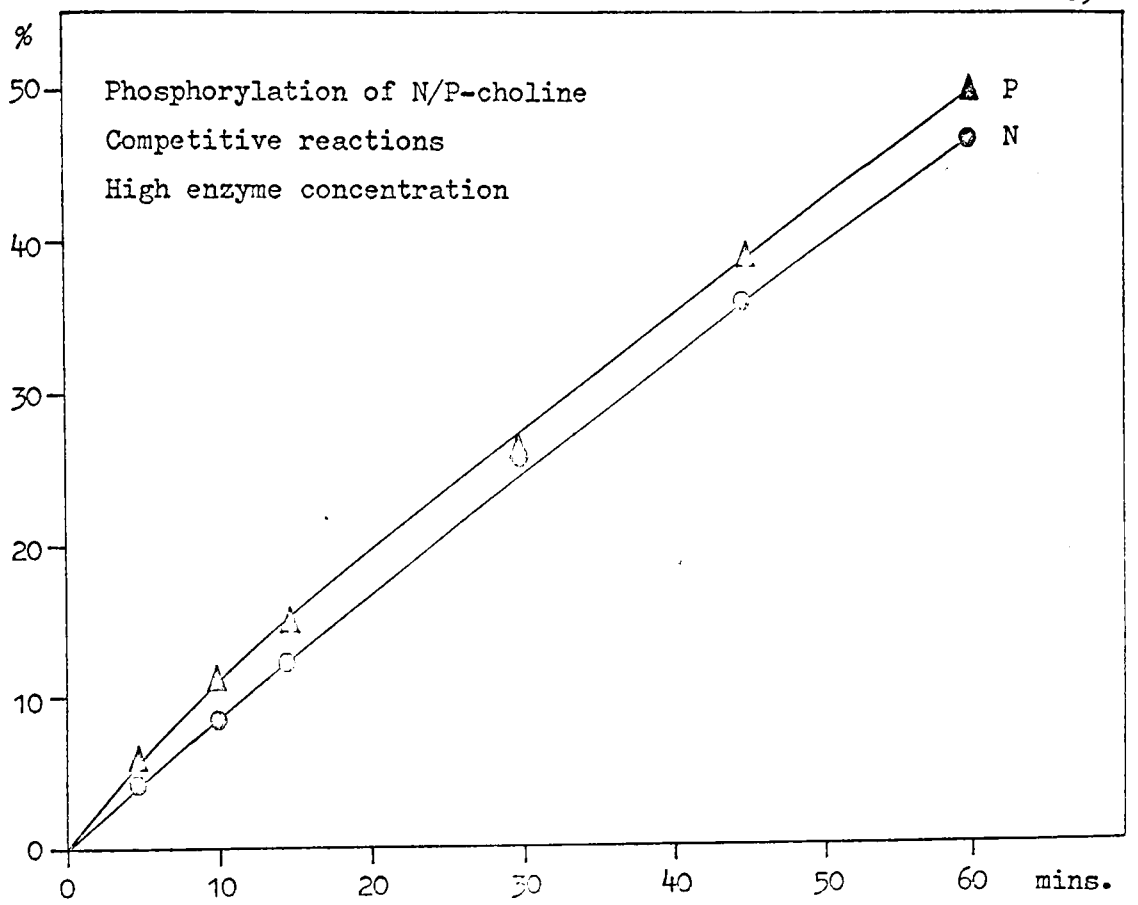
On the basis of these preliminary results, it appeared that choline phosphokinase behaved identically towards choline and phosphocholine, although it was far more discriminating towards the much bulkier 2-hydroxyethyltri-n-butylphosphonium species.

A series of time-course experiments, involving only choline and phosphocholine, and varied enzyme concentrations, were then carried out. By using tritium-labelled choline and carbon-14 -labelled phosphocholine, it was possible to monitor the rate of phosphorylation of each species in both independent and competitive reactions. Samples were removed at regular time intervals, pipetted into ethanol to stop the reactions, and chromatographed on cellulose TLC plates to separate choline and phosphocholine from their phosphorylated products. 1 cm. horizontal strips of

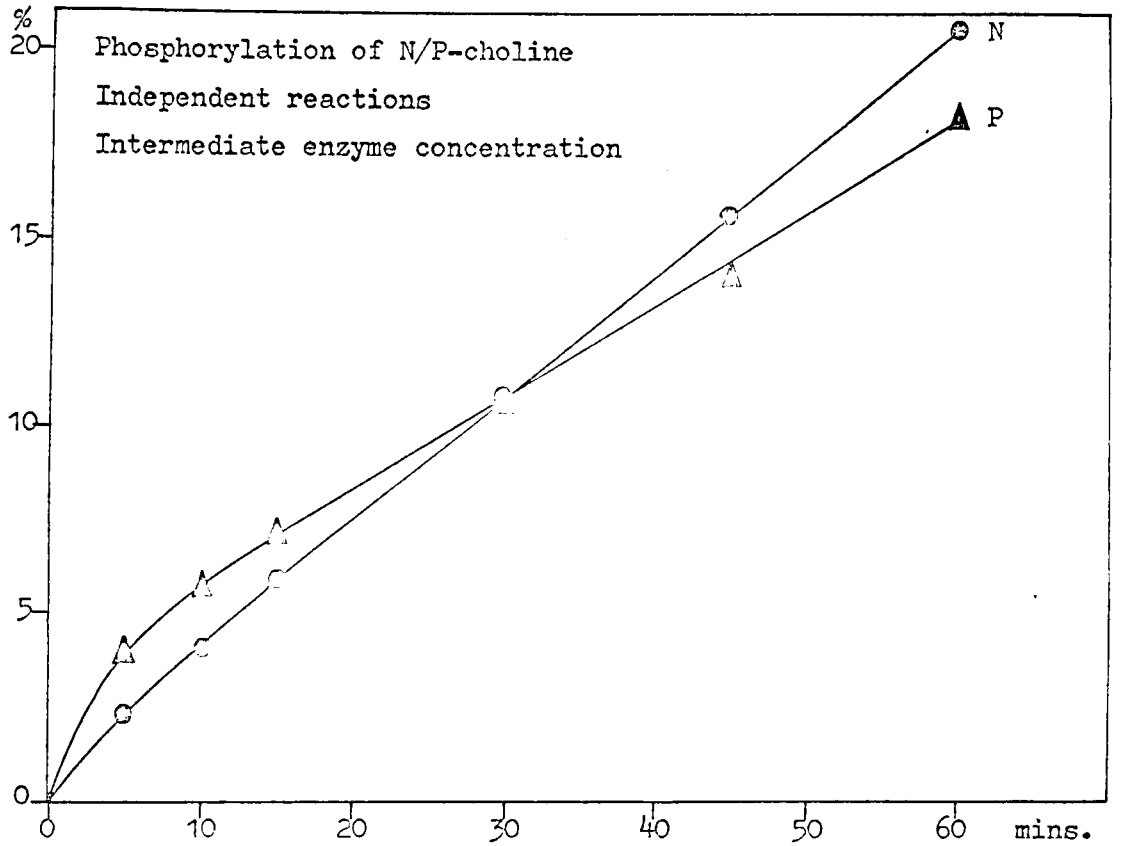
the cellulose layers were scraped into vials containing scintillation fluid, and radioactivity measured. The yield of phosphorylated base could be calculated as the activity of the corresponding area of the chromatogram divided by the total activity of the plate.

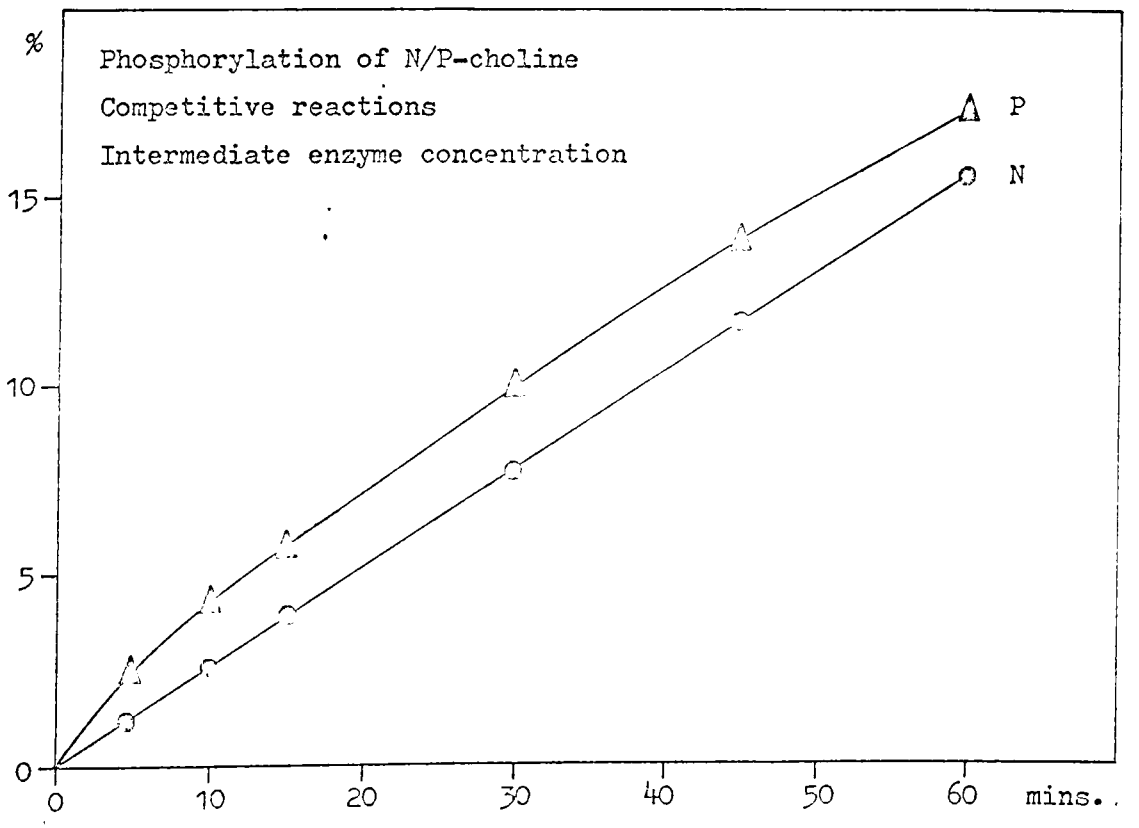
At high enzyme concentrations there was little difference in the rate of phosphorylation of the two substrates, or of degree of phosphorylation after 1 hour, although the enzyme appeared to show slightly greater affinity for phosphocholine than for choline in the competitive experiment, and the reverse in the independent incubations. Moreover, the amount of total substrate phosphorylated was identical in both cases.





A similar effect was observed at intermediate enzyme concentrations; although in both cases the initial rate of phosphorylation of phosphocholine was the higher, the rates became very similar after 5 or 10 minutes. Again, after 1 hour, slightly more choline had been phosphorylated in the independent incubations, and slightly more phosphocholine in the competitive experiment:

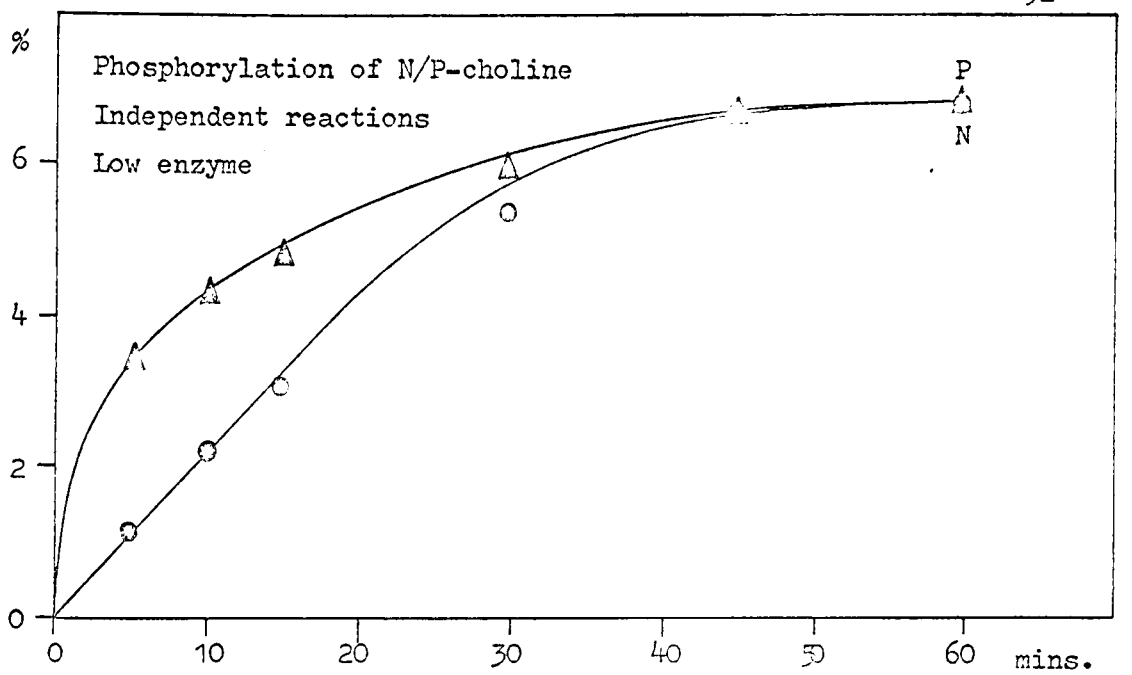

%
20
15
10
5
0
Phosphorylation of N/P-choline
Independent reactions
Intermediate enzyme concentration
N
P
0
10
20
30
40
50
60
mins.

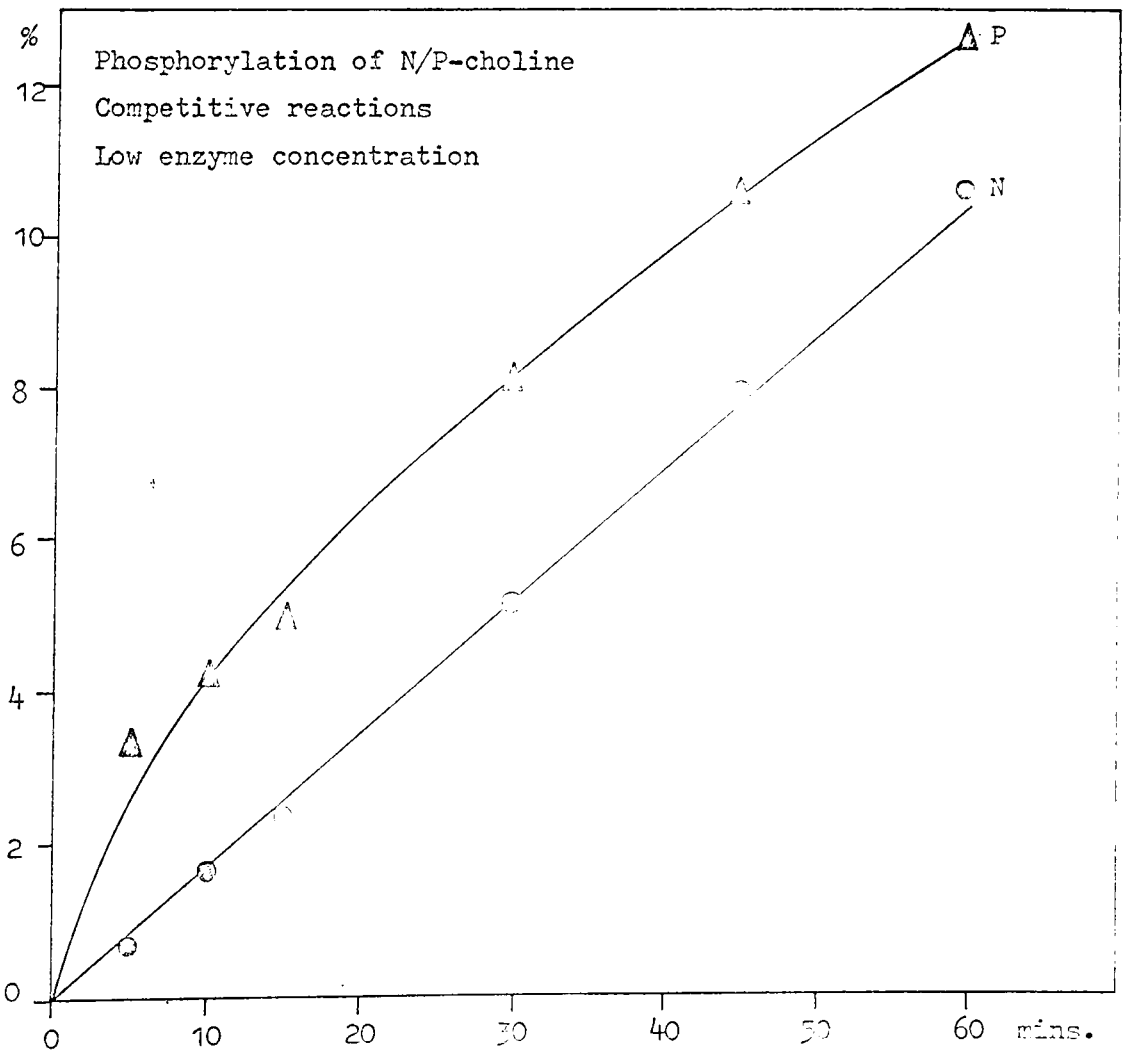

%
15
10
5
0
Phosphorylation of N/P-choline
Competitive reactions
Intermediate enzyme concentration
P
N
0
10
20
30
40
50
60
mins.

At low enzyme concentrations, the differences in the affinity of choline kinase for the two enzymes were more pronounced, especially in the independent experiment. Here the initial rate of phosphorylation of phosphocholine was about three times that of choline; the rates equalized after seven minutes, and the rate curves merged after 45 minutes, giving identical final, and limiting, yields of product. In the competitive experiment, phosphocholine again exhibited the greater initial rate of phosphorylation; the rates equalized after 15 minutes, and remained equal.

The effect of choline kinase from rat liver was also investigated; a homogenate of fresh rat liver in aqueous sodium chloride was prepared and centrifuged for 90 minutes at 25,000 rpm, and the supernatant was used in the incubations according to the method of Schneider et al.[9] Once again, the yields of phosphorylcholine and phosphorylphosphocholine after 1 hour were almost identical.

Thus it appears that the behaviour of choline kinase towards choline and phosphocholine is very similar, although in competitive experiments phosphocholine appears to be the preferred substrate, especially in the initial stages of the reaction.


%
Phosphorylation of N/P-choline
Independent reactions
Low enzyme
P
N
6
4
2
0
0
10
20
30
40
50
60
mins.


%
Phosphorylation of N/P-choline
Competitive reactions
Low enzyme concentration
P
N
12
10
8
6
4
2
0
0
10
20
30
40
50
60
mins.

(ii) Phosphorylcholine - cytidyltransferase.

The enzyme catalyzing the formation of CDP-choline from phosphoryl-

$$CTP + PHOSPHORYLCHOLINE \rightleftharpoons CDP\text{-}CHOLINE + PP_i$$

choline and CTP was first discovered, in liver, by Kennedy and Weiss.[4] In the present experiments, however, the source of the enzyme used was the supernatant of a rat liver homogenate prepared according to Schneider et al.,[6-9] rather than the somewhat purer preparation of Kennedy and Weiss. Before use, the enzyme source was 'aged' either by warming at 38° for 4 hours, or by storing at 0° for 4 days, thus increasing the activity approximately 5-fold.[7] Fiscus and Schneider[8] claim that this increases the concentration of lysolecithin in the homogenate, and that the enzyme is somehow lysolecithin-dependent. The substrates were (methyl-^{3}H)-phosphorylcholine and (1,2-^{14}C)-phosphorylphosphocholine, prepared from labelled base and choline kinase in the previous experiment and purified by ion-exchange chromatography.

For independent experiments, the reaction mixtures, containing enzyme preparation, CTP, magnesium ion, buffer (pH 7.5) and substrate, were sampled at regular time intervals, as in part (i). The samples were pipetted into ethanol and chromatographed on cellulose plates,

effectively separating substrate from product after 3 elutions in a n-butanol-ethanol-acetic acid-water (8:2:1:3) system. The yield of CDP-choline or CDP-phosphocholine was calculated as the radioactivity of the product spot (located under ultraviolet light) divided by that of the whole chromatogram.

Large-scale preparations of labelled CDP-choline and CDP-phosphocholine were made in the same way, for use in the next enzymic stage; the products were purified by ion-exchange chromatography as for the synthetic compounds (Chapter 1).

For competitive reactions, total substrate concentration was kept constant, while the relative proportions of phosphorylcholine and phosphorylphosphocholine were varied; these reactions were only sampled after 1 hour.

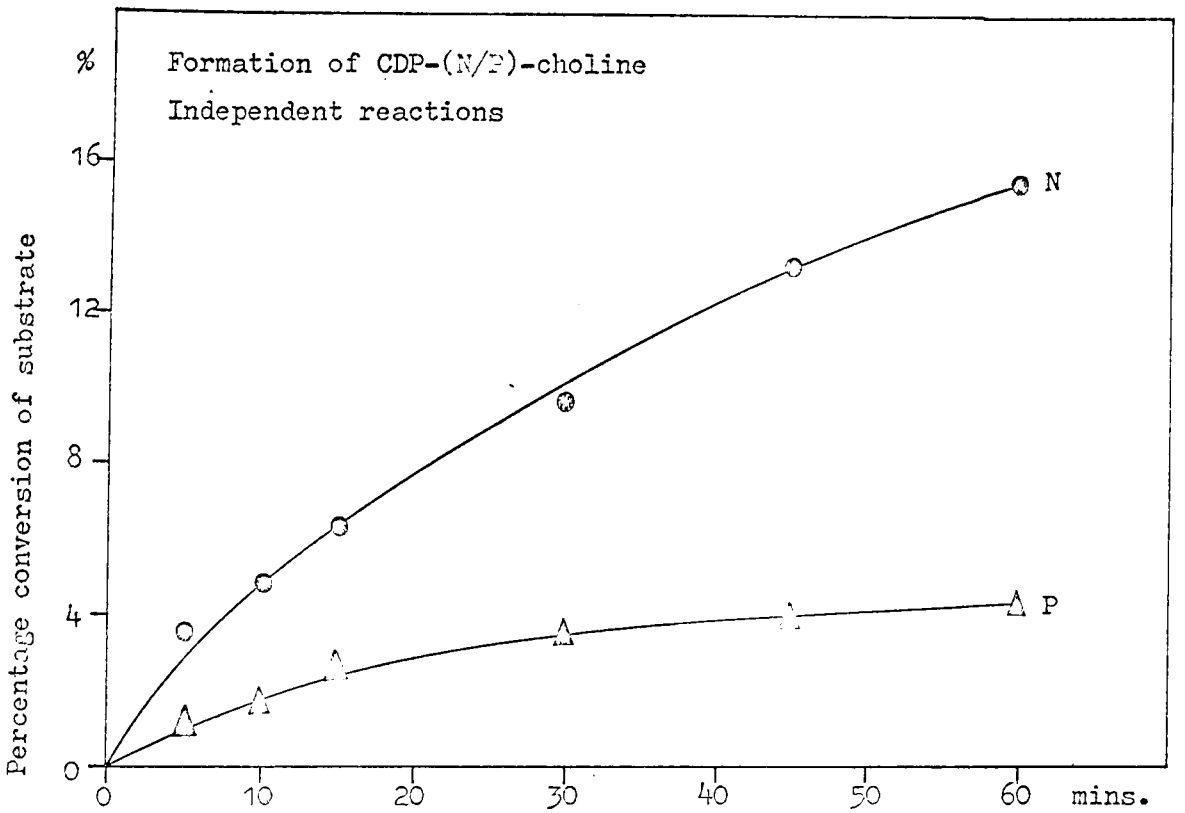
From the graphs of rate of formation of CDP-derivatives, it is seen that phosphorylcholine - cytidyltransferase is far more specific than choline phosphokinase, with an appreciably greater affinity for phosphoryl-choline than phosphorylphosphocholine:

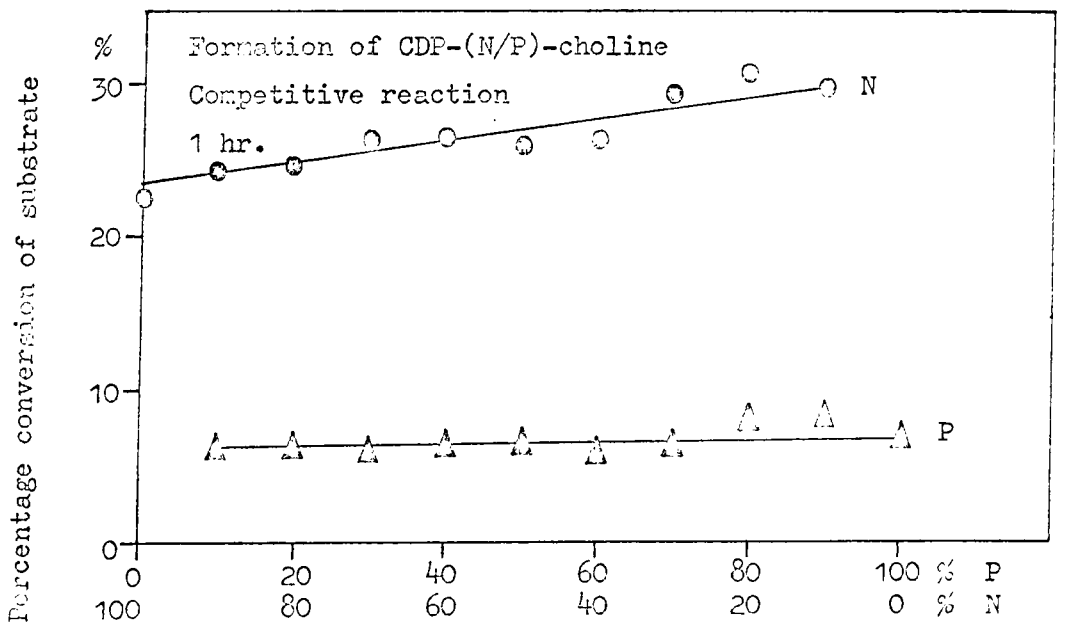
Relative rate of formation of CDP-derivative: initial N/P ratio = 3.0

Yield of CDP-derivative after 1 hour: N/P ratio = 3.6

However, from the results of the competitive experiments it is clear that neither substrate inhibits the conversion of the other; indeed,

high phosphorylphosphocholine levels cause a perceptible increase in the degree of conversion of phosphorylcholine.





(iii) Phosphorylcholine - diglyceridetransferase.

This enzyme, also first isolated from liver by Kennedy and Weiss,[4] catalyzes the transfer of phosphorylcholine from CDP-choline to 1,2-diglyceride:

CDP-choline + 1,2-DIGLYCERIDE $\rightleftharpoons$ PHOSPHATIDYLCHOLINE + CMP

The reaction is reversible, and specific for L-diglyceride.

The main difficulty encountered in the study of this enzyme in vitro is in obtaining the diglyceride in a suitable form; at least one of the acyl chains must be unsaturated, possibly because of the relative ease of emulsion formation. Thus Devar and Mudd[287] have found that 1,2-distearoyl-L-glycerol is totally inactive as a substrate. The problem was solved by the use of natural 1,2-L-diglyceride, prepared by the action of phospholipase C on egg-yolk lecithin; before use it was sonicated in an aqueous solution of the detergent Triton X-100. The final detergent concentration in the reaction mixture was only 0.02%, since the presence of detergent is inhibitory;[4] any solution to the diglyceride emulsion problem is in effect a compromise, between increased choline incorporation through better diglyceride dispersion, and decreased incorporation due to inhibition by the detergent.

The enzyme source used in this work was a crude rat liver homogenate prepared according to the method of Schneider and Behki.[6] The reaction mixture contained enzyme source, diglyceride dispersion, magnesium ion, buffer (pH 7.5), and labelled CDP-choline or CDP-phosphocholine. Competitive incubation mixtures contained a constant quantity of total substrate, while the relative proportions of the two analogues were varied.

Incubations took place at 37°; independent reaction mixtures were sampled over a period of an hour, and competitive mixtures only after 1 hour. Samples were pipetted into ethanol, and phospholipid was extracted with a carbon tetrachloride-ethanol-aqueous potassium chloride system.[6] The dried phospholipid extracts were then redissolved in scintillation fluid and monitored for radioactivity.

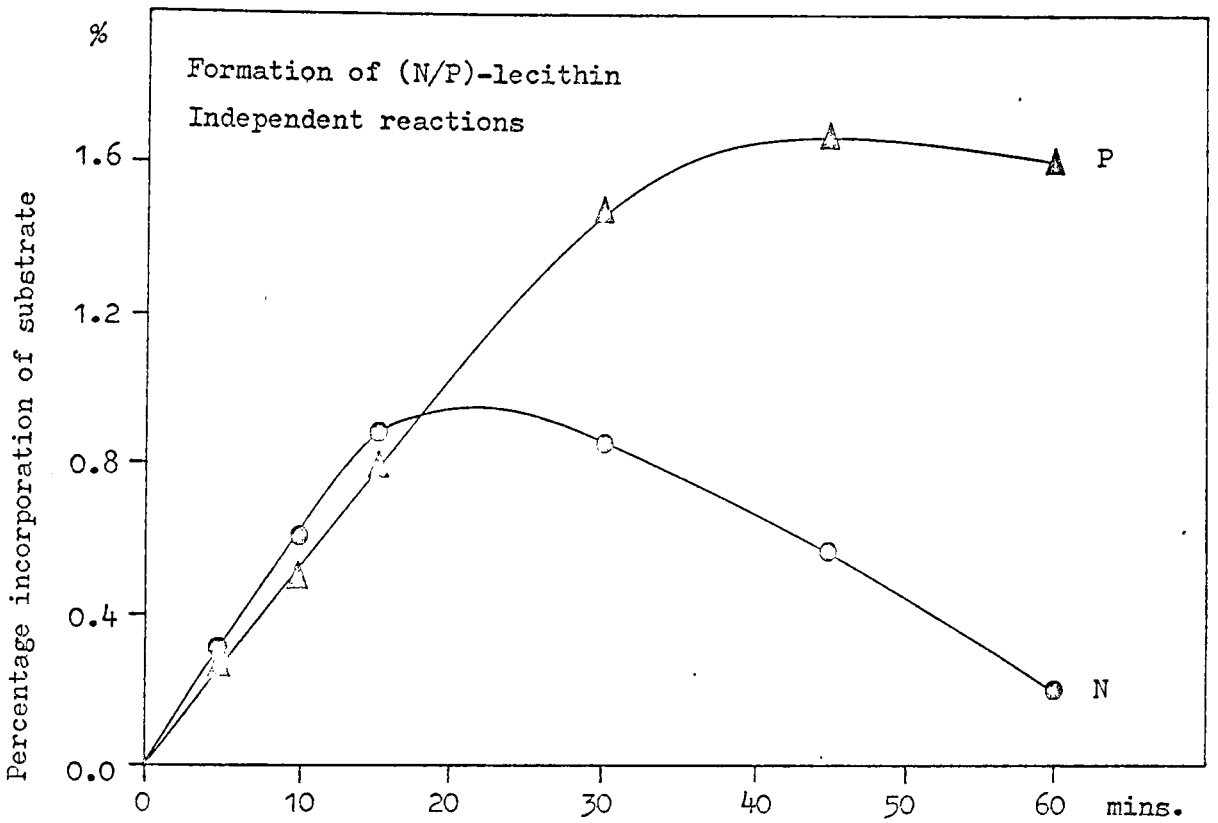
Graphs of choline and phosphocholine uptake against time revealed that initial rates of incorporation into phospholipid were almost identical. However, the rate of ^{3}H-choline incorporation began to decline after 15 minutes, and became negative after 22, whereas the rate of ^{14}C-phosphocholine incorporation did not start to decline for 30 minutes, and only became negative after 44.

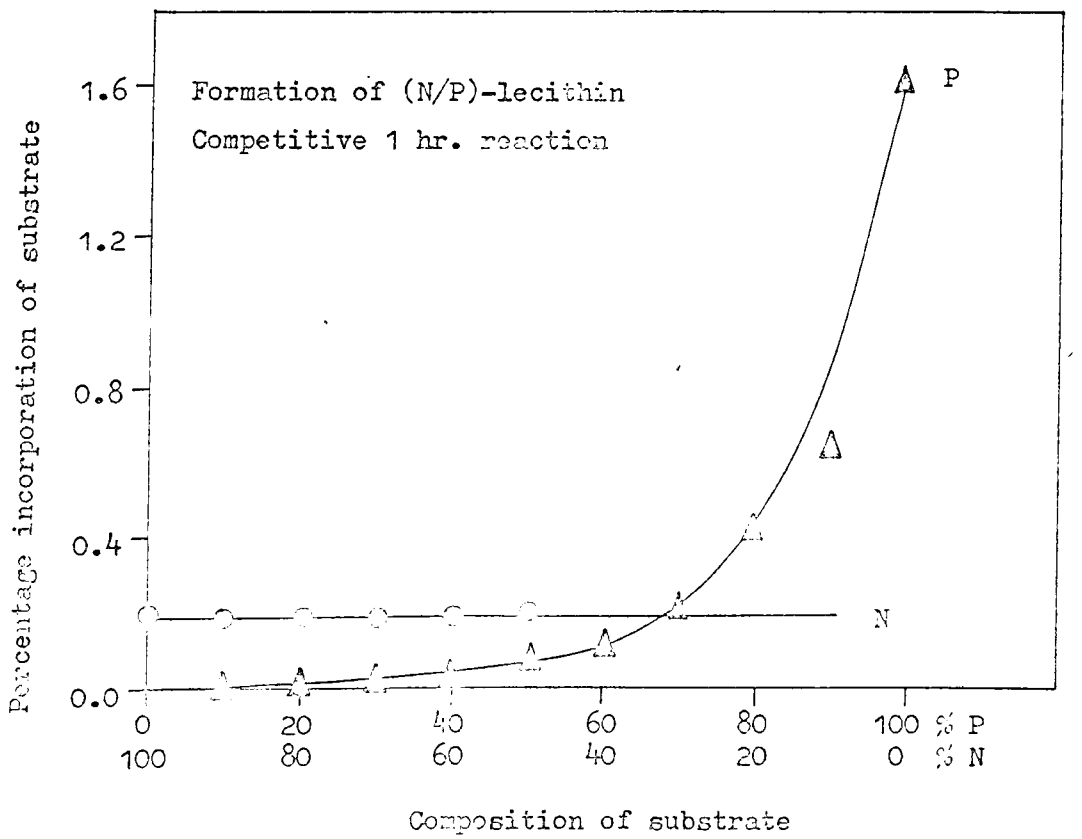
Kennedy and Weiss[4] showed that the reaction was reversible, and this reversibility would appear to account for the loss of label from lecithin and its phosphonium analogue after 22 and 44 minutes respectively. Although the affinity of the enzyme for both substrates appears to be

almost identical in the forward reaction, the reverse reaction appears to be far more favourable towards the degradation of lecithin than towards the degradation of its phosphonium analogue. This is reflected in the 2-fold delay in the start of net degradation of phosphatidylphosphocholine, and the much lower rate of this process.

Although Schneider and Behki[6] claimed that incorporation of labelled choline into lecithin by this reaction reached a plateau after 15 minutes, their published graph shows a decline after 12; no points are plotted beyond 15 minutes. It would seem that the lecithinprepared by these workers started to undergo the same degradative process unobserved.

Since incorporation measurements in the competitive experiments were made only after 1 hour, the phosphatidylcholine formed had already undergone considerable degradation. However, it was still possible to observe from the radioactivity measurements that choline uptake was independent of CDP-phosphocholine concentration, whereas phosphocholine uptake was cosiderably inhibited by the presence of CDP-choline. Schneider and Behki[6] made a similar observation with the incorporation into lecithin of deoxy-CDP-choline; again this was strongly inhibited by CDP-choline, and a similar inhibition curve was obtained.


%
Formation of (N/P)-lecithin
Independent reactions
Percentage incorporation of substrate
1.6
1.2
0.8
0.4
0.0
P
N
0
10
20
30
40
50
60
mins.

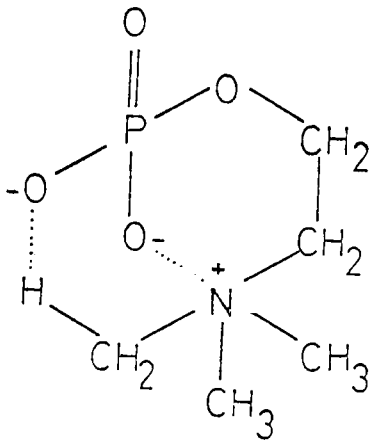

Formation of (N/P)-lecithin
Competitive 1 hr. reaction
Percentage incorporation of substrate
1.6
1.2
0.8
0.4
0.0
P
N
0 100
20 80
40 60
60 40
80 20
100 % P
0 % N
Composition of substrate

(iv) Loss of tritium label from (methyl-^{3}H)-phosphorylcholine.

When the water was removed from a dilute solution of (methyl-^{3}H)-phosphorylcholine which had been stored at 0° and pH 7.5 for 6 months, only 7% of the radioactivity remained in the phosphorylcholine, while 93% appeared in the aqueous distillate.

A similar effect was observed with phosphorylcholine which had been freshly prepared by the action of choline phosphokinase on (methyl-^{3}H)-choline. After deproteinization, the reaction mixture was adjusted to pH 8.0 to ensure that the product was in the dianion state; it was then chromatographed on (H) cation-exchange resin followed by (formate) anion-exchange resin. After removal of water from the product, only 1.4% of the radioactivity remained with the latter, the rest having distilled with the water.

It was therefore apparent that exchange was taking place between the protons of the methyl groups and those of the solvent; since the effect was only observed when phosphorylcholine was in the dianion state, it is conceivable that the exchange process involves a bicyclic intermediate:



Such a structure is an extension of the idea of the internally stabilized cyclic zwitterion postulated from PMR and mass spectral data (Chapter 2 (iii) and (v)); however, this bicyclic structure could only be assumed by the dianion, the only species present at 8.0 pH.

CHAPTER 4 : INCORPORATION OF PHOSPHOCHOLINE INTO P815Y CELLS

P815Y was a line of neoplastic mouse mast cell, and was cultured in medium, formulated by Fischer and Sartorelli,[288] containing essential amino acids, vitamins, salts and other nutrients, and up to 10% horse serum; the choline content was 1.5 mg. per litre (11 μM).

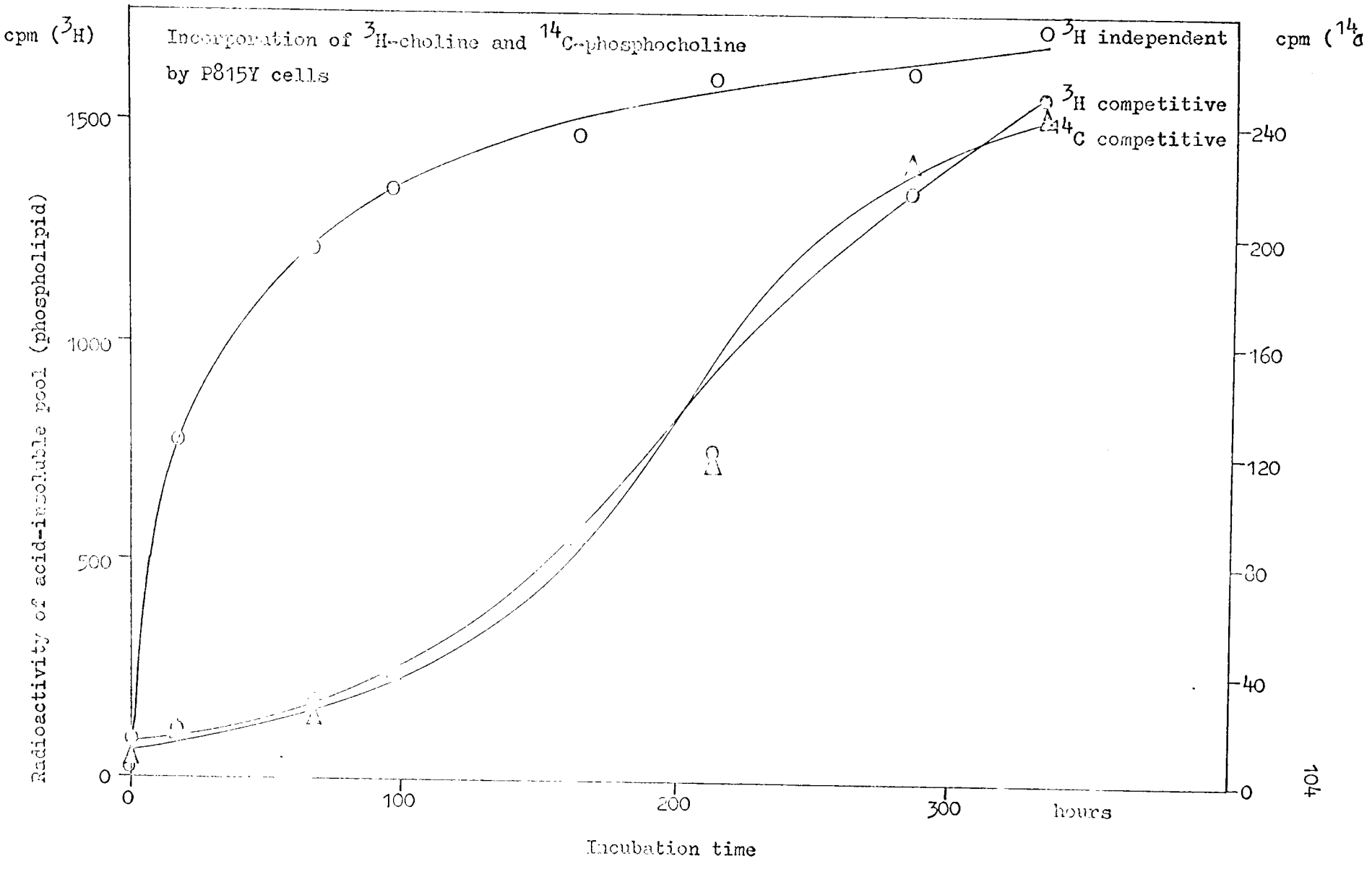
Although conditions of sterility were maintained at as high a level as possible, much time was lost and many experiments invalidated through random infection of the cell cultures by extraneous yeasts, fungi and bacteria.

Preliminary experiments were carried out to ascertain whether addition of phosphocholine to the medium had any adverse effect on cell growth; tubes of cells were cultured for several days in medium with phosphocholine or choline concentrations of 0.05 to 400 mM. Samples were withdrawn after $2\frac{1}{2}$ and 6 days, and cell density was measured by means of a Coulter counter. It was found that choline concentrations above 1 mM began to retard cell growth, and totally prevented it at 400mM, whereas phosphocholine had no obvious effect even at this extremely high concentration.

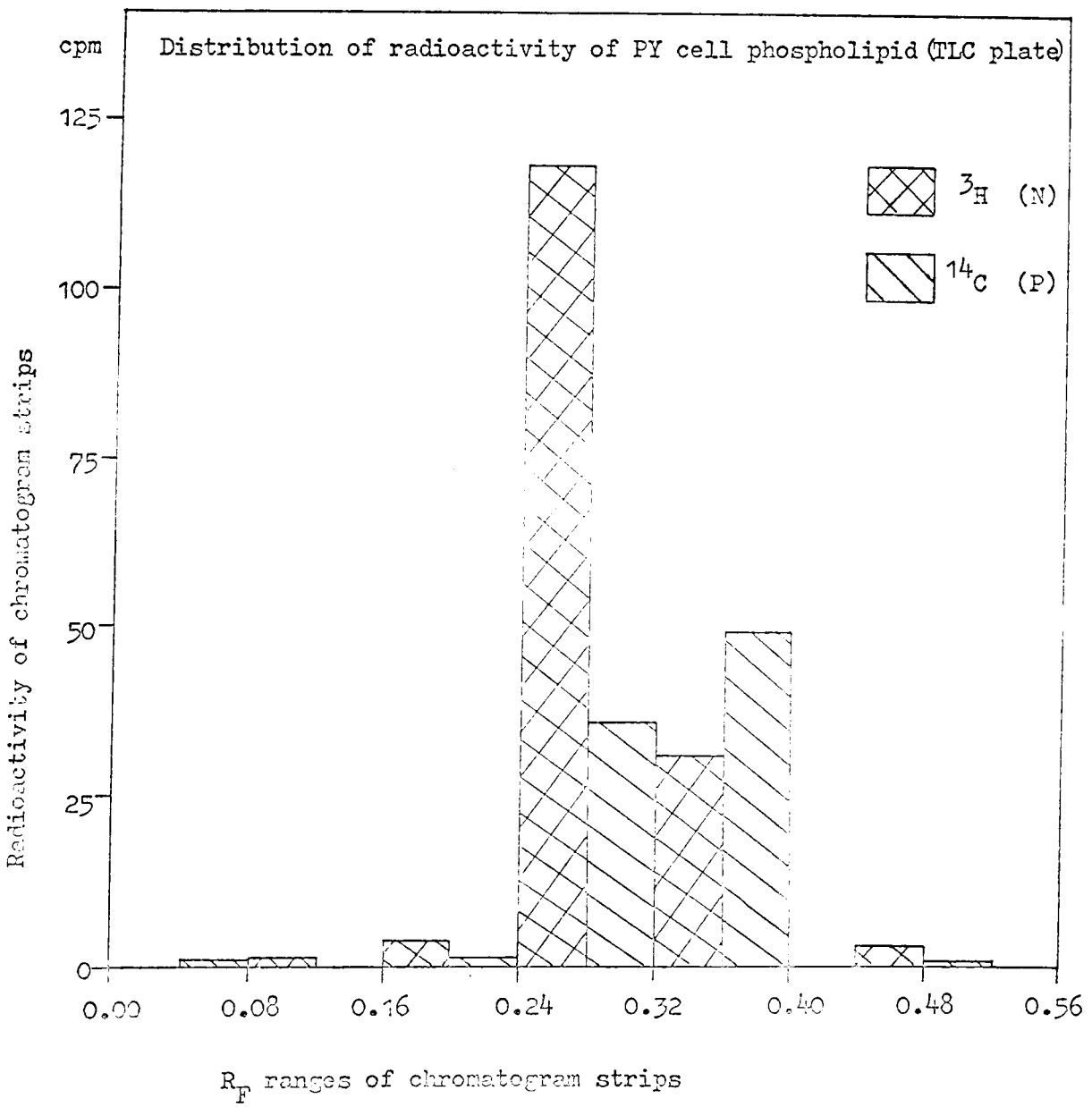
Four aliquots of cells were then cultured in medium containing (methyl-^{3}H)-choline chloride of negligible molarity but high specific activity; two also contained (1,2-^{14}C)-phosphocholine chloride, of low

specific acivity, at a concentration of 1.8 mM. The cultures were sampled over a period of 2 weeks; samples were pipetted into aqueous trichloroacetic acid, and the precipitate filtered, washed carefully and counted for radioactivity. Thus phospholipid derivatives of choline and phosphocholine would be in the acid-insoluble pool (precipitate) while the precursors - choline, phosphorylcholine, CDP-choline and the phosphonium analogues - would remain in the acid-soluble pool (filtrate). In this way the uptake of labelled choline and phosphocholine into PY cell phospholipids could be monitored. Plots of incubation time against radioactivity of acid-insoluble pool showed that the presence of phosphocholine had a marked effect on the uptake of radioactive label, and therefore the commencement of normal cell growth, inhibiting the latter for several days. Only towards the end of the incubation did the level of ^{3}H-choline incorporation approach that observed in the controls, and the incorporation of phosphocholine followed a similar route.

The cells from one of the cultures in phosphocholine-enriched medium (ca. 2.6 X 10^{6} cells) were centrifuged, resuspended and washed several times, combined with a ten-fold excess of cells grown in isotope-free medium, recentrifuged, and extracted with a chloroform-methanol system. The extracted lipids were chromatographed on a silica TLC plate, which was developed with iodine vapour. The plate surface was divided into horizontal strips, which were scraped off and monitored for radioactivity.


cpm (^{3}H)
Incorporation of ^{3}H-choline and ^{14}C-phosphocholine
by P815Y cells
^{3}H independent
^{3}H competitive
^{14}C competitive
cpm (^{14}C
1500
1000
500
0
240
200
160
120
80
40
0
0
100
200
300
hours
Radioactivity of acid-insoluble pool (phospholipid)
Incubation time
10⁴

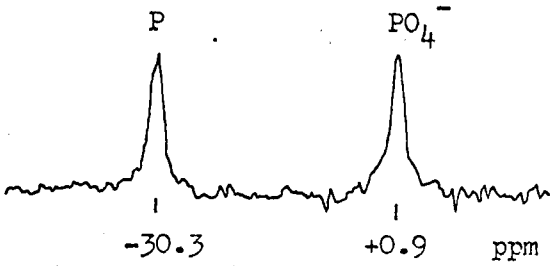
The ^{3}H maximum occurred where the lecithin spot had been identified, while the ^{14}C maximum was very close, but at a slightly higher R_F. This was the first positive proof that phosphocholine had been incorporated into the phospholipids of cell membranes.



Large-scale PY cultures were then prepared; two were grown from 9ml. batches containing approximately 5 X 10^5 cells per ml., in medium containing 10% horse serum, but no extra choline or added phosphocholine. The volume of the cultures was doubled every day by the addition of fresh medium; when volumes of 100 ml. had been reached, different medium, containing only 4% serum and also (methyl-^{3}H)-choline of negligible molarity, was used. Since the serum contained an undetermined quantity of free choline, and also phospholipids which might exchange with those in the cell membranes, it was desirable to reduce its concentration without inhibiting growth. The choline chloride concentration of half of the new medium was increased 4-fold, while enough (1,2-^{14}C)-phosphocholine chloride was added to the other half to give a final phosphocholine/choline ratio of 3:1.

The two cultures were allowed to grow in the new media until final volumes of 1 l. had been reached . The cell concentrations were measured and found to be almost identical, indicating that there had been no net growth inhibition due to addition of phosphocholine.

The cells from each culture were centrifuged and extracted as before, and the phospholipids chromatographed on preparative silica TLC plates. The lecithin bands were removed and extracted, and the extracts investigated both by ^{31}P-NMR and by radioactivity counting; samples of the medium were also measured for radioactivity. The ^{31}P-NMR spectrum of the phosphocholine-incubated cell extract appeared thus:



the phosphate/phosphonium peak area ratio being approximately 1.38:1, indicating 100 molecules of phosphatidylphosphocholine to every 38 of phosphatidylcholine present in the cell phospholipids. Since sufficient phosphocholine had been added to the medium originally to give a phosphocholine /choline ratio of 3:1, that is 100:33, the two bases seem to have been incorporated into the cells in virtually the same ratio, although this does not take into account the phospholipid of cells grown in the original phosphocholine-free medium; the effect of the latter would have been reduced by normal phospholipid turnover.

Radioactivity measurements yielded similar results. The $^{3}H/^{14}C$ ratios in the medium (1.31) and in the phospholipid extract (1.34) were extremely close, indicating that choline and phosphocholine had been incorporated in almost exactly the same proportions as those in which they had been present in the medium.

It therefore appears that phosphocholine and choline are equally acceptable for incorporation into the phospholipids of P815Y cells.

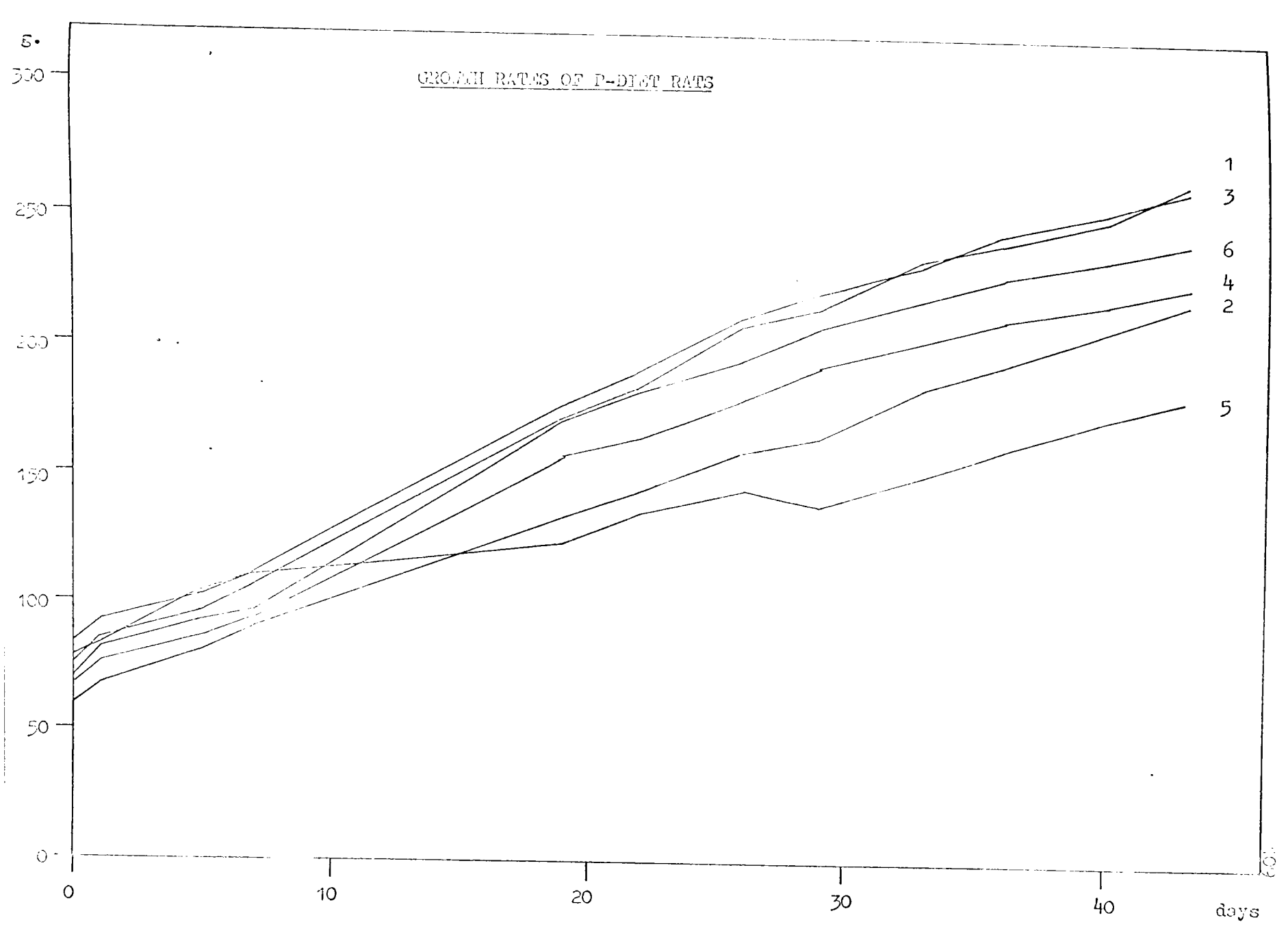
CHAPTER 5 : IN VIVO EXPERIMENTS WITH PHOSPHOCHOLINE, AND ITS EFFECT ON THE CHOLINE-DEFICIENT WEANLING RAT.

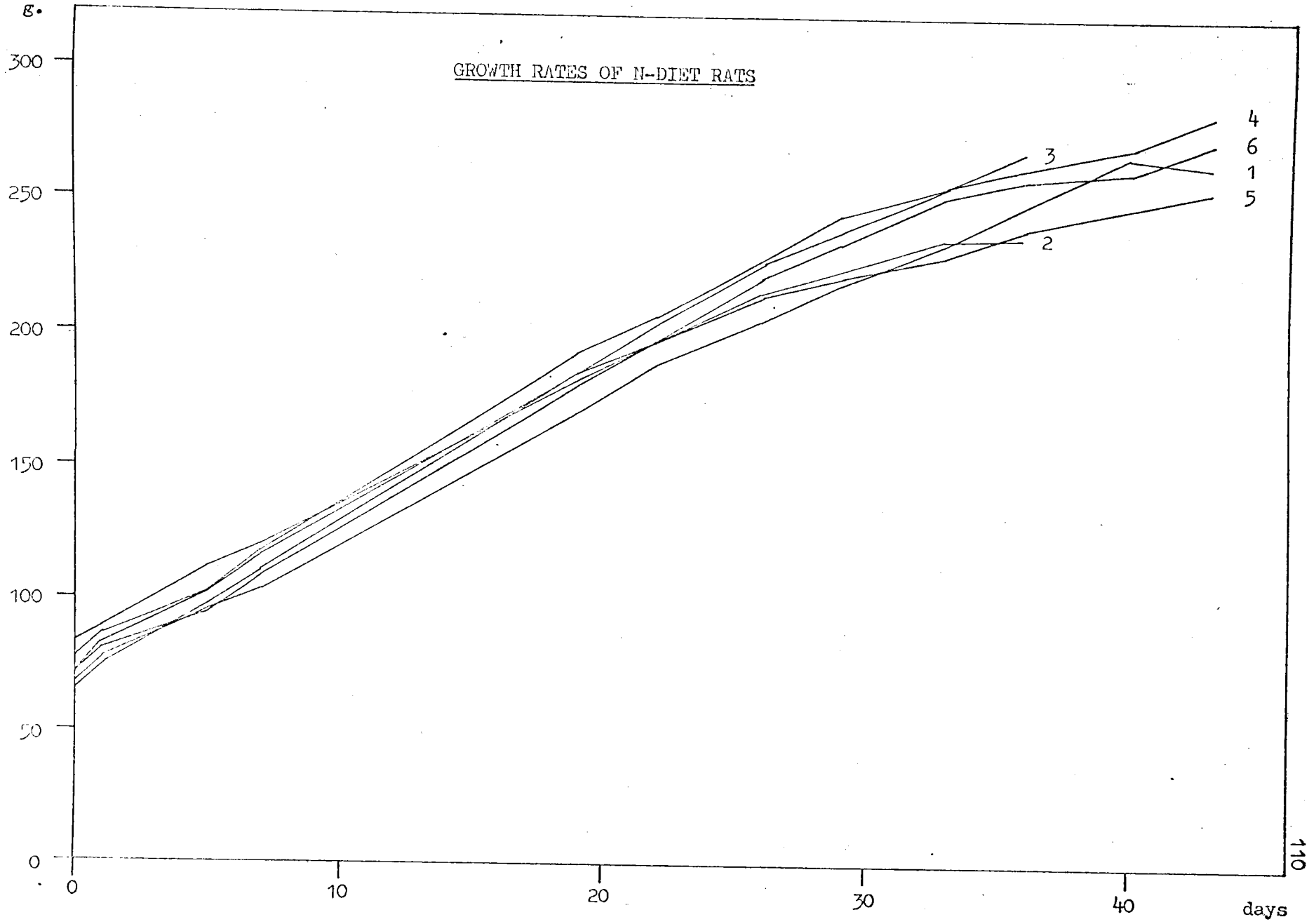
(i) Method.

A choline-deficient rat diet was prepared from essential vitamins and salts, sugar, starch, cod-liver oil, and vitamin-free, choline-free casein; the casein, as the sole source of protein, would have provided the methionine necessary for lecithin synthesis *via* the sequential methylation pathway.[22-8] To this was added (methyl-^{3}H)-choline chloride of high specific activity and negligible molarity. The diet was then divided into two halves; to one half was added sufficient unlabelled choline chloride to compensate fully for the original deficiency in the food mix, and to the other half was added an equimolecular quantity of (1,2-^{14}C)-phosphocholine chloride, of low specific activity.

Twelve male weanling Wistar rats were divided into two groups of six, designated 'N' and 'P'; N received the diet supplemented with choline, and P that supplemented with phosphocholine. This food, and drinking water, were supplied *ad libitum*. The animals were kept three to a cage, and were weighed twice a week.

After 7 days, rat P-5 caught an infection and did not recover, nor


GROWTH RATES OF P-DIET RATS
g.
300
250
200
150
100
50
0
0
10
20
30
40
days
1
3
6
4
2
5


g.
GROWTH RATES OF N-DIET RATS
300
250
200
150
100
50
0
0
10
20
30
40
days
110
4
6
1
5
3
2

gain weight at the original rate, for another 3 weeks; consequently he was still underweight when killed. Rats N-2 and N-3 died after 36 days, and although a post-mortem was inconclusive, their deaths were considered to be due to respiratory infection and unconnected with diet composition.

After a total of 43 days, rats P-2 and N-1 were killed by a veterinary surgeon, who then conducted an autopsy. There were found to be no obvious abnormalities, and no organisms of pathogenic significance: neither of the animals showed the slightest signs of choline deficiency, such as fatty liver or renal haemorrhage, and neither had deteriorated from their original high-quality condition ("Grade 4"). Only their livers were removed for lipid extraction.

From growth curves and the table of weight gain, it can be seen that the growth of the rats was affected only slightly by the change to a phosphocholine-enriched, choline-deficient diet; this difference might even have been due to the very unpleasant odour of trimethylphosphine emanating from the phosphocholine, thus rendering the P-diet less appetizing.

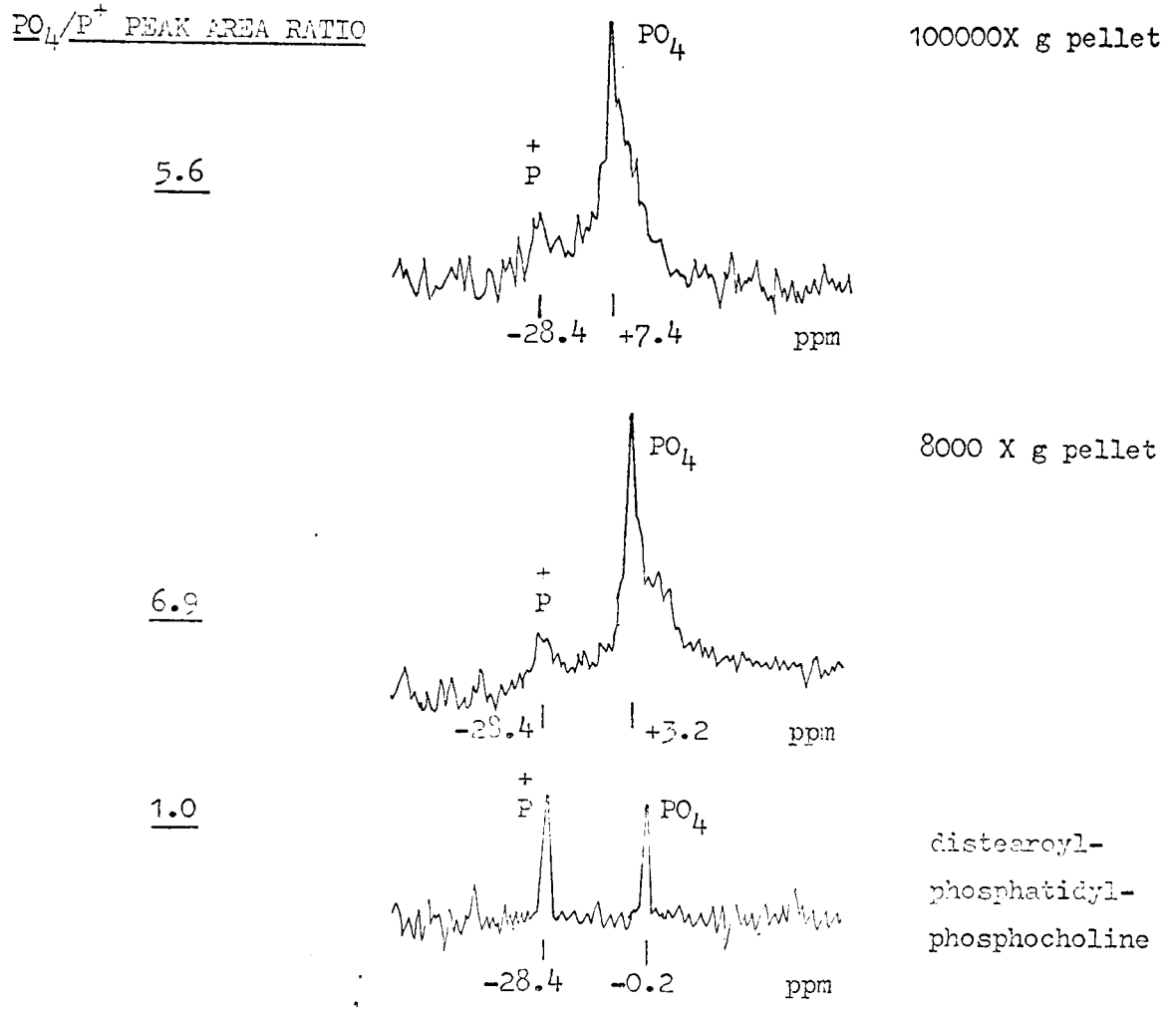
NO. OF RATS	DIET	MEAN BODY WEIGHT (g.)		PERCENTAGE WEIGHT
		INITIAL	FINAL	GAIN (%)
4	N	74	270	269
5	P	70	238	240
1 (sick)	P	84	178	112

The eight remaining animals were killed by stunning and decapitation, and their livers, lungs, kidneys and brains removed and stored frozen under nitrogen until required. Small samples of two livers (P-1 and N-6) were homogenized in sucrose and centrifuged twice; the low-speed pellets (8000 X g, 30 mins.) contained cell debris, nuclei and mitochondria, while the high-speed pellets (100000 X g, 1 hour) contained the microsomal fraction.

Each liver in turn, and pooled representative samples of the other organs from the two groups, were extracted under nitrogen with hot ethanol and chromatographed on columns of alumina and silica in order to remove all but the neutral phospholipids (lecithin, sphingomyelin, traces of lysolecithin, and any phosphorus analogues of these which had been formed). After ^{31}P-NMR spectra had been recorded, some of the extracts (P-rat brain, kidney, lung, and livers P-3 and P-5) were separated into their component phospholipids by preparative TLC on silica gel. Thus it was found that two elutions in a chloroform-methanol-water-0.880 ammonia system (57:20:2:1) were sufficient to separate lecithin from its phosphonium analogue to an extent of about 95%.

(ii) ^{31}P-NMR spectra of liver homogenate pellets.

The spectra of the pellets, resuspended in distilled water, gave an unequivocal indication that phosphonium ion was present in the tissues of

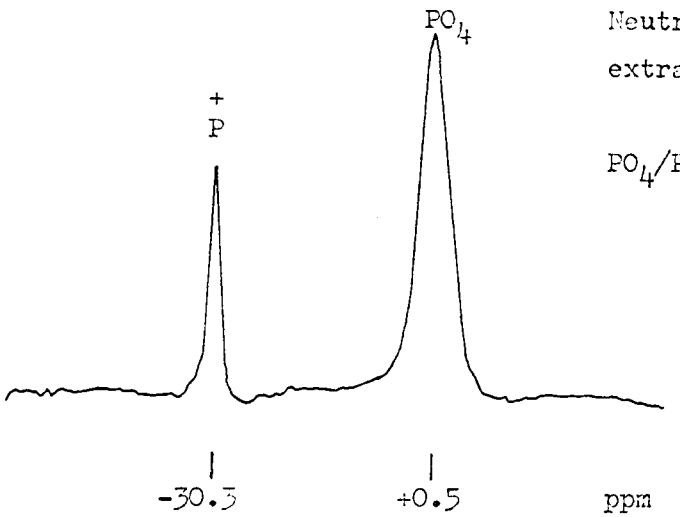


the P-diet rat; only the phosphate signal was present in the spectra from the pelletsof the P-diet rat.

(iii) ^{31}P-NMR analysis of liver phospholipid extracts.

^{31}P-NMR spectra of the neutral liver phospholipids of P-rats showed at

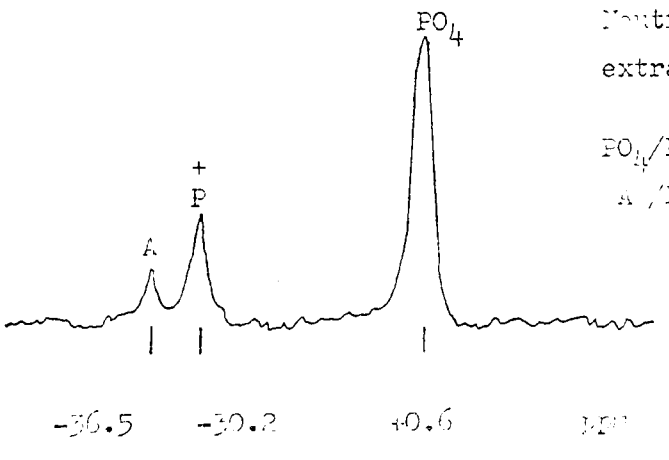
least two signals, readily attributable to phosphonium and phosphate; all N-rat extracts showed a single, phosphate signal.



Neutral phospholipid extract of liver P-1

PO_4/P^+ peak area ratio 3.7

A similar spectrum was obtained from liver P-2; however, the spectra of the other four P-rat liver extracts (3-6) showed a third signal at lower field, designated 'A':

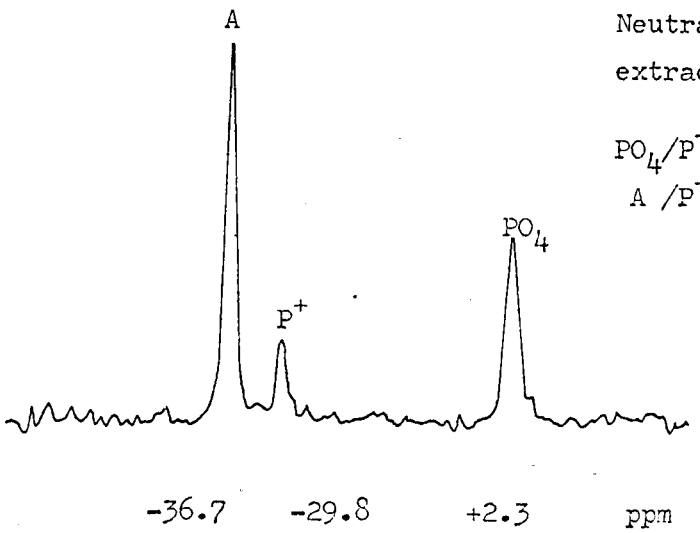


Neutral phospholipid extract of liver P-4

PO_4/P^+ peak ratio 3.5

A $/P^+$ peak ratio 0.46

The third signal, A, was approximately half the area of the phosphonium signal in the spectra of extracts P-3, P-4 and P-6. In the case of the sick rat, P-5, however, the signal from A was the largest in the spectrum:



Neutral phospholipid extract of liver P-5

PO_4/P^+ peak ratio 3.7

A $/P^+$ peak ratio 5.2

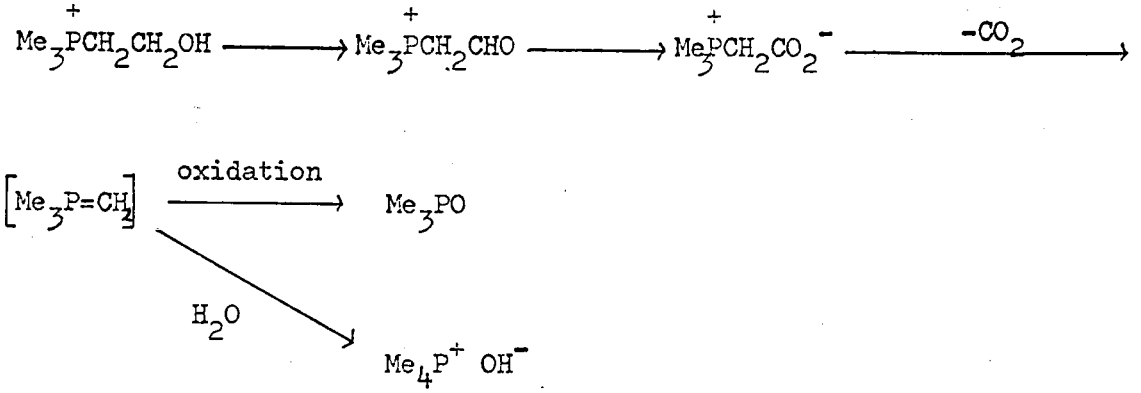
Since signal A appeared at too low a field for phosphocholine itself (chemical shift -25.9 ppm), it is possible that it was due to a degradation product of phosphocholine, such as phosphobetaine aldehyde (LXXX), phosphobetaine (LXXXI) or trimethylphosphine oxide (LXXXII):

$Me_3\overset{+}{P}CH_2CHO$ (LXXX)

$Me_3\overset{+}{P}CH_2CO_2^-$ (LXXXI)

Me_3PO (LXXXII)

originating from the oxidation sequence:



Since it has already been shown (Chapter 2 (iv)) that trialkylphosphine oxides absorb at much lower field, and since a species with a net positive charge like phosphobetaine aldehyde (LXXX) would not have survived the chromatographic processes, it is tentatively suggested that phosphobetaine may have been responsible for the signal A. Although free phosphobetaines in the form (LXXXI) are unstable, readily decomposing to tetraalkylphosphonium salts as shown above,[289] betaine decomposition is a function of pH, and therefore derivatives such as the hydrochloride (XXXII) are quite stable,

$$Me_3\overset{+}{P}CH_2CO_2H \quad Cl^-$$

(XXXII)

and have even been tested for lipotropic activity.[185] The fact that rat liver is capable of oxidizing the arsenic analogue of lecithin to arseno-

betaine (XXXIII) is well established, and although Mann et al.[196] could not isolate the intermediate arsenobetaine aldehyde (XXXVII) they claimed to have 'trapped' it as the 2,4-dinitrophenylhydrazone.

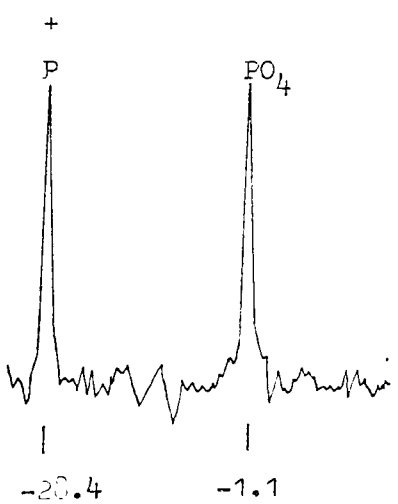
$Me_3\overset{+}{A}sCH_2CO_2H \quad Cl^-$ (XXXIII)

$Me_3\overset{+}{A}sCH_2CHO \quad Cl^-$ (XXXVII)

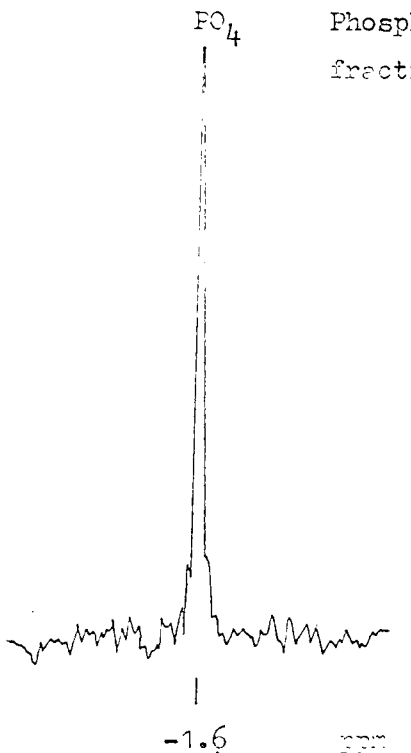
The liver extracts of rats P-3 and P-5 (sick) were subjected to preparative TLC on silica gel, as described in (i); thus 'natural' lecithin and its phosphonium analogue were separated for the first time:

Phosphatidylphosphocholine fraction, liver P-3

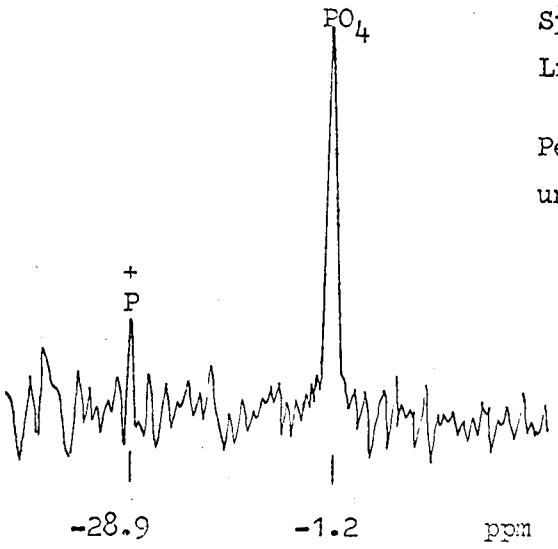
Peak area ratio 1:1



Phosphatidylcholine fraction, liver P-3



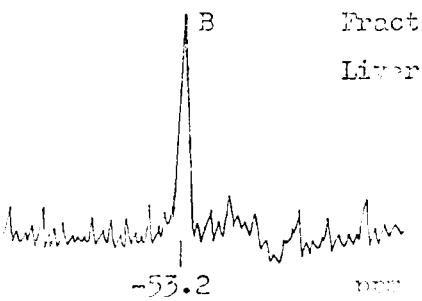
Phosphocholine incorporation into the sphingomyelin fraction seemed to be appreciably less than that into lecithin, however:



Sphingomyelin fraction
Liver P-3

Peak area ratio
uncertain

but nowhere on either plate could a fraction producing signal A be found. However, the spectrum of a fraction extracted from the origin of the liver P-5 (sick rat) chromatogram exhibited a very low-field signal at -53.2 ppm, designated 'B', which suggested the presence of trimethylphosphine oxide Me_3PO (LXXXII) (*cf.* n-butylphosphine oxide at -51.8, Chapter 2(iv)).



Fraction from TLC origin
Liver P-5

Thus phosphobetaine, if it can indeed be identified with A, may have been oxidized on the TLC plate to trimethylphosphine oxide; it seems very likely that the source of signal B is a degradation product of the source of signal A, since the latter was the largestpeak in the spectrum of extract P-5, and no other fractions producing additional NMR signals could be found on the chromatogram.

It is still uncertain why the liver extract of the sick rat contained such a large quantity of phosphocholine degradation product, although it is possible that its liver lipids were experiencing an abnormally high rate of turnover, with a consequent build-up of phosphocholine metabolite. It should be noted, however, that the degree of phosphocholine incorporation into the liver lipids of this rat, as calculated from relative peak areas in the ^{31}P-nmr spectrum, was very close to the average of all the P-rats. Thus measurement of relative peak areas yielded the following information:

RAT	RELATIVE PEAK AREAS (P^+=1)		PERCENTAGE OF PHOSPHOLIPID
	PO_4	'A'	CONTAINING PHOSPHOCHOLINE, %.
P-1	3.7	——	27
P-2	3.6	——	28
P-3	4.9	0.48	20
P-4	3.5	0.46	29
P-5	3.7	5.20	27
P-6	3.9	0.42	26

Thus the incorporation of phosphocholine into neutral phospholipids was fairly consistent at about 26%. This was confirmed by weighing the chromatographed fractions of liver P-3; if the molecular weights of phosphatidylcholine, phosphatidylphosphocholine and sphingomyelin are assumed to be roughly equivalent, the following composition figures are obtained:

P-3 FRACTION	WEIGHT mg.	WEIGHT AS PERCENTAGE OF TOTAL: Phospholipid	Lecithin
Phosphatidyl-phosphocholine	7.5	22.5	28
Phosphatidyl-choline	19.1	57.5	72
Sphingomyelin	6.3	19	—
Lysolecithin	0.5	1	—

The approximate figure of 22.5% phosphocholine-containing phospholipid obtained by this very rough method is in good agreement with the figure of 20% derived from relative peak areas in the NMR spectrum. Similarly, the values obtained for liver P-5 were 25% and 27% respectively.

If it is assumed that all phospholipid present in the animals at the start of the experiment had been degraded and replaced over the 43 day period it would appear that about 70% of lecithin was being formed by sequential methylation of phosphatidylethanolamine;[24-8] this is a well-established route in the liver,[14] since no dietary choline, only phosphocholine, was available for the cytidine pathway, and methionine for transmethylation was readily available in the dietary casein.

It should be noted that McArthur and Lucas[181-3] arrived at a figure of 29% for the incorporation of 2-hydroxyethyltriethylammonium salts (XXX)

$$Et_3\overset{+}{N}CH_2CH_2OH \quad X^-$$

(XXX)

into the liver lecithin of choline-deficient rats after 40 days, implying that 71% of the lecithin was formed by other routes. It is therefore conceivable that 30% is near the limit of incorporation *via* the cytidine pathway under these conditions. However, such a theory makes no allowance for changes in the rates of turnover of phospholipids (including those already formed) when the source of dietary choline is suddenly removed, or for the possibility of recycling of the choline which results from such phospholipid degradation.

It appears that little sphingomyelin is being formed by the established[18]

direct CDP-pathway, since its phosphocholine content is very low, and it is therefore possible that the indirect pathways observed by Sinclair[30] and by Treble et al.[31-2] are being utilized. This may be due to the fact either that these pathways are more generally utilized than is at present thought, or that the enzyme phosphorylcholine-ceramide transferase has little affinity for CDP-phosphocholine.

The proposed indirect pathways are:[30]

Ethanolamine ——————→ Phosphatidylethanolamine (direct synthesis

Phosphatidylethanolamine ——→ Phosphatidylcholine (methylation pathway)

Phosphatidylcholine ——————→ CDP-choline (reversal of synthesis)

CDP-choline ——————→ Sphingomyelin (direct synthesis)

with the direct route

CDP-phosphocholine ——————→ "Phosphosphingomyelin"

as a minor pathway only. These findings appear to be supported by some observations of Welch,[184-5] who supplemented the diet of choline-deficient rats with arsenocholine; he reported strong arsenic lines in the carbon-arc spectra of lecithin extracts, but only weak lines from sphingosinephosphoryl-choline extracts.

(iv) ^{31}P-NMR analysis of other organ extracts.

The spectra of total neutral phospholipid extracts of P-rat brain, lung and kidney showed striking dissimilarities: (overleaf). Although there was no trace of the signal A, attributed to phosphobetaine, both lung and (especially) kidney extracts were found to contain large proportions of the degradation product giving signal B, attributed to trimethylphosphine oxide; that both A and B signals were due to phosphonium derivatives was confirmed by the absence of all but the phosphate signal from the spectra of all N-rat extracts.

Several workers[46-9] have shown that the major portion of orally-administered choline is degraded to trimethylamine oxide by the bacteria of the intestinal tract, and rapidly excreted in the urine. It is therefore highly likely that ingested phosphocholine suffers the same fate, being oxidized to trimethylphosphine oxide in the intestine and also excreted in the urine:

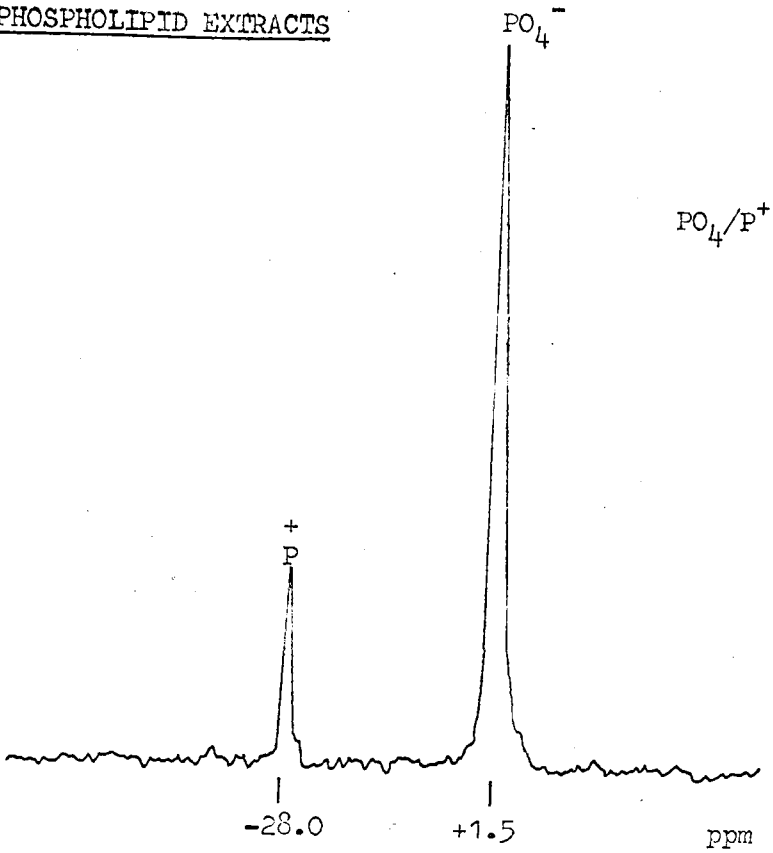
$$\text{Dietary phosphocholine} \xrightarrow{\text{intestine}} \text{trimethylphosphine oxide, } Me_3PO$$

$$\xrightarrow[\text{blood}]{\text{absorbed via}} \text{blood } Me_3PO \xrightarrow[\text{kidneys}]{\text{via}} \text{urine } Me_3PO$$

thus explaining its presence in the kidney in such large quantities.

BRAIN

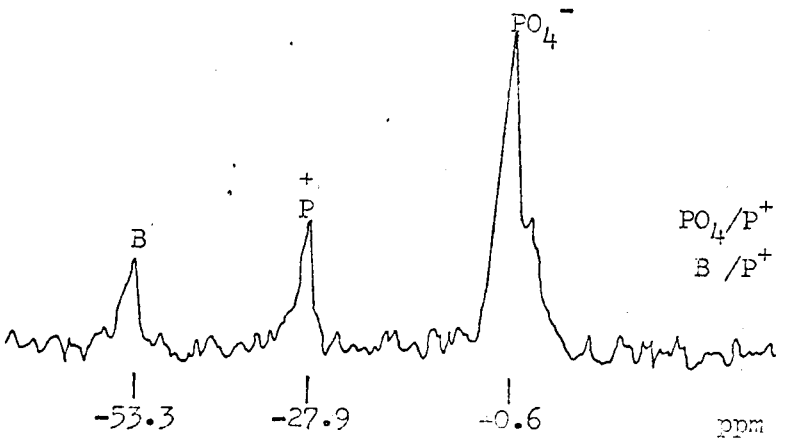
PO_4/P^+ peak area ratio 5.0



LUNG

PO_4/P^+ peak area ratio 3.7

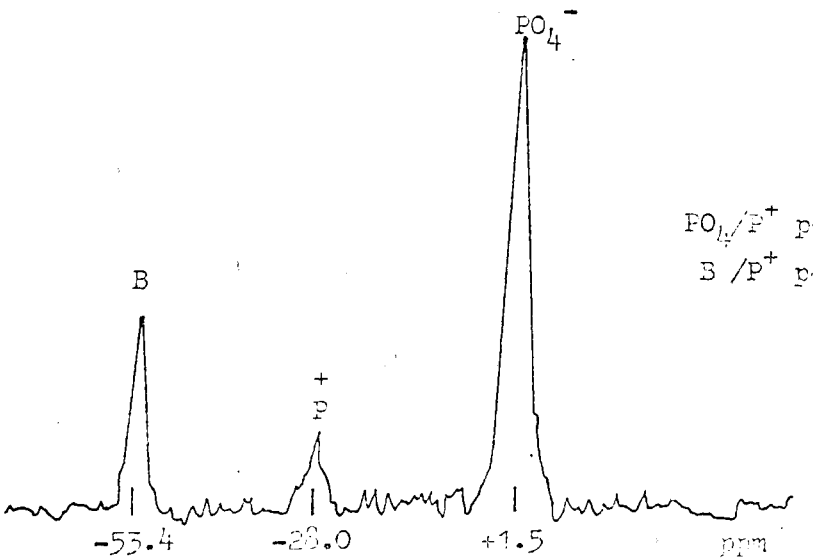
B $/P^+$ peak area ratio 0.92



KIDNEY

PO_4/P^+ peak area ratio 8.4

B $/P^+$ peak area ratio 2.5



Trimethylphosphine oxide would have been extracted with the lecithin in the procedure used, since it is neutral, polar and soluble in chloroform-methanol mixtures. It also appears that phosphocholine is oxidized to trimethylphosphine oxide in the lung, although the mechanism here possibly involves the degradation of phosphocholine when freed by phospholipid turnover. No obvious oxidation, either to phosphobetaine or trimethylamine oxide, appears to take place in the brain.

All three extracts were then subjected to preparative TLC as before, and the constituent phospholipids separated. A fraction producing a very low-field signal, corresponding to B, was isolated from the origin of the chromatogram of kidney lipid. In addition, it was later found that this fraction exhibited no ^{14}C activity, confirming the suggestion that the (1,2-^{14}C)-2-hydroxyethyl side chain, and not one of the methyl groups, had been lost through oxidation to the phosphine oxide.

From the weights of individual phospholipid fractions, and also the relative peak areas of the ^{31}P-NMR spectra, the following composition figures are obtained for phosphatidylphosphocholine:

ORGAN	WEIGHT AS PERCENTAGE OF TOTAL: PHOSPHOLIPID %	LECITHIN %	N/P-LECITHIN RATIO	PHOSPHOLIPID CONTAINING LECITHIN AS P-ANALOGUE (NMR) %
Brain	20	25	3.0	20
Kidney	13	17	4.7	12
Lung	25	33	2.0	27

Once again there is fair agreement between the phosphocholine content of phospholipids as calculated from weights of recovered material (first column) and from ^{31}P-NMR peak areas (last column). In all four organs investigated, a large proportion (67-83%) of lecithin has originated via pathways not utilizing dietary phosphocholine and the cytidine intermediate; of several possible alternatives - base exchange, utilization and recycling of choline from the original phospholipid, sequential methylation - the latter seems the most likely, but this would be in contrast to the claim of Bjørnstad and Bremer[14] that the methylation pathway is of significance only in the liver.

Sphingomyelin was again found to contain unexpectedly low levels of phosphocholine, approximately 6% in kidney and 11% in brain; the relative peak areas for lung and liver could not be measured with any degree of accuracy.

(v) 270 MHz pulsed Fourier transform PMR of purified phospholipids.

Samples of P-rat brain phosphatidylphosphocholine, phosphatidylcholine and sphingomyelin were submitted for 270 MHz PMR spectroscopy, and their structures were confirmed. The characteristic methyl doublet of the trimethylphosphonium group was quite apparent in the spectrum of phosphatidyl-phosphocholine at 8.0 τ; its presence in the spectrum of sphingomyelin indicated a phosphocholine incorporation of 5-10% (*cf.* 11% by ^{31}P-NMR above).

270 MHz PMR SPECTRA OF RAT BRAIN PHOSPHOLIPIDS

ASSIGNMENT	SPHINGOMYELIN	PHOSPHATIDYL-CHOLINE	PHOSPHATIDYLPHOSPHOCHOLINE	
			RAT BRAIN	SYNTHETIC
CH=CH	4.62	4.63	4.65	——
CH.OCO	——	4.80	4.81	4.82
N.C.CH_2OP	5.73	5.71	——	——
P.C.CH_2OP	——	——	5.75	5.78
CH_2.OCO	——	5,62, 5.87	5.61, 5.87	5.68, 5.92
CH_2.OP	6.02	6.06	6.08	6.08
=C.HC(.C.N).O	6.02	——	——	——
NCH_2.C.OP	6.24	6.25	——	——
$N(CH_3)_3$	6.72	6.68	——	——
C=C.CH_2.C=C	——	7.15	7.19	——
P.CH_2.C.OP	——	——	7.25	7.30
CH_2CO_2; CH_2CON	7.73	7.70	7.74	7.75
$P(CH_3)_3$	(8.00)**	——	7.93-7.98*	7.97-8.02*
CH_2.C=C	8.00	7,97	7.98	——
CH_2.C.CO_2; CH_2.C.CON	8.48	8.41	8.45	8.37
$-(CH_2)_n-$	8.71	8.72	8.75	8.76
$-C_n-CH_3$	9.12	9.09	9.14	9.14

*$J_{(P.CH_3)}$ = 14.5 Hz

**Represents ca. 5-10% of phosphonium analogue.

(vi) Radioactivity measurements of phospholipid extracts.

Radioactivity measurements were made on samples of all extracts, both before and after preparative TLC, and on samples of the diet. Since the P-diet had contained ^{3}H-labelled choline of negligible molarity but high specific activity, which was assumed to have come to equilibrium with the choline already present in the rats, and ^{14}C-labelled phosphocholine, changes in the $^{3}H/^{14}C$ ratio from diet to organ phospholipid might indicate a preference for one analogue or the other in the cytidine pathway, although they might also indicate different rates of turnover of the resulting phospholipid However, several assumptions must be made; firstly, that phosphocholine derivatives are not methyl donors (confirmed by Welch and Welch[185]), and that any phosphocholine incorporated into phospholipids is utilized *via* the cytidine pathway; secondly, that any loss of labelled methyl groups from (methyl-^{3}H)-choline through transmethylation is negligible in comparison with utilization of the complete molecule via the cytidine pathway (since there is no shortage of methionine); and thirdly, that base-exchange effects may be disregarded.

If all these assumptions are made, the various phospholipids investigated may be placed in the order of preference for choline (over phosphocholine) of the enzyme systems involved in their biosynthesis:

SAMPLE	CHOLINE/PHOSPHOCHOLINE UTILIZATION RATIO* FOR CYTIDINE PATHWAY
Brain phospholipid	1.33
Liver phospholipid	1.95-2.25
Lung phospholipid	2.35
Liver sphingomyelin	3.22
Kidney phospholipid	3.61
Lung sphingomyelin	10.0
Brain sphingomyelin	13.0
Kidney sphingomyelin	16.3

*(Based on P-diet $^{3}H/^{14}C$ ratio = 1.00. "Phospholipid" here refers to the mixture of lecithins and sphingomyelin prior to preparative TLC.)

One tentative inference is that renal enzyme systems exhibit a greater degree of specificity towards bases utilized for phospholipid synthesis, although this idea is an oversimplification and other factors are undoubtedly involved. It also appears that, in general, phosphoryl-choline-ceramidetransferase is considerably more discriminating than -diglyceridetransferase, thus supporting the conclusion from the ^{31}P-NMR investigation and also explaining Welch's[184-5] observation of different arsenic intensities in the arc-spectra of lecithin and sphingosine-phosphorylcholine.

These results hardly support the suggestion of Welch and Landau,[190] however, that "the lipotropic action (of a choline analogue) might 'spare', for the protection of the more sensitive kidney tissue, small amounts of choline formed as a result of the synthetic activity of the young rat": the difference in the choline/phosphocholine utilization ratios of liver and kidney lecithin is not that significant.

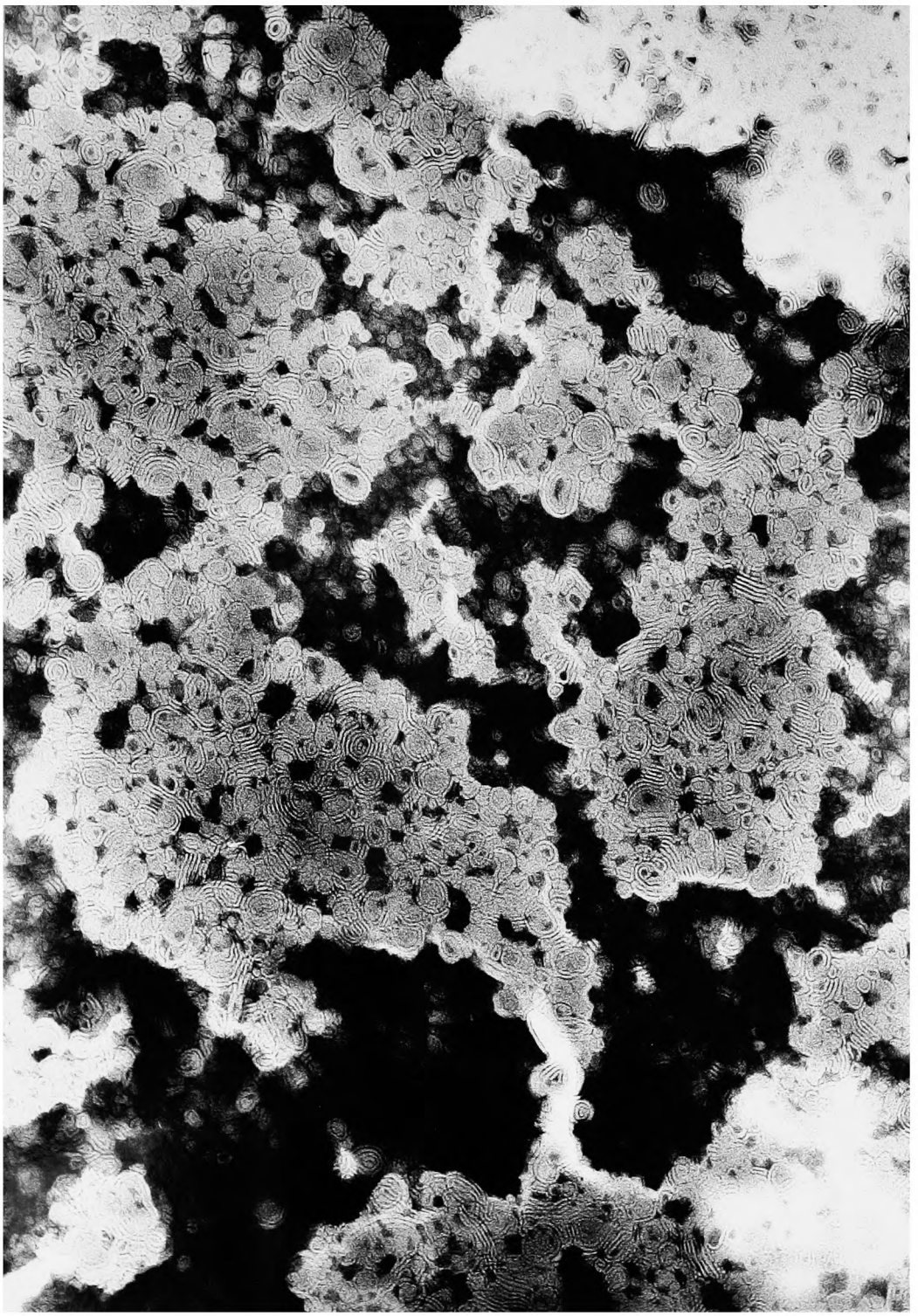
(vii) Lamellar and single-bilayer structure of rat-brain phosphatidylphosphocholine liposomes.

Negatively- and positively-stained liposomes were prepared as described earlier (Chapter 2 (vi)); electron microscopy of the negatively-stained samples (overleaf) revealed extensive lamellar structure, as for natural lecithin, with many single-bilayer liposomes visible. Positive staining indicated a mean micellar diameter (550-600 Å) intermediate between those of distearoylphosphatidylphosphocholine and its tri-n-butyl analogue (micrograph in Chapter 2(vi)).

(viii) GLC analysis of lecithin fatty acid distribution.

Samples of P-rat and N-rat phosphatidylcholine, and of P-rat phosphatidylphosphocholine, from liver, brain, lung and kidney, were subjected to methanolysis by the method of Hübscher *et al.*,[200] the fatty acid methyl

1000 Å



NEGATIVELY - STAINED RAT BRAIN PHOSPHATIDYLCHOLINE LIPOSOMES.

MAGNIFICATION 120,000 X

(PP. 79-80, 30)

esters so formed were then chromatographed at 196° on a poly(ethylene glycol adipate) column, and peaks were identified by comparison with chromatograms of methyl ester standards and, in a few cases, by reference to published data.

Since the samples of lecithin were not freshly extracted, but had spent an appreciable time before methanolysis in chloroform and non-stabilized deuteroform solution while spectrometer time became available and spectral data were accumulated, there may have been some oxidation of the polyenoic fatty acids, even though samples were kept at low temperatures under nitrogen wherever possible. Moreover, the method gave no indication of the original positional distribution of the fatty acids, although the general tendency is for saturated acids to occur at the 1-position and unsaturated at the 2-position of the glycerol moiety of lecithin from these tissues.[292]

From the results overleaf, it is possible to observe the fatty acid distribution in three species of lecithin:

P-PPC, P-rat phosphatidylphosphocholine thought to be synthesized by the cytidine pathway alone;

P-PC, P-rat phosphatidylcholine, synthesized by other pathways, the most probable being transmethylation;

N-PC, N-rat phosphatidylcholine, 'normal' lecithin synthesized by both cytidine and transmethylation pathways.

The outstanding observation is the enormous increase in the proportion of

RELATIVE DISTRIBUTION OF FATTY ACIDS

(Palmitic = 100)

		KIDNEY			LUNG			BRAIN			LIVER		
ACID	CODE	P-PPC	P-PC	N-PC	P-PPC	P-PC	N-PC	P-PPC	P-PC	N-PC	P-PPC	P-PC	N-PC
Myristic	14:0	1.8	1.7	1.0	6.1	6.8	7.3	0.5	0.7	0.8	2.3	2.2	1.6
Palmitic	16:0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Palmitoleic	16:1	11.2	7.0	7.7	13.0	14.4	17.5	5.1	4.8	*	28.0	33.3	15.9
Stearic	18:0	594.5	17.4	38.8	10.4	8.0	8.1	63.4	37.9	40.3	46.7	37.9	42.2
Oleic	18:1	59.4	34.2	44.7	23.5	18.5	19.1	102.0	82.9	76.6	94.8	92.9	57.8
Linoleic	18:2	3.4	3.8	3.0	0.7	0.7	0.4	0.7	0.5	0.8	14.8	10.6	9.8
Linolenic	18:3	63.5	4.3	1.0	4.6	1.6	0.3	1.7	1.7	0.9	1.8	0.5	0.5
Arachidonic	20:4	117.9	9.3	1.0	10.8	2.6	0.9	4.0	1.3	0.0	2.0	2.6	1.5

* unresolved

stearic, arachidonic and linolenic acids in kidney P-PPC, at the expense of palmitic, oleic and linoleic acids. General observations are that in lung, brain and liver, fatty acid distributions are very similar in N-PC and P-PC, and even P-PPC shows only slightly elevated levels of stearic and arachidonic acids. In kidney, N-PC and P-PC still differ only slightly through a depressed level of stearic acid in P-PC.

If the hypothesis that P-PC is formed mainly via the sequential methylation pathway is correct, it is found to be in direct conflict with suggestions[242, 293-4] that the product of this pathway is a lecithin subspecies rich in stearate and poly-unsaturated fatty acids: there is seen to be no such difference between P-PC and the 'normal' lecithin, N-PC. It has similarly been suggested that the product of the cytidine pathway is a subspecies rich in oleic and linoleic acids; if P-PPC has indeed been formed in this way, as is thought, the suggestion is not borne out in the case of three organs, and in the fourth (kidney) it is greatly contradicted.

Of the four tissues chosen for examination, the kidney seems to be the least typical in its phospholipid composition. Thus it has been seen that it incorporates less phosphocholine into lecithin and sphingomyelin than the other organs, suggesting that alternative biosynthetic pathways are utilized to a greater extent; it contains large quantities of a phosphocholine degradation product, possibly trimethylphosphine oxide; its phosphorylcholine-diglyceridetransferase and -ceramidetransferase appear to be somehow more specific (based on radioactivity measurements);

and the fatty acid distribution in the phosphatidylphosphocholine that has been formed is atypical and unexpected. It has been noted[163] that this period just after weaning, in rats, is the time of greatest susceptibility to renal haemorrhage, and of greatest activity in building up renal phospholipids.

The maintenance of the correct degree of membrane fluidity may provide a reason for this abnormal renal fatty acid distribution. Chapman et al.[102, 267] have pointed out that fatty acid behaviour should not be discussed independently of the polar goups attached attached to the phosphatides under consideration, and that variation of fatty-acid distribution may be part of some feed-back mechanism related to a need for a constant optimum fluidity on the part of the membrane in order that it should carry out its various functions at a given temperature. Thus the fluidity requirements of a membrane can be satisfied by variation of chain length, unsaturation or branching, or by a change of polar head group.

If the fluidity of lecithin is a critical factor in satisfactory renal membrane function, therefore, it would explain the increase in fatty acid chain length observed in phosphatidylphosphocholine, from palmitic (16:0) to stearic (18:0), since the transition temperature of phosphatidylphosphocholine has been shown to be lower than that of phosphatidylcholine for a given fatty acid distribution (Chapter 2(i)). Moreover, de Pury and Collins[295] have suggested that arachidonyl lecithins form more stable memb-

ranes than do the more saturated ones, which could also explain the change from oleic (18:1) and linoleic (18:2) acids to linolenic (18:3) and arachidonic (20:4) acids. Finally, in support of this hypothesis,[295] Trewhella and Collins[247] have recently shown that 1-stearoyl-2-arachidonyl-lecithin has the slowest rate of turnover of any lecithin subspecies present in the liver of the normal rat.

It is therefore suggested that the great susceptibility to renal haemorrhage exhibited by weanling rats on a choline-deficient diet is connected with the need to maintain a critical membrane fluidity in the kidney.

EXPERIMENTAL DETAILS

MATERIALS AND EQUIPMENT

Infrared spectra were recorded on a Perkin-Elmer 257 grating spectrophotometer. PMR spectra were recorded at 60 MHz by the author on a Perkin-Elmer R12 spectrometer, and at 90 MHz by J.M.Smith and 270 MHz by R.W.Barker on the Bruker HFX-90 and -270 spectrometers of the Oxford Enzyme group; chemical shifts were measured relative to internal standards of tetramethylsilane (TMS) for organic solvents, and sodium 3-(trimethylsilyl)-propionate (TSS) in aqueous solutions.

^{31}P-NMR spectra were recorded by R.W.Barker using the pulse Fourier transform technique, with a 5T superconducting magnet and a spectrometer and analog Fourier analyzer constructed in the laboratory of Dr. R.E. Richards. The operating frequency was 84.5 MHz, and chemical shifts were measured relative to a 87% orthophosphoric acid external reference standard.

Mass spectra were recorded on an AEI MS9 spectrometer by D.Perrin under the supervision of Dr. R.T.Aplin. Insertion temperatures are given, and major peaks are presented in order of decreasing magnitude, being measured as a percentage of the size of the base peak. The other recording parameters were: ionization potential 70 ev, ion trap

current 100 μamps, accelerating voltage 8 kv. The samples were applied in chloroform solution to the direct insertion probe.

Electron micrographs were obtained on an AEI 801 microscope by Dr. S.Knutton, who also prepared the thin-sections; the photographs were printed by Mrs. D.Jackson.

Radioactivity was monitored on an Intertechnique SL30 liquid scintillation counter, with window settings of 009-070 (^{3}H) and 350-550 (^{14}C), for the experiments in Chapter 3, and on an LKB Wallac 8100 liquid scintillation counter for the remainder of the work. The window settings of the latter were 2-6 (1.26-4.00 kev) (^{3}H) and 13-25 (15.8-317 kev) (^{14}C), with respective efficiencies of 12% and 68% determined by the use of ^{3}H- and ^{14}C-labelled hexadecane standards. Scintillation vials were of linear polyethylene (New England Nuclear, Boston); scintillation fluid was a dioxan solution of naphthalene (20 $g.l.^{-1}$) and 2,5-diphenyloxazole (PPO) (5 $g.l.^{-1}$).

Melting points of non-hygroscopic compounds, and transition temperatures of lecithin analogues, were measured by means of a Köffler heating stage; other melting points, including those of the lecithin analogues, were measured in sealed capillary tubing immersed in silicone oil.

Methyl esters of fatty acids were chromatographed on a Pye Series 104 gas-liquid chromatograph, using a 5-foot glass column with a

stationary phase of poly (ethylene glycol adipate), operating at 196°. The carrier gas was nitrogen, under a pressure of 60 psi and with a flow rate of 60 ml.min.$^{-1}$. The apparatus was equipped with a flame ionization detector, and was connected to a Servoscribe pen recorder operating at a chart speed of 60 cm.hr.$^{-1}$.

All ion-exchange resins were of the Amberlite type (Rohm and Haas, Philadelphia) and were washed thoroughly before use with distilled or deionized water; only analytical-grade resin was used. Analogues of CDP-choline were chromatographed using an LKB Ultrorac 7000 fraction collector, fitted with an automatic ultraviolet measuring device monitoring absorption at 254 nm.

Silica gel for column chromatography was of 60-120 mesh (British Drug Houses), and alumina was of type H (Laporte); the former was activated before use by heating at 120° for 2 hours.

TLC was conducted on Macherey-Nagel MN-polygram sil N-HR/UV$_{254}$ silica gel pre-coated plastic sheets, containing a component fluorescing at 254 nm. Cellulose MN-300 plates were of glass; preparative TLC plates were also of glass, coated with silica to a thickness of 1 mm., and were pre-eluted with methanol before use. Three solvent systems were used:

Solvent system A: chloroform-methanol-water (2:3:1)

Solvent system B: chloroform-methanol-0.880 ammonia-water (57:20:1:2)

Solvent system C: n-butanol-ethanol-acetic acid-water (8:2:1:3)

The majority of microanalyses were carried out by Alfred Bernhardt Mikroanalytisches Laboratorium, Elbach über Engelskirchen, West Germany, although a few were by Dr. Strauss and staff, Dyson Perrins Laboratory, Oxford.

All water used was singly distilled, except where otherwise stated. All chemicals, except those used in the synthesis of reagents, were of analytical grade where obtainable. All solvents were distilled before use; removal of solvents under reduced pressure was achieved using a Büchi Rotavapor rotary evaporator. Diethyl ether was dried with sodium wire. Acetone was dried by the method of Smith et al.[296] using Linde type 4A molecular sieve. Carbon tetrachloride and ethyl acetate were dried with anhydrous magnesium sulphate, filtered and redistilled. Acetonitrile was dried by boiling under reflux with calcium hydride, followed by distillation from the same reagent. 1,4-Dioxan was dried by boiling under reflux with calcium hydride, followed by distillation from sodium wire. Ethanol and methanol were dried using magnesium, after the method described by Vogel.[297] Pyridine, analytical grade, was dried over potassium hydroxide, followed by distillation from fresh reagent.

Stearic acid ("specially pure") was obtained from British Drug Houses. Trimethyl- and tri-n-butylphosphine were from the Aldrich Chemical Co., Milwaukee, Wisconsin. Nucleotides were purchased from the Sigma London Chemical Company, and (methyl-^{3}H)-choline from The Radiochemical Centre,

Amersham; (1,2-^{14}C)-2-bromoethanol was from New England Nuclear, Boston. 1,2-L-Diglyceride and phospholipase C were from the Koch-Light Laboratories For the rat diet, starch and vitamin-free casein were Fisons laboratory reagents, cod-liver oil was from British Drug Houses and sugar was Tate and Lyle granulated; the salt mixture was made up by the staff of the Animal House in this Department. P815Y cells were kindly provided by Dr. C.A.Pasternak, as were the facilities for cell culture.

Modified Zinzadze spray for the detection of phosphate esters.[298-9]

A modification by Dittmer and Lester[298] of the original method of Zinzadze[299] was used.

To 25N sulphuric acid (1 l.) was added molybdenum trioxide (40.11 g.); the mixture was boiled gently until the trioxide dissolved (ca. 2 hours), giving a greenish-yellow solution, "Solution I", which became deep blue after standing overnight.

To a portion of Solution I (500 ml.) was added powdered molybdenum metal (1.78 g.), and the mixture was boiled gently for 15 minutes to give "Solution II"; the latter was cooled and decanted from the residue. Equal volumes of Solutions I and II were mixed, and poured into water, with stirring. The final solution was greenish-yellow in colour.

Phosphate esters on cellulose or silica thin-layer chromatograms showed up rapidly as blue spots on a white background when sprayed with this reagent; development could be accelerated by drying the plate in a stream of hot air, and then holding it over a steam-bath for a few seconds.

Lipid detection spray.

The spray reagent devised by Jones et al.[300] was employed for lipid detection. The composition was Rhodamine B (100 mg.), 2',7'-dichlorofluorescein (35 mg.), diethyl ether (150 ml.), 95% aqueous ethanol (70 ml.) and water (16 ml.). Chromatograms were sprayed and allowed to dry before examination under ultraviolet light (350 nm.). Lipids fluoresced as orange or orange-purple spots on a green background.

Amberlite CG-400 and IRA-400 (formate) anion-exchange resins.

The method of conversion of the chloride form of the exchange resin to the formate was that devised by Hurlbert et al.[253] for Dowex 1 resin.

The appropriate Amberlite resin was packed as an aqueous slurry in a glass column fitted with a sintered glass support. The resin was washed before use with ca. 8 bed volumes of a mixture of 6N aqueous formic acid and 1N aqueous sodium formate until chloride ion was no longer eluted, then with 5 bed volumes of 88% aqueous formic acid, and finally with deionized water until the effluent was no longer acidic.

CHAPTER 1 : CHEMICAL SYNTHESES

Phosphocholine chloride (2-hydroxyethyltrimethylphosphonium chloride) (XXIVa).[136, 140]

Trimethylphosphine (24.8 g., 326 mmole), 2-chloroethanol (29.8 g., 370 mmole) and anhydrous ethanol (25 ml.) were thoroughly mixed in a glass bomb of total capacity 250 ml. in an atmosphere of nitrogen. The bomb was sealed, secured in a steel case and heated in a bath of boiling water, in the open air, for 6 hours. It was allowed to cool, first in cold water and then in an acetone/dry ice bath, before it was unsealed. The contents were transferred to a flask and ethanol, and excess of 2-chloroethanol, were removed by distillation under reduced pressure. The solid white residue was recrystallized from anhydrous ethereal ethanol to give phosphocholine chloride (XXIVa) (45.2 g., 88.6%) as long, colourless, extremely deliquescent needles which decomposed without melting above 250°. (Found: C, 38.2; H, 9.2; Cl, 22.4; P, 19.6. Calc. for $C_5H_{14}ClOP$: C, 38.35; H, 9.0; Cl, 22.6; P, 19.8%. PMR (D_2O): τ 5.76-6.30 ($P.C.CH_2.O$, 2 protons, pair of triplets); 7.30-7.72 ($P.CH_2.C.O$, 2 protons, pair of overlapping triplets); 7.98-8.22 (methyl, 9 protons, doublet). $J_{(P.C.CH_2)}$ =

20.0 Hz; $J_{(\underline{P}.\underline{CH_2}.C)}$ = 12.9 Hz; $J_{(\underline{P}.\underline{CH_3})}$ = 14.4 Hz; $J_{(H.H)}$ = 6.2 Hz ^{31}P-NMR (H_2O): ppm -25.9 (P^+).)

Renshaw and co-workers[136, 140] have only published chlorine analyses for this compound; Lemmon et al.[301] claim satisfactory analyses, but do not quote any figures.

(1,2-^{14}C)-Phosphocholine chloride.

(1,2-^{14}C)-2-Bromoethanol (LII) (1 mCi, 19.5 mg., 0.16 mmole, specific activity 6.4 mCi.mmole^{-1}, ca. 60% with 2 isotopic atoms per molecule), diluted with unlabelled 2-chloroethanol (300 mg., 3.72 mmole), was thoroughly mixed with trimethylphosphine (0.60 ml., excess) and anhydrous ethanol (0.5 ml.) under nitrogen in a glass bomb of capacity 8 ml. The bomb was sealed, kept at 100° for 6 hours, cooled and unsealed as above. Removal of ethanol, excess trimethylphosphine and any unreacted 2-chloroethanol by heating under reduced pressure (100°, 15mm) gave a mixture of phosphocholine bromide and chloride as a white solid. The distillate was monitored for radioactivity with a portable scintillation counter, but the quantity of labelled starting material that it contained was negligible. The crude product was dissolved in water (40 ml.) and allowed to percolate through a column of Amberlite IRA-400 (Cl) anion-exchange resin (10 ml.); the latter was washed with water until the radioactivity of the eluate had become negligible, giving a total

volume of 500 ml. Water was removed under reduced pressure; the resulting white solid was dried in vacuo over phosphorus pentoxide and recrystallized from anhydrous ethereal ethanol to give (1,2-^{14}C)-phosphocholine chloride (502 mg., 3.21 mmole, 82.5%) as colourless needles. Radioactivity measurements indicated a specific acivity of 190 μCi.mmole^{-1}.

Phosphocholine iodide (XXIVb).

To a solution of sodium iodide (9.6 g., 64 mmole) in anhydrous acetone (2 l.) was added phosphocholine chloride (XXIVa) (10.1 g., 64 mmole), and the mixture was boiled under reflux for 12 hours. The solution was filtered and the solvent removed by distillation under reduced pressure to give a very pale yellow solid; recrystallization from anhydrous ethereal ethanol gave 2-hydroxyethyltrimethylphosphonium iodide (phosphocholine iodide) (XXIVb) (12.4 g., 77.5%) as colourless needles which decomposed without melting above 250°. The product was far less hygroscopic than the starting material. (Found: C, 24.4; H, 5.7; I, 51.4; P, 12.2. $C_5H_{14}IOP$ requires: C, 24.2; H, 5.7; I, 51.2; P, 12.5%.)

2-Iodoethanol.[302]

Sodium iodide (1 kg., 7.3 mole) and 2-chloroethanol (500 g., 6.2 mole) were added with stirring to hot, anhydrous acetone (3 l.); the mixture was boiled under reflux, with vigorous stirring, for 6 hours. The resulting dark brown solution was filtered , and the solvent removed under reduced pressure. The residual oil was purified by vacuum distillation to give 2-iodoethanol (544 g., 51%) as a dense, dark brown liquid, b.p. 79-80°/13 mm., n_D^{20} 1.5724.

Mercer[302] gives b.p. 80-83°/15 mm., n_D^{20} 1.5702; Wieland and Sakellarios[303] give b.p. 85-88°/25 mm., while Karvonen[304] gives n_D^{20} 1.5704.

2-Hydroxyethyltri-n-butylphosphonium iodide (LIIIb).

The method was similar to that used by Waters [249] for the preparation of the 3-hydroxypropyl homologue.

Tri-n-butylphosphine (101.1 g. of 98% purity, 0.5 mmole), 2-iodoethanol (86.2 g., 0.5 mmole) and anhydrous ethanol (500 ml.) were boiled under reflux, in an atmosphere of nitrogen, for 2 days. Ethanol was removed by distillation under reduced pressure; the residual oil was dissolved in water (3.5 l.) and the aqueous solution washed with ether (3 X 1l.). Removal of water under reduced pressure gave a colourless

oil; this was dried *in vacuo* over phosphorus pentoxide for several days, yielding 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb) (175.5 g., 96%) as a colourless, deliquescent wax, m.p. 50-52°. (Found: C, 45.0; H, 8.6; I, 34.2; P, 8.3. $C_{14}H_{32}IOP$ requires: C, 44.9; H, 8.6; I, 33.9; P, 8.3%. PMR ($CDCl_3$): τ 5.70-6.23 (P.C.CH_2.O, 2 protons, pair of triplets); 5.80 (OH, 1 proton, singlet, disappears on addition of D_2O); 7.11-8.00 (P.CH_2.C.O overlapping with n-Bu protons α to P, 8 protons. complex); 8.00-8.82 (n-Bu methylene β and γ to P, 12 protons, complex); 8.82-9.20 (methyl, 9 protons, complex). $J_{(P.C.CH_2.O)}$ = 19.6 Hz; $J_{(P.CH_2.C.O)}$ = 11.6 Hz; $J_{(H.H)}$ = 5.8 Hz. ^{31}P-NMR: ppm -33.7 ($CHCl_3$) or -35.0 (H_2O).)

Witschard and Griffin[248] give m.p. 113-6° for a new compound which they claim to be 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb), obtained in an "acceptable" yield of 20% by quaternization in the absence of solvent, and analyzing according to theory. However, they report their compound to be non-hygroscopic, crystallizing from water. This is in obvious disagreement with the above findings, and also with those of Waters,[249] who prepared the 3-hydroxypropyl homologue and reported it to be very hygroscopic, m.p. 40° after drying *in vacuo* over phosphorus pentoxide for 2 days.

2-Hydroxyethyltri-n-butylphosphonium chloride (LIIIa).

A solution of 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb) (2.24 g.) in water (50 ml.) was allowed to percolate through a column of Amberlite IR-400 (Cl) anion-exchange resin (50 ml.), which was then thoroughly eluted with water (500 ml.). Removal of solvent from the eluate under reduced pressure gave 2-hydroxyethyltri-n-butylphosphonium chloride (LIIIa) (1.60 g., 94%) as a colourless gum, n_D^{20} 1.5025, which would not crystallize even when stored under high vacuum over phosphorus pentoxide for several weeks.

Witschard and Griffin[248] report this as a 'viscous oil which could not be crystallized'.

Tri-n-butylphosphine oxide (LV).[249]

Tri-n-butylphosphine (4.4 g.), 3% aqueous hydrogen peroxide (50 ml.) and acetone (250 ml.) were kept at 40° for 3 hours; solvent was removed under reduced pressure, and the residue recrystallized from petroleum (b.p. 80-120°) to give tri-n-butylphosphine oxide (3.0 g., 63%) as a colourless solid, m.p. 65°. (PMR ($CDCl_3$): τ 8.00-8.85 (n-Bu methylene, 18 protons, complex); 8.85-9.22 (methyl, 9 protons, complex). ^{31}P-NMR ($CHCl_3$): ppm -51.8 (cf. $(n\text{-Bu})_3P$, +31.3). IR: ν_{max} (nujol mull):

1150 $cm.^{-1}$ (P-O stretch).)

Waters[249] gives m.p. 65°; Addison and Sheldon[305] give m.p. 67°.

Phenylphosphorodichloridate (XIX) and diphenylphosphorochloridate (XVIII).

Phenol (94 g., 1 mole) and phosphoryl chloride (153.5 g., 1 mole) were boiled under reflux for 21 hours, and the product distilled under reduced pressure. The fraction boiling at 123-127°/18 mm. (123 g.) was collected and redistilled to give phenylphosphorodichloridate (XIX) (99.0 g., 47%) as a colourless liquid, b.p. 123-4°/15 mm., n_D^{20} 1.5241.

Hands[306] gives b.p. 125-127.5°/18 mm., n_D^{22} 1.5225; Hayakawa et al.[307] give b.p. 107-110°/10 mm., n_D^{25} 1.5315.

Further distillation of the residue from the above preparation gave diphenylphosphorochloridate (XVIII) (27.8 g., 10.4%) as a colourless liquid, b.p. 196-8°/14 mm., n_D^{20} 1.5500.

Hands[306] gives b.p. 208-210°/18 mm., n_D^{20} 1.5499; Hayakawa et al.[307] give b.p. 159-62°/6 mm., n_D^{25} 1.5489.

2,2,2-Trichloro-1,3,2-benzodioxaphosph(v)ole (catechyl phosphotrichloride) (LX).[250, 308]

Catechol (LIX) (200 g., 1.82 mole) was added to a stirred suspension of phosphorus pentachloride (475 g., 2.28 mole) in anhydrous benzene (2.5 l

over a period of 2 hours. The mixture was boiled under reflux for 3 hours, and hydrogen chloride was evolved. Benzene and excess of phosphorus pentachloride were removed by distillation under reduced pressure. Vacuum distillation of the residual brownish-green oil gave a pale yellow oil, b.p. 138-42°/14 mm., which solidified in the receiver; recrystallization from benzene yielded 2,2,2-trichloro-1,3,2-benzodioxaphosph(v)ole (LX) (323 g., 72.5%) as pale yellow plates, m.p. 60-62°, b.p. 139-42°/14 mm., which were extremely sensitive to moisture.

Anschütz[308] gives b.p. 132°/11 mm., m.p. 61-2°; Khwaja et al.[250] give b.p. 104°/1.3 mm.

o-Phenylene phosphorochloridate (LVIII).[250, 308]

2,2,2-Trichloro-1,3,2-benzodioxaphosph(v)ole (LX) (170 g., 0.69 mole) was warmed gently with acetic anhydride (73.4 g., 0.72 mole); acetyl chloride was evolved and removed by distillation. The mixture was heated on a water-bath for 20 minutes to complete the reaction. Vacuum distillation of the residue gave o-phenylene phosphorochloridate (LVIII) (89.5 g., 68%) as a colourless oil, b.p. 126-128°/14 mm., solidifying in the receiver; recrystallization from light petroleum (b.p. 40-60°)/benzene (3:1) gave large, colourless prisms, m.p. 59-60°.

Anschütz[308] gives b.p. 122°/12 mm., 58-9°; Khwaja et al.[250] give b.p. 80-1°/1.2 mm., m.p. 59-61°.

Dimethylphosphoramidic dichloride (LVI).[306]

Dimethylamine hydrochloride (100 g., 1.23 mole) was boiled under reflux with phosphoryl chloride (600 g., 3.90 mole) for 18 hours. Fractional distillation of the product under reduced pressure gave dimethylphosphoramidic dichloride (LVI) (162 g., 82%) as a colourless liquid, b.p. 78-80°/15 mm., n_D^{20} 1.4620.

Hands[306] gives b.p. 84-86°/14 mm., n_D^{20} 1.4647; Tolkmith[309] gives n_D^{20} 1.4640, and Dickey et al.[311] give b.p. 80-82°/18 mm.

Bis-(dimethylamido)-phosphoryl chloride (LVII).[306]

A solution of phosphoryl chloride (134.8 g., 0.85 mole) in dry ether (500 ml.) was added, over a period of 45 minutes, to a stirred solution of anhydrous dimethylamine (158.5 g., 3.5 mole) in dry ether (500 ml.) at ca. -70°; stirring was continued at 0° for a further 12 hours. The dense white precipitate of dimethylamine hydrochloride was removed by filtration: removal of ether and distillation of the residual oil gave bis-(dimethylamido)-phosphoryl chloride (LVII) (66.8 g., 45%) as a colourless liquid, b.p. 117-8°/15 mm., n_D^{20} 1.4669.

Hands[306] gives b.p. 120-121°/15 mm., n_D^{25} 1.4630; Tolkmith[309] gives n_D^{20} 1.4670, and Lester gives[310] b.p. 118°/16 mm.

Methylphosphorodichloridate (LXVI).[312]

The method was that of Mizuma et al.,[312] a modification of the preparations devised by Wichelhaus[313] and by Saunders et al.[314]

Anhydrous methanol (32.0 g., 1 mole) was added dropwise over a period of 30 minutes to a solution of phosphoryl chloride (153.3 g., 1 mole) in dry ether (200 ml.) at 0°. The mixture was stirred for 2 hours at 0°, and for 12 hours at room temperature (20°). Ether was removed under reduced pressure, and the residue distilled to give methyl phosphorodichloridate (LXVI) (101.1 g., 68%) as a colourless liquid, b.p. 46-9°/14 mm., n_D^{20} 1.4318.

Mizuma[312] gives b.p. 57-60°/18-20 mm.

Attempted phosphorylation of 2-hydroxyethyltri-n-butylphosphonium iodide with polyphosphoric acid.

The method was a modification of that devised by Plimmer and Burch[104] for the phosphorylation of choline chloride.

2-Hydroxyethyltri-n-butylphosphonium iodide (LIIIb) (13.4 g., 36 mmole) was added in small portions to a mixture of orthophosphoric acid (40.0 g., 410 mmole) and phosphorus pentoxide (4.0 g., 28 mmole). The mixture was heated under reflux for 6 hours, and allowed to cool. Crushed ice was added, and the resulting solution poured into ice-water

(ca. 1.5 l.). The solution was thoroughly shaken with an aqueous paste of calcium hydroxide until it was alkaline to phenolphthalein, and was filtered from the resulting calcium phosphate and excess of calcium hydroxide. Water was removed by distillation under reduced pressure until the volume had dropped to 100 ml., when precipitated calcium carbonate was removed by filtration. The solution was allowed to percolate through a column of Amberlite IR-120 (H) and IRA-400 (formate) ion-exchange resins, which was then thoroughly washed with water (900 ml.). Removal of solvent from the total eluate gave a colourless liquid (598 mg.) which PMR and ^{31}P-NMR, and infrared spectroscopy, showed to be the hydrate of tri-n-butylphosphine oxide (LV), and not the desired phosphate (LIV). (PMR (neat): τ 8.08-8.79 (n-Bu methylene, 18 protons, complex); 8.79-9.23 (methyl, 9 protons, complex). ^{31}P-NMR (neat): ppm -47.5. IR: ν_{max} (liquid film) 1150 cm.$^{-1}$ (P-O stretch).)

Phenyl 2-(tri-n-butylphosphonio)-ethyl phosphate, inner salt (LXII).

The general procedure was that devised by Bird[107] for the synthesis of phosphorylcholine (II).

Quinoline (1.29 g., 10 mmole) in acetonitrile (1 ml.) was added dropwise to a solution of phenylphosphorodichloridate (XIX) (2.11 g., 10 mmole) in acetonitrile (1 ml.) at 0°. This mixture was slowly added,

under anhydrous conditions, to a stirred solution of 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb) (3.74 g., 10 mmole) in acetonitrile (4 ml.) at 0° in the dark. Stirring was continued for 2 hours at 0°, then for 10 hours at room temperature (20°). The mixture was again cooled to 0°, and pyridine (2.5 ml.) and water (0.5 ml.) added. The mixture was stirred at room temperature for 1 hour, and the solvents removed under reduced pressure. The residue was dissolved in water(150 ml.), and the solution allowed to percolate through a column of Amberlite MB-3 monobed resin (100 g. dry weight), which was then thoroughly eluted with water. Solvent was removed from the total eluate (1.25 l.) under reduced pressure, giving a colourless oil; this crystallized when dried in vacuo over phosphorus pentoxide, giving phenyl 2-(tri-n-butylphosphonio)-ethyl phosphate, inner salt (LXII) (1.84 g., 46%) as a colourless, hygroscopic wax, m.p.91-3°. TLC (silica, solvent system A) showed a single spot at R_F 0.55, blue when sprayed with the Zinzadze reagent. (Found: C, 59.7; H, 8.9; P, 15.3. $C_{20}H_{36}O_4P_2$ requires: C, 59.7; H, 9.0; P, 15.4%. PMR ($CDCl_3$): τ 2.68-320 (aromatic, 5 protons, complex); 5.49-6.12 ($P.C.CH_2.O.P$, two protons, pair of overlapping quartets); 7.18-8.15 ($P.CH_2.C.O$ and n-Bu methylene α to P^+, 8 protons, complex); 8.15-8.90 (n-Bu methylene β and γ to P^+, 12 protons, complex); 8.90-9.30 (methyl, 9 protons, complex). $J_{(P.C.CH_2.O)}$ = 19.4 Hz; $J_{(P.CH_2.C.O)}$ = 11.6 Hz; $J_{(H.H)} \approx J_{(P.O.CH_2)} \approx$ 5.4 Hz. ^{31}P-NMR ($CHCl_3$): ppm -34.0 (P^+);

+4.8 (PO_4^-); peak separation 36.9.)

In a second preparation, using 158 mmole (59.0 g.) of phosphonium salt the yield of product was 43% (27.3 g.).

Attempted hydrogenolysis of phenyl 2-(tri-n-butylphosphonio)-ethyl phosphate (LXII).

An attempt was made to employ the procedure devised by Bird[107] for the hydrogenolysis of phenyl phosphorylcholine to phosphorylcholine (II).

Adam's catalyst (100 mg.) was shaken under hydrogen in glacial acetic acid (14.1 g.) until hydrogen uptake ceased. To this was added a solution of phenyl 2-(tri-n-butylphosphonio)-ethyl phosphate, inner salt(LXII) (3.00 g.) in glacial acetic acid (25.1 g.) containing 5% w/w dry hydrogen chloride, but no further uptake of hydrogen was observed. TLC of the reaction mixture indicated the presence of starting material as the sole phosphate ester.

Attempted hydrolysis of the inner salt of phenyl 2-(tri-n-butylphosphonio)-ethyl phosphate (LXII).

The method was that employed by Baer et al.[105-6, 284] to hydrolyze diphenyl phosphorylcholine to phosphorylcholine (II).

A solution of phenyl 2-(tri-n-butylphosphonio)-ethyl phosphate, inner

salt, (LXII) (10.0 g.) and barium hydroxide octahydrate (35.5 g.) in water (250 ml.), containing n-octanol (0.2 ml.) to prevent foaming, was boiled under reflux for 2½ hours. The solution was diluted to 500 ml. with water, and a stream of carbon dioxide was passed through the solution until the latter reached pH 8. The barium carbonate formed was removed by filtration through a glass sinter coated with a 0.5 cm. layer of Hyflo Super Cel. The solution was concentrated to 50 ml., extracted with ether (3 X 20 ml.) to remove phenol and n-octanol, and treated with an equal volume of ethanol, whereupon small, colourless platelets (2.6 g.) crystallized out. PMR indicated that this was the barium salt, not of the phosphonium phosphate (LIV) as expected, but of phenyl phosphoric acid. Moreover, the ethereal extracts were found to contain only a mere trace of phenol, indicating that the hydrolysis had produced phenyl phosphoric acid (LXIII) and phosphonium salt (LIII). Thus removal of solvent from the barium phenyl phosphate mother liquor gave a colourless gum (8.02 g.); PMR spectroscopy identified it as the 2-hydroxyethyltri-n-butylphosphonium salt of phenyl phosphoric acid (LXIV), and microanalysis provided confirmation. The yield was 95%. Ion-exchange chromatography of a sample of the compound on Amberlite IR-120 (H) gave phenyl phosphoric acid, identical in spectral properties and m.p. (97-98°) to a sample prepared by Hands.[306] Ion-exchange chromatography of another sample on Amberlite IRA-400 (Cl) gave a colourless gum

identical in spectral properties and refractive index to 2-hydroxyethyl-tri-n-butylphosphonium chloride (LIIIa). (Found for 2-hydroxyethyltri-n-butylphosphonium phenyl phosphate: C, 60.9; H, 10.1; P, 13.8. $C_{34}H_{69}O_6P_3$ requires: C, 61.2; H, 10.3; P, 13.9%. PMR (D_2O): ~ 2.65-3.03 (phenyl, 5 protons, complex); 5.88-6.38 ($P.C.CH_2$, 4 protons, pair of triplets); 7.41-9.30 ($P.CH_2.C.O$ and n-Bu, 58 protons, complex).)

o-Hydroxyphenyl 2-(tri-n-butylphosphonio)-ethyl phosphate, inner salt (LXV).

A solution of o-phenylene phosphorochloridate (8.52 g., 50 mmole) in acetonitrile (30 ml.) was added slowly to a stirred solution of 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb) (19.7 g., 50 mmole) and pyridine (20 ml.) in acetonitrile (15 ml) at 0°. Stirring was continued at 0° for 2 hours, and at room temperature (22°) for 10 hours. The mixture was again cooled to 0°; water (5 ml.) was added, with stirring, and solvents were removed under reduced pressure after 1 hour. Traces of pyridine were removed by azeotroping with toluene. The residual brown oil was dissolved in water (250 ml.) and allowed to percolate through a column of Amberlite IR-120 (H) and IRA-400 (formate) (200 ml. of each) ion-exchange resin, which was thoroughly eluted with water (2 l.). Removal of solvent under reduced pressure left a colourless gum, which when stored under high vacuum over phosphorus pentoxide at 50° crystallized out to give o-hydroxyphenyl 2-(tri-n-butylphosphonio)-ethyl phosphate,

inner salt, (LXV) (8.17 g., 37%) as a colourless wax, m.p.112°. (Found: C, 57.4; H, 8.7; P, 14.85. $C_{20}H_{36}O_5P_2$ requires: C, 57.4; H, 8.7; P, 14.8%. PMR ($CDCl_3$): τ -0.54 (phenolic -OH, 1 proton, broad singlet, disappears on addition of D_2O); 2.95-3.55 (aromatic, 4 protons, complex); 5.45-6.10 (P.C.CH_2.O, 2 protons, pair of overlapping quartets); 7.38-8.27 (P.CH_2.C.O overlapping with n-Bu methylene α to P^+); 8.27-8.90 (n-Bu methylene β and γ to P^+, 12 protons, complex); 8.90-9.30 (methyl, 9 protons, complex). $J_{(P.C.CH_2.O)}$ = 18.1 Hz; $J_{(P.CH_2.C.O)}$ = 11.5 Hz; $J_{(H.H)}$ ≈ $J_{(P.O.CH_2)}$ ≈ 5.4 Hz. ^{31}P-NMR ($CHCl_3$): ppm -34.1 (P^+); +2.1 (PO_4^-); peak separation 36.2 ppm.)

Attempted oxidation of o-hydroxyphenyl 2-(tri-n-butylphosphonio)-ethyl phosphate by the method of Khwaja et al.[250]

2% Bromine water (168 ml., 21 mmole) was added to a stirred solution of o-hydroxyphenyl 2-(tri-n-butylphosphonio)-ethyl phosphate (LXV) (1.25 g., 3 mmole) in 0.2M aqueous barium acetate (130 ml.) at 20°. An orange-yellow precipitate was formed almost immediately, but soon collected on the bottom of the flask as a dark-red gum. Stirring was continued for 30 minutes, and the solution was decanted and extracted with ether (3 X 100 ml.). The aqueous layer was allowed to percolate through a column of Amberlite IR-120 (H) (100 ml.) and IRA-400 (formate) (100 ml.) ion-exchange resin, which was then thoroughly eluted with water (1.5 l.). Removal of

solvent from the total eluate left a brown gum (10 mg.) with no discernible PMR spectrum.

2-(Tri-n-butylphosphonio)-ethyl phosphate, inner salt (LIV).

Methylphosphorodichloridate (LXVI) (3.73 g., 25 mmole) was added dropwise, with stirring, to a solution of 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb) (9.36 g., 25 mmoles) in pyridine (10 ml.) at 0°. Stirring was continued for 12 hours, during which time the solution was allowed to reach room temperature (21°). It was cooled to 0°, water (1.5 ml.) was added, and stirring continued for another hour. Unreacted pyridine was removed by azeotroping with toluene under reduced pressure. The resulting yellow oil was dissolved in water (400 ml.), and the solution allowed to percolate through a column of Aberlite IR-120 (H) (125 ml.) and IRA-400 (formate) (125 ml.) ion-exchange resin, which was thoroughly eluted with water (2 l.). Solvent was removed from the eluate under reduced pressure, leaving a colourless oil; this, after drying *in vacuo* over phosphorus pentoxide for 2 days, gave 2-(tri-n-butylphosphonio)-ethyl phosphate, inner salt, (LIV) (3.27 g., 40%) as a colourless, hygroscopic wax, m.p. 127-133°(decomp.). TLC (silica, solvent system A) gave a single spot at R_F 0.60, blue when sprayed with the Zinzadze reagent. (Found: C, 51.5, 51.6; H, 9.8, 9.8; P, 18.9. $C_{14}H_{32}O_4P_2$ requires: C, 51.5; H, 9.9; P, 19.0%. PMR (D_2O): τ 5.55-6.15 (P.C.CH_2.O.P, 2 protons, broad

complex); 7.25-8.15 ($P.CH_2$., 8 protons, complex); 8.15-8.85 (n-Bu methylene β and γ to P^+, 12 protons, complex); 8.85-9.28 (methyl, 9 protons, complex). (CF_3CO_2H): τ 5.02-5.62 ($P.C.CH_2.O$, 2 protons, pair of overlapping quartets); 7.13-7.53 ($P.CH_2.C.O$, 2 protons, pair of overlapping triplets); 7.40-8.12 (n-Bu methylene α to P^+, 6 protons, complex); remainder of spectrum as in D_2O. $J_{(\underline{P}.C.\underline{CH_2}.O.P)}$ = 18.3 Hz; $J_{(\underline{P}.\underline{CH_2}.C.O.P)}$ = 11.9 Hz; $J_{(H.H)} \approx J_{(\underline{P}.O.\underline{CH_2})} \approx$ 5.9 Hz. ^{31}P-NMR ($CHCl_3$): ppm -35.0 (P^+); +4.2 (PO_4^-); peak separation 39.2. (CF_3CO_2H): ppm -35.0 (P^+); +5.0 (PO_4^-); peak separation 40.0.)

Phosphorylation with dimethylphosphoramidic dichloride (LVI).

Quinoline (12.92 g., 100 mmole) in acetonitrile (20 ml.) was added dropwise to a stirred solution of 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb) (37.4 g., 100 mmole) in acetonitrile (40 ml.) at 0°. To this mixture was added dimethylphosphoramidic dichloride (LVI) (16.2 g., 100 mmole) in acetonitrile (20 ml.) dropwise, with stirring, at 0° in the dark. Stirring was continued for 12 hours while allowing the mixture to reach room temperature (20°). The solution was again cooled to 0°; pyridine (25 ml., 310 mmole) was added, followed by water (6 ml.). Stirring was continued for another hour. Acetonitrile and excess of pyridine were removed under reduced pressure, the pyridine being azeotroped with toluene. The residual brown gum was dissolved in water (500 ml.)

and boiled under reflux with pre-washed Amberlite IR-120 (H) cation-exchange resin (25 g. dry weight). When cool, the solution was allowed to percolate through a column of Amberlite IR-120 (H) (250 ml.) and IRA-400 (formate) (250 ml.) ion-exchange resin, which was then washed thoroughly with water (2.5 l.). Removal of solvent from the eluate under reduced pressure left a colourless gum, which crystallized when dried in vacuo over phosphorus pentoxide for two days to give 2-(tri-n-butylphosphonio)-ethyl phosphate, inner salt, (LIV) (5.2 g., 16%) as a colourless wax, identical in physical and spectral properties to that prepared in the preceding experiment.

No attempt was made to isolate the dimethylphosphoramide intermediate (LXVIII).

Phosphorylphosphocholine (2-(trimethylphosphonio)-ethyl phosphate, inner salt) (XLIX).

Methylphosphorodichloridate (LXVI) (1.49 g., 100 mmole) was added dropwise to a stirred solution of phosphocholine iodide (XXIVb) (2.48 g., 100 mmoles) in pyridine (10 ml.) and acetonitrile (10 ml.) at 0° in the dark. Stirring was continued for 12 hours, during which time the temperature was allowed to rise to 21°. The solution was again cooled to 0°, water (3 ml.) was added, and stirring continued for an hour. Solvents were removed under reduced pressure, with the addition of toluene

to facilitate the removal of pyridine. The residual gum was dissolved in water (300 ml.) and allowed to percolate through a column of Amberlite IR-120 (H) (100 ml.) and IRA-400 (formate) (100 ml.) ion-exchange resin, which was then washed thoroughly with water (1.5 l.). Removal of solvent from the eluate gave a colourless gum; this was dissolved in anhydrous ethanol (100 ml.) which was removed under reduced pressure to give phosphorylphosphocholine (2-(trimethylphosphonio)-ethyl phosphate, inner salt) (XLIX) (626 mg., 31.3%) as a white powder which decomposed without melting above 180°. It was not deliquescent. (Found: C, 29.8; H, 6.8; P, 30.7. $C_5H_{14}O_4P_2$ requires: C, 30.0; H, 7.0; P, 30.95%. PMR (D_2O): τ 5.47-610 (P.C.CH_2.O, 2 protons, pair of quartets); 7.19-7.60 (P.CH_2.C.O, 2 protons, pair of triplets); 7.97-8.21 (methyl, 9 protons, doublet). $J_{(P.C.CH_2.O.P)}$ = 20.3 Hz; $J_{(P.CH_2.C.O.P)}$ = 12.8 Hz; $J_{(P.CH_3)}$ = 14.0 Hz; $J_{(H.H)} \approx J_{(P.O.CH_2)} \approx$ 6.0 Hz. ^{31}P-NMR (H_2O): ppm -27.3 (P^+); -0.8 (PO_4^-); peak separation 26.5.)

A pH curve was established by the titration of a 0.1M aqueous solution (30 ml.) with 0.4M aqueous sodium hydroxide, using an E.I.L pH meter (results in text, Chapter 1).

It should be noted that pyridine may react explosively with methylphosphorodichloridate if mixing takes place above 0°.

Tri-n-butylphosphonium analogue of CDP-choline (2-(tri-n-butylphosphonio)-ethyl cytidine-5'-pyrophosphate) (LXXI).

The procedure was that adapted by Kennedy[108] from the work of Khorana and co-workers.[109-11]

Dicyclohexylcarbodiimide (XXI) (20.6 g., 100 mmole) was added to a stirred solution of 2-(tri-n-butylphosphonio)-ethyl phosphate (LIV) (490 mg., 1.5 mmole) and cytidine-5'-monophosphate (LXIX) (648 mg. of demihydrate, 1.95 mmole) in water (10 ml.) and pyridine (75 ml.) at 37°. Stirring at 37° was continued for 10 days, during which time further portions of dicyclohexylcarbodiimide (XXI) were added (2 X 10.3 g., 2 X 50 mmole, after 3 and 5 days). The semi-solid reaction mixture was shaken with water (200 ml.) and filtered, the precipitated dicyclohexyl-urea (LXX) being washed with several portions of water (4 X 50 ml.). The combined washings and filtrate were extracted with ether (3 X 400 ml.). The aqueous layer was adjusted to pH 8.5 by the addition of 1N aqueous potassium hydroxide, and was made up to 500 ml. with water. The solution was chromatographed on a column of Amberlite CG-400 Type II (200 mesh) (formate) anion-exchange resin, 20 cm. high and 2 cm. in diameter. The column was first washed thoroughly with water (500 ml.) to remove residual pyridine, and was eluted with a gradient of 0.00-0.04N aqueous formic acid according to the method of Hurlbert et al.[253]; the mixing chamber initially contained 1.5 l. of water, and the upper

reservoir 0.04N aqueous formic acid. 15 ml. fractions were collected at the rate of 8 per hour for 24 hours. Three peaks were observed on the fraction collector recorder chart, and the fractions corresponding to each peak were combined. Removal of water from the first fraction gave a glassy solid, identified from the PMR spectrum as 2-(tri-n-butyl-phosphonio)-ethyl cytidine-5'-pyrophosphate, inner salt, (LXXI) (12.5 mg., 1.3%). The contents of the second and third fractions were tentatively identified as dicytidyl pyrophosphate and unreacted CMP (LXIX) respectively (Insufficient sample for microanalysis. PMR (D_2O): see table in text, Chapter 2 (iii) - cf. spectra of cytidine and derivatives.[271, 315-6] ^{31}P-NMR (H_2O): ppm -34.9 (P^+); +9.1 (PO_7^-); peak separation 44.0.)

CDP-phosphocholine (2-(trimethylphosphonio)-ethyl cytidine-5'-pyro-phosphate) (L).

The procedure was identical to that used in the preceding experiment; phosphorylphosphocholine (XLIX) (300 mg., 1.5 mmole) was coupled to CMP (LXIX) (648 mg. of demihydrate, 1.95 mmole) using dicyclohexylcarbodiimide (XXI) (total of 41.2 g., 200 mmole). Three fractions were obtained by ion-exchange chromatography of the product, and the first yielded CDP-phosphocholine (2-(trimethylphosphonio)-ethyl cytidine-5'-pyrophosphate) (L) (180 mg., 24%) as a colourless glass which decomposed without melting above 190°. The monosodium salt was obtained by neutralization of an

aqueous solution with dilute aqueous sodium hydroxide; unlike the sodium salt of CDP-choline (III)[317] that of CDP-phosphocholine was found to be soluble in aqueous ethanol, and was therefore recrystallized from ethanolic ether as a white powder, m.p. as for the free acid. (Found for the monosodium salt: C, 29.4; H, 5.1; N, 7.45. $C_{14}H_{25}N_3NaO_{11}P_3.2.5\ H_2O$ requires C, 29.4; H, 5.3; N, 7.35%. PMR (D_2O): see table in text, Chapter 2 (iii). ^{31}P-NMR (H_2O): ppm -27.4 (P^+); +10.4 ($P_2O_7^-$); peak separation 37.8.)

Stearic anhydride (LXXV).[254]

The procedure was that of Lapidot et al.[254] To a stirred solution of stearic acid (5.69 g., 20 mmoles) in dry carbon tetrachloride (50 ml.) was added dicyclohexylcarbodiimide (XXI) (2.06 g., 10 mmoles) in dry carbon tetrachloride (50 ml.). Stirring at room temperature (23°) was continued for 15 hours. The solution was filtered to remove dicyclohexylurea (LXX), the solvent was removed under reduced pressure, and the residue recrystallized from ether to give stearic anhydride (LXXV) (4.00 g., 72.5%) as colourless glistening plates, m.p. 70-71°. (Found: C, 78.8; H, 12.5. Calc. for $C_{36}H_{70}O_3$: C, 78.5; H, 12.8%)

Zetsche and Fredrich[318] give m.p. 70-72°.

Tetraethylammonium stearate (LXXVI) and tetraethylammonium DL-glycero-3-phosphate (LXXIV).

To a stirred solution of stearic acid (2.85 g., 10 mmole) in ethanol (150 ml.) was added 25% aqueous tetraethylammonium hydroxide (5.88 g. of solution, 10 mmole). Solvents were removed under reduced pressure; the residue was dried under high vacuum over phosphorus pentoxide and recrystallized from dry ether to give tetraethylammonium stearate (LXXVI) (3.61 g., 89%) as colourless microcrystals, m.p. 108-10°.

The disodium salt of DL-glycero-3-phosphoric acid was converted to the free acid by passage of an aqueous solution through Amberlite IR-120 (H) cation-exchange resin; removal of the solvent gave the product as a colourless gum. To a stirred solution of this free acid (1.72 g., 10 mmole) in ethanol (150 ml.) was added 25% aqueous tetraethylammonium hydroxide (5.88 g. of solution, 10 mmole). Removal of solvents under reduced pressure gave tetraethylammonium DL-glycero-3-phosphate (LXXIV) (3.10 g.), in quantitative yield, as a colourless solid, m.p. 150° (decomp.).

Distearoylphosphatidic acid (1,2-distearoyl-DL-glycero-3-phosphoric acid) (LXXIII).[254]

The method was that of Lapidot et al.[254] Monotetraethylammonium DL-glycero-3-phosphate (LXXIV) (301 mg., 1 mmole) in methanol (10 ml.)

was added to a stirred solution of tetraethylammonium stearate (4.13 g., 10 mmole) in methanol (30 ml.); the solvent was removed under reduced pressure and the residual oil dried at 50° under high vacuum over phosphorus pentoxide for 2 days. To the product was added a solution of stearic anhydride (5.51 mg., 10 mmole) in dry carbon tetrachloride (40 ml.) The solution was boiled under reflux for 3 hours, the solvent was removed under reduced pressure and the residue was dissolved in chloroform-methanol (2:1 v/v) (30 ml.). Pyridine (25 ml.) and water (6 ml.) were added, and the mixture was stirred at room temperature for 1 day. The solvents were removed under reduced pressure; the residue was dissolved in chloroform-methanol (1:1 v/v) (20 ml.) and passed through a column of Sephadex LH-20 lipophilic gel filtration medium (150 X 4 cm.). The column was eluted with chloroform-methanol (1:1 v/v) with a flow rate of 30 ml. per hour; fractions were collected at the rate of 2 per hour. Phosphate esters were detected by spotting fraction samples on silica TLC plates and spraying with the Zinzadze reagent. The fractions containing phosphatidic acid were combined, and the solvent removed. The residue was redissolved in chloroform-methanol-water (50:45:5 v/v) at 2°, and the solution was allowed to percolate through a column of Amberlite IRC-50 (H) cation-exchange resin (10 g. dry weight) maintained at 2°. The column was thoroughly eluted with the same solvent mixture (500 ml.) and the solvents removed from the total eluate under reduced

pressure; the white residue was dried under high vacuum over phosphorus pentoxide for 2 days, and recrystallized from anhydrous ethyl acetate to give 1,2-distearoyl-DL-glycero-3-phosphoric acid (LXXIII) (415 mg., 59%) as a white powder, m.p. 70-1°.

Uhlenbroek and Verkade[319] give m.p. 70-1°. In later syntheses of analogues of lecithin, it was found preferable to use phosphatidic acid prepared on a large scale by the phosphorylation of distearin.[255-6]

1,2-Distearoyl-DL-phosphatidyl choline (distearoyllecithin; 1,2-distearoyl-DL-glycero-3-phosphorylcholine) (LXXII).[114]

The general method adopted for the synthesis of lecithin and its analogues was a modification of that devised by Aneja and Chadha.[114]

1,2-Distearoyl-DL-glycero-3-phosphoric acid (LXXIII) (705 mg., 1 mmole) and choline iodide (I) (462 mg., 2 mmole) were mixed, and dried overnight under high vacuum over phosphorus pentoxide. The mixture was dissolved in dry acetonitrile (10 ml.) and dry pyridine (25 ml.); a solution of dried triisopropylbenzenesulphonyl chloride (XXII) (909 mg., 3 mmole) in pyridine (5 ml.) was added, and the solution was stirred at 50-60° for 2 hours, then at room temperature (22°) for 24 hours. Water (2 ml.) was added, and the stirring continued for 30 minutes. Solvents were removed under reduced pressure, toluene being added to form an

azeotropic mixture with pyridine. The residual reddish-brown gum was dissolved in chloroform-methanol-water (50:45:5 v/v) (100 ml.) and allowed to percolate through a column of Amberlite IRC-50 (H) (50 g.) and IR-45 (OH) ion-exchange resin which had previously been washed with the solvent mixture; elution continued with the addition of a further 700 ml. of solvent mixture. Solvents were removed from the total eluate under reduced pressure, and the residual brown solid was dissolved in chloroform (3 ml.) and chromatographed on a 1 m. X 20 cm. X 1 mm. preparative TLC plate (silica gel H, solvent system B, 1 elution). The band containing lecithin (identified by the spraying of a 1 cm. vertical strip with the Zinzadze reagent) was scraped off and eluted with chloroform-methanol (50 ml. each of 9:1, 8:2 and 7:3 consecutively). Removal of solvents from the eluate left a white solid which was recrystallized from dry dioxan, filtered, washed with anhydrous ether and dried to give 1,2-distearoyl-DL-glycero-3-phosphorylcholine (LXXII) (319 mg., 40%) as a white powder, transition temperature 79-82°, m.p.233° (decomp.). TLC (solvent system B, silica, Zinzadze spray) gave a single blue spot, R_F 0.45. (PMR ($CDCl_3$): as lit.[114] ^{31}P-NMR ($CHCl_3$): ppm -0.2 (PO_4^-). IR: see table in text (Chapter 2 (ii)). MS: m/e (250°) 395 (base peak), 267 (99%), 341 (97%), 606 (62%), 98 (44%), 451 (36%), 396 (32%), 382 (32%), 97 (32%).)

Aneja and Chadha[114] report m.p. 234-5° (for the L-compound).

1,2-Distearoyl-DL-phosphatidylphosphocholine (1,2-distearoyl-DL-glycero-3-phosphorylcholine) (LI).

The initial procedure was identical to that used in the preceding experiment, except that tenfold quantities were used, namely phosphatidic acid (7.05 g., 10 mmole), phosphocholine iodide (XXIVb) (4.96 g., 20 mmole) and triisopropylbenzenesulphonyl chloride (XXII) (9.09 g., 30 mmole). However, after the ion-exchange step the resulting yellow solid was chromatographed on a column of silica gel (700 g.) using a gradient of chloroform containing an increasing percentage of methanol (0-50%). Samples of fractions were tested for phosphate esters by TLC (silica, solvent system B, Zinzadze spray); fractions thought to contain phosphatidylphosphocholine (LI) were combined, and the solvents removed under reduced pressure. The resulting white solid was recrystallized from dry dioxan to give 1,2-distearoyl-DL-glycero-3-phosphorylphosphocholine (LI) (4.53 g., 56%) as a white powder, transition temperature 65-74°, m.p. 135-137° (decomp.). TLC (as above) gave a single spot, R_F 0.51. (Found: C, 62.8; H, 10.4; P, 7.2. $C_{44}H_{88}O_8P_2.2H_2O$ requires: C, 62.7; H, 11.0; P, 7.35%. PMR ($CDCl_3$): ^{31}P-NMR; IR: see tables in text (Chapter 2, (iii), (iv) and (ii) respectively). MS: m/e (240°) 341 (base peak), 267 (96%), 98 (40%), 340 (24%), 606 (20%), 359 (20%), 129 (20%).)

Tri-n-butylphosphonium analogue of 1,2-distearoyl-DL-phosphatidylcholine (1,2-distearoyl-DL-glycero-3-phosphoryl-(2'-tri-n-butylphosphonio)-ethane) (LXXIX).

The entire procedure was identical to that used for the preparation of distearoylphosphatidylcholine (LXXII) except that double quantities were used, namely phosphatidic acid (LXXIII) (1.41 g., 2 mmole), 2-hydroxyethyltri-n-butylphosphonium iodide (LIIIb) (1.50 g., 4 mmole) and triisopropylbenzenesulphonyl chloride (XXII) (1.82 g., 6 mmole). Recrystallization of the crude product from dry dioxan gave the tri-n-butylphosphonium analogue of 1,2-distearoyl-DL-glycero-3-phosphorylcholine (LXXIX) (619 mg., 33%) as a white powder, transition temperature 58-65°, m.p. 138-9°. TLC (silica, solvent system B, Zinzadze spray) gave a single blue spot, R_F 0.95. (Found: C, 65.8; H, 10.7; P, 6.25. $C_{53}H_{106}O_8P_2.2H_2O$ requires: C, 65.8; H, 11.4; P, 6.4%. PMR ($CDCl_3$): τ 4.68-5.15 (CHOCO, 1 proton, complex); 5.54-6.42 ($OCH_2.C.P$, CH_2OCO, glycerol CH_2OP, 6 protons, complex); 7.12-7.45 ($O.C.CH_2P$, 2 protons, complex); 7.55-8.07 ($2CH_2CO_2$, n-Bu methylene α to P^+, 10 protons, complex); 8.16-8.98 (n-Bu methylene β and γ to P^+, 2 stearate $-(CH_2)_{15}$, 72 protons, complex); 9.02-9.35 (n-Bu and stearate methyl, 15 protons, complex). ^{31}P-NMR ($CHCl_3$): ppm -34.6 (P^+); -0.2 (PO_4^-); peak separation 34.4. IR: see table in text (Chapter 2 (ii)).

CHAPTER 2 : PROPERTIES OF THE PHOSPHORUS ANALOGUES

(vi) Electron microscopy of liposomes of distearoyllecithins.

The procedure was derived from that of Batzri and Korn.[276]

For negative staining, warm 10 mM methanolic or ethanolic solutions (0.25 ml.) of distearoylphosphatidylcholine (LXXII), its trimethyl- and tri-n-butylphosphonium analogues.(LI) and (LXXIX), and rat brain phosphatidylphosphocholine (Chapter 5), were rapidly injected into warm 1% aqueous sodium phosphotungstate (pH 7.0) (2.25 ml.) with the aid of a preheated 1 ml. Hamilton syringe. The preparations were allowed to cool, and were examined by electron microscopy on carbon-coated 200 mesh copper grids. Micrographs were taken at instrumental magnifications of 16000, 25000 and 40000.

For positive staining, ethanolic liposome preparations were made up in water rather than sodium phosphotungstate. Equal volumes of 2% aqueous osmium tetroxide were added; the preparations were left for 2 days at room temperature, and centrifuged. The pellets were dehydrated by repeated soaking for periods of 10 to 30 minutes, in aqueous ethanol of decreasing water content (25% to 0%), in absolute ethanol (3 X) and finally in propylene oxide (3 X). The vesicles were

embedded in Spurr resin, which was left to set at 70° overnight. Thin serial sections (500-600 Å) were cut by means of an LKB Ultrotome III ultramicrotome fitted with a glass knife. The sections were mounted on carbon-coated 200 mesh copper grids, and were stained with 25% methanolic uranyl acetate; this was followed by lead staining in the case of the rat brain preparation. Electron micrographs were taken at magnifications of 10000 and 40000.

(vii) Hydrolysis of lecithins by phospholipase C.

To each of four beakers (10 ml.) labelled A, B, C and D were added aliquots (1.58 μmole) of the following lecithins:

A) Egg-yolk lecithin.

B) Distearoylphosphatidylcholine (LXXII).

C) Distearoylphosphatidylphosphocholine (LI).

D) Tri-n-butylphosphonium analogue of distearoylphosphatidylcholine (LXXIX).

Each sample was dissolved in chloroform (1 ml.) containing cetyltrimethylammonium bromide (3 μg., 8.2 nmole), and the solvent was removed under a stream of nitrogen; 50 mM aqueous tris buffer (1.50 ml., pH 7.2) was added, and the contents of each beaker were sonicated for 10 seconds at 6.5 μ peak-to-peak amplitude in an MSE sonicator. To each sample was added 10 mM calcium chloride in 1 mM aqueous tris buffer (0.1 ml., pH 7.4);

at zero time a solution of phospholipase C in 50mM aqueous tris buffer (0.01 mg. in 0.1 ml., pH 7.2) was added, and incubation was allowed to proceed at 37°. Samples (0.2 ml.) were withdrawn from the reaction mixtures over a period of 1 hour, and were pipetted into tubes containing chloroform-methanol (2:1, v/v) (2 ml.). These solutions were thoroughly extracted with water (1 X 0.4 ml.) followed by the upper phase of a chloroform-methanol-water (10:5:3) system (3 X 0.4 ml.). Each set of aqueous extracts was combined, and all aqueous and organic phases evaporated to dryness and digested with 72% aqueous perchloric acid (0.8 ml.) at 220° for 1 hour. Phosphate content was then determined by a modification of the method of Bartlett[320] as follows.

1-Amino-2-naphthol-4-sulphonic acid (0.5 g.) was dissolved in 15% aqueous sodium bisulphite solution (200 ml.), and anhydrous sodium sulphite (1.0 g.) was added; the solution was filtered.

The perchloric acid digestions were made up to 5.0 ml. with water. Duplicate phosphate standards (1 µg. to 5 µg. phosphorus in 1 ml. water) and a water blank (1.0 ml.) were mixed with 72% aqueous perchloric acid (0.8 ml.) and also made up to 5 ml. with water. To each tube was added 5% aqueous ammonium molybdate (0.4 ml.); the solutions were mixed well with a vortex mixer, the aminonaphtholsulphonic acid solution (0.4 ml.) added, and mixing repeated. The solutions were diluted to 10 ml. with water, mixed once more, heated for 7 minutes on a boiling water bath, and

allowed to cool to room temperature. Optical density of each solution at 830 nm. was measured on a Unicam SP 500 spectrophotometer. A standard curve was plotted, phosphate concentrations in aqueous and organic phases calculated, and percentage hydrolyses estimated thus:

$$\text{Percentage hydrolysis by enzyme} = \frac{\text{Phosphate in aqueous phase}}{\text{Phosphate in organic phase}} \times 100\%$$

TIME (mins.)	PERCENTAGE OF HYDROLYSIS, %.			
	A	B	C	D
0	0.0	0.0	0.0	0.0
5	13.4	18.5	15.2	3.9
10	23.3	26.5	23.3	6.6
15	33.0	28.9	26.6	7.6
30	53.3	29.6	27.7	8.8
60	77.1	31.1	29.5	8.9

Degree of hydrolysis was plotted as a function of time (see text).

CHAPTER 3 : IN VITRO METABOLISM OF PHOSPHOCHOLINE

(i) Choline Phosphokinase.

(a) Peparation of enzyme extract. The method used was a slight modification of that devised by Wittenberg and Kornberg.[3] Dried Brewers' Bottom yeast (Sigma London Cemical Co.) (75 g., 100 ml.) was suspended in 0.1 M aqueous sodium carbonate (300 ml.) and stirred at 31° for 4 hours. The suspension was centrifuged for 15 mins. at 2500 rpm (1900 X g). To the supernatant fluid (200 ml.) was added a solution of ammonium sulphate (350 g.) in ice-cold water (800 ml.), and the mixture was left to stand overnight at 2.5°; all subsequent operations were carried out at this temperature unless otherwise stated. The precipitate so formed was collected by centrifugation for 30 mins. at 20000 rpm (50000 X g), and was dissolved in water (200 ml.). Sulphate ion was removed by passage of the solution through a column of Amberlite CG-400 (formate) anion-exchange resin, 2.5 cm. in diameter and 8 cm. in height. The effluent was adjusted to pH 7.2 with 2N aqueous sodium hydroxide. Calcium phosphate gel (British Drug Houses, prepared by the method of Keilin and Hartree[321]) (80 ml., containing 2.4 g. of dry weight) was centrifuged for 5 minutes at 1000 rpm (300 X g), and excess of water was

removed by decantation. The gel was added to the Amberlite effluent with gentle stirring. After standing for 5 mins., the gel was centrifuged for 15 mins. at 2500 rpm, washed once with 0.01M phosphate buffer (pH 7.0) (200 ml.) and centrifuged again. The supernatant fluid was discarded, and the gel washed with 0.05M phosphate buffer (pH 7.0, 2 X 100 ml.) and centrifuged as before. To the supernatant fluid was added ammonium sulphate (84 g.), and the solution was left to stand overnight, this second precipitation process being introduced to remove phosphate ions. The precipitated protein was collected by centrifugation for 30 mins. at 20000 rpm and was dissolved in 0.05M aqueous glycylglycine buffer (pH 8.5, 40 ml.). From the results of subsequent incubation experiments, the enzyme preparation was found to possess an activity of 3050 units (76 units per ml.): 1 unit of choline phosphokinase activity is defined[3] as that amount of activity which causes the removal of 1 μmole of free choline per hour. The preparation was stored at -13° until required.

(b) Preliminary investigation of activity. The incubation mixture contained ATP, disodium salt pentahydrate (1.81 g., 2.90 mmole), L-cysteine (1.08 g., 8.90 mmole), magnesium chloride hexahydrate (1.22 g., 6.00 mmole), tris buffer (0.727 g., 6.00 mmole) and choline phosphokinase in glycylglycine buffer (30 ml.), all in aqueous solution, adjusted to pH 8.5 by the addition of 2N aqueous sodium hydroxide, of total volume 600 ml.

To an aliquot of 200 ml. (solution A) was added choline chloride (I) (0.124 g., 0.890 mmole); to a second aliquot of 200 ml. (solution B) was added phosphocholine chloride (XXIVa) ((0.139 g., 0.890 mmole); to a third aliquot of 100 ml.(solution C) was added 2-hydroxyethyltri-n-butylphosphonium chloride (LIIIa) ((0.126 g., 0.445 mmole), while the remainder of the incubation mixture (100 ml., solution D) was the control. The solutions were incubated for 2 hours at 20° with shaking, and were deproteinized by heating to 90°, the residue being removed by centrifugation. The solutions were adjusted to pH 5 with 2N hydrochloric acid, and the precipitate of cystine formed by air oxidation of cysteine was removed by filtration. A mixed-bed Amberlite ion-exchange resin was made up using equal parts of IR-120 (H) and IRA-400 (formate); solutions A and B were chromatographed using 44 ml. each of the resin mixture, which was then washed through with 2 l. of water, while C and D were chromatographed on half the scale.

(c) Assay by the method of Hayashi et al.[286] Standard solutions of phosphorylcholine (II), phosphorylphosphocholine and 2-(tri-n-butyl-phosphonio)-ethyl phosphate (LIV), of concentrations 0.5, 1.0, 2.0, 3.0 and 4.0 mM, were made up. The volumes of solutions A and B were reduced to 200 ml., and those of C and D to 25 ml. Duplicate samples (1 ml.) of the standard and test solutions were pipetted into centrifuge tubes (10 ml.); to each tube was added molybdenum blue solution (an equal-

volume mixture of 12% aqueous phosphomolybdic acid and a 2% solution of stannous chloride in 2.5N hydrochloric acid) (0.4 ml.). The tubes were shaken well and kept at 0° overnight; the contents were centrifuged at 2000 rpm for 10 mins., and the supernatant solutions decanted and discarded The remaining blue precipitates were resuspended in a wash solution (2.5 ml.) of 0.5% stannous chloride in 0.1N hydrochloric acid, and centrifuged as before. The supernatant solutions were again decanted and discarded, and the washing process repeated twice. The precipitates were dissolved in 10 ml. portions of an equal volume mixture of acetone and 2.5N hydrochloric acid, and allowed to stand for 30 mins. Absorbance was measured at 725 nm. on a Unicam SP 1800 spectrophotometer:

SOLUTION	YIELD OF PHOSPHORYL DERIVATIVE		(MOLARITY OF SOLUTION)
	mmole	%	
A (200 ml.)	0.408	46	2.04 mM
B (200 ml.)	0.408	46	2.04 mM
C (25 ml.)	0.013	2.9	0.51 mM

(d) Characterization of products. Solutions A-C were evaporated to dryness, and the residues redissolved in deuterium oxide. The NMR spectra of A and B were identical to those of phosphorylcholine (II) and phosphorylphosphocholine (XLIX) respectively. Removal of water from solution C had left only 9 mg. of colourless gum; the NMR spectrum

appeared to be the same as that of 2-(tri-n-butylphosphonio)-ethyl phosphate (LIV), although the signal was rather weak.

(e) Use of labelled substrates. An incubation mixture was made up as in (b), using one-fifth quantities in a total volume of 120 ml. To one half of the solution was added choline chloride (37.2 mg., 0.266 mmole) and (methyl-^{3}H)-choline of negligible molarity (200 μCi, specific activity 15 Ci.mmole^{-1}). To the remaining 60 ml. was added (1,2-^{14}C)-phosphocholine chloride (41.6 mg., 0.266 mmole, ca. 50 μCi). These two solutions were divided up as follows, and solutions of choline phosphokinase added at zero time in the quantities indicated.

CODE	CHOLINE SOLUTION (ml.)	PHOSPHOCHOLINE SOLUTION (ml.)	ENZYME SOLUTION (ml.)
X_N	25	—	1.25
X_P	—	25	1.25
X_{NP}	5	5	0.50
Y_N	10		0.20
Y_P		10	0.20
Y_{NP}	5	5	0.20
Z_N	10		0.067
Z_P		10	0.067
Z_{NP}	5	5	0.067

Thus solutions X contained 50 µl. of enzyme solution per ml., solutions Y 20 µl. per ml., and solutions Z 6.7 µl. per ml. Similarly, flasks N contained choline only, flasks P phosphocholine only, and flasks NP an equimolar mixture of the two; total substrate concentrations were identical in all solutions.

The solutions were incubated at 20°, with shaking. Samples (0.5 ml.) were withdrawn over a period of 1 hour, and were pipetted into tubes containing aliquots (1 ml.) of ethanol. The tubes were centrifuged for 5 mins. at 2000 rpm, and samples of each supernatant solution were chromatographed on cellulose TLC plates (solvent system C, 2 elutions). The plates were dried in a stream of warm air, and each chromatogram was divided into 1 cm. horizontal strips from origin to solvent front. The strips were scraped into vials containing scintillation fluid (7 ml.), and these were counted for periods of 10 mins. each. The R_F's of substrates and products in this system were found to be: choline, 0.63; phosphorylcholine, 0.26; phosphocholine, 0.71; phosphorylphosphocholine, 0.34. Yields of phosphorylated products were calculated as the activity of the product spot divided by that of the whole chromatogram, after allowing for background radiation. Percentage yield was plotted as a function of time for each solution (see text, Chapter 3).

PERCENTAGE YIELDS OF PHOSPHORYLATED PRODUCTS

SOLUTION	TIME (mins)	SUBSTRATE N choline %	P phosphocholine %	NP choline %	NP phosphocholine %
X	5	5.5	6.5	4.4	5.7
	10	10.5	10.2	8.8	11.3
	15	16.0	14.0	12.8	14.6
	30	30.7	26.4	25.5	26.5
	45	41.1	36.8	36.1	39.3
	60	51.6	46.2	47.0	50.2
Y	5	2.3	4.0	1.3	2.5
	10	4.1	5.7	2.6	4.4
	15	5.9	7.1	4.0	5.9
	30	10.8	10.7	7.8	10.0
	45	15.8	14.1	11.8	13.9
	60	20.6	18.3	15.6	17.4
Z	5	1.20	3.50	0.70	3.35
	10	2.23	4.39	1.67	4.23
	15	3.02	4.84	2.40	4.93
	30	5.35	5.90	5.17	8.10
	45	6.78	6.78	7.96	10.60
	60	6.96	6.96	10.61	12.61

The remainder of solutions X_N and X_P were allowed to incubate for a further 18 hours; they were deproteinized at 90° for 5 mins., centrifuged at 2000 rpm for 10 mins., adjusted to pH 5 with 2N hydrochloric acid, filtered, and passed through ion-exchange columns containing Amberlite IR-120 (H) (7 ml.) and IRA-400 (formate) (7 ml.). These were eluted with water until the radioactivity of the eluate had become negligible; when the eluates had been checked for radiochemical purity by the TLC method used above, samples were monitored for radioacivity, and the volumes subsequently adjusted to give 1.00mM aqueous solutions of (methyl-^{3}H)-phosphorylcholine (68.0 ml.) and (1,2-^{14}C)-phosphoryl-phosphocholine (54 ml.), to be used as labelled substrates for phosphoryl-choline - cytidyltransferase.

(f) Choline phosphokinase from rat liver. An adult male Wistar rat was stunned and decapitated; the liver was quickly removed, and homogenized with ice-cold 0.154M aqueous sodium chloride (4 volumes) in an homogenizer of the Potter-Elvehjem type with a loose-fitting teflon pestle. The homogenate was centrifuged for 90 mins. at 25000 rpm and 2° in the SW 25-3 rotor of the Spinco model L ultracentrifuge. The supernatant layer was decanted and stored at -2° for 24 hours.

The reaction mixture, as formulated by Schneider,[9] contained ATP, disodium salt pentahydrate (112.2 mg., 180 μmole), magnesium acetate

(193 mg., 900 μmole), succinic acid (177 mg., 1.50 mmole), tris buffer (182 mg., 1.50 mmole) and disodium glycero-1-phosphate (259 mg., 1.20 mmole) in a total volume of 39 ml. adjusted to pH 7.5. To half (19.5 ml.) (solution N) was added choline chloride (3.94 mg., 28.2 μmoles) mixed with (methyl-^{3}H)-choline chloride of negligible molarity (100 μCi, specific activity 15 Ci.mmole^{-1}). To the remaining 19.5 ml (solution P) was added (1,2-^{14}C)-phosphocholine chloride (4.42 mg., 28.2 μmole, ca. 5 μCi). Portions (6.5 ml.) of solutions N and P were mixed to give solution NP, containing equimolar quantities of choline and phosphocholine; all 3 solutions were now of the same volume (13 ml.). 20% (v/v) rat liver supernatant (12 ml.) was added to each solution, and the mixtures were incubated at 37° for 30 mins., with shaking. They were then deproteinized at 90° for 5 mins. and centrifuged; samples were chromatographed and counted as in (e), in triplicate.

PERCENTAGE YIELDS OF PHOSPHORYLATED PRODUCTS

SUBSTRATE				
N	P	NP	NP	
Choline	Phosphocholine	Choline	Phosphocholine	
%	%	%	%	
25.3	27.2	25.0	27.8	
24.6	27.7	24.9	27.5	
25.7	28.8	25.9	27.9	
25.2	27.9	25.3	27.9	(Mean)

(ii) Phosphorylcholine-cytidyltransferase.

The method was that of Schneider et al.[7-9] A rat liver homogenate was prepared and centrifuged as in the previous section, and the supernatan activated by shaking at 37° for 4 hours. The reaction mixture contained CTP (341 mg. of sodium salt, 660 μmole), ATP (449 mg. of disodium salt pentahydrate, 720 μmole), magnesium acetate (723 mg., 3.6 mmole), disodium DL-glycero-1-phosphate (1.04 g., 4.8 mmole), tris buffer (727 mg., 6 mmole) and succinic acid (709 mg., 6 mmole) in aqueous solution, adjusted to pH 7.5, of total volume 36 ml. Aqueous solutions of (methyl-^{3}H)-phosphoryl-choline and (1,2-^{14}C)-phosphorylphosphocholine (1 μmole per ml.), prepared in the previous section, were also adjusted to pH 7.5. The following reaction mixtures were made up, enzyme being added at zero time.

FLASK NO.	CTP SOLUTION ml.	PHOSPHORYL-CHOLINE ml.	PHOSPHORYLPHOSPHO-CHOLINE ml.	ENZYME ml.
1	12	40	—	48
2	12	—	40	48
3	0.3	0.1	0.9	1.2
4	0.3	0.2	0.8	1.2
5	0.3	0.3	0.7	1.2
6	0.3	0.4	0.6	1.2
7	0.3	0.5	0.5	1.2

8	0.3	0.6	0.4	1.2
9	0.3	0.7	0.3	1.2
10	0.3	0.8	0.2	1.2
11	0.3	0.9	0.1	1.2

The mixtures were incubated at 37°, with shaking. Samples (0.1 ml.) were withdrawn from flasks 1-2 over a period of an hour, and were pipetted into aliquots (0.1 ml.) of 6% aqueous perchloric acid. After 1 hour, all reactions were inactivated by the addition of 60% perchloric acid (6 ml. in flasks 1-2, 0.15 ml. in flasks 3-11). Duplicate samples from each flask were chromatographed on cellulose as in section (i)(e), but using 3 elutions. Cytidine derivatives were located under ultraviolet light (254 nm.). The chromatograms were divided into 1 cm. strips as before, and their radioactivity measured. In this system the R_f's were found to be: CDP-choline and CDP-phosphocholine, ca. 0.15; phosphorylcholine, 0.34; phosphorylphosphocholine, 0.44. Percentage yields of CDP-derivatives were calculated and plotted as functions of time or substrate composition.

PERCENTAGE YIELDS OF CDP-DERIVATIVES

TIME mins.	YIELD OF CDP-CHOLINE (Flask 1) %	YIELD OF CDP-PHOSPHOCHOLINE (Flask 2) %
5	3.6	1.1
10	4.9	1.7
15	6.4	2.6
30	9.7	3.6
45	13.4	3.9
60	15.6	4.4

FLASK no.	YIELD OF CDP-CHOLINE µMOLE	%	YIELD OF CDP-PHOSPHOCHOLINE µMOLE	%
2	—	—	6.9	6.9
3	3.0	30.0	7.4	8.2
4	6.2	31.0	6.4	8.0
5	8.9	29.6	4.6	6.4
6	10.6	26.5	3.5	5.9
7	13.0	26.1	3.2	6.4
8	16.2	26.8	2.5	6.3
9	18.7	26.7	1.9	6.2
10	20.0	25.0	1.3	6.4
11	22.1	24.5	0.6	6.4
1	22.8	22.8	—	—

The contents of flasks 1-2 were passed through columns of Amberlite IR-120 (H) cation-exchange resin (40 ml.) which were then washed thoroughly with water to give total eluate volumes of 1.5 l. The solutions were reduced to 100 ml., and passed through columns of Amberlite CG-400 (formate) (40 ml., 2 X 12.5 cm.). Fractions (50 ml.) were collected and monitored for radioactivity. Elution with water, to remove unchanged phosphorylcholine or phosphorylphosphocholine, was followed by gradient elution with 0-0.04N aqueous formic acid after the method of Hurlbert et al.[253] Elution continued until radioactivity of the eluate had fallen to background level; fractions were checked for cytidine content by absorption at 284 nm., and those with positive absorption were checked for radiochemical purity by cellulose TLC as above. Satisfactory fractions were combined, and the volumes adjusted to give (^{3}H-methyl)-CDP-choline (27.8 ml.) and (1,2-^{14}C)-CDP-phosphocholine (10 ml.) at a final concentration of 0.22 mM, to be used as substrates for phosphorylcholine-diglyceride transferase.

(iii) Phosphorylcholine-diglyceridetransferase.

The general procedure of Schneider and Behki[6] was adopted. An adult male Wistar rat was stunned and decapitated, and the liver homogenized in ice-cold distilled water (2.75 volumes) in the usual way, giving an homogenate of ca. 270 mg. of liver per ml. Natural 1,2-L-

diglyceride (100 mg., ca. 160 μmole) was emulsified at 0° in 0.2% aqueous Triton X-100 (10 ml.) using an MSE sonicator (5 seconds at 6.5 μ peak-to-peak amplitude). The reaction mixture contained magnesium acetate (129 mg., 600 umole), succinic acid (118 mg., 1.00 mmole) and tris buffer (121 mg., 1.00 mmole) in aqueous solution, adjusted to pH 7.5, of total volume 10 ml. 0.22 mM aqueous (methyl-^{3}H)-CDP-choline and (1,2-^{14}C)-CDP-phosphocholine, prepared as above, were also adjusted to pH 7.5 before use. Incubation mixtures were made up as follows, the 27% aqueous rat liver homogenate being added at zero time.

TUBE NO.	REACTION MIXTURE	CDP-CHOLINE ml.	CDP-PHOSPHO-CHOLINE ml.	DIGLYCERIDE ml.	ENZYME ml.
1	2.0	4.0	—	1.0	3.0
2	2.0	—	4.0	1.0	3.0
3	0.5	0.1	0.9	0.25	0.75
4	0.5	0.2	0.8	0.25	0.75
5	0.5	0.3	0.7	0.25	0.75
6	0.5	0.4	0.6	0.25	0.75
7	0.5	0.5	0.5	0.25	0.75
8	0.5	0.6	0.4	0.25	0.75
9	0.5	0.7	0.3	0.25	0.75
10	0.5	0.8	0.2	0.25	0.75
11	0.5	0.9	0.1	0.25	0.75

The mixtures were incubated at 37°, with shaking. Samples(1 ml.) were withdrawn from tubes 1-2 over a period of 1 hour and pipetted into aliquots (4 ml.) of ethanol in centrifuge tubes. Additional 2.5 ml. samples were withdrawn after 1 hour and pipetted into ethanol (10 ml.). The reactions in tubes 3-11 were stopped after 1 hour by the addition of ethanol (10 ml.). The tubes were centrifuged and the ethanol decanted. The pellets were extracted a further three times with the same volumes of ethanol (4 or 10 ml., as above), the combined ethanolic extracts were mixed with carbon tetrachloride (8 or 20 ml.), and the mixtures equilibrated with 2M aqueous potassium chloride (24 or 60 ml.), the upper layers being discarded. The lower layers were washed twice more with potassium chloride and once with water. The organic layers were finally transferred to scintillation vials, and the solvent removed under a stream of nitrogen. Scintillation fluid (7 ml.) was added, and the radioactivity assayed against duplicate standards of original substrate; it was assumed[6] that in the presence of excess diglyceride, the formation of of labelled phospholipids other than lecithin was negligible. Percentage yields of lecithin and its phosphonium analogue were plotted as functions of time or substrate composition. It was difficult to measure ^{3}H levels from tubes 3-6, as they were very low, and obscured by overspill from the ^{14}C channel of the scintillation counter.

TIME	INCORPORATION OF ^{3}H-CHOLINE		INCORPORATION OF ^{14}C-PHOSPHOCHOLINE	
mins	mμmole	%	mμmole	%
5	0.28	0.32	0.25	0.28
10	0.54	0.61	0.44	0.50
15	0.77	0.88	0.70	0.80
30	0.76	0.86	1.30	1.48
45	0.50	0.57	1.48	1.68
60	0.18	0.20	1.43	1.62

FLASK	INCORPORATION OF ^{3}H-CHOLINE		INCORPORATION OF ^{14}C-PHOSPHOCHOLINE	
NO.	mμmoles	%	mμmole	%
2	——	——	3.562	1.620
3			1.288	0.650
4			0.717	0.408
5			0.310	0.202
6			0.149	0.112
7	0.220	0.200	0.088	0.080
8	0.260	0.197	0.032	0.036
9	0.282	0.183	0.022	0.033
10	0.330	0.187	0.007	0.016
11	0.383	0.193	0.005	0.023
1	0.431	0.196	——	——

(iv) Loss of tritium from (methyl-^{3}H)-phosphorylcholine.

Choline kinase was isolated from yeast for a second time, in the same quantities as before, and a portion (2 ml.) Of the glycylglycine solution was incubated with (methyl-^{3}H)-choline chloride (0.8 mCi of negligible molarity) and unlabelled choline chloride (37 mg., 0.27 mmole) in a total volume of 60 ml., in the usual manner. TLC and scintillation counting indicated a yield of (methyl-^{3}H)-phosphorylphosphocholine of ca. 60% after 1 hour The mixture was deproteinized, and the solution filtered, adjusted to pH 8.0 and passed through a column of Amberlite IR-120 (H) (20 ml., upper bed) and IRA-400 (formate) (20 ml., lower bed) ion-exchange resin. The column was washed well with water, and a total eluate of 500 ml. collected; at this point the radioactivity of the solution being eluted from the column was negligible. Water was removed under reduced pressure on a rotary evaporator, and the residue was redissolved in more water. Scintillation counting of 0.1 ml. samples of eluate, redissolved product, and water in the rotary evaporator receiver showed that approximately 98.6% of the activity had been lost from the phosphorylcholine solution during evaporation, and this had appeared in the water in the receiver; this could only be explained in terms of tritium exchange between the product and the solvent, and consequent formation of ^{3}HHO.

SAMPLE	VOLUME ml.	ACTIVITY OF 0.1 ml cpm	ACTIVITY OF TOTAL cpm
Eluate	500	19280	9.6×10^7
Solvent	500	18660	9.3×10^7
New solution	50	2700	1.35×10^6

Attention was turned to the remainder of the aqueous solution of (methyl-^{3}H)-phosphorylcholine prepared 6 months earlier and stored since then at pH 7.5, 0° (sections (i) (e) and (ii)). A sample (0.1 ml.) was counted, the water was removed from the rest of the solution, and the residue redissolved in the original volume of fresh water; 0.1 ml. samples of the solvent and fresh solution were also counted. Results indicated a 93% loss of label from the sample.

SAMPLES (identical volumes)	ACTIVITY OF 0.1 ml., cpm.
Original solution	12050
Solvent	11020
Fresh solution	834

CHAPTER 4 : INCORPORATION OF PHOSPHOCHOLINE INTO PY CELLS

(i) Cell culture.

P815Y neoplastic mouse mast cells were the line used. The culture medium was that formulated by Fischer and Sartorelli,[288] and was obtained in powdered form from Grand Island Biological Co.; it was made up with doubly distilled water in 20 l. batches, giving a choline concentration of 1.5 mg. of the chloride per 1 l. At this stage sodium hydrogen carbonate (1.125 $g.l.^{-1}$), benzyl penicillin (250 $mg.l.^{-1}$) and streptomycin chloride (50 $mg.l.^{-1}$) were added. The medium was sterilized by filtration through a microporous porcelain filter, 0.3 μ absolute retention size, and was stored in 1 l. sterile screw-capped bottles at 2.5° until required. Aseptic horse serum, type HA 92H (Tissue Culture Services, Slough) was added just before use, in the quantities stated for each experiment. The pH of the medium was adjusted by adding sufficient sterile 0.1N hydrochloric acid to change the phenol red indicator to a buff colour (pH 7.0).

All operations involving live cells were carried out under sterile conditions, with sterile equipment. Heat-stable equipment was sterilized by autoclaving at 15 psi for 15 mins. Solutions were sterilized by filtration through oxoid membrane filters, 0.45 μ pore size.

(ii) Effect of addition of choline and phosphocholine on cell growth.

Into each of 11 screw-capped tubes were pipetted aliquots (4.5 ml.) of PY cell culture in medium containing 10% horse serum (ca. 10^5 cells per ml.) followed by aqueous solutions (0.5 ml.) of unlabelled choline or phosphocholine chloride, of concentrations between 0.5mM and 4.0M. The cultures were incubated at 37°; after 2½ and 6 days, samples (0.2ml.) were withdrawn from each tube and pipetted into 10 ml. beakers containing centrifuged 0.9% aqueous sodium chloride. The contents of the beakers were stirred well, and cell concentrations measured on a Type A Coulter counter set to take 0.5 ml. samples. The optimum instrument settings for counting these cells, as shown by C.A.Pasternak using a haemocytometer,[322] were threshold 50, scale expansion factor 5. The following cell counts, uncorrected for count loss due to coincident passage of cells, are the mean of 4 or 5 readings.

After 2½ days, the contents of the tube containing 80mM choline were still buff (neutral), indicating little cell growth, while the contents of the tube containing 400mM choline were red (alkaline), indicating no growth; all other cell cultures were yellow (acid), indicating reasonable cell growth.

SALT ADDED	FINAL CONCENTRATION (mM)	CELLS PER 0.01 ml. CULTURE	
		$2\frac{1}{2}$ days	6 days
Phosphocholine chloride	0.05	2328	5928
	0.20	3300	6100
	4.00	3305	5613
	80.0	4880	6870
	400	3412	6280
Choline chloride	0.05	3518	7840
	0.20	3401	7677
	4.00	2686	6272
	80.0	1585	3781
	400	1039	1002
(Distilled water)	——	3459	7994

(iii) Effect of addition of phosphocholine on choline incorporation.

The medium contained 10% horse serum. To each of 4 screw-capped tubes was added cell culture (5 ml., ca. 10^5 cells per ml.) and a solution of (methyl-^{3}H)-choline chloride in Fischer's medium (0.1 ml of negligible molarity, 10 μCi, specific activity 1 Ci.mmole^{-1}). Tubes 1-2 were capped; to tubes 3-4 was added a solution of (1,2-^{14}C)-phosphocholine chloride (1.4 mg.) (1.7 μCi) in Fischer's medium (0.1 ml.). Incubation took place at 37°; samples (0.1 ml.) were withdrawn from the cultures over a period of 2 weeks, and were pipetted into tubes of ice-cold 5% aqueous trichloroacetic acid (2 ml.). The precipitates so formed were collected on Whatman GFC 2.1 cm. glass-fibre filter papers supported on metal discs, and were washed with more ice-cold 5% aqueous trichloroacetic acid (2 X 5 ml.). The filter papers were sucked dry by water pump, were placed in vials containing scintillation fluid (7 ml.), and radioactivity was measured. Activity is shown in counts per minute (cpm), corrected for background radiation and interchannel overspill, and averaged between duplicate samples.

TIME	ACTIVITY OF SAMPLE PRECIPITATE (MEAN CPM) TUBES 1-2	TUBES 3-4		
	^{3}H	^{3}H	^{14}C	$^{3}H/^{14}C$
0	37	86	8	(11)
17	774	115	17	6.8
67	1221	175	23	7.6
96	1352	248	39	6.4
164	1480	570	90	6.3
214	1604	749	117	6.5
266	1619	1354	225	6.0
334	1719	1573	244	6.5

(iv) TLC of PY CELL PHOSPHOLIPIDS.

The contents of tube 4 from the above experiment (4.4 ml., 5.7 X 10^5 cells per ml.) were centrifuged on a bench machine; the pellet was combined, after several washings, with fresh, isotope-free cell culture (50 ml.) (ca. 5 X 10^6 cells) and recentrifuged. The new pellet was extracted with warm chloroform-methanol (2:1 v/v) (2 ml.) and the resulting phospholipid extract was washed once with water (0.4 ml.) and three times with 0.4 ml. portions of the upper phase of a chloroform-methanol-water (10:5:3) system. The resulting solution was reduced in volume and

chromatographed by silica TLC (glass plate, silica gel H, solvent system B, 1 elution). The position of lecithin was identified by development in iodine vapour; once the iodine had evaporated, the plate was divided into 1 cm. horizontal strips which were scraped into vials containing scintillation fluid (7 ml.) and monitored for radioactivity. The majority of both ^{3}H and ^{14}C activity was located in the region of the lecithin spot, although the ^{14}C maximum occurred at slightly higher R_F than ^{3}H. Counts were corrected for background and interchannel over-spill.

R_F	ACTIVITY IN COUNTS PER 10 MINS.	
	^{3}H	^{14}C
0.00-0.08	0	7
0.08-0.16	10	0
0.16-0.24	35	11
0.24-0.32	1182	358
0.32-0.40	310	491
0.40-0.48	2	29
0.48-1.00	3	2

(v) Large-scale culture in phosphocholine-enriched medium; ^{31}P-NMR.

Large-scale cell cultures were prepared as follows, using, initially, medium containing 10% horse serum. The medium was warmed to 37° before

use; all incubations were carried out at this temperature. Cultures were not stirred.

Day 1. Medium (4.5 ml.) was added to each of 2 screw-capped tubes containing PY cell culture (9 ml., ca. 5 X 10^5 cells per ml.), giving a total volume of 27 ml.

Day 2. The total volume was increased to 40 ml. with fresh medium.

Day 3. The entire culture was transferred to a 100 ml. sterile screw-capped bottle, and the volume increased to 60 ml.

Day 4. The volume was increased to 90 ml.

Day 5. The culture was transferred to a 250 ml. screw-capped conical flask, and was doubled in volume. Two 1l. batches of new medium were prepared, designated N and P. To both was added (methyl-^{3}H)-choline chloride (50 μCi of negligible molarity, specific activity 16.5 Ci.mmole^{-1}). To N was added unlabelled choline chloride (4.5 mg.), thus increasing the choline content of the medium fourfold. To P was added an equimolecular amount of (1,2-^{14}C)-phosphocholine chloride (5.05 mg., 6.1 μCi, specific activity 190 μCi.mmole^{-1}), giving a choline/phosphocholine ratio of 1:3. Horse serum (40 ml.) was added to both N and P, giving a concentration of 4% (v/v).

Day 7. Half of the culture (90 ml.) was transferred to a second 250 ml. flask; one was labelled N, the other P. Both cultures were doubled in volume by the addition of the appropriate new medium (90 ml.).

Day 9. The cultures were transferred to two 1l. screw-capped conical flasks, again labelled N and P, and the volumes were doubled to 2 X 360 ml.

Day 11. The volumes were doubled to 2 X 720 ml.

Day 12. The volumes were made up to 2 X 1l.

Day 13. Coulter counter measurements gave cell densities of 1.66×10^5 per ml. for culture N, and 1.74×10^5 for culture P; thus the flasks contained 1.66×10^8 and 1.74×10^8 cells respectively. Three-quarters of each culture (750 ml.) were centrifuged at 2500 rpm for 15 minutes, and the medium discarded. The pellets were resuspended in fresh, isotope-free medium (100 ml.) and centrifuged again; the process was repeated. The pellets were extracted with warm chloroform-methanol (2:1 v/v) (5 ml.) under nitrogen, and the resulting phospholipid extracts were washed once with water (1 ml.) and three times with the upper phase of a chloroform-methanol-water system (10:5:3 v/v). The solvents were removed under reduced pressure, and the residues chromatographed on silica gel H preparative TLC plates (solvent system B, single elution). The lecithin band was located by development with iodine vapour; the iodine was allowed to evaporate, and each lecithin band was scraped from the plate and eluted with chloroform-methanol (1:2) (50 ml.) followed by methanol alone (50 ml.). The solvents were removed under reduced pressure, giving lecithin residues (ca. 7 mg.) from each culture as colourless gums. When ^{31}P-NMR spectra had been recorded, the gums were

dissolved in warm dioxan (1 ml.) and one-fifth of each solution (0.2 ml.) was pipetted into a vial containing scintillation fluid (7 ml.) and monitored for radioactivity. Samples of each medium (0.1 ml.) were also counted; measurements are given corrected for background radiation and interchannel overspill.

^{31}P-NMR ($CHCl_3$): ppm +0.3 (PO_4^-) for extract N.

+0.9 (PO_4^-) and -30.3 (P^+) for extract P:

PO_4^-/P^+ ratio of peak areas = 1.38

RADIOACTIVITY MEASUREMENTS:

SAMPLE	ACTIVITY (cpm)		$^3H/^{14}C$
	^3H	^{14}C	
Medium N	942	—	—
Medium P	966	765	1.31
Extract N	63410	—	—
Extract P	31665	23623	1.34

CHAPTER 5 : IN VIVO EXPERIMENTS WITH PHOSPHOCHOLINE

(i) Method.

A choline-free rat diet, based on recent tables[323] of nutrient requirements for the growing rat, was prepared as follows:

Water-soluble vitamins:

	mg.
Cyanocobalamin (B_{12})	0.1
Folic acid	2.0
Niacin (nicotinic acid)	300
Pantothenic acid (B_3)	174
Pyridoxine (B_6)	24
Riboflavin (B_2)	50
Thiamine (B)	26

Ethanol-soluble vitamin:

Biotin	2.0

To a solution of the water-soluble vitamins in distilled water (90 ml.) was added biotin (2.0 mg.) in ethanol (8 ml.) and (methyl-^{3}H)-choline chloride of negligible molarity (2 mCi, specific activity 16.5 Ci.mmole^{-1}) in ethanol (2 ml.). The solution was divided into 2 equal portions of 50 ml., designated N and P. To solution N was added

unlabelled choline chloride (8.140 g., 58.3 mmole); to solution P was added unlabelled phosphocholine chloride (9.029 g.) and (1,2-^{14}C)-phosphocholine chloride (91.2 mg., 110 μCi, specific activity 190 μCi. mmole^{-1}) (total phosphocholine chloride 9.120 g., 58.3 mmole). The dietary salt mixture (Dept. of Biochemistry Animal House formulation) had the following composition:

	g.
Sodium chloride	104
Magnesium sulphate (dried)	166
Sodium dihydrogen phosphate	204
Dipotassium hydrogen phosphate	272
Calcium hydrogen phosphate	324
Ferrous citrate	70
Calcium lactate	780
Potassium iodide	0.1
Cupric sulphate	2.0
Manganese chloride	5.0

1 kg. Batches of foodstuff (enough for 6 rats for ca. 1 week) were made up as follows. Casein (choline- and vitamin-free) (220 g.), salt mixture (50 g.), starch (340 g.), and granulated sugar (340 g.) were emptied into the bowl of an electric food mixer, in the order indicated, and were mixed thoroughly for 5 minutes. Vitamin solution, either N or P, (5 ml.) was added from a pipette, followed by cod-liver oil B.P. (50 ml.

containing α-tocopherol (vitamin E) acetate (1.2 units per ml., 1 unit = 1 mg. dl form), and stirring was continued for another 10 minutes. The mixture was stored at -5°.

12 four-week old Wistar white male weanling rats, 3 to a cage, were used in the experiment. 6 received a phosphocholine diet (standard mix with vitamin solution P), and 6 a choline diet N; they were weighed twice a week. Food mixes were made into an edible form by adding sufficient water to give a dough-like consistency. Both food and drinking water were unlimited. Rat P-5 caught an infection after 7 days and did not gain weight at the original rate for another 22 days. Rats N-2 and N-3 died after 36 days; autopsy revealed little, but their deaths were considered to be due to respiratory infection. After 43 days 8 of the 10 remaining rats were stunned and decapitated, and their livers, brains, kidneys and lungs rapidly removed and deep-frozen under nitrogen until required. 1 rat from each group (P-2 and N-1) was taken to the Veterinary Diagnostic and Advisory Laboratory (Oxford University Dept. of Physiology) for slaughter and autopsy; the latter revealed "no abnormalities, and no organisms of pathogenic significance". Their livers were removed by the veterinary surgeon and were stored with the organs of the other animals.

Each liver was allowed to thaw out in ethanol (30 ml.); it was cut into small pieces with scissors, and homogenized with a Silverson mixer/emulsifier unit. The homogenate was extracted with boiling ethanol

under an atmosphere of nitrogen in a Soxhlet apparatus for 6 hours. Ethanol was removed under reduced pressure, and the residue taken up in light petroleum (b.p. 40-60°, 30 ml.) and filtered, after the method of Lucas and Ridout.[163] Solvent was again removed under reduced pressure and the resulting dark brown gum weighed and chromatographed on a column of alumina (1 g. per 10 mg. liver extract), using an eluant of chloroform-methanol-water (46:46:8), in order to remove non-neutral phospholipids. A total of 10 column-volumes of eluate was collected, and the solvent removed under reduced pressure. The residual gum was weighed and re-chromatographed on a column of silica gel (1 g. per 10 mg. extract); the column was first eluted with chloroform (10 column-volumes) in order to remove non-phospholipids such as cholesterol and triglycerides, and then with methanol (10 column volumes). The total methanol eluate was combined and the solvent removed to give a pale yellow gum. TLC (silica gel H, solvent system B) followed by development with iodine vapour, Rhodamine B-dichlorofluorescein reagent, and finally Zinzadze reagent, revealed the presence of only lecithin, sphingomyelin and sometimes traces of lysolecithin.

Other organs were then pooled (3 selected from each group) and extracted in exactly the same way.

(ii) ^{31}P-NMR spectra of liver homogenate pellets.

Before extraction, small samples (1.0 g.) of livers P-1 and N-6 were removed and homogenized for 5 minutes in an homogenizer of the Potter-Elvehjem type with a loose-fitting ground-glass pestle, in an aqueous solution (10 ml.) of 0.25M sucrose, 0.9% sodium chloride, 1.5 mM magnesium chloride and 10 mM tris buffer of pH 7.4. The homogenates were centrifuged at 11000 rpm (8000 X g) for 30 minutes in the 40(5) Spinco rotor of a Beckman L ultracentrifuge. The pellets, containing cell debris, nuclei, and mitochondria, were resuspended in distilled water and their ^{31}P-NMR spectra recorded. The supernatant fluids were recentrifuged at 40000 rpm (100000 X g) for 1 hour, and the pellets, containing the microsomal fractions, were resuspended in distilled water for NMR analysis as before (spectra in text, Chapter 5 (ii)).

(iii) ^{31}P-NMR analysis of liver phospholipid extracts.

^{31}P-NMR spectra were recorded for all the liver extracts, in chloroform solution (see text). Preparative TLC (silica gel H, solvent system B, 2 elutions) was then applied to extracts P-3 and P-5, and the constituent phospholipids (phosphatidylphosphocholine, phosphatidylcholine, sphingomyelin and traces of lysolecithin, in order of decreasing R_F) were weighed, and spectra recorded where there was sufficient sample. A fraction isolated from the origin of plate P-5 gave rise to a very low-field NMR signal, 'B'.

(iv) ^{31}P-NMR analysis of other organ extracts.

Spectra were recorded as for liver (see text). Preparative TLC was then applied to all three extracts (kidney, brain and lung), and the fractions weighed as before. Spectra were recorded for some of the kidney and brain fractions, but the lung extract provided insufficient material for this. A fraction exhibiting a very low-field NMR signal was isolated from the origin of the kidney extract plate (P-diet rats).

(v) 270 MHz PMR of purified rat-brain phospholipid extracts.

Samples of phosphatidylphosphocholine, phosphatidylcholine and sphingomyelin isolated from P-diet rat brain extract, after preparative TLC, were dissolved in deuterochloroform, and their 270 MHz PMR spectra recorded by the pulse Fourier transform method. Peak assignments were made with the aid of published data,[65, 87, 114] and the results were compared with the spectra of synthetic phosphatidylphosphocholine (see table in text).

(vi) Radioactivity measurements of phospholipid extracts.

Extract samples were weighed, and, where possible, dissolved in hot dioxan to give approximate concentrations of 4 mg.ml.$^{-1}$. Aliquots (0.5 ml.) were pipetted into vials containing dioxan-based scintillation

fluid (7 ml.) and were counted for 10 minute periods. Samples of the original radioactive vitamin solutions were diluted 100-fold with distilled water (to reduce quenching of the scintillant by the ingredients) and 0.1 ml. samples were counted in the same way. The readings below are corrected for background radiation and interchannel overspill:

EXTRACT	ACTIVITY cpm		COUNTS RATIO
	^{3}H	^{14}C	$^{3}H/^{14}C$
Vitamin soln. P	3724	2920	1.275
Vitamin soln. N	3723	0	—
Liver P-1	2380	868	2.74
" P-2	2267	914	2.48
" P-3	2317	807	2.87
" P-4	2103	734	2.87
" P-6	1875	734	2.56
" N-6	1764	0	—
P-lung	2577	860	3.00
P-kidney	2716	590	4.60
P-brain	1990	1174	1.70

SPHINGOMYELIN SOURCE	ACTIVITY cpm		COUNTS RATIO
	^{3}H	^{14}C	$^{3}H/^{14}C$
Liver P-3	2417	583	4.14
Liver P-5	2432	596	4.08
Lung (P)	2400	188	12.8
Kidney (P)	2268	109	20.8
Brain (P)	2243	135	16.6

There was no ^{14}C activity in samples extracted from the preparative TLC plate origins of liver P-5 and P-kidney.

(vii) Electron microscopy of rat-brain phosphatidylphosphocholine liposomes.

(See Chapter 2(vi) for preparative details.)

(viii) GLC analysis of lecithin fatty acid distribution.

The methanolysis procedure was similar to that of Hübscher et al.[290] Samples (2 mg.) of P-diet phosphatidylphosphocholine (P-PPC), P-diet phosphatidylcholine (P-PC) and N-diet phosphatidylcholine (N-PC) from

FATTY ACID COMPOSITION OF RAT PHOSPHOLIPIDS BY GLC

PERCENTAGE OF TOTAL MEASURED FATTY ACIDS, BY PEAK AREA (%)

CODE	RETENTION VALUE*	LIVER			LUNG			BRAIN			KIDNEY		
		P-PPC	P-PC	N-PC	P-PPC	P-PC	N-PC	P-PPC	P-PC	N-PC	P-PPC	P-PC	N-PC
iso-14:0	0.47												0.04
14:0	0.56	0.72	0.72	0.65	3.54	4.37	4.61	0.18	0.29	0.34	0.17	0.95	0.50
15:0	0.74	0.49	0.46	0.44	0.59	0.73	0.75	0.10	0.14	0.13	0.19	0.47	0.27
iso-16:0	0.85	0.11	0.17	0.11	0.42	0.50	0.39	0.01	0.03	0.11	***	***	0.33
16:0	1.00	31.70	33.46	41.40	57.60	64.30	63.35	34.30	42.10	44.20	9.48	54.70	49.60
16:1	1.13	8.90	11.15	6.58	7.51	9.26	11.10	1.77	1.67	***	1.07	3.82	3.75
17:0	1.34	0.41	0.22	0.27	0.25	0.19	0.25	0.33	0.32	0.46	0.22	0.25	0.32
iso-18:0	1.56	0.80	0.67	0.53	0.26	0.15	0.34	0.13	0.13	0.29	***	***	0.27
18:0	1.79	14.83	12.68	17.45	5.99	5.16	5.13	21.80	15.95	17.80	56.40	9.54	19.20
18:1	2.01	30.00	31.10	23.90	13.59	11.91	12.10	35.00	34.92	33.90	5.64	18.70	22.20
18:2	2.39	4.68	3.56	4.04	0.44	0.43	0.25	0.25	0.23	0.34	0.80	2.08	1.50
18:3	3.09	0.57	0.17	0.20	2.63	0.90	0.22	0.64	0.72	0.40	6.02	2.36	0.47
** 20:1	3.56	0.83	0.52	0.48	0.87	0.40	0.57	1.81	2.34	2.15	0.00	0.47	0.49
20:4	4.98	0.65	0.88	0.63	6.26	1.70	0.56	1.36	0.54	0.00	11.19	5.06	0.49
** 22:0	5.32	1.05	1.06	0.93	0.00	0.00	0.20	2.32	0.65	0.00	3.84	0.61	0.55
** 20:5	6.72	4.29	3.15	2.41	0.00	0.00	0.14	0.00	0.00	0.00	5.00	0.97	0.00

*Based on 16:0 = 1.00 **Tentative attribution ***Unresolved peak

lung, kidney, brain and liver were dissolved in chloroform (0.5 ml.) in 20 ml. test-tubes. Chloroform-methanol (2:1, v/v) (0.5 ml.) and 0.5N methanolic sodium hydroxide (0.5 ml.) were added, the solutions were thoroughly mixed using a vortex mixer, and were left at 22° for 15 mins. Ether (6 ml.) and 0.1N hydrochloric acid (4 ml.) were added, and the samples were mixed as before. The tubes were centrifuged at 2000 rpm. for 5 mins. on a bench machine, and the aqueous layers were removed and discarded. Solvents were removed from the organic layer under a stream of nitrogen, and the residues were redissolved in ether (0.1 ml.), and chromatographed on a column of poly (ethylene glycol adipate) at 196°. Sample size was 4-6 µl., and analysis time was approximately 30 mins. to arachidonate. Retention volumes were measured, and tabulated relative to palmitate; peak identification was achieved by comparison with chromatograms of methyl ester standards, and by reference to published data.[291] Peak areas were estimated using the method recommended by Scott and Grant,[324] i.e. peak height X width at half height.

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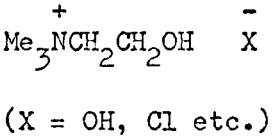
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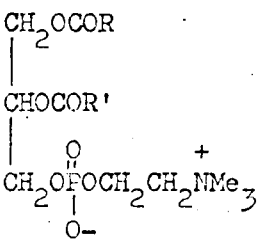
ABSTRACT

The biological role of choline (I), and the metabolic pathways involved in the synthesis and degradation of lecithin (II) and other phosphatides, have been discussed. Reference has been made to current ideas on the properties of phospholipids, both in cell membranes and in isolation, and to the physical methods required for their evaluation.



(X = OH, Cl etc.)

(I)



(II)

The published literature on the biological properties of choline analogues has been reviewed. Although the earliest investigations, mainly by Hunt and Renshaw,[1] were concerned solely with pharmacological properties, the observation by Best et al.[2] of such consequences of choline deficiency as fatty liver and renal haemorrhage stimulated the search for substances which could effectively replace choline in the diet, and these lipotropic compounds included the phosphorus (III) and arsenic (IV) analogues. Welch[3] claimed to have isolated the arsenic analogue of lecithin from the tissues of rats fed a choline-deficient arsenocholine (IV)-supplemented diet, but his evidence was not conclusive

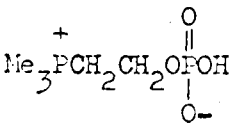
$Me_3\overset{+}{P}CH_2CH_2OH\ \ \overset{-}{Cl}$ (III)

$Me_3\overset{+}{As}CH_2CH_2OH\ \ \overset{-}{Cl}$ (IV)

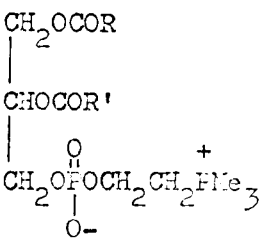
by modern standards. Moreover, knowledge of the lipotropic role of phosphocholine (III) was confined solely to the fact that its administration allayed the symptoms of choline deficiency. It was therefore decided to undertake a thorough chemical and biological investigation of this compound. The tri-n-butylphosphonium analogue (V) was used in all exploratory synthetic work, however, for reasons of convenience and economy.

$(n\text{-}Bu)_3\overset{+}{P}CH_2CH_2OH\ \ \overset{-}{X}$ (V)

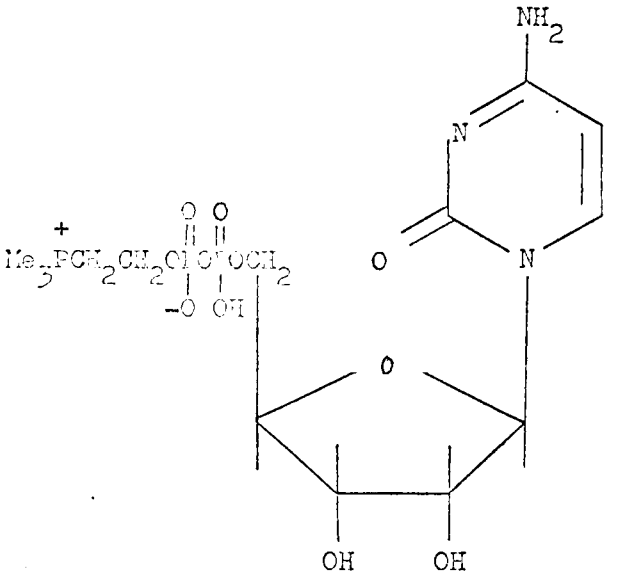
The chemical investigation involved the synthesis of the phosphorus analogues of those derivatives of choline involved in the cytidine pathway to lecithin, namely phosphorylphosphocholine (VI), cytidine-5'-diphosphate phosphocholine (CDP-phosphocholine) (VII) and phosphatidylphosphocholine ("phospholecithin") (VIII). Of these, the synthesis of phosphorylphosphocholine proved the most difficult, as the standard methods[4-6] of choline phosphorylation were unsuccessful when applied to the phosphorus analogue. Use was eventually made of a novel phosphoryl-



(VI)

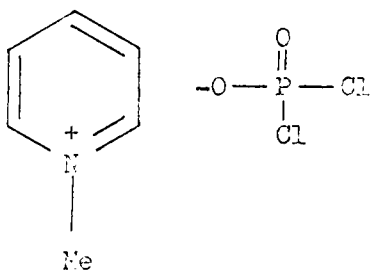


(VIII)

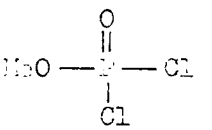


(VII)

ating agent, a complex (IX) formed by methylphosphorodichloridate (X) in pyridine, discovered, by chance, by Smrt and Catlin.[7] Thus both phosphocholine and its tri-n-butyl homologue were phosphorylated in fair yield (31-40%) in a single-stage process.



(IX)

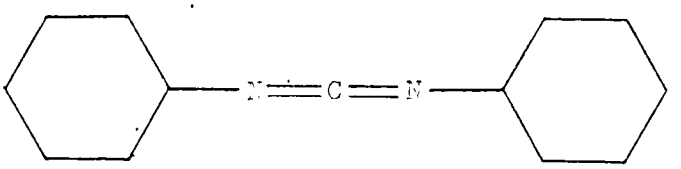


(X)

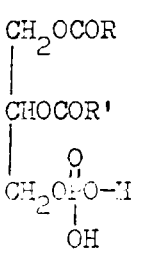
The synthesis of CDP-choline, in 24% yield, was a straightforward adaption of the method of Kennedy,[8] and involved the condensation of

phosphorylphosphocholine (VI) with cytidine-5'-monophosphate in the presence of dicyclohexylcarbodiimide (XI).

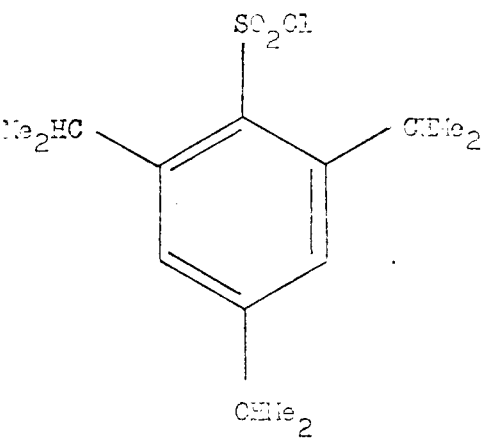
Well-established methods of lecithin synthesis were considered unsuitable for the phosphonium compound, but a recent method of Aneja and Chadha,[9] involving the condensation of choline and phosphatidic acid (XII) in the presence of triisopropylbenzenesulphonyl chloride (XIII) proved to be satisfactory, and in this way both 1,2-distearoyl-DL-phosphatidylphosphocholine (VIII, $R'' = -(CH_2)_{16}CH_3$) and its tri-n-butyl homologue were prepared in reasonable yields (33-35%).



(XI)



(XII)



(XIII)

Some of the properties of these synthetic "phospholecithins" were compared with those of distearoylphosphatidylcholine; thus the phosphorus analogues were found to exhibit lower transition temperatures, and remarkably lower melting points (ca. 135°), than the nitrogen compound (m.p. 233°). Both the trimethylphosphonium and trimethylammonium compounds appeared to form similar spherical or ellipsoidal single-bilayer liposomes in aqueous dispersion, but those of the tri-n-butylphosphonium compound were unusually long and thin, like collapsed vesicles; this is possibly due to the less hydrophilic nature of the tri-n-butylphosphonium group. Both trimethyl compounds were attacked to a similar extent by phospholipase C, while the enzyme showed a far lower affinity for the tri-n-butylphosphonium lecithin.

Since the phosphorus-31 nucleus has a spin of $\frac{1}{2}$, and couples with the proton, both proton magnetic resonance (PMR) and ^{31}P nuclear magnetic resonance (^{31}P-NMR) studies of the phosphonium analogues were found to be of interest. Thus the methyl proton signal in the PMR spectrum of phosphocholine (III) is a doublet with a coupling constant of ca. 14.5 Hz, and is characteristic of all the trimethylphosphonium derivatives. This property later proved to be a useful method of structure confirmation when phosphatidylphosphocholine (VIII), synthesized in vivo, was isolated from rat tissues. As phosphorylphosphocholine (VI) and its derivatives were probably the first compounds to be

prepared containing both a phosphonium and a phosphate group, they provided a unique opportunity for the simultaneous study of two very different phosphorus groups by ^{31}P-NMR.

The use of ^{3}H-labelled choline and ^{14}C-labelled phosphocholine provided a means of investigating the relative affinity of the enzymes of the cytidine pathway for phosphonium compounds. Thus it was found that phosphocholine was at least as acceptable as choline to the enzyme choline phosphokinase:

$$\text{ATP} + \text{choline} \longrightarrow \text{phosphorylcholine} + \text{ADP}$$

and was eventhe preferred substrate in the initial stages of competitive incubations near enzyme-saturation level. Phosphorylcholine-cytidyl-transferase:

$$\text{CTP} + \text{phosphorylcholine} \rightleftharpoons \text{CDP-choline} + \text{pyrophosphate}$$

was far more specific, and initial rates of conversion of the phosphorylated bases to the CDP derivatives indicated a threefold preference for the natural substrate. In the case of phosphorylcholine-diglyceride-transferase:

$$\text{CDP-choline} + \text{diglyceride} \rightleftharpoons \text{lecithin} + \text{CMP}$$

both phosphorus and nitrogen substrates were equally acceptable in the forward reaction, but phosphatidylphosphocholine, once formed, appeared far less susceptible to degradation by the reverse reaction.

The *in vitro* investigation was extended to cell culture, using the P815Y line of neoplastic mouse mast cell. The cells showed similar growth characteristics in choline-supplemented and in phosphocholine-supplemented media, and extraction of lecithin from cells grown in the latter led to the conclusion that both bases were equally acceptable; radioactivity readings showed similar choline/phosphocholine ratios in the medium and in the extracted lecithin. Moreover, measurement of the relative areas of phosphate and phosphonium peaks in the ^{31}P-NMR spectrum of the extract confirmed this finding.

Finally, an *in vivo* study was undertaken using choline-deficient weanling rats. One group of animals was provided with a choline-free ^{14}C-phosphocholine-supplemented diet ("P-diet"), while a control group received food containing a full complement of choline (N-diet); the two analogues were provided in equimolecular quantities to their respective groups. Both diets contained ^{3}H-labelled choline of negligible molarity and high specific activity. The animals were killed, and their livers, kidneys, lungs and brains removed, after a period of 7 weeks. Neutral phospholipid was extracted from the organs, and preparative thin-layer chromatography (TLC) of these extracts led to the isolation of biosynth-

esized phosphatidylphosphocholine (VIII), free from phosphatidylcholine and identified by NMR and ^{31}P-NMR; the latter gave a spectrum bearing two signals of equal intensity, from phosphonium and phosphate. Measurement of relative peak areas in the ^{31}P-NMR spectra of some of the extracts gave a measure of the level of phosphocholine incorporation, varying from 33% in lung lecithin to 6% in kidney sphingomyelin. Although the effect of lecithin already present in the tissues at the start of the experiment was unknown, it was suggested that much of the phosphatidylcholine in the P-diet rats originated from sequential methylation of phosphatidylethanolamine, a route well-established in the liver but claimed to be of little significance in other tissues.[10]

It was also suggested that sphingomyelin was being synthesized mainly by an indirect route,[11] from CDP-choline formed reversibly from this methylation-derived phosphatidylcholine, and not by a direct pathway involving CDP-phosphocholine.

Additional signals in the ^{31}P-NMR spectra of extracts from P-rats (but not N-rats) were attributed to phosphobetaine (XIV) produced by oxidation of phosphocholine in the liver, and trimethylphosphine oxide (XV) in the kidney, originating from phosphocholine oxidized by the bacteria of the intestinal tract. Similar liver oxidation of arsenocholine had been observed by Mann et al.,[12] while the oxidation of choline to trimethylamine oxide in the intestine, and its excretion in

the urine, was first reported by Davies.[13]

$Me_3\overset{+}{P}CH_2CO_2H \quad \overset{-}{X}$ (XIV)

Me_3PO (XV)

Comparison of $^3H/^{14}C$ ratios in the P-diet itself and in the phospholipid extracts gave a possible indication of the differing preferences of some enzyme systems for choline and phosphocholine, although it was necessary to make several basic assumptions.

Comparison of the relative fatty acid composition in the lecithin and "phospholecithin" of the four organs investigated showed little difference between the two in liver, lung and brain, but a profound difference in the kidney. Thus renal phosphatidylphosphocholine contained greatly elevated levels of stearic and polyunsaturated acids, and it was suggested that this might be due to a need to maintain a critical optimum membrane fluidity, since it had already been noted that a change from ammonium to phosphonium produced a lecithin with a lower transition temperature. Thus a change from palmitic to stearic acid increased the transition temperature, while a change from oleic and linoleic acids to polyunsaturates increased the membrane stability.[14]

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