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A PROCEDURE FOR THE EVALUATION OF IN VIVO LIPID BIOSYNTHESIS IN MOUSE BRAIN: THE EFFECTS OF CYSTEINE, MOLYBDATE, DIMETHYLAMINOETHANOL, GAMMA - HEXACHLOROCYCLOHEXANE, AND 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ON LIPID BIOSYNTHESIS IN MOUSE BRAIN IN VIVO.

The City University of New York, Ph.D., 1973
Biochemistry

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By Brenda Breuer

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

June, 1973

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

May 22, 1973
date

Thomas H. Haines
Chairman of Examining Committee

May 22, 1973
date

Aaron Lukton
Executive Officer

Dr. Max Hamburgh

Dr. Abraham Mazur

Dr. Neil McKelvie
Supervisory Committee

The City University of New York

Abstract

A PROCEDURE FOR THE EVALUATION OF *IN VIVO* LIPID BIOSYNTHESIS IN MOUSE BRAIN: THE EFFECTS OF CYSTEINE, MOLYBDATE, DIMETHYLAMINOETHANOL, *GAMMA* - HEXACHLORO-CYCLOHEXANE, AND 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ON LIPID BIOSYNTHESIS IN MOUSE BRAIN *IN VIVO*

by

Brenda Breuer

Adviser: Dr. Thomas H. Haines

The *in vivo* incorporation patterns of $(2-^{14}C)$-acetate, $(U-^{14}C)$serine, $^{32}PO_4^{\equiv}$, and $^{35}SO_4^{=}$, into the lipids of mouse brain have been established. It was demonstrated that the relative distribution of the radioactive label was similar when the precursor injected was $^{32}PO_4^{\equiv}$ or $(2-^{14}C)$-acetate. $(U-^{14}C)$serine was shown to be especially useful for studying the formation of phosphatidylserine and the sphingolipids. The normal incorporation patterns were altered by injecting potential enzyme inhibitors.

(1) Cysteine depresses phosphatidylserine formation. Other metabolic changes observed in the presence of cysteine are similar to those caused by dithioerythritol, a sulfhydryl reagent.

(2) Injection of a low concentration of molybdate into mouse brain, significantly lowers the

biosynthesis of phosphatidylserine, phosphatidic acid, sphingomyelin, and phosphatidylcholine. It depresses the formation of phosphatidylinositol to a smaller extent. The changes in phosphatidylethanolamine and sulfatide biosynthesis are negligible.

The formation of phosphatidic acid in mice injected with a higher dosage of molybdate is similar to that found in the control animals. The specific activities of the remaining lipids are drastically inhibited.

(3) Dimethylaminoethanol depresses the formation of phosphatidylserine, sphingomyelin, phosphatidycholine, and phosphatidylethanolamine. A negligible depression in the biosynthesis of phosphatidic acid and phosphatidylinositol is also observed.

(4) *Gamma*-hexachlorocyclohexane activates the synthesis of phosphatidylcholine. It depresses that of phosphatidylserine and phosphatidylethanolamine. Phosphatidylinositol biosynthesis is slightly increased by the compound.

(5) The formation of phosphatidylserine, phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine appears to be enhanced in the presence of 3,4-dihydroxybutyl-1-phosphonate.

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Introduction

The processes of the mind, such as "learning" and "remembering," are current subjects of neurochemical investigations. For example, such studies have demonstrated a relationship between the biosynthesis of brain proteins to learning. Puromycin inhibits protein synthesis *in vivo* by being incorporated into growing polypeptides and released from polysomes as peptidyl-puromycin (Flexner and Flexner, 1968[a]). In addition, puromycin, injected intracerebrally into mice, appears to interfere with the consolidation of memory (Flexner and Flexner, 1968[b]). The time course of the effects of puromycin suggests that memory is formed in at least three stages (Barondes and Cohen, 1966). The first stage extends for a number of minutes after learning, and is unaffected by puromycin; a second phase, inhibited by temporal injections of puromycin, lasts for several days; and a third phase, which is influenced by more diffuse intracerebral injections of puromycin.

Studies with another inhibitor of protein synthesis, acetocycloheximide, also indicate that there are three stages of memory (Flexner *et al.*, 1966). Mice injected intracerebrally with acetocycloheximide, before or immediately after learning, show no impairment of memory in the first period. Subsequently, there is a

period, during which the expression of memory is lost, followed by a final stage in which memory returns.

In addition to providing a deeper insight into normal processes, such as memory, biochemical investigations have correlated neurochemical abnormalities to mental retardation diseases. Such studies have dealt with sphingolipidoses, a group of many neuronal diseases, each of which is caused by a deficiency of an enzyme responsible for the catabolism of a specific sphingolipid (Morell and Braun, 1972).

As an example, classical Tay-Sachs disease, one of the sphingolipidoses, is an inherited disorder of sphingolipid metabolism (Klenk, 1939). Patients afflicted with this disease usually die within the first three years of life. The accompanying disorders are both physical and mental. Abilities which already have been learned, such as lifting the head, grasping and sitting, are lost again. Neurological symptoms, like convulsions and exaggerated startled response to noise precede the general damage to the brain. Patients having this disease suffer from dementia and blindness before death (Sandhoff *et al.*, 1971). Biochemical studies have shown that the disease is accompanied by an accumulation of ceramide-glucose-galactose (N-acetylneuraminic acid)-N-acetylgalactosamine ((Tay-Sachs ganglioside), (Svennerholm, 1962, 1963)). The cause of the abnormal storage of this compound was found

to be a defect in the catabolic enzyme, hexosaminidase A (Sandhoff _et al_., 1971).

A second example, Niemann-Pick disease (also classified among the sphingolipidoses) has been characterized by an accumulation of sphingomyelin, resulting from a deficiency of sphingomyelinase (Sloan _et al_., 1969).

Other examples of sphingolipidoses and their defective enzymes are Metachromatic Leukodystrophy (sulfatidase), Gaucher's disease (β-glucosidase), and Fabry disease (α-galactosidase).

The relationships between protein formation in the brain to memory, and between aberrations in sphingolipid metabolism to neuronal disorders illustrate how different chemicals in the brain contribute to mental processes and to mental health. It was thus thought that ascertaining the roles played by other neuronal substances might prove to be invaluable.

Lipids are a major constituent of the brain, accounting for approximately forty per cent of the brain's dry weight (Maggio _et al_., 1972), and they are found almost exclusively in the membranes. Furthermore, since each membrane type has a unique combination of lipids (Table 1), the importance of specific membranes may be ultimately elucidated by regulating the production of their major lipid components. For example, cardiolipin is almost unique to the mitochondrial membrane, as are gangliosides

Lipid	Neuron	Glia	Mito-chondria	Synap-tosome	Myelin	E.R.
* PL	430[1]	640[1]	460[1]	570[1]	1410[1]	m[3]
PC	200[1]	270[1]	210[1]	230[1]	450[1]	
PE	140[1]	240[1]	170[1]	220[1]	600[1]	
PI	24[1]	20[1]	24[1]	15[1]	21[1]	m[2]
PS	32[1]	50[1]	28[1]	55[1]	170[1]	m[4]
SM	34[1]	56[1]	32[1]	46[1]	170[1]	
Chol	250[1]	380[1]	160[1]	280[1]	1050[1]	+[3]
Cer	20[1]	34[1]		18[1]	530[1]	
Su	4[1]	8[1]		6[1]	160[1]	
Gang	4.7[1]	9.5[1]	3.6[1]	24.6[1]		
CL	s[2]		m[2]			s[2]

Table 1. Distribution of lipids in subcellular fractions of brain.

1 - according to Hamberger and Svennerholm, 1971; values are in terms of micromoles/gram protein in rabbit cerebral cortex.

2 - according to Eichberg *et al.*, 1964.

3 - according to Bradford *et al.*, 1964.

4 - according to McMurray and Dawson, 1969.

s - a slight amount of the lipid in the particular fraction has been found.

m - much of the lipid in the particular fraction has been found.

+ - the lipid has been found in the particular fraction. Values underlined indicate in which subcellular fraction the lipid predominates.

---

* The following are the abbreviations used in this report: APS - Adenosine-5'-phosphosulfate; cer - cerebrosides; CH - cerebrosides with hydroxy-fatty acids; chol - cholesterol;

to the synaptosomal membrane, and sphingolipids (except for the gangliosides) to the myelin membrane. This predominance of a particular lipid (or of a class of lipids) in a specific membrane may indeed provide a means of controlling the formation of the membrane.

Thus the subject for this project was established. It was to test chemicals that one would reasonably _expect_ should alter normal lipid biosynthesis for their _actual_ effects on the formation of these lipids. To undertake such a project the following would be essential: (1) to be able to separate the individual brain lipids, (2) to determine the normal biosynthetic patterns of these lipids, and finally (3) to select and to test compounds that could potentially induce changes in lipid metabolism for their _actual_ effects.

After having thus defined the problem, an appropriate procedure for handling the investigation was formulated by adopting different techniques that had been employed in independent investigations.

CL - cardiolipin; CN - cerebrosides with normal fatty acids; CNS - central nervous system; 3,4-DBP - 3,4-dihydroxybutyl-1-phosphonate; DMAE - dimethylaminoethanol; dpm - disintegrations per minute; DTE - dithioerythritol; E.R. - endoplasmic reticulum; FFA - free fatty acids; gang - gangliosides; HCC - hexachlorocyclohexane; MGDG - monoglycosyl diglyceride; NL - nonpolar lipids; O, OR - origin; PA - phosphatidic acid; PAPS - 3'-phosphoadenosine-5'-phosphosulfate; PC - phosphatidylcholine; PE - phosphatidylethanolamine; PI - phosphatidylinositol; PL - phospholipid; PR - proteins; PS - phosphatidylserine; SH - sulfatides with hydroxy-fatty acids; SM - sphingomyelin; SN - sulfatides with normal fatty acids; Su - sulfatides; TLC - thin layer chromatography.

Firstly, the brain lipids were separated quite successfully using the two-dimensional thin layer chromatographic procedure of Rouser *et al*. ((1967); (Figure 1)).

Secondly, it was found that radioactive precursors had proved to be an invaluable tool in other *in vivo* metabolic investigations. Ganglioside biosynthesis was studied by following the incorporation of labelled sphingosine derivatives, CMP-N-acetylneuraminic acid-^{14}C (Kanfer and Brady, 1967), and N-acetyl-^{3}H-mannosamine (Kanfer and Ellis, 1971) into gangliosides. The metabolic relationship between cerebrosides and sulfatides was investigated with ^{14}C-galactose (Hauser, 1964), and aspects of phospholipid metabolism were studied with sodium dihydrogen^{33}P-phosphate (Friedel and Schanberg, 1971). Thus, for this study the normal biosynthetic patterns were established by following the incorporation into brain lipids of ^{14}C-acetate, ^{32}P-phosphate, ^{14}C-serine, and ^{35}S-sulfate.

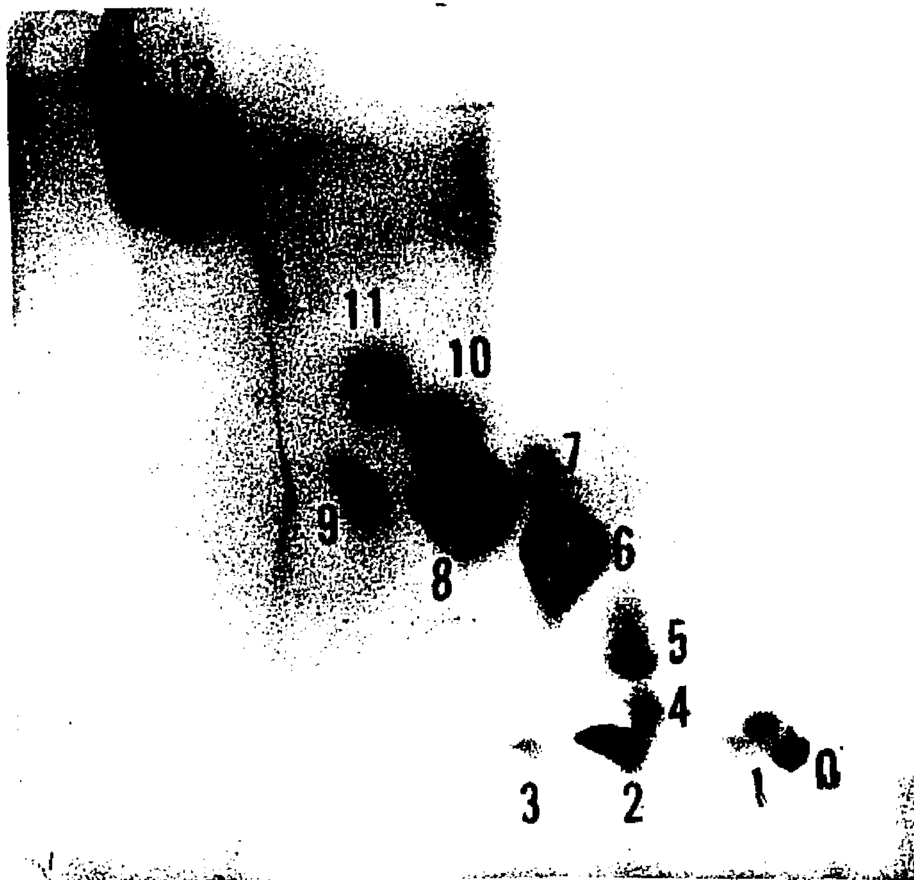
These precursors were injected intracerebrally according to the procedure of Barondes (1968). In order to determine specific activities (disintegrations per minute/microgram phosphate) of the individual phospholipids, phospholipid determinations (according to Rouser, 1970) were performed directly on the spots scraped off the thin layer chromatographic plates.

Finally, chemicals that had proved to inhibit the synthesis or degradation of specific lipids *in vitro* were

Figure 1. Thin layer chromatograph of brain lipids.

0 - origin; 1 - proteins and gangliosides; 2 - PS; 3 - PA; 4 - PI; 5 - SM; 6 - PC and SH; 7 - SN; 8 - PE; 9 - CL; 10 - CH; 11 - CN; 12 - NL.

The solvent system for the first (vertical) direction is chloroform-methanol-concentrated (28%) aqueous ammonia (65:35:5), and for the second (horizontal) direction chloroform-acetone-methanol-glacial acetic acid-water (5:2:1:1:0.5).



tested for their *in vivo* effects. (This criterion did not apply to the investigation of dimethylaminoethanol, which was tested for its *in vitro* effects on lipid metabolism subsequent to the completion of the *in vivo* investigations reported herein). In addition, all the chemicals tested met the following specifications:

(1) The substance was stereochemically similar to the natural substrate of the target enzyme.

(2) The substance was sufficiently different from the enzyme's substrate so that it would not react further to give product.

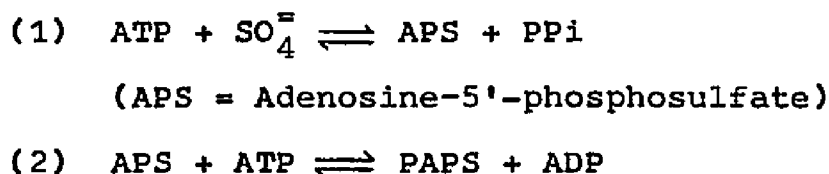
(3) The substance would not reasonably be expected to affect other activities of the brain.

Cysteine, molybdate, dimethylaminoethanol, *gamma*-hexachlorocyclohexane, and 3,4-dihydroxybutyl-1-phosphonate thus qualified to be tested for their *in vivo* effects on lipid metabolism.

Cysteine, the first substance to be tested, inhibits the synthesis of dihydrosphingosine in particulate systems from both *Hasenula ciferri* (Braun and Snell, 1967; 1968) and rat brain (Brady and Koval, 1958). The activation of serine by pyridoxal phosphate is necessary for the formation of dihydrosphingosine, and apparently cysteine, sterically and chemically similar to serine, depletes the system of pyridoxal phosphate.

Molybdate was tested for its *in vivo* effects on the formation of brain lipids, because it had inhibited the *in vitro* sulfation of cerebrosides to form sulfatides.

The sulfation is dependent upon the presence of PAPS (3'-phosphoadenosine-5'-phosphosulfate), the synthesis of which involves the following reactions:



(1) $ATP + SO_4^{=} \rightleftharpoons APS + PPi$

(APS = Adenosine-5'-phosphosulfate)

(2) $APS + ATP \rightleftharpoons PAPS + ADP$

The first reaction is enzymatically catalyzed by ATP sulfyrase, and the second by ATP kinase. In bacterial (Pasternak, 1962) and yeast (Wilson and Bandurski, 1958) systems, molybdate inhibits reaction (1) by forming adenosine-5'-molybdophosphate, an unstable compound which is subsequently hydrolyzed to AMP and PPi; consequently, sulfatide formation is depressed.

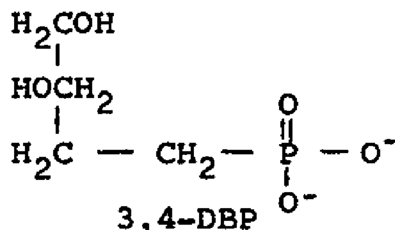
It was thought that dimethylaminoethanol (DMAE) might inhibit the biosynthesis of choline-containing lipids because: (1) in yeast, DMAE is a substrate for choline phosphokinase, the enzyme that catalyzes the synthesis of phosphorylcholine (Wittenberg and Kornberg, 1953), and (2) the formation of PC in the brain does not involve stepwise methylation (Ansell and Spanner, 1971). (Figure 7A)

Gamma-hexachlorocyclohexane (γ-HCC) is an analog of mucoinositol. Hokin and Brown (1969) demonstrated its inhibitory effects on (1) the acetylcholine-stimulated synthesis of PI in cerebral cortex, and on (2) PA-inositol transferase in the microsomal fraction of guinea pig brain.

In the above two systems the metabolism of PA and PC was unaffected. On the other hand δ-HCC, having the configuration that corresponds to myoinositol (which is the configuration of inositol in PI), not only inhibits PI formation, but also dramatically depresses the biosynthesis of PA and PC.

Thus, because of its greater specificity as an inhibitor *in vitro*, the *gamma* analog was chosen for an *in vivo* investigation of its effect on the metabolism of brain phospholipids.

In *Clostridium perfringens* phospholipase C is dramatically inhibited by a phosphonate-containing analog of lecithin (3,4-dioctadecoxybutylphosphonylcholine). This inhibition seems to be dependent, at least in part, upon specific features of molecular geometry (Rosenthal and Pousada, 1968). It therefore seemed worthwhile to study the effects of an analog of glycerol phosphate, 3,4-dihydroxybutyl-1-phosphonate (3,4-DBP), on the *in vivo* metabolism of mouse brain phospholipids.



Experimental

Materials: Unless otherwise stated, all radioactive compounds were purchased from International Chemical Nuclear, and all other chemicals from Fisher Scientific and were reagent grade. Dowex 50 was supplied by J. T. Baker, N. J. Male mice (CF1 strain) were obtained from Carworth Farms. Intracerebral injections were performed with microsyringes purchased from Glenco. Thin layer chromatographic (TLC) plates (Redi Cotes) and lipid standards were supplied by Supelco. Ground glass homogenizers and repipets were obtained from Will Scientific. Equipment and supplies for liquid scintillation counting were supplied by Nuclear Chicago. Spectrophotometric readings were taken either with a Gilford (Model 240) or with a Zeiss (Model PMQIII) Spectrophotometer.

Dr. B. Tropp kindly supplied the lithium 3,4-dihydroxybutyl-1-phosphonate.

Injection Procedure: To ensure that the injected solutions reached the brain and were not excluded therefrom by the blood-brain barrier, all injections were administered intracerebrally (Barondes, 1968). Mice, slightly anaesthesized with ether, were mounted on a stereotaxic instrument (Scientific Prototype, N. Y.). Their scalps were incised and reflected, and holes, three millimeters deep, were bored on both sides of the skull, two millimeters

anterior and two millimeters medial to the angle formed by the intersection of the insertion of the temporal and occipital muscles. Five to seven microliters of a solution of a radioactively labelled lipid precursor and a potential inhibitor of lipid biosynthesis was injected into each site with a microsyringe (gauge 26). To minimize refluxing (leakage), the solution was injected at the rate of one microliter every fifteen seconds, and after injecting the desired volume the syringe remained in place for sixty seconds before it was slowly removed. Immediately afterward filter paper was placed upon the site of injection to absorb any leakage of the solution. The percentage of leakage was determined by counting the filter paper in a liquid scintillation counter (Nuclear Chicago, Model 725).

Lipid Extraction and Thin Layer Chromatography (TLC) of Brain Lipids. Mice were sacrificed and brains excised three hours after injection (Moser and Karnovsky, 1959). The brains were homogenized in a ground glass homogenizer with seven complete passes in chloroform: methanol 2:1 (six milliliters per brain), and the whole lipid extract was concentrated in a flash evaporator. An aliquot of the extract was separated into its individual lipids by two-dimensional TLC on Redi Cotes (Supelco, Supelco Park, Pa.), using chloroform: methanol:concentrated (28%) aqueous ammonia (65:35:5) for the first direction, and chloroform:acetone:methanol:glacial acetic acid:water (5:2:1:1:0.5) for the second direction (Rouser *et al.*, 1967). Spots were visualized either by

charring, in which case plates were sprayed with a solution of 25 per cent sodium bisulfate: 3% sulfuric acid (v/v) and then heated for one hour at 180°C; alternately the plates were exposed to iodine vapors (Figure 1).

Radioactive lipids were located by autoradiography. When the lipids were labelled with ^{14}C the plates were sprayed with Omnispray (New England Nuclear), exposed to Kodak non-screen X-ray film, and placed into light-proof cassettes. The cassettes were kept at dry-ice temperature for two to four weeks before the films were developed. Because of the high energy of the beta emissions of ^{32}P, Omnispray was not necessary for TLC's of lipids labelled with this isotope. The plates were exposed to Kodak non-screen film, and placed into cassettes for one to two days at room temperature before the films were developed.

Determination of Radioactivity Incorporated into Brain Lipids. Radioactive counting was done with either a gas flow counter (Nuclear Chicago) or with a Nuclear Chicago liquid scintillation counter (Model 725). In the latter case radioactive spots were scraped off TLC plates into counting vials containing ten milliliters of toluene: Spectrafluor ((10:0.42, v/v), Nuclear Chicago)). The samples were counted for radioactivity, and the disintegrations per minute (d p m's) were calculated using the instrument's external standard method.

Phosphorus Determination for Phopholipids. All phosphate determinations were done according to the procedure of Rouser (1970). Individual lipid spots were scraped off TLC plates into 18 x 125 millimeter test tubes. 0.5ml hydrochloric acid was added to each sample, and the test tubes were heated in a sand bath at 180°C for ten minutes. After the test tubes had cooled, 0.65ml of perchloric acid (70%) was added to each sample, and the tubes were heated at 180°C until the samples were colorless. After cooling, reagents added in order were: 3.3ml water, 2.5% ammonium molybdate (0.5ml), and 10% ascorbic acid (0.5ml.). Color was developed by heating the tubes in a boiling water bath for five minutes. Adsorbent was sedimented by brief centrifugation. The color intensity was determined at 797mμ in a Gilford (Model 240) or Zeiss (Model PMQ III) spectrophotometer.

Identification of phospholipid bases. For studying the effects of dimethylaminoethanol on the formation of brain lipids, it was deemed desirable to hydrolyze the phospholipids and separate the bases therefrom. Following TLC of a brain lipid extract, plates were air dried for one minute, and spots were visualized by exposure to iodine vapors for thirty seconds. Spots were outlined, and the plates were air dried for an additional five minutes. The spots were scraped off individually into glass-stoppered bottles. Polar lipids were extracted successively with ten milliliters of each: (1) methanol-5% water, (2) methanol, and (3) chloroform. Less polar lipids were extracted once

with ten milliliters of methanol and twice with ten milliliters of chloroform. The extracts of each sample were passed through glass-scintered filters into a round-bottom flask, pooled and evaporated to dryness in a flash evaporator.

Subsequently, each sample was refluxed in 6N HCl for twenty-four hours, and then evaporated to dryness. Thereafter, the samples were treated according to the procedure described by Ansell and Spanner (1971). The samples were placed in a vacuum desiccator over KOH for several hours. They were then dissolved in a half milliliter of water and extracted with ether. The water soluble fraction was evaporated to dryness in a flash evaporator.

An appropriate volume of methanol was then added to the residue, and the glycerol moiety and the phospholipid bases were separated by TLC. Two-dimensional TLC was used for the hydrolysates of the spots that usually corresponded to phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), using chloroform:methanol:concentrated (28%) aqueous ammonia (65:35:4) for the first direction, and methanol:acetone:HCl (70:20:3) (a variation of a solvent system proposed by Speed and Richardson (1968)) for the second direction. A two-dimensional system was also used for the phosphatidylinositol (PI) hydrolysate; chloroform:methanol:concentrated (28%) aqueous ammonia (65:35:4) was used for the first direction, and methanol:acetone:glacial acetic acid

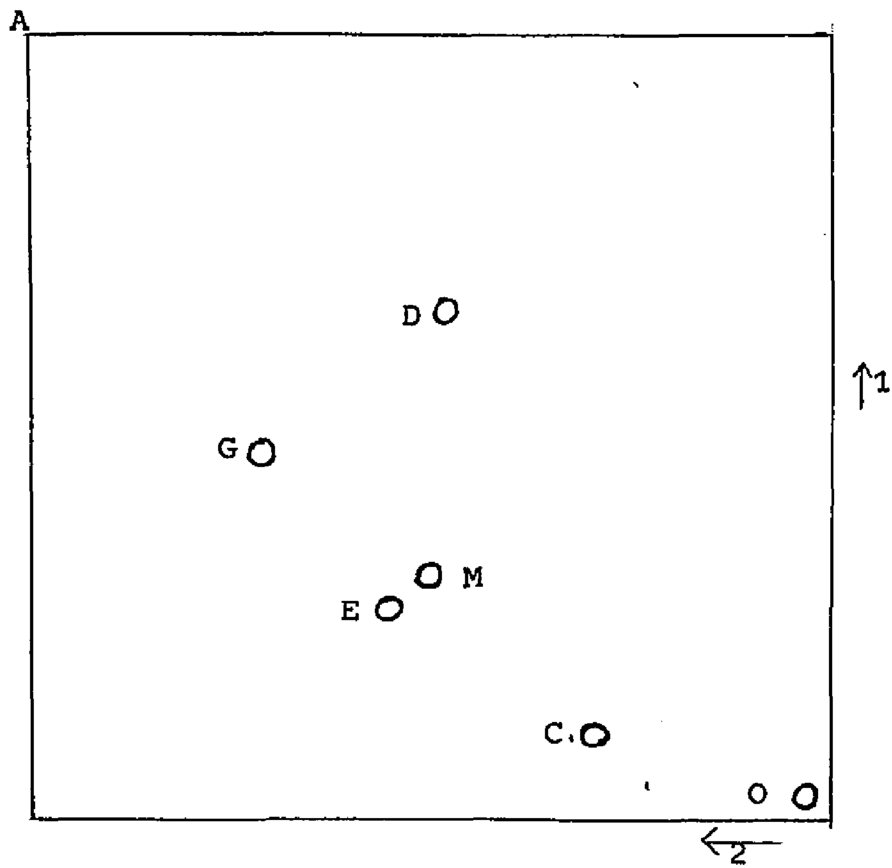
(90:10:10) for the second direction. The solvent system for the phosphatidylserine (PS) hydrolysate is methanol: acetone:glacial acetic acid (11:1:1).

The TLC's of the phospholipid hydrolysate products are shown in Figure 2.

Preparation of sodium 3,4-dihydroxy-butyl-1-phosphonate. Sodium 3,4-dihydroxybutyl-1-phosphonate was prepared by applying a sample of lithium 3,4-dihydroxybutyl-1-phosphonate to a column of Dowex 50 (X8· H^+ form). The column was washed with 35ml. of water. The effluent was titrated quantitatively with 0.10N NaOH, and subsequently evaporated to dryness in a flash evaporator. The residual salt was dissolved in a desired volume of water.

Figure 2. Thin layer chromatographs of the products of the hydrolysis of brain phospholipids.

A - The solvent system for the first direction is chloroform-methanol-concentrated (28%) aqueous ammonia (65:35:4), and for the second direction is methanol-acetone-hydrochloric acid (70:20:3).

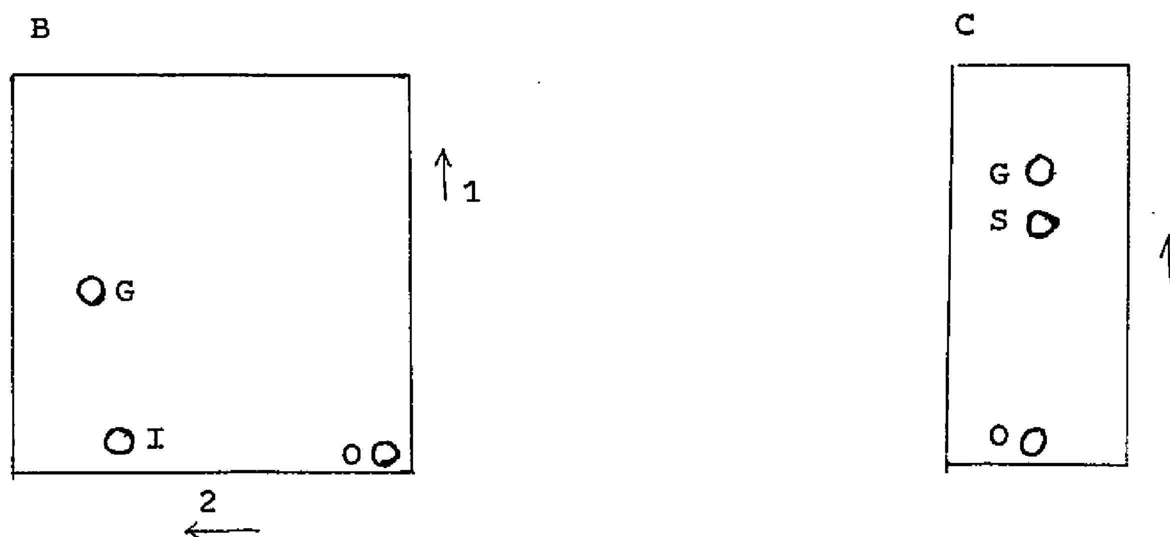


	R_f ↑ 1	R_f ← 2
O (Origin)	O	O
C (choline)	0.06	0.32
M (monomethyl-ethanolamine)	0.22	0.46
E (ethanolamine)	0.19	0.49
G (glycerol)	0.35	0.67
D (dimethyl-ethanolamine)	0.49	0.43

Figure 2. Thin layer chromatographs of the products of the hydrolysis of brain phospholipids. (Continued)

B - The solvent system for the first direction is chloroform-methanol-concentrated (28%) aqueous ammonia (65:35:4), and for the second direction is chloroform-acetone-glacial acetic acid (90:10:10).

C - The solvent system is chloroform-acetone-glacial acetic acid (11:1:1).



	R_f ↑ 1	R_f ← 2
O (origin)	0	0
I (inositol)	0.06	0.70
G (glycerol)	0.44	0.75

	R_f
O (origin	0
S (serine)	0.61
G (glycerol)	0.71

Results

Percentage of (2-^{14}C) acetate metabolized to $^{14}CO_2$. The purpose of this experiment was to determine whether the level of radioactive $^{14}CO_2$ exhaled by mice injected with (2-^{14}C)acetate warranted the use of NaOH traps as a general procedure.

Two mice, each injected with 16 microcuries of (2-^{14}C)acetate, were placed in flasks hooked up to a series of water and 6N sodium hydroxide traps, the last trap being connected to a water aspirator. The animals were sacrificed three hours after injection, and aliquots were taken from each trap to determine the $^{14}CO_3^{=}$ content. 0.24 millimoles of Na_2CO_3 was added to each aliquot, and the pH adjusted to 10.3. The $CO_3^{=}$ was precipitated with 0.15M $BaCl_2$,by centrifuging at 35,000 g. The precipitate was then suspended in ethanol, transferred to planchets, dried, and counted for radioactivity in a gas flow counter. The dpm of the exhaled $^{14}CO_2$ accounted for 13.2 per cent of the injection. This level of counting indicates that all animals injected with (2-^{14}C) acetate should be kept in cages connected to NaOH traps.

Specific activities of the lipids in the brain of the mouse: Figure 1 is a two-dimensional TLC of mouse brain lipids. Quantitative values of the relative distribution of the

phospholipids are presented in Table 2. These values agree well with those Hogan et al. (1970) reported for mouse brain, and Sun (1972) and Friedel et al. (1970) reported for rat brain. The distribution of intracerebrally injected ^{32}P and of lipid-phosphate among the brain lipids is shown in Figure 3. As can be seen, PS and SM, myelin lipids (Table 1), have the lowest specific activities (dpm /μg lipid-phosphate). This is in accord with the relatively low biochemical turnover of myelin (Spohn and Davison, 1972). The high specific activity of PI supports the hypothesis that its functional role is one that is actively involved in synaptosomal transmission (Friedel et al., 1971). One would have predicted the high turnover of PA, as it is a precursor of phosphatides, and it also has been implicated in synaptosomal transmission. PC, a ubiquitous phospholipid, has a relatively intermediate specific activity.

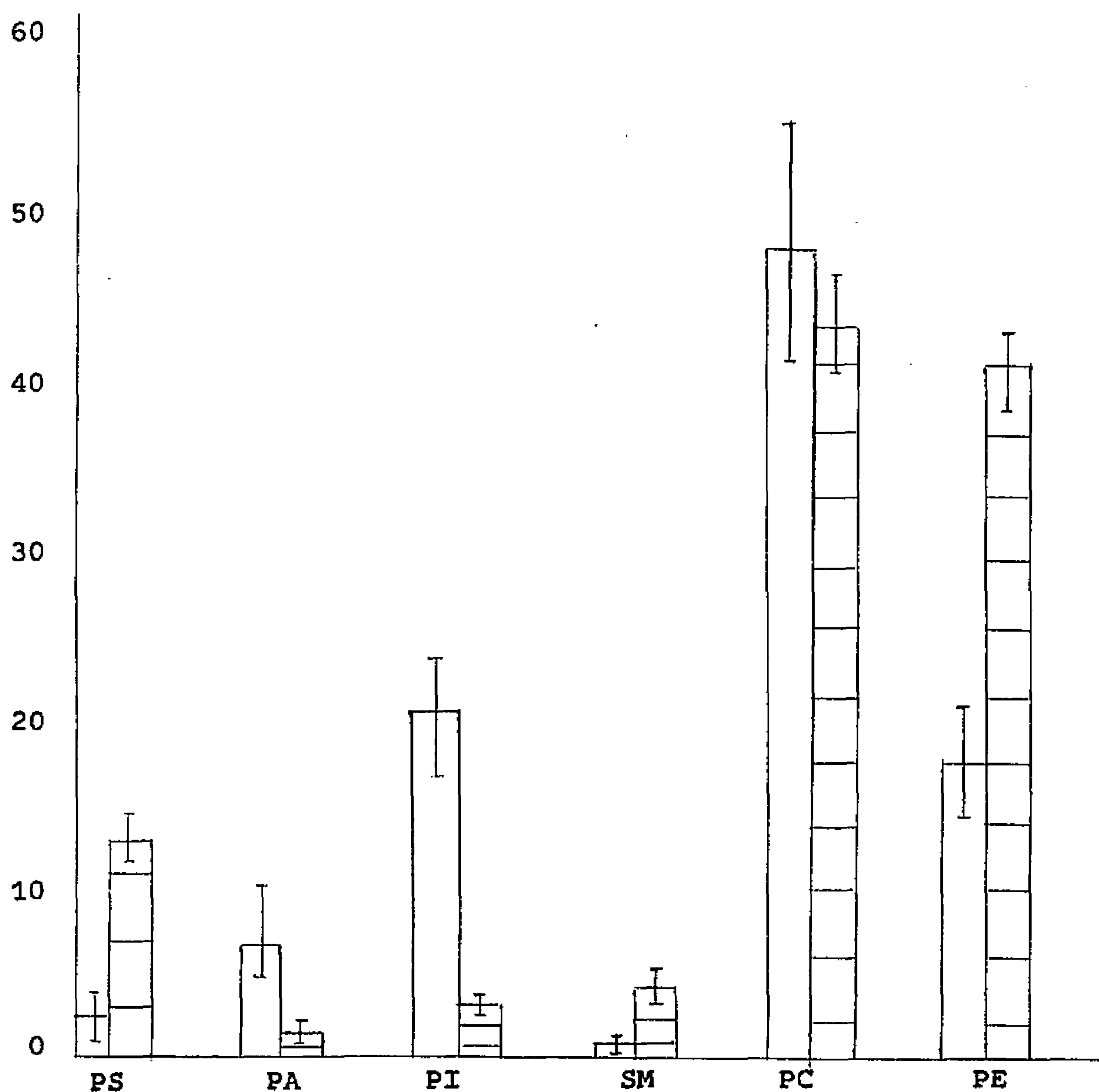
In the TLC system employed, phosphatidylethanolamine and the ethanolamine plasmalogens are not separated. Therefore the specific activity of PE reflects an average of that for phosphatidylethanolamine, a ubiquitous lipid, whose specific activity should probably resemble that of PC; and that of the ethanolamine plasmalogens, a lipid that is most highly concentrated in the myelin, and therefore would be expected to have a lower specific activity.

Table 2. Per cent distribution of phospholipids in mouse brain.

(These values are based on phosphate determinations performed on six mice.)

lipid	PS	PA	PI	SM	PC	PE
percentage distribution	12.5± 0.9	1.9± 0.7	3.1± 0.2	3.7± 0.7	41.5± 2.3	39.1± 2.5

Figure 3. The relative distribution of intracerebrally injected ^{32}P and of lipid-phosphate among the brain lipids. Clear bars represent percentage ^{32}P dpm in total lipid. Striped bars represent percentage lipid-phosphate in total lipid.



In vivo incorporation of (2-^{14}C)acetate, (U-^{14}C)serine, $^{32}PO_4^{\equiv}$, and $^{35}SO_4^{=}$: The autoradiograms of the TLC of brain lipids of mice injected with sodium (2-^{14}C)acetate, (U-^{14}C)serine, and sodium dihydrogen^{32}P-phosphate are shown in figures 4, 5 and 6, respectively. The *in vivo* distribution of these precursors and of $^{35}SO_4^{=}$ among the individual brain lipids is summarized in Table 3. The labelling pattern obtained with (2-^{14}C)acetate agrees well with that reported by Winterbourn (1971). In order to facilitate the comparison of the incorporation patterns of acetate and serine with that of the phosphate, the values of the patterns for the ^{14}C precursors are also presented as per cent of total dpm in phospholipids. The labelling of the lipids with (2-^{14}C) acetate is quite similar to that with the $^{32}PO_4^{\equiv}$. On the other hand, (U-^{14}C)-serine is predominantly incorporated into PS, and to a significant, but much smaller extent, into PE.

The effects of dithioerythritol and of cysteine on the *in vivo* incorporation of (2-^{14}C)acetate into brain lipids: Because cysteine had proved to be a potent inhibitor *in vitro*, it was decided to test its effects in our *in vivo* system. Dithioerythritol (DTE) was coinjected with cysteine to prevent the oxidation of the latter to cystine. It was thus necessary to determine the effect of DTE, a sulfhydryl reagent, on lipid metabolism. Three twenty-one day old mice were used in this experiment. Each was injected with

Figure 4. Autoradiogram of brain lipids of mouse injected with (2-^{14}C)acetate.

The solvent system for the first (vertical) direction is chloroform-methanol-concentrated (28%) aqueous ammonia (65:35:5), and for the second (horizontal) direction chloroform-acetone-methanol-glacial acetic acid-water (5:2:1:1:0.5).

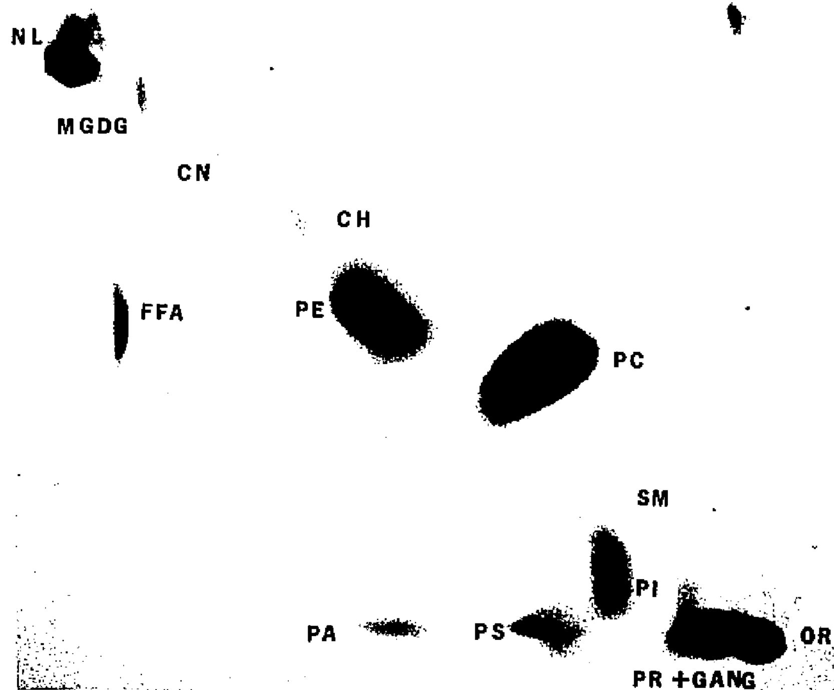


Figure 5. Autoradiogram of brain lipids of mouse injected with (U-^{14}C)serine.

The solvent system for the first (vertical) direction is chloroform-methanol-concentrated (28%) aqueous ammonia (65:35:5), and for the second (horizontal) direction chloroform-acetone-methanol-glacial acetic acid-water (5:2:1:1:0.5).

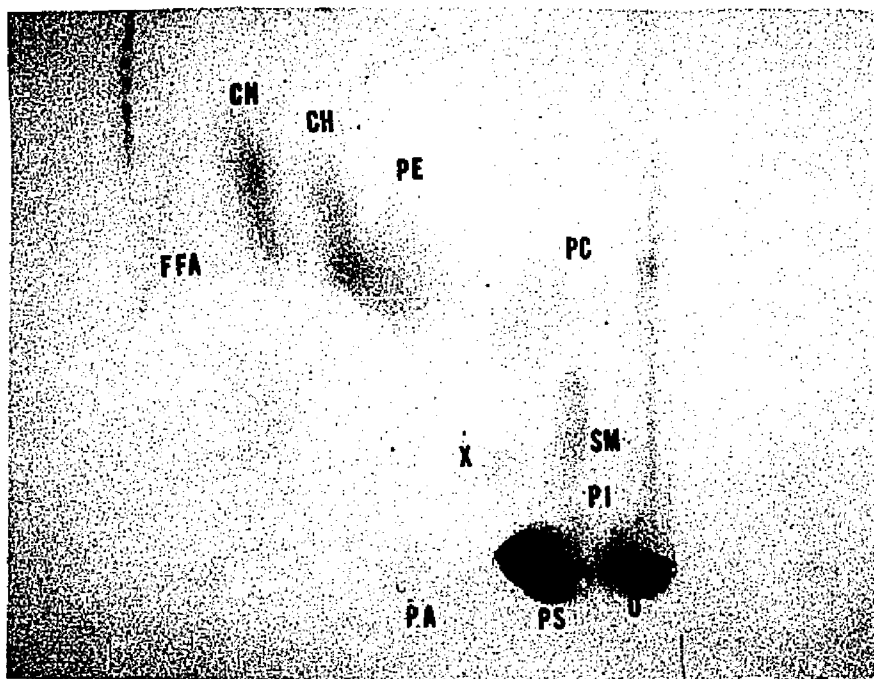
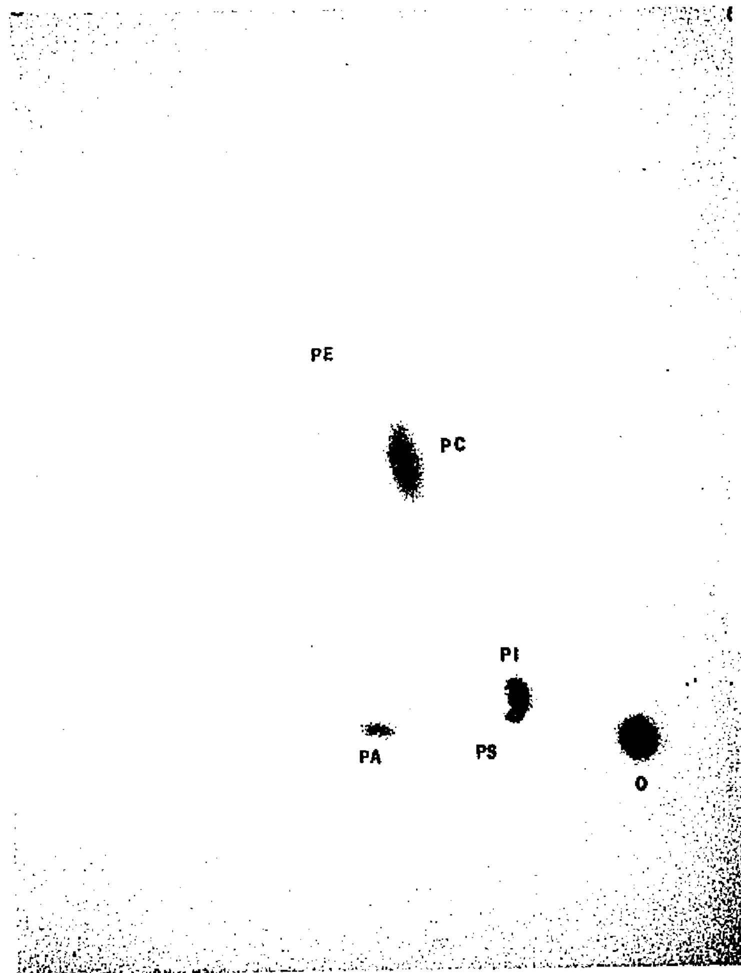


Figure 6. Autoradiogram of brain lipids of mouse injected with $NaH_2{}^{32}PO_4$.

The solvent system for the first (vertical) direction is chloroform-methanol-concentrated (28%) aqueous ammonia (65:35:5), and for the second (horizontal) direction chloroform-acetone-methanol-glacial acetic acid-water (5:2:1:1:0.5).



	$(2\text{-}^{14}C)$-acetate 30 day old [1] [a]	$(U\text{-}^{14}C)$-serine 21 day old mice [4] [b]	$^{32}PO_4^{=}$ 28 day old mice [9]	$^{35}SO_4^{=}$ 21 day old litter mates [3] [d]
PS	1.8(2.5)	65.4±5.0 (73.2)	1.9±0.9	
PI	12.2(16.8)	2.7±1.5 (3.0)	18.5±3.2	
PA	1.9(2.6)	0.5±0.2 (0.6)	8.0±1.9	
SM	0.4(0.6)	4.3±1.0 (4.8)	0.6±0.2	
PC	43.7(46.0)	5.0±1.3 (5.6)	53.9±5.2	
PE	13.6(18.7)	11.4±0.5 (12.8)	15.9±2.2	
CH	2.6	4.4±0.6		
CN		3.0±1.3		
SN	0.5	0.3±0.1		80±10
SH				20± 9
choles-terol	21.2			
FFA	1.8			

Table 3. In vivo incorporation of $(2\text{-}^{14}C)$acetate, $(U\text{-}^{14}C)$-serine, $^{32}PO_4^{=}$, and $^{35}SO_4^{=}$:

Values are given as per cent of dpm in total lipid. Figures in parentheses ( ) denote per cent of dpm in total phospholipids. Numbers in brackets [ ] are number of mice in experiment.

a) Mouse injected with 48 microcuries $(2\text{-}^{14}C)$acetate and sacrificed one hour after injection.

b) Mice injected with 30 microcuries $(U\text{-}^{14}C)$serine, and sacrificed three hours after injection.

c) Mice injected with 12 microcuries $^{32}PO_4^{=}$, and sacrificed three hours after injection.

d) Mice injected with 10 microcuries of $^{35}SO_4^{=}$ (New England Nuclear) and were sacrificed four hours after injection.

48 μc_i of (2-^{14}C)acetate. In addition the first received 4 μl of 0.15N NaCl; the second, 4 μl of a solution containing 30 μg DTE/ μl 0.15N NaCl; and the third, 4 μl of a solution containing 30 μg DTE and 43 μg cysteine/ μl 0.15N NaCl. The concentration of the injected cysteine in the brain was 10mM.* The mice were sacrificed two hours after injection.

The results of the experiment are summarized in Table 4. In the presence of DTE the percentage of labelled carbon falls in SM, PE, and cholesterol, while it rises in PS, PC and FFA. When the effect of injecting DTE and cysteine is compared to that of injecting DTE alone, it is found that the percentage of radioactive carbon found in PS decreases, as it does in SM. In the former the depression might well be attributed to the similarity between cysteine and serine (rather than to the sulfhydryl group), since the effect of the sulfhydryl reagent, DTE, is to enhance the relative amount of ^{14}C in PS. In the latter, however, the effect and the degree of the effect are similar to what could be expected from simply a dosage of DTE that was greater than that used in the control. FFA is the only group showing an increase in its percentage of radioactive carbon. The relative incorporation of ^{14}C into cerebrosides is the same whether DTE is injected alone or whether it is coinjected with cysteine.

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* In a preliminary experiment the volume of the mouse brain was found to be 0.3ml.

	Mouse I	Mouse II	Mouse III
PS	3.7	4.7	3.2
SM	3.2	1.2	0.5
PC	45.6	56.3	56.0
PE	14.7	5.1	4.9
cer		1.5	1.4
chol	24.9	16.2	16.8
FFA	1.2	3.3	5.0

Table 4. The effect of DTE and cysteine on the *in vivo* incorporation of $(2-^{14}C)$acetate into brain lipids.

Values are given as per cent dpm in total lipid. Each of the three mice was injected with 48 μc_i of $(2-^{14}C)$acetate. In addition Mouse I received 4 µl of 0.15N NaCl, Mouse II - 4 µl of a solution containing 30 µg DTE/ µl 0.15N NaCl, and Mouse III - 4 µl of a solution containing 30 µg DTE and 43 µg cysteine/ µl 0.15N NaCl. The mice were sacrificed 2 hours after injection.

The effects of DTE and of cysteine on the *in vivo* incorporation of (U-^{14}C)serine into brain lipids: Three groups of mice were treated in the following manner: Each mouse in each group was injected with 30 μc_i of (U-^{14}C)serine. In addition, those in group one, the control, received 4 μl of 0.15N NaCl, group II mice received 4 μl of a solution containing 30 μg DTE/ μl 0.15N NaCl, and the animals of group III received a solution containing 30 μg DTE and 43 μg cysteine/ μl 0.15N NaCl. Mice were sacrificed two hours after injection. The results are summarized in Table 5.

The trend seen with DTE in this experiment is comparable to that in the (2-^{14}C)acetate experiment. As in the latter case, the relative incorporation of ^{14}C increases slightly in PS, while it decreases in SM and PE. With regard to PC one cannot draw any definite conclusion; the incorporation of serine into PC is so small, that any small differences caused by experimental technique represent substantial relative differences. In addition to the above changes, it appears that mice treated with DTE have a lower percentage of ^{14}C-serine incorporated into cerebrosides.

As in the previous experiment, mice treated with DTE and cysteine have a smaller percentage of their ^{14}C in PS than do mice treated with DTE alone. Also there is no significant difference between the two groups of animals in their relative incorporation of ^{14}C into cerebrosides.

	Group I [4]	Group II [2]	Group III [2]
PS	65.4±5.0	71.2±6.2	48.2±5.3
SM	4.3±1.0	1.7±0.4	2.0±0.4
PC	5.0±1.3	3.7±0.5	4.7±1.2
PE	11.4±0.5	8.3±0.04	7.1±0.8
cerebrosides	7.4±1.9	4.1±0.4	4.6±0.4

Table 5. The effect of DTE and cysteine on the *in vivo* incorporation of (U-^{14}C)serine into brain lipids.

Values are given as per cent dpm in total lipid. Each mouse received 30 μc_i of (U-^{14}C)serine. In addition, Group I, the controls, received 4 µl of 0.15N NaCl, Group II mice received 4 µl of a solution containing 30 µg DTE/ µl 0.15N NaCl, and Group III mice received 4 µl of a solution containing 30 µg DTE and 43 µg cysteine/ µl 0.15N NaCl. Mice were sacrificed two hours after injection.

Effect of molybdate on the *in vivo* incorporation into brain lipids of (2-^{14}C)acetate, (U-^{14}C)serine, $^{32}PO_4^{\equiv}$, and $^{35}SO_4^{=}$:

(1) Effect on the incorporation of (2-^{14}C)acetate: The effect of intracerebrally injected molybdate on the incorporation of (2-^{14}C)acetate into brain lipids of 30 day old mice is summarized in Table 6. The concentration of the injected molybdate in the brain was 1.0mM in mouse B and 2.0mM in mouse C.

In the presence of $MoO_4^{=}$ the percentage of total radioactive carbon rises in PI, PA, cholesterol and sulfatides, while it falls in PC, PE, and cerebrosides.

(2) Effect on the incorporation on (U-^{14}C)serine: Thirty microcuries of (U-^{14}C)serine were coinjected with sodium molybdate into twenty-one day old littermates. The resulting concentration of molybdate in the brain was 2.0mM. The control group was similarly treated, except that each of its mice received 0.15N NaCl instead of the molybdate. The animals were sacrificed three hours after injection, and the brains of each group were pooled. The results of the experiment are summarized in Table 7. The percentage of labelled PI and sulfatides rose, while that of SM, CH (cerebrosides with hydroxy fatty acids), and CN (cerebrosides with normal fatty acids) decreased.

During the course of this project it became evident that changes in the label-distribution patterns may result from any of a number of possibilities. For

	Mouse A	Mouse B	Mouse C
PS	1.8	2.2	2.0
PI	12.2	14.6	17.9
PA	1.9	1.9	2.5
PC	43.7	43.0	31.6
PE	13.6	9.9	11.1
cer.	2.6	1.4	.9
chol	21.2	24.4	31.0
Su	0.5	0.7	2.2

Table 6. Effect of molybdate on the incorporation of ($2-^{14}C$) acetate into mouse brain lipids.

Values are given as per cent dpm in total lipid. The mice received the following injections: Mouse A - 4 µl 0.15N NaCl and 48 μc_i ($2-^{14}C$)acetate; Mouse B - 4 µl 0.075M $Na_2MoO_4 \cdot 2H_2O$ and 48 μc_i ($2-^{14}C$)acetate; Mouse C - 4 µl 0.15M $Na_2MoO_4 \cdot 2H_2O$ and 48 μc_i ($2-^{14}C$)acetate.

	Control 4	Experimental 3
PS	65.4 ± 5.0	69.8 ± 4.3
PA	0.5 ± 0.2	0.6 ± 0.1
PI	2.7 ± 1.5	4.6 ± 0.2
SM	4.3 ± 1.0	2.2 ± 0.5
PC	5.0 ± 1.3	5.2 ± 0.8
PE	11.4 ± 0.5	11.1 ± 0.4
CH	4.4 ± 0.6	1.7 ± 0.5
CN	3.0 ± 1.3	1.3 ± 0.3
SN SH	0.3 ± 0.1	0.6 ± 0.1

Table 7. Effect of molybdate on the *in vivo* incorporation of (U-^{14}C)serine into brain lipids.

Values are given as per cent dpm in total lipid. Control mice received intracerebral injections of 6 µl (U-^{14}C)-serine (30 μc_i) and 4 µl of 0.15N NaCl. The experimental group received 6 µl of (U-^{14}C)serine (30 μc_i) and 4 µl of 0.15M $Na_2MoO_4 \cdot 2H_2O$. Mice were sacrificed 3 hours after injection.

example, in the experiment described above, the changes may have been caused by an increase in the biosynthetic rates of PI and sulfatides, or from a depressed rate of the incorporation of (U-^{14}C)serine into SM, CH, and CN; or finally they may have resulted from any combination of these possibilities.

One way to clarify the exact nature of the effect of an injected compound is to report the data in terms of specific activities. Therefore, in subsequent experiments results for each phospholipid are reported as dpm/microgram lipid-phosphate.

(3) Effect on the incorporation of $^{32}PO_4^{\equiv}$ and $^{35}SO_4^{=}$: Twenty-one day old male littermates were divided into three groups, I, II, and III, each group consisting of three mice. Group I mice, the controls, received intracerebral coinjections of 6 microcuries of $NaH_2{}^{32}PO_4$, 10 microcuries $Na_2{}^{35}SO_4$ (New England Nuclear) and 4 µl 0.15N NaCl. Groups II and III were similarly treated, except that in place of the NaCl, animals of Group II received 4 µl of 0.6M Na_2MoO_4 (8mM with respect to the brain), and those of Group III received 4 µl of 2.50M Na_2MoO_4 (33.3mM with respect to the volume of the brain); (at this higher concentration the mice seemed to be physically ill). Mice were sacrificed 3.5 hours after the injection.

The results of the experiment are summarized in Table 8. Table 8A shows that the only differences in the

Table 8. Effect of molybdate on the *in vivo* incorporation of $^{32}PO_4^{\equiv}$ and $^{35}SO_4^{=}$ into mouse brain lipids.

Each mouse of Group I was injected with 6 μc_i of $^{32}PO_4^{\equiv}$, 10 μc_i of $^{35}SO_4^{=}$ and 4 µl of 0.15N NaCl. Groups II and III were similarly treated, except that in place of the NaCl animals of group II received 4 µl of 0.60M $Na_2MoO_4 \cdot 2H_2O$ (8mM with respect to the volume of the brain), and those of Group III received 4 µl of 2.50M $Na_2MoO_4 \cdot 2H_2O$ (33.3mM with respect to the volume of the brain). Mice were sacrificed 3.5 hours after injection.

Table 8A reports results as percentage dpm in total phospholipids. The results of Table 8B are specific activities, i.e., dpm/ µg phosphate for each compound.* Table 8C reports the results as percentage specific activity of the specific activity of the control.

A	Group I [3]	Group II [3]	Group III [3]
PS	3.3 ± 0.5	1.5 ± .2	3.4 ± 1.0
PA	5.1 ± 0.9	6.6 ± 1.2	18.8 ± 0.3
PI	19.8 ± 1.1	24.3 ± 2.2	39.5 ± 5.8
PC	43.4 ± 0.2	40.1 ± 2.1	19.6 ± 2.8
PE	21.5 ± 1.1	18.3 ± 0.7	12.0 ± 2.8
Sulfatide	26.5 ± 0.6	34.5 ± 3.4	47.9 ± 8

B	Group I	Group II	Group III
Sulfatide	108	100	26
PS	78.0	27	15
PA	2126	1220	2125
PI	2684	2291	601
SM	51	30	--
PC	404	213	30
PE	175	156	14

C	Group I (%)	Group II (%)	Group III (%)
PS	100	34.2	19.4
PA	100	57.4	100
PI	100	85.4	22.4
SM	100	58.2	--
PC	100	52.8	7.5
PE	100	89	7.8
Sulfatide	100	93	24

* Sulfatide results expressed as dpm sulfatide/total µg phospholipid-phosphate on the TLC plate.

distribution of the labelled phosphate between Groups I and II are in PS (a decrease in Group II) and in PI (an increase). The relative incorporation of $^{35}SO_4^{=}$ into sulfatides is higher in Group II. However, the _biosynthetic_ _rates_ of _all_ the phospholipids _have_ _decreased_ (Tables 8 Band 8C), with that of PA, SM, and PC being depressed by similar degrees, but that of PS being inhibited to a greater degree, and of PI to a lesser degree. Sulfatide formation appears unaffected.

The pattern of distribution of $^{32}PO_4^{=}$ in Group III is dramatically different from that of the control. The actual biosynthesis has been almost completely inhibited in PC and PE (92% depression; Table C) and substantially decreased in the remaining lipids. Surprisingly, the specific activity of PA has returned to the level of the control.

The effect of dimethylaminoethanol on the in vivo incorporation of $NaH_2{}^{32}PO_4$ into brain lipids: Thirty day old mice were intracerebrally injected with 16 microcuries of NaH_2-$^{32}PO_4$. In addition each of the control mice received two microliters of 0.15N NaCl, and each of the experimental animals received two microliters of DMAE (i.e., 1.88 mg.). The concentration of DMAE in the brain was 0.07M. This massive dosage caused cerebral hemhorraging. However, it is of interest to note that the biosynthesis of PI, usually characterized by a high incorporation of ^{32}P, was

completely inhibited.

The effect of a lower dose of DMAE was also tested. Mice were injected with 2 μc_i of $NaH_2{}^{32}PO_4$. In addition each of the control group received 2 μl of 0.15N NaCl, and each of the experimental group received 3.33 μg of DMAE (a concentration of 117μM in the brain).

The results are summarized in Table 9. The formation of choline-containing lipids was not specifically inhibited. Rather there was an <u>overall</u> depression of phospholipid biosynthesis; the formation of PS, SM, PC, and PE were inhibited by similar degrees (37 - 57% inhibition), while that of PA and PI were not affected as severely (16 - 30% depression).

To be certain that none of the compounds studied contained dimethylaminoethanol (or any metabolites therefrom), an experiment was conducted in which twenty-eight day old mice were coinjected with 2 μc_i of $NaH_2{}^{32}PO_4$ and 0.15 μc_i of (1,2-^{14}C) DMAE [which was equivalent to 3.6 μg of (1,2-^{14}C) DMAE (New England Nuclear)] , and sacrificed three hours after injection. However, there were no ^{14}C counts in the lipids, nor were there any in the products of the acid hydrolysis of the phospholipids.

<u>The effect of gamma-hexachlorocyclohexane on in vivo phospholipid metabolism</u>: Twenty-nine mg. of γ-HCC (Hooker, N. Y.) were ultrasonicated in 1.0 ml of water, and 6 μl. of

A	Control [9]	Experimental [5]
	% of dpm	in total lipid
PS	2.4 ± 0.8	2.9 ± 1.1
PA	9.5 ± 4.1	10.6 ± 0.5
PI	20.9 ± 3.7	26.9 ± 5.4
SM	0.8 ± 0.3	1.3 ± 0.5
PC	46.0 ± 6.9	39.1 ± 4.2
PE	17.9 ± 2.4	17.7 ± 5.7

B	Control [2]	Experimental [2]	
	dpm/ µg phosphate	dpm/µg phosphate	% change resulting from DMAE
PS	111 ± 22	56 ± 4	- 50%
PA	2693 ± 246	2256 ± 169	- 16%
PI	5090 ± 790	3578 ± 752	- 30%
SM	150 ± 47	65 ± 13	- 57%
PC	265 ± 29	166 ± 21	- 37%
PE	127 ± 14	70 ± 16	- 45%

Table 9. The effect of a mild dose of DMAE on the *in vivo* incorporation of $^{32}PO_4^{\equiv}$ into brain lipids.

Twenty-eight day old mice were injected with 2 μc_i of NaH_2-$^{32}PO_4$. In addition, the controls each received 2 µl of 0.15N NaCl, and the experimental mice each received 3.33 µg DMAE (a concentration of 117µM in the brain). Mice were sacrificed 3 hours after injection.

the resulting solution were intracerebrally injected into each of four twenty-one day old mice. The final concentration of the compound in the brain was 2mM. The results are summarized in Table 10. Surprisingly, HCC-treated mice showed a slight increase in the incorporation of ^{32}P into PI. The specific activities of PS and PE are reduced by 60% and 42%, respectively, while that of PC shows an increase of 55%.

The effect of 3,4-dihydroxybutyl-1-phosphonate (3,4-DBP) on *in vivo* phospholipid biosynthesis: Forty-one day old mice were intracerebrally injected with two microcuries of $NaH_2{}^{32}PO_4$. In addition, the injection solution of the controls contained 10 microliters of 0.15N NaCl, while that of the experimental mice contained 10 microliters of a solution of 16.1 µg 3,4-DBP/ µl (the concentration of 3,4-DBP in the brain was 2.5mM). The brain of each mouse was treated individually for determining the specific activities of the phospholipids. The results shown in Table 11 indicate a general increase in the specific activities of the phospholipids. However, the results for the effect on PA and SM were inconsistent.

The large average mean deviations in the experimental group may be attributed to the different resulting concentrations of 3,4-DBP in the individual mouse brains. The concentration is a function of the size of the brain, and the amount of the injected compound

	Specific activities (dpm/µg P)		% change caused by HCC
	Control mice	HCC-treated mice	
PS	319	129	-60%
PI	5804	6900	+19%
PC	1512	2350	+55%
PE	822	474	-42%

Table 10. The effect of *gamma*-hexachlorocyclohexane (γ-HCC) on *in vivo* phospholipid formation.

Each group, the control and the HCC-treated mice, consisted of four twenty-one day old males. Each mouse was injected with 12 microcuries of $NaH_2{}^{32}PO_4$; in addition, the controls were injected with 6 microliters of 0.15N NaCl, and the experimentals with 6 microliters of a suspension of 29 µg of HCC/ µl. (Thus, the experimentals had a final concentration of 2mM HCC in the brain.) Mice were sacrificed three hours after injection, and the brains of each group were pooled.

	Control (5)	Experimental (5)	
	dpm/ μg P	dpm/ μg P	change caused by 3,4-DBP
PS	95 ± 26	123 ± 25	+ 29%
PI	2252 ± 269	3176 ±1261	+ 41%
PC	284 ± 28	397 ± 142	+ 40%
PE	186 ± 25	272 ± 87	+ 46%

Table 11. The effect of 3,4-dihydroxybutyl-1-phosphonate (3,4-DBP) on *in vivo* phospholipid biosynthesis.

Forty-one day old mice were intracerebrally injected with two microcuries of $NaH_2{}^{32}PO_4$. In addition, the injection solution of the controls contained ten microliters of 0.15N NaCl, while that of the experimental mice contained ten microliters of a solution of 16.1 micrograms 3,4-DBP/microliter. (The concentration of 3,4-DBP in the brain was 2.5mM.) The brain of each mouse was treated individually for determining the specific activities of the phospholipids. The values are reported as dpm/microgram phosphate ± a.m.d.

retained by the brain. The weight of the lipids extracted by the chloroform-methanol (2:1) homogenization of the brains varied from 21 mg to 36 mg, and the amount of leakage of injected material varied from 10% - 30%. The resulting difference in the concentration of 3,4-DBP compounded by the possibility of differing threshold dosages from one mouse to another could very well account for the large average mean deviations of the specific activities of the phospholipids reported for the experimental group.

Discussion

The blood-brain barrier and the choice of injection technique: Over seventy years ago it was observed that the brain did not stain with intravenously injected vital dyes, and that it had a remarkable resistance to bacteria and toxins circulating in the blood (Bakay, 1957). These observations led to the belief that there was a powerful mechanism protecting the brain. Studies with ^{74}As, ^{76}As, ^{82}Br, ^{14}C, ^{64}Cu, ^{38}Cl, ^{131}I, ^{42}K, ^{24}Na, ^{32}P, ^{35}S, and ^{136}Zn indicate that the uptake of these isotopes from the plasma by the central nervous system (CNS) is markedly slower than the uptake of the same ions by other organs. In contrast, injections of ^{32}P into cerebrospinal fluid results in a rapid absorption of the tracer by the brain. Once in the nervous tissue, substances stay there much longer than in any other organ, indicating that perhaps the blood-brain barrier acts as an obstacle in both directions, i.e., the uptake into, and the release from the brain.

Although the site and nature of the blood-brain barrier is problematical, it has been hypothesized that the barrier is due to a layer of glia membranes, enmeshed in mucopolysaccharides, which apparently surrounds the cerebral capillaries (Brierley, 1957).

Only a few substances do permeate the barrier easily. In addition, *certain* parts of the CNS incorporate large amounts of various substances from the circulating blood. This may account for the contradictory findings dealing with the biochemical activity of the myelin membrane. Intraperitoneal injections of lipid precursors indicate that the turnover of myelin lipids is extremely slow. However, intracerebral injections of the same precursors show a greater turnover (Ansell, 1972).

Because it was the goal of this project to study and to attempt to manipulate lipid metabolism of the brain, and in light of the characteristics of the blood-brain barrier described above, all injections were administered intracerebrally.

In vivo labelling patterns of brain lipids obtained with (2-^{14}C)acetate, (U-^{14}C)serine, and $^{32}PO_4^{\equiv}$: The similarity between the distribution patterns of (2-^{14}C)acetate and $^{32}PO_4^{\equiv}$ among the brain phospholipids (Table 3) indicates that transacylation probably does not occur in three hours to a very significant degree *in vivo*; rather the incorporation of both precursors appears to reflect *de novo* synthesis of these phospholipids.

(U-^{14}C)Serine is useful in studying the turnover of PS, PE, SM, and cerebrosides. As can be seen (Table 3) PS accounts for about 65% of the total lipid radioactivity incorporated from intracerebrally injected (U-^{14}C)serine.

PE, having the second highest relative incorporation, has approximately twice as much label as PC. Since the *de novo* synthesis of PC is higher than that of PE (as can be seen from the greater incorporation into PC of labelled acetate and phosphate) it seems that it is the ethanolamine moiety of PE that accounts for most of its radioactivity. Thus, in this system, decarboxylation of serine seems to occur to a significant extent.

The advantage in using serine to study the turnover of SM and cerebrosides is that the relative incorporation of serine into these sphingolipids is greater than that of acetate or phosphate.

In vivo inhibition studies with dimethylaminoethanol: DMAE was considered to be a potential inhibitor of PC biosynthesis since (1) it serves as a substrate for choline phosphokinase in yeast (Wittenberg and Kornberg, 1953), and (2) PC in brain is not formed by stepwise methylation of PE (Ansell and Spanner, 1971). However, the results indicate (Table 9) that the inhibitory effect of DMAE is not limited to the biosynthesis of PC. The fact that DMAE depresses the formation of PS and PE as well as that of PC, and that the effect in all three cases is of similar magnitude is especially interesting in light of Kanfer's suggestion that there is a single protein responsible for the *direct* incorporation of serine, choline, and ethanolamine into their respective phospholipids

(Kanfer, 1972). In Kanfer's particulate system of rat brain, he notes a number of similarities in the incorporation of these bases. The uptake of all three are: inhibited by EDTA, stimulated by Ca^{2+} and isoserine; in addition, the bases are competitive inhibitors. Kanfer also tested the effect of 2.5mM DMAE on his system, and found that the incorporation of the three bases was inhibited, although by different degrees. (The uptake of choline was depressed by 87%, that of serine by 70%, and of ethanolamine by only 15%).

It is not surprising that the *in vivo* biosynthesis of sphingomyelin is also severely affected by DMAE, as CDP-choline is a precursor in its biosynthetic route (Fujino and Negishe, 1968; Figure 7B). On the other hand, there was a relatively small effect on the metabolism of PA and PI, both of which are independent of the cytidine pathway (Figure 7A).

In vivo inhibition studies with molybdate: In light of the well documented inhibitory effect of molybdate on sulfatide formation *in vitro* (Pasternak, 1962; Wilson and Bandurski, 1958), it was especially attractive to test its effects *in vivo*; for if it were demonstrated that molybdate specifically inhibits the *in vivo* biosynthesis of sulfatides in the brain, we might then have a tool for actually manipulating myelin formation--as sulfatides are almost entirely localized in this membrane (Table 1). It was

also clear, however, that molybdate might inhibit the formation of phospholipids from $^{32}PO_4^{\equiv}$.

The data of Tables 8B and 8C shows that when molybdate is intracerebrally injected so that its concentration in the brain is 8mM, there is a significant drop in phospholipid formation, while the change in sulfatide biosynthesis seems negligible.

At a higher concentration of molybdate, the inhibition of phospholipid biosynthesis is greater, and sulfatide formation is also depressed significantly. PA stands out as an exception in this pattern. Its specific activity in the presence of 33.3mM molybdate equals that of the control.

The effect on the phospholipids may involve formation of a complex between molybdate and phosphate (which is the basis of quantitative phosphate analyses). However, since molybdate reacts with so many different compounds (such as oxalic, lactic, and tartaric acids; sugars, polyalcohols, and phosphate (Killefer and Linz, 1954)) it would be extremely difficult to propose a mechanism that could account for its *in vivo* effects on lipid formation in mouse brain.

Metabolic changes in brain phospholipids caused by γ-hexachlorocyclohexane: Much research has been involved with the elucidation of the metabolism and of the functional aspects of PI. It has been proposed that the

metabolic activity of PI is associated with the control of axonal and synaptosomal permeability (Friedel and Schanberg, 1971). This control may be mediated through acetylcholine (ACh) or other neurotransmitters, which activate the hydrolysis of PI by a phosphodiesterase, thus depolarizing the membrane and permitting neuronal transmission (Durell and Garland, 1969).

In light of the important postulated role ascribed to PI, it was of great interest to learn of the inhibition by _γ_-HCC of (1) PI turnover in lymphocytes exposed to phytohemagglutinin (PHA) (Fisher and Mueller, 1971), and of (2) ACh-stimulated PI synthesis in cerebral cortex slices (Hokin and Brown, 1969). As PA and PC turnover are not affected in the latter system, it seems that the inhibition by _γ_-HCC is specific for PI formation. On the other hand, _δ_-HCC, having the "myo" configuration, depresses the formation of PA and PC as well as PI, while the _α_ and _β_ configurations have no effect on phospholipid biosynthesis.

The inhibitory effect of _γ_-HCC was apparent only in the presence of a stimulant (i.e., PHA or ACh). When no ACh was added the phospholipid turnover in brain slices treated with _γ_-HCC was similar to that of the controls. However, in mouse brain much of the _in vivo_ incorporation of ^{32}P into PI might be due to such stimulation, as the environment provides a constant source

of stimulation for live animals. The effect of intracerebrally injected γ-HCC on phospholipid biosynthesis was therefore studied. Once again, the data of Table 10 shows that the manner in which a compound will act *in vivo* cannot be predicted with much certainty from its actions *in vitro*. The incorporation of ^{32}P into PI is not depressed by γ-HCC; to the contrary, a slight activation is indicated. It also activates the incorporation of ^{32}P into PC, while it depresses the turnover of PE and PS.

It seems that the effects of γ-HCC may not be caused by its structural similarity to inositol.

Changes in phospholipid metabolism caused by 3,4-dihydroxybutyl-1-phosphonate: Investigations by Rosenthal and Pousada (1966) of the effects of a phosphonate-containing analog of PA (2-hexadecoxy-3-octadecoxypropylphosphonate) on phosphatidate phosphohydrolase yielded grossly inconsistent results. In a number of experiments some inhibition was observed, while in others some degree of activation was observed. When the substrate was added after a thirty-minute preincubation of the enzyme with the analog, the gross inconsistencies were eliminated; yet it was difficult to reproduce very closely the degree of inhibition from one experiment to another.

On the other hand, the inhibition by phosphonate-containing analogs of lecithin (Rosenthal and Pousada, 1968) of *Clostridium perfringens* phospholipase C was consistent

and conclusive whether or not the enzyme was preincubated with the analog before the substrate was added.

If one assumes that the *in vivo* system used in this project, (1) 3,4-DBP is acylated and (2) that the bases are incorporated to form the phosphonyl analogs of the normally occuring brain phospholipids, then if phospholipid hydrolysis were inhibited by these analogs, one should see an increase in the specific activities of these phospholipids. The enhancement would be explained as follows: Upon injecting $^{32}PO_4^{\equiv}$ into the brain there is a very high turnover of (^{32}P)phospholipids until an equilibrium is reached with the acid-phosphate pool. However, if the phosphonyl analogs formed from 3,4-DBP inhibit phospholipase C, then the ^{32}P-phospholipids would accumulate, resulting in higher specific activities.

The procedure used for this study, i.e., coinjecting the analog with the radioactive precursor, may be considered analogous to the studies of Rosenthal and Pousada (1966, 1968), where their phosphonyl analogs were coincubated with the enzyme and substrate. In their system the effects upon the hydrolysis of PA were grossly inconsistent (1966), while those of PC were conclusive (1968). Similarly, in this study the effect of the phosphonyl analog on PA metabolism is unclear, while the results with the other glycerophosphatides do suggest an increase in specific activities. In both the *in vivo* and *in vitro* cases, PA, as a precursor of other

glycerophosphatides, probably reaches equilibrium with the acid-phosphate pool before the other phospholipids. This rapidity may account for the necessity of preincubating the enzyme with the analog in order to eliminate the gross inconsitencies otherwise characteristic of PA.

Throughout this experiment, the mice were treated individually, and the specific activities were calculated for each brain. The results in Table 11 demonstrate that although animals within a group were treated as similarly as is technically feasible, the specific activities of the phospholipids varied from mouse to mouse. In the control group, the relative mean deviations ranged from ten to twenty-seven per cent (these values are based on determinations performed on five mice, an admittedly small, statistical sample); therefore, changes in average specific activities resulting from an injection of 3,4-DBP were considered significant if the difference from the average control value was approximately thirty per cent or more.

The relative mean deviations in the experimental group ranged from twenty to forty per cent. These high values indicate that the number of variables affecting lipid metabolism increase by injecting an inhibitor. Firstly, the degree of the effect of the inhibitor varies from one animal to another. Secondly, because of the limitations of the experimental technique, the amount of leakage of the injection solution is different for each animal. Thus the relative mean deviation rises as one increases the number of factors influencing lipid metabolism.

Summary

A procedure has been established for investigating the effects of potential inhibitors on *in vivo* lipid biosynthesis in mouse brain. It was formulated by adopting different, applicable techniques that had been employed in independent investigations. Thus, it includes methods for intracerebral injections, isolation and separation of brain lipids, and determining the specific activities of these lipids.

Following this procedure, the normal incorporation patterns of several radioactive precursors into brain lipids of the mouse have been established, and have subsequently been altered by intracerebral injections of potential inhibitors of lipid formation. The entire study was conducted *in vivo*, so that the results yielded might ultimately be applied to investigations of the functional roles lipids play in behavioral manifestations (for example, learning and memory).

Many of the compounds tested had already been investigated *in vitro*. However, the results of this project demonstrate that *in vitro* metabolic changes caused by a particular inhibitor do not necessarily occur *in vivo*; and if they do occur, they are usually accompanied by other, unreported changes. For example, it has been reported that *in vitro*, molybdate inhibits sulfatide

formation. But when the concentration of injected molybdate is comparable to that used in the *in vitro* studies, sulfatide formation is barely affected; rather, phosphatide formation is depressed. Only at higher dosages of molybdate is sulfatide formation significantly inhibited.

Another example of the possible inconsistencies between the effects a compound produces *in vitro* and its effects *in vivo* is illustrated by studies with *gamma*-hexachlorocyclohexane. Although this substance inhibits the formation of phosphatidylinositol *in vitro*, the results of this project indicate an enhancement of PI synthesis by γ-HCC *in vivo*.

The *in vivo* effects of dimethylaminoethanol are comparable to those observed *in vitro*. In both cases the biosynthesis of PS, PC, and PE are depressed. In addition, the degree of inhibition of sphingomyelin is also significant. The inhibitory effect is much smaller on phosphatidic acid and phosphatidylinositol metabolism.

The apparent *in vivo* enhancement of phospholipid formation in the presence of 3,4-dihydroxybutyl-1-phosphonate is consistent with the reported *in vitro* inhibitory effect on phospholipase C by phosphonate-containing analogs of phospholipids.

A diagrammatic summary of the effects of the compounds investigated is presented in figure 8.

Continuing the investigation of the effects of other potential inhibitors of lipid biosynthesis may provide interesting topics for future research. For example, electron microscopic studies of the effects of these inhibitors on membranes may prove fruitful. Furthermore, this area of study can be approached from an anatomical point of view, studying each section of the brain individually and determining which part is most affected by the inhibitors. Lastly, it may prove rewarding to study the behavioral effects that accompany the biochemical and morphological changes induced by the inhibitors.

Figure 7A: Summary of the biosynthetic routes of the major glycerophosphatides of the brain.

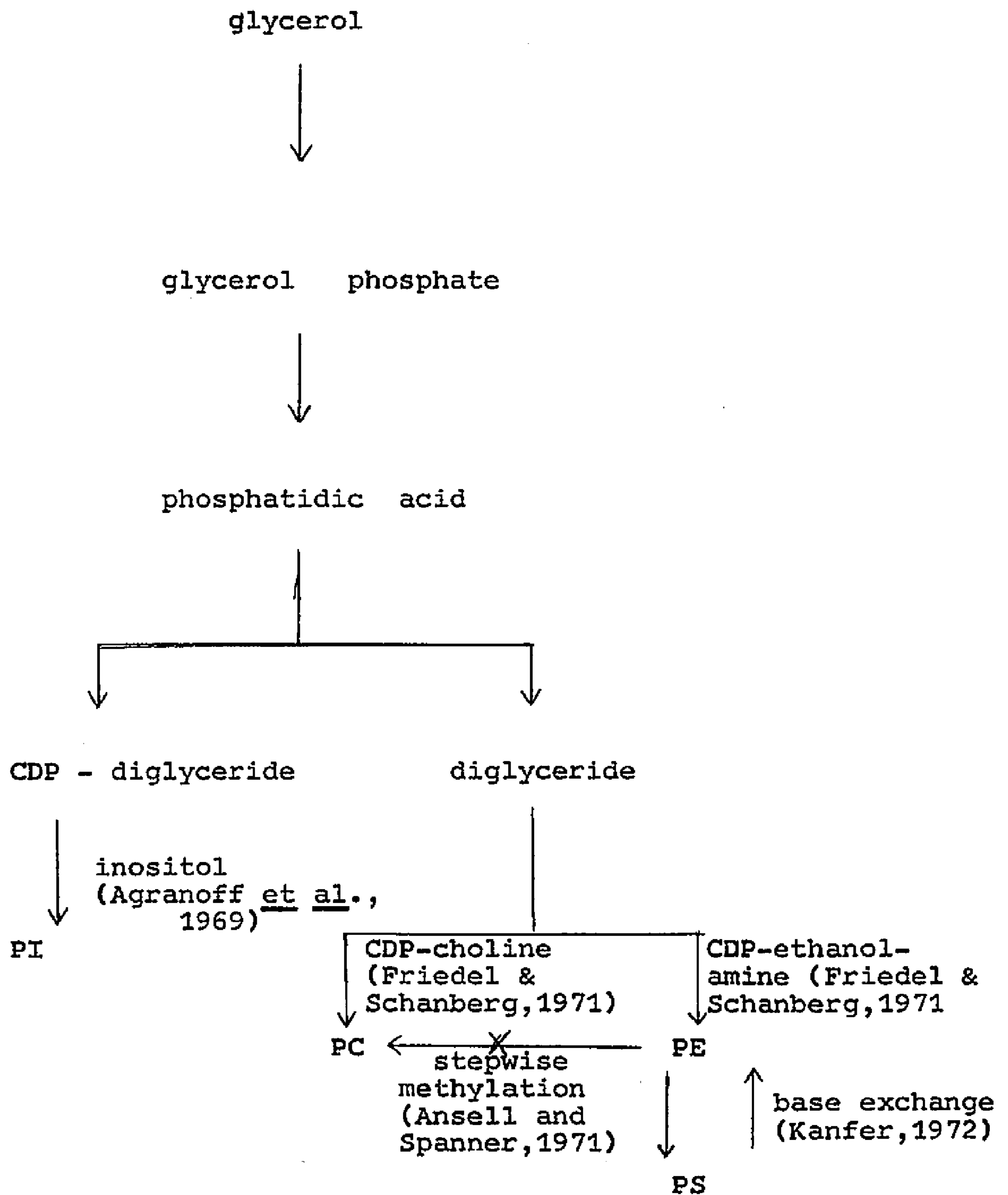

Figure 7B: Summary of the biosynthetic routes of the major sphingolipids of the brain.

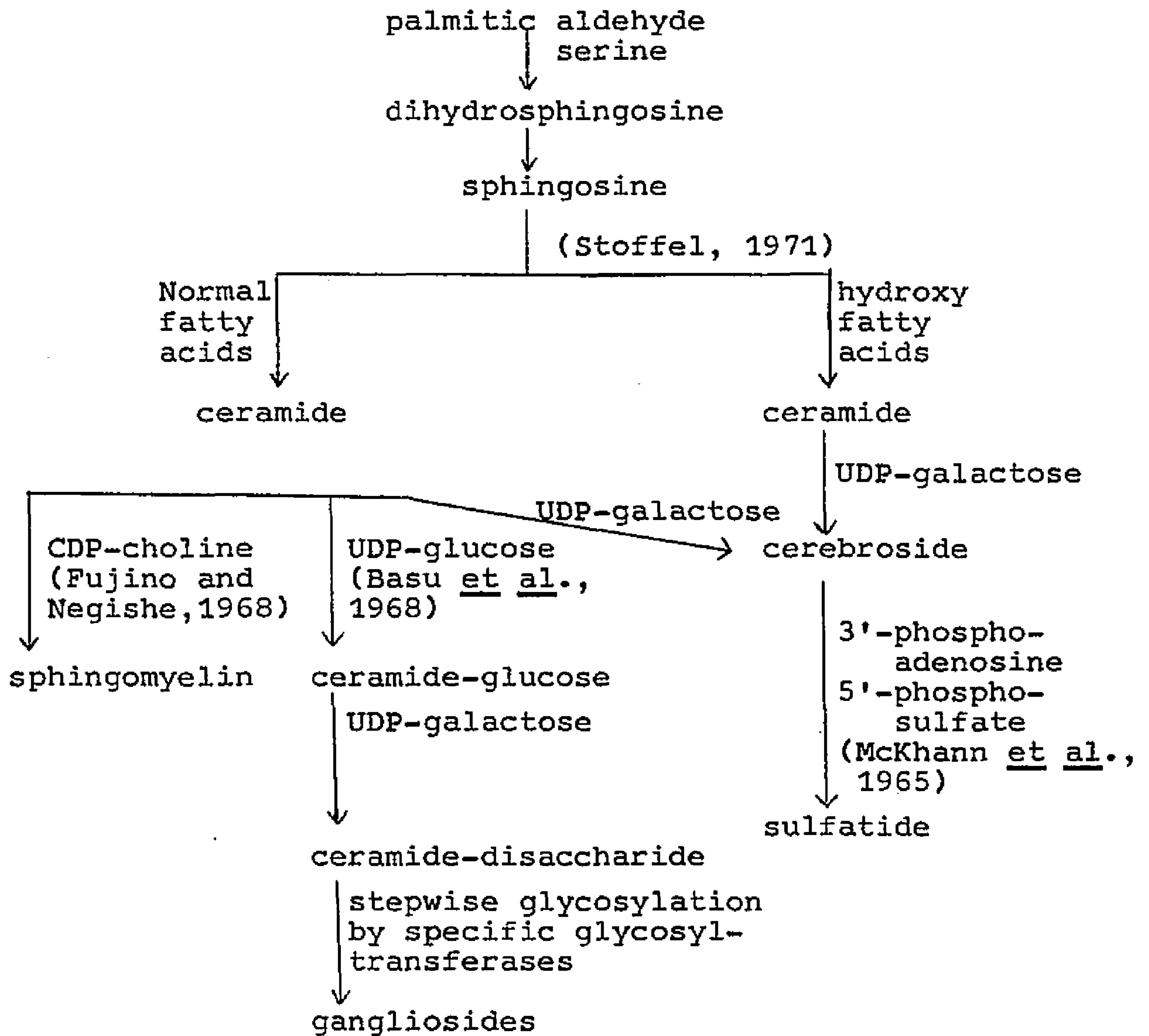

Figure 8: Schematic summary of the effects of different compounds on *in vivo* phospholipid metabolism.

Legend:

+ change (%)	Symbol	- change (%)	Symbol
0 - 14	—	0 - 14	—
15 - 30	$\uparrow$	15 - 30	$\downarrow$
31 - 50	$\boldsymbol{\uparrow}$	31 - 50	$\boldsymbol{\downarrow}$
51 - 75	$\Big\uparrow$	51 - 75	$\Big\downarrow$
> 75	$\boldsymbol{\Big\uparrow}$	> 75	$\boldsymbol{\Big\downarrow}$

Changes reported are with respect to the control values.

	8mM $MoO_4^{=}$	33.3mM $MoO_4^{=}$	117µM DMAE	2mM γ-HCC	2.5mM 3,4-DBP
PS	$\Big\downarrow$	$\boldsymbol{\Big\downarrow}$	$\boldsymbol{\downarrow}$	$\Big\downarrow$	$\uparrow$
PA	$\boldsymbol{\downarrow}$	—	$\downarrow$		
PI	$\downarrow$	$\boldsymbol{\Big\downarrow}$	$\downarrow$	$\uparrow$	$\boldsymbol{\uparrow}$
SM	$\boldsymbol{\downarrow}$		$\Big\downarrow$		
PC	$\boldsymbol{\downarrow}$	$\boldsymbol{\Big\downarrow}$	$\boldsymbol{\downarrow}$	$\Big\uparrow$	$\boldsymbol{\uparrow}$
PE	—	$\boldsymbol{\Big\downarrow}$	$\boldsymbol{\downarrow}$	$\boldsymbol{\downarrow}$	$\boldsymbol{\uparrow}$
Su	—	$\boldsymbol{\Big\downarrow}$			

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