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LIPIDS AND DEVELOPMENT—

DEPOSITION AND METABOLISM OF PHOSPHOINOSITIDES

IN CHICKEN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

by

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A Thesis

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ABSTRACT

Lipid composition of chick brain and sciatic nerve was determined during development and the active periods of myelin deposition were defined by galactolipid deposition in both tissues. Special interest was focused on polyphosphoinositides (PPI = DPI + TPI) levels and metabolism.

Both DPI and TPI were present in equimolar amounts in premyelination embryonic brain. The concentrations of these lipids increased dramatically during the period of active myelination while only TPI continued to increase thereafter. The lipid composition of sciatic nerve closely reflected that of myelin. PPI were absent initially, their accumulation paralleled that of galactolipids, and the increase was much greater than for any other lipid. The concentrations of DPI remained constant after the period of most active myelination while TPI and galactolipids continued to increase. The changes were more distinct in nerve than brain and provide evidence for long term maturational changes in the myelin content of TPI. These data support the earlier suggestion that brain contains both a myelin pool (principally TPI) and a non-myelin pool (consisting of DPI and a small portion of TPI) of PPI but that only the myelin associated pool is present in nerve.

The enzymes responsible for PPI metabolism (PI kinase, DPI kinase, TPI phosphatase and TPI phosphodiesterase) were also characterized and their activities measured in both chick neural tissues as a function of age. DPI kinase was inhibited by high concentrations of ATP and low concentrations of TPI.

Both kinase activities were stimulated by the non-ionic detergent (Cutscum), and the ratio of detergent to protein in the reaction mixture was both critical and different in the two tissues. Assay systems were developed to permit the measurement of the two TPI phosphohydrolase activities independent of each other and of other non-specific phosphatases. A complex relationship between enzyme, substrate, divalent cations and CETAB (cationic detergent) was observed. TPI phosphodiesterase was shown to require Ca^{2+} for activity while the Mg^{2+} requiring TPI phosphatase was inhibited by this ion.

Unlike earlier studies in rat brain, a marked increase in PI kinase activity coincided with the onset of myelination in chick brain as did DPI kinase and TPI phosphatase activities. The increase in TPI phosphodiesterase activity occurred earlier and all four activities remained high in adult brain. In embryonic sciatic nerve, PI and DPI kinases exhibited peaks of activity in the middle of the period of most active myelination and appeared to be correlated with the rate of TPI deposition. Values in adult nerve were very low. The activities of both hydrolytic enzymes were not related to myelination. TPI phosphatase activity changed little while TPI phosphodiesterase activity was greatest in young nerves and steadily decreased during maturation. These data suggest synthesis of PPI during maturation which are not subject to rapid metabolic turnover in adult nerve.

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ABBREVIATIONS AND TERMINOLOGY

Lipids

CPG	Choline phosphoglycerides, unless otherwise specified includes (i) diacyl derivatives (phosphatidyl); (ii) alk-1-enyl-acyl derivatives (phosphatidal or plasmalogen) and (iii) alkyl, acyl derivatives (ether form).
EPG	Ethanolamine phosphoglycerides, unless otherwise specified includes all forms as described for CPG.
SPG	Serine phosphoglycerides, unless otherwise specified includes all forms as described for CPG.
IPG	Inositol phosphoglycerides includes PI, DPI, TPI and inositol plasmalogens.
PA	Phosphatidic acids (1, 2-diacyl-sn-glycerol-3-phosphate).
PC	Phosphatidylcholines (1, 2-diacyl-sn-glycerol-3-phosphorylcholine).
PE	Phosphatidylethanolamines (1, 2-diacyl-sn-glycerol-3-phosphorylethanolamine).
PS	Phosphatidylserines (1, 2-diacyl-sn-glycerol-3-phosphorylserine).
PI	Phosphatidylinositols (1, 2-diacyl-sn-glycerol-3-phosphoryl-1-myo-inositol).
DPI	Diphosphoinositides (1, 2-diacyl-sn-glycerol-3-phosphoryl-1-myo-inositol-4-phosphate).
TPI	Triphosphoinositides (1, 2-diacyl-sn-glycerol-3-phosphoryl-1-myo-inositol-4, 5-diphosphate).
PPI	Polyphosphoinositides (DPI + TPI).
Lyso	A prefix used to designate lack of one fatty acid in the lipid molecule i.e. lyso-PE: PE lacking one fatty acid on the glycerol moiety.

Abbreviations and Terminology cont.

PG	Phosphatidylglycerols (1, 2-diacyl-sn-glycerol-3-phosphorylglycerol).
DPG	Diphosphatidylglycerols (cardiolipin; bis-(1, 2-diacyl-sn-glycerol-3-phosphoryl)-1', 3'-sn-glycerol).
Sph	Sphingomyelins (N-acyl-erythro-sphingosine-1-phosphorylcholine).
Cer	Cerebrosides (1-O-(β-D-galactopyranosyl)-N-acyl-D-erythro-sphingosine).
Sulf	Sulfatides (Sulfuric acid esters of cerebrosides).
CDP-DG	Cytidine diphosphate diglycerides (CDP-diglycerides).

Solvents

C	Chloroform
M	Methanol
A	Acetone
AA	Acetic acid (glacial)
W	Water

Enzymes

Gal-DH	D-Galactose dehydrogenase (D-galactose: NAD^+ oxidoreductase, EC1.1.1.48).
PIK	Phosphatidylinositol kinase (ATP: phosphatidyl-inositols-4-phosphotransferase, EC2.7.1.67).
DPIK	Diphosphoinositide kinase (ATP: diphosphoinositides-5-phosphotransferase, EC 2.7.1.68).
TPI phosphatase	Triphosphoinositide phosphatase (phosphatidyl-myo-inositol-4, 5-bisphosphate phosphohydrolase, EC 3.1.3.36).

Abbreviations and Terminology cont.

TPI phosphodiesterase
Triphosphoinositide phosphodiesterase (Triphosphoinositide inositol-tris-phosphohydrolase, EC 3.1.4.11).

Others

CNS	Central nervous system
PNS	Peripheral nervous system
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
NAD^+	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
BSA	Bovine serum albumin
EDTA	Ethylenediaminetetra-acetate
EGTA	Ethyleneglycol-bis-(ß-aminoethyl ether)N, N'-tetraacetate
DEAE-cellulose	Diethylaminoethylcellulose
Tris	2-Amino-2-hydroxymethylpropane-1, 3-diol
Tris-HCl	Tris dissolved in water and titrated with HCl to desired pH at the required temperature (temperature at which it was later used)
ACh	Acetylcholine
ADP	Adenosine, 5'-diphosphate
ATP	Adenosine 5'-triphosphate
CMP	Cytidine 5'-monophosphate
CDP	Cytidine 5'-diphosphate
t.l.c.	Thin-layer chromatography
d.p.m.	Disintegrations per minute
c.p.m.	Counts per minute

Abbreviations and Terminology cont.

Rf	Ratio of the distance a compound moves to the distance the solvent moves.
P	Phosphorus (e.g. lipid-P)
Pi	Inorganic phosphate
Po	Organic phosphate
PPi	Inorganic pyrophosphate
S.D.	Standard deviation

I. GENERAL INTRODUCTION

A. LIPIDS OF THE NERVOUS SYSTEM

1. Introduction

The biochemistry of lipids, particularly of complex lipids, has been an integral part of the study of the nervous system since the 19th century. The discovery that phosphorus was bound to fatty substances was made as early as 1811 by L. N. Vauquelin on material extracted from brain with ethanol. The isolation of a phosphorus-containing lipid from egg-yolk and brain which he later called "lecithin" was accomplished by Gobley in 1850. It was, however, J. L. W. Thudichum (1884) who made most of the early advances in phospholipid chemistry and provided the classical terminology. Since then, the intensive studies of the nervous system and of lipids generally have overlapped and contributed to each other. The independent and important contributions to phospholipid and sphingolipid chemistry by Leven (1914, 1916) and Ernst Klenk (1929), the identification and determination of the structure of phosphatidylserine from brain by Folch (1914, 1948) and recent studies on polyphosphoinositides from the same source (Brockerhoff and Ballou, 1961a; Dawson and Dittmer, 1961) are among many examples. A number of reviews on the chemistry and metabolism of lipids in the nervous system have recently appeared (Ansell and Hawthorne, 1964; Rouser and Yamamoto, 1969; Eichberg et al., 1969; Davison, 1970; Ramsey and Nicholas,

1972; Rouser et al., 1972; Ansell, 1973; Bowen et al., 1974).

2. The Nature of Lipids in the Nervous System

The most abundant lipids of the nervous system are cholesterols, the phosphoglycerides and the sphingosine-containing lipids. Other lipids which are present in very small quantities are free fatty acids, neutral glycerides (mono-, di- and triacylglycerols), galactosyl diacylglycerols (Steim, 1967) and some other sterols (cholestenol, Δ-7 cholestenol, 7-dehydrocholesterol).

The principal phosphoglycerides are those containing choline (choline phosphoglycerides, CPG), ethanolamine (ethanolamine phosphoglycerides, EPG), and serine (serine phosphoglycerides, SPG) as nitrogenous bases linked via a phosphate diester to 1, 2-diacyl glycerol. The phosphoglycerides occur mostly as diacyl derivatives, to a much lesser extent as monoacyl derivatives (lyso phosphatides), as alk-1-enyl derivatives (plasmalogens) and as alkyl-acyl derivatives (ether forms). Possible exceptions here are the alk-1-enyl derivatives of EPG which are also present in abundance in certain tissues. The major diacyl phospholipids are phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylserines (PS). Phosphatidylinositols (PI) and polyphosphoinositides (PPI), even though the latter are minor components, are of major importance and will be extensively described later. Other minor phosphoglycerides of nervous tissue are phosphatidic acids (PA), phosphatidylglycerols (PG,

Wells and Dittmer, 1966a), phosphatidylglycerol phosphates (PGP; Wells and Dittmer, 1966b) and diphosphatidylglycerols (DPG).

The sphingolipids are derivatives of sphingosine, an eighteen-carbon chain with hydroxyls at carbons 1 and 3, an amino group at carbon 2 and a double bond between carbons 4 and 5. Dihydrosphingosine and eicosasphingosine (containing a 20-carbon chain) have also been reported to occur (Moscatelli and Mayers, 1965). The major sphingolipids, sphingomyelins (Sph), cerebrosides (Cer) and sulfatides (Sulf) are derivatives of ceramide (sphingosine having a fatty acid attached to the amino group). Esterification of the ceramide primary alcohol to phosphorylcholine yields sphingomyelin. Cerebrosides consist of ceramides whose primary alcoholic group is linked by a glycosidic bond to a monosaccharide (principally galactose). Sulfatides possess a sulfate ester at the 3 position of the galactose moiety of cerebrosides (Yamakawa et al., 1962; Stoffyn and Stoffyn, 1963). Other minor cerebroside-like components with acyl groups at carbons 3 and 6 of the galactose, or an ether-linked, 1, 2-unsaturated, long, fatty chain at the 3 position of sphingosine have also been reported (Norton and Brotz, 1963; Kochetkov et al., 1963; Klenk and Löhr, 1967; Kishimoto et al., 1968). Ceramide polyhexosides and the gangliosides are more complex sphingoglycolipids with oligosaccharide chains. These lipids are present in much smaller quantities. Structures of some lipids commonly found in the nervous system are shown in Fig. 1.

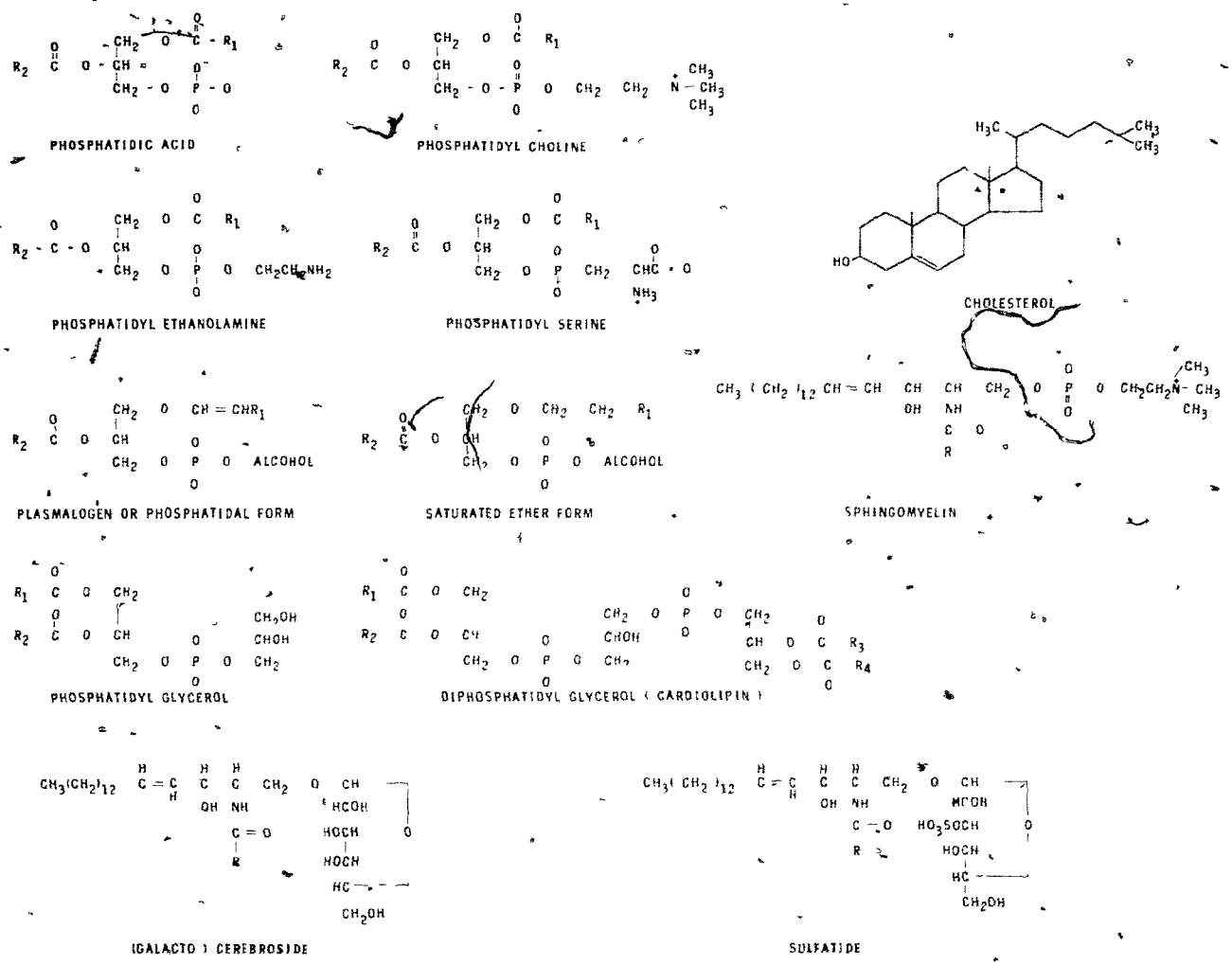

Fig. 1: Structures of Some Lipids Present in Nervous Tissue.

The phosphoglycerides and other lipids exist in brain associated with proteins in various ways. These associations are to some extent reflected in the ease with which the lipids may be extracted from tissues. A fresh tissue extract made with neutral chloroform-methanol (C-M, 2:1, v/v) contains nearly all the lipids, with the possible exception of triphosphoinositides (TPI) and to a lesser extent of diphosphoinositides (DPI), accompanied by a considerable amount of protein. These so-called proteolipids (Folch-Pi and Lees, 1951) contain phospholipids, Chol and Cer that may be associated with the protein '*in vivo*' or have become associated with the protein during isolation procedures (Folch-Pi, 1964; Zand, 1968). The protein moiety of proteolipids has been shown to have an abundance of non-polar amino acids, appreciable amounts of tryptophan, methionine and cystine and a relatively low content of acidic and basic amino acids (Folch-Pi and Lees, 1951; Chatagnon *et al.*, 1953; Wolfgram, 1967; Folch-Pi and Stoffyn, 1972). Proteolipids are especially concentrated in the white matter of brain and are thought to represent structural elements of the myelin sheath (Folch-Pi, 1955).

Phosphatidopeptides represent another fraction containing PPI as the principle lipids (LeBaron *et al.*, 1962), which can be extracted with acidified organic solvents (Folch, 1952; LeBaron and Folch, 1956; LeBaron and Rothlender, 1960). The lipids in phosphatidopeptides account for only a small part of the inositol phosphoglycerides (IPG) of the brain since PI (which is the major component of IPG) is extracted in neutral

solvents. The protein moiety of phosphatidopeptides is rich in acidic and basic amino acids and is considered to be linked to the lipids by electrostatic bonds (Le Baron, 1963). The distribution and functional significance of phosphatidopeptides are unknown; however, it has been suggested that these protein-bound phosphoinositides act as a bridge between the protein and less closely associated lipids in some of the membrane structures of brain white matter (Le Baron et al., 1962).

3. Distribution of Lipids in the Nervous System

Development in microdissection and cell fractionation methods and the increasing refinement of analytic techniques for lipids have led in the recent past to the accummulation of rather comprehensive data concerning lipid distribution and concentration. For the present purpose, studies of lipid composition of nervous tissue other than phosphoinositides will be described only briefly. The lipid composition of well-defined microanatomical regions of brain, of subcellular fractions other than myelin, and of the nervous system of lower vertebrates and invetebrates is described elsewhere (Krep et al., 1963; McMurray et al., 1964; Rouser and Yamamoto, 1969; Ansell, 1973).

a. Distribution of Lipids in Central Nervous System (CNS)

The CNS is composed principally of two cell types: the nerve cells (neurones) and the glial cells. Areas rich in myelinated fibres are referred to as "white matter" while those

areas rich in cell bodies together with their non-myelinated fibres (both dendrites and unmyelinated axons) constitute the "gray matter". Early extensive investigations of the lipid composition of gray and white matter, spinal cord and peripheral nerve were performed by several workers (Johnson et al., 1948, 1949; Brante, 1949; McColl and Rossiter, 1952). Summarized results of these studies and others are available (LeBaron and Folch, 1957; Ansell, 1961; Rossiter, 1962; Ansell and Hawthorne, 1964). Recently, the major lipid composition of adult nervous tissues of various species has been tabulated (Eichberg et al., 1969; Porcellati, 1969; White, 1973).

In general, the major categories of lipids i.e. sterols, sphingolipids and phosphoglycerides constitute almost all of the lipids of the normal brain. Lipids represent about 40 - 60% and phospholipids some 20 - 25% of the dry weight of adult mammalian brain. On the basis of unit weight, neural tissues contain the largest amount of cholesterol, glycolipids and phospholipids in the animal body. Triacylglycerols are essentially absent from normal brain and cholesterol esters are found only during early development. Only traces of other sterols are present in adult mammalian brain. Free fatty acids are quantitatively minor components of brain lipids (Bazán and Joel, 1970), and almost all the fatty acids occur esterified in glycerolipids and sphingolipids. All fatty acids so far characterized are even-numbered, contain 16 - 26 carbon atoms and are predominantly unsaturated.

Although the CNS is rich in certain phospholipids

such as ethanolamine plasmalogens and Sph, no phospholipid is unique to this tissue. In order of abundance, the phospholipids present are: EPG, CPG, Sph, SPG and IPG, together with such phospholipids as DPG and alkyl ether phospholipids (Svennerholm and Thorin, 1962; Wells and Dittmer, 1967) which are found only in small amounts. Much of the EPG of the nervous tissue, unlike that of other tissues, is in the form of the aldehydogenic plasmalogens (phosphatidal ethanolamines). Only small amounts of serine, choline and inositol plasmalogens are present (Webster, 1960; Dawson, 1960; Dawson et al., 1962; Rouser et al., 1963; Eichberg et al., 1964; Wells and Dittmer, 1967).

The total lipid and phospholipid content of the white matter is very much greater than that of gray matter. There is almost three times as much lipid in white matter as in gray matter on a wet weight basis and twice as much on a dry weight basis. Both white and gray matter contain the same individual lipids but in different proportions. Gangliosides are characteristic of gray matter where they are found in five to six times greater amounts than in white matter. When represented as a percent of total lipid, white matter is relatively richer in Chol, much richer in galactolipids and relatively poorer in most phospholipids than gray matter. Galactolipids constitute 25 to 30% of the lipids in white matter, but only 5 to 10% in gray matter. Phospholipids account for two-thirds of the total lipid of gray matter, but less than half in white matter. Plasmalogens represent 75 to

80% of EPG in white matter but less than half of these phospholipids in gray matter. Proteolipids occur at the greatest concentration in brain white matter; values in gray matter are only one-fifth as high (Finean et al., 1957a; Folch et al, 1958).

Except for the enrichment of gangliosides in gray matter (Norton et al., 1966; see also Ramsey and Nicholas, 1972), all other differences in lipid composition between gray and white matter appear to be due mostly to the presence of myelin in the latter. The lipid composition of the non-myelin fractions of bovine white matter also differs considerably from that of the myelin portion. For example, lipids such as PS, Sulf, and to a lesser extent PC are more concentrated in the non-myelin portion while Chol, EPG (plasmalogens) and Sph are concentrated in the myelin portion of white matter (Norton and Autilo, 1966). The lipid composition of the non-myelin portion of white matter also differs considerably from that of gray matter. In particular, the galactolipid content of the non-myelin portion of bovine white matter is threefold that of gray matter (Norton and Autilio, 1966).

b. Distribution of Lipids in Peripheral Nervous System (PNS)

The total lipid and phospholipid content of peripheral nerves is higher than that of whole brain but lower than that of spinal cord. The sum total of Chol, phospholipids and Cer normally amounts to about 95% of the total lipid in the PNS. The remainder may be triacylglycerols

which have been found only in trace amounts in lobster (Richards, 1955) and chicken (Joel et al., 1967) nerves. Much higher values for this lipid have also been reported in chicken and cat sciatic nerves along with small amounts of mono- and diacylglycerols, although this may reflect a large contribution of the epineural and other adjacent structures (Berry et al., 1965; Berry and Cevallos, 1966). The proteolipid content of various mammalian sciatic nerves appears similar but large differences can be found when compared with other peripheral nerves of the same species (Finean et al., 1957a; Folch-Pi et al., 1958; Adams and Taqan, 1961; Wolfgram and Rose, 1961; Amaducci, 1962). For example, human spinal roots contain about 4 times the amount of proteolipids present in human sciatic nerves and about 15 times that found in the brachial plexus. The values for phosphatidopeptides have not been reported for the PNS. Small amounts of free fatty acids have been reported in nerves (Berry et al., 1965; Berry and Cevallos, 1966; O'Brien et al., 1967).

The principal galactolipids of peripheral nerves are Cer; the contribution of gangliosides to total galactolipids is almost negligible. No sterols other than Chol have been detected in peripheral nerves. Free Chol is abundant and cholesterol esters occur in small quantities (Heald et al., 1964; Berry et al., 1965; Berry and Cevallos, 1966; O'Brien et al., 1967). In most nerves, Sph are present in higher concentration than Cer (Johnson et al.,

1948; Branté, 1949; McCaman and Robins, 1959; O'Brien et al., 1967), although not consistently (Evans and Finean, 1965). The lipid composition of the PNS, when compared to that of brain and spinal cord, shows interesting differences. About twice as much Sph and less Cer and Sulf are found in the PNS as compared to the CNS (O'Brien et al., 1967).

The differences in lipid composition of unmyelinated and myelinated peripheral nerves have also been reported (Berry et al., 1965; Sheltawy and Dawson, 1966). Unmyelinated or poorly myelinated nerves, such as those of crab, lobster and mammalian splenic and vagus nerves, contain about one-sixth of the total lipid or phospholipid of myelinated peripheral nerve. The myelinated mammalian peripheral nerves (and also spinal cord) are enriched in lipids similar to those of white matter of the brain (e.g. galactolipids, Chol, Sph, and plasmalogens), while unmyelinated nerves (e.g. splenic) resemble gray matter (Branté, 1949). The major plasmalogens of peripheral nerve are ethanolamine lipids as in the case for spinal cord (Webster, 1960); however, small amounts of serine and choline plasmalogens have also been reported (Porcellati and Mastrantonio, 1964; Sheltawy and Dawson, 1966). Myelinated peripheral nerves are also enriched in PS and TPI compared to unmyelinated nerve but contain lower quantities of PC. However, the total choline-containing phospholipids are relatively constant in both myelinated and unmyelinated peripheral nervous tissue in spite of the differences in the concentrations of the

individual choline-containing lipids. This suggests variation in the tightness of packing of membrane lipid bilayers without, however, affecting the net surface charge balance (Sheltawy and Dawson, 1966). Myelinated peripheral nerve contains a far greater concentration of Chol than unmyelinated nerve due to the abundance of this lipid in the myelin sheath (Johnson et al., 1948; Brante, 1949; Evan and Finean, 1965). The fatty acid pattern of lipids from myelinated nerve generally resembles that of brain white matter, while the pattern from unmyelinated nerve resembles that of gray matter (Light and Easton, 1967).

Differences in lipid concentrations among similar nerves of various animal species have also been observed. For example, Chol and phospholipid concentrations of human sciatic nerve are lower than that of corresponding myelinated nerves of other species. Tables of detailed results are available (see Porcellati, 1969).

c. Lipid Composition of the Central and Peripheral Nervous System Myelins

The myelin sheath is a greatly extended and presumably modified plasma membrane which is wrapped around the nerve axon in a spiral fashion making a multilamellar structure. The myelin membranes originate from, and are part of, the Schwann cell in the PNS and the oligodendroglial cell in the CNS. When the brain tissue is gently homogenized in isotonic media, the myelin sheath is broken into fragments

and the compacted lipid-rich membranes of the myelin proper can be separated by suitable techniques. Myelin can be readily prepared from central white matter in large amounts where it represents about 50% of the total dry weight. A great deal of work has been done on myelin lipid composition and is briefly described here. Early studies of the lipid composition of myelin were done on crude fractions of tissues in which myelin was presumed to be concentrated (Branté, 1949; Brodie and Bain, 1952; Peterson and Schou, 1955; Biran and Bartley, 1961). The results of these studies, although of considerable value, should now be regarded only as indicative of myelin composition. Recent developments in differential and density gradient centrifugation (De Robertis et al., 1962, Eichberg et al., 1964; Whittaker, 1965) coupled with morphological characterization by electron microscopy (Gray and Whittaker, 1962) and improved methods for the separation and measurement of various lipids have permitted the precise analysis of purified myelin or myelin concentrates from both the CNS and PNS of a variety of animal species: human (Cuzner et al., 1965a; O'Brien and Sampson, 1965a, b; Norton et al., 1966; Gerstl et al., 1967), squirrel monkey (Saimiri sciureus) (Horrocks, 1967), cow (Hulcher, 1963; Autilio et al., 1964; Cuzner et al., 1965a; Norton and Autilio, 1966; Soto et al., 1966; O'Brien et al., 1967), rat (August et al., 1961; Seminario et al., 1964; Cuzner et al., 1965a; Nussbaum and Mandel, 1965; Evans and Finean,

1965), guinea pig (Laatsch et al., 1962; Eichberg et al., 1964; Evans and Finean, 1965), rabbit, pigeon, frog and dogfish (Evans and Finean, 1965; Cuzner et al., 1965a).

The results of such studies have shown that the same lipids are present in CNS myelin of all species investigated and also that the relative proportions of the individual lipids in the various species are in fairly close agreement, especially considering the variety of techniques used for isolating myelin. These lipid patterns are distinct from those obtained for other subcellular fractions. In most species examined, lipids such as Chol, Cer, ethanolamine plasmalogens and Sph are found to be enriched in myelin. These lipids although typical of myelin cannot be considered to be located exclusively in this structure (O'Brien et al., 1965a; Norton and Autilio, 1966).

The major sterol of myelin lipids is Chol and 80% of the white matter Chol is in myelin (Norton and Autilio, 1966). Small quantities of Chol esters and desmosterol have also been reported in bovine and rat brain myelin (Young and Hulcher, 1966; Smith et al., 1967; Banik and Davison, 1967). Of the galactolipids, Cer are enriched in myelin fraction of white matter while Sulf are mostly in the non-myelin fraction (Norton and Autilio, 1966), e.g. in axons (Devries and Norton, 1974). The presence of small amounts of gangliosides has also been reported in purified bovine brain myelin (Norton and Autilio, 1966; Soto et al., 1966).

Myelin is relatively deficient in PC and PE when compared to other membranous structures, otherwise PC is the second most abundant phosphoglyceride after EPG. The EPG amount to about 10% of the dry weight of myelin and their concentration is virtually the same for CNS and PNS myelins (Evans and Finean, 1965; O'Brien et al., 1967; Horrocks, 1967). Most of the plasmalogens of myelin are found in the EPG fractions (Branté, 1949; Webster, 1960), comprising at least 80% of this fraction in purified myelin (Norton and Autilio, 1965; O'Brien and Sampson, 1965a) and in one case about 100% (Cuzner et al., 1965a). Inositol plasmalogen content is very low, while serine plasmalogens account for less than 10% of the total SPG. The content of total SPG, CPG and IPG was found to be more variable than EPG. Usually, PI content is the lowest, followed by PS and then PC. Traces of PA and DPG were also reported in CNS myelin (Eichberg et al., 1964; Cuzner et al., 1965b). Sphingomyelins are relatively concentrated in myelin; 38% of rat brain Sph (Nussbaum et al., 1963) and 70% of the bovine white matter Sph are in the myelin (Norton and Autilio, 1966). Proteolipids are present largely in myelin, although synaptic endings and mitochondria also contain some proteolipid protein (Lees, 1966; Radin et al., 1967).

The lipid composition of both CNS and PNS myelin is generally similar but not identical, perhaps a reflection of their different cellular origins (Norton and Autilio,

1966; O'Brien et al., 1967). Evan and Finean (1965) have compared the lipid composition of CNS and PNS myelin from both the rat and guinea pig. Little difference was found in the phospholipid composition, but a significantly higher phopholipid:cholesterol ratio was evident in peripheral myelin. A considerably higher galactolipid content was also observed in guinea pig sciatic nerve myelin. Results with other species, however, generally indicate that there are more galactolipids and less Sph in CNS myelin than PNS myelin (Norton and Autilio, 1966; O'Brien et al., 1967; Norrocks, 1967; see also Johnson et al., 1948 ; McCaman and Robins, 1959; Schmidt et al., 1966). For comparative purposes, lipid composition data of both CNS and PNS myelin of several species is presented in Table 1.

B. LIPIDS AND THE DEVELOPING NERVOUS SYSTEM

1. Introduction

The nervous system develops through several overlapping periods, each defined by one major event in brain growth and/or structural maturation. These periods are artificial at best, when applied to a dynamic system. In addition, the nervous system is heterogeneous in development from region to region in terms of time, type of cells and complexity of interaction among these different cells. The events of nervous system development have often been divided into stages for ease of discussion and organization.

Table 1. Lipid Composition of CNS and PNS Myelins *

	Ox		Squirrel Monkey		Guinea Pig		Rat	
	Brain (a)	Spinal roots (b)	Spinal cord (c)	Brachial Plexus (c)	Brain (d)	Sciatic nerve (d)	Brain (d)	Sciatic nerve (d)
Total Lipid-P	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Chol	1.32	0.77	1.30	1.08	1.00	0.64	1.11	0.83
Total Galactolipids	0.63	0.22	0.65	0.46	-	-	-	-
Cer	0.52	-	-	-	0.45	1.15	0.39	0.33
Sulf	0.07	-	-	-				
EPG	0.44	0.27	0.43	0.38	0.42	-	0.48	0.37
CPG	0.25	0.24	0.19	0.14	0.25	-	0.24	0.24
Sph	0.15	0.22	0.18	0.23	0.13	0.34	0.18	0.20
SPG	0.14	0.27	0.17	0.19	0.20		0.10	0.19
IPG	0.02							
Plasmalogens	0.35	-	0.35	0.28	-	-	-	-
Total Lipid-P/ Chol	0.76	1.30	0.77	0.92	1.00	1.56	0.90	1.20

* Data given as mol proportions relative to total lipid-P.
Data recalculated from: (a), Norton and Autilio, 1966; (b), O'Brien et al., 1967; (c), Horrock, 1967; (d), Evan and Finean, 1965.

Recently, Davison and Dobbing (1968) described four stages which serve to correlate major changes in all species (see Table 2).

2. Biochemical Changes During Development

a. Changes in Brain Weight, Nucleic Acids and Protein

The most apparent index of brain growth is weight. Depending upon the species, the most rapid growth may occur just before, after, or at time of birth. The rate of growth varies greatly in different anatomical regions (e.g. cerebellum shows the highest change in rate of growth; the spinal cord the most gradual) as does the time at which the period of most rapid growth occurs. The increase in brain solids reflects the increase in membranous structures and a decrease in water. In rat brain for example, the water content decreases from 90% of the total brain weight at birth to 83% at maturity (Himwick, 1969).

The developmental changes of four major constituents of brain - DNA, RNA, protein and lipid - are summarized for the fore-brain of rat in Fig. 2 a & b. Early proliferation of cells is indicated by synthesis of DNA. As differentiation occurs, DNA replication is followed by increased transcription, then translation of RNA to protein. With the appearance of cell-specific enzymes and structural proteins, each cell type acquires a unique metabolism and morphology. Maturation of such elements as neuronal processes, synaptic endings, and myelin involves deposition of lipids into

Table 2. Stages of Development of the Brain

Period	Process	Completion	
I	Organogenesis and neural multiplication (i.e. total cell number reaches its adult value).	Guinea pig	- 45th day of gestation.
		Rat	- 3rd postnatal day.
		Man	- 25th week of gestation.
		Chick*	- 10 - 12 days of incubation.
		Mouse and cat	- until birth.
IIa	A "growth spurt" i.e. a maturation period of axonal and dendritic growth, glial multiplication and myelination. Development of certain enzyme systems.	Guinea pig	- at birth.
		Pig	- 5 weeks after birth.
		Rat	- 3 weeks after birth.
		Man	- 2 years after birth.
		Chick*	- 2 weeks after hatching.
IIb	Later but overlapping period of growth.		-
III	Mature state.		-
IV	Senile regression, i.e. aging.		-

* see Romanoff (1960).

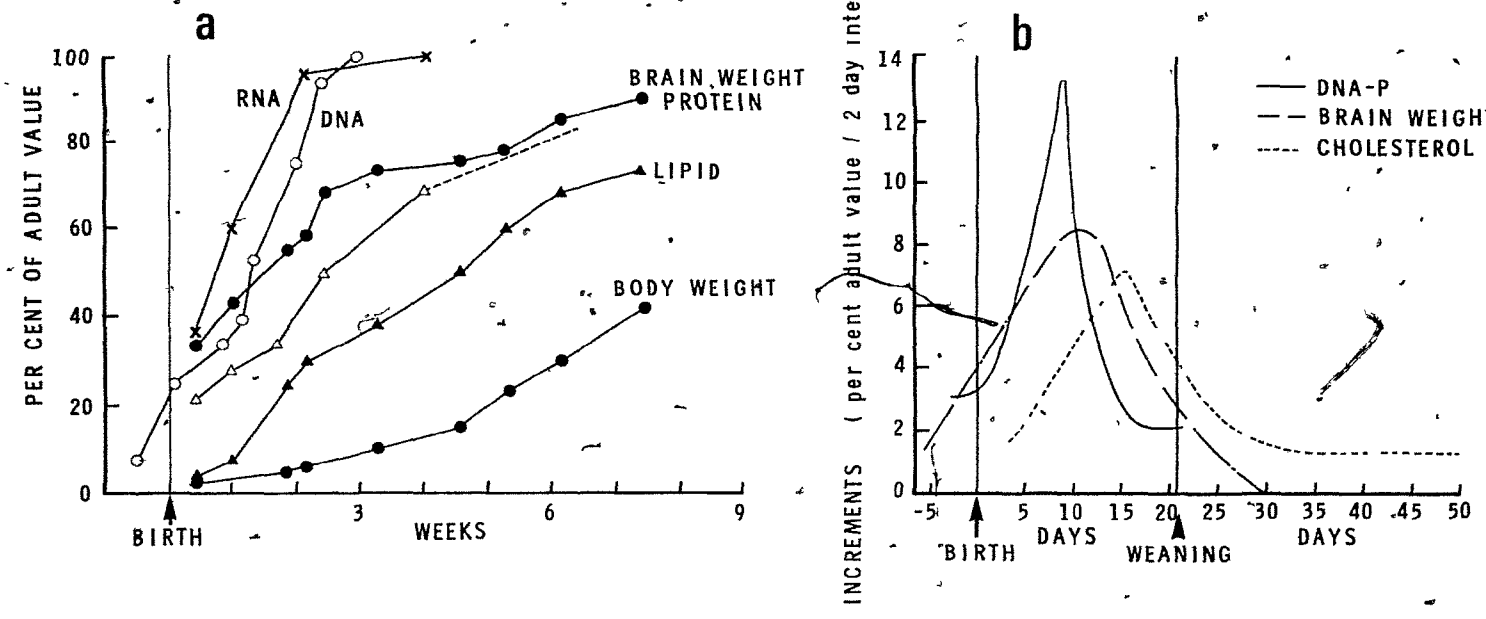

Fig. 2:
(a) Changes in Body Weight and Several Brain Constituents During Development in Rat. Values are expressed as % of maximum (adult) levels. Results for body weight, brain weight, and brain lipid have been calculated from the data of Kishimoto et al., 1965; DNA: Fish and Winick, 1969; RNA: Mandel et al., 1964; and protein: Himwich, 1962, 1969.
(b) Rate of Change in Wet Weight, DNA and Chol of Developing Rat Brain. All values are calculated as increments (% of adult levels) at 2 day intervals (from Davison and Dobbing, 1968).

these membranous structures. Changes in DNA, RNA and also protein content of brain with development have been comprehensively described (see Rapport et al., 1969; 1971).

b. Changes in Lipid Composition

With respect to lipid composition, the development of the nervous system may be divided into three distinct periods: (i) that which precedes visible or histological deposition of myelin, (ii) that of rapid deposition of myelin and (iii) the period which follows and completes maturation. The period of rapid myelination differs from species to species; for example, the guinea pig (also cow, horse, deer) is born virtually fully myelinated, whereas in the rat (also cat, rabbit, dog), myelination occurs after birth (Himwich, 1962). The human infant falls between these groups indicating a partial myelination in utero.

Lipid changes accompanying maturation have been studied extensively in the mammalian CNS. The main object of these has frequently been to obtain information on the composition of myelin at a time when fractionation techniques were inadequate to permit direct analysis of myelin. Rat brain and to a lesser extent mouse brain have been used most often since myelination in these species is a postnatal event, and also, specimens of these animals of known age can be more readily obtained in the required quantities. Data on man, clearly of greatest interest and importance, are less abundant. Most reports deal with postnatal development

of the species in question, although fragmentary information exists on the lipid composition during embryonic life, expecially in the chick.

The most complete analyses for twenty-one lipid classes in rat brain at eight ages (3 to 330 days postnatal) have been reported by Wells and Dittmer (1967). Other similar studies, although providing less complete data, are also available (Greany, 1961; Cuzner and Davison, 1968; see also Ansell and Hawthorne, 1964). These investigators attempted to distinguish those lipids which are characteristically found in myelin from those which are considered to reside in all membranes in the brain cells.

A simplified table indicating changes in lipid concentration with respect to myelination in rat brain is presented in Table 3. The lipids are grouped on the basis of their period of most rapid change relative to myelination: some lipids undergo marked shifts before myelination begins; others show marked, moderate or small increases during myelination. When allowance is made for the differences in temporal scale, these changes are in general similar in all species. Information regarding deposition of some of the lipid components during development in various other mammalian species is available: cholesterol concentration (Mandel and Bieth, 1951; Kishimoto et al., 1965; Davison and Wajda, 1959; Spence and Wolfe, 1967), early occurance of desmosterol (Kritchevsky et al., 1965; Banik and Davison, 1967), plasmalogens, although not for all lipid components (Stammler et al., 1954; Erickson and Lands,

Table 3. Changes in Lipids During Development in Rat Brain*

Group	Lipid	% of Adult Level at 3 Days	Time of Max. Increase	Localization or Possible Role
I	Sterol esters	?	Prenatal	Fatty acid donor?+
	Gangliosides	27.0	3 - 12 d	Neuronal process
II	Cerebrosides	0.2	6 - 42 d	Myelin primarily
	Sphingomyelins	5.6	6 - 24 d	Myelin primarily
	Triphosphoinositides	7.5	12 - 42 d	Myelin primarily
	Phosphatidic acids	10.0	12 - 42 d	Metabolic intermediate
	Galactosyldiglycerides	3.0		Myelin primarily
	Inositol plasmologens	9.0		Myelin primarily
III	Choline plasmalogens	12.0	18 - 42 d	Myelin primarily
	Ethanolamine plasmalogens	17.0	6 - 24 d	Myelin primarily
	Phosphoglyceryl ethers++	15.9	6 - 24 d	All membranes
	Phosphatidylserines	34.0	Gradual	All membranes
	Cardiolipin	34.5	6 - 42 d	All membranes
	Cholesterols	26.0	Gradual	Myelin primarily
	Plasmalogenic acid	16.7	6 - 12 d	All membranes
IV	Phosphatidylcholines	59.0	Gradual	All membranes
	Phosphatidylethanolamines	50.0	Gradual	All membranes
	Phosphatidylinositols	55.0	Gradual	All membranes
V	Sulfatides	3.3	6 - 24 d	All membranes
	Diphosphoinositides	5.0	3 - 18 d	Intermediate in TPI metabolism
	Phosphatidylglycerol-phosphates	50.0**	12 - 18 d	Metabolic intermediate

continued on next page

Table 3 Legend

* Data were recalculated from those of Wells and Dittmer (1967); the table is taken in part from Benjamins and McKhann (1972). The lipid content of rat brain was examined at 3, 6, 12, 18, 24, 42, 180, and 330 days after birth. The sterol esters decreased after birth; no significant data was obtained after 3 days (at 3 days 2 μmol/g wet wt.).

** % of maximum level at 12 days. Phosphatidylglycerolphosphate decreased after that time to lowest levels in 180 and 330 days.

+ See Mickel and Gilles, 1970.

++ Mainly containing ethanolamine.

1959; Korey and Orchen, 1959; Bieth et al., 1961; Freysz et al., 1963), phospholipids (Cumings et al., 1958; Edgar and Smits, 1959; Davison and Wajda, 1959; Balakrishnan et al., 1961; Freysz et al., 1963; Siek and Newburgh, 1965; Clausen et al., 1965; O'Brien and Sampson, 1965a, b; Kreps et al., 1966; Myant and Cole, 1966; Rouser et al., 1967), sphingomyelins (Bieth et al., 1961; Ansell and Spanner, 1961; Marshall et al., 1966), cerebrosides and sulfatides (Cuming et al., 1958; Uzman and Rumley, 1959; Kishmoto and Radin, 1959; Bakke and Comatzer, 1961; Davison and Gregson, 1962; Garrigson and Chargaff, 1963; Clausen et al., 1965; Kishimoto et al., 1965 ; O'Brien and Sampson, 1965a; Menkes et al., 1966a; Maker and Hauser, 1967; Spence and Wolf, 1967; Hauser, 1968), gangliosides (Pritchard and Cantin, 1962; Pritchard, 1963; James and Fotherby, 1963; Burton et al., 1963; Garrigan and Chargaff, 1963; Kishimoto et al., 1965 ; Marker and Hauser, 1967; Spence and Wolf, 1967).

Very little is known about changes in lipid composition during development in the PNS. The work described in this thesis provides information in this area.

The fatty acid patterns within the lipid classes of CNS have also been shown to undergo significant changes but these are less pronounced than the changes in the lipid concentrations. Generally, in phospholipids, there is a noticeable shift from the medium chain fatty acids towards longer chains and greater unsaturation. In glycolipids, the most noticeable shift, besides the increase in chain length

and degree of unsaturation, is a steady increase in long chain hydroxy-fatty acids (see Eichberg et al., 1969). The information on PNS is quite scanty. However, the general trend appears likely to be more or less the same as in CNS (Banik et al., 1968).

c. Changes in Myelin Lipid Composition

The first report that the lipid composition of myelin changed during development (Horrocks et al., 1966) was treated with scepticism. It has now been proven convincingly by several groups (Cuzner and Davison, 1968; Eng and Noble, 1968; Horrocks, 1968) that the rodent brain myelin when first deposited has a very different composition from that of the adult. The myelin lipid changes are those typical of whole brain. Essentially, in rat brain myelin, the relative proportions of galactolipids, cholesterol and ethanolamine plasmalogen increase from about 15 days of age as the relative proportions of CPG decrease. The very small amount of desmosterol declines, but the other lipids remain constant. In addition, the polysialogangliosides decrease and the monosialogangliosides increase to 90% of the total gangliosides. This change is not complete until the rat is about 2 months old.

"Early myelin" preparations from developing brains of rodents also contain less cerebrosides and more phospholipids than mature myelin. This early myelin resembles more closely the cell membranes from which it is derived than mature myelin. Davison et al. (1966) and Banik et al. (1968) suggest that this

early myelin is a mixture of mature myelin and "pro-myelin" derived directly from the oligodendroglial plasma membranes. In fact, the "pro-myelin" component was later shown to be poor in cerebrosides and to contain a higher proportion of shorter chain fatty acids in the phosphoglycerides than mature myelin (Banik and Davison, 1969). This "pro-myelin" fraction is likely a transition state between the oligodendroglial plasma membrane and compact myelin.

For more detailed coverage of developmental changes in the lipids of myelin and other subcellular fractions along with fatty acid patterns of the lipids, the reader is referred to the following articles: (Eichberg et al., 1969; Rouser et al., 1972).

C. PHOSPHOINOSITIDES

1. Introduction

Inositol was first isolated by Scherer (1850) from muscle extracts and was found later in other animal and plant tissues (Müller, 1857; Thudichum, 1884). It was identified as hexahydroxycyclohexane (Maquenne, 1887). Of the 9 possible stereoisomers, the most abundant one in nature is myo-inositol (in which all but one of the hydroxyls are equatorial).

Myo-inositol occurs both free and combined as phospholipids in animal and plant tissues, and as phytin

(mixed Mg^{2+}-Ca^{2+} salt of phytic acid - the hexaphosphate) in plants. It is synthesized by cyclization of glucose (Fischer, 1944; Chen and Charalampous, 1963, 1964a, b, 1965, 1966; Pina and Tatum, 1967) and can be metabolized to glucose (Stetten and Stetten, 1946; Moscatelli and Larner, 1946) via D-glucuronic acid (Charalampous and Lyras, 1957; Charalampous, 1959, 1960; Richardson and Axelrod, 1959; Burns et al., 1959). Myo-inositol is considered to be a lipotrophic agent (Gavin and McHenry, 1941; Gavin et al., 1943; Abels et al., 1943; Best et al., 1951a, b) and was also found to protect dividing fibroblasts from mitotic poisons in tissue culture (Chargaff et al., 1948). It is essential for the survival and growth of cultured mammalian cells (Eagle et al., 1957; Geyer and Chang, 1957; Eagle et al., 1960; Charalampous et al., 1961). However, its most important role seems to be in eukaryotic cell membranes as a constituent of phospholipids.

Thudichum (1884) did not recognize inositol as a lipid constituent. It was first shown by Anderson (1930) to be a component of the phosphatides of tubercle bacilli. Later, an inositol monophosphate was found among the hydrolysis products of soy bean phosphatides (Klenk and Sakai, 1939) and brain lipids (Folch and Wooley, 1942; Burmaster, 1949). The crude myo-inositol - containing phospholipid complex of brain was described by Folch and Sperry (1948) as a group of heterogenous compounds all of which contain glycerol, inositol, phosphoric acid and fatty acids. An

inositol-containing phospholipid was reported earlier in rat liver (Macpherson and Lucas, 1947) and was subsequently isolated from wheat germ and heart muscle (Faure and Morelec-Coulon, 1953, 1954). This lipid yielded inositol monophosphate upon hydrolysis (McKibbon, 1954; Hawthorne, 1955a) and exhibited molar proportions for glycerol, phosphorus, inositol and fatty acid of 1:1:1:2. Folch (1949a, b) prepared "Brain diphosphoinositide" ("Folch DPI") by fractional precipitation of ox brain cephalin*. This acidic lipid, obtained as the Ca^{2+} and Mg^{2+} salts, exhibited a phosphorus/inositol ratio of about 2. For many years, it was thought to be a single compound and the only phosphoinositide in brain. A number of later studies reported the presence of inositol monophosphate among the hydrolysis products of brain lipids but these observations were usually dismissed as arising from the further hydrolysis of inositol diphosphate (Hawthorne and Charagaff 1954; Hawthorne, 1955b; Hutchinson et al., 1956). Hokin and Hokin (1958) were the first to demonstrate the presence of PI in brain. Hörhammer et al. (1958) showed the presence of lyso-PI in ox brain. Later, using counter-current distribution and paper chromatographic techniques, several phosphoinositides including PI were revealed in the "Folch DPI" fraction (Hörhammer et al., 1959, 1960; see also Kemp et al.,

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*In this thesis the term "cephalin" is used to denote the alcohol insoluble fraction of tissue phospholipids rather than an earlier usage which implied a single phospholipid, phosphatidylethanolamine.

1961). Dawson and Dittmer (1961) showed that this fraction contained PI, DPI and TPI. This finding was simultaneously and independently supported by others (Brockerhoff and Ballou, 1961a; Tomlinson and Ballou, 1961; Grado and Ballou, 1961) and later confirmed by Ellis et al. (1963). A tetraphosphoinositide also was reported in brain (Klenk and Hendricks, 1961; Santiago-Calvo et al., 1963) but subsequent careful analysis showed this compound to be TPI (Hendrickson and Ballou, 1964).

2. Structure and Properties of Phosphoinositides

a. Structure of Phosphatidylinositols

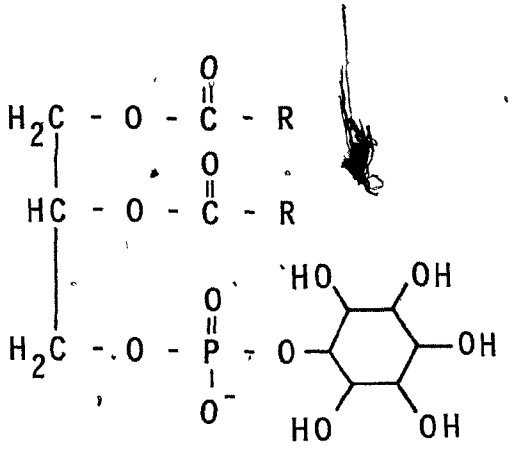
Lipid preparations described as "glycerol inositol phosphatidic acids" were first isolated from wheat germ and ox heart muscle (Faure and Morelec-Coulon, 1953, 1954, 1958; Morelec-Coulon and Faure, 1958a, b, c). These preparations contained glycerol, phosphorus, inositol and fatty acids in the molar ratio of 1:1:1:2 and the structure of a "monophosphoinositide" was proposed (Fig. 3a). Similar inositol phosphatides were isolated from a variety of other sources (Scholfield and Dutton, 1954; Okuhara and Nokayama, 1955; McKibbon, 1954, 1956). A series of chemical studies (Hawthorne, 1955b; McKibbon, 1956; Hanahan and Olley, 1958; Brockerhoff and Hanahan, 1959; Hawthorne and Hübscher, 1959; Kemp et al., 1959) confirmed the structure (Fig. 3a) first proposed by Faure and Morelec-Coulon (1953). The optical rotation of enzymatically produced α, β-diglyceride (1, 2-diacylglycerol) from beef liver monophosphoinositide was shown to be the

same as similarly prepared 1, 2-diacylglycerol from naturally-occurring lecithin and synthetic D-2, 3-diglycerides (Brockerhoff, 1961) thereby establishing the L configuration of the "phosphatidyl" group.

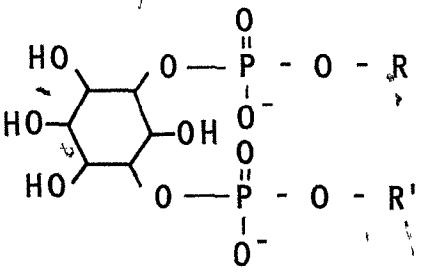
There are six possible sites of attachment of phosphate group to inositol. From a stereochemical point of view, the most probable is the axial hydroxyl at position 2 (Hawthorne, 1955b; Folch and LeBaron, 1956; Hanahan and Olley, 1958). However, the isolation of optically active inositol-1-phosphate (inositol-2-phosphate is inactive) from the hydrolysis products of phosphoinositides of several sources (Pizer and Ballou, 1959a, b; Ballou and Pizer, 1959; Hawthorne et al., 1960a, b) firmly established the structure of monophosphoinositide as 1-phosphatidyl-L-myo-inositol (Fig. 4a). The chemical synthesis of this lipid containing a phosphatidyl group linked to the 1-position of inositol proved difficult; however, a successful synthesis has been reported (Klyaschitskii et al., 1969).

b. Structures of Polyphosphoinositides

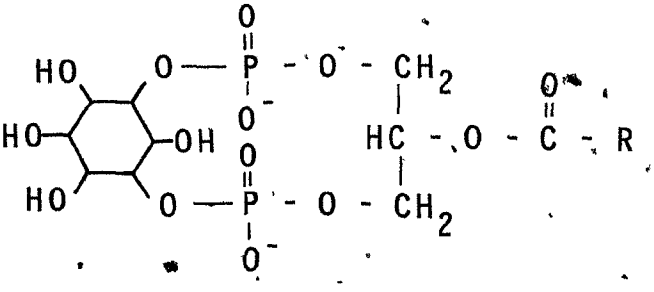
Acid hydrolysis of "Folch DPI" yielded equimolar amounts of fatty acid, glycerol and inositol diphosphate which periodate oxidation showed to be substituted in the meta position (Folch, 1949a, b). On the basis of these studies and evidence that the phosphate groups in the parent lipid were present as diesters (only one titratable group per



a. MPI
(FAURE and MORELEC-COULON, 1958)



b. (FOLCH, 1949)



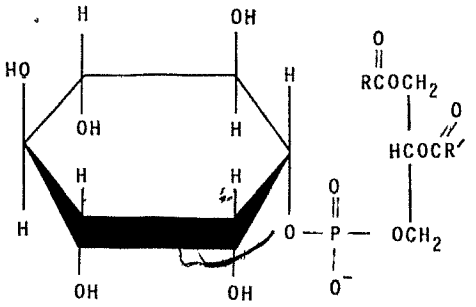
c. (HAWTHORNE, 1955b)

" FOLCH DPI "

Fig. 3: Early Proposals for the Structures of the Phosphoinositides.

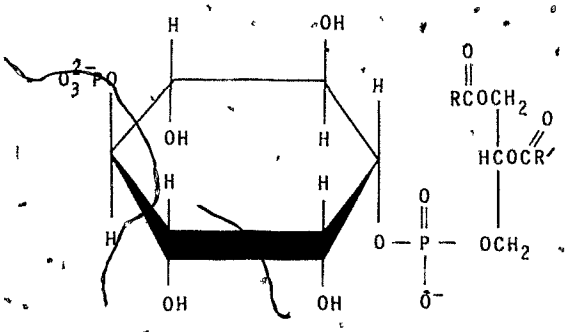
phosphorus atom), a structure was proposed (Fig. 3b, R and R' denote unknown residues). Folch and LeBaron (1956) suggested that one of the two unknown residues may be a "monoglyceride". Hawthorne (1955b) proposed a cyclic structure (Fig. 3c) and/or a polymeric structure in which inositol diphosphate residues were linked to "monoglyceride" (see also Folch and LeBaron, 1956). Ion-exchange chromatography of the water-soluble products obtained from "Folch DPI" by alkaline, acid and enzymic hydrolysis provided evidence for the presence of three inositides, one of which was a "monophosphoinositide" (Kemp et al., 1960, 1961). A series of subsequent studies of the products of acid and alkaline hydrolysis demonstrated the presence of lipids containing inositol-1, 4-diphosphate and inositol-1, 4, 5-triphosphate in the "Folch DPI" fraction (Grado and Ballou, 1961; Tomlinson and Ballou, 1961). The deacylation products of mild alkaline hydrolysis were shown to be 1-(glyceryl-phosphoryl)-L-myo-inositol, 1-(glyceryl-phosphoryl)-L-myo-inositol-4-phosphate and 1-(glyceryl-phosphoryl)-L-myo-inositol-4, 5-diphosphate (Ellis et al., 1960; Ellis and Hawthorne, 1961a; Brockerhoff and Ballou, 1961a). Independently, Dawson and Dittmer (1961) showed the "Folch DPI" fraction to contain three phosphoinositides and succeeded in isolating 90-95% pure TPI. The structures for DPI and TPI are now firmly established (Fig. 4b, c). Recently, Russian workers have synthesized DPI by condensing 1, 2:4, 5-dicyclohexylidene-myo-inositol with phosphoryl-chloride and 1,2-distearoylglycerol (Klyashchitskii et al., 1969).

a MONOPHOSPHOINOSITIDE (PHOSPHATIDYLINOSITOL)



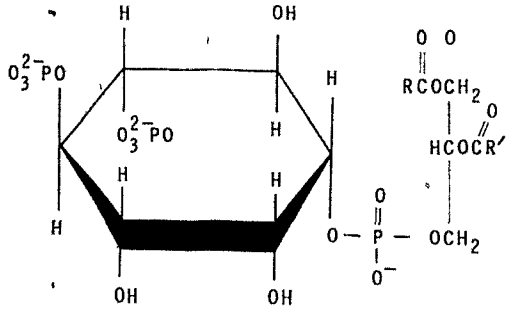
1,2-DIACYL-SN-GLYCERO-3-PHOSPHORYL-1-MYO-INOSITOL

b DIPHOSPHOINOSITIDE



1,2-DIACYL-SN-GLYCERO-3-PHOSPHORYL-1-MYO-INOSITOL-4-PHOSPHATE

c TRIPHOSPHOINOSITIDE



1,2-DIACYL-SN-GLYCERO-3-PHOSPHORYL-1-MYO-INOSITOL-4,5-DIPHOSPHATE

Fig. 4: Structures of Phosphoinositides.

c. Nomenclature

The only inositol so far found as a lipid constituent is myo-inositol. By analogy with the term "phosphoinositides" originally suggested by Folch and Sperry (1948) for phospholipids containing inositol, all the three major inositol lipids of brain were conveniently named as monophosphoinositide, diphosphoinositide and triphosphoinositide. Recent work has indicated the occurrence of molecular species which vary in fatty acid composition for all three phosphoinositides as well as other phospholipids. It now is preferred to use the plural of all names to indicate a family of each phosphatide. By analogy with phosphatidylcholines, Hawthorne (1960a) suggested "phosphatidylinositols" as a preferable name. Following this, the higher phosphorylated derivatives of phosphatidylinositols (PI) would be phosphatidylinositol-4-phosphate (DPI) and phosphatidylinositol-4, 5-diphosphates (TPI). "Phosphatidyl" denotes a specific chemical structure, 1, 2-diacyl-sn-glycerophosphoric acid. The symbol "sn" refers to a stereospecific numbering system (Hirschmann, 1960) that provides a much more precise designation of the structure of glycerol derivatives and is strongly recommended by the IUPAC-IUB Commission (1968). The structural names for PI, DPI and TPI would be: 1, 2-diacyl-sn-glycero-3-phosphoryl-1-myo-inositol, 1, 2-diacyl-sn-glycero-3-phosphoryl-1-myo-inositol-4-monophosphate and 1, 2-diacyl-sn-glycero-3-phosphoryl-1-myo-inositol-4, 5-

diphosphate, respectively (Strickland, 1973). Lyso-PI by analogy with the other lyso-phosphoglycerides designate PI having no fatty acid esterified at the 1 or 2 position of glycerol (i.e. 1-, or 2-acyl-sn-glycero-3-phosphoryl-1-myo-inositol). In this dissertation the names PI (phosphatidylinositols), DPI (phosphatidylinositol-4-phosphates), TPI (phosphatidylinositol-4, 5-diphosphates) and PPI (polyphosphoinositides, designating DPI and TPI) will be used for the sake of convenience.

d. Isolation Procedures and Determination

Phosphatidylinositols are the only phosphoinositides which can be easily extracted with neutral C-M mixtures. Polyphosphoinositides appear to be bound rather tightly to tissue proteins. Isolation of PPI requires treatment of the tissue to disrupt these inositide-protein bounds. In early studies pretreatment of the tissue with acetone and ethanol or with 0.3 N-perchloric acid prior to extraction of all lipids with petroleum ether (Folch, 1942, 1949a), or chloroform-ethanol mixtures (Santiago-Calvo et al., 1963) was used. Tissues have also been subjected to prolonged extraction with diethyl ether in a Soxhlet apparatus in order to solubilize these lipids (Wagner et al., 196la, 1962). The best and most widely used system is that of Dittmer and Dawson (1961) who first extracted the bulk of phospholipids with neutral C-M mixtures and then

extracted the inositide-protein complex from the tissue residue with C-M mixtures containing HCl. This method is a modification of one originally described by Folch (1952) to prepare phosphatidopeptides (LeBaron et al., 1962). Less effective systems have substituted NH_4OH (Rouser et al., 1963), salts, organic acids or chelating agents for HCl (LeBaron et al., 1963; Dawson and Eichberg, 1965; Dittmer and Douglas, 1969; Michell et al., 1970). LeBaron et al., (1963) and Dawson and Eichberg (1965) determined that no additional phosphoinositides remained in the tissue after extraction with C-M-HCl (400:200:1.5, by vol.). Unfortunately, a portion of the DPI is also extracted during treatment with neutral C-M mixtures. This loss is minimized by raising the proportion of methanol in the solvent used for the first extraction (Wells and Dittmer, 1967; Dittmer and Douglas, 1969). Extraction of all the lipids together with C-M-HCl has greater disadvantages. Determination of the PPI is made very difficult since they represent very minor proportions of the total phospholipids and also, the alk-1-enyl ether linkages are hydrolyzed making the extract unsuitable for plasmalogen determinations. Procedures involving pretreatment with acetone result in low recoveries. Furthermore, TPI is degraded to DPI during acetone treatment (Dawson and Eichberg, 1965; Wells and Dittmer, 1965; Dittmer and Douglas, 1969). Another critical factor in the quantitative recovery of the PPI is the necessity of avoiding the rapid post-mortem breakdown which occurs

just prior to extraction (Kerr et al., 1964; Dawson and Eichberg, 1965; Wells and Dittmer, 1965; Eichberg and Hauser, 1967). The most effective method is to immediately freeze the tissue in liquid nitrogen within seconds of death.

There are two approaches to quantitative determination of PPI in tissue extracts. The first depends upon the direct separation of phosphoinositides followed by chemical analysis (usually measurement of phosphorus content). Separation has been achieved by chromatography on formaldehyde-treated paper (Wagner et al., 1963), on columns of DEAE-cellulose (Hendrickson and Ballou, 1964), on Silica Gel impregnated paper (Santiago-Calvo et al., 1964) and on thin layers of calcium-free Silica Gel treated with potassium oxalate (Gonzales-Sastre and Folch-Pi, 1968). The second approach depends upon the isolation of acid or alkaline hydrolysis products of lipids either by two-dimensional paper chromatography and ionophoresis (Dawson and Dittmer, 1961; Dawson et al., 1962) or by anion-exchange chromatography (Hubscher and Hawthorne, 1957; Ellis et al., 1963; Lester, 1963; Dittmer and Douglas, 1969). The direct analysis of intact phosphoinositides is preferable since hydrolytic procedures are tedious, require utmost precautions and must be corrected for losses resulting from further hydrolytic cleavage of the measured products (Brockerhoff, 1963; Dawson and Eichberg, 1965; Wells and Dittmer, 1965, 1966).

e. Interesting Physical Characteristics

Although the bulk of the membrane phospholipids are zwitterionic, the phosphoinositides are anionic at physiological pH and hence contribute to the negative charge on membranes and to their ion-binding properties. Phosphoinositides are miscible in water and exist in solution as micelles with ionic groups oriented towards the aqueous phase. The apparent micellar weight of both DPI and TPI (measured in 0.1 M N-ethylmorpholin buffer, pH 8, by ultracentrifugation) was estimated to be 78,100 (Hendrickson, 1969). This is smaller than the values obtained for gangliosides (257,000; Gammack, 1963) and sonicated PC (1.3×10^6; Gammack et al., 1964). Phosphatidylinositols, having a much lower charge density, form large aggregates in aqueous media (Hendrickson, 1969).

Anionic lipids and TPI in particular bind divalent cations avidly, but without great specificity for individual ionic species; affinities for monovalent cations are much lower. At physiological pH, TPI possess five negative charges and two free hydroxyl groups per molecule making them quite hydrophilic. In the presence of high concentrations of univalent cations (Na^+, K^+) or lower concentrations of divalent cations (Mg^{2+}, Ca^{2+}), the hydrophilic nature is reduced by counter-ion binding and precipitation from aqueous solution occurs (Thompson and Dawson, 1964b). Cations also cause redistribution of PPI into the non-polar phase of the biphasic solvent system

of Folch et al. (1957) and the counter-current isolation procedure of Kerr et al. (1963). The amount of Ca^{2+} or Mg^{2+} required to shift TPI into the non-polar phase is quite specific and is 2 equiv. per mol of TPI. The affinity for divalent cations is quite high. Triphosphoinositides can compete effectively with EDTA at pH 7.2 and even the more effective chelating agent, cyclohexan-1, 2-diaminetetra-acetate, is required at 5 times the concentration of TPI to dissociate the salt completely. The affinity for Ca^{2+} is some 2 - 2.5 times as great as for Mg^{2+}. Possibly, this is due to a more thermodynamically stable stereochemical configuration for the dicalcium complex (Dawson, 1965). Gel filtration of mixtures of TPI and a variety of proteins have indicated the formation of weak to moderately strong complexes in the absence of divalent metal ions. The formation of complexes by these highly anionic lipids with anionic serum proteins or ATP suggests that hydrophobic interactions may be important (Hendrickson, 1969). In the presence of metal ions, stronger complexes are formed which are largely insoluble in the aqueous or organic solvents (Dawson, 1965; Hendrickson, 1969). Electrostatic interactions are important in this case as well as in the binding of very basic proteins (Palmer and Dawson, 1969). Phosphoinositides, like all other anionic lipids, also interact strongly with a variety of amphipathic cations such as alkylamines, local anaesthetics (e.g. tetracaine, procaine) and phenothiazine tranquillizers (Dawson and Hauser, 1970;

Papahadjopoulos, 1972; Ito and Ohnishi, 1974).

Some of the physical properties in aqueous media and the ion-binding properties of purified phosphoinositides have been described in some detail (Dawson, 1965; Hendrickson and Fullington, 1965; Fullington and Hendrickson, 1966; Fullington, 1967; Hauser and Dawson, 1967, 1968; Abrahamson et al., 1968; Hendrickson and Reinertsen, 1969, 1971; Hauser et al., 1969; Hendrickson, 1969; Dawson and Hauser, 1970). In view of the key role of Ca^{2+} in neurotransmitter-receptor interactions (Triggle, 1972), the interaction of PPI with divalent cations, including Ca^{2+}, may be of important physiological significance (see Section I.C.6).

3. Distribution of Phosphoinositides in the Nervous System

a. Anatomical Distribution

Phosphatidylinositols occur in most mammalian tissues where they represent about 2 - 12% of the total phospholipids (0.5 - 2.5 μmol/g tissue). In mammalian CNS and PNS, the concentration is 1.0 - 3.5 μmol/g tissue and 0.1 - 1.6 μmol/g tissue respectively or about 0.5 - 4% of the total phospholipids. The largest concentrations of DPI and TPI occur in nervous tissue. Little confidence can be placed in the values reported, particularly in early studies since (i) they are subject to rapid post-mortem destruction (Kerr et al., 1964; Dawson and Eichberg, 1965; Dittmer and Douglas, 1969), (ii) quantitative extraction

is difficult, (iii) extraction procedures have varied greatly in efficiency and (iv) being minor components of the total phospholipids, they are difficult to measure accurately. The phosphoinositide content of normal mature nervous tissue of some animal species studied by several laboratories is compiled in Table 4.

Since the initial demonstration of PPI in cerebral tissue (Folch, 1949a), many efforts have been made to determine their localization. Folch and LeBaron (1951) described the inositol-containing "phosphatidopeptides" fraction in brain and suggested an association with specific proteins. However, this fraction may be an artifact produced by degradation or modification of an original tissue component (Hawthorne, 1960a). The concentration of PPI is higher in white matter while similar concentrations of the PI occur in white and gray matter (Höhammer et al., 1960; LeBaron et al., 1963). Sheltawy and Dawson (1969a) obtained similar results with guinea pig brain but pointed out that the PPI remaining after post-mortem breakdown during dissection was being measured and the distribution could be distorted by differing rates of degradation in the two tissue fractions. The enrichment of PPI in white matter has generally been interpreted to mean that they are selectively localized in myelin. However, it has also been suggested that they may be concentrated in some closely associated structures of the fibre tracts other than the myelin sheath (Amaducci et al., 1962; LeBaron et al., 1963).

Table 4. Phosphoinositide Content of Adult Nervous Tissue

Animal	Ref.	PI		DPI		TPI	
		µmol wet wt.	% Total Lipid-P	nmol/g wet wt.	% Total Lipid-P	nmol/g wet wt.	% Total Lipid-P
Brain							
Rat[a]	1	2.7	4.4	287.4	0.9	99.4	0.5
Rat[b]	2,3	-	3.1	195.2	0.6	397.8	1.9
Rat[c] (330 days old)	4	2.4	3.5	200.0	0.3	390.0	0.6
Guinea pig[d]	5,3	-	2.9	177.4	0.6	584.9	2.9
Cat[e]	6	1.2	1.8	61.3	0.2	271.0	1.3
Cat[e]	6a	-	-	112.9	0.3	200.0	0.9
Ox[f]	7,3	-	3.2	119.4	0.2	450.5	3.2
Peripheral Nerve[g]	8						
Lobster (claw)	8	0.2	1.5	8.1	0.1	43.0	1.0
Lobster (leg)	8	0.1	0.6	1.6	trace	32.3	0.6
Cow Splenic	8	0.5	3.6	-	-	2.2	trace
Rabbit Sciatic	8	0.7	1.1	32.3	trace	537.6	2.6
Sheep Sciatic	8	1.6	3.0	145.2	0.5	462.4	2.6
Monkey Sciatic	8	0.3	0.5	96.8	0.4	569.9	3.2
Hen Sciatic	8	0.5	0.8	21.0	0.1	258.1	1.5

continued on next page

Table 4 Legend

(a), Lyophilized tissues were extracted, phosphoinositides determined after formaldehyde-treated paper chromatography of intact lipids. (b), Frozen in liquid N_2 immediately after decapitation, phosphoinositides determined after acid hydrolysis followed by paper chromatography and ionophoresis. (c), Frozen in dry ice after decapitation and dissection (± 30 sec after death), phosphoinositides analyzed after mild alkaline methanolysis followed by ion-exchange chromatography. (d), Thiopentone anesthesia, brain fixed *in situ* with liquid N_2, analyzed as in (b). (e), Cerebral hemispheres, brain chilled on ice, analyzed as in (a). (e'), Cerebral hemispheres, head frozen in liquid N_2, analyzed as in (a). (f), 45 min after death on ice, analyzed as in (b). (g), All nerves were frozen in liquid N_2 immediately after separation; hens, urethane anesthesia; monkeys, pentobarbitol anesthesia; lobster, cooled under ice for 2h; Sciatic nerves from rabbit and sheep ± 5 min after death and cow splenic nerve ± 10 - 15 min after death; analyzed as in (b) and/or as in (a).

(1), Wagner *et al.*, 1963. (2), Wuthier, 1966. (3), Dawson and Eichberg, 1965. (4), Wells and Dittmer, 1967. (5), Eichberg *et al.*, 1964. (6), Rossiter and Palmer, 1965. (6a), Palmer, 1965, Ph.D. thesis, University of Western Ontario. (7), Dawson *et al.*, 1962. (8), Sheltawy and Dawson, 1966.

b. Subcellular Distribution

Studies of the subcellular distribution of PPI in nervous system must also be interpreted with caution for the same reasons stated in preceding section (I.C.3.a). The prolonged centrifugation procedures provide even greater opportunity for post-mortem breakdown of PPI and, until recently, it has been difficult to be certain that the subcellular fractions obtained are not contaminated by other membranous structures.

Eichberg and Dawson (1965) fractionated guinea pig forebrain homogenates and reported a two- to threefold enrichment of PPI in myelin. However, only slightly more than 50% of the original inositide was recovered in the isolated fractions. Other subcellular fractions contained little or no PPI. They also reported that in the purified myelin, the amounts of Ca^{2+} plus Mg^{2+} exhibited a close acid-base equivalence to the PPI present, thus providing further support to the concept that these phospholipids exist in tissue as complexes with divalent cations. Additional support for the localization of PPI in myelin structures comes from the finding of high concentrations of these lipids in myelinated vertebrate nerves and low concentrations in poorly myelinated or unmyelinated nerves (Sheltawy and Dawson, 1966). Thus, despite technical difficulties, most studies of nervous tissue so far have suggested that PPI are selectively localized in myelin and/or some closely associated structure.

c. Phosphoinositides and the Developing Nervous System

Folch and coworkers (1959) reported the appearance of a trypsin-resistant protein residue believed to contain DPI during the period of myelination in postnatal rat brain. LeBaron et al. (1962) also found the TPI-rich phosphatidopeptide fraction to increase during myelination. Many comprehensive developmental studies of the lipids in the CNS of several mammalian species have since appeared but most do not report values for PPI. Polyphosphoinositides have been analyzed in postnatal rat brain in several laboratories with essentially similar results (Rossiter and Gardiner, 1966; Eichberg and Hauser, 1967b; Wells and Dittmer, 1967). Significant quantities of DPI and TPI were found as early as 2 - 3 days of age. The concentrations increased slowly up to 10 days (the time at which histologically recognizable myelin is first seen; Folch et al., 1959) and then increased rapidly. The presence of PPI before significant myelination has occurred suggests that they may be present in extramyelin structures as well. The pattern of deposition after 10 days is similar to that of such characteristically myelin lipids as the Cer. Phosphatidylinositols were present in much higher concentration at the earliest ages studied and showed only moderate changes in concentration throughout development.

Polyphosphoinositides have not been studied in the developing PNS. Such a study forms a part of this thesis.

4. Phosphoinositides Outside the Nervous System

Phosphatidylinositols, as noted earlier, are found in most mammalian tissues as well as in a variety of plants and micro-organisms. Studies of this lipid and its complex derivatives (phosphatidylinositol-di-tetra and pentamannosides found in Mycobacteria and other sugar-containing phosphoinositides of plants) will not be described here (see Hawthorne, 1960a; Ballou and Lee, 1966; Carter, 1966; Ambron and Pieringer, 1973).

Di- and triphosphoinositides were initially detected in brain but subsequently found in many other tissues. Hörhammer et al. (1961) seem to have been the first to detect DPI and TPI in tissues other than brain. The amounts found were considerably smaller (Wagner et al., 1963). Early work indicated the presence of PPI in rat kidney cortex (Andrade and Huggins, 1963) and in dog adrenals (Lo Chang and Sweeley, 1963). Since then DPI and/or TPI have been measured quantitatively or have been detected by incorporation of radioactive precursors in rat liver (Hölzl and Wagner, 1964; Dawson and Eichberg, 1965; Michell et al., 1967; Kiselev, 1969), pig liver (Kfoury and Kerr, 1964), rat kidney (Andrade and Huggins, 1964; Dawson and Eichberg, 1965), rat lung, spleen, heart, skeletal muscle, testis and pancreas (Hölzl and Wagner, 1964; Harwood and Hawthorne, 1969a), Erlich ascites tumor cells (Palmer, 1965), human, dog, rabbit, sheep and swine erythrocyte membranes (Harwood and Hawthorne, 1969a;

Schneider and Kirschner, 1970; Palmer and Verpoorte, 1971; Verpoorte and Palmer, 1974), rabbit polymorphonuclear leukocytes (Garrett and Redman, 1975) and several avian tissues (Santiago-Calvo et al., 1964). Polyphosphoinositides have been reported in subcellular fractions from rat liver, kidney cortex, heart, skeletal muscle and testis (Garbus et al., 1963; Galliard and Hawthorne, 1963; Hawthorne and Michell, 1966; Harwood and Hawthorne, 1969a; Tou et al., 1968, 1969, 1970). Among micro-organisms, only the protozoan Crithidia fasciculata (Palmer, 1973) and the yeast Saccharomyces cerevisiae (Lester and Steiner, 1968; Steiner and Lester, 1972; Talwalker and Lester, 1974) have been shown to contain PPI.

Where quantitative analysis have been reported, the concentrations of PPI outside the nervous system are generally much lower than in brain. When expressed as a percent of total phospholipids, the proportions of both DPI and TPI are similarly quite low compared to brain with the exception of erythrocyte membranes and protozoa. The concentrations of PPI in various tissues, where possible have been converted to a common mode of representation (n mol/g wet wt. and % of total lipid-P) and are compiled in Table 5. Values reported in earlier studies are sometimes quite low due to the use of inadequate analytical procedures then available which have since been improved considerably.

Table 5. Concentrations of Polyphosphoinositides Outside the Nervous System

Tissue	Ref.	DPI		TPI	
		nmol/g wet wt.	% total Lipid-P	nmol/g wet wt.	% total Lipid-P
Kidney (rat)	1	69.4	-	59.1	-
" "	2	44.0	0.3	29.4	0.3
" "	10	40.0	-	30.0	-
Liver (rat)	1	19.4	-	8.6	-
" "	3	30.6	-	38.7	-
" "	10	20.0	-	30.0	-
" (pig)	4	280.0	-	-	-
Lung (rat)	1	37.1	-	-	-
" "	2	50.8	0.4	33.9	0.3
" "	10	20.0	-	0.0	-
Heart (rat)	2	36.0	0.30	15.7	0.2
Intestine (rat) (small)	10	40.0	-	40.0	-
Pancreas (rat)	10	30.0	-	60.0	-
Spleen (rat)	10	I0.0	-	-	-
Testis (rat)	10	20.0	-	-	-
Ehrlich ascite cells (rabbit)	5	159.7^{+}	0.2	236.6^{+}	0.4
Erythrocyte membranes (human)	7	-	0.6	-	0.9
	8	4.3^{++}	-	6.1^{++}	-
Erythrocyte membrane (swine)	9	0.3*	-	2.6*	-
Crithidia fasciculata (Protozoa)	6	-	1.8**	-	0.7**

continued on next page

Table 5 Legend

(+), represented as nmol/10 cc packed cells; (++), nmol/mg protein; (*), nmol/mg dry wt.; (**), values are of logarithimic phase cells.

(1), Dawson & Eichberg, 1965; (2), Wagner et al., 1963; (3), Kiselev, 1969; (4), Kfoury and Kerr, 1964; (5), Palmer (Ph.D. Thesis), 1965; (6), Palmer, 1973; (7), Palmer and Verpoorte, 1971; (8), Verpoorte and Palmer, 1974; (9), Schneider and Kirschner, 1970; (10), Dittmer and Douglas, 1969.

5. Metabolism of Phosphoinositides

a. Turnover Studies

The use of isotopically labelled precursors has contributed much to the understanding of phospholipid metabolism and its physiological significance. Using appropriate precursors, the ability of tissue slices and cell-free preparations to synthesize phospholipids *in vitro* was demonstrated in many early studies (see reviews: Kennedy, 1956, 1957a, b, 1961a, b; Hokin and Hokin, 1956; Rossiter, 1957; Rossiter and Strickland, 1959, 1960). The synthesis is energy dependent with ATP as the source of phosphatide phosphorus (McMurray *et al.*, 1957b). With the advent of suitable isolation and analytical techniques, extensive studies of individual phospholipids were begun (Dawson, 1954a). The incorporation of ^{32}Pi into brain phosphoinositides and PA was found to be greater than that into the other phospholipids (Dawson, 1954a, b; Hokin and Hokin, 1955). The early chromatographic methods, however, did not adequately separate the phosphate esters derived from PI and DPI, so only DPI was thought to occur in brain. It was subsequently shown that PI are more highly labelled from ^{32}Pi, [1-^{14}C]glycerol and [^{3}H]inositol than the other phosphoglycerides with the exception of PA (Hokin and Hokin, 1958b). Phosphatidylinositols are also readily labelled both *in vivo* and *in vitro* in other tissues as well as in brain

(Hokin and Hokin, 1958a, b; Pritchard, 1958; Agranoff et al., 1958; Thompson et al., 1959; Brockerhoff and Ballou, 1962b; Palmer and Rossiter, 1964).

A very high rate of phosphate turnover was observed for the phosphatidopeptide fraction from various animal tissues including brain, in both in vivo and in vitro experiments (Davidson and Smellie, 1952; Findley et al., 1954a, b; Crosbie et al., 1954; Hutchison et al., 1956; Huggins, 1959; Huggins and Cohn, 1959; LeBaron et al., 1960, 1962). The specific radioactivity was 10 - 15 times that of other phospholipids and second only to nucleotide phosphorus. Most of the radioactivity was found in the monoester phosphate groups of DPI and TPI (Wagner et al., 1961; Ellis and Hawthorne, 1961b; Brockerhoff and Ballou, 1961b), and this greatly exceeded that of the diester phosphate (Brockerhoff and Ballou, 1962a). The specific radioactivities of the poorly labelled diester phosphate of the three phosphoinositides were in the order: PI>DPI>TPI. Similar results were obtained following incorporation of [^{3}H]inositol or [2-^{14}C]glycerol (Brockerhoff and Ballou, 1962b; Rossiter and Palmer, 1966a) suggesting that the synthesis of PPI occurs by successive phosphorylation of PI. A similar high metabolic turnover rate of the monoesterified phosphate groups of PPI has also been observed outside of the nervous system e.g. kidney (Huggins and Cohn, 1959; Andrade and Huggin, 1964), Ehrlich ascite tumor cells (Rossiter and Palmer, 1966b), erythrocytes (Schneider and

Kirshner, 1970; Peterson and Kirshner, 1970; Palmer and Verpoorte, 1971; Verpoorte and Palmer, 1974), several other mammalian tissues (Santiago-Calvo et al., 1964), yeast (Steiner and Lester, 1972; Talwalker and Lester, 1973) and protozoa (Palmer, 1973).

The rapid partial depletion of the PPI content of brain after death is well documented (Kerr et al., 1964; Dawson and Eichberg, 1965; Wells and Dittmer, 1965; Eichberg and Hauser, 1967b). The concentrations fall quickly and attain a constant value after several minutes. When these lipids are prelabelled with ^{32}Pi in vivo, a time dependent decrease in the specific radioactivity of TPI is observed while that of the acid-soluble phosphorus pool, PI and PA, remains unchanged. The rate and magnitude of the fall in PPI content and specific radioactivity appear to depend on the stage of development, being appreciably faster and more extensive in younger rat and guinea pig brains (Sheltawy and Dawson, 1969a). These observations suggest the presence of two metabolically distinct pools of PPI, one with a higher turnover rate which is preferentially degraded after death and a more stable fraction. The concentration of the stable fraction appears to increase with age (Dawson, 1969).

Most phospholipids in myelin, including PI and PA, exhibit a very slow rate of turnover when compared to whole brain. However, PPI isolated from a purified myelin

fraction were found to have essentially the same specific radioactivity (following in vivo incorporation of ^{32}Pi) as the PPI isolated from the whole brain (Eichberg and Dawson, 1965). Thus, the monoester phosphate groups of the PPI appear to be very active metabolically even within the compact myelin structure.

b. Biosynthesis

(i) Phosphatidylinositols

In 1939 Chargaff suggested a pathway for phospholipid biosynthesis in which phosphoethanolamine or phosphocholine was combined with 1, 2-diacylglycerol. Kennedy and Weiss (1956) came to the same conclusion and demonstrated the requirement for cytidine-5'-triphosphate (CTP) in the biosynthesis of PC, PE, PG, Sph and possibly the plasmalogenic phosphatides (Kiyasu and Kennedy, 1960). The participation of CTP in PI synthesis in rat brain was demonstrated by McMurray et al. (1957a, b). By analogy to the synthetic pathways for the other phosphatides, inositol phosphate or CDP-inositol was an expected intermediate. However, attempts to show the phosphorylation of inositol by tissue extracts in the presence of ATP were unsuccessful (Hawthorne, 1960a, Paulus and Kennedy, 1960), although inositol monophosphate was in fact found in several tissues (Hubscher and Hawthorne, 1957).

Based largely on studies with ^{32}P-labelled glycerol-3-phosphate and phosphatidic acid, PI was shown

to be formed by the transfer of a phosphatidyl group from the liponucleotide CDP-diglyceride (CDP-DG) to inositol. Incorporation of glycerol-3-phosphate requires the presence of a suitable acylating system as well as CTP (Fig. 5, P.67 reaction 1, 2, 3 & 4 or 5; Agranoff et al., 1958; Paulus and Kennedy, 1958, 1959, 1960; Rossiter et al., 1960; Thompson et al., 1963). This de novo synthesis of PI differs from that for PC in that phospholipid-P comes from glycerophosphate and that inositol is incorporated directly. The scheme of Paulus and Kennedy and of other workers, quoted earlier, supported Agranoff's pathway (Fig. 5, reaction 1, 6, 5) except that CTP, rather than CDP-choline (formed by reversal of reaction 7), is involved in the formation of CDP-DG. Phosphatidic acid, the precursor of CDP-DG can also be formed by reaction (8), (9) & (10) (Strickland, 1962; Pieringer and Hokin, 1962) but their relative importance is unknown.

Incorporation of labelled inositol into phospholipids may also occur without a source of energy or cytidine nucleotide by an exchange reaction, the significance of which is not clear since no net synthesis occurs. The reaction is dependent upon Mn^{2+} ions and stimulated by CMP in a phosphate buffer. This reaction, earlier noted by Agranoff et al. (1958) has been explained by Paulus and Kennedy (1958, 1959) as a reversal of reaction (4) or by the reversible action of phospholipase D (Fig. 5, reaction 11); however, phospholipase D activity on phosphoinositides

has not been demonstrated in animal tissues.

The enzyme phosphatidate cytidylyltransferase (CTP:phosphatidate cytidylyltransferase, EC 2.7.7.41) responsible for CDP-DG formation (Fig. 5, reaction 2), although originally proposed by Paulus and Kennedy (1960), was actually demonstrated later in liver (Carter and Kennedy, 1966) and in embryonic chick brain (Petzold and Agranoff, 1966). The enzyme appears to be primarily in the mitochondrial fraction in chick brain, but is localized in the microsomes of guinea pig liver. Carter (1968) and McCaman and Finnerty (1968) have also presented evidence for a similar synthesis of CDP-DG in E. coli and in Micrococcus cerificans.

The enzyme CDP-diglyceride inositolphosphatidyltransferase (CDP-diglyceride:myo-inositol phosphatidyltransferase, EC 2.7.8.11) responsible for subsequent formation of PI (Fig. 5, reaction 5) has been studied extensively in rat kidney (Agranoff et al., 1958; Prottey and Hawthorne, 1967), liver (Paulus and Kennedy, 1960) and brain (Agranoff, 1957; Thompson et al., 1963; Benjamins and Agranoff, 1969). In guinea pig brain, the highest specific activity was in a crude microsomal fraction. When a variety of chemically synthesized CDP-DG with fatty acids of varying chain length were tested, the brain microsomal preparations preferentially incorporated the shorter chain derivatives (e.g. the incorporation of CDP-didecanoin was about six times higher than of

CDP-dipalmitin). With CDP-didecanoin as the lipid acceptor, only myo-inositol, of all the nine possible isomers, was incorporated into PI. None of the myo-inositol monophosphates served as substrates (Benjamin and Agranoff, 1969). Similar experiments with rat brain microsomes demonstrated a preference for CDP-DG having an unsaturated fatty acid at the 2 position (Bishop and Strickland, 1970).

(ii) Polyphosphoinositides

The earlier work on the biosynthesis and turnover of inositides in brain gave confusing results because methods were not then available for the separation of PI, DPI and TPI. Later studies, however, not only revealed the chemical structures of the brain inositides, but also provided better understanding of the incorporation studies with labelled precursors. On the basis of the molecular similarities between the brain phosphoinositides and the information from labelling studies (see Section I.C.5.a), it was proposed that the biosynthesis of TPI proceeds by a stepwise phosphorylation of PI (Fig. 5, reaction 12 & 13).

Direct evidence for successive phosphorylation of PI was sought by incubating rat brain mitochondria with ^{32}P-labelled PI in phosphate buffer with the cofactors suggested by McMurray et al.(1957b) without success (see review, Hawthorne and Kemp, 1964). However, similar experiments with kidney and liver mitochondria incubated with ^{32}Pi for short periods (5 min.) yielded labelled DPI.

and a small amount of TPI (Garbus et al., 1963; Galliard and Hawthorne, 1963). The process required Mg^{2+} but not exogenous substrate and was inhibited by dinitrophenol, azide, cyanide, antimycin A, dicumarol, gramicidin and oligomycin (Michell et al., 1964; Hajra et al., 1965; Galliard et al., 1965).

Enzymatic hydrolysis of the labelled lipid revealed that all of the radioactivity was in the 4-phosphate of the DPI, thus indicating that the lipid was formed by phosphorylation of PI. However, it was not clear whether the phosphate donor was ATP or a high energy precursor of ATP. Subsequently, Hokin and Hokin (1964) reported the labelling of the monoesterified phosphates of PPI in erythrocytes from [γ-^{32}P]ATP. Simultaneously, Colodzin and Kennedy (1964) provided evidence for the enzymatic phosphorylation of PI from ATP in a microsomal preparation of rat brain (Fig. 5, reaction 12). Later, the enzyme PI kinase (ATP:phosphatidylinositol-4-phosphotransferase, EC 2.7.1.67) responsible for the phosphorylation of PI was characterized in brain microsomes and the product was identified as DPI (Colodzin and Kennedy, 1965). Other nucleoside triphosphates can not be substituted for ATP. Systems synthesizing DPI from PI have now been demonstrated in liver (Galliard et al., 1965; Hajra et al., 1965; Michell and Hawthorne, 1965; Michell et al., 1967), brain (Kai and Hawthorne, 1966; Kai et al., 1966b), kidney (Colodzin and Kennedy, 1965; Lou et al., 1968) and several

other tissues (Colodzin and Kennedy, 1965). In the absence of detergents, only endogenous substrate (PI) appears to be phosphorylated.

The PI kinase requires Mg^{2+} (or to a lesser extent Mn^{2+}) ions for optimal activity. It is sensitive to sulfhydryl reagents and is inhibited by Ca^{2+} ions in the presence and absence of Mg^{2+}. Certain detergents activate PI kinase. It has a subcellular distribution similar to that of 5'-nucleotidase in rat brain and liver and also to that of Na^{+}/K^{+}-stimulated ATPase in rat brain, both plasma membrane markers (Kai et al., 1966b; Michell et al., 1967; Harwood and Hawthorne, 1969a). Like acetylcholinesterase, it appears to be concentrated in the outer membrane of guinea pig brain synaptosomes (Harwood and Hawthorne, 1969b) and has been found in the chromaffin-granule membrane of bovine adrenal medulla (Phillips, 1973; Muller and Kirshner, 1975). The microsomal fraction of rat kidney cortex also contains a PI kinase which may be partially solubilized by non-ionic detergents (Tou et al., 1969). It has been described in the soluble fraction of Saccharomyces cerevisiae (Talwalkar and Lester, 1974) and recently been located on the cytoplasmic surface of the erythrocyte membrane (Garrett and Redman, 1975).

Synthesis of TPI accompanies the formation of DPI from PI in brain, kidney and erythrocytes but not in liver and chromaffin granules. The first direct evidence that DPI is phosphorylated by ATP was provided in rat

brain by Kai and Hawthorne (1966) and Kai et al. (1966a). The enzyme DPI kinase (ATP:diphosphoinositides 5-phosphotransferase, EC 2.7.1.68) required exogenous DPI, ATP, and Mg^{2+} for maximum activity and the product was identified as TPI by chromatographic separation of the intact lipids (Fig. 5, reaction 13). Its subcellular distribution in rat brain resembled that of 6-phosphogluconate dehydrogenase, a characteristic enzyme of the soluble fraction (Kai et al., 1968). However, it has been detected in purified myelin from rabbit sciatic nerve (Iacobelli, 1969) while in rat kidney cortex it is predominantly localized in the plasma membrane fraction (Tou et al., 1970). It is also located on the cytoplasmic surface of the erythrocyte membrane (Garrett and Redman, 1975). DPI kinase can be partially purified by ammonium sulfate fractionation, treatment with ethanol at -15°C, and chromatography on Sephadex G200 (Kai et al., 1968). Detergents do not seem to stimulate this kinase, nor do thiol groups appear necessary for activity. At the optimum Mg^{2+} concentrations, both PI and DPI kinases are inhibited by Ca^{2+}, but only the latter enzyme can be partly activated by Ca^{2+} in the absence of Mg^{2+}. Neither kinase is activated by Na^{+} or K^{+} ions; in fact, a considerable loss of DPI kinase activity was observed in their presence. Only DPI kinase has been reported to be stimulated by acetylcholine (Kai et al., 1968).

The three phosphoinositides exhibit very similar

fatty acid distributions and, as had already been described, (see Section I.C.5.a), the PPI are formed directly from and are rapidly interconvertible with PI. However, the proportion of arachidonic acid-containing species is greater in PI (Holub et al., 1970; Luthra and Sheltawy, 1972). Metabolic studies have shown that PI rapidly take up arachidonic acid presumably via deacylation and acyl transfer reactions but do not equilibrate as rapidly with PPI (Baker and Thompson, 1972). Acylation of lyso-PI by rat brain microsomes has been demonstrated to be selective for arachidonic acid while acylation of lyso-lecithin is not (Baker and Thompson, 1973). This cycle may have some significance in controlling the availability of free arachidonic acid for prostaglandin synthesis.

c. Catabolism

(i) Introduction

Early studies by Sloane-Stanley (1951; see also 1953) showed brain suspensions to be capable of hydrolyzing "Folch DPI" to organic (Po) and inorganic phosphates (Pi). Rodnight (1956) extended this work using homogenates of distinct anatomical regions of the brain, sciatic nerve and spinal cord and tissue homogenates of kidney, spleen and liver. He showed that Ca^{2+} added to the incubation medium stimulated the release of acid-soluble Po. In the absence of added Ca^{2+}, the release of Pi always exceeded that of Po.

He suggested the presence of two enzymes acting simultaneously on the same substrate, a diesterase which he believed to release inositol monophosphate and a monoesterase, yielding Pi. He considered that his experimental findings made it unlikely that inositol monophosphate is a further hydrolytic product of originally released inositol diphosphate. However, the complex nature of the substrate used precluded any definitive interpretation.

(ii) Phosphatidylinositols

Phosphatidylinositols may be deacylated to yield glycerophosphorylinositol (Fig. 5, reaction 14) which can be further degraded to glycerophosphate and inositol (reaction 15) and subsequently to glycerol and Pi (reaction 16). The diester phosphate bond of PI may also be hydrolyzed to yield 1, 2-diacylglycerols and inositol phosphate as products (reaction 17).

Dawson (1958, 1959) found that phospholipase preparations from Penicillium notatum catalyze the hydrolysis of PI by reactions (14), (15) and (16). An ox pancreas preparation was also shown to liberate inositol phosphate from PI (Fig. 5, reaction 17), but the enzyme concerned was not specific for phosphoinositides. Kemp et al. (1959; 1961; see also Hawthorne and Kemp, 1960) purified a system of enzymes from rat liver which hydrolyzes PI both ways (Fig. 5, reaction 14 and 17). This preparation required Ca^{2+} ions for activity and was specific for inositol lipids

but released both inositol phosphate and glycerophosphoryl-inositol from PI. Evidence was presented which precluded the possibility that glycerophosphorylinositol is first produced and then degraded further to inositol phosphate and glycerol. When "Folch DPI" was used as the substrate, no Pi was released, in contrast to the results with brain homogenates (Sloane-Stanley, 1953; Rodnight, 1956). Further studies with subcellular fractions from rat liver have shown that the supernatant fraction contains only the phospholipase C-like activity of phosphoinositide inositolphosphohydrolase (Fig. 5, reaction 17). Presumably the particulate fraction was responsible for the deacylation reactions yielding glycerophosphorylinositol. A similar enzyme (diesterase) was reported in guinea pig intestinal mucosa (Atherton et al., 1966), where the activity was found to be 30 - 50 times greater than in liver. It was enriched in the cytoplasm and exhibited an absolute requirement for Ca^{2+}. Diethyl ether, which is known to activate certain phospholipases including those which hydrolyze PPI (see following Section, page 65), had no effect (Atherton and Hawthorne, 1968). Under optimum conditions for PI hydrolysis, this enzyme also degrades PPI to inositol di- and triphosphates. It is not active with other phospholipids and releases no Pi from PPI. Similar enzymes have been reported in guinea pig brain (Friedel et al., 1967) and the supernatant fraction of rat brain (Thompson, 1967). Recently it has been shown that the cleavage of the diester phosphate bond of PI results in the simultaneous

production of approximately equal proportions of inositol-1-phosphate and inositol 1:2 cyclic phosphate (Dawson et al., 1971; Lapetina and Michell, 1973). A specific phosphodiesterase is also reported which converts the cyclic compound to inositol-1-phosphate (Dawson and Clarke, 1972).

(iii) Polyphosphoinositides

The PPI may be degraded by two enzymes which were originally described in aqueous extracts from brain acetone powders (Thompson and Dawson, 1962a, b, 1964a, b; Dawson and Thompson, 1964). The enzyme TPI phosphatase (phosphatidyl-myo-inositol-4, 5-biphosphate phosphohydrolase, EC 3.1.3.36), removes successively the monoesterified phosphate of TPI to yield first DPI and then PI (Change and Ballou, 1967; Prottey et al., 1968). The other enzyme, TPI phosphodiesterase (Triphosphoinositide inositol-tris-phosphohydrolase; EC 3.1.4.11), cleaves the phosphodiester bond of either TPI or DPI to give 1, 2-diacylglycerols and inositol tri- or diphosphate respectively. Recent evidence suggests that the initial formation of cyclic inositol di- and triphosphates, similar to that of inositol 1:2 cyclic phosphate from PI cleavage, does not occur (Lapentina et al., 1975). On the basis of similarity in fatty acid distribution between PPI and 1, 2-diacylglycerols, the cleavage of the diester phosphate of PPI has been suggested to be the major source of the small pool of 1, 2-diacylglycerols in brain (Keough et al., 1972).

The activity of TPI phosphatase (Fig. 5, reaction

18, 19) is depressed by the addition of diethyl ether while the TPI phosphodiesterase (Fig. 5, reaction 20, 21) is stimulated. This diesterase differs from the phosphoinositide phosphodiesterase described by Thompson (1967) in that there is no requirement for Ca^{2+} which can be replaced by Mg^{2+} (Thompson and Dawson, 1964b). TPI phosphatase, partially purified from ox brain, does not catalyze the cleavage of phosphate from PA, glycerophosphate, ATP and various sugar phosphates. It has an absolute requirement for Mg^{2+} (or Mn^{2+}) both in ox brain preparations and in rat kidney homogenates (Dawson and Thompson, 1964; Lee and Huggins, 1968a). Unlike the acetone powder extracts of Dawson and Thompson (1964), the enzyme from the dialyzed supernatant fraction of rat brain was inhibited by Na^{+} and K^{+} ions (Salway et al., 1967).

Early fractionation studies indicated that the TPI phosphatase was localized in the cytoplasmic fraction of rat and guinea pig brain (Salway et al., 1967; Harwood and Hawthorne, 1969b). Reassessment of the activities in the presence of a "pH 5 supernatant" activator gave a subcellular distribution very similar to that of 5'-nucleotidase, a plasma membrane marker (Sheltawy et al., 1972). TPI phosphatase is also a particulate enzyme in kidney cortex and appears to be assbciated with the Golgi complex (Lee and Huggins, 1968a; Cooper and Hawthorne, 1975). TPI phosphodiesterase is also largely a particulate enzyme in brain having a distribution similar to that of 5'-nucleotidase

Fig. 5: Reactions in the Metabolism of Phosphoinositides

GP + 2 fatty acyl CoA ⟶ PA + 2 CoASH.....(1)

PA + CTP ⟶ CDP-DG + PPi.....(2)

CDP-DG + enzyme ⟶ enzyme-P-diglyceride + CMP.....(3)

Enzyme-P-diglyceride + inositol $\xleftrightarrow{Mn^{2+}}$ PI + enzyme.....(4)

CDP-DG + inositol ⟶ PI + CMP.....(5)

PA + CDP-choline ⟶ CDP-diglyceride + phosphorylcholine.....(6)

CDP-choline + 1, 2-diacyl-glycerols ⇌ PC + CMP.....(7)

1, 2-diacylglycerols + ATP ⟶ PA + ADP.....(8)

Monoacylglycerol + ATP ⟶ lyso-PA + ADP.....(9)

Lyso-PA + fatty acyl CoA ⟶ PA + CoA.....(10)

PI + H_2O ⟶ inositol + PA.....(11)

PI + ATP ⟶ DPI + ADP.....(12)

DPI + ATP ⟶ TPI + ADP.....(13)

PI + $2H_2O$ ⟶ GPI + 2 fatty acid....(14)

GPI + H_2O ⟶ GP + inositol.....(15)

GP + H_2O ⟶ Pi + glycerol.....(16)

PI + H_2O ⟶ 1, 2-diacylglycerols + inositol phosphate....(17)

TPI + H_2O ⟶ DPI + Pi.....(18)

DPI + H_2O ⟶ PI + Pi.....(19)

TPI + H_2O ⟶ 1, 2-diacylglycerols + inositol triphosphate.(20)

DPI + H_2O ⟶ 1, 2-diacylglycerols + inositol diphosphate..(21)

GP, glycerol-3-phosphate; GPI, glycerophosphorylinositol

(Keough and Thompson, 1970, 1972). The TPI phosphodiesterase of kidney cortex is found predominantly in the supernatant fraction (Tou et al., 1973). During the fractionation of protozoa (C. fasiculata) the TPI phosphodiesterase follows the distribution of protein while the TPI phosphatase is concentrated in the soluble fraction (Palmer, 1973a, 1976).

d. Metabolism of Phosphoinositides in Developing Brain Homogenates

Brain homogenates from developing rats, when incubated with the appropriate precursor or substrate and all the necessary cofactors, displayed some differences in the ability to synthesize or catabolize phosphoinositides as a function of age. If these lipids are associated with myelin, increased activity of the enzymes might be expected to accompany myelinogenesis. The only report in rat brain describing the CDP-DG transferase (the enzyme responsible for PI synthesis) activity as a function of age failed to show any definite correlation with myelination. This result was therefore consistent with the presence of PI in other membranes as well as myelin (Salway et al., 1968). The PI kinase activity was reported to be greatest at 5 days of age and fell during subsequent development (Eichberg and Hauser, 1967). The results of Salway et al. (1968) are rather inconsistent for PI kinase. The two independent experiments reported for PI kinase yielded

essentially contradictory results for the period of slow maturation. One experiment was, more or less, in accord with the results of Eichberg and Hauser (1967a) and showed a declining activity. The other showed no change in enzyme activity after one day of age. Both experiments, however, showed an initial increase which preceded the period of active myelination, suggesting an early synthesis of DPI. Small amounts of DPI but no TPI have already been reported in rat brains as young as 36-48 hours postnatal (Eichberg and Dawson, 1965).

DPI kinase activity increases during the period of active myelination and continues to rise throughout subsequent development (Salway et al., 1968). This is consistent with the observation of Rossiter and Gardiner (1966) that TPI concentration increased more rapidly than DPI or PI in this period.

Catabolic enzymes of phosphoinositides have also been studied in postnatal rat brain by the same two laboratories. Hauser et al. (1967) found similar activities for both TPI phosphatase and TPI phosphodiesterase in extracts of acetone powders of brains from new-born and adult rats. In the second report, TPI phosphodiesterase was not measured and the results for TPI phosphatase are not in accord with the earlier report. Salway et al. (1968) found the TPI phosphatase activity in rat brain homogenates to increase during the active period of myelination (8-20 days postnatal). Further work is needed

to resolve these discrepancies.

Phosphoinositide metabolic enzyme activities have only been studied in brain and no information is available on PNS of any species. This dissertation will provide this information.

6. Physiological Significance of Phosphoinositides

a. Introduction

The universal presence of inositol phosphoglycerides in all eukaryote cell membranes and their rapid metabolic turnover suggest an important physiological role. There is indirect evidence which suggests their involvement in a variety of membrane functions. For example, the enzymic characteristics of Na^+/K^+-dependent ATPase (and transport of various solutes) are severely impaired in inositol-deficient mammalian cells (Charalampous, 1971); the activity of the Ca^{+2} activated ATPase in erythrocytes varies with the concentration of PPI (Buckley and Hawthorne, 1972); secretion of lipoproteins by liver and of protein by yeast depends upon an adequate supply of inositol (Matile, 1966; Yogie and Kotaki, 1969); DPI have been implicated in arsenate transport in yeast (Cérbon, 1970); ATP dependent contractile activity of mitochondria shows a specific requirement for PI (Vignais et al., 1964); cell-wall formation is impaired in inositol-deficient yeasts (Posternak, 1966) and glycosides of inositol lipids may be involved in the synthesis of certain polysaccharides

(Tanner, 1967).

The following discussion will be limited to a very brief consideration of the possible role of inositol lipids in nerve transmission and, for polyphosphoinositides, in more general functions of the plasma membrane. More comprehensive descriptions are available (Dawson, 1969; Kai and Hawthorne, 1969; Hawthorne and Kai, 1970; Hawthorne, 1973; Michell, 1975).

b. Phosphatidylinositols and Nerve Transmission

When stimulated, many tissues exhibit an increased turnover of PI and often PA as measured by the incorporation of ^{32}Pi (Hokin and Hokin, 1953, 1955, 1958a, b, c; Hokin et al., 1960; Pumphrey, 1969). The effect is coincident with the application and duration of the stimulus. A recent tabulation of stimuli (including several neurotransmitters) which provoke enhanced metabolism of PI in a number of tissues is available (see Michell, 1975). Many studies have suggested that the enhanced PI turnover in response to extra-cellular stimuli in nervous tissue is associated either directly or indirectly with synaptic transmission (Hokin and Hokin, 1958c; Redman and Hokin, 1964; Durell and Sodd, 1964), but does not accompany the conduction of impulses along the nerve trunk (Lapetina and Michell, 1973a). The effect had been ascribed to the cleavage of PI by a diesterase followed by reutilization of the diacylglycerol rather than to increased de novo synthesis

(Hokin, 1967; Durell et al., 1969; De Robertis, 1971a; Michell, 1973a, 1974; Freinkel and Dawson, 1973) or changes in the turnover of other precursors of PI (Hokin and Hokin, 1959; Hollander et al., 1970; Schacht and Agranoff, 1973; Yagihara et al., 1973).

More recently, the stimulus dependent depletion in PI has been shown to be accompanied by increases in 1, 2-diacylglycerols and PA (Hokin, 1974; Hokin-Neaverson, 1974; Bansbach et al., 1974). The effect is localized most probably in post-synaptic structures (Hokin, 1965, 1966a; Larrabee and Leicht, 1965; Larrabee, 1968) although synaptic vesicles and other membranous subfractions have not been decisively excluded (Lapetina and Michell, 1972; Lunt and Pickard, 1975). The exact mechanism by which transmitter-induced PI turnover is involved in synaptic transmission is not yet clear. It is possible that the cleavage of diester phosphate of membrane-bound PI could regulate ion transport indirectly by altering the properties of the post-synaptic membrane. De Robertis and his group (De Robertis et al., 1967; La Tarre et al., 1970; De Robertis, 1971b; Lunt et al., 1971) considered the change in membrane permeability to result from the binding of ACh to ACh-receptor proteolipids with PI cleavage occurring as a subsequent secondary event. A critical review of this field has been published recently by Michell (1975).

c. Polyphosphoinositides and Plasma Membrane Function

(i) General Considerations

Polyphosphoinositides have generally been regarded as myelin constituents. Their presence in unmyelinated immature nervous tissues and in some mature unmyelinated invertebrate nerves possessing at most a single bimolecular layer of lipid (Geren and Schmitt, 1954) suggests that they are also present in extramyelin structures. It has been suggested that the PPI may occur in the axolemma or orientated adjacent to or on the surface of the myelin, as well as buried in the myelin sheath (see Section I.C.3.b). The various enzymes responsible for their metabolism are also located at this site or in the adjacent cytosol. In view of their co-location with enzymes at the cell surface, the high metabolic turnover of their monoesterified phosphate groups in many tissues and the correlation of PPI concentrations with the adenylate energy charge in yeast (Talwalkar and Lester, 1973), it seems probable that they have some important physiological significance to plasma membrane function.

Several investigators have suggested that the PPI are directly or indirectly concerned with the active transport of cations by nervous tissue (Dawson, 1966; Standefer and Samson, 1967; Hawthorne and Kai, 1970). Experiments with brain slices (Palmer and Rossiter, 1965; Hayaski et al., 1966) and with subcellular particles or

purified enzymes have shown some effects of Na^+ and K^+ ions on ^{32}P incorporation into PPI and on the activity of certain enzymes involved in phosphoinositide metabolism. However, kinetic experiments and arguments failed to link PPI turnover directly to ion transport (Hokin and Hokin, 1964; Schneider and Kirschner, 1970; Peterson and Kirschner, 1970). No direct involvement of PPI has been described in the ATPase system which constitutes the "Na pump" (Hokin and Hokin, 1964; Glynn et al., 1965). In this system, a phosphorylated protein is a better candidate for the "carrier" molecule (McIlwain, 1963).

(ii) Polyphosphoinositides and the Permeability of the Axonal Membrane

There is some evidence that membrane bound Ca^{2+} blocks the "pores" in the axolemma through which Na^+ passes during depolarization. Calcium has been shown to enter the axoplasm of the squid giant axon during stimulation, suggesting that Ca^{2+} discharge from the membrane makes it more permeable to Na^+ (Hodgkin and Keynes, 1957). Involvement of PPI in Ca^{2+} transport or binding of Ca^{2+} to membrane which alters permeability to univalent cations is a more attractive suggestion (Dawson, 1966). In fact, considerable amounts of bound divalent cations have been shown to be associated with isolated DPI and TPI (Kerr et al., 1964). TPI has a greater affinity than EDTA for Ca^{2+} and forms a stable complex with acidic protein in its presence (Dawson, 1965). In purified myelin, there exists

a close equivalence between the number of PPI monoester phosphate groups and the total Ca^{2+} and Mg^{2+} content (Eichberg and Dawson, 1965). Generally, PPI synthesizing enzymes like other kinases are dependent on Mg^{2+} (Ca^{2+} ions are inhibitory in the presence of optimum Mg^{2+}) and TPI phosphatase is activated by Mg^{2+} ions. All these observations, coupled with the location of metabolically active PPI in excitable tissue, led Kai and Hawthorne (1969) and Hawthorne and Kai (1970) to suggest a tentative scheme linking PPI metabolism with membrane Ca^{2+} content and indirectly with the permeability of the axonal membrane to monovalent ions. They suggested that Ca^{2+} ions bound to DPI or TPI molecules might bridge and obstruct an ion channel through the membrane. Removal of the phosphoinositide monoester phosphate groups responsible for Ca^{2+} binding would make the membrane more permeable since PI have a much lower affinity for Ca^{2+}. Therefore, excitation of the membrane resulting in dephosphorylation of PPI would allow passive transport of Na^{+}. The hypothesis appears to be in accord with the location of the necessary enzymes in brain. The original proposal, however, did not recognize the significance of DPI-TPI interconversion. Hendrickson and Reinertsen (1971) suggested that DPI-TPI interconversion might itself be adequate to markedly modify Ca^{2+} binding at membrane surfaces and also to control intercellular concentrations of free Ca^{2+}. This conclusion was based on observations that glycerolphosphoryl-

inositol-4, 5-diphosphate has a considerably higher affinity for Ca^{2+} and Mg^{2+} than glycerolphosphoryl-inositol-4-phosphate, presumably because it could use the two phosphate groups on the inositol ring to form more stable chelates with divalent metal ions (Hendrickson and Reinertsen, 1969). Conversion of TPI to DPI was calculated to release 2/3 of the chelated divalent ions. However, a criticism of both models arises from the observation that the isolated PPI do not show the necessary marked specificity for binding of Ca^{2+} over other divalent cations (Hendrickson and Reinertsen, 1969; Dawson and Hauser, 1970). It would be expected that in the absence of any hypothetical modulatory factor, the PPI would bind far less Ca^{2+} than Mg^{2+} since the tissue concentrations of Mg^{2+} are much higher than Ca^{2+} concentrations (see for example, Rasmussen et al., 1972; Triggle, 1972).

If the PPI are involved in the regulation of the flux of Na^{+} ions, stimuli which evoke action potentials would be expected to increase the turnover of PPI phosphate groups. A number of studies are available reporting variable results. Stimulants such as pentylenetetrazol or electroconvulsive shocks resulted in increased labelling of PPI in gold fish brain (Schacht and Agranoff, 1972a). Norepinephrine and γ-aminobutyric acid have no specific effect on the PPI but higher concentrations of 5-hydrotryptamine may increase DPI labelling (Hokin, 1966a, 1970). Electrical stimulation of brain slices has no effect on

PPI (Pumphrey, 1969). Electric stimulation of preganglionic nerves does increase the incorporation of ^{32}Pi into PI of the ganglion and the effect is post-synaptic but the effects on PPI have been variable. Prolonged stimulation of the ganglia was without effect (White and Larrabee, 1973). Other electrical stimulation studies in nerves probably showed increased turnover of TPI, although it was difficult to demonstrate reliably (Birnberger et al., 1971; Salway and Hughes, 1972; White et al., 1974; see also White and Larrabee, 1973). ACh was shown to have no effect on PPI labelling in brain preparations (Palmer and Rossiter, 1965; Hokin, 1966b; Yagihara and Hawthorne, 1972) and in one instance, it even resulted in reduced labelling (Schacht and Agranoff, 1972b). To accommodate these findings, Kai and Hawthorne (1969) suggested that, if DPI or TPI take part in the events associated with synaptic transmission, they are more likely to be involved with presynaptic membrane. Arrival of the nerve impulse at the presynaptic terminal would be associated with the hydrolysis of monesterified phosphate groups of PPI and release of Ca^{2+} into the cytoplasm. The Ca^{2+} released would then initiate changes in the synaptic vesicle leading to the release of ACh into synaptic cleft (ACh release is dependent upon Ca^{2+} ions; Triggle, 1965). Therefore, ACh would not affect PPI turnover, as it does PI turnover, most likely, in post-synaptic structures.

Agents which block synaptic or axonal trans-

mission or which reduce the rate of rise of the action potential might be expected to inhibit the turnover of PPI phosphate groups. Studies using such agents have yielded essentially negative results. White and Larrabee (1973) reported that hexachlorocyclohexanes which block impulse conduction have little effect on PPI turnover. Similarly, tetrodotoxin, cinchocaine and ouabain have no effect on PPI metabolism in excited nerves at concentrations sufficient to abolish conduction (Salway and Hughes, 1972; White et al., 1974). However, the latter authors criticized the tetrodotoxin observation suggesting that the drug selectively blocks the outside of the channels through which Na^+ enters during depolarization. This need not interfere with the chemical events inside of the membrane such as a release of membrane bound Ca^{2+}. Therefore, it is not a good argument against the participation of PPI in the control of membrane permeability. In order to explain the preponderance of negative evidence, White and Larrabee (1973) have suggested that only very small localized changes in PPI metabolism may occur which cannot be detected when diluted by the larger pool of unaffected PPI.

(iii) Diphosphoinositides/Triphosphoinositides Interconversion and the Generation of Action Potential

Control of membrane permeability (to Na^+ or K^+) via control of Ca^{2+} binding by the TPI/DPI ratio has

remained an attractive suggestion. Torda (1972a, b; 1973a, b, c; 1974) claims to have evidence of a more direct nature in support of this hypothesis. She has proposed a mechanism for the fast generation of action potentials at cholinergic synapses. The sequence of events is initiated by the interaction of released acetylcholine with nicotinic cholinergic receptors on the post-synaptic membrane. Activation of these receptors causes changes in ion-conductances in the post-synaptic membrane which generate the action potential. She suggests that ACh combines with the regulatory subunit of TPI phosphatase causing dissociation of the active catalytic subunit resulting in the conversion of TPI to DPI. Release of Ca^{2+} from the membrane is followed by permeability changes. The regulatory subunit is claimed to be the same as or similar to the ACh receptor proteolipid described earlier (De Robertis et al., 1967; La Torre et al., 1970).

It is known that dopamine is an inhibitory transmitter which exerts its actions by raising the intracellular cyclic AMP concentration in the post-synaptic neuron (Greengard and Kebabian, 1974): the effect of cyclic AMP is to hyperpolarize the post-synaptic membrane and thus make it less sensitive to cholinergic stimulation. Torda attributed repolarization to the action of DPI kinase. She envisaged an inhibitory subunit of this enzyme which binds cyclic AMP, the result being its dissociation from the catalytic subunit.

At present, the entire experimental support of this hypothesis comes from the work of Torda. She has claimed to have isolated the regulatory and catalytic subunits of the two enzymes (TPI phosphatase, DPI kinase) and demonstrated their interactions with ACh and cyclic AMP *in vitro*. The enzyme subunits and activators in various combinations were applied extracellularly and intracellularly to cholinergically innervated neuron preparations and the electrical response recorded. This work must be treated skeptically for many reasons. Technical discrepancies occur i.e. measuring TPI phosphatase activity in a phosphate buffer by determining the release of inorganic phosphate. Even assuming the enzyme preparations to be legitimate, such preparations (prepared by the methods of Thompson and Dawson, 1964a; Dawson and Thompson, 1964) are far from pure and are subjected to extremely denaturing conditions during subunit isolation. No information on the composition or characteristics of "purified" preparations is given. The electrophysiological results are also suspect on several grounds; most notably, it is difficult to envisage how the membrane-bound inositide pool which is interconverted between DPI and TPI can have its inositol headgroup accessible both to TPI phosphatase applied extracellularly and to DPI kinase applied intracellularly. This would require the unlikely rapid translocation of the highly polar lipid and would imply that PPI are not located asymmetrically in the membrane. Also, the hyperpolarization effect of cyclic AMP and of the

catalytic subunit of DPI kinase required exogenous substrate. This would not be expected since only the DPI molecules associated with the ion-gate should be involved. Furthermore the whole scheme is at variance with the body of evidence showing that the nicotinic cholinergic receptor system can be adequately described as an acetylcholine-stimulated ionophoric membrane protein consisting of identical subunits (De Robertis and Schacht, 1974). A detailed criticism of these studies is available (Michell, 1975). Clearly, further experimentation from other laboratories is necessary to resolve the discrepancies.

(iv) Role of Phosphoinositides in Other Physiological Processes

Little effort has been made to assign a function to the cleavage of the diester phosphate bonds of all phosphoinositides (Durell and Garland, 1969). The cyclic 1, 2-inositol phosphate which results from PI cleavage has been suggested to act as second messenger analogous to cyclic AMP for those stimuli which provoke enhanced PI turnover (Michell and Laptina, 1972, 1973; Laptina and Michell, 1973a), although later information has refuted this suggestion (Freinkel and Dawson, 1973). Michell (1975) has presented a speculative diagram indicating possible relationships between PI breakdown and other receptor-linked phenomena.

Outside the nervous system, only two indications

of the involvement of PPI in physiological processes have been reported. Phagocytosis by polymorphonuclear leukocytes has long been known to increase the incorporation of radioactive precursors into PI. The effect, due to increased synthesis, has recently been reported for DPI and TPI as well (Tou and Stjernholm, 1974).

In erythrocyte membranes, PPI have been shown to bind Ca^{2+}. It was suggested that control of the membrane concentrations of PPI may regulate intracellular Ca^{2+} levels (Buckley and Hawthorne, 1972). Although membranes with higher PPI concentrations do exhibit greater Ca^{2+}-ATPase activity, the PPI are not directly involved in the active transport of Ca^{2+} out of erythrocytes (Buckley, 1974). Factors affecting Ca^{2+} metabolism may be of some interest in view of a report that Ca^{2+}-ATPase is depressed in erythrocyte membranes from cystic fibrosis patients (Horton et al., 1970). Both reduced Ca^{2+}-ATPase (Feig and Guidotti, 1974) and elevated affinity for Ca^{2+} (Eichberg et al., 1971) have been reported for hereditary spherocytosis erythrocytes. The levels of PPI and the capacity to form PPI in erythrocytes in these conditions are unknown.

II. MATERIALS AND METHODS

A. MATERIALS

1. Animals and Tissues

Fertilized eggs, 5 month old chickens, and mature hens (16 - 20 months) of the Shaver "Starcross No. 288" line of White Leghorn were obtained from Lone Pine Farm Ltd., Berwick, N. S. Eggs were incubated in a commercial incubator (Humidaire Incubator Co., New Madison, Ohio, U.S.A.) at constant temperature (37°C) and humidity (100%). The eggs were automatically turned every 2 hours. After 19 days, the eggs were transferred to a non-rotating incubator and maintained at the same temperature and humidity. Only those chicks which hatched on the 21st day of incubation were used. The hatched chicks were provided with water and housed in the incubator for 2 days. They were then transferred to cages maintained at 22°C and provided with water and chick starter feed (Maritimes Co-operative Services Ltd., Moncton, N.B.). Alternating 12 h periods of light and darkness were maintained regardless of season.

Bovine brains and spinal cords for use as a source of DPI and TPI were purchased from St. Louis Serum Co., East St. Louis, Illinois, U.S.A. They were quick-frozen at the time of collection and packed in solid CO_2 for shipment to the laboratory where they were stored at -20°C.

2. Chemicals

All chemicals and solvents were reagent grade and were used without further purification except where indicated. Methanol was redistilled when used in the isolation of TPI (Sheltawy et al., 1972). Reagent grade orcinol (Fisher Scientific Co. Ltd., Montreal, P.Q.) was recrystallized twice from benzene. An insoluble brown oil was removed from the hot benzene solution by filtration through silicone-treated paper (Whatman # IPS). Orcinol crystals were washed with cold benzene, dried in a vacuum desiccator and stored in the dark at -20°C.

D-Galactose: NAD^+ Oxidoreductase (EC 1.1.1.48, Gal-DH) from Pseudomonas fluorescens and NAD^+ were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Sodium [γ-^{32}P]ATP was obtained from Amersham-Searle Corp., Toronto, Ont., and carrier free $H_3{}^{32}PO_4$ from Atomic Energy of Canada Ltd., Ottawa, Ont. Cutscum (iso-octylphenoxypolyoxyethanol) was obtained from Fisher Scientific Co., Ltd., Montreal, P.Q.

3. Lipids

Chromatographically pure lipids (Cer, Sulf, Chol, PC, lyso-PC, PE, PS and PI) for use as standards were purchased from Serdary Research Laboratories, London, Ont. When used as the substrate in the PI kinase assay, pig liver PI were dissolved in C-M (2:1, v/v) and shaken with 0.2 vol. of

0.9% (w/v) NaCl. The lower phase was then shaken twice with "synthetic upper phase" (chloroform-methanol-water, C-M-W, 3:48:47, by vol.) containing 0.3% (w/v) NaCl. The lower phase was dried *in vacuo*, and the PI stored in chloroform at -20°C.

4. Polyphosphoinositides

a. Preparation of Triphosphoinositides

Triphosphoinositides are not available commercially. They were prepared from bovine brains and/or spinal cords by two methods. Very pure TPI for use as the substrate in enzymatic reactions were prepared by a procedure which also yielded pure DPI (to be described later). For chromatographic purposes as a marker and for use as a carrier in enzymatic assay procedures, TPI were prepared by the relatively simple method of Dittmer and Dawson (1961). In this procedure highly acidic phospholipids including DPI and TPI are recovered from the tissue residue with acidified organic solvent mixtures after preliminary removal of other lipids in neutral solvents. Triphosphoinositides are purified by utilizing their ability to bind to proteins and by fractional precipitation of their sodium salts from methanol. Thin-layer chromatographic analysis showed the product to contain TPI with some DPI (less than 8%) as the only contaminant.

b. Preparation of Pure Di- and Triphosphoinositides

The preparation of very pure DPI and TPI can be

divided into 4 stages:

(i) Preparation of Crude "Inositol Phosphatide" Fraction

The procedure is essentially the same as described by Folch (1949a, b) with two modifications. First, the initial homogenate of bovine brain in acetone was maintained at room temperature for 45 min to permit the partial conversion of TPI to DPI (Wells and Dittmer, 1965; Dittmer and Douglas, 1969). This increased the yield of DPI which are present in smaller amounts than TPI. Second, the volumes were reduced when possible in order to conserve extracting solvents. The initial product of this procedure, "cephalin", represents the ethanol and acetone insoluble fraction of the diethyl ether extract of an acetone powder of bovine brain and/or spinal cord. The yield was 20 - 22 g of cephalin powder per kg of initial tissue. The crude "inositol phosphatide" fraction is that portion of the "cephalin" which is precipitated from chloroform by the addition of 1.45 vol. of ethanol. The yield of this fraction was about 5 - 7 g per kg of initial tissue. Thin-layer chromatographic analysis of all the precipitates and solvent washes obtained during the entire course of this procedure indicated that no appreciable loss of PPI occurs (see Appendix I.a). However, such preparations contain large amounts of water-soluble contaminants (Folch 1942) as well as other phospholipids.

(ii) Purification of Crude "Inositol Phosphatide" Fraction and Conversion to Sodium Salts

The preparation and purification of the "Folch DPI" fraction from the crude "inositol phosphatide" fraction as described by Folch, (1949a, b) is both time-consuming and is accompanied by considerable losses of PPI. Instead, the crude "inositol phosphatide" fraction was first washed in the biphasic system containing acid and precipitated as the sodium salts from methanol. The procedure is a modification of the system employed by Dittmer and Dawson (1961) and Thompson and Dawson (1964a) for the partial purification of TPI.

The lipid (about 5 - 8 g) was dissolved in 100 ml of chloroform to which was added 50 ml of methanol. A biphasic system was produced by adding 0.2 vol. of N-HCl and thoroughly mixed. The lower phase was recovered (centrifugation for 20 min at 10,000 x g) and the procedure was repeated. The above procedure was again repeated with 0.2 vol. of distilled water replacing N-HCl. The lower phase was dried in vacuo and the residue dissolved in 20 ml of chloroform. The solution was placed in ice bath and 140 ml of cold redistilled methanol added. After 10 min, any insoluble material was removed by centrifugation and discarded if the amount was small. Otherwise, the precipitate was dissolved in a reduced volume of chloroform and the above procedure was repeated. The supernatant ("washed PPI") was

chilled on an ice-bath and neutralized by the addition of ice-cold 0.1 N-methanolic NaOH. After 30 min at -20°C, the precipitated phosphatides, consisting mainly of TPI with little DPI, were collected by centrifugation. Addition of acetone to the supernatant precipitated more phospholipids, principally DPI and PS (see Appendix I.b). Almost complete precipitation of the remaining DPI and TPI was achieved when the ratio of acetone/methanol was 1 (Table 6). The methanol and methanol-acetone insoluble precipitates ("washed Na-PPI" fraction) were pooled, dissolved in chloroform and stored at -20°C.

(iii) DEAE-Cellulose Batch Treatment

In order to facilitate the preparation of pure DPI and TPI by DEAE-cellulose column chromatography, an enriched sodium polyphosphoinositide fraction ("enriched Na-PPI") was first prepared. Substantial amounts of PS and PI were removed from the "washed Na-PPI" fraction by batch treatment with DEAE-cellulose.

DEAE-cellulose (Whatman Microgranular DE-32) was washed and converted to the acetate form (Hendrickson and Ballou, 1964). 100 g of this preparation was suspended in C-M-W (20:9:1, by vol.) and the slurry poured into a Buchner funnel (7 x 6.5 cm I.D. with coarse grade fritted disc). The solvent was drained off and the "washed Na-PPI" fraction (50 mg P) in 50 ml C-M-W (20:9:1, by vol.) was poured evenly over the DEAE-cellulose. The sample was allowed to soak

Table 6. Fractionation of "Sodium Inositol Phosphatide" Fraction in Methanol and Methanol-Acetone: Composition of Various Fractions

Fraction		Phosphatides			
		TPI	DPI	Others*	Total
I. Washed crude PPI	mg P	41.0	16.8	92.3	150.1
	% fraction	27.3	11.2	61.5	100.0
II. Methanol Insoluble Na-PPI	mg P	37.9	9.4	40.1	87.4
	% fraction	43.4	10.8	45.9	100.0
III. Methanol-acetone Insoluble Na-PPI	mg P	0.9	6.4	5.9	13.2
	% fraction	6.8	48.5	44.7	100.0
Na-PPI (II + III)	mg P	38.8	15.8	46.0	100.6
	% fraction	38.6	15.7	45.7	100.0
	% recovery	94.6	94.0	49.8	67.0

* PI + SPG and small amounts of EPG & CPG

Methanol was added to washed crude PPI in chloroform and after neutralization with methanolic-NaOH, the precipitate was collected. An equal vol. of acetone was added to the supernatant and the second precipitate collected. Individual fractions were then analyzed by t.l.c.

slowly into the DEAE-cellulose which then was eluted with solvent containing increasing concentrations of ammonium acetate. In a preliminary experiment, 500 ml of ammonium acetate of concentration less than 0.2 M eluted no TPI, a small amount of DPI and more than half of the other phosphatides. By increasing the volume of ammonium acetate to 1 liter and reducing its concentration to 0.05 M, it was possible to remove substantial quantities of the other phospholipids with little loss of PPI (Table 7). The PPI were then eluted with 500 ml of 0.8 M-ammonium acetate. This "enriched PPI" fraction was freed from ammonium acetate by three successive washes in the biphasic system against upper phases containing 1M-$MgCl_2$ (Tou et al., 1968). The PPI were then converted to sodium salts by washing with acid followed by neutralization in methanol and precipitation from methanol-acetone as described in the preceding section (pp. 81-82).

(iv) DEAE-Cellulose Column Chromatography

A slurry of washed DEAE-cellulose (acetate form) in C-M-W (20:9:1, by vol.) was poured into a solvent resistant SR-25 column (2.5 x 100 cm I.D., Pharmacia, Upsalla, Sweden) and packed to a constant height (80 cm) under pressure. The "enriched Na-PPI" fraction (1 - 2 g) was applied to the column in the same solvent. The column was eluted with a concave gradient of ammonium acetate rather than the linear gradient employed by Hendrickson and Ballou (1964). A six-chambered gradient former (Varigard, Buchler Instruments Inc., Fort Lee,

Table 7. Preparation of Enriched Polyphosphoinositides Fraction by DEAE-Cellulose Batch Technique

Ammonium Acetate*	Volume (ml)	Phosphatides (mg P) TPI	DPI	Others**	Total
I. None	200	-	-	4.3	4.3
II. 0.05 M	1000	0.7	1.1	10.1	11.9
III. 0.80 M	500	17.4	5.9	7.4	30.7
Lipid-P applied		19.4	7.9	23.0	50.3
Lipid-P recovered		18.1	7.0	21.8	46.9
% recovery		93.3	88.6	94.8	93.2
% composition of fraction III		56.7	19.2	24.1	100.0

* Lipid fractions were eluted from DEAE-cellulose with C-M-W (20:9:1, by vol.) containing ammonium acetate at concentrations stated. Individual fractions were washed in biphasic system against upper phase containing 1M-$MgCl_2$ and P estimated after t.l.c.

** PI + SPG + small amounts of CPG & EPG.

New Jersey, U.S.A.) was used, each chamber containing 400 ml of C-M-W having the following concentrations of ammonium acetate: 0.1, 0.1, 0.1, 0.25, 0.45 and 0.7 M. Fractions of 14 ml were collected and examined by t.l.c. to locate DPI and TPI. The chromatographic profile of these fractions is shown in Fig. 6. Ammonium acetate was removed from the fractions containing DPI and TPI and the phosphoinositides converted to sodium salts as described earlier (see pp. 81-82). The sodium salts of pure DPI and TPI preparations were stored in chloroform at -20°C. When examined by t.l.c., these pure phosphoinositide preparations were free of contamination by Pi or other phosphatides (Fig. 7). The column was regenerated by washing with 1 M-ammonium acetate in C-M-W followed by a large volume of C-M-W.

c. "Carrier Polyphosphoinositides" Preparations

"Carrier polyphosphoinositides" for use in enzyme assays was a mixture of di- and triphosphoinositides (approx. 40% DPI & 60% TPI). These preparations were composed of those fractions from the DEAE-cellulose column which contained both DPI and TPI and/or the "enriched Na-PPI" fraction obtained by the initial batch treatment with DEAE-cellulose.

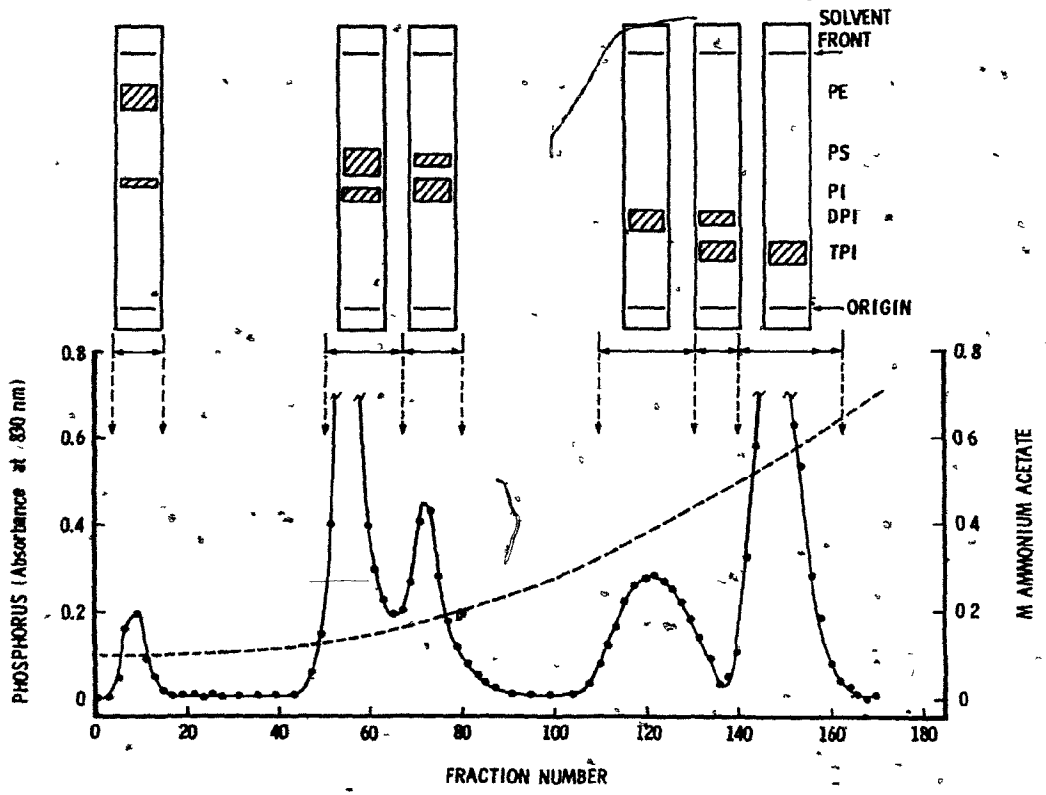


Fig. 6: DEAE-Cellulose Column Chromatography of Bovine Brain "Enriched Sodium Polyphosphoinositide" Fraction. Fractions were examined by t.l.c. using Silica Gel HR plates containing oxalate, developed in C-A-M-AA-W (40:15:13:10:7, by vol.). (●), Phosphorus measured; (---), gradient of ammonium acetate (calculated).

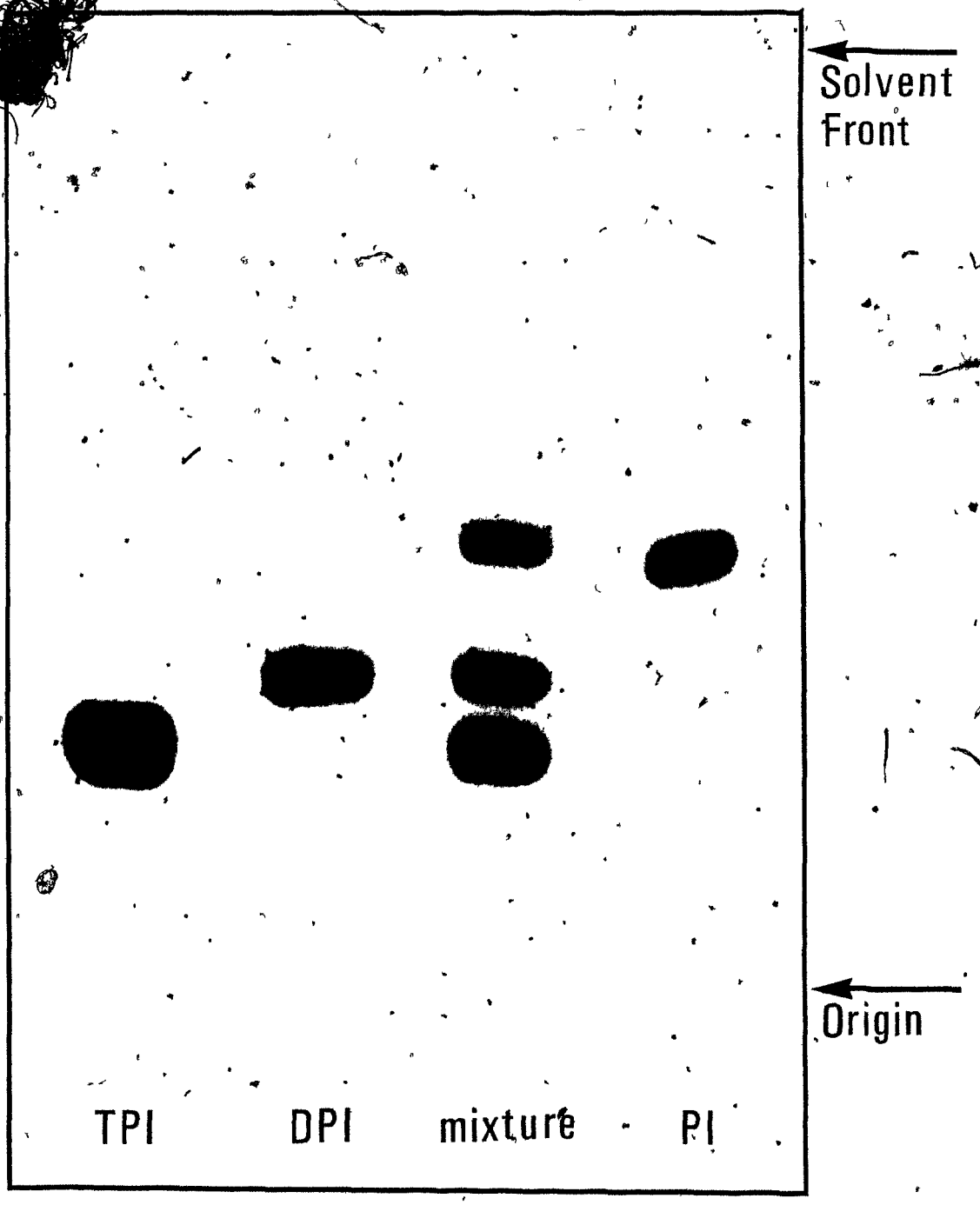


Fig. 7: Thin-Layer Chromatography of Pure DPI and TPI Prepared by DEAE-Cellulose Column Chromatography. Lipids (in µg P: TPI, 8; DPI, 5; PI, 5 and mixture of three lipids in the same amounts) were chromatographed on Silica Gel H plate impregnated with potassium oxalate (see Methods 4b & c).

B. METHODS

1. Preparation of Tissue for Lipid Analysis

Embryos, chicks and adult chickens were killed by decapitation. Brains were quickly removed, blotted dry and immediately frozen in liquid nitrogen. Sciatic nerves were dissected from the upper thigh. The nerves were cut proximally just below the spinal ganglia and distally just above the knee joint. The nerves were then quickly freed from connective tissue, rinsed with saline, blotted dry and immediately frozen in liquid nitrogen. Recovery of a whole brain or a pair of nerves from embryos and younger chicks was achieved in less than 1 min after death. The interval between death and freezing of the isolated tissues was greater for adult birds but less than 1.5 min for brain and 3 min for sciatic nerves. Both brain and sciatic nerves were weighed in the frozen state. Approximately 1 g of pooled tissue was used for each analysis. Nerves from up to 20 dozen embryos were required at the earlier ages and 4 dozen from 8 day chicks. Similarly, several brains were pooled from young embryos.

2. Extraction and Purification of Lipids

a. Extraction of Lipids from Chick Brain and Nerve

Lipids were extracted by procedures basically similar to those used previously for studies of whole brain

and nerve (Wells and Dittmer, 1967; Sheltawy and Dawson, 1969a). Lipid extracts were prepared immediately after collection of tissue. The weighed, frozen samples were placed in 15 ml of C-M (1:1, v/v) and homogenized in a Potter-Elvehjem homogenizer as soon as they began to soften. After 15 min, the tissue residue was recovered by centrifugation and re-extracted twice with 10 ml of C-M (2:1, v/v). The ratio of chloroform to methanol in the combined extracts was adjusted to 2:1 (v/v).

Polyphosphoinositides were extracted from the tissue residue with 10 ml of C-M-conc. HCl (200:100:1, by vol.). After 15 min at 37°C, the residue was recovered by brief centriguation and re-extracted twice with 10 ml portions of the same solvent.

b. Purification of Lipid Extracts

Non-lipid contaminants were removed from the C-M and C-M-HCl extracts in the biphasic system of Folch et al. (1957). The C-M (2:1, v/v) extract was shaken with 0.2 vol. of 0.9% (w/v) NaCl. The lower phase, including any interfacial material to which cerebrosides adhere, was washed twice with "synthetic upper phase" (C-M-W, 3:48:47, by vol.) containing 0.3% (w/v) NaCl. The upper aqueous phase was carefully removed and discarded. The final lower phase was clarified by adding sufficient methanol and dried in vacuo.

The residue was dissolved in moist chloroform and filtered through glass fiber paper. This washed purified lipid extract was stored at -20°C.

The crude C-M-HCl extract was shaken with 0.2 vol. of N-HCl. The upper phase and any interfacial material were discarded. The lower phase was neutralized with ammonia vapour, washed twice with "synthetic upper phase" containing 0.2% (w/v) $CaCl_2$ and dried in vacuo. The lipids were dissolved in C-M-W (75:25:2, by vol.; Sheltawy and Dawson, 1966).

3. Chemical Analysis of Lipid Extracts

a. Cholesterol Determination

Duplicate aliquots of the lipid extracted with neutral C-M containing 0.2 - 0.5 µmol lipid-P were transferred to 6 ml test tubes and evaporated to dryness. Samples were thoroughly mixed with 1 ml of isopropanol on a Vortex mixer. The tubes were covered with parafilm and left overnight to allow complete dissolution. Standards containing 0.05 - 0.20 mg Chol per ml of isopropanol were also prepared. The total Chol were then measured by the method of Block et al. (1966) as adapted for the Technicon Autoanalyzer (Method N-24a, 1966). This method is based on the formation of a colored complex when sterols having a 5-ene, 3-β-ol structure are mixed with a reagent containing ferric chloride in a mixture of acetic

and sulfuric acids. Both free and esterified Chol are measured. The Chol content of the lipid extracts were determined from the standard curve (Fig. 8).

b. Galactolipids Determination

(i) Orcinol Method

The galactose content of both brain and sciatic nerve lipids extracted with neutral C-M mixtures was determined by the procedure of Hess and Lewin (1965). In this method an orcinol-H_2SO_4 reagent reacts directly with galactolipid galactose and does not necessitate initial hydrolysis of the lipid. All glassware used for the analysis was thoroughly cleaned with chromic acid cleaning solution followed by several washes with tap and distilled water. When dry, it was stored out of contact with paper or other carbohydrate-containing material. These precautions were essential for reproducibility. The orcinol reagent was prepared using recrystallized orcinol (see Materials, p. 85). The reagent was stable for one week when stored in the dark at -20°C.

Aliquots (in triplicate) of the lipid extracts containing 0.5 - 4.0 µg of galactolipid galactose were placed in 12 ml round-bottom tubes fitted with glass stoppers. The solvent was evaporated under dust free nitrogen. The procedure of Hess and Lewin (1965) was followed except that 3 ml of cold (-20°C) orcinol reagent was added to each tube and

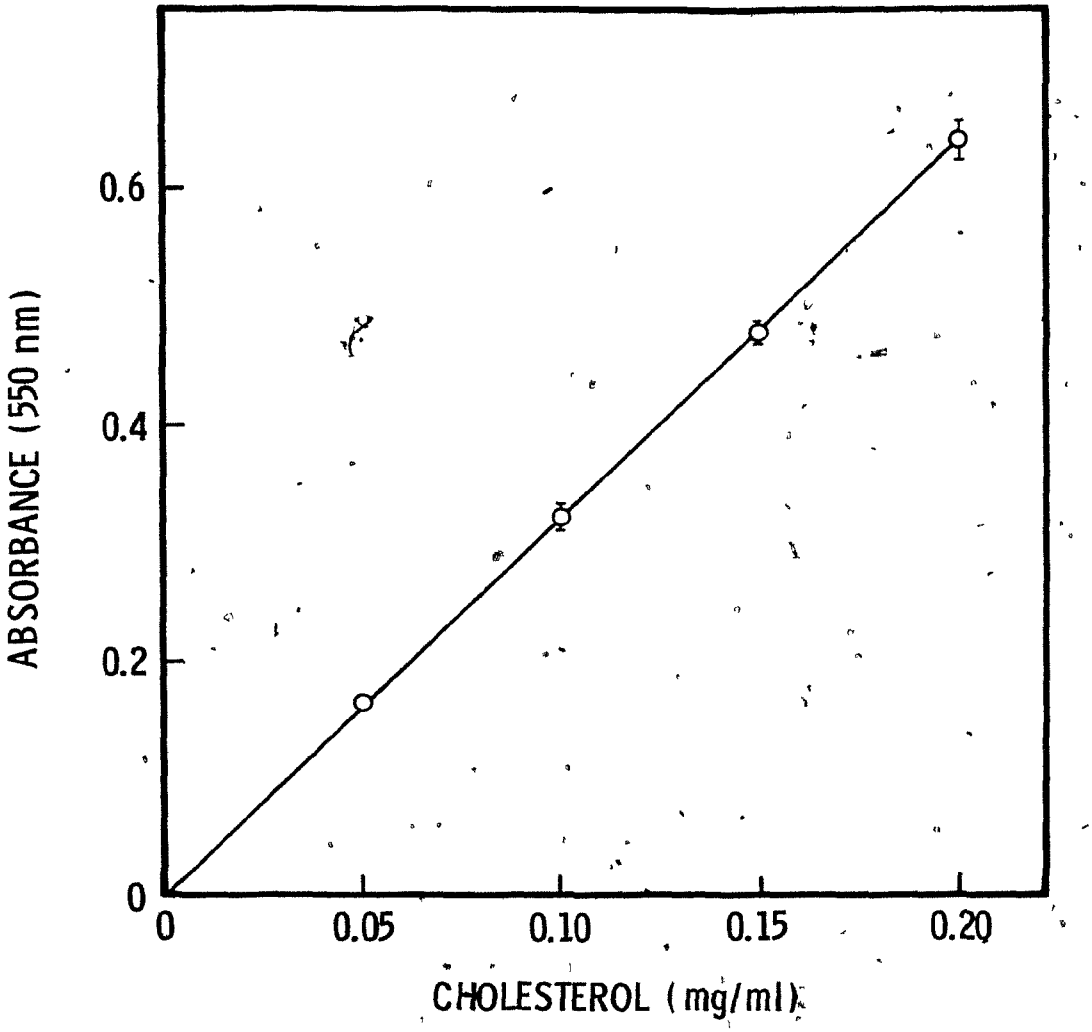


Fig. 8: Cholesterol Standard Curve as Determined with the Technicon Autoanalyzer. Values represent the mean ± S.D. (where S.D. is of sufficient magnitude to plot) for 7 determinations.

the heating in a metal block at 100°C was increased to 40 min. This was found to be necessary for maximum color yield. The galactose standard curve obtained by this method is shown in Fig. 9. Galactolipid content was calculated by multiplying the galactose content by a factor of 4.6 (Svennerholm, 1956; Soto et al., 1966). The molar content of galactolipids was determined by assuming a mean molecular weight of 846 (Soto et al., 1966).

(ii) Galactose Dehydrogenase Method

Measurement of galactolipids by this method requires initial hydrolysis of the lipid. The galactose content is then measured enzymatically using Gal-DH. The oxidation of galactose to galactonic acid is measured by following the reduction of NAD^+ spectrophotometrically. The assay has been described by Finch et al. (1969) and was followed with the exception of buffer concentration which was reduced from 500 to 200 mM. The requirement that the reaction go to completion was satisfied after 30 min when 0.2 µmol or less of galactose was present. A linear standard curve was obtained (Fig. 10).

It was necessary to establish the optimum conditions for hydrolysis of the lipid samples since it was not described by Finch et al. (1969). In these experiments, 1 µmol cerebrosides (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) dissolved in C-M (2:1, v/v) was placed in a glass vial and the solvent evaporated

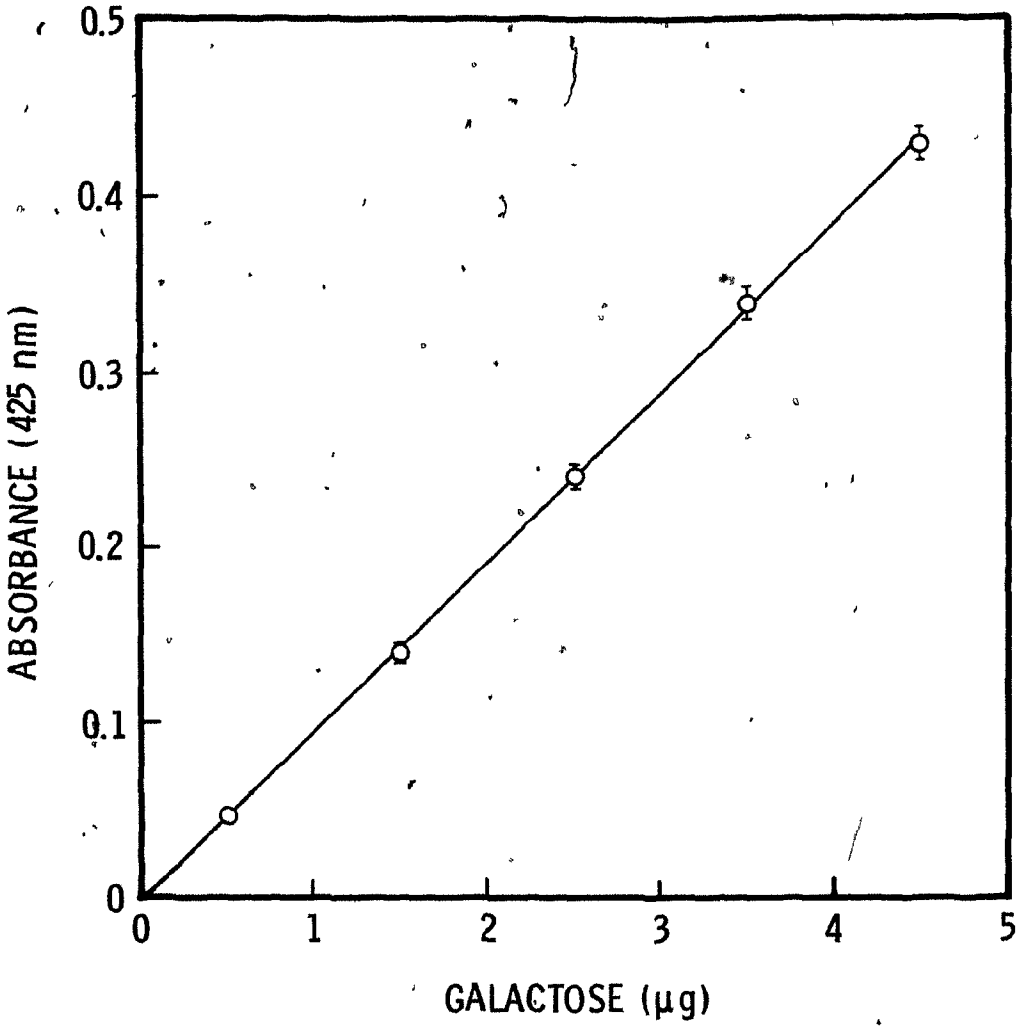


Fig. 9: Galactose Standard Curve as Determined by the Orcinol-H_2SO_4 Method. Values represent the mean ± S.D. (where S.D. is of sufficient magnitude to plot) for 7 determinations.

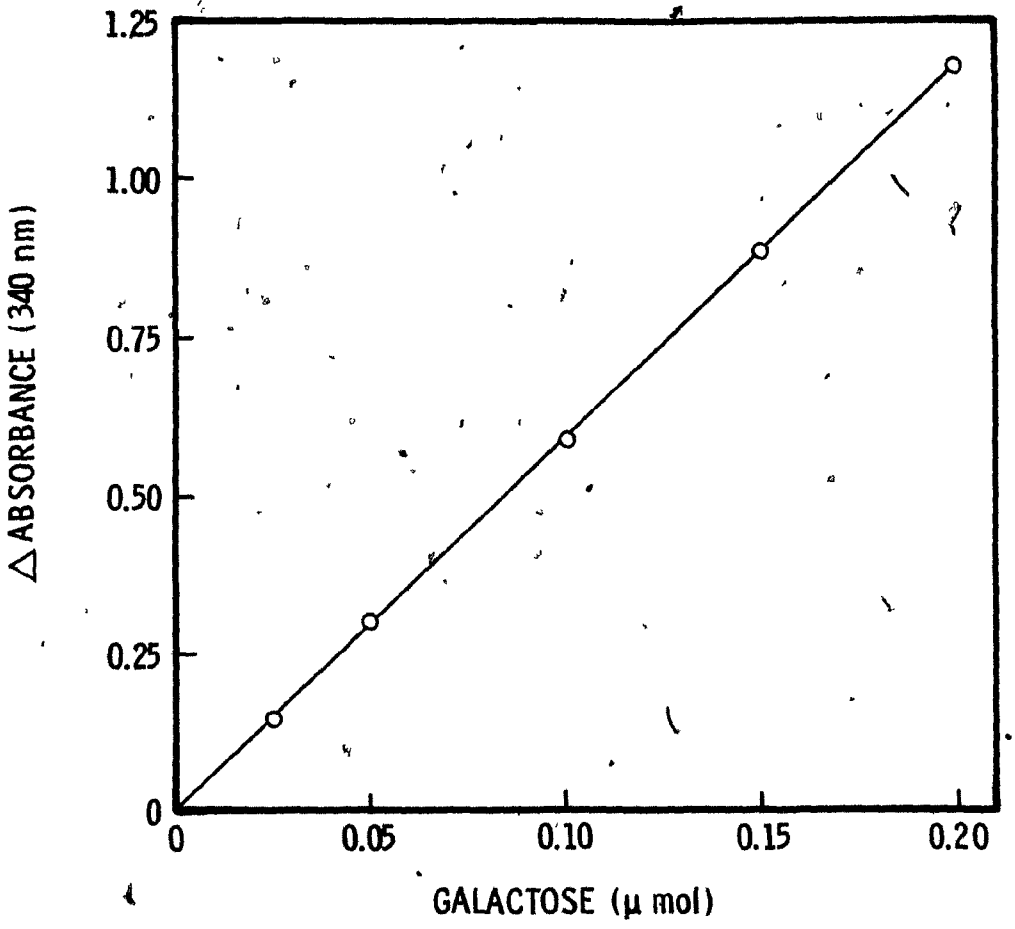


Fig. 10: Galactose Standard Curve as Determined by Gal-DH Assay. Values represent the mean ± S.D. (where S.D. is not of sufficient magnitude to plot) for 5 determinations.

under nitrogen. Ethanol (Svennerholm, 1956) could not be used to solubilize the sample because its presence in the final hydrolysate was found to affect the enzymatic assay (i.e. 20 - 25% inhibition at a final concentration of 2 - 5%, v/v, ethanol). The cerebrosides were therefore suspended in 0.5 ml water by vigorous mixing and gentle warming. To this was added 2 ml of dilute H_2SO_4. The vial was sealed and heated in an oven. After cooling, the hydrolysate was neutralized with a calculated amount of 14M-NH_4OH, diluted to a known volume and filtered through pre-washed glass fiber paper. The pH was checked with indicator paper. The results of hydrolysis in 2 and 3N-H_2SO_4 studied as a function of both time and temperature are given in Appendix II.a. The optimum conditions were 3N-H_2SO_4 at 100°C for at least 3 h for standards containing 0.2 - 1.5 µmol of cerebrosides. Prolonged hydrolysis (7 - 10h) resulted in lower recoveries. When standard galactose solutions were treated similarly, the recovery declined during the first few hours of heating (Appendix II.b). This loss was not observed with cerebrosides or when bovine serum albumin (BSA, 1 mg) was added to a galactose standard. However, BSA increased the blank absorption in the subsequent enzymatic assay. When brain lipid extracts were hydrolyzed under standard conditions (no BSA), similar blank absorbance was observed which increased with increasing time of hydrolysis. The optimum time of hydrolysis for maximum yield of galactolipid galactose varied from 3 to 6 h

for lipid extracts of chick brain and sciatic nerve of different ages. Each lipid sample (duplicate) was therefore hydrolyzed for at least three different time periods. A typical time study pattern for both brain and nerve lipid extracts is shown in Appendix II.c. It was confirmed that glucose and sucrose (alone or in combination) did not interfere with analysis of galactose standards or lipid hydrolysates.

(iii) Determination of Sulfatide Content

The lipids which were extracted from both brain and sciatic nerve with neutral C-M mixtures were chromatographed on small columns of Florisil (Rouser et al., 1961). Cholesterol, ceramide and phospholipid fractions were discarded and the content of the sulfatides-containing fraction estimated spectrophotometrically by the method of Kean (1968). This procedure is based upon the formation of a complex, between the water-soluble cationic dye Azure A and anionic sulfolipids, which is preferentially extracted into chloroform.

4. Analysis of Phospholipids by Chromatographic Procedures

a. Thin-Layer Chromatography of Phospholipids Extracted with Neutral Chloroform-Methanol

The washed preparations, originally extracted with C-M mixtures, were separated by t.l.c. on precoated

Silica Gel HR plates (20 x 20 cm, 250 μm thick layer; Analtech Inc., Newark, Delaware). These plates were stored packed in their original boxes and were not further heat-activated before use unless they had been stored for a long time and failed to provide adequate resolution. In this case, they were reactivated at 120°C for 45 min. Lipid samples dissolved in chloroform were applied as thin bands (10 - 15 μg lipid-P/cm of plate) with Hamilton syringes. The plates were developed in paper-lined chromatographic tanks containing developing solvent which had been allowed to equilibrate for at least 30 min in a temperature-controlled cabinet at 21 - 22°C. The developing solvent consisted of chloroform-methanol-acetic acid-water (C-M-AA-W, 100:45:20:7, by vol.). The solvent was replaced after two plates had been developed. The developed chromatoplates were dried in air and the lipids located by exposure to iodine vapour or spraying with the acid-molybdate reagent of Dittmer and Lester (1964). Charring with 98% (w/w) H_2SO_4 - 37% (w/w) formaldehyde (97:3, v/v) was used when a permanent record of a plate was desired. Ninhydrin (1, 2, 3-triketohydrindene hydrate) solution in acetone (0.2%, w/v) was occasionally used for detecting phospholipids containing free amino groups.

The solvent system clearly separated EPG, SPG, PI, CPG, Sph and lyso-PC (Fig. 11). Neutral lipids (mono- di- and triacylglycerols, cholesterols, free fatty acids etc.) and other phospholipids (PA, DPG etc.) migrated

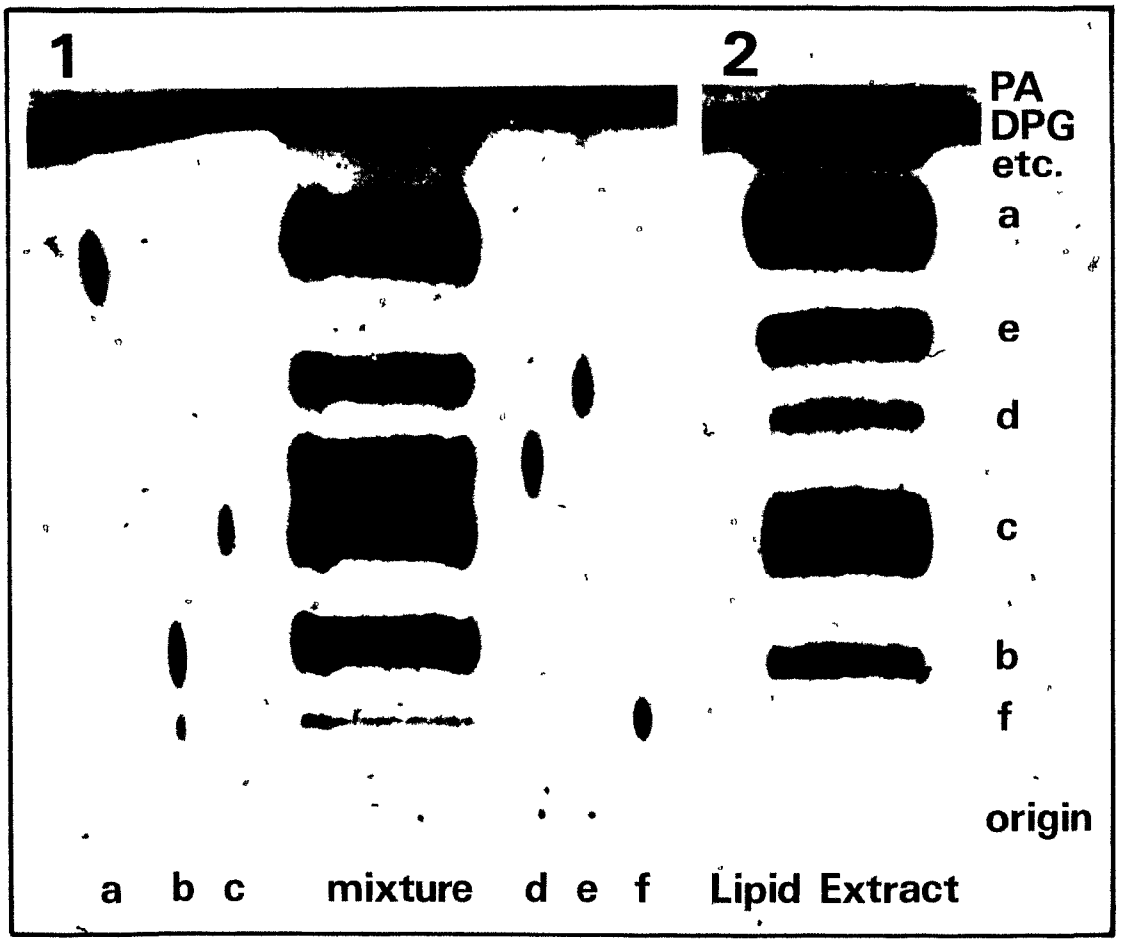


Fig. 11: Thin-Layer Chromatography of Lipids Extracted with Neutral Chloroform-Methanol.
1. Reference phospholipids: (a) PE, (b) Sph, (c) PC, (d) PI, (e) PS, (f) lyso-PC. Mixture consists of all reference phospholipids (40 µg P).
2. 10 day chick brain lipid extract (30 µg P).
Precoated Silica Gel HR plates (250 µm thick layer) were used, plates developed with C-M-AA-W (1, 100:45:20:7; 2, 100:45:20:8, by vol.). Lipids were charred with H_2SO_4-formaldehyde solution (97:3, v/v) at 100°C for 1 hour.

at the solvent front. Resolution was sensitive to variations in temperature and also varied with different batches of pre-coated plates. The proportions of water and methanol in the solvent were the major factors controlling separation and these were altered slightly in order to achieve optimum resolution. For example, low Rf values for EPG (lower than 0.7) were increased by increasing the proportion of water or acetic acid. The proportion of methanol determined the separation of CPG and PI. Separation of PI and SPG was a function of the proportions of both methanol and water.

b. Thin-Layer Chromatography of Phospholipids Extracted with Acidified Chloroform-Methanol

The washed phospholipid preparations, originally extracted with acidified C-M, were separated on thin-layers of Silica Gel H containing oxalate (Gonzalez-Sastre and Folch -Pi, 1968). In initial studies, acid-washed glass plates (20 x 20 cm) were coated with a uniform layer (250 µm) of Silica Gel H (E. Merck A.G., Darmstadt, Germany) which was prepared as a slurry of 30 g Silica Gel in 70 ml of 1% (w/v) potassium oxalate. Preparative layers (500 µm) were prepared from a slurry containing 52 g of Silica Gel in 85 ml of 1% potassium oxalate. Plates were coated using an adjustable Desaga Model S-II applicator (Canadian Laboratory Supplies, Montreal, P.Q.). In later work, precoated Silica Gel HR plates (20 x 20 cm, 250 µm thick

layer, Analtech Inc., Newark, Delaware, U.S.A.) were sprayed with a 1% aqueous solution of potassium oxalate until the surface was wet. The plates were dried in air at room temperature and activated at 120°C for at least 30 min before use. The lipids were dissolved in C-M-W (75:25:2, by vol.) or C-M-conc. HCl (200:100:0.1, by vol.) and applied as narrow bands (5 - 7 µg lipid-P/cm). When C-M-HCl was used, the bands were neutralized by brief exposure to ammonia vapour. The plates were developed at room temperature in a paper-lined chamber containing either n-propanol-4N-NH_4OH (2:1, v/v; Gonzalez-Sastre and Folch-Pi, 1968) or chloroform-acetone-methanol-acetic acid-water (C-A-M-AA-W, 40:15:13:10:7, by vol.). Rf values of phospholipids in these systems are given in Appendix III.a. The Rf values were different from the published values (Gonzalez-Sastre and Folch-Pi, 1968); however, the relative position of the bands was the same.

c. Identification and Analysis of Phospholipids Separated by Thin-Layer Chromatography

Phospholipids were isolated by preparative t.l.c. The individual lipid bands were located by brief exposure to iodine vapour. After the iodine had dissipated, the bands were scraped onto a Glassine Powder Paper (E. Lilly and Co., Indiana, U.S.A.) and transferred to 25 ml conical centrifuge tubes. The phospholipids (except phosphoinositides) were extracted (Skipski et al., 1964)

by suspending the Silica Gel with vigorous agitation on a Vortex mixer (Scientific Industries, Springfield, Massachusetts, U.S.A.) in two successive portions of the developing solvent, then in methanol and finally in M-AA-W (95:1:5, by vol.). Each extract was recovered by brief centrifugation. The solvent composition of the pooled extracts was adjusted to that of the biphasic system of Folch et al. (1957) containing NaCl as described earlier. Phosphoinositides were recovered from Silica Gel by three successive extractions of C-M-conc. HCl (200:100:1, by vol.). After neutralization with ammonia vapour, the extract was washed in a biphasic system containing $CaCl_2$ as described earlier (see Methods, p. 98). The isolated phospholipids were tentatively identified by comparing their chromatographic mobilities with those of known standards by t.l.c. using several neutral (Wagner et al., 1961a), acidic (Skipski et al., 1964; Getz et al., 1970) and basic solvent systems (Abramson and Blecher, 1964; Kuhn and Lynon, 1965), and in two dimensional systems of Rouser et al. (1969) and Getz et al. (1970). As an additional means of identification, the isolated lipids were also examined in the sequential hydrolytic procedure of Dawson et al. (1962). For inositol-containing lipids, the phosphorus to inositol ratio was determined also.

For quantitative analysis, the Silica Gel containing the located lipid bands was scraped from the t.l.c. plates. The phosphorus content was measured after

digestion with perchloric acid (see Methods, 5.a).

d. Ethanolamine Plasmalogen Determination

Ethanolamine plasmalogen content was determined in lipid preparations extracted with neutral C-M mixtures by rechromatography of the isolated EPG (see Methods, 4.a) following acid hydrolysis of the alk-1-enyl acyl analogue to 1-lysophosphatidyl-ethanolamine (Paltauf, 1971). The EPG fraction was isolated by preparative t.l.c. and washed in the biphasic system as described in the preceding section. This lipid was spotted on a Silica Gel HR plate and exposed to HCl vapour. After removal of HCl fumes under a stream of nitrogen, the chromatogram was developed, PE and lyso-PE visualized and the phosphorus content determined as described elsewhere (see Methods, 5.a).

5. Other Analyses

a. Phosphorus Estimation

Phosphorus was determined by the method of Bartlett (1959) after digestion with $HClO_4$ to convert organic phosphates to inorganic phosphate. The method is based on the reaction of inorganic phosphate with ammonium molybdate to form a "phosphomolybdate" complex which, when reduced, is blue.

Aliquots (duplicate) of the washed lipid extracts containing 0.5 - 10.0 µg P were transferred to 18 x 150

mm glass test tubes and organic solvents removed by evaporation. Phosphorus-containing areas of thin-layer chromatograms (25 - 300 mg Silica Gel) were scraped off and transferred to tubes. One ml of 70 - 72% (w/w) $HClO_4$ and 2 glass beads were added to each tube and the samples were digested for 25 min on an electric heating rack mounted on a shaker in a fume hood. The tubes were cooled to room temperature and phosphorus estimated by the method of Bartlett (1959). Tubes containing Silica Gel were centrifuged briefly after color development and the absorbance of the supernatants measured. The samples were corrected for paper or Silica Gel blanks. Recovery of reference phospholipids after t.l.c. and phosphorus determination is given in Appendix III.b.

b. Inositol Estimation

Samples of phosphoinositides were hydrolyzed in 6N-HCl at 105°C for 16 h in sealed vials. The hydrolysates were dried in vacuo over KOH pellets, the residues dissolved in 2 ml H_2O and the fatty acids removed by two extractions with diethyl ether. Traces of ether were removed at 37°C under stream of air and aliquots (duplicate) were taken for phosphorus estimation. Inositol content was determined chemically by the method of Lornitzo (1968). This method is based upon the controlled oxidation of inositol with chromate to form inosose which reacts

with furfural in the presence of H_2SO_4 to form a colored compound (probably 1, 2, 3, 5-tetrahydroxybenzene). All the parameters of the method were checked. It was necessary to increase the oxidation time to 12 min from the recommended 8 min in order to obtain the maximum color yield. A typical standard curve is shown in Fig. 12.

c. Nucleic Acids Analysis

The nucleic acid content of chick brain and sciatic nerve was determined by spectrophotometry. After extraction of lipids the tissue residue was washed successively with 10 ml portions of C-M (2:1, v/v), 95% ethanol and ice-cold 10% (w/v) trichloracetic acid, then dehydrated with ethanol and diethyl ether. The product was digested in N-KOH at 37°C for 16 h and the nucleic acids determined spectrophotometrically (Santen and Agranoff, 1963).

d. Protein Estimation

Protein content of chick brain and nerve homogenates was determined by the method of Lowry et al. (1951) using BSA as standard.

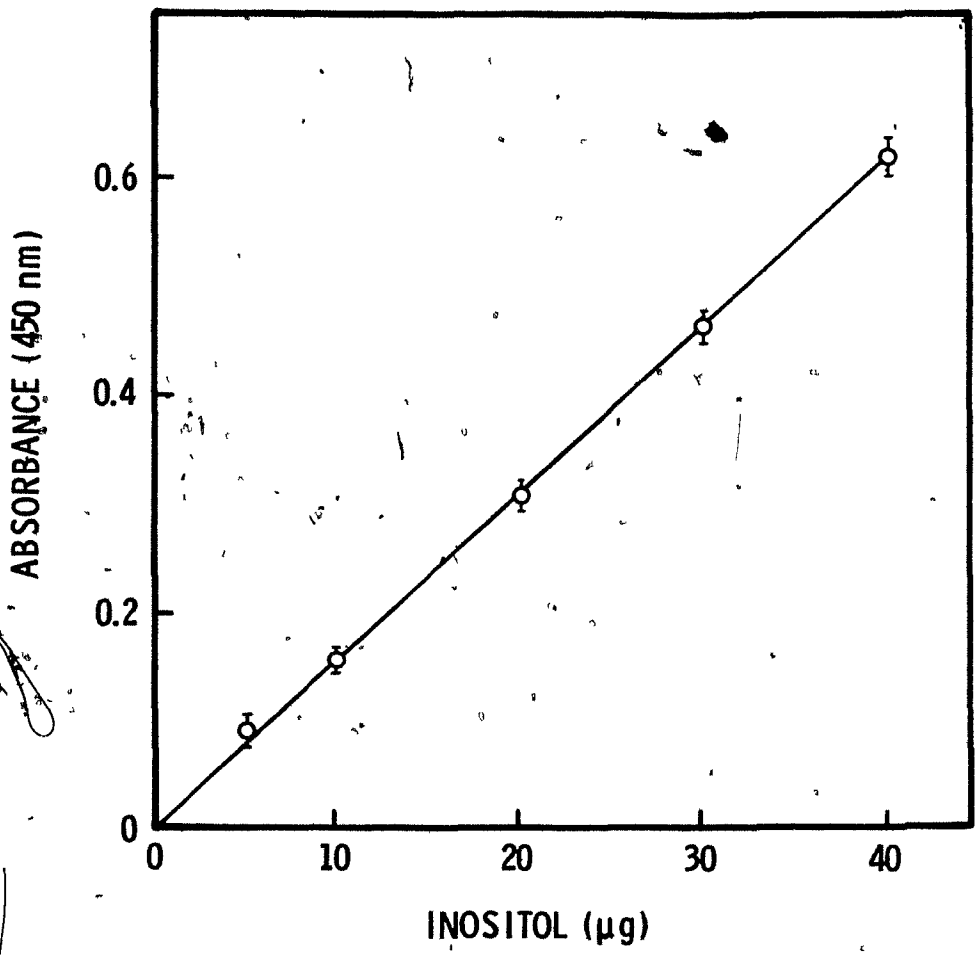


Fig. 12: Inositol Standard Curve by the Chemical Method. Values represent the mean ± S.D. for 10 determinations.

6. Enzyme Assays

a. Preparation of Tissue Homogenates

Unless otherwise specified, all preparations were maintained at 0 - 4°C. Chicks and embryos were killed by decapitation. Brains were removed and 10% (w/v) homogenates in 0.32 M-sucrose containing 0.5 mM - Tris-HCl (pH 7.4) were prepared with a Polytron (Kinematica, Luzern, Switzerland). Sciatic nerves were dissected from up to 5 doz embryos and collected in buffered sucrose. Connective tissues were stripped from the nerves and a 15% (w/v) homogenate prepared by initial treatment with a Polytron followed by grinding in a Potter-Elvehjem homogenizer. The final suspension was diluted to give a 10% (w/v) homogenate.

b. Phosphoinositide Kinases

Phosphatidylinositol kinase (PIK) and diphosphoinositide kinase (DPIK) activities were assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the appropriate products. After several preliminary experiments, the following assay procedures were adopted.

The incubation mixture for PIK measurements contained 45 mM - Tris-HCl (pH 8.3), 30 mM-$MgCl_2$, 5 mM-[γ-^{32}P]ATP (5 - x 10^6 d.p.m./assay), 1 mM-PI and 0.1 ml of a 5% (w/v) brain homogenate or 10% (w/v) nerve homogenate in a total volume of 0.5 ml. Buffer, $MgCl_2$, ATP

and PI were mixed together and preincubated at 37°C for 2 min. The reactions were started by addition of the tissue homogenates (warmed to 37°C by preincubation for 2 min) and incubation continued for 15 min. The same system was employed for DPIK except that 0.5 mM-DPI was used as the substrate and the pH of the incubation mixture was maintained at 7.4. In experiments where non-ionic detergent (Cutscum) was added to the incubation mixtures, the following Cutscum to protein ratios were used for optimum activity: PIK (brain and nerve), 7:1; DPIK (brain), 0.6:1; DPIK (nerve), 3:1. Optimum substrate requirements remained the same except in the case of DPIK (nerve) where 1 mM-DPI was used in place of 0.5 mM as described above.

In both assays the reactions were stopped by adding 2 ml of ice-cold 0.3 N-methanolic HCl followed by 2 ml of chloroform. After 10 min at 4°C, a biphasic system was formed by adding 2 ml of chloroform containing "carrier PPI" (aprox. 30 µg P) and 1 ml of 1N-HCl. The upper phase and the interfacial layer were carefully removed and discarded. The lower phase was washed twice with 2.25 ml of "synthetic upper phase" (C-M-W, 3:48:47, by vol.) containing 0.02% (w/v) $CaCl_2$. The washed extracts were taken to dryness under nitrogen, dissolved in 0.5 ml of C-M-W (75:25:2, by vol.) or C-M-conc. HCl (200:100:0.1, by vol.) and applied as 2 cm bands to t.l.c. plates coated with Silica Gel HR containing oxalate. Two successive additional washings of the tubes with solvent were

required for quantitative transfer of the samples. The polarity of the developing solvent was increased to move the phosphoinositides further up the chromatogram (C-A-M-AA-W, 40:15:13:12:8, by vol.) and to reduce possible contamination with residual radioactive [γ-^{32}P]ATP. Areas containing DPI and TPI were located by a brief exposure to iodine vapour and scraped into counting vials containing 1 ml water. Following addition of 10 ml of Aquasol (New England Nuclear, Boston, Massachusetts, U.S.A.), ^{32}P was measured by scintillation counting (Unilux I, Nuclear Chicago, Des Plaines, Illinois, U.S.A.).

c. Phosphoinositide Hydrolases

Triphosphoinositide phosphatase and triphosphoinositide phosphodiesterase activities were assayed by measuring the release of inorganic and organic phosphate respectively. To accommodate a large number of samples, an autoanalyzer was used for these determinations. After several preliminary experiments, the following assay procedures were adopted.

(i) Triphosphoinositide Phosphatase

The incubation mixture contained 45 mM - Tris-HCl (pH 7.2), 0.2 M-KCl, 2 mM-EGTA, 1 mM-$MgCl_2$, 2.8 mM-TPI, 5.6 mM-CETAB and 0.05 ml of a 2% (w/v) brain or 10% (w/v) nerve homogenate in a total volume of 0.25 ml. All components except TPI-CETAB were preincubated together

in 2 ml centrifuge tubes for 10 min at 0 - 4°C, then warmed to 37°C. Triphosphoinositides were dried under nitrogen and thoroughly suspended in water. The reactions were started by addition of TPI or, when required, with the TPI-CETAB mixture (prepared by mixing CETAB with the TPI solution). The incubation was continued for 20 min at 37°C. The reactions were stopped by the addition of 0.05 ml of ice-cold 10% (w/v) BSA containing 10 mM-EDTA followed by 0.2 ml ice-cold 10% (w/v) $HClO_4$. The tubes were chilled on an ice bath for 15 min after thorough mixing on a Vortex mixer. Following centrifugation, 0.2 ml duplicate aliquots of each supernatant were diluted to 1 ml with water and Pi determined as described in the following section. Zero time controls were run simultaneously and test values were corrected.

(ii) Analysis of Inorganic Phosphate Using the Autoanalyzer

Inorganic phosphate was determined by the method of Van Belle (1970) using an autoanalyzer (Technicon Corp., Ardsley, New York, U.S.A.). The method measures the spectral shift which occurs when the dye methyl green reacts with phosphomolybdate in dilute acid. The advantage of this procedure is that the sample is exposed only briefly to relatively dilute acid (1.2 N-HCl) without heating. The danger of hydrolyzing organic phosphates in the sample is greatly reduced while retaining a sensitive response to Pi.

The manifold for the determination of Pi is outlined in Appendix IV.a. Sample tracings of Pi analysis (standards, reproducibility, peak separation and continuous uptake) are shown in Appendix IV.b.

The presence of $HClO_4$ up to 1% (w/v) concentration and metal ions up to 3 mM were reported not to affect the response (Van Belle, 1970). It was confirmed that $MgCl_2$, KCl, EGTA and $HClO_4$ at concentrations encountered in the assay procedure had no effect on the response (Appendix IV.c) and that increased acidity (more than 1%, w/v, of $HClO_4$) of the final solution decreased the response. Slight alkalinity increased the response. The high sensitivity of the method permitted the samples to be diluted sufficiently to avoid interference. The presence of TPI (at a concentration of 2.8 mM) in the assay mixture does not interfere with the recovery of added Pi (Appendix IV.d). When CETAB was added to standard Pi solutions, the response was greatly reduced. At 0.1 and 0.3 mM the response fell to 76% and 28% of the control respectively. At 0.5 mM or higher, peak deformation and baseline tagging occurred. However, the presence of CETAB (up to 5.6 mM) in the incubation mixture had no effect, suggesting that most, if not all, of the CETAB is precipitated along with the BSA by the $HClO_4$. The remaining amount (if any) is too small to affect the response (Appendix IV.d).

(iii) Triphosphoinositide Phosphodiesterase

The reaction mixture contained 45 mM - Tris-HCl (pH 7.2), 0.2 M-KCl, 0.2 mM-$CaCl_2$, 2.8 mM-TPI, 5.6 mM-CETAB and 0.05 ml of a 2% (w/v) brain or nerve homogenate in a total volume of 0.25 ml. After preincubation at 37°C for 10 min, the reactions were started by the addition of a TPI or TPI-CETAB mixture and the incubation continued for 7 min. Reactions were stopped with 0.15 ml of ice-cold 15% (w/v) BSA followed immediately by 0.1 ml ice-cold 20% (w/v) $HClO_4$. After mixing on a Vortex mixer, the tubes were chilled for 15 min in an ice-bath. Following centrifugation, 0.2 ml aliquots of the supernatants were taken for Pi analysis by Autoanalyzer. Aliquots (0.2 ml) were also taken for total phosphate analysis as described below. The release of Po was then calculated by difference and used as a measure of TPI phosphodiesterase activity. Zero time controls were run simultaneously and test values were corrected.

(iv) Analysis of Total Phosphate Using the Autoanalyzer

Aliquots (0.2 ml) from the above assay were digested with 0.25 ml of 72% (w/w) $HClO_4$ for 12 min with constant shaking. After cooling to room temperature the volumes were diluted to 4 ml and Pi (now representing the total-P of the sample) was measured by the automated version of the method of Fiske and Subbarow (1925) which is less sensitive to the presence of $HClO_4$ than the method of Van Belle (1970) described earlier (see p. 118). The principle is the same as that

described for the procedure of Bartlett (1959). To increase the sensitivity, the volume of sample taken into the system was increased over that in the standard method (Technicon N-4b). Compensation for the volume change was achieved by reducing the volume of acid-molybdate and increasing the concentration of this reagent.

The manifold for the determination of phosphate is outlined in Appendix IV.e. Example tracings of analyses (standards, reproducibility, peak separation and continuous uptake) are shown in Appendix IV.f. The response of the method was not adversely affected by $HClO_4$ at concentrations higher than those encountered (Appendix IV.g).

Triphosphoinositides and TPI-CETAB in assay controls gave values for the release of acid-soluble phosphate when the amount of added BSA was low (equal to that used in the TPI phosphatase assay). This suggested that TPI were not completely precipitated. The effect was greater with TPI-CETAB mixtures. The problem was avoided by increasing the amount of BSA added. Appendix IV.h shows the effectiveness of the various systems employed. The effect of CETAB was increased when Mg^{2+} or EDTA was added to BSA solution but could be minimized by using 0.15 ml of 15% (w/v) BSA alone.

III. RESULTS

A. CHEMICAL COMPOSITION OF THE DEVELOPING CHICKEN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

1. Investigation of Lipid Extracts

The technique used to extract lipids was similar to that of Wells and Dittmer (1967) and Sheltawy and Dawson (1969a). The neutral C-M mixtures are commonly used to extract neutral glycerides, glycerophosphatides (but not PPI), cholesterols and glycolipids. The PPI are effectively extracted with C-M mixtures containing HCl (Dawson and Eichberg, 1965). It was confirmed in the present study that three successive extractions each with 10 ml portion of neutral C-M mixtures (for 1 - 1.5 g tissue) effected almost complete removal of usual lipids with the exception of PPI from chick neural tissues. Additional extracts were found to contain less than 2% of the extractable lipid-P (Table 8). Polyphosphoinositides were recovered from the C-M extracted tissue residue with acidified solvents. Again, three successive extractions with 10 ml portions of solvent were sufficient for almost complete removal of these lipids (Table 9). Further extractions increased the recovery by less than 3%.

The neutral C-M lipid extracts, after purification in a biphasic system (Folch et al., 1957), were fractionated by a relatively simple one-dimensional t.l.c. technique developed for this purpose (see Methods). All the major glycerophosphatides e.g. Sph, CPG, PI, SPG and

Table 8. Yield of Lipid Phosphorus in Successive Extracts Made with Chloroform-Methanol

Extract No.	Brain		Sciatic Nerve	
	µgP/g wet wt.	% total Lipid-P	µgP/g wet wt.	% total Lipid-P
1	426.1	33.6	395.7	23.3
2	733.1	57.8	1076.6	63.4
3	90.0	7.1	193.6	11.4
4	15.2	1.2	23.8	1.4
5	3.8	0.3	8.5	0.5
6	N.D *	N.D	N.D	N.D
Total	1268.2	100.0	1698.2	100.0

Values are means of duplicate analyses. Chick neural tissues (21 day embryonic) were extracted once with 10 vol. C-M (1:1, v/v) followed by successive extractions with 10 vol. each of C-M (2:1, v/v). The extracts were washed in biphasic system and phosphorus determined.

* N.D - Not detectable

Table 9. Yield of Lipid Phosphorus in Successive Extracts Made with Chloroform-Methanol-conc. HCl.*

Extract No.	Brain		Sciatic Nerve	
	µgP/g wet wt.	% total Lipid-P	µgP/g wet wt.	% total Lipid-P
1	23.2	75.2	44.4	66.4
2	4.0	12.9	14.3	21.4
3	1.8	5.8	5.3	7.9
4	0.5	1.6	1.3	1.9
5	0.1	0.3	0.3	0.5
6	N.D#	N.D	N.D	N.D
Residue**			trace	
IFM+	1.3	4.2	1.3	1.9
Total	30.9	100.0	66.9	100.0

Values are means of duplicate analyses.

* 4 day old chick brain and sciatic nerve were first extracted 3 times with neutral C-M mixtures followed by successive extractions (10 vol. each) with C-M-conc. HCl (200:100:1, by vol.). Extracts were washed in the biphasic system containing calcium and phosphorus determined.

** Residue after 6th extraction was boiled in acetone and then in ethanol, dried and extracted once more with C-M-HCl. Extract was washed in the biphasic system.

+ Interfacial material obtained during washings of successive extracts with N-HCl, pooled, rinsed with C-M (2:1, v/v) and treated as above (**).

N.D. - Not detectable

EPG were well separated (see Fig. 11). No lyso-phospholipids could be detected in lipid extracts although lyso-PC move well behind Sph in this system. Other minor phospholipids such as phosphatidylglycerols, phosphatidylglycerol phosphates, PA and DPG were not separated and moved together at the solvent front. The recovery of phospholipids from t.l.c. was 99.3 ± 2.3%(±S.D.,51 determinations). Characterization of the major phospholipids separated by this system is described in the Methods. This t.l.c. system, which gave adequate and reproducible separation of phospholipids, was favored over two-dimensional t.l.c. systems (Rouser et al., 1969; Getz et al., 1970) where the quantity of sample which could be applied is limited and over the one-dimensional system of Skipski et al. (1964) which, in this laboratory, did not resolve PI and PS satisfactorily.

The lipids extracted with acidified C-M were separated on Silica Gel H t.l.c. plates impregnated with oxalate (see Methods). These lipid extracts contained DPI, TPI, PI and PS (occasionally traces of PE). The phospholipids, isolated by preparative t.l.c., were identified by co-chromatography with authentic standards in several solvent systems (see Methods). The amounts of PI and PS, although very small, were added to the values for these lipids in the extracts made with neutral solvents. The recovery of phospholipids after t.l.c. was 95 - 98%. The identity of isolated phosphoinositides was

confirmed by determining their phosphorus to inositol ratios (Table 10).

It has been known for some time that the PPI are hydrolyzed post mortem if the necessary precautions to freeze the tissue immediately after death are not taken. In several early experiments, chick neural tissues were frozen immediately in liquid N_2 after death and stored at -20°C for 5 - 30 days before extraction of the lipids. The values for PPI were much lower than when the tissues were dissected into liquid N_2 and extracted immediately (Table 11). Recoveries of other phospholipids were unaffected. Therefore the lipids were extracted from all tissues immediately after collection in liquid N_2.

2. Chemical Composition

The prime objective of this study was to investigate changes in the phospholipid composition, particularly the phosphoinositides, in relation to the maturation of the CNS and PNS of the chicken. The relative merits of the different ways in which analytical data from developing tissues are best presented have often been discussed and none are totally satisfactory (McIlwain, 1959; Rouser et al., 1972). All lipid analyses in this study are presented relative to wet weight. As background to lipid analyses and to permit other modes of evaluation of this data, several other parameters were also measured. The weights of both brains and sciatic nerves are given in Table 12. Although speed of dissection

Table 10. Characterization of Phosphoinositides: Phosphorus to Inositol Ratios

Phospholipid	Phosphorus:Inositol (molar ratio)
PI*	1.07 ± 0.10(6)
DPI	2.03 ± 0.17(3)
TPI	3.06 ± 0.16(3)

Values are the mean ± S.D. for (n) determinations. Phosphoinositides, isolated by preparative t.l.c., were hydrolyzed in acid solution. Phosphorus and inositol were estimated as described in Methods.

* No significant difference in phosphorus to inositol ratio was observed for PI extracted with neutral or acidified C-M mixtures.

Table 11. Effect of Storage of Frozen Tissue on the Levels of Polyphosphoinositides

	Brain (19 day embryo)		Sciatic Nerve (4 day chick)	
	Fresh+	Frozen*	Fresh	Frozen**
C-M Extract (μgP/g wet wt.)	1075.7	1109.8	2377.8	2340.9
C-M-HCl Extract (μgP/g wet wt.)	12.6	7.0	54.6	31.0
C-M-HCl Extract components (nmol/g wet wt.)				
PS	28.1	20.0	153.0	168.7
PI	24.5	21.9	90.0	102.6
DPI	33.9	14.0	32.3	21.6
TPI	89.7	50.7	459.0	191.6

Data presented as means of at least 3 determinations except the values for C-M extract are means of 10 determinations.

\+ Extracted same day.

* Kept at -20°C for 11 - 17 days; ** kept at -20°C for 19 - 25 days.

to permit rapid freezing of tissues was the primary concern, care was taken to remove all of the sciatic nerve lying along the length of the femur. The values for nerve therefore represent an anatomic unit. The protein content of brain and sciatic nerve was also determined and is presented in Table 12. RNA and DNA were measured as an indication of changes in protein synthesizing activity and cell numbers (Table 13). Tissues from old birds (16 - 20 months) were analyzed to permit consideration of the concentration changes relative to adult levels as recommended by Wells and Dittmer (1967).

Myelination in the chick CNS commences at the 12th - 14th day of embryonic development (El-Eishi, 1967) while in sciatic nerve it begins a little later at 15 - 17 days (Geren, 1954). The period of most rapid myelination occurs in the following week during which the mass of the brain doubles (Kurihara and Tsukada, 1968). In the present study the weight of the brain increased by 70% from 15 day embryo to 2 day chick; the protein content was increased only by 51% over a similar period. The increase in weight was greater in sciatic nerve (88 - 90%) while the protein content increased by 33% over the period from 17 days of embryonic age to 4 days after hatching. The smaller increase in protein content relative to the increase in weight reflects rapid deposition of lipid-rich myelin membranes which is more apparent in sciatic nerve. This is consistent with the decrease in nucleic acid

Table 12. Protein Content and Weight of Developing Chick Brain and Sciatic Nerve*

	Whole Brain		Sciatic Nerve	
Age (days)	wet. wt. (g/brain)	Protein (mg/g wet wt.)	wet wt. (g/50 nerves)	Protein (mg/g wet wt.)
Embryo				
11	0.165±0.002(10)	48.4±1.1(3)	-	-
13	0.280±0.016(10)	47.0±2.4(3)	-	-
15	0.489±0.011(10)	51.0±2.1(6)	0.147±0.013(3)	32.7±7.6(4)
17	0.647±0.031(10)	60.6±3.8(6)	0.208±0.020(3)	43.7±2.5(5)
19	0.729±0.013(10)	67.0±1.2(6)	0.335±0.040(3)	47.6±2.4(6)
21	0.781±0.013(10)	75.7±1.9(8)	0.453±0.035(3)	52.8±2.0(5)
Chick				
2	0.827±0.011(10)	77.0±3.1(8)	0.545±0.024(3)	56.8±1.4(5)
4	0.885±0.032(10)	78.2±2.3(7)	0.630±0.028(3)	58.0±2.7(6)
8	0.934±0.030(10)	83.5±3.3(7)	0.600±0.050(3)	59.5±4.3(5)
21	-	83.8±4.1(6)	-	61.6±5.9(5)
140-180	2.240±0.140(11)	-	7.140±1.070(10)	-
Adult	3.480±0.260(7)	87.7±7.2(6)	11.790±3.420(6)	38.2±7.5(1)

* Data expressed as mean ± S.D. (number of determinations).

concentrations during maturation (Table 13). Most of the decrease occurred during the periods mentioned above, the decrease being about 15% for brain and 44% for sciatic nerve. The reduction of RNA concentration was greater than DNA concentration in both tissues. Such differences are obvious when RNA/DNA and protein/DNA ratios of both tissues are compared (Table 14). The former ratio decreased for both brain and sciatic nerve, the decrease being 11% and 29% respectively. The protein/DNA ratio is higher for brain than sciatic nerve and increased 1.5 - 2.0 fold during such period. These differences are probably indicative of an increase in cell mass without cell proliferation. As might be expected, the sciatic nerve exhibited a greater decrease in nucleic acid concentrations, relatively greater increase in weight and a lesser increase in protein content than brain since it contains proportionately less cytoplasm and more myelin.

3. Lipid Analyses

Galactolipids were measured primarily to provide a chemical index of myelination. Galactolipid measurements made by the two methods gave the same average values (Fig. 13). The enzymic method was more time-consuming and required a larger sample but is more specific and generally gave less variable results than the more widely used orcinol reaction. The galactolipids recorded in Fig. 13 represent only those which are present in the washed lipid extracts

Table 13. Nucleic Acid Content of Developing Chick Brain and Sciatic Nerve*

	Whole Brain		Sciatic Nerve	
	RNA mg/g wet wt.	DNA mg/g wet wt.	RNA mg/g wet wt.	DNA mg/g wet wt.
Embryo				
11	2.23±0.08(3)	1.99±0.05(3)	-	-
13	2.19±0.07(3)	1.48±0.02(3)	-	-
15	2.17±0.08(8)	0.91±0.24(8)	2.22±0.08(14)	1.64±0.06(4)
17	2.14±0.13(8)	0.88±0.20(8)	1.92±0.13(4)	1.49±0.11(4)
19	1.89±0.07(4)	0.67±0.03(4)	1.55±0.10(4)	1.37±0.10(4)
21	1.88±0.06(6)	0.68±0.02(6)	1.49±0.12(4)	1.33±0.13(4)
Chick				
2	1.94±0.10(6)	0.73±0.09(6)	1.22±0.07(6)	1.13±0.08(6)
4	1.69±0.03(4)	0.72±0.04(4)	0.92±0.15(4)	1.00±0.20(4)
8	1.22±0.06(7)	0.62±0.06(7)	0.48±0.07(3)	0.81±0.03(3)
140-180	1.00±0.03(3)	0.49±0.03(3)	0.26±0.05(3)	0.44±0.03(3)
Adult	1.01±0.06(4)	0.23±0.02(4)	0.35±0.06(5)	0.29±0.06(5)

* Data expressed as mean ± S.D. (number of determinations)

Table 14. RNA/DNA and Protein/DNA Ratios in Developing Chick Brain and Sciatic Nerve

Age (days)	Whole Brain		Sciatic Nerve	
	RNA/DNA	Protein/DNA	RNA/DNA	Protein/DNA
Embryo				
11	1.12	24.3	-	-
13	1.48	31.8	-	-
15	2.39	56.0	1.35	19.9
17	2.43	68.9	1.29	29.3
19	2.82	100.0	1.13	34.7
21	2.77	111.0	1.12	39.7
Chick				
2	2.66	106.0	1.08	50.3
4	2.35	109.0	0.92	58.0
8	1.97	135.0	0.59	124.0
140-180	-	-	0.59	-
Adult	4.39	381.0	1.21	109.0

Ratios are calculated from the data presented in Tables 12 & 13.

and could be expected to consist of Cer, Sulf and galactosyl diglycerides. Gangliosides partition into the upper phase washes and were not recovered. Galactosyl diglycerides have been shown to be very minor components of the galactolipid fraction in both brain and sciatic nerve (Wells and Dittmer, 1967; Inoue et al., 1971; Yates and Wherrett, 1974) and were not measured separately. Sulfatides represented 5% of the galactolipids prior to hatching and increased thereafter attaining 12% at 8 days and 21% in both the 5 month and adult brains. The proportion of galactolipid represented by Sulf in sciatic nerve remained constant at 21% for all ages. A considerable proportion (30% of adult) of the galactolipid was already present in the 15 day embryo brain and the increase to adult levels occurred over a short 5 day span beginning with the 18 day embryo. Very little increase occurred after the chicks were 4 days old. The values reported in Fig. 13 for brain are identical to those in an earlier study (Garrigan and Chargaff, 1963) in which the oldest brain analyzed was from a 1.5 day old chick. In nerve the change was much greater and occurred over a longer time period. Only 12% of the adult concentration was present in 15 day embryo nerves rising to 90% in the 8 day chick nerves. Since Cer constitute the major portion of the galactolipids, the deposition of total galactolipids in both chick neural tissues could be used to define the period of most active myelination: 18 day chick embryo to 1 day chick for brain

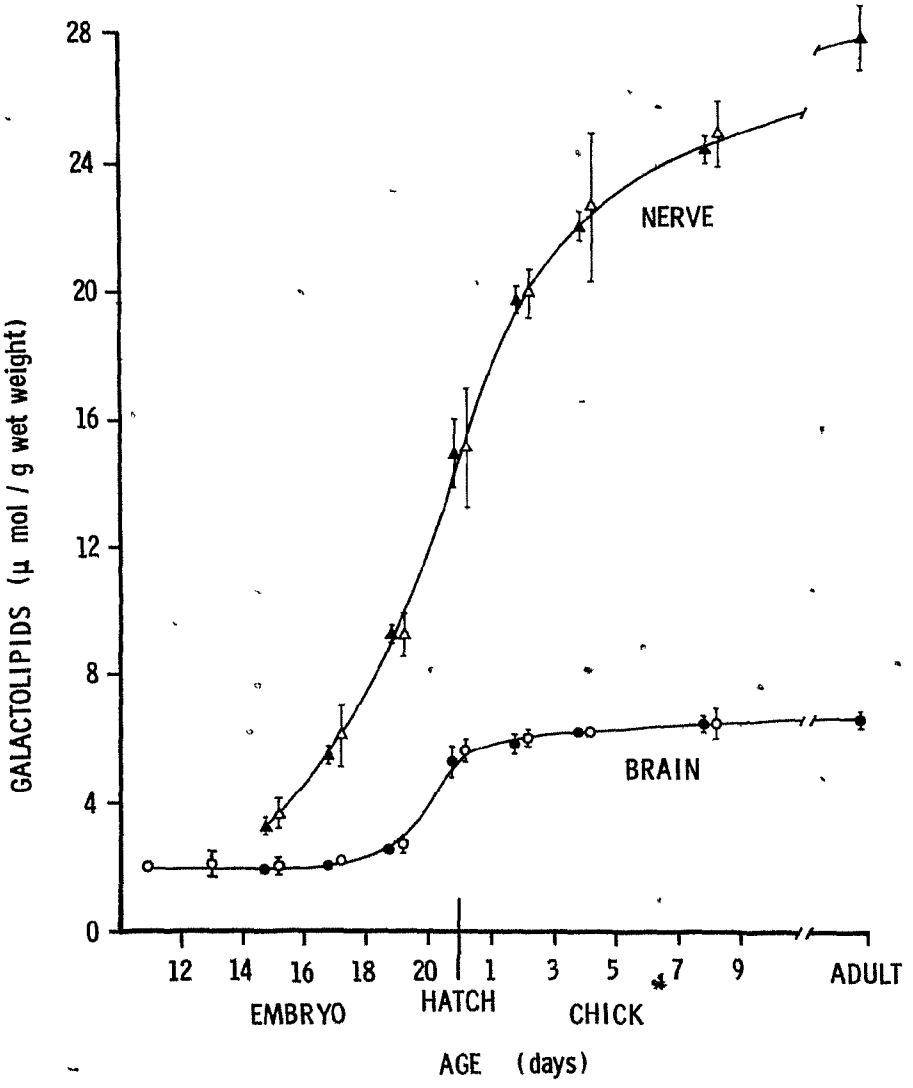


Fig. 13: Deposition of Galactolipids (Cerebrosides + Sulfatides + Galactosyl Diglycerides) in Developing Chick Brain and Sciatic Nerve. Values are means ± S.D. (where S.D. is of sufficient magnitude to plot) of 3 to 5 determinations. Galactolipids were determined by both the orcinol method (open symbols) and the D-galactose: NAD^+ oxidoreductase method (solid symbols).

and 17 day chick embryo to 4 day chick for sciatic nerve.

Both the Chol and total phospholipid concentrations (Table 15) increased rapidly between the 16 day embryo and the 4 day chick. This was followed by a slower rate of increase to the adult concentrations. The initial concentrations were the same in brain and nerve but the rate of increase and the adult values were greater in sciatic nerve. The molar Chol/total phospholipids ratio changed very little in brain throughout maturation. In sciatic nerve, the ratio increased slightly during or close to the period of active myelination (15 day embryo to 8 day chick) and changed very little thereafter (Table 16). The obvious increase in galactolipid/phospholipid ratio of brain occurred over a 3 day span between 19 - 21 days of embryonic age and remained unchanged during subsequent development. The sciatic nerve exhibited an increase in this ratio from late embryonic age to 8 days after hatching and thereafter showed no change. These observations indicate that the 3 major catagories of lipids (i.e. Chol, galactolipids and phospholipids) accumulate during development with relatively different rates but the major deposition of these lipids occurs over a short time period and is generally coincident with the myelinogenesis.

The concentrations of phospholipid classes in developing chick brain and sciatic nerve are given in Tables 17 and 18. Changes occurring throughout development in the nerve and brain were similar although their magni-

Table 15. Total Phospholipid and Cholesterol Concentrations in Developing Chick Brain and Sciatic Nerve

	μmol/g wet weight*			
	Brain		Sciatic Nerve	
Age (days)	Cholesterol	Phospholipid	Cholesterol	Phospholipid
Embryo				
11	10.2±0.6(3)	22.3±0.8(3)	-	-
13	11.4±0.4(3)	22.9±0.5(3)	-	-
15	13.5±1.8(3)	27.0±1.1(14)	16.5±3.2(3)	24.6±4.5(7)
17	17.1±2.1(3)	28.3±1.2(13)	23.8±2.4(3)	38.9±3.7(10)
19	21.0±1.8(3)	34.7±3.3(13)	35.5±3.6(2)	47.1±5.0(11)
21	23.9±2.2(4)	41.5±2.2(19)	46.1±4.3(3)	54.3±4.5(10)
Chick				
2	26.3±1.9(5)	44.2±1.9(16)	52.5±5.5(5)	62.5±4.9(13)
4	28.0±1.9(3)	46.3±1.9(13)	60.0±2.9(3)	76.7±6.8(13)
8	31.2±1.5(6)	46.9±1.6(7)	66.6±3.8(3)	71.8±2.6(3)
12	29.2±1.2(4)	51.8±2.3(8)	-	-
140-180	37.7±1.0(4)	52.1±5.6(9)	72.5±5.1(4)	81.8±10.0(6)
Adult	40.0±0.9(5)	59.3±0.9 (5)	76.7±0.7(5)	88.0±1.9(5)

*Data expressed as mean ± S.D. (number of determinations)

Table 16. Cholesterol/Phospholipid and Galactolipid/Phospholipid Ratios in Developing Chick Brain and Sciatic Nerve

Age (days)	Cholesterol /Phospholipid		Galactolipid /Phospholipid	
	Brain	Sciatic Nerve	Brain	Sciatic Nerve
Embryo				
11	0.457	-	0.088	-
13	0.498	-	0.090	-
15	0.500	0.671	0.073	0.140
17	0.604	0.612	0.073	0.150
19	0.605	0.754	0.077	0.198
21	0.576	0.849	0.133	0.278
Chick				
2	0.595	0.840	0.135	0.318
4	0.605	0.782	0.135	0.293
8	0.665	0.928	0.138	0.345
140-180	0.724	0.886	-	-
Adult	0.675	0.872	0.123	0.348

Data calculated from Fig. 13 and Table 15.

tude was generally greater in nerve. Choline phospholipid increased by less than 50%, achieving adult levels in the 8 day chick brain while no change was observed in sciatic nerve. The proportion of total EPG represented by the plasmalogen form was 41.1% in the 11 - 13 day embryo brain which then increased in the following 8 days to reach the adult value of 51.6% at 21 days. The ethanolamine plasmalogen content of embryonic sciatic nerve was initially 41.1% of total EPG in the 15 day embryo. The proportion of this lipid then increased rapidly to 46.6% and 58.5% in the 19 day embryo and 2 day chick nerve respectively. In this tissue the adult proportion of 73.2% was achieved 8 days after hatching. In contrast to brain the concentration of PE in sciatic nerve changed very little during development and virtually all of the increase seen in the concentration of total EPG was due to the plasmalogen form. The increase in SPG and Sph that occurs in both tissues was not restricted to the period of active myelination. Phosphatidylinositols increased in both tissues, the increase being greater in nerve and not restricted to the period of active myelination. The phospholipids which moved at the solvent front on t.l.c. (PA, DPG, etc.) showed a general increase in brain but remained about the same in sciatic nerve throughout development. Both PPI were present in 15 day embryo brain and increased greatly up to the 4 day chick. After this period, DPI declined slightly while TPI increased steadily throughout subsequent maturation. Neither PPI were detectable in

Table 17. Phospholipid Composition of Developing Chicken Brain*

Age (days)	n	µmol/g wet weight						nmol/g wet wt.	
		CPG	EPG	SPG	Sph	Others**	PI	DPI	TPI
Embryo 11	3	10.2±0.90	6.1±0.3	2.0±0.1	0.5±0.10	0.8±0.1	1.4±0.40	13.2± 1.3	48.2± 6.4
13	3	10.8±0.50	6.9±0.4	2.3±0.1	0.4±0.10	0.8±0.2	1.1±0.20	13.6± 0.9	49.6± 4.8
15	3	12.2±0.50	7.9±0.4	2.8±0.2	1.1±0.20	1.0±0.1	1.5±0.20	15.2± 2.4	54.5±10.8
17	4	13.5±0.30	8.9±0.2	3.2±0.3	1.5±0.30	0.8±0.1	1.4±0.10	24.5± 6.6	72.8± 9.1
19	3	14.7±0.40	11.3±0.1	4.2±0.3	1.6±0.03	1.0±0.1	1.6±0.03	33.2± 6.9	89.7±15.6
21	6	18.2±0.70	13.6±0.5	4.2±0.2	1.8±0.30	1.1±0.1	1.8±0.04	67.9±13.9	122.0±25.1
Chick 2	3	18.8±0.04	14.3±0.3	4.5±0.1	2.0±0.03	1.2±0.1	1.8±0.03	100.0±11.8	116.7±16.6
4	3	18.9±0.60	15.1±1.5	5.0±0.4	2.5±0.30	1.4±0.2	1.5±0.04	116.1±13.2	207.7±23.9
8	7	18.9±0.90	15.9±0.4	5.3±0.2	2.5±0.20	1.6±0.4	2.4±0.08	101.6± 3.6	269.0± 9.1
12	3	19.2±1.50	18.1±1.4	5.4±0.4	3.7±0.50	-	2.2±0.30	142.3± 4.8	381.0± 6.9
140-180	3	19.7±2.00	22.2±1.6	6.1±0.5	3.9±0.40	1.9±0.5	2.2±0.20	102.1± 7.3	546.4±15.2
Adult	5.	21.1±0.60	22.6±0.3	7.0±0.2	4.6±0.30	2.2±0.2	1.8±0.20	115.8±16.5	620.9±13.2

* Data presented as means ± S.D. for n determinations.

** Represents µmol lipid-P, includes phosphatidylglycerols, phosphatidylglycerol phosphates, PA and DPG.

Table 18. Phospholipid Composition of Developing Chicken Sciatic Nerve*

Age (days)	n	µmol/g wet weight						nmol/g wet weight	
		CPG	EPG	SPG	Sph	Others**	PI	DPI	TPI
Embryo 15	3	12.9±0.10	8.0±0.10	3.0±9.10	2.4±0.05	1.1±0.1	1.5±0.03	ND+	ND
17	3	13.5±0.05	11.3±0.30	4.2±0.20	5.0±0.20	1.6±0.2	1.6±0.08	13.1± 1.0	74.8± 5.5
19	3	13.9±0.05	13.6±0.05	5.8±0.02	7.1±0.03	2.0±0.4	1.7±0.01	21.5± 2.9	175.2± 21.6
21	3	14.3±1.10	19.3±1.00	7.8±0.50	9.7±0.40	2.2±0.7	1.7±0.20	30.9± 2.1	292.7± 18.2
Chick 2	3	14.1±0.40	22.5±0.60	8.1±0.20	11.3±0.30	2.3±0.6	1.8±0.04	37.3±4.8	363.5± 41.1
4	4	16.5±0.30	31.5±0.30	12.3±0.10	16.3±0.20	2.1±0.1	2.3±0.30	31.9± 2.8	459.1± 41.8
8	3	15.0±0.20	26.6±0.30	11.9±0.10	13.7±0.30	2.5±0.6	2.3±0.10	45.2± 2.6	538.2± 6.9
140-180	3	14.9±1.50	30.5±1.00	16.1±0.70	20.3±0.09	2.4±0.7	3.4±0.80	68.3± 5.1	772.3± 40.4
Adult	5	12.9±0.30	30.5±1.10	16.8±1.40	22.0±1.30	2.2±0.6	3.6±0.10	67.2±15.0	875.3±126.2

* Data presented as means ± S.D. for n determinations.

** Represent µmol lipid-P, includes phosphatidylglycerols, phosphatidylglycerol phosphates, PA and DPG.

\+ Not detectable.

the 15 day embryo nerve. Both were deposited rapidly during the period of active myelination followed by a steady increase to the adult level. Sciatic nerve exhibited a characteristically low DPI/TPI ratio which has been observed by others (Sheltawy and Dawson, 1966).

B. PHOSPHOINOSITIDE KINASES OF THE DEVELOPING CHICKEN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

1. Investigation of Assay Procedure

Assay procedures for both PIK and DPIK have been described for use with homogenates of a variety of tissues (Hokin and Hokin, 1964; Michell and Hawthorne, 1965; Colodzin and Kennedy, 1965; Hajra et al., 1965, 1968; Kai et al., 1966, a, b, 1968; Michell et al., 1967; Tou et al., 1968, 1970; Harwood and Hawthorne, 1969a, b; Iacobelli, 1969). In these systems, the source of enzyme was incubated with the appropriate substrate in the presence of [γ-^{32}P]ATP and the appearance of radioactivity in the isolated product measured. The conditions of incubation were variable and somewhat different means of isolating the reaction products were employed. Final separation of phosphoinositides was achieved chromatographically on formaldehyde-treated paper (Kai et al., 1966, 1968), silicic acid-impregnated glass fiber paper (Tou et al., 1968, 1970) or thin-layers of Silica Gel H impregnated

with potassium oxalate (Gonzales-Sastre and Folch-Pi, 1968).

a. Measurement of Radioactivity

The most convenient method for the radioassay of phospholipids following separation by t.l.c. is the direct counting by liquid scintillation spectrometry of the lipid-containing Silica Gel scraped from the plate. The recovery of radioactivity in ^{14}C- and ^{3}H-labelled lipids varies with the class of phospholipid and the composition of the scintillation mixture (Webb and Mettrick, 1972). Smaller variations in counting efficiency would be expected with ^{32}P; however, the PPI adhere more tenaciously to Silica Gel than other phospholipids. Both toluene and xylene-based scintillation systems were investigated using ^{32}P-labelled PPI obtained from a standard DPIK assay system in which DPI was also labelled by the action of DIK on endogenous PI. The phosphoinositides were extracted and separated by t.l.c. as described in "Methods". The DPI and TPI bands were scraped into vials containing scintillation mixtures. The recovery of radioactivity in the xylene-based mixture (Aquasol) was better than that achieved in the toluene-based scintillation mixture even when the Silica Gel was maintained in suspension by the addition of sufficient Cab-o-Sil (Packard Instrument Co., Downers Grove, Illinois, U.S.A.) to form a stable gel (Table 19). Addition of water to Aquasol marginally increased the

Table 19. Recovery of Radioactivity from ^{32}P-labelled Polyphosphoinositides Absorbed onto Silica Gel

Scintillation Mixture	Radioactivity (c.p.m.) net
Toluene+ (10 ml)	1250
Toluene++ (10 ml) + Cab-o-Sil	1364
Aquasol* (10 ml)	1558
Aquasol (10 ml) + 1 ml water	1616
Aquasol (10 ml) + 2 ml water	1618
Aquasol** (10 ml) + 3.5 ml water	1616

+ Toluene based scintillation mixture containing 4g/l 2, 5-diphenyloxazole and 100 mg/l 1, 4-bis-2-(5-phenyl oxazolyl)-benzene.

++ Sufficient Cab-o-Sil added to form a stable gel.

* Xylene-based scintillation mixture.

** Stable gel.

recovery. Formation of a gel with larger quantities of water (3.5 ml) offered no further improvement. A scintillation mixture consisting of 10 ml Aquasol and 1 ml water was adopted.

b. Isolation of Reaction Products

Procedures for the extraction of reaction products from the reaction mixture were investigated using the standard DPIK incubation mixture (see Methods). In the method of Kai et al. (1968) the reaction was stopped and the lipids extracted by the addition of 3.75 vol. of C-M (1:2, v/v) to give a monophasic system. After 15 min at room temperature, a biphasic system was produced by the addition of 1.25 vol. of chloroform containing 1 mg of carrier TPI and 1.25 vol. of 2M-KCl containing 0.5M-sodium phosphate buffer (pH 7.4). The lower chloroform phase was recovered and washed with 4.75 vol. of synthetic upper phase (C-M-W, 3:48:47) containing KCl and sodium phosphate buffer. In the studies of this system, a mixture of DPI and TPI (representing about 50 µg of lipid-P per tube) served as a carrier and the total radioactivity present in the washed lower phase was measured. Several difficulties were encountered (Table 20). Extracts of control tubes lacking DPI contained small amounts of radioactivity which could arise from DPI formation utilizing endogenous PI as the substrate and/or incomplete removal of [γ-^{32}P]ATP and/or formation of radioactive products by other reactions

involving the ATP. Extracts of control tubes lacking enzyme also contained radioactivity suggesting the incomplete removal of [γ-^{32}P]ATP. An insoluble particulate material was present in the extracts. Removal of this material by centrifugation before or after washing in the biphasic system or through glass wool as described by Garbus et al. (1963) did not result in any improvement. The controls were still unacceptably high (Table 20).

The extraction system employed by Iacobelli (1969) for the assay of DPIK in rabbit sciatic nerve myelin was therefore investigated. This procedure uses acidified solvents to ensure complete extraction of PPI followed by the removal of water-soluble products in the biphasic system of Folch et al. (1957). The kinase reaction was stopped by the addition of 2.5 ml of ice-cold 0.1 N-HCl in methanol followed by 5 ml of chloroform containing 1 mg carrier-TPI. After 15 min at 37°C, 1.38 ml of 1N-HCl was added and the resulting lower chloroform layer washed once with 0.5 vol. of synthetic upper phase containing N-HCl in place of water. A similar system was employed by Tou et al. (1970) except that a biphasic system was created by the addition of 10 ml 1M-$MgCl_2$ and the chloroform phase was washed three times with 10 ml each portions of 1M-$MgCl_2$.

Initial trials with the procedure of Iacobelli (1969) yielded somewhat lower blank values than that obtained by the procedure of Kai et al. (1968). The controls were still unacceptably high and insoluble material

Table 20. Radioactivity in Washed Lipid Extract from Phosphoinositide Kinase Assay

Assay System[+] (omissions)	Radioactivity in Washed Lipid Extracts			
	With Particles	Removal of Particles		
		Centrifugation		Filtration[**]
		Before wash[++]	After wash[*]	
None	1365	658	1118	1109
Substrate (DPI)	170	115	115	150
Homogenate	998	328	837	991

+ DPIK system of Kai et al. (1968) as described in the text.

++ Particles removed by centrifuging the monophasic system after addition of C-M (1:2, v/v), see text.

* Particles removed by centrifuging final washed lipid extract.

** Particles removed by filtering the biphasic system through glass wool as described by Garbus et al. (1963).

persisted in the final washed lipid extract which would interfere with quantitative transfer of lipids for chromatography. The presence of the insoluble material was avoided by increasing the concentration of acid in the methanolic-HCl used to stop the reaction from 0.1 N to 0.3 N. The initial biphasic system was then produced by adding chloroform and 1N-HCl as described by Iacobelli (1969). Several procedures for washing the lower phases were compared using extracts of unincubated assay controls (Table 21) in order to assess the removal of non-lipid radioactive compounds. One wash of the lower phase with acidified synthetic upper phase as described by Iacobelli (1969) gave low blank values, approximately twice the background of the counter. However, PPI are soluble to some extent in acidified upper phase solutions. This potential loss was overcome by using $CaCl_2$ in the upper phase wash solutions. Two washes reduced the blank counting rate to background levels (25 - 30 c.p.m.) and this procedure was tentatively adopted. A similar washing procedure was employed by Eichberg and Hauser (1969) who stopped the reaction and extracted lipids by adding 19 vol. of C-M (2:1, v/v) containing 0.25% concentrated HCl. This procedure gave somewhat variable results since centrifugation often did not effectively remove the insoluble residue from the homogenate and particulate material persisted in washed lower phase. The KCl-PO_4 buffer system of Kai et al. (1968) was least effective in removing ^{32}P from the lipid extracts.

Table 21. Comparison of Biphasic Washing Procedures on the Removal of Water-soluble ^{32}P-labelled Compounds from the Unincubated Phosphoinositide Kinase Assay Controls

Omissions	No. of Washes	Wash Solution	Radioactivity
None (1)	1	Acid upper phase (2)	72
Homogenate	1	Acid upper phase	51
None	1	Ca^{2+} upper phase (3)	118
Homogenate	1	Ca^{2+} upper phase	127
None	2	Ca^{2+} upper phase	28
Homogenate	2	Ca^{2+} upper phase	29
None	2	KCl-PO_4 upper phase (4)	150
Homogenate	2	KCl-PO_4 upper phase	138

(1) Homogenate added after addition of 0.3 N-HCl in methanol to complete incubation mixture.

(2) Synthetic upper phase (C-M-W, 3:48:47, by vol.) prepared with N-HCl in place of water (Iacobelli, 1969).

(3) Synthetic upper phase containing 0.02% (w/v) $CaCl_2$.

(4) Synthetic upper phase prepared with 2M-KCl-0.5M-sodium phosphate buffer (pH 7.4) in place of water (Kai et al., 1968).

Previous work (Kai, et al., 1966, 1968; Tou et al., 1968, 1970) had shown that PIK and DPIK activities cannot be measured independently in crude homogenates by measuring ^{32}P incorporation into total lipid extracts. The assay systems differ only in the requirement for exogenous substrates. In the DPIK assay system, the endogenous PI present in the crude homogenate, is also phosphorylated to DPI. Triphosphoinositides are labelled also in PIK assay system by phosphorylation of the reaction product (DPI) and/or small amounts of endogenous DPI. It is therefore necessary to separate the reaction products in order to isolate the two activities. Thin-layer chromatography on oxalate-treated Silica Gel HR was used to separate the reaction products. The polarity of the developing solvent mixture was increased (C-A-M-AA-W, 40:15:13:12:8, by vol.) in order to move phosphoinositides farther up the chromatogram and reduce the possibility of contamination with residual [γ-^{32}P]ATP. Almost all of the radioactivity was found in the DPI and TPI bands and only trace quantities were detected at or close to the origin of the chromatogram. Little or no radioactivity was found anywhere else although distinct lipid bands were present at the solvent front and at the positions expected for PI and PS.

The overall recovery of added carrier PPI in the modified system (see above or Methods) was compared with that obtained using systems reported by others. The standard

DPIK incubation system was used. The lipids in all washed extracts were separated by t.l.c. Using 2 mg BSA in place of tissue homogenate, the best recovery was obtained with the modified system (Table 22). Using an active brain homogenate, the modified extraction procedure yielded the greatest recovery of radioactive PPI while the radioactivity in the control tubes remained at background levels (25 - 30 c.p.m.). The reproducibility of this system was assessed in 10 repetitive assays. The following measurements of radioactivity (c.p.m. ± S.D.) were obtained: Control (no homogenate), 27 ± 3; DPI, 1053 ± 55; TPI, 2671 ± 15.

2. Characteristics of Phosphoinositide Kinases

Both PI and DPI were readily phosphorylated by homogenates of chick brain and sciatic nerve (Table 23). As noted earlier, simultaneous synthesis of DPI occurred in DPIK assays. Synthesis of TPI also occurred in the PIK assays to a lesser extent. Kinetic parameters (Vmax, Km, etc.) of some enzymes are known to change during postnatal development (i.e. Bell and Ecobichon, 1975). Therefore, it was necessary to investigate each aspect of the assay systems to ensure that they were suitable for homogenates of both chick brain and nerve at all ages.

a. Phosphatidylinositol Kinase.

(i) Basic Assay Characteristics

The pH profile for PIK of chick brain showed

Table 22. Recovery of Polyphosphoinositides in Phosphoinositide Kinase Assay Systems

Method	% Recovery of polyphosphoinositides (DPI + TPI) +	Radioactivity ++ (c.p.m.)
Modification of Iacobelli (1969)	92.6	1714
Iacobelli (1969)	88.6	1569
Tou et al. (1968)	72.6	-
Kai et al. (1968) *	51.6	166

Results are means of two determinations.

+ Active homogenate replaced by 2 mg BSA. Added DPI-TPI recovered after t.l.c.

++ Similar experiment containing 0.1 ml of 10% (w/v) 2 day old chick brain homogenate and 0.9 mM-DPI in the reaction mixture. Radioactivity measured in DPI + TPI after t.l.c.

* Presence of particulate material in washed lipid extracts hampered application of the lipid to t.l.c. plates.

Table 23 Phosphoinositide Kinase Activities in Chick Brain and Sciatic Nerve Homogenates

	Kinase Activity*, nmol formed/min/mg protein							
	Brain				Nerve			
	pH 7.4		pH 8.3		pH 7.4		pH 8.3	
	DPI	TPI	DPI	TPI	DPI	TPI	DPI	TPI
Basic Assay System*	0	0	0	0	0	0	0	0
+ Homogenate	0.50	0.48	1.49	0.46	0.20	0.08	0.23	0.07
+ Homogenate + PI (1 mM)	-	-	1.65	0.49	-	-	0.35	0.09
+ Homogenate + DPI (0.5 mM)	0.85	1.50	-	-	0.24	0.32	-	-

* Contains 40 mM - Tris-HCl buffer, 30 mM-$MgCl_2$ and 5 mM-[γ^{32}-P]ATP (2.38 x 10^6 c.p.m.) in a total volume of 0.5 ml. 3 day old chick brain (0.341 mg protein) and nerve (0.46 mg protein) homogenates were used.

only a single peak of activity at pH 8.3 (Fig. 14). The relative activity at pH 7.4 was lower than reported for either rat brain microsomes (Colodzin and Kennedy, 1965) or rat kidney cortex (Tou et al., 1968). The pH profile for chick sciatic nerve exhibited the same activity between pH 7.35 and 8.5 raising the possibility of an additional pH 7.4 enzyme in this tissue (Fig. 14) as has been reported in mammalian liver (Harwood and Hawthorne, 1969a).

The effect of varying concentrations of PI on DPI formation is shown in Fig. 15a. Without added PI, there was a considerable synthesis of DPI. Only marginal stimulation was observed when exogenous PI were added to the incubation mixtures but the extent of stimulation varied slightly with tissue and age. Higher concentrations (up to 4 mM-PI) have produced no inhibition in both chick brain and sciatic nerve homogenates. Subsequently, 1 mM-PI was routinely used.

In chick neural tissues, optimum activity was obtained with 5 mM-ATP in the presence of added $MgCl_2$ (Fig. 15b). Higher concentrations of ATP than 7 - 8 mM produced some inhibition in chick brain. The "apparent Km" for ATP was 0.95×10^{-3} M (Fig. 16). The response to added $MgCl_2$ was variable; however, optimum activity was obtained for all preparations above 20 mM (Fig. 17). Only brain homogenates exhibited slight inhibition by Mg^{2+} concentrations above 40 mM under standard assay conditions. The Mg^{2+} and ATP requirements were related (Fig. 18a, b). At low Mg^{2+}

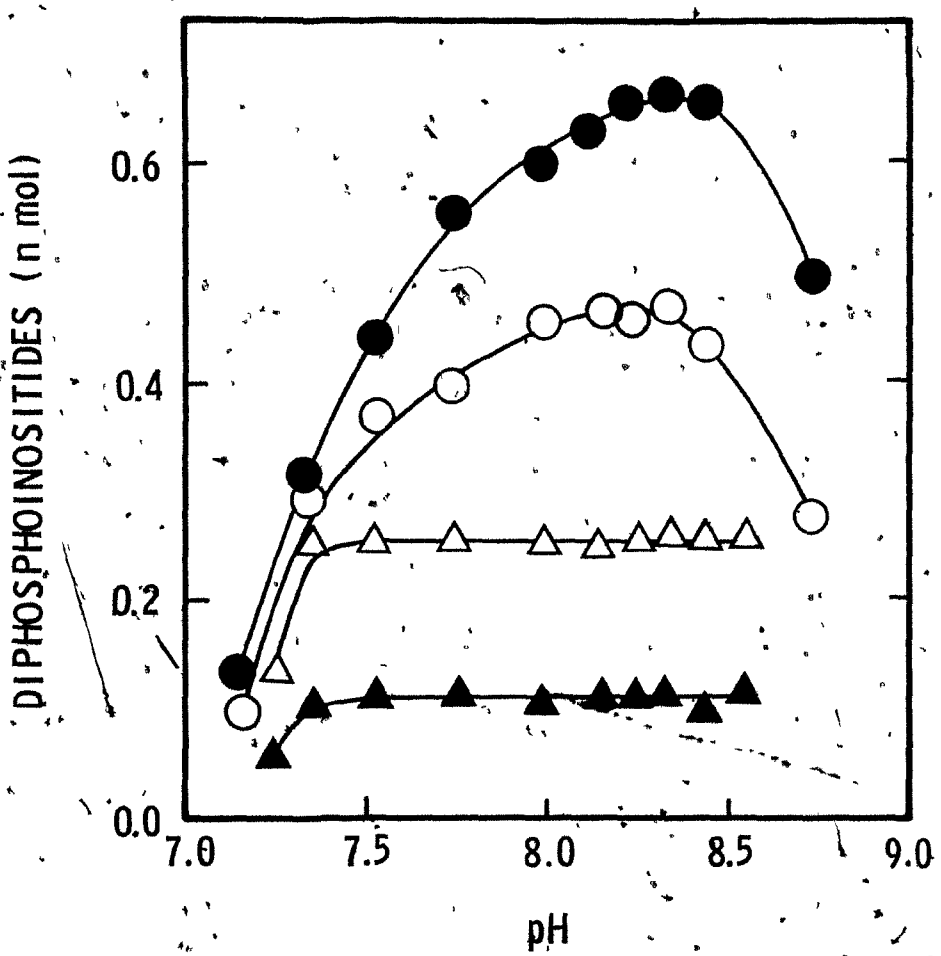


Fig. 14: Effect of pH on PIK Activity. Standard assay procedure using homogenates from: O, 15 day embryonic chick brain (0.74 mg protein); ●, 4 day chick brain (0.31 mg protein); Δ, 16 day embryonic chick sciatic nerve (0.37 mg protein), and ▲, 49 day chick sciatic nerve (1.14 mg protein). Product expressed as nmol/assay.

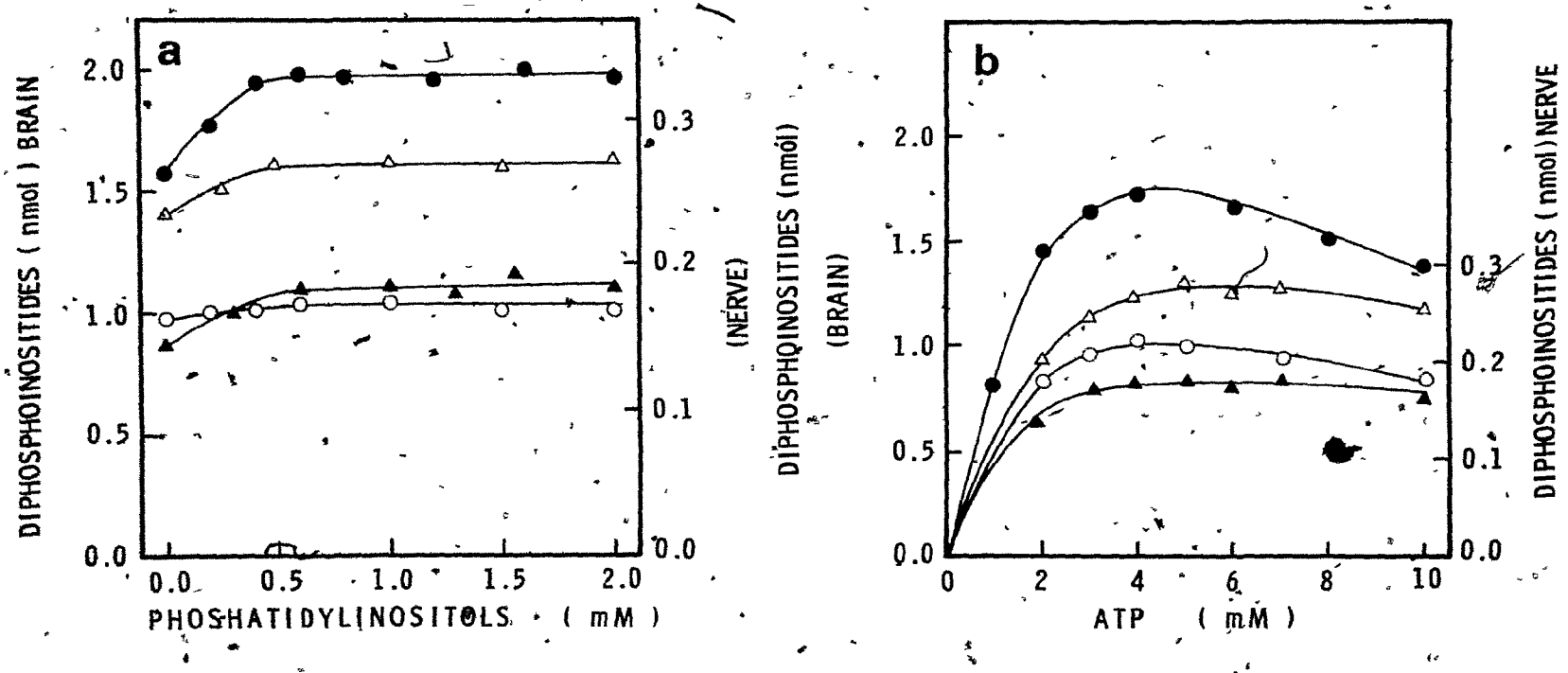


Fig. 15:

(a) Effect of Substrate (PI) Concentration on PIK Activity. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain (1.25 mg protein); ●, 52 day chick brain (0.91 mg protein); Δ, 16 day embryonic chick sciatic nerve (0.37 mg protein) and ▲, 20 day chick sciatic nerve (1.50 mg protein).

(b) Effect of ATP Concentration on PIK Activity. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain (1.25 mg protein); ●, 52 day chick brain (0.81 mg protein); Δ, 16 day embryonic chick sciatic nerve (0.37 mg protein), and ▲, 20 day chick sciatic nerve (1.50 mg protein).

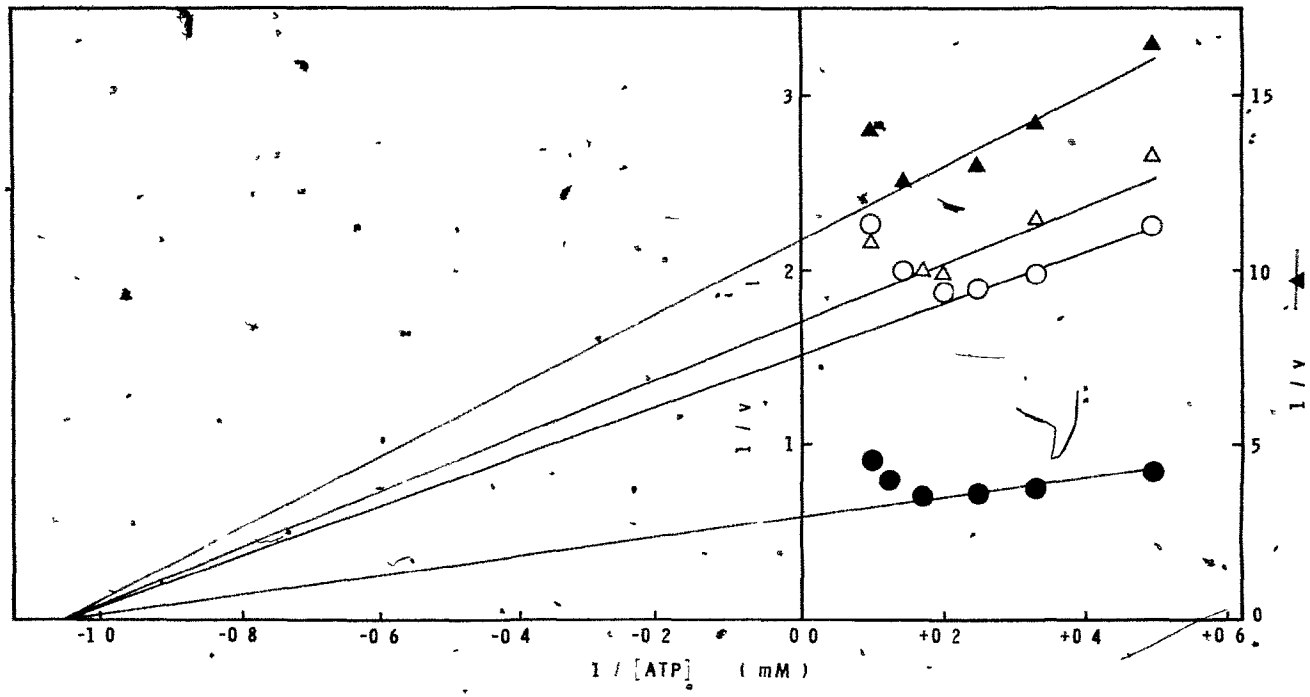


Fig. 16: The Dependence of Chick Brain and Sciatic Nerve PIK Activity on ATP Concentrations. Data recalculated (nmol DPI formed/min/mg protein) and symbols taken from Fig. 15b. Double-reciprocal plot.

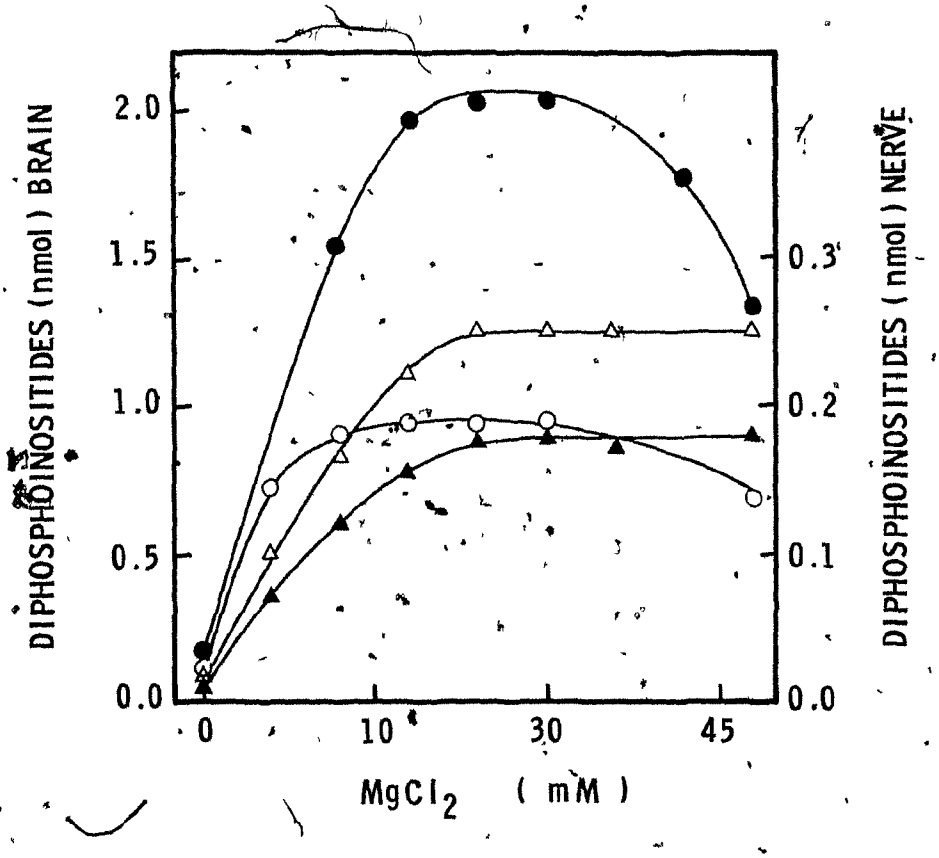


Fig. 17: Effect of $MgCl_2$ on PIK Activity. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain (1.25 mg protein); ●, 52 day chick brain (0.91 mg protein); △, 16 day embryonic chick sciatic nerve (0.37 mg protein); ▲, 20 day chick sciatic nerve (1.50 mg protein).

concentrations (5 mM), maximum activity was observed with 2 mM-ATP and higher concentrations caused inhibition (Fig. 18a). This activity was much lower than observed at higher concentrations of both ATP and Mg^{2+}. A similar effect was observed for the simultaneous production of TPI. The ratio of Mg:ATP is important since inhibition by excess ATP was overcome by raising the Mg^{2+} concentration. The requirement for $MgCl_2$ also appeared to shift slightly toward lower concentrations when no exogenous substrate was provided (Fig. 18b).

The component used to start the PIK reaction was of some importance (Table 24). Attempts to start the reaction with ATP gave the lowest values. Initiation by the addition of substrate (PI) was not possible since the reaction with endogenous substrate gave high zero time controls (tubes in which the reaction was stopped after the preincubation period) and the reaction in the following 1.5 min yielded low values. In the absence of added Mg^{2+}, the endogenous reaction during the preincubation period was much reduced but the activity measured after the addition of Mg^{2+} was also low. The highest activities were obtained when the reaction was started by the addition of homogenate suggesting that the prior mixing of the ATP and Mg^{2+} is advantageous.

Under standard assay conditions, the reaction rate was constant only for 2 min after which there was little net synthesis of DPI (Fig. 19a). Similar results

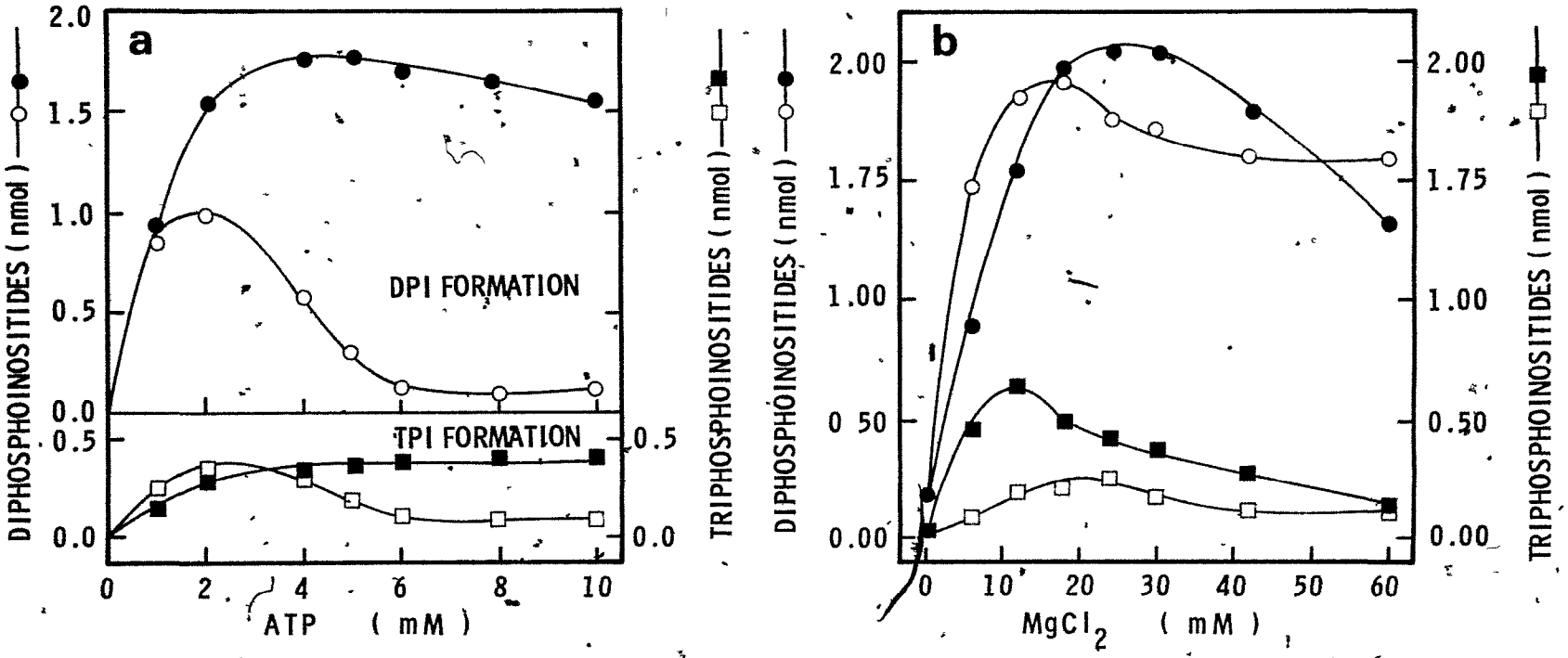


Fig. 18:
(a) Effect of ATP Concentration on PIK Activity with Two Different Concentrations of $MgCl_2$. 31 day chick brain homogenate (0.83 mg protein) was assayed under standard conditions with: ●, ■, 30 mM $MgCl_2$; ○, □, 15 mM $MgCl_2$. Lower graph indicates simultaneous synthesis of TPI.
(b) Effect of $MgCl_2$ Concentration on PIK Activity in the Absence or Presence of Exogenous Substrate (PI). 52 day old chick brain homogenate (0.95 mg protein) was assayed under standard conditions with 1 mM-PI (open symbols) and with no added PI (closed symbols). Simultaneous production of TPI is indicated by squares.

Table 24. Initiation of Phosphatidylinositol Kinase Reaction

Reaction Initiator*	PI Kinase Activity (nmol DPI formed)
Homogenate (no PI in reaction)	1.27
Homogenate	1.53
Homogenate + PI (mixed together)	1.37
PI (zero time control = 0.93 nmol DPI)	0.91
ATP	0.72
$MgCl_2$ (zero time control = 0.08 nmol DPI)	0.84

* Assays contained 45 mM - Tris-HCl (pH 8.3), 5 mM-[γ-^{32}P]ATP, 30 mM-$MgCl_2$, 1 mM-PI and 0.1 ml of 10% (0.63 mg protein) 2 day chick brain homogenate. All components except those used to initiate the reactions were mixed together at 4°C and warmed up to 37°C for 2 min before the reactions were started. Assays in which homogenate and [γ-^{32}P]ATP were preincubated together required a zero time control (stopped after preincubation period) to correct for synthesis with endogenous substrate. Reactions were terminated after 1.5 min.

have been reported for rat brain and kidney cortex (Colodzin and Kennedy, 1965; Kai et al., 1966b; Tou et al., 1968) although these investigators continued to use longer incubation times in their standard assay systems. In the present study, a very short incubation time of 1.5 min was used and a linear response to protein concentration was seen over the range of 0.25 - 1.5 mg protein (Fig. 19b). No loss of PIK activity was observed when homogenates were stored at -20°C for up to 14 days (Table 25).

(ii) Effect of Thiol Groups, Chelating Agents and Reaction Products

Table 26 shows the effects of various additives on PIK activity. In contrast to earlier reports (Kai et al., 1966b; Tou et al., 1969) reduced glutathione inhibited the reaction. Sodium-EDTA (2 mM) has been included in Mg^{2+} fortified assay mixtures by others since stimulation was observed (Kai et al., 1966b; Tou et al., 1968) and/or the proportionality to protein concentration was extended (Kai et al., 1966b). The effect had been attributed to the preferential removal of Ca^{2+} (over Mg^{2+}) and/or other heavy-metal ions (i.e. Cu^{2+} inhibits kinase) and also to the inhibition of TPI phosphohydrolases. In chick neural tissues, EDTA had no effect at low concentrations but larger amounts were inhibitory, probably as a result of the lowered Mg^{2+} concentrations. Chick brain PIK was inhibited by one reaction product (ADP) but not by the other. DPI actually

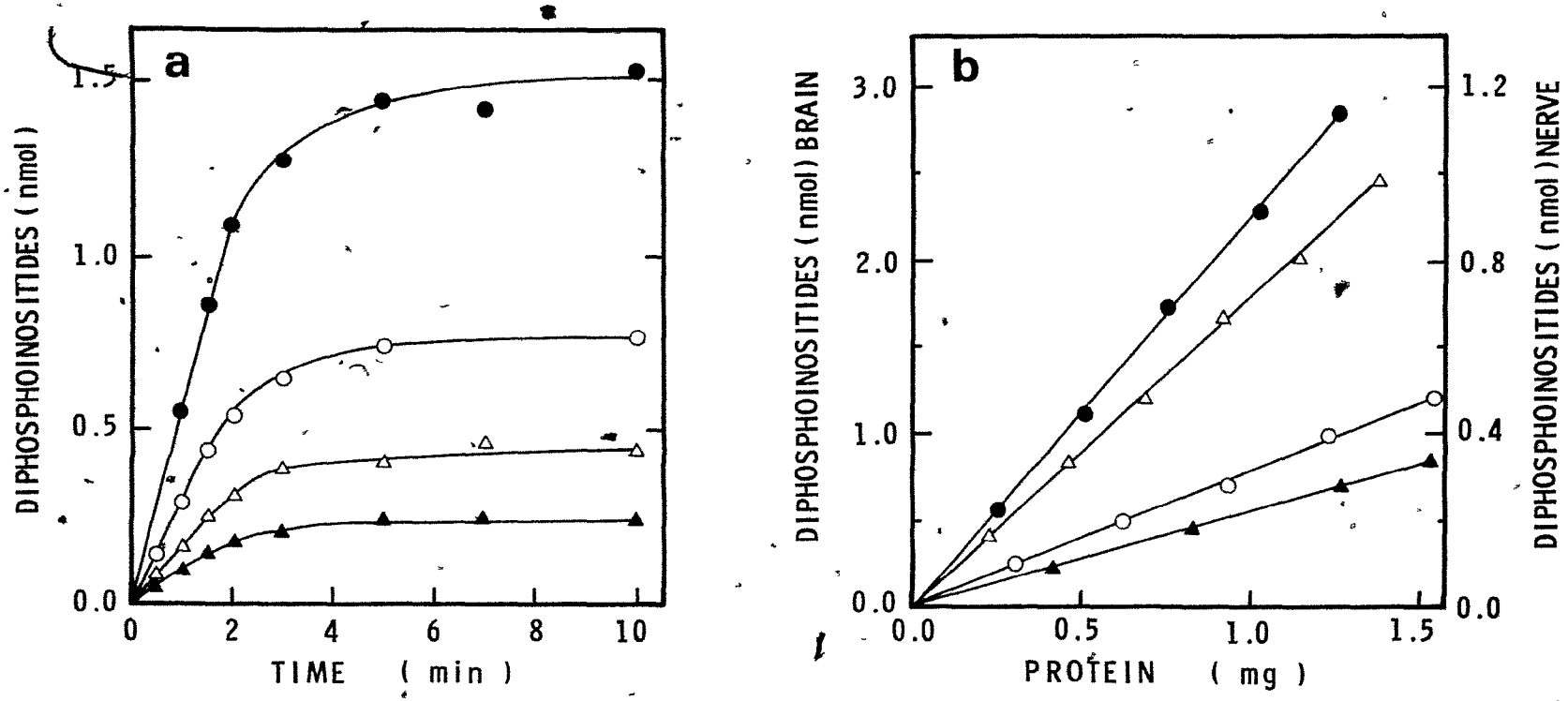


Fig. 19:
(a) Time-Course of PIK Reaction. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain (0.50 mg protein); ●, 4 day chick brain (0.37 mg protein); △, 16 day embryonic chick sciatic nerve (0.375 mg protein) and ▲, 4 day chick sciatic nerve (0.45 mg protein).
(b) Effect of Protein Concentration on PIK Activity. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain; ●, 8 day chick brain; △, 16 day embryonic chick sciatic nerve and ▲, 24 day chick sciatic nerve.

Table 25. Stability of PI Kinase Activity of Chick Brain Homogenate

Time of Storage* (days)	DPI Synthesized (nmol/min/mg Protein)
0	1.59
2	1.57
5	1.60
8	1.56
10	1.58
14	1.56
20	1.31

Standard assay procedure using homogenate of 15 day chick brain. Results are means of duplicate analyses.

* Homogenate was kept at -20°C for various days in separate tubes and thawed only once before use.

Table 26. PI Kinase Activity in Chick Brain Homogenates: Effect of Various Additives

Addition		Percent of Control
EDTA	1.2 mM	96
	4.0 mM	82
	6.0 mM	31
EGTA	1.0 mM	106
	2.0 mM	99
GSH	5.0 mM	93
	10.0 mM	77
	20.0 mM	64
TPI	0.05 mM	114
	0.10 mM	115
	0.20 mM	122
DPI	0.10 mM	110
	0.50 mM	112
ADP	1.0 mM	85
	2.0 mM	77

Standard assay conditions using homogenates of 21 day chick brain.

produced a small but consistent stimulation (10 - 12%). Triphosphoinositides also produced similar stimulation of PIK activity in both chick brain and sciatic nerve homogenates.

(iii) Effect of Cutscum

Cutscum, a non-ionic detergent, has been reported to stimulate PIK activity in both neural and non-neural tissues (Colodzin and Kennedy, 1965; Kai et al., 1966b; Michell et al., 1967; Tou et al., 1968, 1969; Harwood and Hawthorne, 1969a). This effect was confirmed for chick neural tissues. In preliminary experiments, concentrations of Cutscum (2%) similar to those employed by Kai et al. (1966b) were used to check the basic parameters of the assay system. Cutscum did not alter the pH profile for either tissue and the requirements for ATP and Mg^{2+} remained the same (Fig. 20a, b, c). The stimulatory effect of Cutscum was found to be related to protein concentration (Fig. 21a, b). As noted previously (Tou et al., 1969), simultaneous production of TPI was inhibited at all detergent concentrations. Inhibition of DPI formation at high detergent: protein ratios was not as pronounced in sciatic nerve. Maximum activity was consistently obtained at a ratio of 7:1 for homogenates of both chick brains and sciatic nerves of all ages. When the optimum detergent:protein ratio was maintained, Cutscum did not extend the linearity of DPI formation with time (Fig. 22a), as has been observed for rat brain (Kai et al., 1966b), nor did it alter the linearity

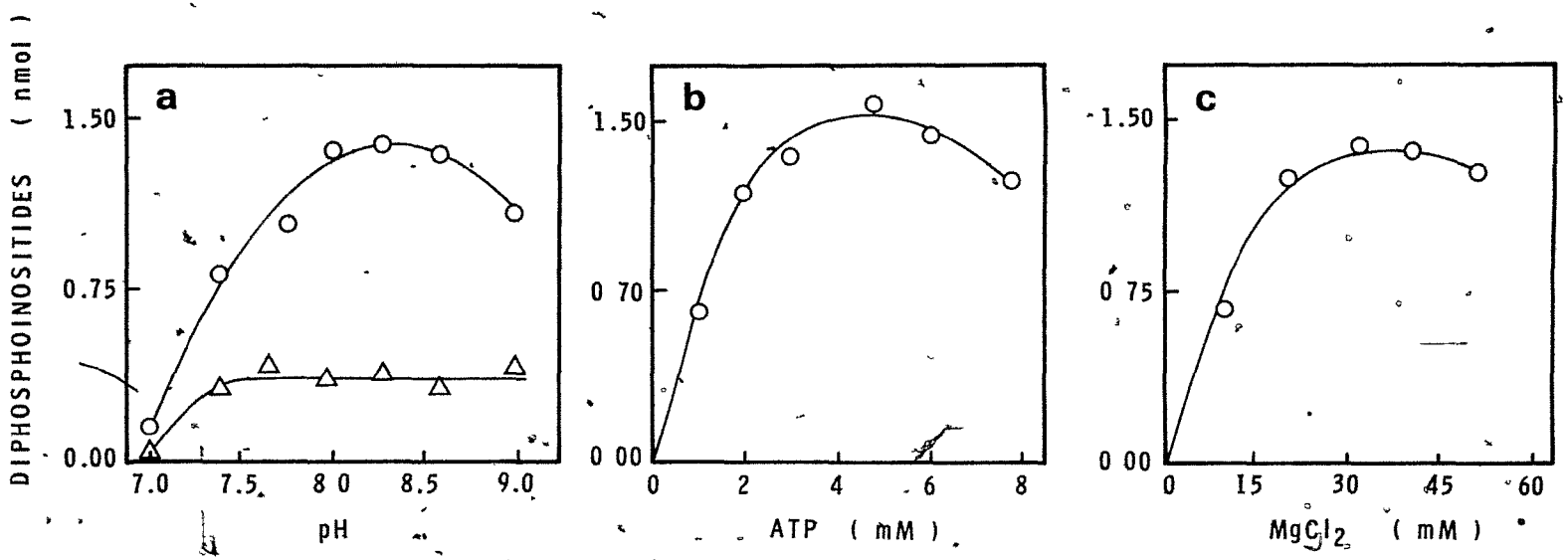


Fig. 20: Effects of pH(a), ATP(b) and $MgCl_2$(c) on PIK Activity. Standard assay conditions. using 2% Cutscum with 3 min incubation time. o, 8-day chick brain (0.95 mg protein); ∆, 11 day chick sciatic nerve (0.61 mg protein).

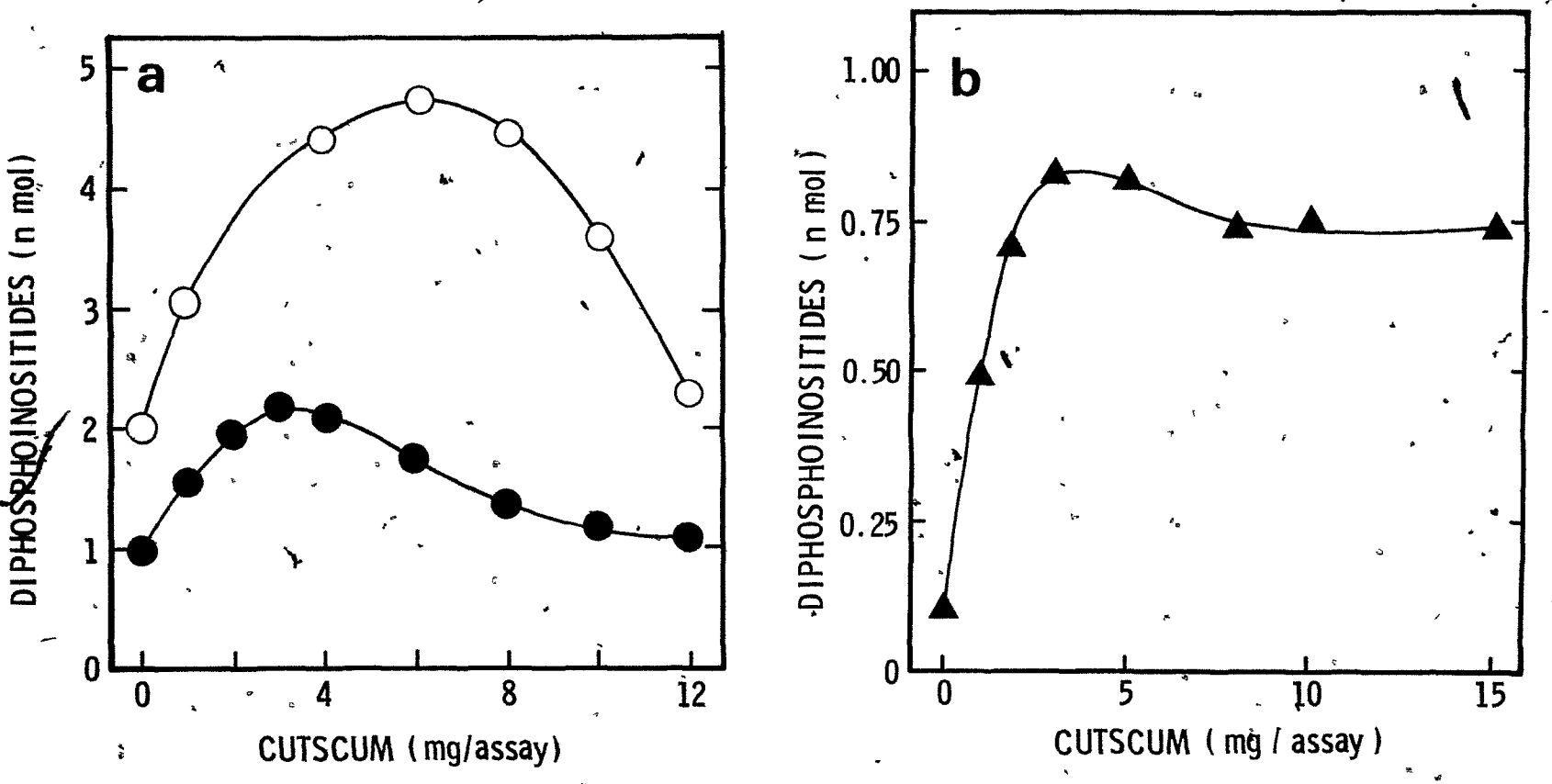


Fig. 21:
(a) Effect of Cutscum on Chick Brain PIK Activity. Standard assay procedure was used with homogenate of 35 day old chick brain containing: ●, 0.43 mg protein and O, 0.85 mg protein.
(b) Effect of Cutscum on Chick Sciatic Nerve PIK Activity. Standard assay procedure was used with homogenate of 2 day chick sciatic nerve containing 0.486 mg protein. Homogenate used was frozen for 26 days at -20°C.

with protein concentration (Fig. 22b). The response to added substrate was changed (Fig. 23a, b). Cutscum was inhibitory at very low concentrations of exogenous PI in chick brain. A similar effect has been reported in erythrocyte membrane PIK (Buckley and Hawthorne, 1972). The activity was dependent upon the concentration of exogenous substrate in both brain and sciatic nerve homogenates. The stimulation observed at higher PI concentrations was considerably greater for sciatic nerve than brain.

b. Diphosphoinositide Kinase

(i) Basic Assay Characteristics

Both chick brain and sciatic nerve preparations exhibited little activity below pH 7 (Fig. 24a). Maximum activity occurred at pH 7.4 with relatively little decline at higher pH values. The optimum requirement for ATP was 5 mM in the presence of 30 mM-$MgCl_2$ (Fig. 24b). The "apparent Km" for ATP was 0.95×10^{-3} M (Fig. 25). Concentrations higher than 6 mM strongly inhibited the reaction. Magnesium was required and the maximum activity was obtained with concentrations above 20 mM (Fig. 26a). All preparations exhibited little DPIK activity in the absence of added substrate (Fig. 26b). Maximum activity was observed with 0.4 - 0.5 mM-DPI in both chick brain and sciatic nerve homogenates. Higher concentrations of DPI produced some inhibition in chick brain; the inhibition was greater

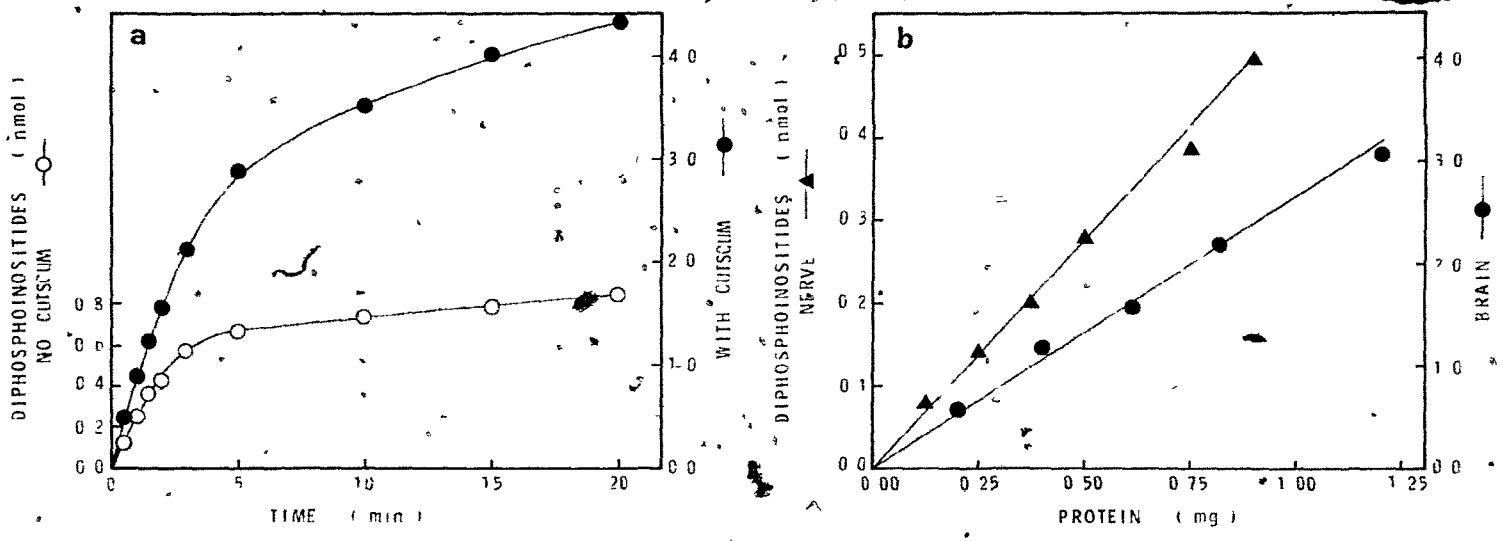


Fig. 22:
(a) Time-Course of PIK Reaction. Standard assay conditions using homogenates from 2 day chick brain (0.41 mg protein; homogenate used was frozen for 20 days at -20°C) without Cutscum (O) and with Cutscum/protein ratio of 7/1(●).
(b) Effect of Protein Concentration on PIK Activity. Standard assay conditions with Cutscum/protein ratio of 7/1. Homogenates from 2 day chick brain (frozen for 20 days at -20°C) and sciatic nerve (frozen for 26 days at -20°C) were used.

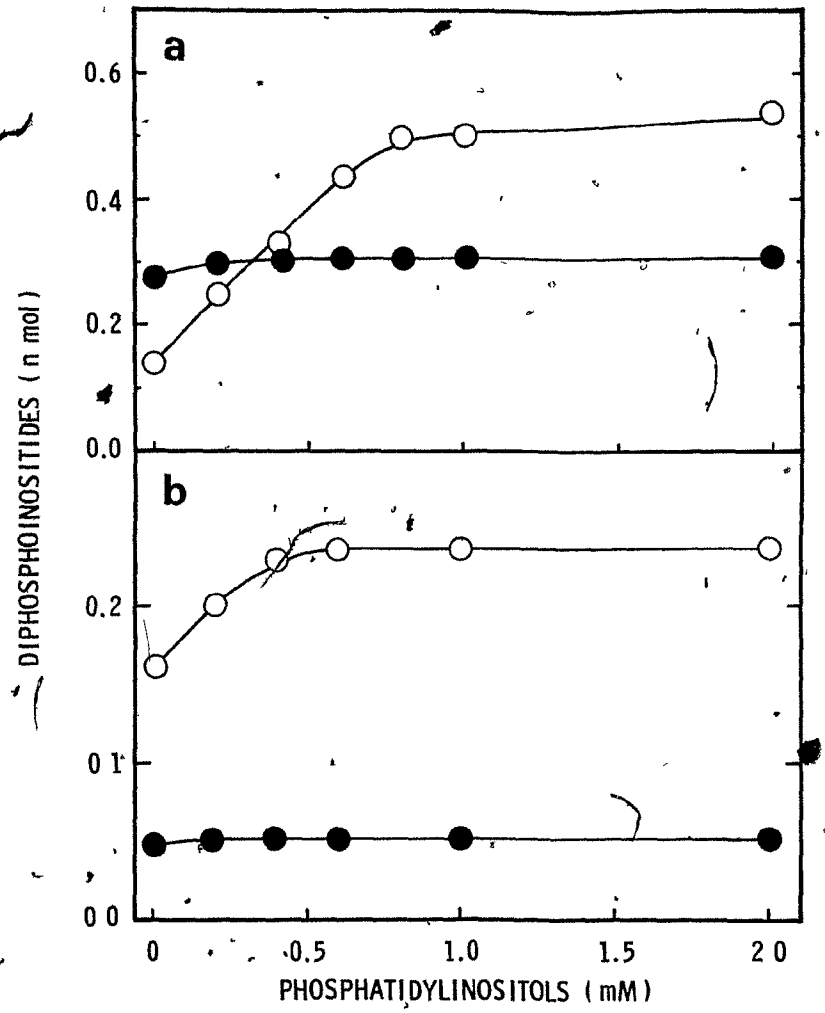


Fig. 23: Effect of Cutscum on the Substrate Dependence of PIK. Standard assay conditions without Cutscum (●) and with Cutscum/protein ratio of 7/1 (O). (a)-homogenate of 21 day embryonic chick brain, 0.48 mg protein (frozen for 18 days at -20°C). (b)-homogenate of 30 day chick sciatic nerve, 0.48 mg protein (frozen for 30 days at -20°C).

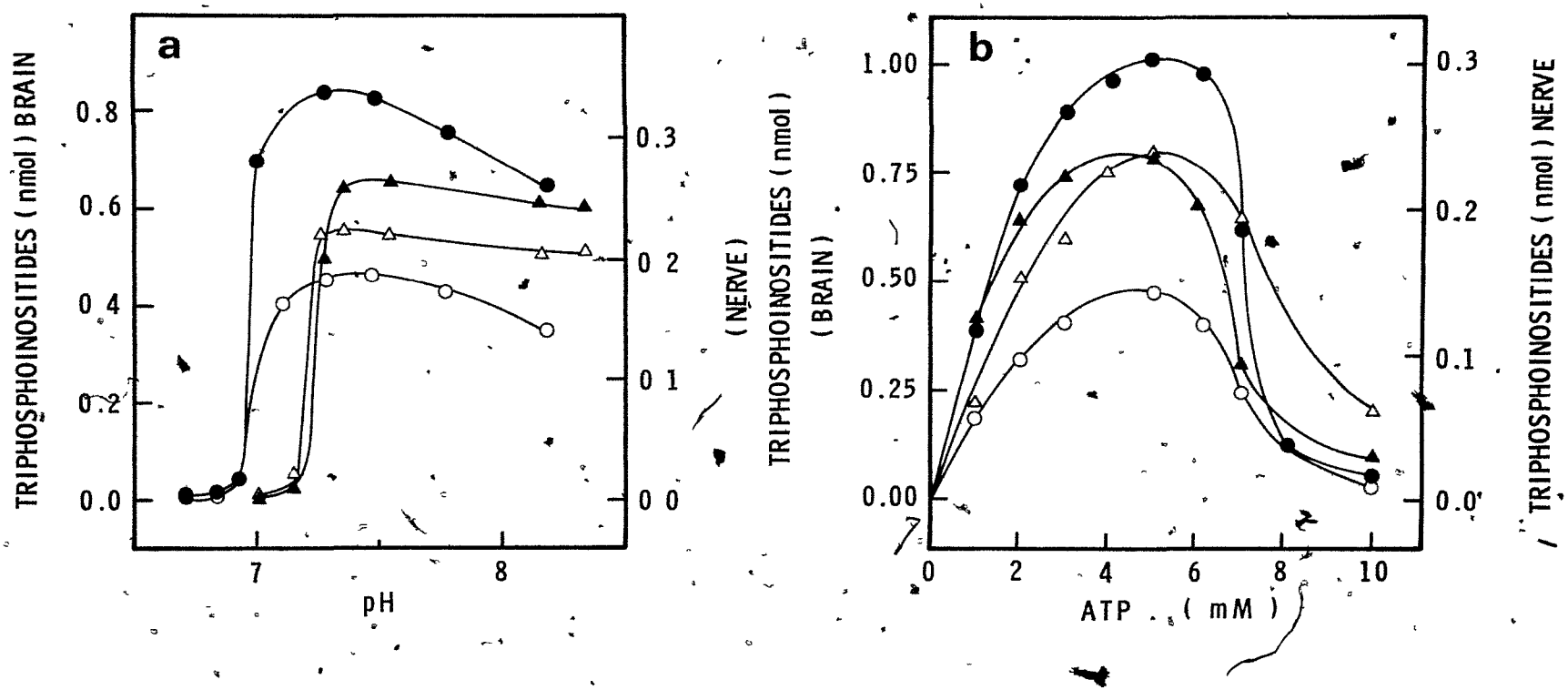


Fig. 24:
(a) Effect of pH on DPIK Activity. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain (0.56 mg protein); ●, 19 day chick brain (0.38 mg protein); ▵, 16 day embryonic chick sciatic nerve (0.38 mg protein) and ▲, 49 day chick sciatic nerve (1.14 mg protein).
(b) Effect of ATP Concentration on DPIK Activity. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain (0.56 mg protein); ●, 16 day chick brain (0.44 mg protein); ▵, 16 day embryonic chick sciatic nerve (0.39 mg protein) and ▲, 49 day chick sciatic nerve (1.14 mg protein).

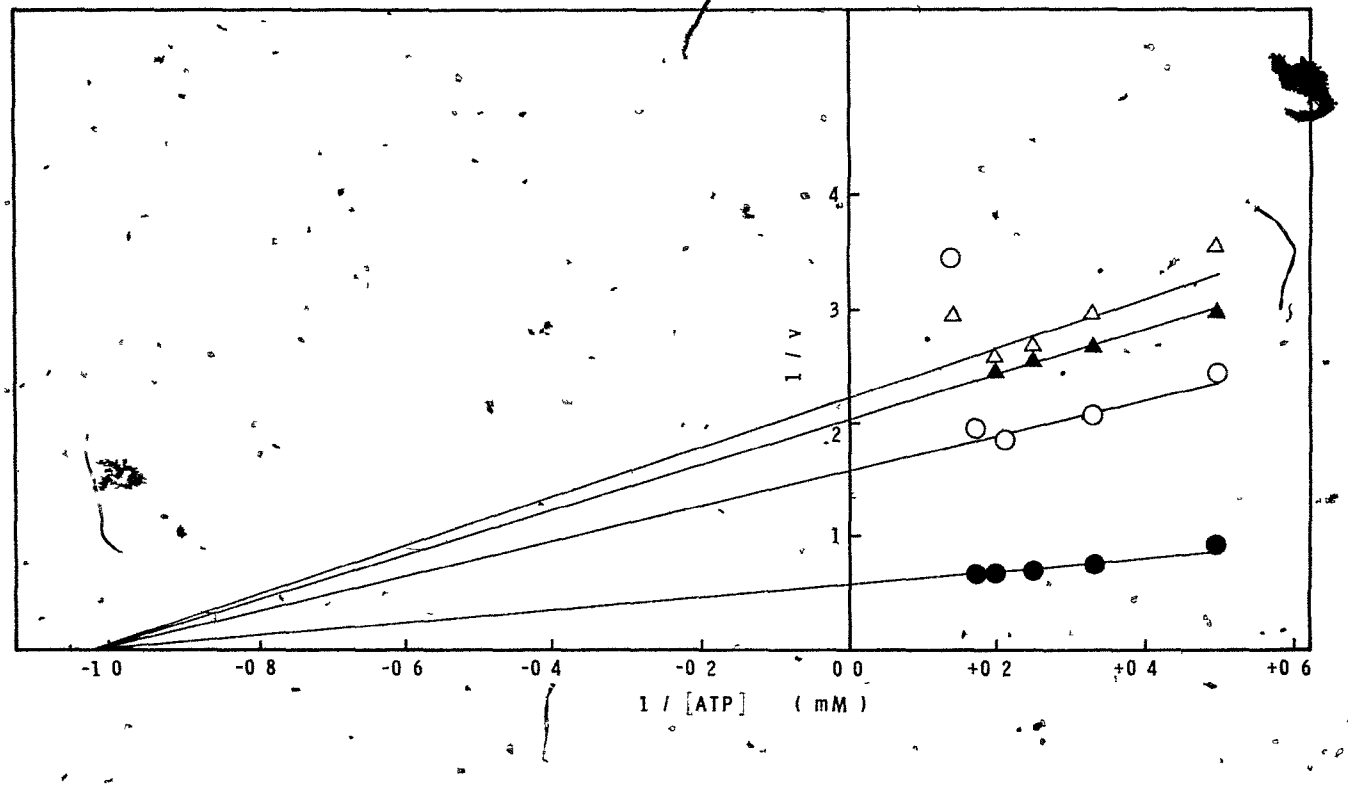


Fig. 25: The Dependence of Chick Brain and Sciatic Nerve DPIK Activity on ATP Concentrations. Data recalculated (nmol TPT formed/min/mg protein) and symbols taken from Fig. 24b. Double-reciprocal plot.

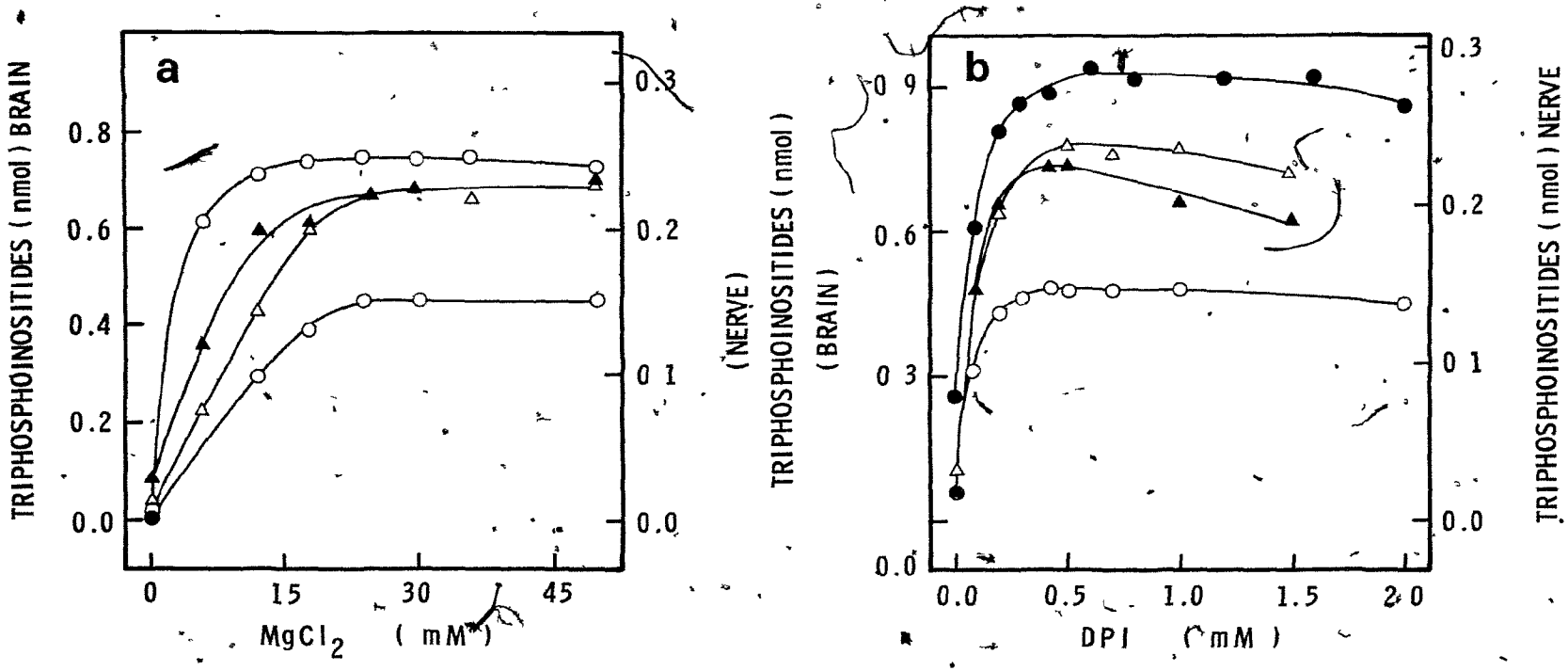


Fig. 26:

(a) Effect of $MgCl_2$ on DPIK Activity. Standard assay procedure using homogenates from o, 15 day embryonic chick brain (0.56 mg protein); ●, 11 day chick brain (0.35 mg protein); ▲, 16 day embryonic chick sciatic nerve (0.39 mg protein) and ▲, 49 day chick sciatic nerve (1.14 mg protein).

(b) Effect of Substrate (DPI) Concentration on DPIK Activity. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain (0.56 mg protein); ●, 31 day chick brain (0.42 mg protein); ▲, 16 day embryonic chick nerve (0.39 mg protein) and ▲, 34 day chick sciatic nerve (1.14 mg protein):

for sciatic nerve and was observed at concentrations higher than 0.6 - 0.8 mM. The "apparent Km" for DPI was about 0.1 mM (Fig. 27). The reaction rate was constant for only a very short time (Fig. 28a) and assays were routinely incubated for 1.5 min. This is a much shorter time than has generally been used by other investigators, although the reaction rates in these studies also declined sharply after few minutes (Kai et al., 1968; Tou et al., 1970).

The order in which the constituents of the incubation system were added was also important. Attempts to initiate the reaction by the addition of [γ-^{32}P]ATP gave higher but variable results and a convex curve was obtained with increasing amounts of DPI. Reactions initiated by the addition of $MgCl_2$ resulted in low activities. Best results were obtained when the reaction was started with the enzyme (Table 27). The same was true for the simultaneous production of DPI at this pH except similar activities were observed when reaction was started with either $MgCl_2$ or ATP. Kai et al. (1968) observed higher DPIK activities when Mg^{2+} was added last since precipitation of Mg-DPI in the absence of protein could be avoided. In the present system a very fine precipitate did form during the brief preincubation period but it was not a less suitable substrate for the enzyme. When assayed under optimum conditions, the activity was proportional to protein concentrations up to 1.0 - 1.4 mg protein/tube

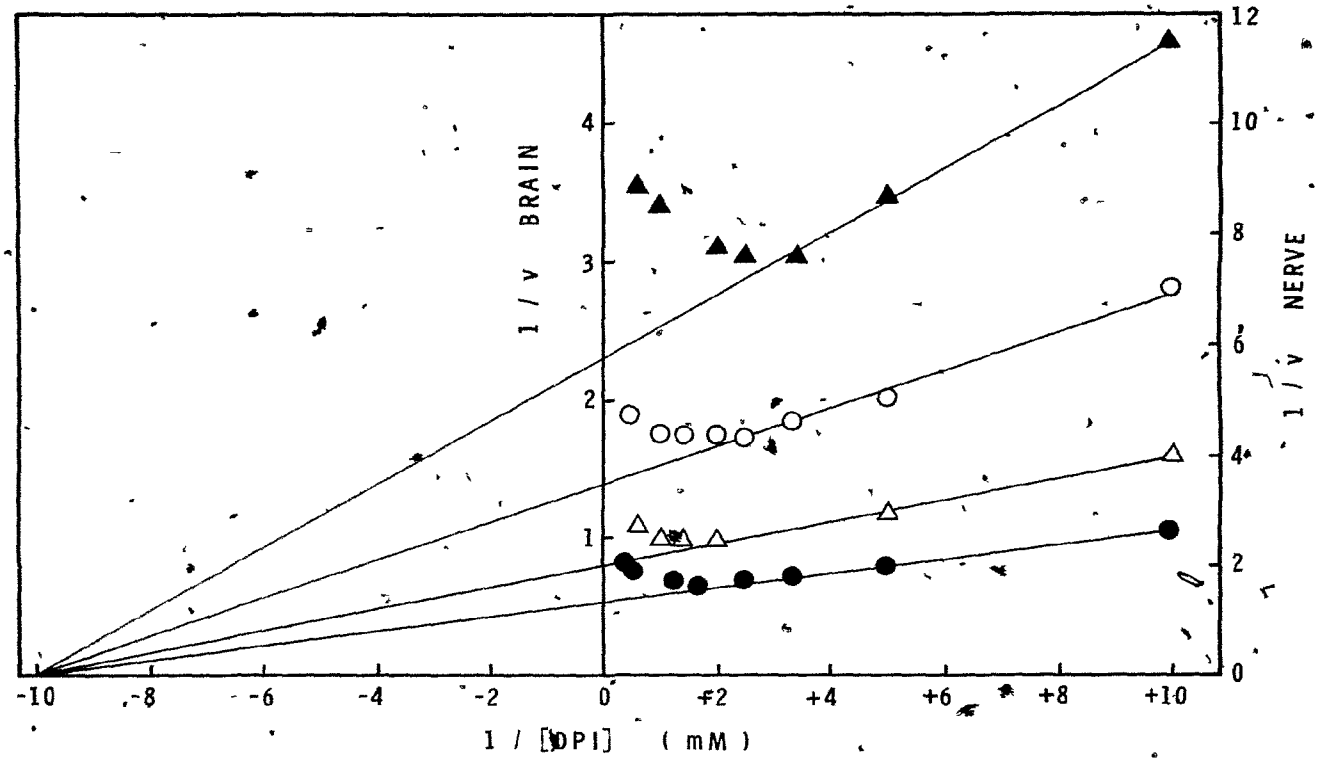


Fig. 27: The Dependence of Chick Brain and Sciatic Nerve DPIK Activity on DPI Concentrations. Data recalculated (nmol TPI formed/min/mg protein) and symbols taken from Fig. 26b. Double-reciprocal plot.

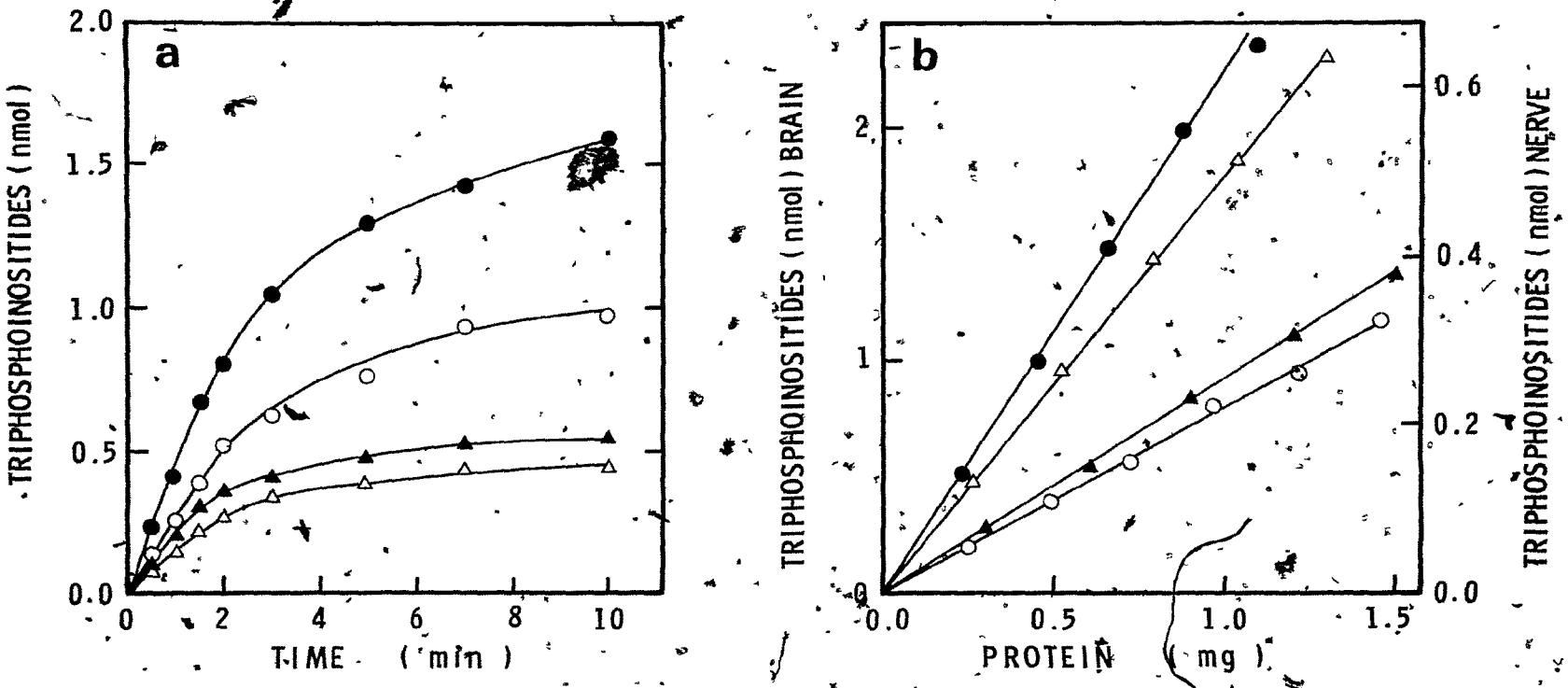


Fig. 28:

(a) Time-Course of DPIK Reaction. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain (0.45 mg protein); •, 4 day chick brain (0.37 mg protein); △, 15 day embryonic chick sciatic nerve (0.39 mg protein) and ▲, 4 day chick nerve (0.90 mg protein).

(b) Effect of Protein Concentration on DPIK Activity. Standard assay procedure using homogenates from: o, 15 day embryonic chick brain; •, 8 day chick brain; △, 16 day embryonic chick sciatic nerve and ▲, 36 day chick sciatic nerve.

Table 27. Initiation of DPI Kinase Reaction

Reaction Initiator *	DPI Kinase cpm	DPI Kinase nmol TPI (net)	PI Kinase (pH 7.4) cpm	PI Kinase (pH 7.4) nmol DPI (net)
DPI	575	0.65	261	0.09
(control)	80		191	
Enzyme	684	0.96	261	0.31
(control)	27		22	
Mg^{2+}	525	0.64	164	0.18
(control)	34		23	
ATP	846	1.07	155	0.18
(control)	26		19	

* Optimum concentrations of all components were used (see Methods). All constituents except component used to initiate the reactions were maintained at 4°C, mixed together and preincubated at 37°C for 2 min. Controls were terminated after the preincubation period. Enzyme source was 36 day chick brain (0.38 mg protein/tube). Reactions were terminated after 1.5 min.

depending upon the age of the chick (Fig. 28b). The activity was stable for 9 - 12 days if the homogenates were stored at -20°C (Table 28).

(ii) Effect of Thiol Groups, Chelating Agents and Reaction Products

Sulfhydryl compounds have been reported to inhibit DPIK (Kai et al., 1968; Tou et al., 1970). In chick brain, DPIK activity was reduced 11% by 5 mM-reduced glutathione (Table 29). Higher concentrations (10 mM) restored the activity to control levels as was observed for rat brain (Kai et al., 1968). The DPIK activity in rat brain homogenates is stimulated by low concentrations of EDTA (2 mM) in the presence of added $MgCl_2$ (Kai et al., 1968). The effect was presumed to be the result of inhibition of catabolic enzymes. However, the DPIK of chick neural tissues was inhibited by similar concentrations of EDTA (Table 29). EGTA, a specific inhibitor of brain and nerve TPI Phosphatase (see Section III.C.1.) only marginally increased the activity, indicating that removal of the labelled products by this catabolic enzyme is not significant. As observed earlier for rat brain and kidney cortex (Kai et al., 1968; Tou et al., 1970), DPI kinases of chick nervous tissues were inhibited by both reaction products (ADP and TPI). The presence of only 0.05 mM-TPI reduced the activity by more than 30% (Table 29).

Table 28. Stability of DPI Kinase Activity of Chick Brain Homogenate

Time of Storage* (days)	Activity (nmol/min/mg Protein) DPIK	PIK (pH 7.4)**
0	1.43	0.648
3	1.45	0.602
6	1.42	0.610
9	1.41	0.620
12	1.43	0.478
15	1.27	0.431

Standard Assay procedure using homogenate of 15 day chick brain. Results are means of duplicate analyses.

* Homogenate was kept at -20°C for various days in separate tubes and thawed only once before use.

** Represent PIK activity (without added PI) under conditions of DPIK assay.

Table 29. DPI Kinase Activity in Chick Brain Homogenates: Effect of Various Additives

Addition		DPI kinase (% of Control)
EDTA	1.2 mM	86
	2.0 mM	79
	4.0 mM	60
	6.0 mM	33
EGTA	1 mM	114
GSH	5 mM	89
	10 mM	102
TPI	0.05 mM	68
	0.10 mM	38
ADP	1 mM	83
	2 mM	16

Standard assay conditions using homogenates of 28 day chick brain (EDTA & EGTA) and 19 day chick brain (GSH, TPI and ADP).

(iii) Effect of Cutscum

Detergents have not generally been included in DPIK assay systems since Cutscum and Triton X-100 have been reported to be inhibitory in rat brain and kidney preparations while sodium deoxycholate has no effect (Kai et al., 1968; Tou et al., 1970). However, Eichberg and Hauser (1969) found that the activity in crude homogenates of rat brain was greatly enhanced by Cutscum. Cutscum was also found to stimulate the DPIK of chick brain and sciatic nerve to a greater extent than observed for PIK under appropriate conditions. In preliminary experiments, Cutscum (1%) did not alter the pH optimum or ATP and Mg^{2+} requirements (Fig. 29a, b, c). However, as was observed for PIK, the ratio of detergent to protein was critical (Fig. 30) and a sharp peak of activity was obtained with brain homogenates at a ratio of 0.6 (Fig. 30). The optimum ratio for nerve was 3.0. When the Cutscum/protein ratio for brain was maintained at the optimum value, maximum stimulation was seen only at low substrate concentrations (Fig. 31a), with apparent inhibition occurring at higher concentrations. The sciatic nerve DPIK was not similarly inhibited but the substrate concentration required to saturate the enzyme was increased by Cutscum (Fig. 31b). Other aspects of the DPIK assay (time and protein curve) were not changed by Cutscum (Fig. 32a, b).

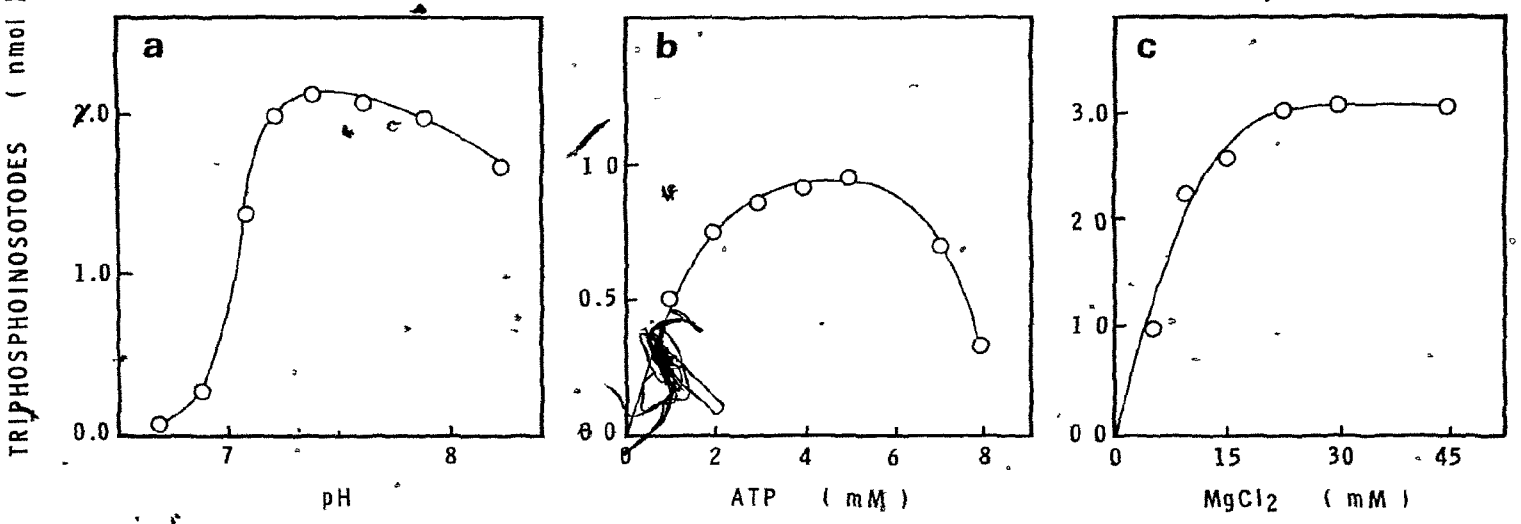


Fig. 29: Effects of pH(a), ATP(b) and $MgCl_2$(c) on DPIK Activity. Standard assay conditions were used with 1% Cutscum but with different incubation times: (a) 5 day chick brain (0.71 mg protein) for 3 min; (b) 19 day embryonic chick brain (0.48 mg protein) for 3 min; (c) 14 day chick brain (0.68 mg protein) for 5 min.

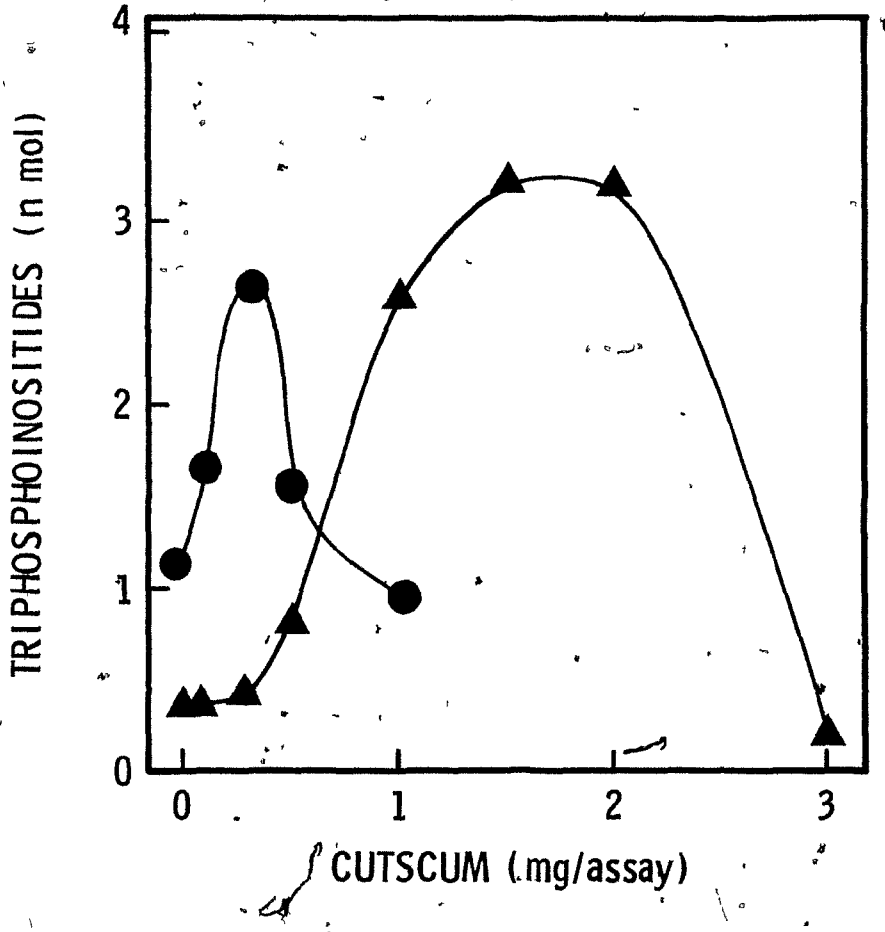


Fig. 30: Effect of Cutscum on DPIK Activity. Standard assay procedure using: ●, 3 day chick brain homogenate (0.54 mg protein) and ▲, 2 day chick sciatic nerve homogenate (0.49 mg protein), frozen for 26 days at -20°C.

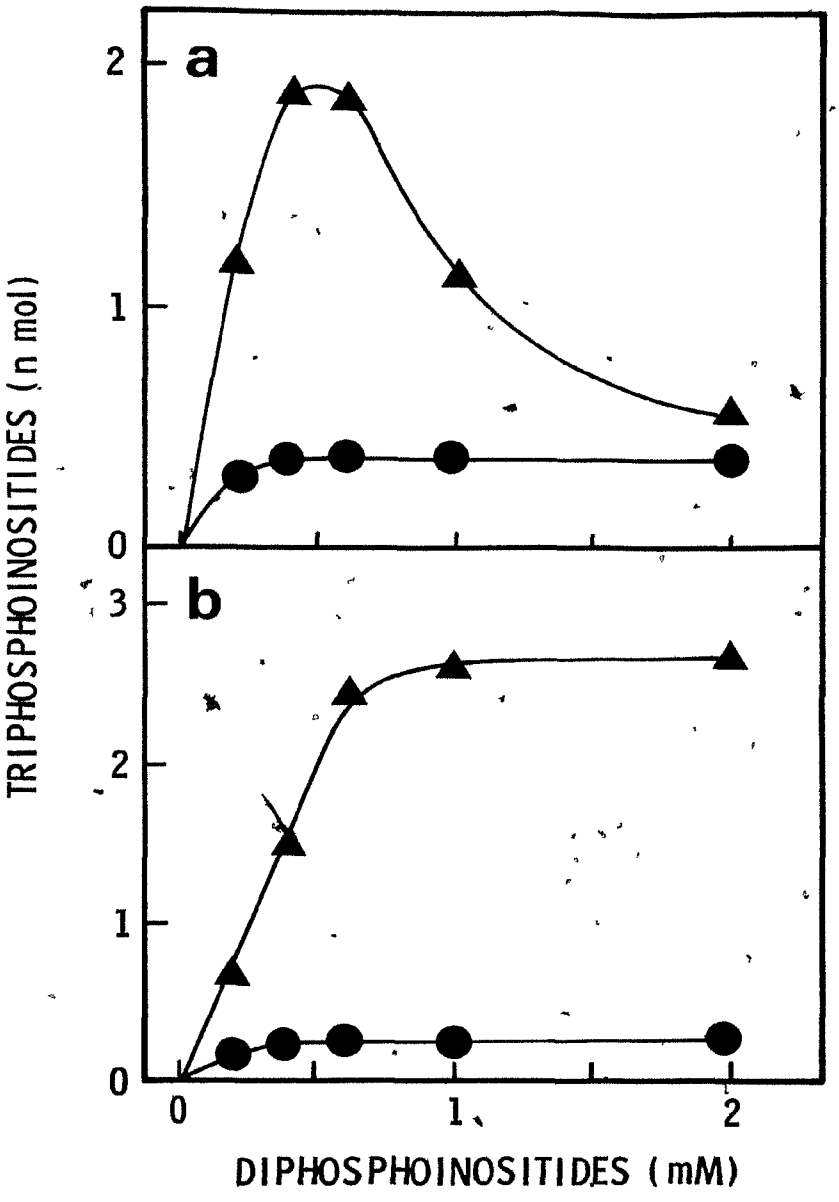


Fig. 31: Effect of Cutscum on the Substrate (DPI) Dependence of DPIK. Standard assay procedure using: (a) homogenate (32 days frozen at -20°C) of 21 day embryonic chick brain, 0.48 mg protein without Cutscum (●) and with a Cutscum/protein ratio of 0.6 (▲); (b) homogenate (30 days frozen at -20°C) of 2 day chick sciatic nerve, 0.48 mg protein without Cutscum (●) and with Cutscum/protein ratio of 3.0 (▲).

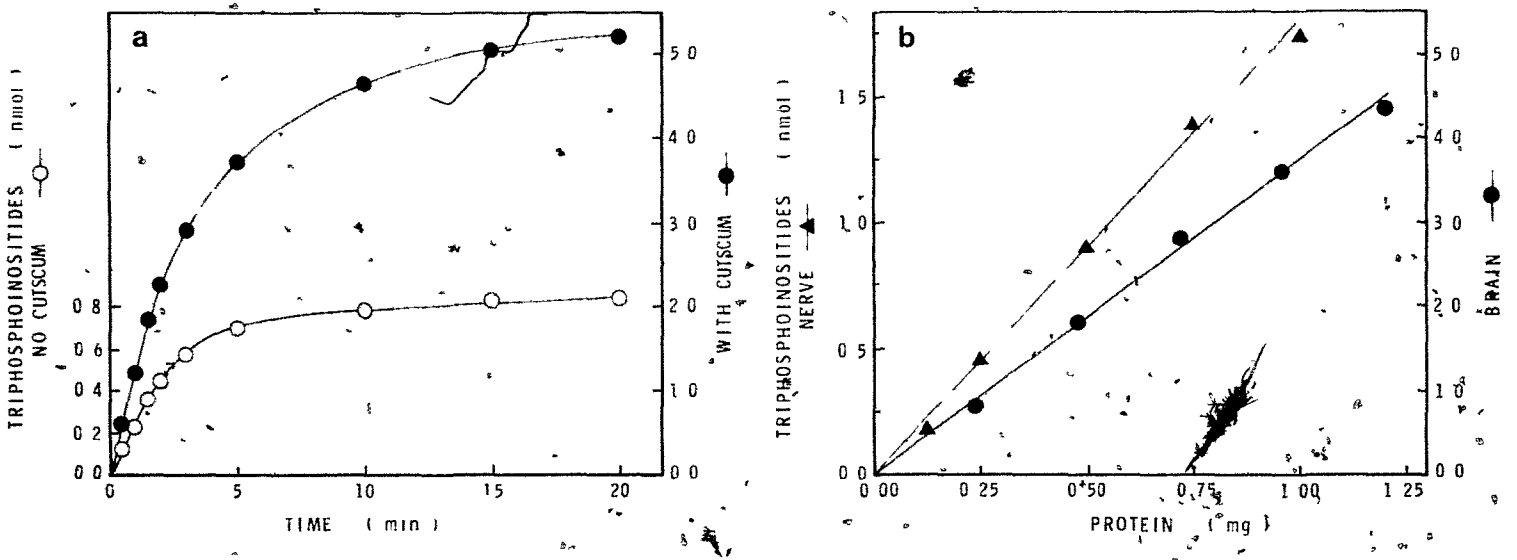


Fig. 32:
(a) Time-Course of DPIK Assay. Standard assay conditions using homogenates from 21 day embryonic chick brain (0.48 mg protein; homogenate used was frozen for 15 days at -20°C) without Cutscum (O), and with Cutscum/protein ratio of 0.6(●).
(b) Effect of Protein Concentration on DPIK Activity. Standard assay conditions with Cutscum/protein ratio of 0.6 for brain and 3.0 for sciatic nerve. Homogenates from 21 day embryonic chick brain (frozen for 10 days at -20°C) and 2 day chick sciatic nerve (frozen for 49 days at -20°C) were used.

C. PHOSPHOINOSITIDE PHOSPHOHYDROLASES OF THE DEVELOPING CHICKEN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

Several assay procedures for both TPI phosphatase and TPI diesterase have been described. The major differences have been the presence or absence of a cationic amphipathic compound such as CETAB and attempts to inhibit competing enzymatic activities. Assay conditions were investigated primarily in homogenates of chick brain (6 - 28 days old). The important features of each assay system were then confirmed to be the same for homogenates of chick brain and sciatic nerve from chick embryos, chicks and adult chickens.

When suspensions of TPI were incubated with chick brain homogenate in a reaction mixture containing Tris-HCl buffer (pH 7.2), release of both inorganic-P and organic-P was observed (Table 30). Addition of CETAB enhanced both activities when an equimolar mixture of substrate and detergent was used to start the reactions. Similar stimulation has been observed by others in several animal species and is believed to be the result of moderation of the negative charge on the substrate (Dawson and Thompson, 1964; Sheltawy et al., 1972; Palmer, 1973a; Cooper and Hawthorne, 1975). Maximum stimulation of both enzymes was observed with 0.2 M-KCl in the presence or absence of CETAB.

Table 30. Effect of KCl and Chelating Agents on the Hydrolysis of Triphosphoinositides by Chick Brain

Additions	TPI Phosphatase (µg Inorganic-P)		TPI Phosphodiesterase (µg Organic-P)	
	A	B	A	B
None*	1.3	2.5	0.4	4.6
KCl 0.05 M	1.5	3.2	1.1	6.8
KCl 0.1 M	1.5	3.5	1.9	9.0
KCl 0.2 M	1.4	3.7	3.2	8.8
EDTA 1 mM	1.1	1.5	ND**	ND
EDTA 4 mM	1.0	1.2	ND	ND
EDTA 10 mM	1.0	1.0	ND	ND
EGTA 0.5 mM	1.7	2.5	ND	ND
EGTA 2 mM	1.7	2.5	ND	ND
EGTA 5 mM	1.7	2.5	ND	ND

* Control contained 45 mM Tris-HCl (pH 7.2) and 1 mM TPI (experiment A) or 1 mM TPI - 1 mM CETAB (experiment B). Additions were preincubated with the buffer and enzyme (0.23 mg protein, 10 day chick brain homogenate) at 0°C for 10 min. All tubes were then incubated at 37°C for 30 min.

** ND - not detectable.

1. Characteristics of TPI Phosphatase

For the accurate measurement of TPI Phosphatase, it is desirable to eliminate or at least minimize competition for the substrate by TPI phosphodiesterase and other non-specific phosphatases. Salway et al. (1967) reported selective inhibition of TPI phosphodiesterase in rat brain by EDTA (4 mM) while others have found TPI phosphatase to be inhibited as well (Lee and Huggin, 1968a, b; Sheltawy et al., 1972). Both enzymes in chick brain were inhibited by EDTA although the effect was smaller for TPI phosphatase. Selective abolition of the diesterase activity was accomplished in the presence or absence of CETAB by the use of EGTA, a highly specific chelating agent of Ca^{2+}. The phosphatase, a Mg^{2+} requiring enzyme (Dawson and Thompson, 1964), was unaffected. In the absence of CETAB, the activity was slightly enhanced by EGTA. This stimulation may be due to the elimination of competition for the same substrate by TPI phosphodiesterase as was suggested earlier for EDTA stimulation in rat brain (Salway et al., 1967) and EGTA stimulation in protozoa (Palmer, 1976). Competition by the diesterase was not apparent in the presence of CETAB since EGTA caused no stimulation of phosphatase activity. Therefore, EGTA was included in the measurement of TPI phosphatase activity. The effects of CETAB were found to be more complex than initially expected. Stimulation of the activity involved a complex relationship between the concentrations of detergent, substrate, Mg^{2+} and homogenate.

In the absence of CETAB, the TPI phosphatase of embryonic chick brain was saturated with 0.2 mM-TPI (Fig. 33a). Similar results were obtained with homogenates of sciatic nerve of all ages where the specific activity was very low (Fig. 33c, d). Older chick brain homogenates exhibited much higher phosphatase activities and smaller amounts of homogenate were used in the assay system. These homogenates required more substrate to saturate the enzyme but were inhibited by TPI concentrations greater than 0.8 mM (Fig. 33b).

Addition of CETAB greatly increased the maximum velocity in both tissues and prevented substrate inhibition in chick brain homogenates. Optimum stimulation occurred at a CETAB/TPI molar ratio of 2 (Fig. 34a, b). The detergent also increased the substrate concentrations required for saturation of the enzyme in both tissues to at least 2 mM. Stimulation by CETAB (Fig. 33a, b, c, d) was greater for embryonic tissues (4 - 8 fold) than for adult tissues (2 - 3 fold).

Sheltawy et al. (1972) assumed that homogenates of guinea pig brain contained sufficient Mg^{2+} to meet the requirements both for an activating ion and to moderate the negative charge on the substrate. This is not a valid assumption for homogenates of chick neural tissues since addition of $MgCl_2$ to the incubation mixture enhanced the TPI phosphatase activity. In the absence of CETAB, a 10 fold increase in activity occurred with 2 mM-Mg^{2+} at the

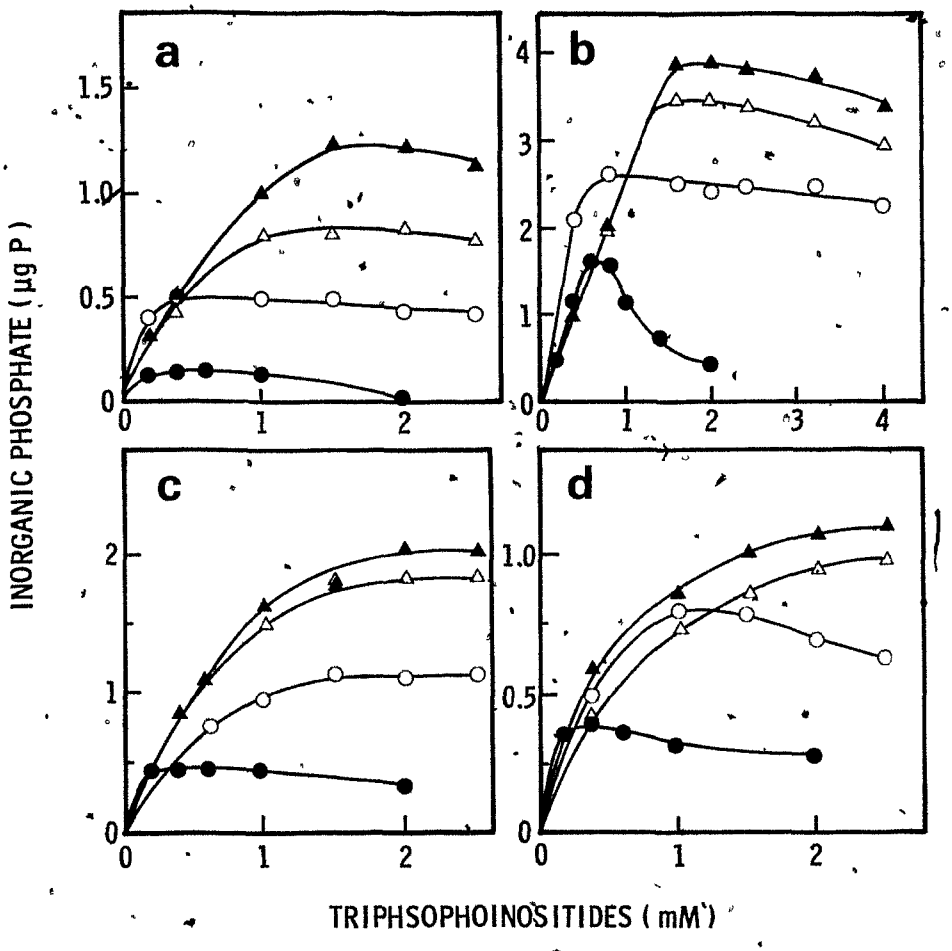


Fig. 33: The Effect of CETAB on the Substrate Dependence of Chick Nervous System TPI Phosphatase Activities. Assays were as described without added $MgCl_2$ using homogenates of (a) 15 day embryonic chick brain (0.12 mg protein); (b) 6 day chick brain (0.19 mg protein; (c) 15 day embryonic chick sciatic nerve (0.20 mg protein and (d) adult chicken sciatic nerve (0.20 mg protein). Assays contained: no CETAB (●); CETAB/TPI ratios of 1/1 (O); 2/1 (▲) and 2.5/1 (△). Reaction product expressed as µg Pi/assay.

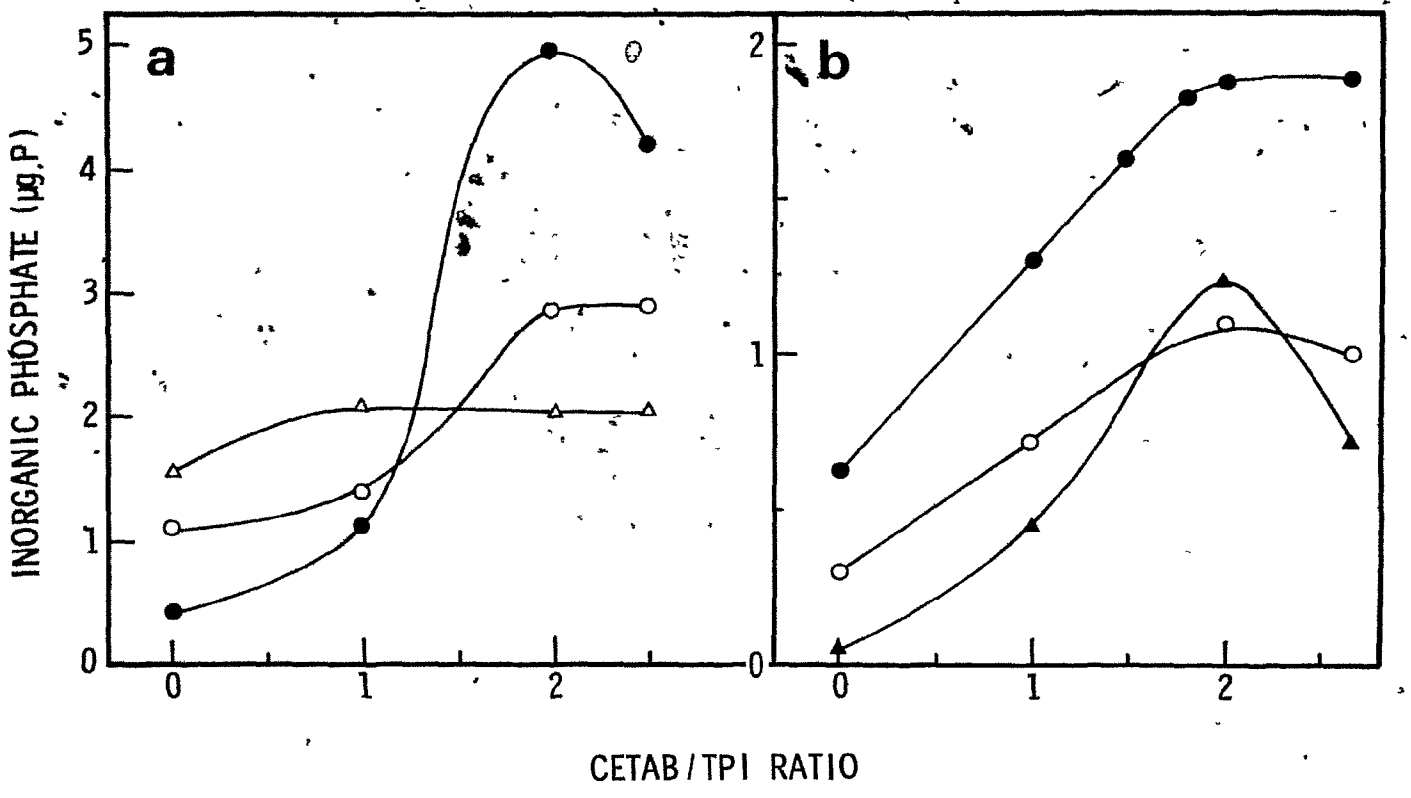


Fig. 34: Effect of CETAB/TPI Ratio on TPI Phosphatase Activity. Standard assay conditions were used without added $MgCl_2$. (a) Homogenates used were from 6 day chick brain containing 0.14 mg protein/assay. Substrate concentrations were: △, 0.6 mM; o, 1 mM; ●, 2 mM. (b) Homogenates used were from: ▲, 17 day embryonic brain (0.07 mg protein); o, adult chicken sciatic nerve (0.20 mg protein) and ●, 15 day embryonic chick sciatic nerve (0.19 mg protein). Substrate concentrations were 2 mM.

optimum substrate concentration of 1 mM (Fig. 35). The requirement was reduced to 1 mM in the presence of CETAB but remained the same for both concentrations of CETAB confirming that the added Mg^{2+} is required for another purpose as well as to neutralize substrate charge. This was also apparent in the failure to achieve a linear relationship of activity with increasing protein concentrations in the absence of Mg^{2+} with or without CETAB (Fig. 36). This is characteristic of the presence of activator in the homogenate (Dixon and Webb, 1964).

The substrate inhibition seen with brain homogenates in the absence of CETAB (Fig. 33b) appears to be the result of inadequate neutralization of the negative charge on the substrate by endogenous divalent cations. Competition by TPI for the Mg^{2+} required by the enzyme may also contribute. Addition of Mg^{2+} (1 mM) did not prevent inhibition by TPI when the Mg^{2+}/TPI ratio became less than about 1.7 (Fig. 35). Maintaining this ratio constant at 2.0 prevented inhibition by high concentrations of TPI but no stimulation of the maximum activity occurred. The Mg^{2+}/TPI ratio was not critical in the presence of CETAB (Fig. 37b) so long as some Mg^{2+} was added (1 mM). Under these conditions, the maximum activity was considerably increased (4 fold). As observed earlier the concentration of substrate required to saturate the system was increased to over 2 mM. The requirements for Mg^{2+} at a CETAB/TPI ratio of 2/1 did not change with

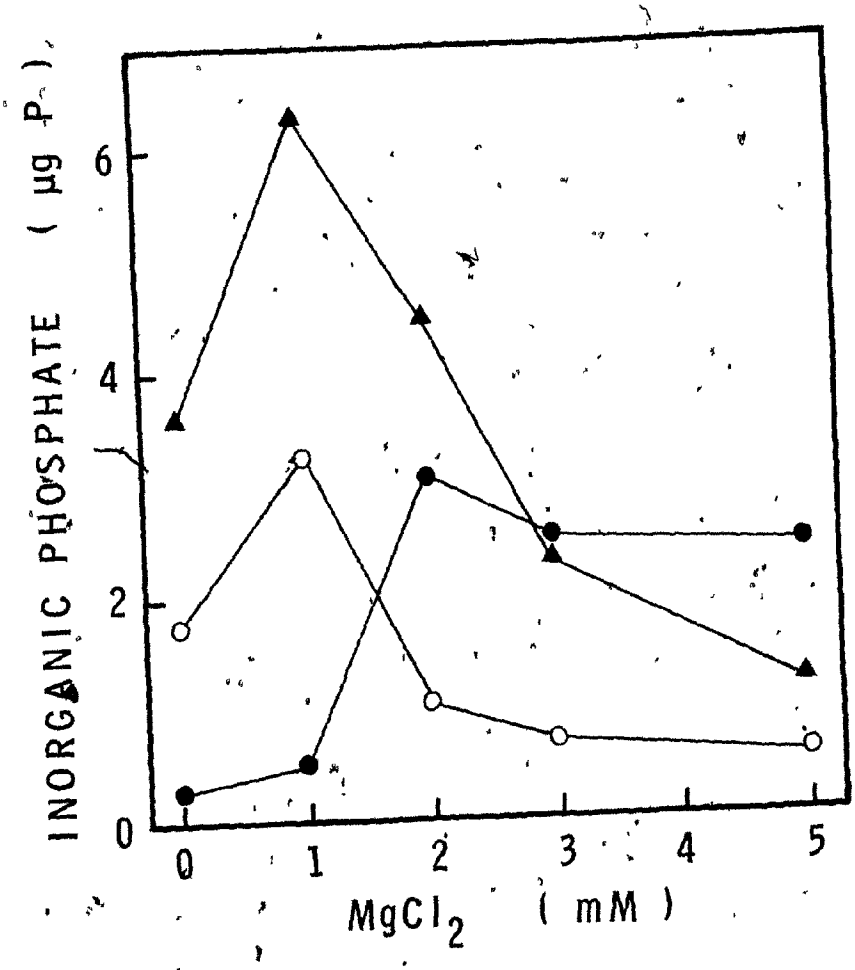


Fig. 35: The Effect of Magnesium on TPI Phosphatase. Assays were as described using a homogenate of 26 day chick brain (0.14 mg.protein) and 1 mM-TPI. The CETAB concentrations were: none (●), 1 mM (o), 2 mM (▲).

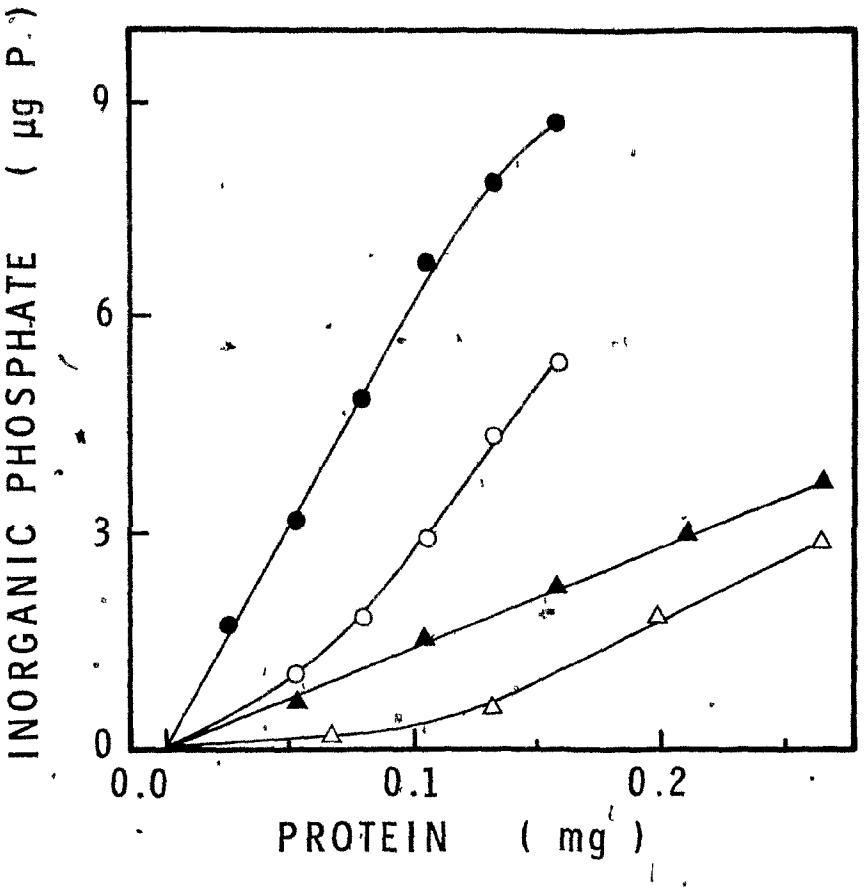


Fig. 36: The Dependence of TPI Phosphatase Activity on the Amount of Homogenate. Activity was measured with a homogenate of whole brain from a 28 day old chick. The assay system was as described using 2 mM-TPI - 4 mM-CETAB with (●) and without (○) 0.8 mM-$MgCl_2$. When CETAB was omitted (triangles) the TPI concentration was reduced to 0.5 mM to avoid substrate inhibition and activity was measured with (▲) and without (△) 1 mM-$MgCl_2$.

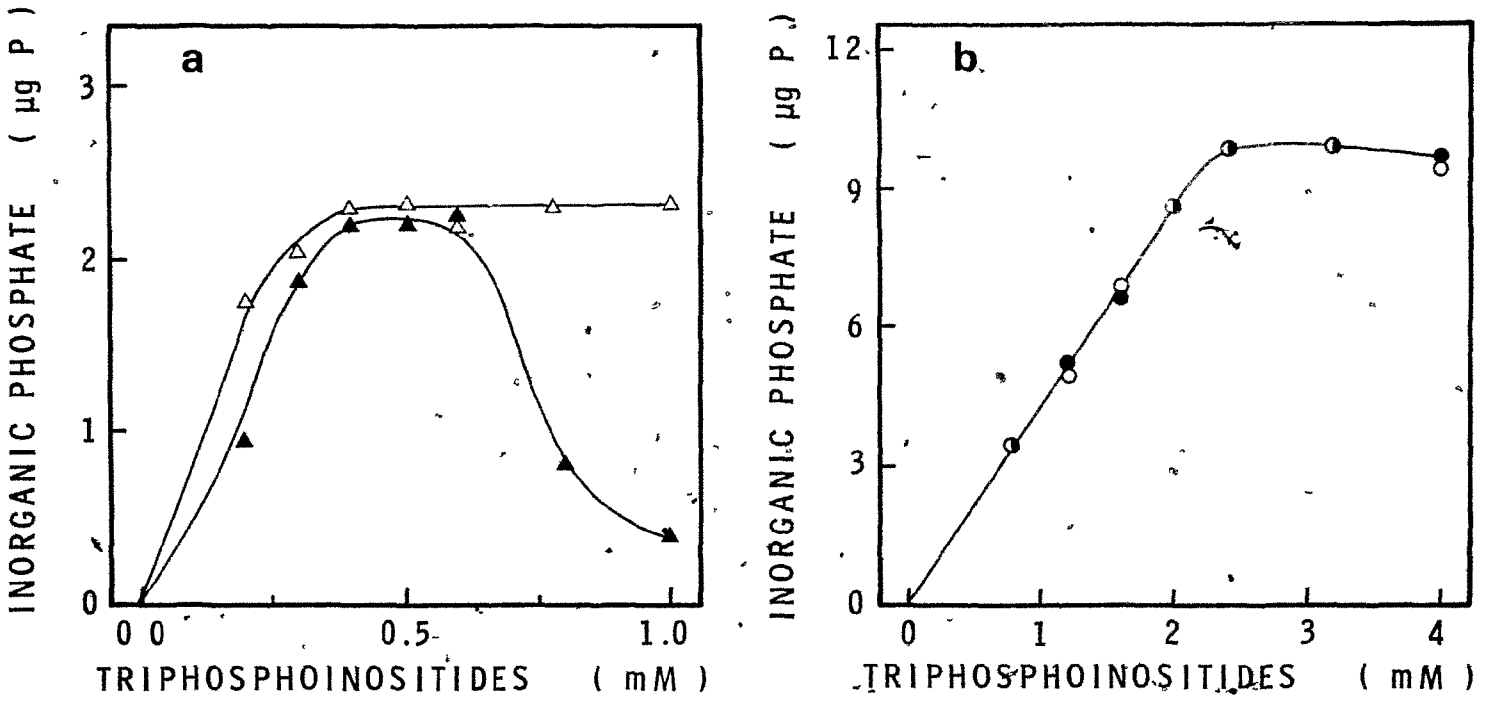


Fig. 37: Effect of Magnesium on the Substrate Dependence of TPI Phosphatase. Assays were as described using a homogenate of 28 day chick brain (0.11 mg protein). (a) In the absence of CETAB activities were measured in the presence of 1 mM-$MgCl_2$ (▲) and with the ratio of $MgCl_2$/TPI maintained at 2/1 (△). (b) Assays containing CETAB with 1 mM-$MgCl_2$ and CETAB/TPI ratio of 2/1 (○) and with constant CETAB/TPI/$MgCl_2$ ratios of 2/1/1 (●).

developmental age. Both brain and nerve phosphatases exhibited a broad peak of activity at 1 mM-Mg^{2+} (Fig. 38). Stimulation was greatest for chick brain homogenates.

The final assay system contained 1 mM-$MgCl_2$ and a saturating concentration of TPI (2.8 mM) with sufficient CETAB (5.6 mM) to give optimum CETAB/TPI ratio of 2. Using these conditions, the response to increasing protein was linear (0.01 - 0.1 mg protein) for homogenates of embryonic and older chick tissues (Fig. 36 & 39a). The time course of the release of inorganic-P was also linear up to at least 20 min (Fig. 39b) and a broad optimum from pH 6.8 - 7.5 was observed (Fig. 40). Thin-layer chromatography of the lipid products of the reaction confirmed that DPI did not accumulate but were dephosphorylated to PI as had been reported for the TPI phosphatase of brain (Sheltawy et al., 1972) and kidney cortex (Lee and Huggins, 1968b).

The presence of acid and alkaline phosphatases represent potential sources of error in TPI phosphatase measurements. Alkaline phosphatase can be inhibited by EDTA (Hubscher and West, 1965) or cysteine (Cooper and Hawthorne, 1975) and acid phosphatases are inhibited by fluoride (Hubscher and West, 1964; Cohen, 1970). EDTA is not appropriate in this assay system since it inhibits the TPI phosphatase. Addition of NaF or cysteine (up to 3 mM) to homogenates of chick neural tissues had no effect on the liberation of inorganic-P from TPI suggesting that neither alkaline nor acid phosphatases contributed to the measured

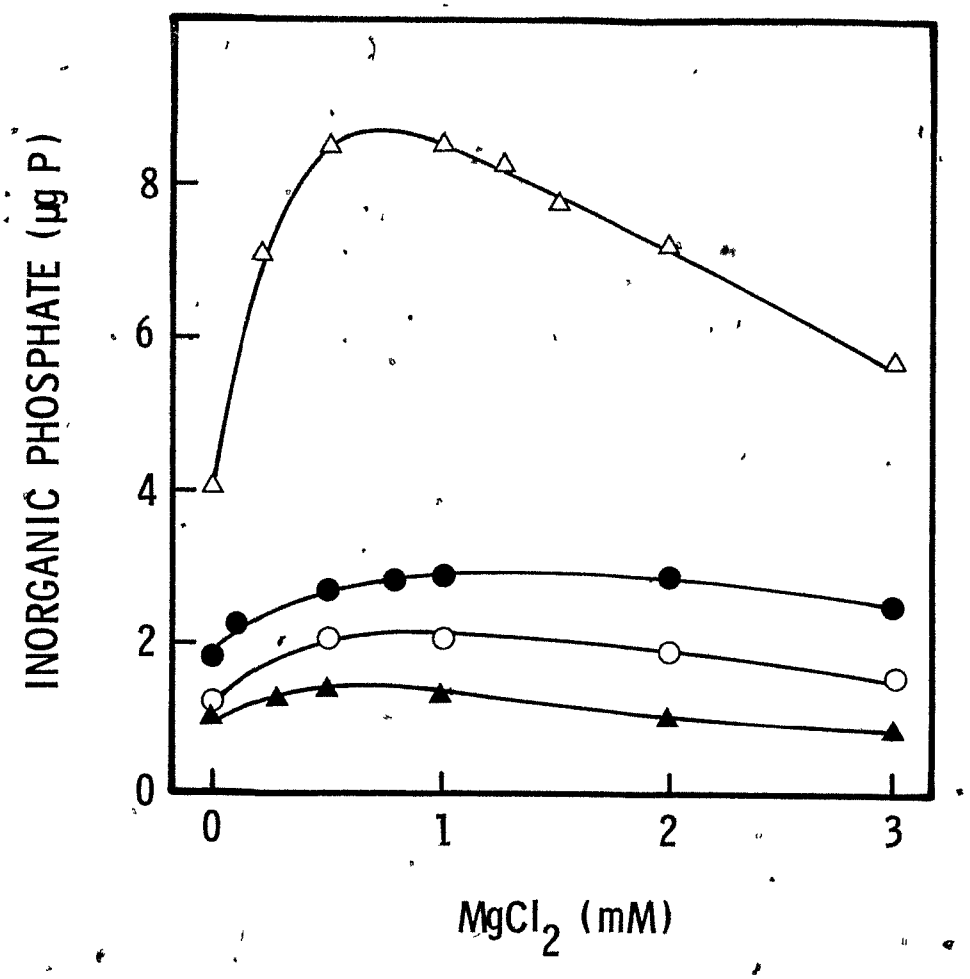


Fig. 38: Effect of $MgCl_2$ on TPI Phosphatase. Standard assay conditions used. Enzyme sources were: △, 18 day old chick brain (0.11 mg protein); ▲, 17 day embryonic chick brain (0.07 mg protein); ○, adult chicken sciatic nerve (0.20 mg protein); ●, 15 day embryonic chick sciatic nerve (0.20 mg protein).

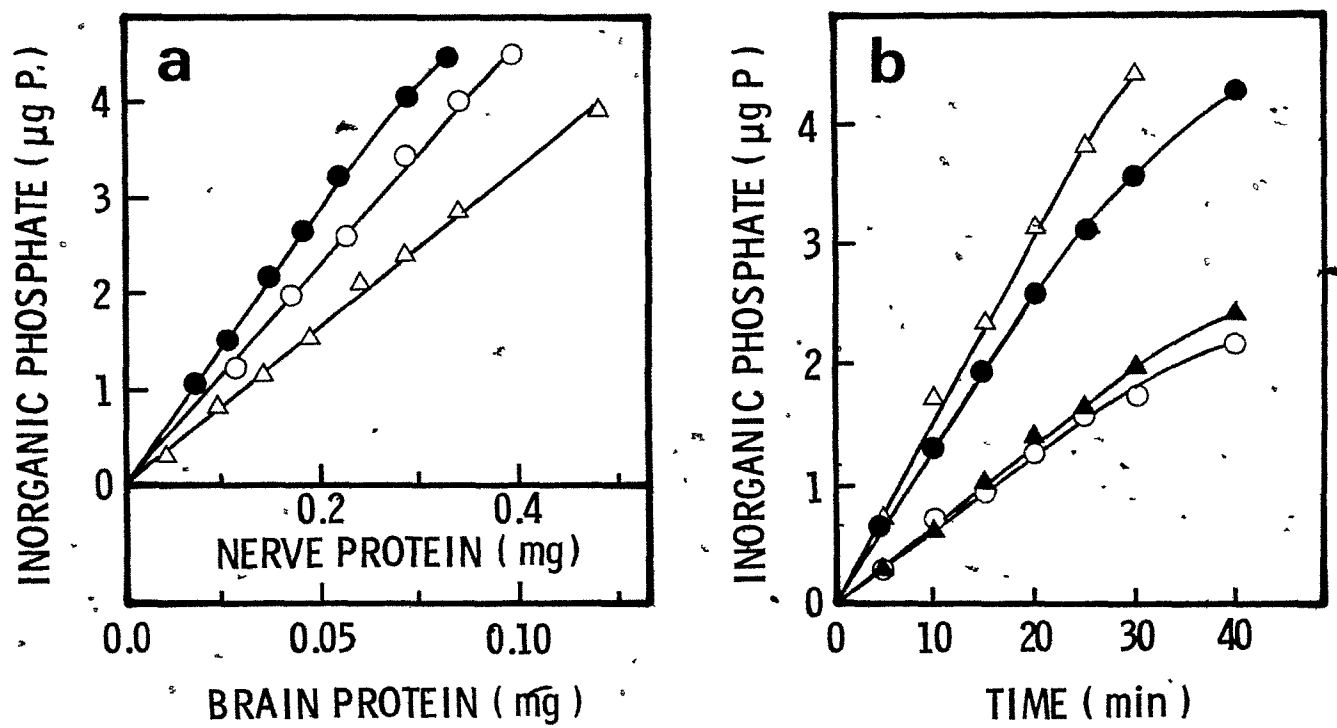


Fig. 39:
(a) Effect of Protein Concentration on TPI Phosphatase Activity. Standard assay conditions were used. Sources of enzyme were: △, 17 day embryonic chick brain, ●, 17 day embryonic chick sciatic nerve; ○, 21 day chick sciatic nerve.
(b) Time-Course of TPI Phosphatase Activity. Standard assay conditions were used. Sources of enzyme were: ▲, 17 day embryonic chick brain (0.07 mg protein); △, 21 day old chick brain (0.06 mg); ●, 17 day embryonic chick sciatic nerve (0.26 mg protein; ○, adult chicken sciatic nerve (0.16 mg protein).

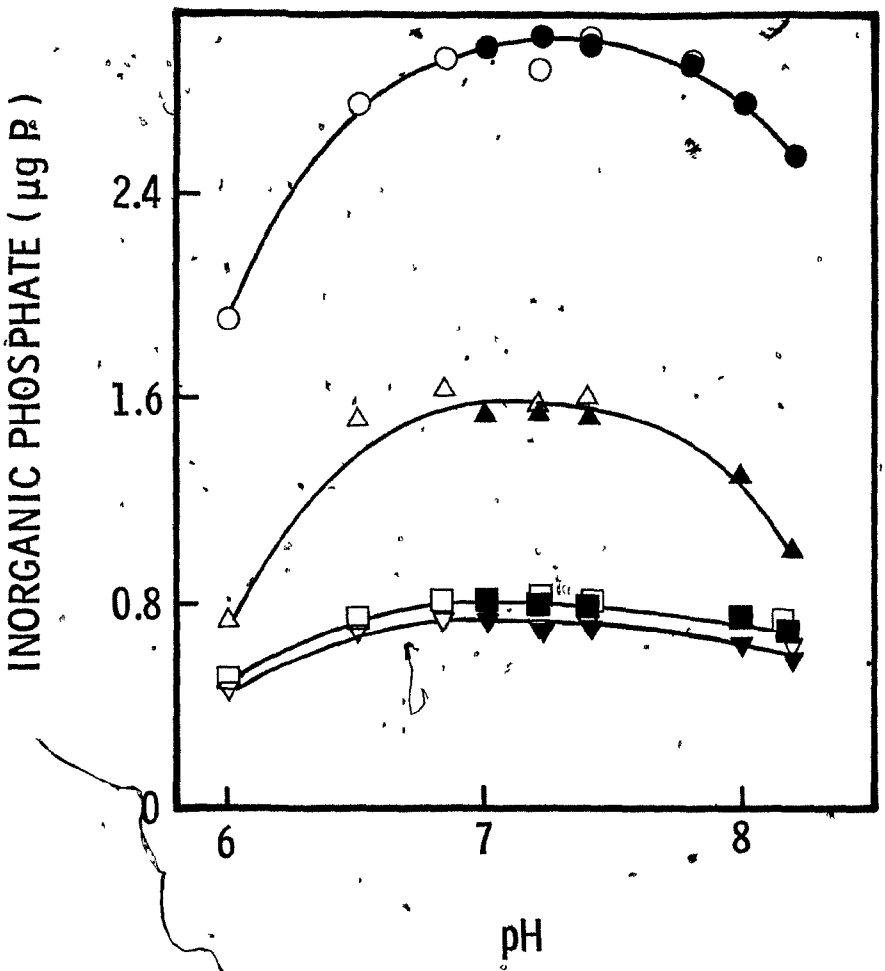


Fig. 40: Effect of pH on TPI Phosphatase. Assays were as described. Sources of enzymes were: ▲, △, 17 day embryonic chick brain (0.07 mg protein); ●, ○, 21 day old chick brain (0.06 mg protein); ■, □, 17 day embryonic chick sciatic nerve (0.19 mg protein); ▼, ▽, adult chicken sciatic nerve (0.20 mg protein). Solid symbols, 45 mM - Tris-HCl; open symbols, 50 mM-HEPES - 50 mM-MES.

activity (Table 31). Higher concentrations of cysteine were slightly inhibitory. Blank values, already low, were marginally reduced by both agents. Stimulation of the activity by CETAB and a sensitive detection system permitted the use of small amounts of protein, thus further reducing the effect of any possible hydrolysis of TPI by non-specific phosphatases. Reduced glutathione, which has been reported to stimulate TPI phosphatase (Dawson and Thompson, 1964), had no net effect in our assay system at up to 15 mM concentrations. Higher amounts (20 mM) were inhibitory (Table 31). The "pH 5 supernatant" fraction (Sheltawy et al., 1972) prepared from either rat brain or chick brain also had very little effect in the presence of CETAB. These agents were therefore not included in the routine assay system.

2. Characteristics of TPI Phosphodiesterase

Stimulation of TPI phosphodiesterase in crude homogenates by KCl has been reported for rat brain (Keough and Thompson, 1970); however, the optimum concentration of KCl was determined only for TPI phosphatase (Dawson and Thompson, 1964; Sheltawy et al., 1972). In chick brain homogenates the effect of KCl was found to be both time and concentration dependent (Fig. 41a). Preincubation of the homogenate with KCl for 10 min was necessary to obtain the maximum effect, particularly at low KCl concentrations. All the chick neural tissues exhibited a similar response

Table 31. Effect of Sodium Fluoride, Cysteine and Reduced Glutathione on TPI Phosphatase Activity

Addition		% of Control
None**		100
NaF	0.5 mM	95
	1.0 mM	99
	1.5 mM	101
	2.0 mM	93
Cysteine	1 mM	102
	3 mM	96
	5 mM	83
Glutathione	10 mM	97
(reduced)	15 mM	102
	20 mM	74

* Standard assay conditions using homogenates from 20 day chick brain, 0.095 mg protein (for NaF & Cysteine) and 21 day embryonic chick brain, 0.08 mg protein (for glutathione).

** Homogenates added after the addition of BSA and $HClO_4$.

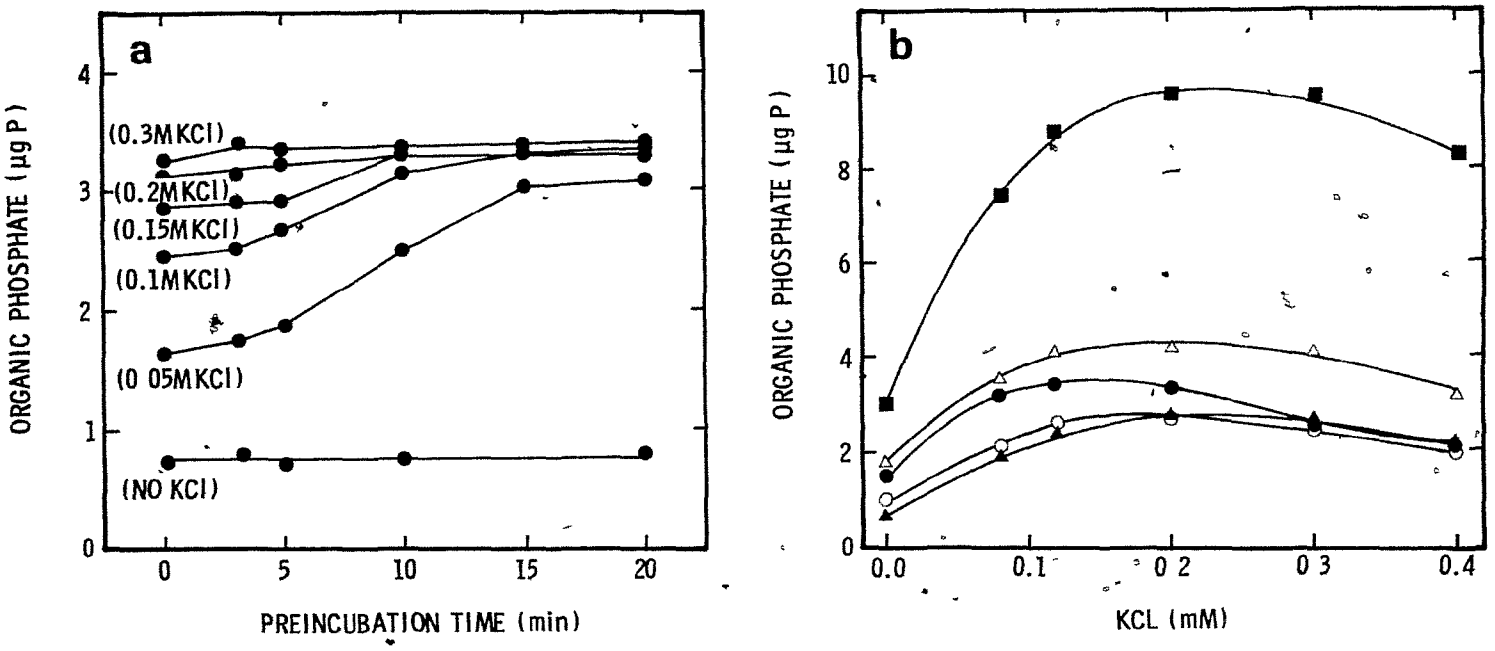


Fig. 41:
(a) The Effect of Pre-Incubation with KCl on TPI Phosphodiesterase. Incubation mixture contained 45 mM-Tris-HCl (pH 7.2), tissue homogenate (16 day embryonic chick brain, 0.13 mg protein) and 1.5 mM-TPI, reaction time 7 min.
(b) The Effect of KCl Concentrations on TPI Phosphodiesterase. Reaction conditions as above but contained 1.0 mM-TPI. Homogenates were used from: ●, 9 day chick brain (0.21 mg protein); ○, 16 day embryonic brain (0.13 mg protein); ▲, adult chicken sciatic nerve (0.20 mg protein); △, 15 day embryonic sciatic nerve (0.19 mg protein). Embryonic chick brain was also assayed with 1.5 mM-TPI and 3 mM-CETAB. Pre-incubation time of 10 min was used.

to KCl, optimum activity occurring with 0.12 - 0.2 M-KCl in the absence of CETAB (Fig. 41b). The optimum was slightly shifted toward higher KCl concentrations in the presence of CETAB and all subsequent assays included 0.2 M-KCl.

Stimulation of brain TPI phosphodiesterase activity by CETAB has been reported (Thompson and Dawson, 1964b; Keough and Thompson, 1972) and in each case the molar ratio of CETAB to TPI was critical for optimum activity. The optimum ratio at any substrate concentration was 2/1 for homogenates of chick brain (Fig. 42a). As reported earlier for bovine brain (Thompson and Dawson, 1964b), no stimulation occurred below a threshold ratio of about 0.5/1. Similar results were observed for embryonic chick brain and for embryonic and adult sciatic nerves (Fig. 42b). In the absence of CETAB, the TPI phosphodiesterase was saturated by 0.5 mM-TPI. Although the total activity was greatly enhanced by CETAB, the apparent affinity of the enzyme for TPI was reduced (Fig. 43a, b, c, d). The enzyme could not be fully saturated at practical substrate concentrations particularly in older chick brain where saturation was not achieved in some preparations up to 5 mM-TPI.

Addition of low concentrations of Ca^{2+} to the crude homogenates caused some increase in activity (Fig. 44a). Higher concentrations resulted in inhibition which was accompanied by obvious precipitation of the substrate. Optimum levels of CETAB were present in these experiments suggesting that the Ca^{2+} was not required for substrate neutralization and that the Ca^{2+} content of the homogenate

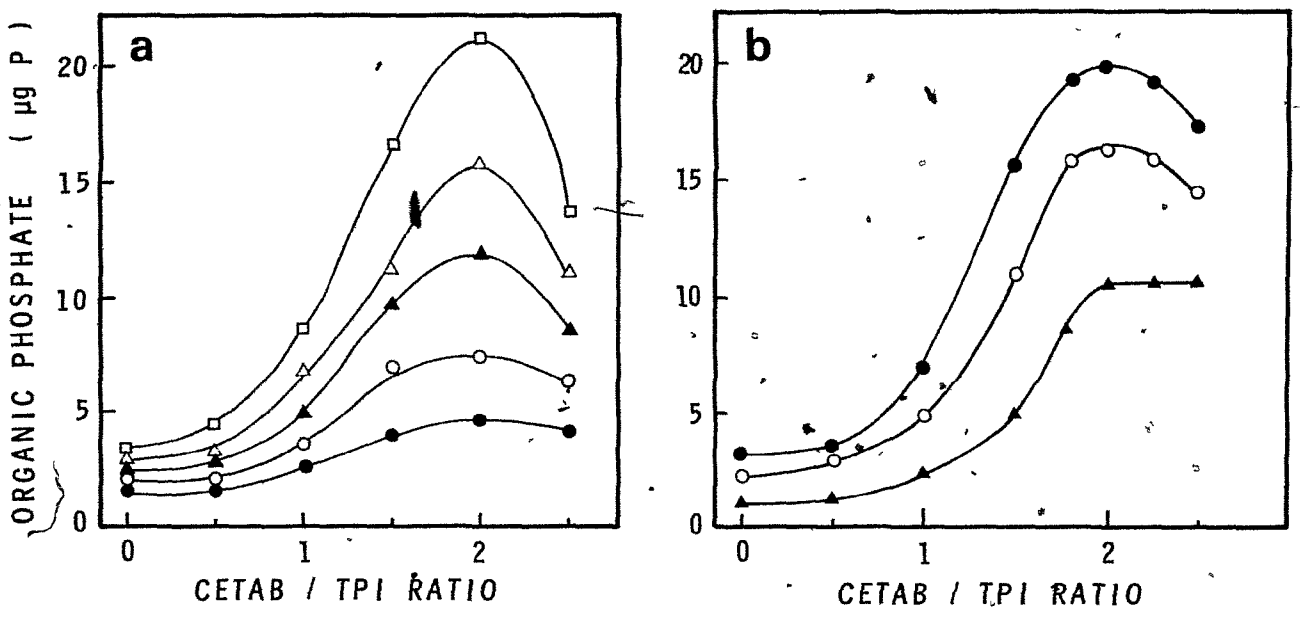


Fig. 42: The Effect of CETAB on TPI Phosphodiesterase Activity in Chick Brain. Assays were as described without added calcium.

(a) The effect of increasing CETAB/TPI ratios using a homogenate of 10 day chick brain (0.12 mg protein). TPI concentrations were: 0.4 mM (●), 1.0 mM (○), 1.5 mM (▲), 2.0 mM (△) and 3.0 mM (□).

(b) The Optimum CETAB/TPI Ratio for TPI Phosphodiesterase Activity. TPI concentration was 2.0 mM and the sources of enzyme were: ▲, 15 day embryonic chick brain (0.06 mg protein); ●, 17 day embryonic chick sciatic nerve (0.09 mg protein); ○, adult chicken sciatic nerve (0.20 mg protein).

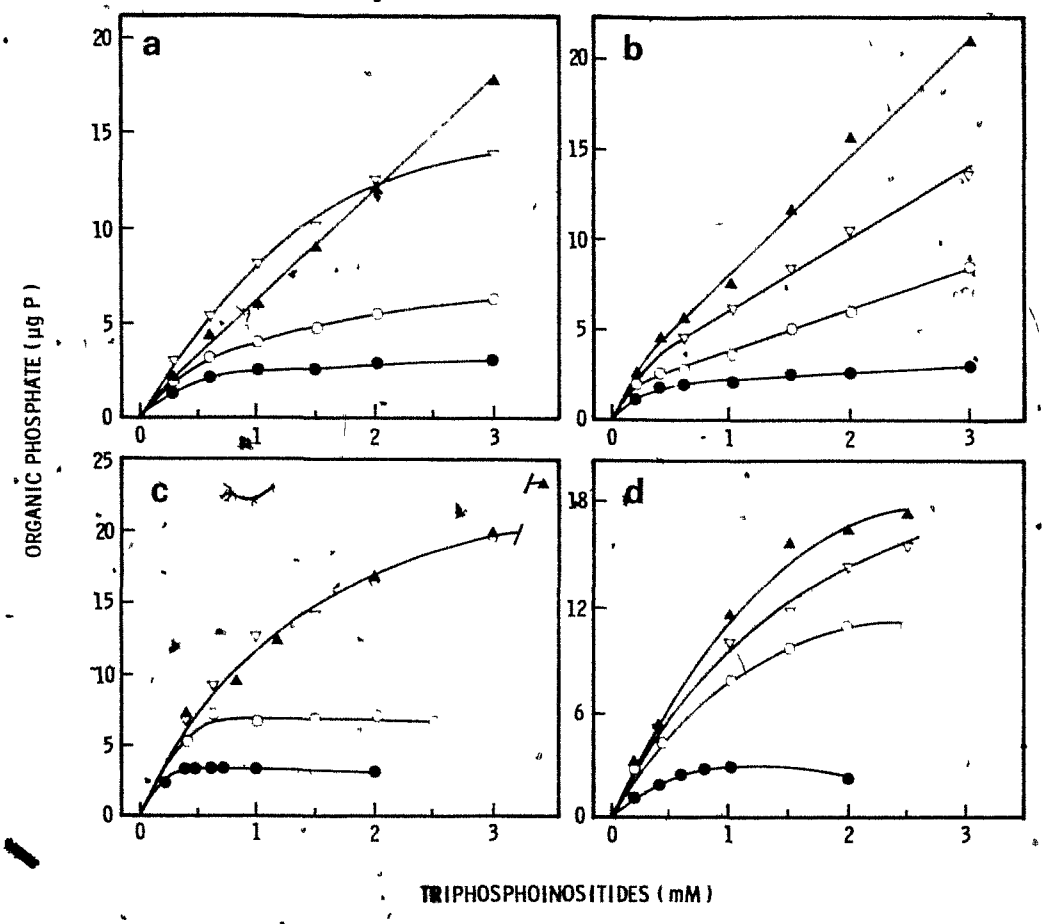


Fig. 43: The Effect of CETAB on Substrate Dependence. Standard assay conditions were used without added calcium. Homogenates from: (a) 15 day embryonic chick brain (0.06 mg protein), (b) 10 day chick brain (0.115 mg protein), (c) 17 day embryonic chick sciatic nerve (0.09 mg protein) and (d) adult chicken sciatic nerve (0.2 mg protein). TPI alone (●); CETAB/TPI ratios of 1/1 (○) for (b) and (c) and 1.5/1 for (a) and (d); 2/1 (▲) and 2.5/1 (▽).

was inadequate for optimum activation of the enzyme. The requirement for Ca^{2+} was dependent to some extent upon prior treatment of the homogenate with KCl. The amount of stimulation by Ca^{2+} was greater but the maximum activity obtained with Ca^{2+} alone was considerably lower than in the presence of KCl. On the other hand, Mg^{2+} was inhibitory. As anticipated, Mg^{2+} stimulated the TPI phosphatase activity in the homogenates. The release of Pi was suppressed by Ca^{2+} (Fig. 44b) thus making it possible to assay the diesterase without significant competition from the TPI phosphatase. Similar observations were made for embryonic brain and both embryonic and adult sciatic nerve (Fig. 45). Optimum activity was obtained with 0.2 mM-$CaCl_2$.

Very low activities were encountered in mature nerve. For the intended developmental study it was desirable to enhance the activity with CETAB. The final assay system contained KCl (0.2 mM), $CaCl_2$ (0.2 mM), CETAB (5.6 mM) and TPI (2.8 mM). Since the phosphodiesterase was not fully saturated the reaction rate could be expected to decrease with time. The reaction rate was in fact constant for only 7 - 10 min (Fig. 46). With this short incubation period the release of organic phosphate was linear with respect to enzyme concentration so long as no more than 25% of the substrate was consumed in the reaction (Fig. 47). Both central and peripheral nervous tissues exhibited the same pH optimum at 7.2 - 7.4 (Fig. 48).

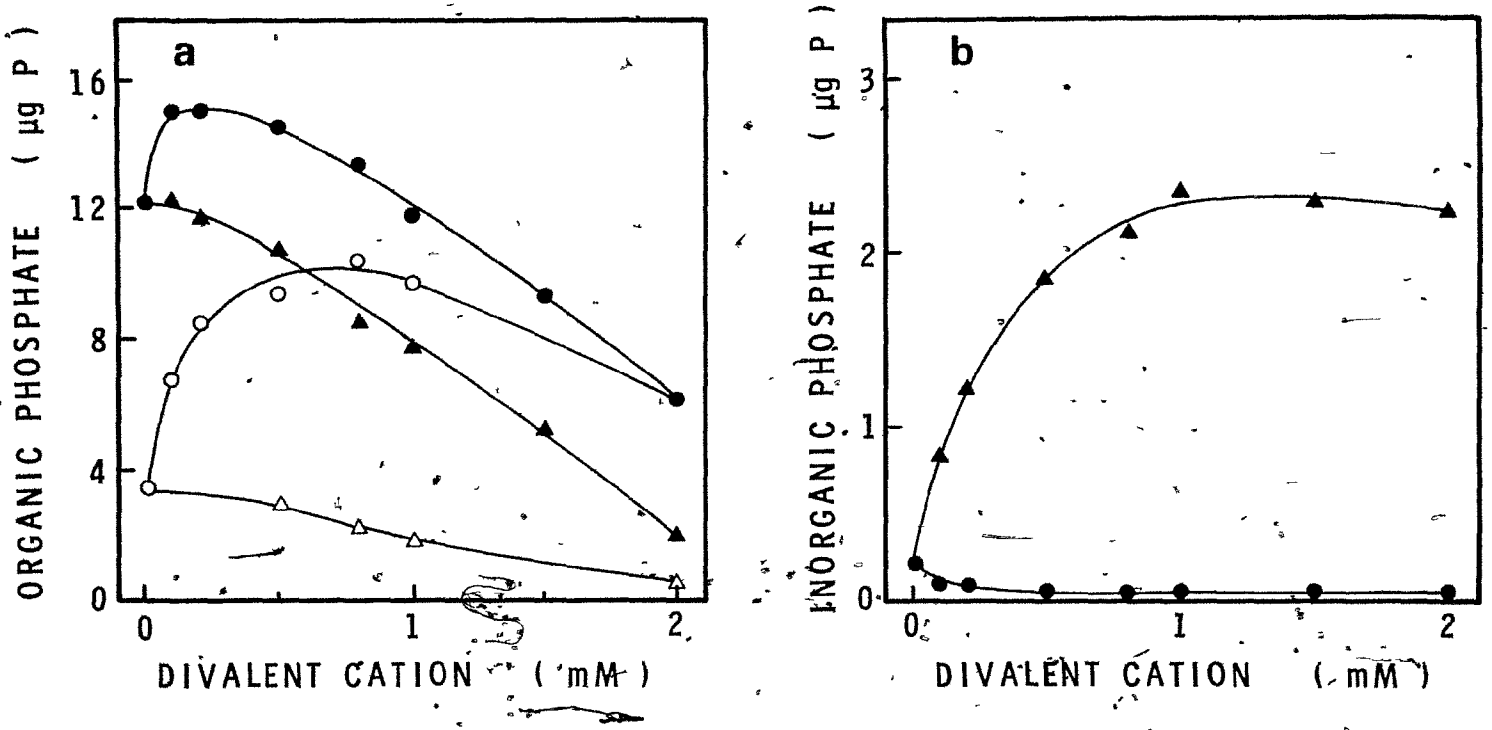


Fig. 44: The Effect of Divalent Cations on Chick Brain TPI Phosphodiesterase. Assays were described with 10 min incubation time using a homogenate of 28 day chick brain (0.05 mg protein). Activity was measured with (solid symbols) and without (open symbols) preincubation of the homogenate with 0.15 M KCl. The effects of increasing concentrations of both $CaCl_2$ (●, ○) and $MgCl_2$ (▲, △) on the release of organic phosphate (a) and inorganic phosphate (b).

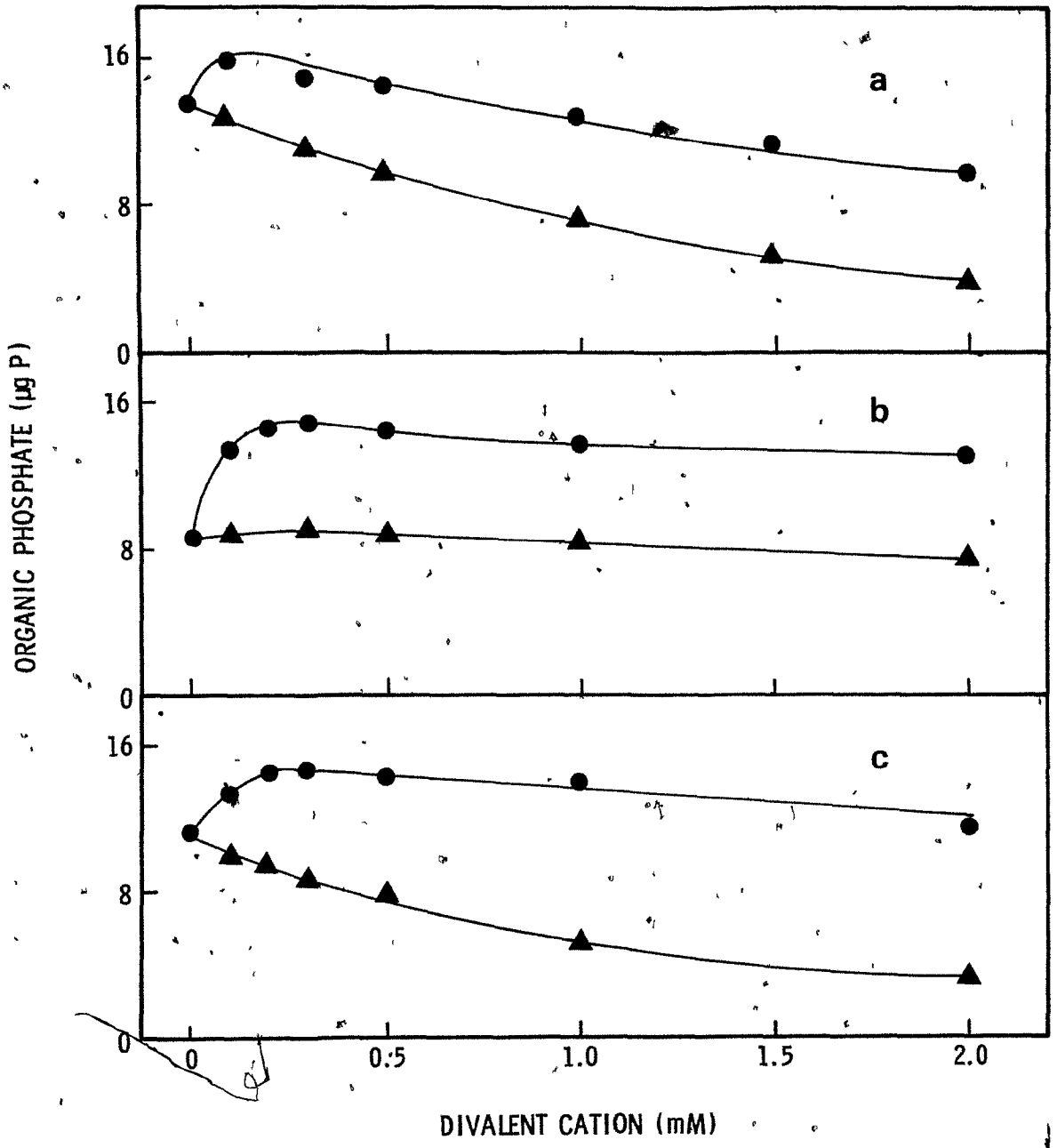


Fig. 45: Effect of Ca^{2+} and Mg^{2+} on the TPI Phosphodiesterase of Chick Nervous Tissues. Standard assay conditions with added $CaCl_2$ (●) or $MgCl_2$ (▲) in the presence of 2.0 M-KCl. Data was obtained with (a) 15 day embryonic chick brain (0.7 mg protein), (b) 17 day embryonic chick sciatic nerve (0.03 mg protein) and (c) adult chicken sciatic nerve (0.08 mg protein).

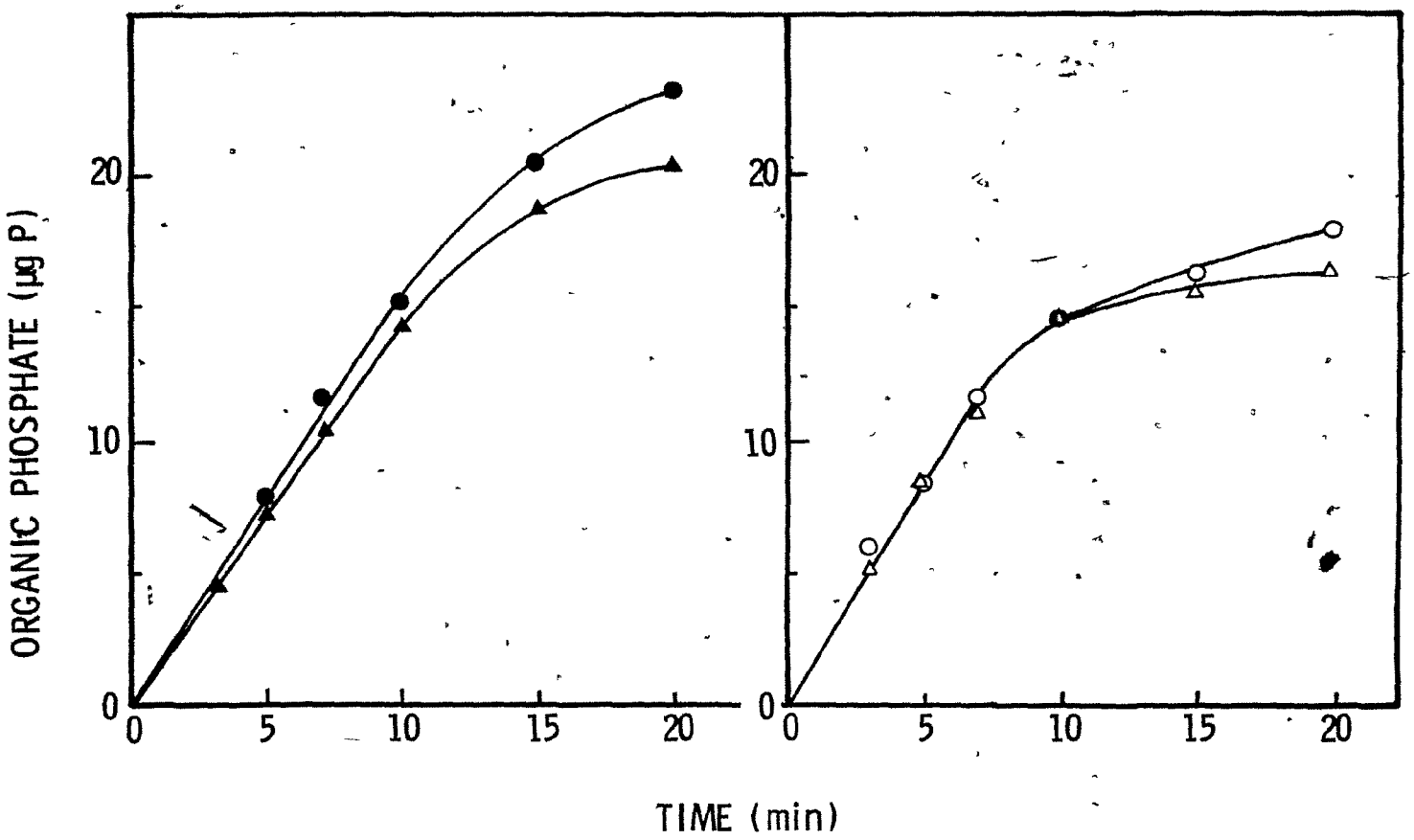


Fig. 46: Time-Course of TPI Phosphodiesterase Action. Assays were as described in "Methods". Enzyme sources were: ●, 28 day old chick brain (0.05 mg protein); o, 17 day embryonic chick brain (0.04 mg protein); ▲, adult chicken sciatic nerve (0.06 mg protein); △, 17 day embryonic chick sciatic nerve (0.04 mg protein).

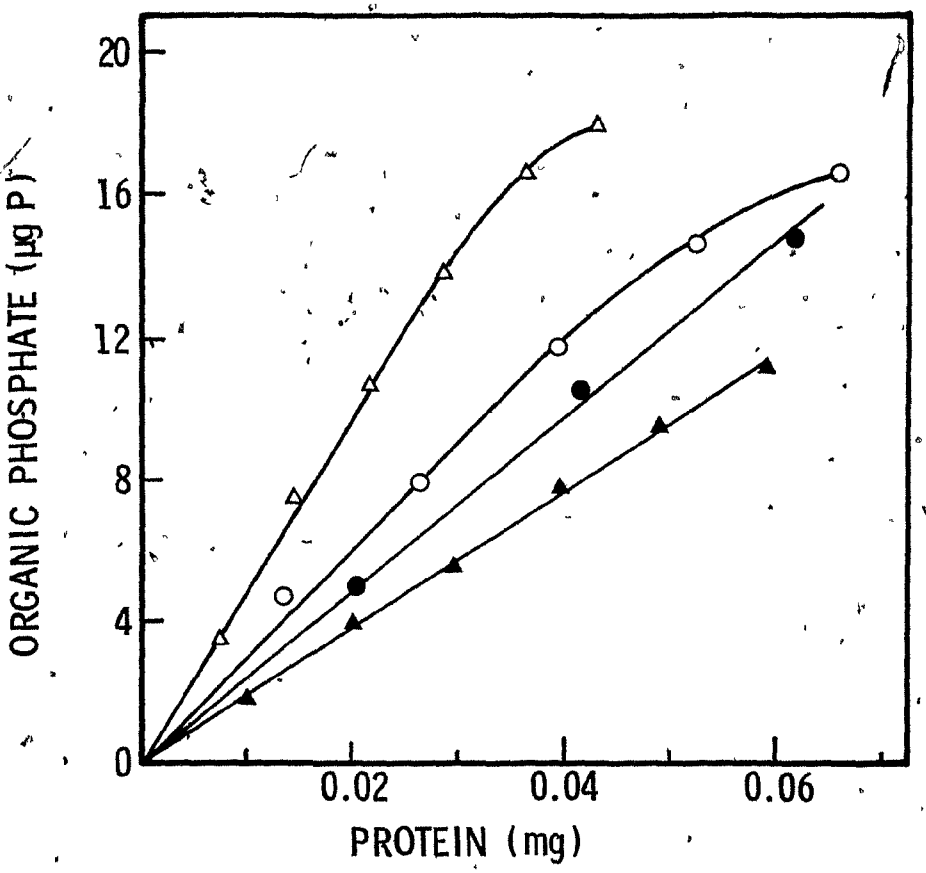


Fig. 47: Dependence of TPI Phosphodiesterase Activity on Enzyme Concentration. Assay conditions were as described in "Methods". Sources of enzyme were: ○, 17 day embryonic chick brain; ●, 28 day old chick brain; △, 17 day embryonic chick sciatic nerve and ▲, adult chicken sciatic nerve.

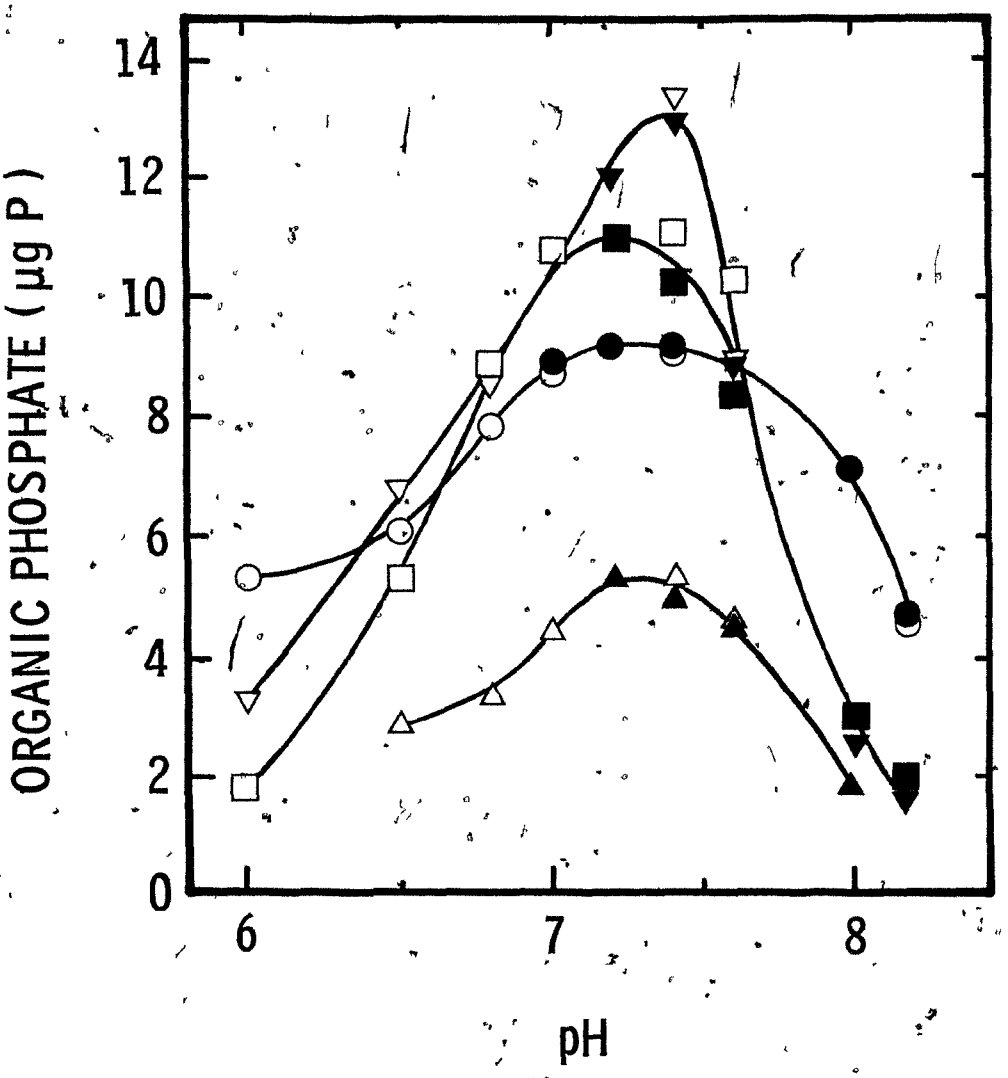


Fig. 48: pH Dependence of TPI Phosphodiesterase. Assay conditions were as described in "Methods" and buffered with 45 mM-Tris-HCl (solid symbols) or 50 mM-HEPES - 50 mM-MES (open symbols). Enzyme sources were: ■, □, 17 day embryonic chick sciatic nerve (0.02 mg protein); ▼, ▽, adult chick sciatic nerve (0.07 mg protein); ▲, △, 17 day embryonic chick brain (0.03 mg protein); ●, o, 21 day old chick brain (0.06 mg protein).

MES: 2-(N-morpholino)ethanesulphonic acid
HEPES:N-2-hydroxy-methyl piperazine-N-2-ethanesulphonic acid

D. POLYPHOSPHOINOSITIDE METABOLISM IN DEVELOPING CHICKEN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

The difficulties of expressing biochemical changes in developing tissues referred to earlier apply equally to enzymatic activities. The usual expression of enzyme activities relative to protein is not ideal since individual brain proteins accumulate at different rates and hence may affect the observed activity. Probably the preferred expression is relative to tissue weight (Rouser et al., 1972) but earlier studies have used activity/mg protein and total activity/whole tissue as well (Salway et al., 1968). Expression of activities in terms of unit DNA is also complex and seldom is used. The data in this study are presented graphically in 3 ways; relative to wet weight, to protein and to anatomical unit i.e. per whole brain or n number of nerve pairs. Unless otherwise indicated, descriptive statements will refer to data expressed relative to wet weight of tissue. Shaded areas in the graphs indicate the period of most rapid myelin accumulation as defined earlier by cerebrosides deposition (see Fig. 13).

1. Polyphosphoinositide Synthesis

Several difficulties were encountered in determining the two phosphoinositide kinase activities in the presence of Cutscum e.g. (i) the amount of Cutscum required for optimum activity varied with the amount of

protein in the homogenate, (ii) the substrate (PI, DPI) requirements were altered etc. Even using the apparent optimum conditions (including suitable Cutscum/protein ratios for both brain and sciatic nerve), the results were less reproducible than those obtained in the absence of detergent. The variability was greatest with homogenates of embryonic tissues (e.g. the stimulation observed in a given homogenate ranged from 8 - 15 fold in 15 day embryonic brain compared to 6 - 9 fold in adult brain). Therefore, the use of Cutscum was initially avoided. Both PIK and DPIK activities in developing chick brain and sciatic nerve are presented in Figs. 49, 50, 51 & 52.

The pH optimum for DPIK is 7.4 in both tissues. In the absence of detergent it is principally endogenous PI which is phosphorylated by PIK at either pH 7.4 or 8.3 and inhibition by DPI does not occur (Fig. 15a, Table 26). Since the reaction conditions were otherwise similar, the DPIK assays provided a measure of PIK activity at pH 7.4 when the incorporation of ^{32}P into DPI was also measured. These values are included in Figs. 49 & 51 since the previous developmental studies of PIK in postnatal rat brain were at pH 7.4 (Salway et al., 1968; Eichberg and Hauser, 1969) and sciatic nerve PIK appeared to be active over a broad range of pH. In this work as in an earlier study (Salway et al., 1968), expression of kinase activities relative to protein and to tissue weight yielded similar developmental patterns. The pattern was even quite similar

when represented relative to the weight of the whole tissue. However, the adult values were always the highest, even though the activities had stabilized or declined when expressed relative to protein or wet weight. This is due to the very large increase in size of the brain and sciatic nerve (see Table 12).

The PIK activity was low in unmyelinated embryonic brain and the 4 - 5 fold increase was coincident with the period of most active myelination (Fig. 49). The activity increased very little after this period. A similar developmental pattern was observed at pH 7.4. The DPIK activity in chick brain also increased dramatically (4 fold) during the period of most active myelination and remained unchanged throughout subsequent maturation (Fig. 50).

The developmental patterns for both PIK and DPIK were very different in chick sciatic nerve (Figs. 51, 52). The PIK activity doubled during the initial phase of myelination and then decreased quickly to premyelination levels (Fig. 51). The activity continued to fall to very low values characteristic of mature nerve. The DPIK activity also increased 2 - 3 fold during the initial phase of myelination and then fell to premyelination levels (Fig. 52).

The fall in PIK and DPIK activities in chick sciatic nerve during maturation was unexpected in view of the continued accumulation of TPI long after the period

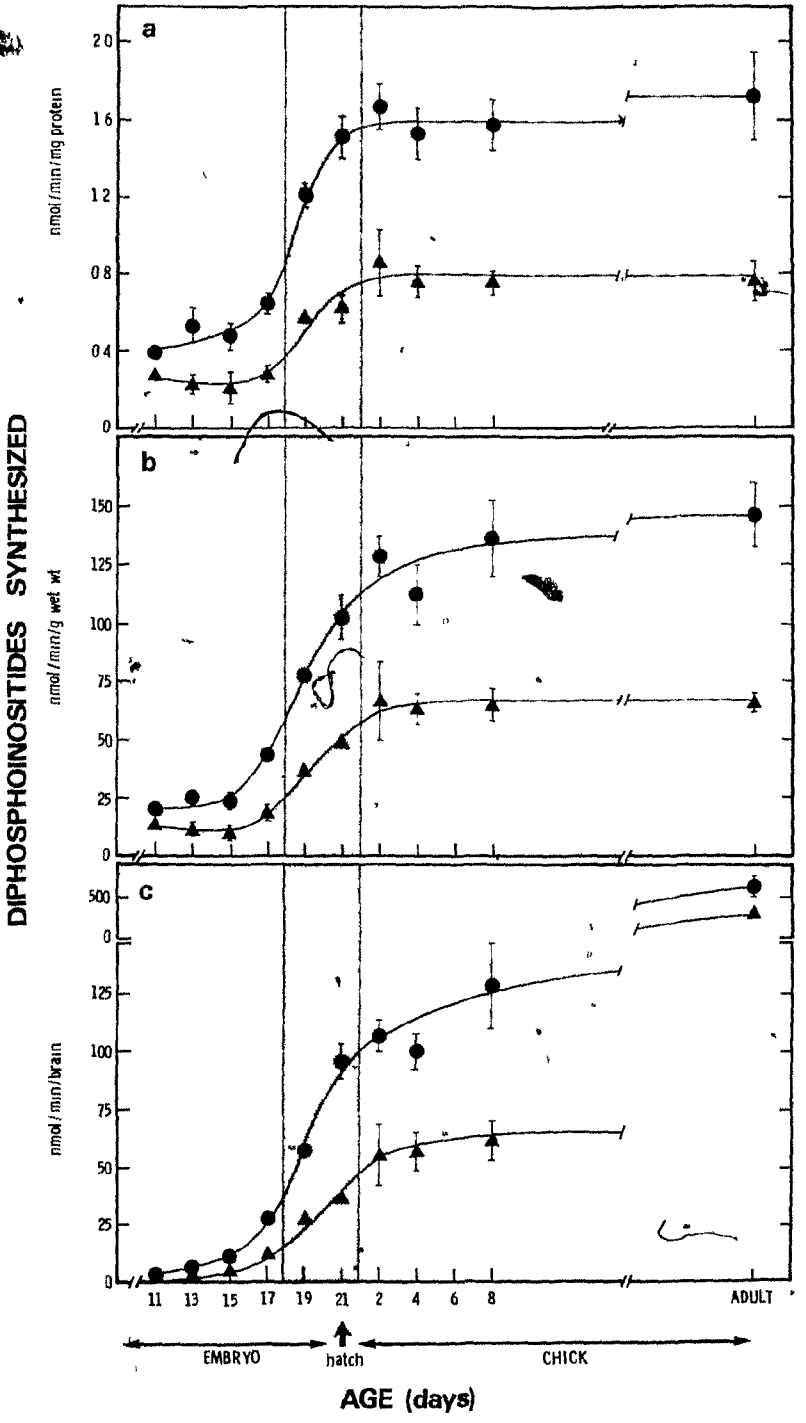


Fig. 49: Phosphatidylinositol Kinase Activity in Developing Chicken Brain. Standard assay conditions were used. (●) activity at pH 8.3, (▲) activity at pH 7.4 obtained from DPI kinase assay (see text). Each value is the mean of at least 3 determinations (different homogenate preparations). The vertical bars represent the standard deviation where it is larger than the symbol. The shaded area represents the period of most rapid myelin accummulation.

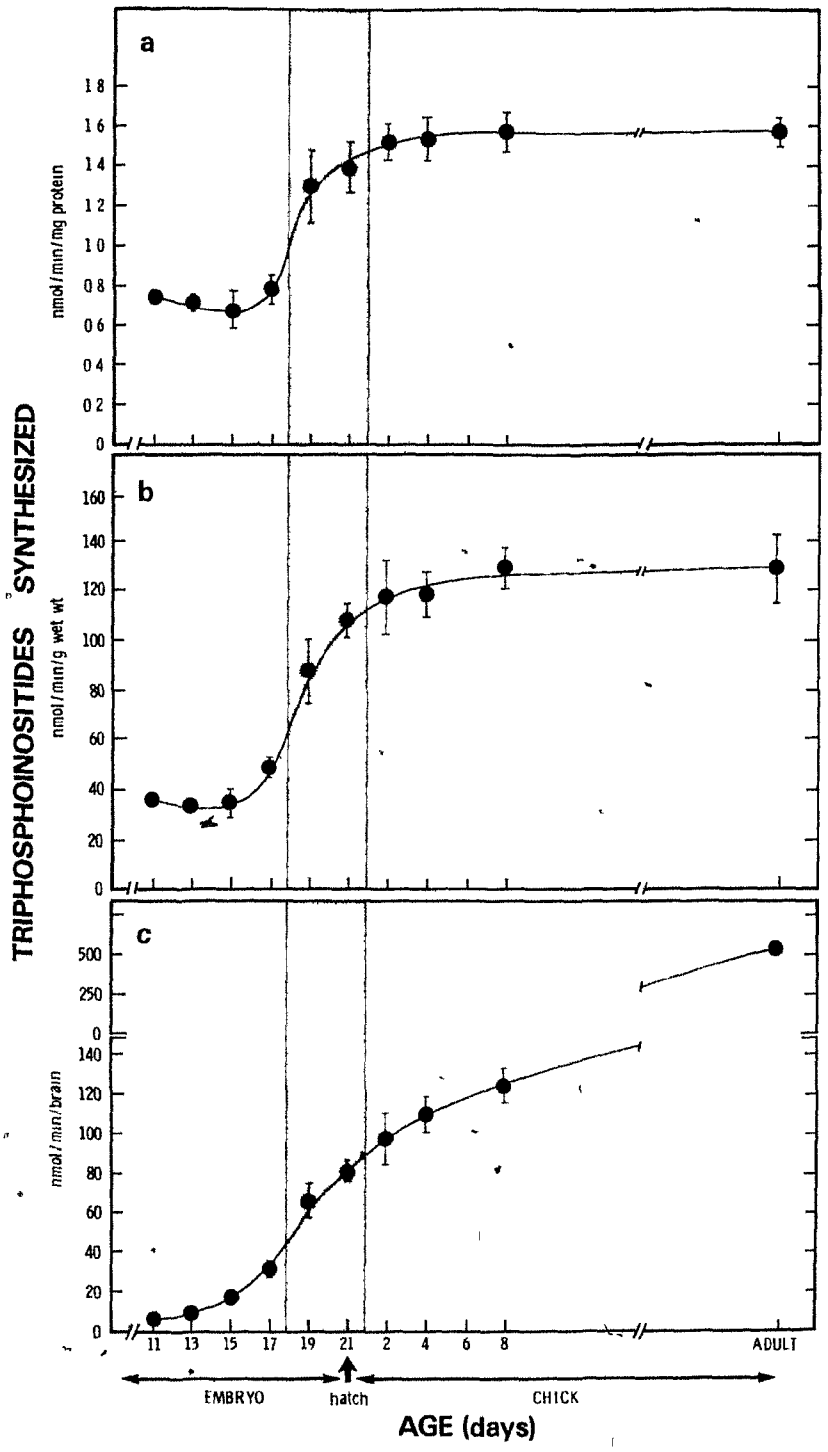


Fig. 50: Diphosphoinositide Kinase Activity in Developing Chicken Brain. Standard assay conditions were used. Each value is the mean of at least 3 determinations (different homogenate preparations). The vertical bars represent the standard deviation where it is larger than the symbol. The shaded area represents the period of most rapid myelin accummulation.

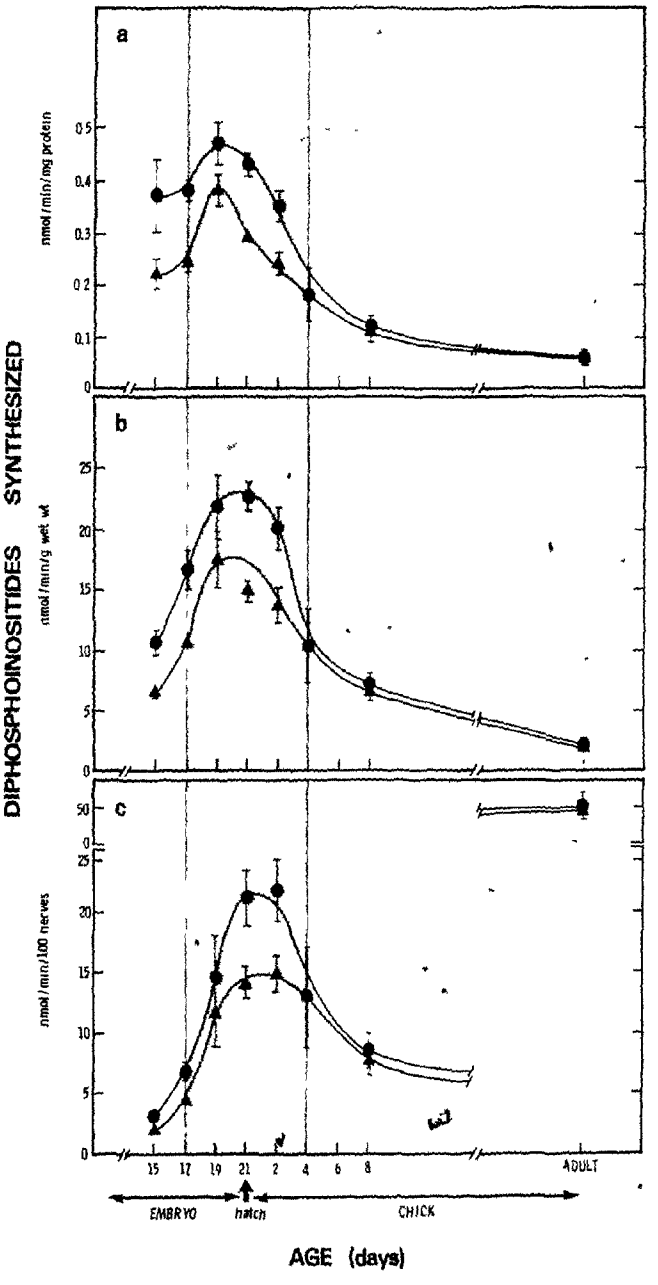


Fig. 51: Phosphatidylinositol Kinase Activity in Developing Chicken Sciatic Nerve. See Fig. 49 for explanation of symbols and description.

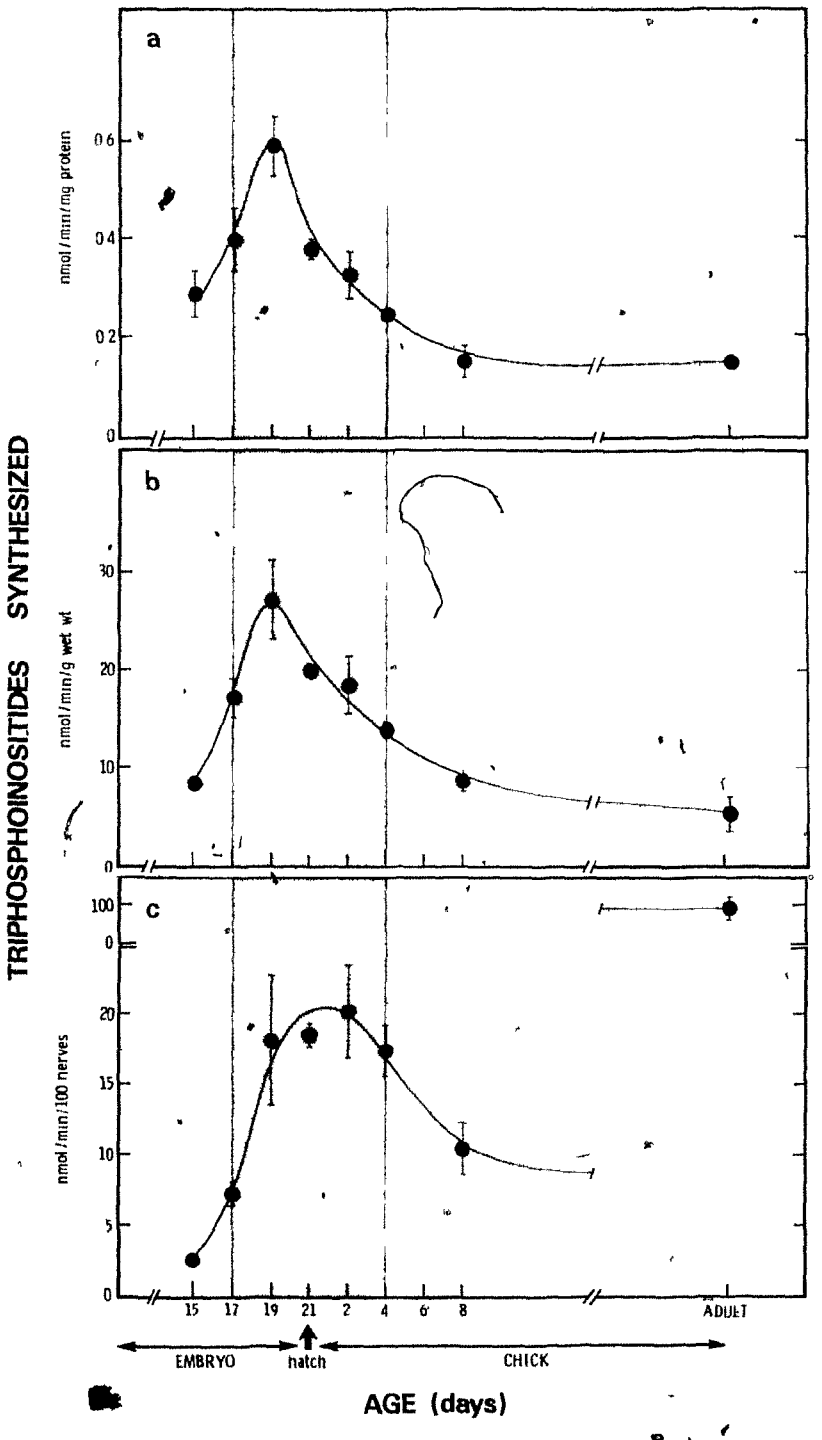


Fig. 52: Diphosphoinositide Kinase Activity in Developing Chicken Sciatic Nerve. See Fig. 50 for explanation of symbols and description.

of most active myelination (see Table 18). It was considered possible that the increasing amounts of compact myelin and connective tissue might reduce access of the exogenous substrates (ATP, DPI) to the enzymes and contribute to the declining activities as the nerve matures. Therefore, the enzymatic activities in both chick brain and sciatic nerve were redetermined in the presence of optimum amounts of Cutscum. The results confirmed that both kinase activities increase during myelination and remain high in chick brain (Fig. 53a, b). The developmental pattern of PIK was identical to that observed without Cutscum except for the higher activities. The DPIK activity increased only 2 fold at the onset of myelination (Fig. 53b). This in part reflects the greater stimulation of embryonic homogenates by Cutscum. It remained constant thereafter. Measurements of PIK and DPIK in the presence of Cutscum also confirmed that these activities decline in sciatic nerve after the period of most active myelination (Fig. 54a, b). However, the developmental pattern was altered. The PIK activity increased during myelination, but the peak of activity occurred later (7 - 10 day chick). The DPIK assays yielded the highest values in unmyelinated nerves followed by a decline to low adult values.

The data obtained with detergent-solubilized system is useful for comparative purposes only, since the tissue homogenates were those used in the initial studies without detergent which had been stored frozen at -20°C

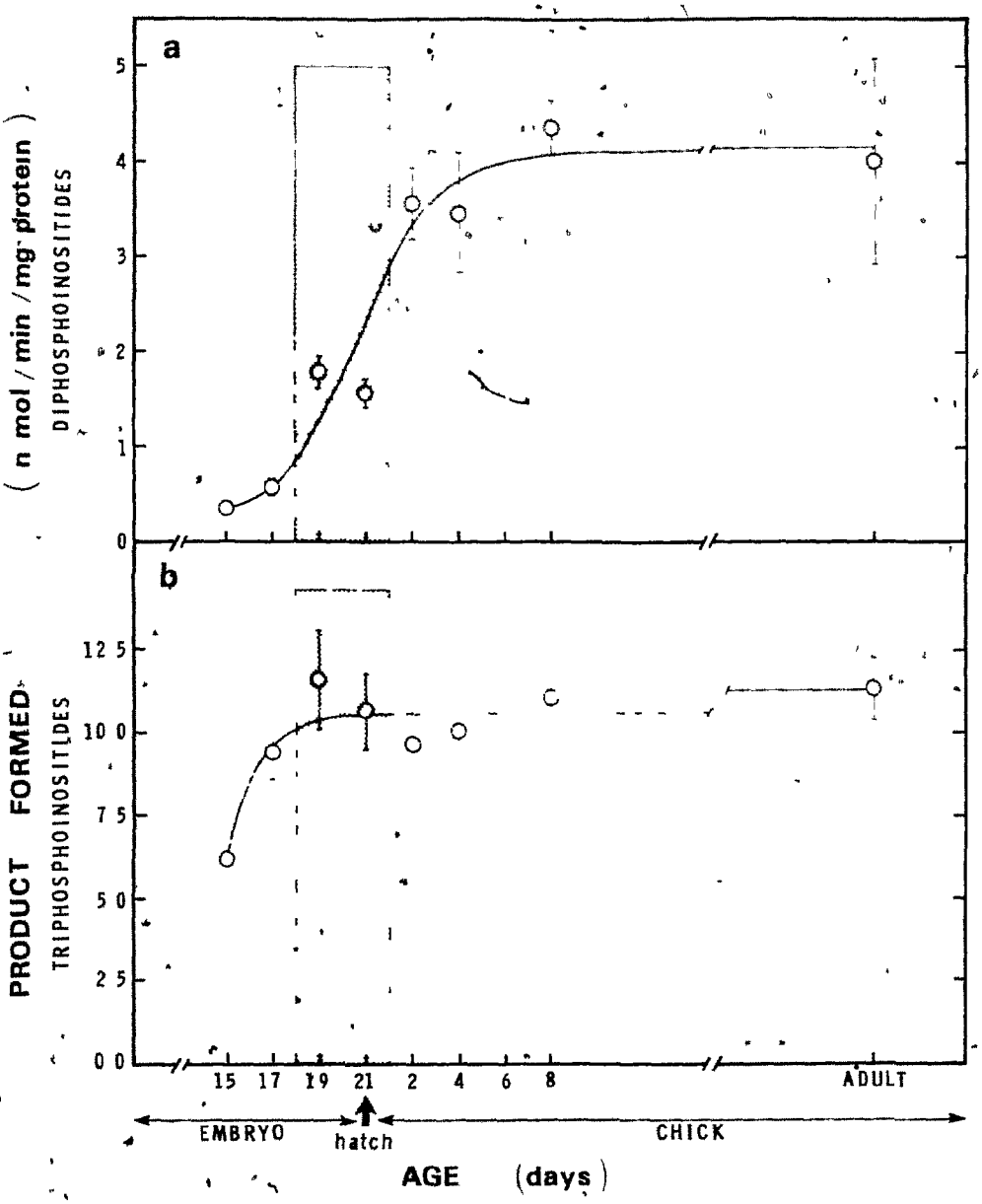


Fig. 53: Phosphatidylinositol Kinase and Diphosphoinositide Kinase Activities in Developing Chicken Brain Assayed in the Presence of Cutscum. Standard assay conditions were used. PIK activity (a) assayed at Cutscum/protein ratio of 7 and DPIK (b) at ratio of 0.6.

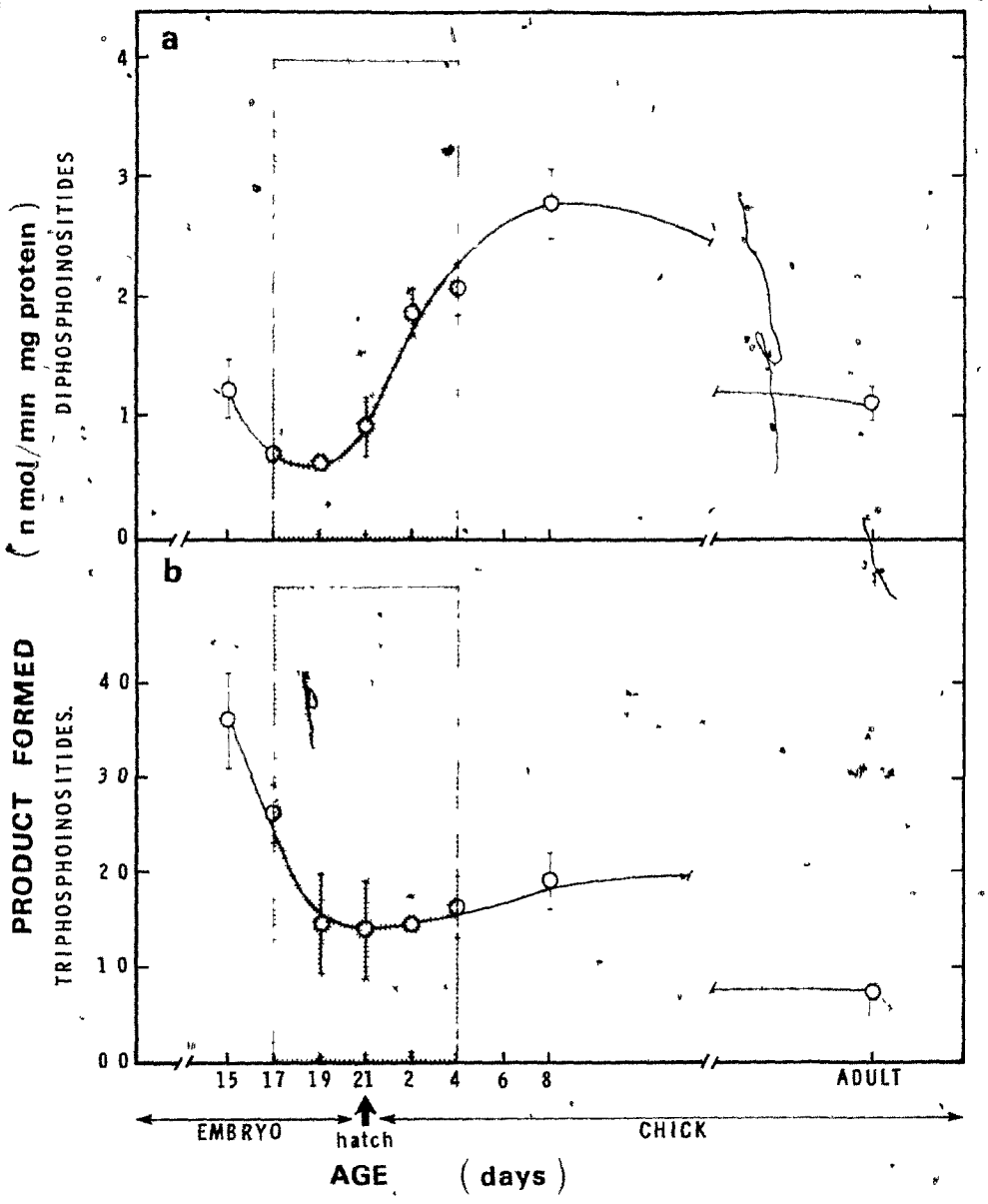


Fig. 54: Phosphatidylinositol Kinase and Diphosphoinositide Kinase Activities in Developing Chicken Sciatic Nerve. Standard assay conditions were used. PIK activity (a) assayed at Cutscum/protein ratio of 7 and DPIK (b) at ratio of 3.

for 30 - 40 days. Considering the many additional variables encountered with Cutscum supplemented assays, the data without detergent is more reliable. The graphs, however, do confirm that the activities of both kinases decrease after the initial period of myelination in nerve but not in brain.

2. Triphosphoinositide Hydrolysis

The maximum level of TPI phosphatase activity in myelinated adult chicken brain (14.1 ± 1.7 µmol inorganic-P released/min/g wet weight) was at least 2 orders of magnitude greater than the phosphoinositide kinase activities (see Figs. 49; 50) and significant activity was present in premyelinated embryonic brain. The rapid increase in the activity occurred over a short period and coincided with the rapid deposition of myelin (Fig. 55). The TPI phosphodiesterase activity, like TPI phosphatase, was 2 orders of magnitude greater than the phosphoinositide kinase activities in chick brain but the increase occurred over a longer period of time and was not specifically correlated with myelination (Fig. 56). This observation is at variance with the results in post-natal rat brain where a closer association of the enzyme activity with myelination has been reported (Keough and Thompson, 1970). If expressed relative to protein, the phosphodiesterase activity rose abruptly 5 days before the onset of myelination and remained constant thereafter.

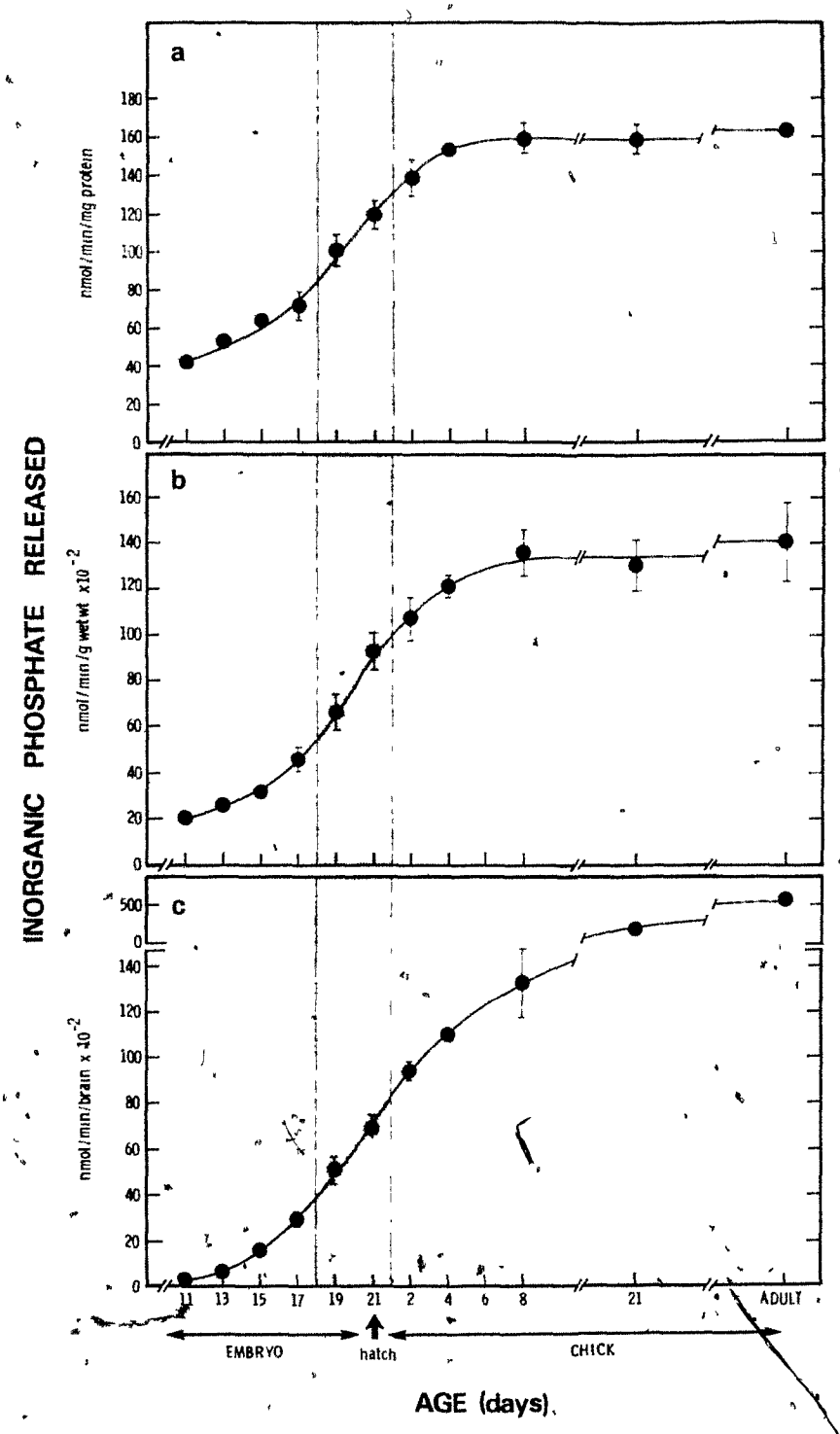


Fig. 55: Triphosphoinositide Phosphatase Activity in Developing Chicken Brain. Standard assay conditions were used. Each value is the mean of at least 3 determinations (different homogenate preparations). The vertical bars represent the standard deviation where it is larger than the symbol. The shaded area represents the period of most rapid myelin accummulation.

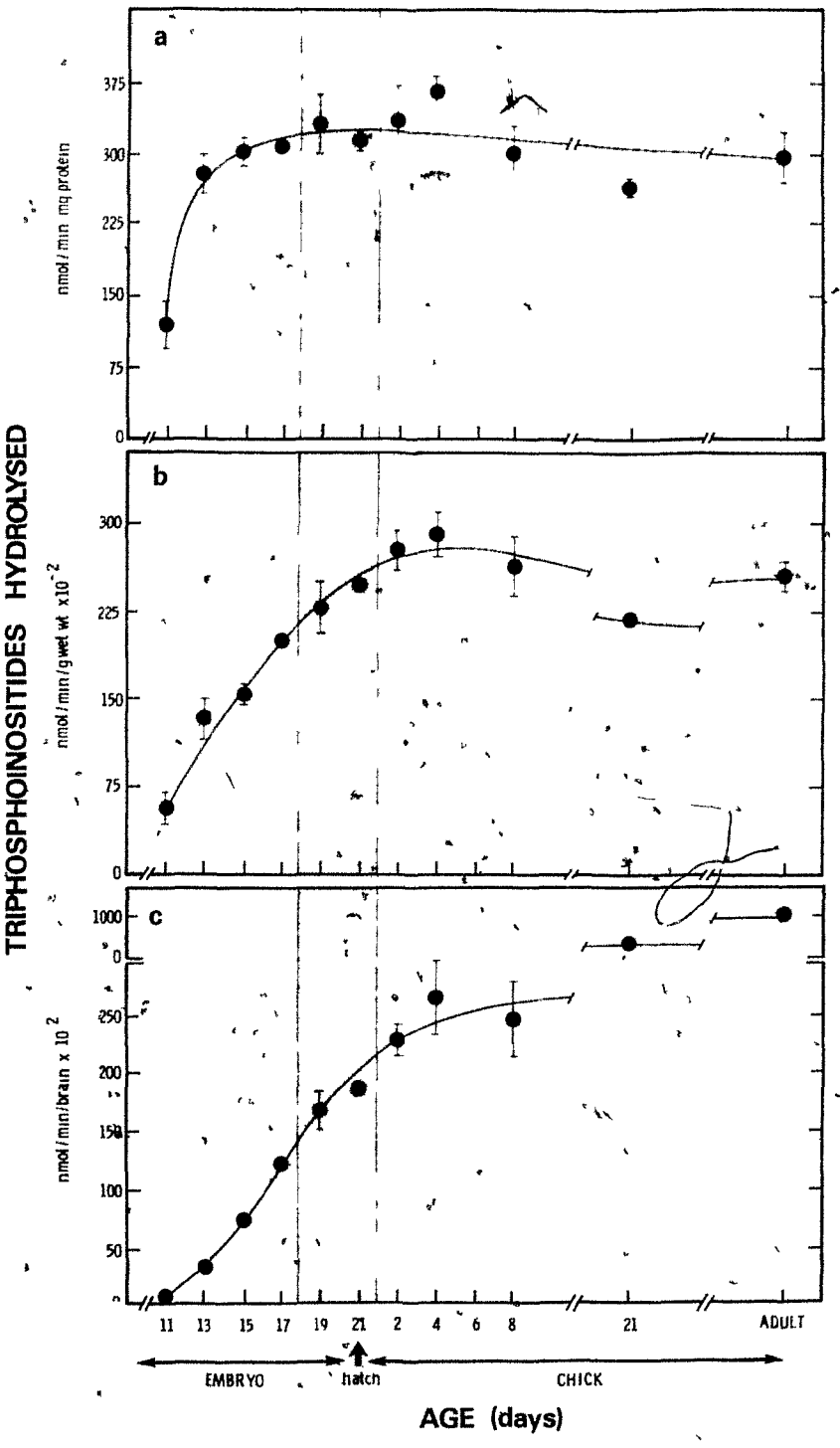


Fig. 56: Triphosphoinositide Phosphodiesterase Activity in Developing Chicken Brain. See Fig. 55 for description.

The developmental patterns of both hydrolytic activities were also very different in sciatic nerve. The TPI phosphatase activity did not change appreciably with age and was lower than the activity in embryonic brain (Fig. 57). Embryonic chick sciatic nerve exhibited high TPI phosphodiesterase activities equal to that found in adult brain (Fig. 58). This activity steadily declined with increasing age with no abrupt change during myelination. The phosphodiesterase activity in adult nerve was comparable to that found in embryonic brain. The total activity of both enzymes in sciatic nerve increased during and after the period of myelination as a consequence of the growth in the size of the nerves (Figs. 57c & 58c).

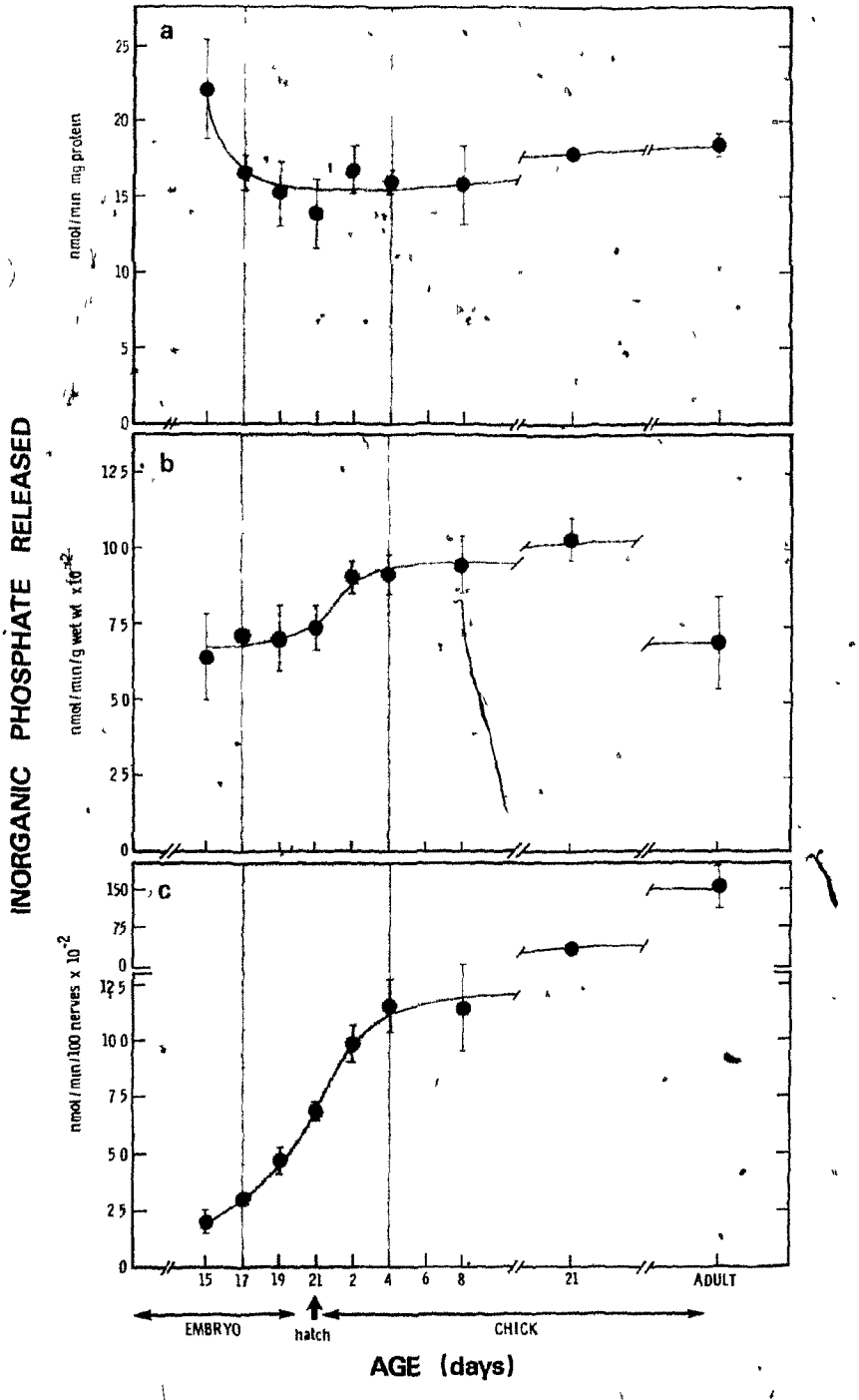


Fig. 57: Triphosphoinositide Phosphatase Activity in Developing Chicken Sciatic Nerve. See Fig. 55 for description.

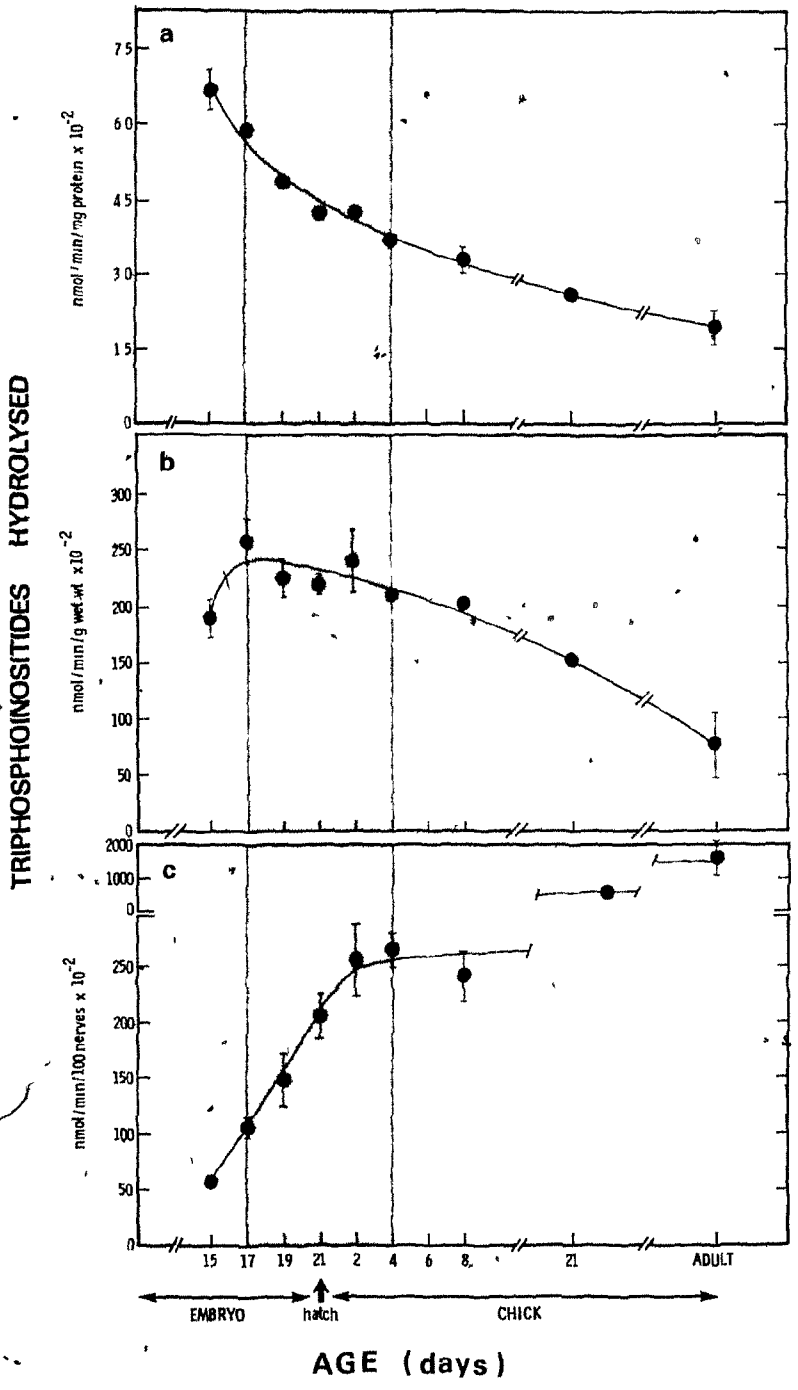


Fig. 58: Triphosphoinositide Phosphodiesterase Activity in Developing Chicken Sciatic Nerve. See Fig. 55 for description.

IV. DISCUSSION

A. CHEMICAL COMPOSITION OF THE DEVELOPING CHICKEN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

1. Periods of Active Myelination

Wells and Dittmer (1967) related lipid changes to the three periods of development in the postnatal rat brain: premyelination (1 - 10 days), rapid myelination (11 - 20 days) and a period of late maturation continuing to adulthood during which myelin accumulation continues at a slower rate. Similarly, these three major developmental stages may be distinguished in both chick brain and sciatic nerve. It is generally accepted that Cer are primarily myelin components (Garrigan and Chargaff, 1963; Wells and Dittmer, 1967; Eng and Noble, 1968; Norton and Poduslo, 1973). Cerebrosides are absent from pre-myelination rat brain (Wells and Dittmer, 1967) although other galactolipids (Sulf and galactosyl diglycerides) are present in small quantities. However, Cer are not completely restricted to myelin. This is particularly evident in embryonic chick brain where 26% of the adult concentration of galactolipids is present prior to the appearance of microscopically demonstrable myelin at 17 - 18 days. Only a small portion of these galactolipids are Sulf (5%). Both the present and earlier work depict a period of active galactolipid deposition in chick embryonic brain, beginning at the 18th day of incubation and continuing until shortly after hatching. In chick embryo sciatic nerve the concentration

of galactolipids was very low at 15 days but increased dramatically over the next 10 days (the proportion represented by Sulf was constant). Similar observations have been recently made for human (Davison et al., 1973) and rabbit sciatic nerves (Yates and Wherrett, 1974). The presence of small quantities of galactolipids in 15 day embryonic chick sciatic nerve suggests that early myelination may have just begun. Their subsequent deposition indicates a slightly longer period of very active myelination in nerve than in brain which continues for 3 or 4 days after hatching. Nucleoside-2′:3′-cyclic phosphate-3′-nucleotidohydrolase (EC 3.1.4.16) is well documented as a myelin marker for CNS although its role in the peripheral nervous system is not as certain. Activity appears in 17 day embryonic chick brain followed by a period of rapid increase lasting until 2 - 3 days after hatching (Kurihara and Tsukada, 1968). Recent studies (Dreiling and Newburgh, 1972; Mezei and Palmer, 1974) have shown this enzyme to be absent in 14 day embryonic nerve and to increase rapidly to maximum activity in the 1 - 2 day old chick. These periods of rapid myelination correlate well with earlier anatomical and histological studies (Geren, 1954; Carpenter and Bergland, 1957; El-Eishi, 1967).

2. Changes in Weight, Nucleic Acids and Protein

The most apparent increase in the weight of both chick neural tissues occurs during the period close

to that of rapid galactolipid deposition. Kurihara and Tsukada (1968) observed doubling of the mass in rat brain during this period. Other workers also observed an increase in chick brain weight similar to the one reported here (Freysz et al., 1971). Shrivastaw and Subba-Roa (1975) reported an increase in weight but their values for the total weight of the chick brain were somewhat smaller than reported here probably because the brain samples they used did not include cerebellum. Lipids, and to a much lesser extent proteins, are deposited at much higher rates during the period of rapid myelination than at any developmental age as a result of the accumulation of lipid-rich membranes. This is reflected by the steady decrease (relative to wet wt.) in nucleic acid concentrations and increase in protein/DNA ratios, in both chick brain and sciatic nerve (see Tables 13 & 14). The decrease in nucleic acid concentrations suggests an increase in cell size without cellular proliferation. These results are in agreement with recent studies of rabbit sciatic nerve and chick brain (Yates and Wherret, 1974; Shrivastaw and Subba-Roa, 1975). The latter workers also noted a marked increase in the DNA content of the chick brain at 2 years of age as compared to the values at 10 days. In the present study, this increase was smaller in brain except that in whole sciatic nerve about a 6 fold increase occurred over the same period. A similar rise in the DNA content of rat brain has been observed and was

interpreted as a reflection of progressive proliferation of glial cells with aging (Vernadakis, 1973). Earlier studies have supported the general idea that the accumulation of DNA in brain is only marginal after hatching in the chicken and after 21 days postnatally in the rat (Winick and Noble, 1965; Margolis, 1969; Zamenhof et al., 1971; Howard, 1973). However, these workers did not analyze very old brains (less than 100 days of age; in one instance 425 days).

3. Lipid Composition

The values for lipids reported here for adult chicken brain are comparable to several previous studies (Sheltawy and Dawson, 1969b). During the initial phases of this investigation, a comprehensive analysis of the phospholipids in chick brain covering the period from 7 day embryo to adult appeared (Freysz et al., 1971). If converted to the same mode of expression their values are very similar to those in Table 17 except for the PPI. They reported almost equimolar concentrations of DPI and TPI in brains of all ages. It is well known that TPI is rapidly degraded after death at least initially to DPI (Sheltawy and Dawson, 1969a). The best analytical procedures for PPI have minimized this loss by freezing the tissues immediately in liquid nitrogen, a precaution not taken by Freysz et al. (1971). However, their values for total PPI were similar to those reported here. The present study also exhibited a higher TPI/DPI ratio for adult chicken than reported by

Sheltawy and Dawson (1966); however, the relative proportions of each class of phospholipid, cholesterol and galactolipid were similar. This may simply reflect the use of different breeds of chicken since such differences have been observed in studies of sciatic nerve cholesterol and cholesterol esters (C. Mezei, private communication).

After the lipid analyses of chick brain and sciatic nerve had been completed, Hauser and Eichberg (1973) reported greatly improved recoveries of DPI when calcium was added to the first solvent in the extraction scheme. Therefore, the values for PPI in Tables 17 & 18, obtained without $CaCl_2$ might not represent the *in vivo* concentrations of these lipids. The effect was confirmed for chick brain by subsequent work by others in this laboratory. The amount of added $CaCl_2$ required for optimum recovery is different than reported for rat brain and decreases with age from 30 - 60 μmol/g of tissue for embryonic brain to 15 μmol/g of tissue for adult brain (Shaikh and Palmer, 1976). It seems likely that the improved recoveries are the result of reduced losses of PPI into the initial extracts with neutral solvents due to enhanced binding of these lipids to tissue proteins. An earlier study (Palmer, 1971) found that divalent cations suppressed the readsorption of acidic phospholipids onto insoluble tissue proteins from C-M when sufficient water was added to result in a two phase system. It is evident that adsorption onto tissue proteins is enhanced by calcium

when the proportion of water is well below that which gives two phases. The effect of $CaCl_2$ also varies with the tissue studied. The addition of even small amounts of $CaCl_2$ has little if any beneficial effect on DPI recoveries from both embryonic and chick sciatic nerves; however, recoveries of TPI are reduced, greatly so in the older nerves. Hauser and Eichberg (1973) noted little effect on PPI recoveries from rat kidney and suggested that the probable reason was the higher calcium content of kidney tissue. Subsequent work in this laboratory showed that this is not a likely explanation for chick nervous tissues. Alternatively, these effects may reflect differing tissue concentrations of acidic and basic proteins which bind PPI, the former via calcium salt bridges (Dawson, 1965; Palmer and Dawson, 1969). Both classes of proteins are known to increase during maturation in brain (Cicero et al., 1970; Roboz-Einstein et al., 1970; Rappoport et al., 1971).

Improved values of chick brain PPI obtained with $CaCl_2$-fortified solvents are presented in Fig. 59. The recoveries of these lipids were increased considerably at all ages but the developmental pattern remained unchanged.

4. Lipids and Development

When the data for the lipid constituents of chick brain are considered relative to their concentration in adult brain they generally fit the classification derived by Wells and Dittmer (1967) for rat brain. Although present

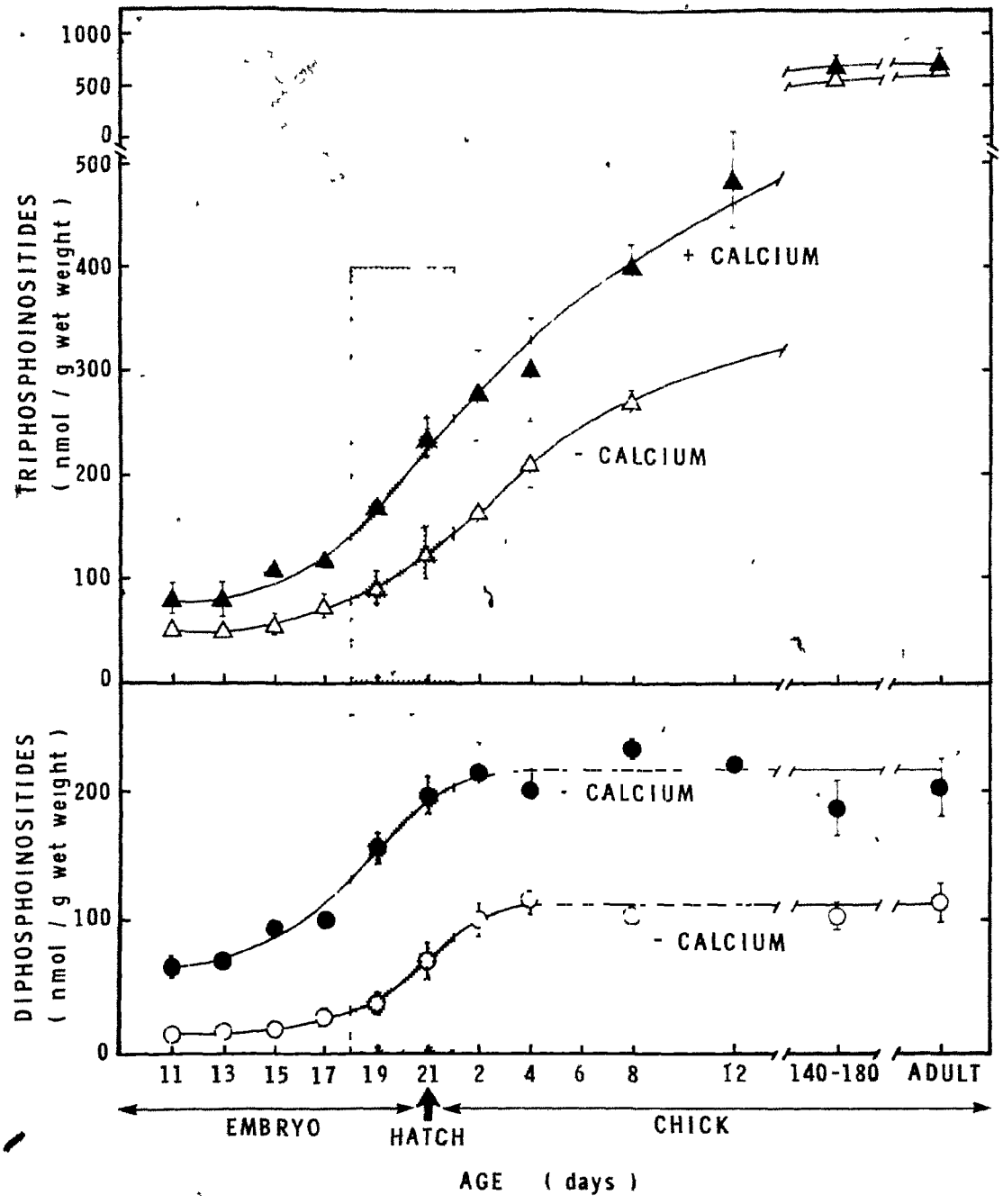


Fig. 59: The Deposition of Polyphosphoinositides in Developing Chicken Brain: Comparison of polyphosphoinositides concentrations determined with and without added $CaCl_2$ in extracting solvents.

in considerable concentration prior to the onset of myelination, EPG increased more rapidly during myelination in chick brain than other components classified as general membrane lipids. This more clearly reflects the higher concentration of ethanolamine plasmalogens found in myelin membranes (Horrocks, 1972). Lipids occurring in very low initial relative amounts which undergo more rapid increases are quantitatively more important components of myelin. To be considered exclusively a myelin constituent a compound must be absent prior to myelination. Only Cer meet these criteria in rat brain (Wells and Dittmer, 1967; Norton and Poduslo, 1973). Polyphosphoinositides deposition has generally been reported to follow a similar pattern to that of Cer in brain, although results have varied with different laboratories and species. The greatly improved yields achieved by adding $CaCl_2$ to the system make earlier values for these lipids obsolete, particularly for DPI in immature brain. DPI were present at 1/3 of the adult concentration before the onset of myelination in chick brain and increased abruptly to adult level within the period of rapid myelination (the same as Cer and Sulf). This pattern suggests that DPI are constituents of both myelin and non-myelin membranes and the concentration in these membranes is probably different but constant. Triphosphoinositides and Sph were the only lipids present at less than 10% of the adult concentration in 11 - 13 day embryonic chick brain.

When expressed relative to the adult concentra-

tions, all the lipids in chick sciatic nerve except CPG and PI behaved in a parallel fashion. They differed only in the amount present in 15 day embryonic nerve with none exceeding 25% of the adult values. Phosphatidylinositols were present at 40% of the adult concentration and increased slowly without any sharp increase during the most active myelination. Phosphatidylcholines were present at the adult concentration before myelination and showed an initial increase followed by a subsequent decline. This lipid is a major component of membranes, including myelin, and its concentration probably reflects the initial deposition of myelin followed by a change in the composition of the myelin during stages of development.

Considerable evidence now available suggests that PPI are principally components of the myelin sheath or an anatomically contiguous structure. The presence of DPI and TPI mostly in a "myelin-rich" subcellular fraction and their 2 - 3 fold enrichment (as a % of total phospholipids) in the highly purified myelin support this suggestion (Eichberg and Dawson, 1965). Further support arises from the observation that substantially higher quantities of these compounds are present in myelinated than unmyelinated peripheral nerves (Sheltawy and Dawson, 1966). The levels of DPI and TPI, as well as several of the enzymes involved in their metabolism, were found to increase in rat brain during development, most rapidly during the period of maximal myelination (Eichberg and Hauser, 1967; Wells and Dittmer, 1967; Salway et al.,

1968; Sheltawy and Dawson, 1969a; Keough and Thompson, 1970). The results of the present study complement the above suggestions. The content of PPI in both chick brain and sciatic nerve increased dramatically during development. The increase was much greater than for most other lipids. In chick brain, the rapid increase in TPI concentration which continued into adulthood supports that they are largely associated with myelin. However, there are indications that an appreciable portion of PPI may be components of non-myelin structures (Hauser et al., 1971). Both PPI are present prior to myelination in rat brain (Eichberg and Hauser, 1973) as well as chick brain. Evidence has also been reported for the presence of small quantities of PPI in brain subcellular fractions other than myelin (Eichberg and Dawson, 1965; Hajra et al., 1968) as well as in astrocytes in cell culture (Hauser et al., 1970). The PPI have also been identified, usually in low quantities, in a wide variety of mammalian tissues in addition to brain and also in protozoa and yeast (see Section I.C.4). The deficiency of TPI is less than that of Cer in the brains of "quaking" mice which are characterized by deficient myelination while DPI are unaffected (Hauser et al., 1971b; Dawson and Clarke, 1971). These data suggest an extramyelin compartment containing DPI and some TPI and a myelin compartment containing mainly TPI which is consistent with earlier studies of white and gray matter (Hauser et al., 1971a). A recent fractionation study employing an alkaline

medium to avoid post-mortem breakdown and a $CaCl_2$ supplemented extraction system has confirmed this impression (Eichberg and Hauser, 1973) and shown that only the myelin PPI increase during maturation of the rat brain. Furthermore, there is considerable evidence that the metabolic activities of the two pools of PPI are different (Sheltawy and Dawson, 1969a; Gonzalez-Sastre et al., 1971). It is not known where the putative non-myelin PPI pool is localized. In nervous system they may be the components of plasma membranes of neurones (including axolemma and synaptic membranes) or localized in other cells (astroglia, ependymal, microglia, etc.).

The increase of PPI content in sciatic nerve during development was greater than brain. Both DPI and TPI were absent before myelination and their deposition closely paralleled that of characteristically myelin lipids (Cer). This suggests that the extra-myelin PPI pool is either absent or very small in peripheral nerve. The very low quantities of both DPI and TPI in partially myelinated/unmyelinated peripheral nerves of lobster and cow support this suggestion (Sheltawy and Dawson, 1966). This is not surprising since peripheral nerve is less complex, having proportionately less non-myelin constituents. Axons represent a small proportion of the nerve and there are fewer cells.

When the lipid data for whole chick brain is expressed as mol percent of total lipid (Table 32), the

Table 32. Lipids of Chick Brain During Development (mol percent of total lipid)*

Lipids / Age (days) →	Embryo						Chick					
	11	13	15	17	19	21	2	4	8	12	140-160	Adult
Chol	31.4	32.6	32.8	35.7	36.6	35.8	35.7	36.0	37.4	34.2	37.9	38.0
Galacto-lipids	6.0	5.6	4.8	4.3	4.6	8.3	8.1	8.1	7.80	8.1	6.9	6.9
Phospho-lipids	62.6	61.8	62.4	60.0	58.8	55.9	56.2	55.9	54.8	57.7	55.2	55.1
CPG	31.4	30.9	29.6	28.2	25.6	27.3	24.9	24.3	22.7	22.5	19.8	20.0
EPG	18.8	19.7	17.0	18.6	19.7	20.4	19.4	19.4	19.1	21.2	22.3	21.5
SPG	6.2	5.6	6.8	6.7	7.3	6.3	6.1	6.4	6.4	6.3	6.1	6.7
Sph	1.5	1.1	2.7	3.1	2.8	2.7	2.7	3.2	3.0	4.3	3.9	4.4
PI	4.3	3.1	3.6	2.9	2.8	2.7	2.4	1.9	2.9	2.6	2.2	1.7
DPI+	0.19	0.19	0.10	0.21	0.27	0.30	0.29	0.26	0.28	0.26	0.19	0.19
TPI+	0.24	0.22	0.26	0.24	0.29	0.35	0.37	0.38	0.48	0.57	0.67	0.67

* Total lipids represent Chol + galactolipids + individual phospholipids

+ Values obtained with $CaCl_2$ (added during first extraction with C-M).

proportion of most lipids (Chol, EPG, SPG, and PI) remains constant. Phosphatidylcholines decrease during the entire time period studied while galactolipids increase only during the active period of myelination. The proportions of Sph and ethanolamine plasmalogens increased. Similar observations have been made for rat brain except that the increase in galactolipid continues long after the period of most active myelination. These changes have been shown to be the result of corresponding maturational changes in the composition of myelin itself rather than simply reflecting the deposition of myelin having a different lipid composition from the other parts of the brain (Norton and Poduslo, 1973). Galactolipid increased very little in chick brain after the period of most active myelination suggesting no change in the galactolipid composition of the myelin. Since the PPI are quantitatively minor components, changes in their proportions reflect their increasing concentrations. The increase in TPI but not DPI continues long after the galactolipids have ceased to change, and could indicate a maturational change in myelin membranes for this lipid as well.

Since myelin sheaths make up a much greater proportion of nerves, the lipid analyses might be expected to reflect more closely the composition of myelin. If expressed as a proportion of total lipids (Table 33), the analyses of whole sciatic nerve resemble the limited lipid composition reported for chick sciatic nerve myelin (Oulton

Table 33. Lipids of Chick Sciatic Nerve During Development (mol percent of total lipid)*

Lipids / Age (days) →	Embryo				Chick				
	15	17	19	21	2	4	8	140-180	Adult
Chol	34.6	36.4	40.5	40.3	40.5	37.1	41.3	39.9	39.5
Galactolipids	7.2	8.9	10.6	13.2	14.7	13.9	15.3	14.9	15.8
Phospholipids	58.2	54.7	48.2	46.5	44.8	49.1	43.4	46.2	45.2
CPG	27.0	20.7	15.9	12.5	10.9	10.2	9.3	8.0	6.7
EPG	16.8	17.3	15.5	16.9	17.3	19.5	16.5	16.4	15.7
SPG	6.3	6.4	6.6	6.8	6.2	7.6	7.4	8.6	8.7
Sph	5.0	7.7	8.1	8.5	8.7	10.1	8.5	10.9	11.3
PI	3.1	2.5	1.9	1.5	1.4	1.4	1.4	1.8	1.9
DPI+	-	0.02	0.03	0.03	0.03	0.02	0.03	0.04	0.04
TPI+	-	0.11	0.20	0.26	0.28	0.28	0.33	0.42	0.45

* Total lipids represent Chol + galactolipids + individual phospholipids.

\+ Values obtained with $CaCl_2$ (added during first extraction with C-M).

and Mezei, 1976). Contributions of non-myelin membranes are evident only in the higher PC, marginally higher Chol and lower galactolipid proportions. The maturational changes are also the same. The most striking change is the progressive reduction in PC and the reciprocal relationship between phosphatidyl and phosphatidal-ethanolamines. The adult values for these lipids are very similar to those reported recently for rat sciatic nerve (Klein and Mandel, 1976). The increase in the proportions of galactolipids and phosphatidalethanolamines is largely complete after the period of most active myelination. The decline of CPG continued after this period as did the increase in the Sph portion. Phosphatidylserines tended to increase slightly while the total phospholipid decreased. Similar trends are also evident in lipid analyses of human fetal sciatic nerve (Davison et al., 1973). Furthermore, the results indicate a continuous increase in the proportion of TPI at later ages suggesting that the concentration of this lipid is not constant and may also exhibit long-term maturational changes in nerve myelin.

Although the association of PPI with myelination is more apparent in peripheral nerve, the confirmation awaits the analyses of the isolated myelin. A suitable procedure which avoids losses of PPI during preparative procedures has recently been suggested (Eichberg and Hauser, 1973).

B. PHOSPHOINOSITIDE KINASES

Published assay procedures for PIK and DPIK were found to be unsatisfactory. As noted by Hajra et al. (1968), the recovery of reaction products was often low. The system used in this study was a modification of the method of Iacobelli (1968) and similar to that used by Eichberg and Hauser (1969). The recovery of DPI and TPI after extraction from the reaction mixture and separation by t.l.c. was 93% and considerably better than the 50 - 75% achieved by the earlier methods.

The reaction rates for both PIK and DPIK in chick neural tissues were constant for only a few minutes (2 - 3 min). A similar time dependence has been observed for rat brain, liver, kidney and ox adrenal chromaffin granule kinases (Kai et al., 1966a, b, 1968; Michell et al., 1967; Tou et al., 1968; Eichberg and Hauser, 1969; Harwood and Hawthorne, 1969a). In spite of this, longer incubation periods were used in most of the earlier studies. The use of a much shorter incubation time in this study (1.5 min) thus provides more reliable measurements of the initial rate. The reaction conditions were otherwise very similar to that used by others.

The characteristics of chick neural tissue PIK and DPIK differ in several respects from those of other tissues and species. Phosphatidylinositol kinase activities having several different pH optima have been reported. Liver contains two enzymes with maximum activity at pH 7.4

and 8.3 (Harwood and Hawthorne, 1969a) while chromaffin granule membranes of bovine adrenal medulla possess peaks of activity at pH 4.9 and 7.7 (Phillips, 1973). The soluble PIK of yeast is active at pH 7.4 (Talwalker and Lester, 1974). The most comprehensive investigations of PIK in rat brain (Kai et al., 1966b), including developmental studies (Salway et al., 1968), have been done at pH 7.4. However, the pH optimum for rat brain and kidney cortex PIK activities is 8.3 (Colodzin and Kennedy, 1965; Tou et al., 1968). As expected, chick brain homogenates exhibited maximum activity at pH 8.3. Chick sciatic nerve homogenates exhibited essentially the same activity over a wide pH range (7.3 - 8.4), indicating the possibility of two enzymes as has been reported for rat liver (Harwood and Hawthorne, 1969a). The developmental changes in PIK were the same at pH 7.4 and 8.3 in both brain and sciatic nerve. This suggests that both tissues possess only a single kinase although the nerve enzyme is active over a much broader pH range. Only rat liver homogenates (Michell et al., 1967) and the soluble PIK of yeast (Talwalker and Lester, 1974) are able to phosphorylate exogenously added substrate. Both chick neural tissues and rat brain homogenates show little ability to do this in the absence of detergent suggesting that only membrane-bound PI is reactive. PI are insoluble and exist as very large micelles in aqueous suspension and may not be accessible to the

enzymes in this form. Exogenous PI are phosphorylated in the presence of detergents. It is not clear whether the detergent acts by solubilizing the substrate (formation of smaller mixed micelles) and/or the membrane-bound enzyme.

High concentrations of PI inhibit PIK in erythrocytes (Hokin and Hokin, 1964), brain microsomes (Colodzin and Kennedy, 1965) and kidney cortex (Tou et al., 1968) but not in rat brain and liver homogenates (Kai et al., 1966a; Michell et al., 1967), yeast (Talwalker and Lester, 1974) or chick neural tissues.

Synthesis of TPI accompanies the formation of DPI from PI in both chick brain and sciatic nerve (see Table 23). Simultaneous synthesis of TPI is also a characteristic of homogenates of rat brain (Kai et al., 1966b), kidney (Tou et al., 1968) and of erythrocytes (Hokin and Hokin, 1964), but not liver or chromaffin granules (Michell et al., 1967; Phillips, 1973).

Both DPI and TPI, when added to the standard PIK incubation mixture, produced some stimulation (10 - 20%) of PIK activity in chick brain homogenates. The effect is smaller than the stimulation reported in rat brain homogenates (Kai et al., 1966b). This may reflect some degree of detergent-like activity of the very polar PPI. It has also been suggested that these lipids compete with the reaction product (labelled DPI) for sites on hydrolytic enzymes. EGTA (an inhibitor of TPI phosphodiesterase) had no effect on PIK reaction rates in chick brain or nerve.

However, hydrolysis of the labelled DPI by TPI phosphatase can not be excluded. DPI is reported to inhibit rat liver and kidney cortex microsome PIK (Michell et al., 1967; Tou et al., 1969) but these observations were made in the presence of Cutscum (Tou et al., 1969) and other additional components (Michell et al., 1967).

Several other important considerations were brought to light. The substantial inhibition of the chick nervous tissue DPIK by relatively low concentrations of TPI places an extra emphasis on the use of pure substrate. The DPI used in some earlier studies contained as much as 25% TPI and may explain the inhibition reported at high substrate concentrations (Kai et al., 1966b). The DPI used in the present investigation did not contain any TPI as judged by t.l.c. (see Fig. 7). Concentrations of ATP higher than 6 mM inhibited chick neural tissue DPIK. In previous studies with rat brain (Kai et al., 1968) and rabbit sciatic nerve myelin (Iacobelli, 1969), inhibition by ATP was not observed at values up to 8 - 12 mM, although it was reported for rat kidney cortex (Tou et al., 1970). This inhibition was attributed to competition between free ATP and Mg:ATP complex for the active site (Tou et al., 1970) since increasing the Mg^{2+} concentration shifted the ATP optimum to higher concentrations.

PIK has frequently been assayed in the presence of non-ionic detergents. The detergents were not included in DPIK measurements since they have generally been

reported to have no effect or to inhibit rat brain and kidney cortex preparations (Kai et al., 1968; Tou et al., 1970). However, Eichberg and Hauser (1969) observed stimulation of both PIK and DPIK activities in rat brain homogenates by Cutscum. Little attention appears to have been paid to the relationship between detergent and protein, as well as to effects of detergent on other parameters of the assay system which were found to vary with the tissue studied (see Results, Section B.2.a.(iii) and b.(iii)). It is clear that the effects of Cutscum are complex when crude homogenates are used. The importance of the detergent/protein ratios implies an effect on the enzyme itself while the altered substrate dependence suggests possible changes in the physical state of the substrate and/or its accessibility.

C. TRIPHOSPHOINOSITIDES PHOSPHOHYDROLASES

Reported assay mixtures for both TPI phosphatase and TPI phosphodiesterase have varied from simple mixtures of homogenate and substrate to complex mixtures containing various additives and requiring special precautions. A major difference has been the addition of CETAB. Undoubtedly the major role of CETAB in these assay systems is to reduce the negative charge on the substrate micelles. This may be essential for enzyme substrate contact since TPI phosphatase from ox brain is negatively charged at physiological pH (Dawson and Thompson, 1964). In aqueous

solution, TPI form small micelles (Thompson and Dawson, 1964) which have a molecular mass estimated at 78100 daltons (Hendrickson, 1969) and a very large negative charge on the surface (Palmer and Dawson, 1969). CETAB, like other amphipathic cations, is believed to become aligned with the TPI molecule so that their polar head-groups are together at the micelle surface. As the CETAB/TPI ratio increases, the net charge on the surface decreases leading ultimately to neutral or nearly neutral particles which form large insoluble aggregates. Although divalent cations can also serve this function to some extent the higher concentrations required frequently result in precipitation of the substrate, a problem which is largely avoided with CETAB (Thompson and Dawson, 1964b; Dawson and Thompson, 1964; Cooper and Hawthorne, 1975). In all cases, the molar ratio of CETAB/TIP is critical. The preferred ratio in different tissues and assay conditions has been variable. Both chick neural tissue TPI phosphatases and phosphodiesterases exhibited optimum activity at a ratio of 2.0. Earlier studies reported optimum ratios of 0.3 and 1.0 for the TPI Phosphatases of ox brain and rat brain respectively (Thompson and Dawson, 1964b; Salway et al., 1967). The preferred ratio reported for phosphodiesterase preparations from ox brain has varied from 1.2 to 2 (Thompson and Dawson, 1964b; Keough and Thompson, 1972). The differences in the optimum CETAB/TPI ratio for various tissues are not fully understood. However, it may simply

be the consequence of differences in enzyme preparations, presence of other phospholipids in varying amounts in tissue homogenates and/or impurities in the detergent itself. These observations have been made for several enzymes (see Helenius and Simons, 1975).

The action of CETAB is more complex since it also appears to alter the affinity of both enzymes for the substrate. Without CETAB, the TPI phosphatases of chick neural tissues as well as mammalian brain (Salway et al., 1967; Sheltawy et al., 1972) are saturated by low TPI concentrations (0.5 mM). The substrate requirement rises to at least 2 mM in the presence of CETAB. This increase, first reported by Salway et al. (1967), had been attributed to the presence of EDTA and large amounts of $MgCl_2$ by Sheltawy et al. (1972). The results with chick neural tissues indicated that it is a consequence of adding CETAB. A similar CETAB induced "apparent" decreased substrate affinity for TPI phosphodiesterase has been noted before. The "apparent Km" value of 1.6 mM for ox brain phosphodiesterases (Keough and Thompson, 1972) measured in the presence of CETAB implies a lower affinity than observed earlier without CETAB in rat brain (Keough and Thompson, 1970) and kidney (Tou et al., 1973). CETAB makes it difficult to achieve saturating conditions at reasonable TPI concentrations during measurements of TPI phosphodiesterase in chick brain and nerve. The concentration used (2.8 mM) was barely adequate and made a shorter reaction

time necessary. The effect may be more apparent than real if the lowered net charge of TPI-CETAB micelles results in larger micelles having less total surface area. However, some preliminary studies of TPI-CETAB micelles in the ultracentrifuge tend to suggest that this is not the case (Dr. Palmer, private communication). It may be that reducing the negative charge permits the micelles to approach the enzyme more easily but that the mixed detergent-TPI micelle is less compatible with the active site in some other aspect. CETAB may also exert an effect directly on the enzyme. The converse has also been observed in kidney extracts where CETAB reduced the saturating concentration of TPI from 2 mM to .1 mM (Cooper and Hawthorne, 1975).

Systems for measuring TPI phosphatase and phosphodiesterase must ensure that the two activities are measured independently of each other and of other non-specific phosphatases and phosphodiesterases. Although TPI phosphatase is monitored by measuring the release of inorganic phosphate, competition for the substrate by TPI phosphodiesterase could reduce the apparent activity. Contribution by non-specific phosphatases must also be considered. Sheltawy et al. (1972) avoided the use of CETAB since it was known to stimulate the diesterase and preferred not to supplement their assay system with Mg^{2+} to avoid stimulation of alkaline phosphatases. It was assumed that the Mg^{2+} present in the homogenate would be sufficient for both activation of

TPI phosphatase and reduction of the negative charge on the substrate. Under these conditions, appreciable hydrolysis of TPI by alkaline phosphatase was considered to be unlikely at neutral pH. In homogenates of chick neural tissues, the TPI phosphatase activity would also be very low in the absence of CETAB and Mg^{2+}. The endogenous Mg^{2+} in chick brain and nerve homogenates is not adequate for maximum stimulation of the TPI Phosphatase even when CETAB is present to reduce the charge on the substrate. Similar results have been observed for rat kidney preparations (Cooper and Hawthorne, 1975). Non-specific phosphatase activity is high in this tissue but the alkaline phosphatase could be effectively inhibited by cysteine and the acid phosphatase by fluoride. However, neither fluoride nor thiol reagents (cysteine, reduced glutathione) had any effect on the release of inorganic phosphate by homogenates of chick neural tissues in our assay system suggesting that non-specific phosphatases were not active or did not act on TPI under these conditions. The presence of CETAB is probably at least partially responsible since it has been found to suppress non-specific phosphatase activity as measured by the hydrolysis of 2-glycerophosphate in protozoa (Palmer, 1976). Furthermore, crude homogenates of rat kidney also exhibit little non-specific activity towards TPI in an assay system containing CETAB (Cooper and Hawthorne, 1975).

Selective inhibition of TPI phosphodiesterase by EDTA

(4 mM) reported in rat brain was not obtained in chick neural tissues. Although the phosphodiesterases were more susceptible, a considerable loss of phosphatase activity also occurred. However, the selective abolition of TPI phosphodiesterase activity by EGTA provides for the independent measurement of phosphatase activity.

The dephosphorylation of TPI by TPI phosphatase results in the formation of PI with DPI appearing only as a transient intermediate in the mammalian tissues (Thompson and Dawson, 1964b; Lee and Huggins, 1968) as well as in the chick neural tissues. The first step is a selective hydrolysis of the monoester phosphate group in the 5 position of the inositol ring (Change and Ballou, 1967; Cooper and Hawthorne, 1975). It is not known if the sequential dephosphorylation of TPI is the function of one or two enzymes. Few studies have used pure DPI as the substrate for this enzyme. Cooper and Hawthorne (1975) have compared the hydrolysis of TPI and DPI in rat kidney cortex homogenates. Small differences in response to dialysis, metal-ion activation and detergent suggested two enzymes. However, addition of DPI to a preparation already saturated with TPI gave very little increase in the reaction rate. Based upon this and similarities in other properties, it was concluded that only one phosphatase is present which is capable of hydrolyzing both DPI and TPI. Recently, protozoal (C. fasiculata) TPI phosphatase has been described which converts TPI to DPI only (Palmer, 1976). This also appears

to be true of the TPI phosphatase activity in the mammalian erythrocyte cytosol (Dr. Palmer, private communication). Obviously, further studies with purified enzyme preparations are needed to establish if two phosphatases are present in these tissues.

A difficulty with TPI phosphodiesterase assay systems has been the selection of conditions which discourage competition from phosphatases. In the initial studies of Thompson and Dawson (1964), about 50% of the total water-soluble phosphate released from TPI by ox brain preparation was inorganic-P. Addition of ether reduced this to 17 - 29%. Similar results were observed by Keough and Thompson (1970) for rat brain homogenates. Thompson and Dawson (1964) achieved selective measurements of TPI phosphodiesterase activity only when ox brain acetone powder extracts were subjected to heat treatment. TPI phosphatase activity was destroyed but a partial loss (40%) of the diesterase activity also occurred. Other attempts at partial purification have also resulted in considerable losses of phosphodiesterase activity (Thompson and Dawson, 1964b;; Tou et al., 1973) and were therefore unsuitable for studies intended to measure total tissue activities. Under the conditions used with chick neural tissues, inorganic-P represented no more than 1% of the total water-soluble phosphate released. This was the result of (i) low Mg^{2+} concentrations which would stimulate phosphatases, (ii) CETAB inhibition of non-specific phosphatases and (iii) addition of Ca^{2+} which

stimulates the diesterase and inhibits the phosphatase.

The specific requirement of TPI phosphatases for Mg^{2+} is well documented (Dawson and Thompson, 1964); however, an absolute cation requirement for TPI phosphodiesterases has been less certain. Thompson and Dawson (1964b) initially reported that both Ca^{2+} and Mg^{2+} stimulated the activity of a partially purified preparation from bovine brain only in the absence of CETAB, suggesting that their prime mode of action is to reduce the negative charge on the substrate. In most studies, no cations have been required in the assay system (Keough and Thompson, 1970, 1972) except when phosphatidylinositol was the substrate (Keough and Thompson, 1972). The rat kidney enzyme is inhibited by both Ca^{2+} and Mg^{2+} unless initially depleted by EDTA treatment; in which case, either cation partially restores activity (Tou et al., 1973). Stimulation of chick nervous tissue TPI phosphodiesterases by Ca^{2+} but not Mg^{2+} in the presence of optimum levels of CETAB and specific inhibition by EGTA, a chelating agent with a high affinity for Ca^{2+}, strongly suggests that these enzymes specifically require Ca^{2+}. Although adding $CaCl_2$ to the assay system did not cause a large increase in the activity, it did suppress the small TPI phosphatase activity supported by the endogenous Mg^{2+}. The catabolism of TPI in chick brain and nerve therefore occurs via a Mg^{2+} dependent phosphatase and a Ca^{2+} dependent phosphodiesterase comparable to that described recently in protozoa (Palmer, 1976).

D. ENZYME ACTIVITIES DURING DEVELOPMENT

1. Phosphoinositide Kinases

Measurements of PIK in the two earlier studies of polyphosphoinositide synthesis in postnatal rat brain were made at pH 7.4 (Salway et al., 1968; Eichberg and Hauser, 1969). The premyelination values are similar to those observed in chick brain at pH 7.4. However, the activity in adult chick brain was at least 3 times greater than in rat brain. Salway et al. (1968) reported the PIK to increase only 2 fold at birth, well before the onset of myelination in the rat. One experimental series showed the activity to remain high while the second series showed it to decrease during and after myelination. The latter observation was supported by the work of Eichberg and Hauser (1969) who found the PIK activity to steadily decrease after birth. The appearance of PIK activity in developing chick brain was different in three respects. The increase in activity was greater (4 - 5 fold), was coincident with onset of myelination and the activity remained high during subsequent maturation. The DPIK activity also showed a dramatic (4 fold) increase during the period of most active myelination and thereafter remained high in chick brain. Salway et al. (1968) observed a similar developmental pattern for this enzyme in postnatal rat brain; however, Eichberg and Hauser (1969) reported an increase in activity during later stages

of myelination. The appearance of PI kinase before DPI kinase in rat brain has been linked to the presence of DPI but not TPI in unmyelinated brain (Salway et al., 1968). This is not likely a valid correlation since embryonic chick brain also contains a relatively large amount of DPI (30% of the adult concentration) and little TPI but exhibits low PIK activity prior to myelination.

Eichberg and Hauser (1969) assayed both kinases in the presence of Cutscum (0.4%) which might account for the differences between their results and those for chick brain. However, assays repeated in the presence of optimum amounts of Cutscum did confirm that both enzyme activities increased during myelination and remained high in adult chick brain.

2. TPI Phosphohydrolases

In a short abstract, Hauser et al. (1967) reported that the activities of TPI phosphatase and TPI phosphodiesterase showed no changes in brain acetone powder extracts from newborn and mature rats. However, detailed studies of these enzymes (Salway et al., 1967; Keough and Thompson, 1970) in developing postnatal rat brain have shown the activities of both enzymes to increase 3 - 4 fold during the period of most rapid myelination (10 - 20 days of age). In chick brain, the increase in TPI phosphatase activity coincided with the deposition of myelin. The activity observed was at least 10 fold greater than reported

for guinea pig brain (Sheltawy et al., 1972), largely as a result of using a maximally stimulated assay system. They were also at least 2 fold greater than values for rat brain obtained in the presence of CETAB (Salway et al., 1968), although a similar developmental pattern was observed. Salway et al. (1968) found the TPI phosphatase activity in rat brain to be very much greater than the DPI and TPI synthetic capacity. The difference was smaller in chick brain but TPI phosphatase was still 2 orders of magnitude greater than either the PIK or DPIK activities. The TPI phosphodiesterase activity was even greater. Unlike rat brain (Keough and Thompson, 1970), the increase in chick brain TPI phosphodiesterase was not coincident with rapid deposition of myelin, although this is true for the other three enzymes of polyphosphoinositide metabolism. TPI phosphatase in chick sciatic nerve remained constant at a value lower than that found in embryonic brain. The highest TPI phosphodiesterase activity was found in the embryonic nerve but the low adult values were comparable to those in embryonic brain. Even these low activities greatly exceeded the activities of the synthetic enzymes. Reported values for PIK and DPIK could be increased through the use of appropriate detergents, although not sufficiently to equal the catabolic activities. Extrapolation of data obtained with detergent-fortified assay systems to the in vivo situation is questionable. Therefore, to what extent these data indicate that the in vivo catabolic activity far exceeds

the synthetic capacity is not clear. If it is true, effective control on the catabolic enzymes must be exerted.

The deposition of TPI correlates well with myelination in both chick brain and sciatic nerve (see Section III.D.1.). Since both PIK and DPIK are necessary for TPI synthesis it is not surprising that their activities increase when this lipid is being deposited in myelin. The catabolic enzyme activities (TPI phosphatase and phosphodiesterase) also rise at or near the period of most active myelination in brain. Both synthetic and catabolic enzyme activities appear to be correlated with the concentration of TPI in this tissue. The continued high synthetic and catabolic activities in adult brain are consistent with the presence of PPI having a high rate of turnover, probably that portion of the total PPI which is located outside of the myelin (Sheltawy and Dawson, 1969a; Gonzalez-Sastre et al., 1971).

This study constitutes the only report concerning all four enzymatic activities in PNS. The activity patterns are quite different than brain. The synthesis of TPI for deposition in sciatic nerve appears to be the result of a transitory increase in synthetic capacity which is restricted to the period of most active myelination. The kinase activities are therefore correlated with the rate of TPI deposition. The TPI phosphatase activity is 10 - 15 times lower than that of brain and does not change during development. Although very high in embryonic nerve, the

diesterase activity falls throughout development to very low levels in mature nerve. The transitory rise in synthetic enzyme activities accompanied by declining hydrolytic capacity in nerve implies the synthesis of a product required for myelin formation but one which is not subject to continuing rapid turnover. This is consistent with evidence that the turnover of PPI is low in nerve (Sheltawy and Dawson, 1969b).

V. SUMMARY

V. SUMMARY

The present study represents a comprehensive analysis of lipids with particular interest on inositol-containing lipids in developing CNS and PNS of the chicken. This study constitutes the first comprehensive lipid analysis of chick sciatic nerve during development and also the first such study of peripheral nerve of any species to include the values for PPI. This work also includes an investigation of the enzymes responsible for the metabolism of the PPI, in the course of which improvements to the assay procedures were developed and several characteristics were investigated in greater detail than attempted previously. Except for the DPIK, this work represents the first description of these activities in the mature peripheral nerve and the first and only report of these enzymes in developing PNS.

Lipid analyses of adult chick brain generally confirmed earlier reports except for the PPI. The TPI/DPI ratio was greater although the total PPI concentration was similar. Since it is well known that the active post-mortem hydrolysis of TPI initially results in DPI accumulation, the higher TPI/DPI ratio can be attributed to the greater precautions taken to minimize post-mortem hydrolysis. The concentrations of the PPI, and to a lesser extent other lipids, were also higher than reported in an earlier analysis of adult sciatic nerves from the chicken and other species.

Sciatic nerve is a less complex tissue than brain in terms of the number and variety of cells which do not possess myelin. For this reason, it was selected as a tissue whose lipid analysis would more closely reflect that of myelin. The lipid analysis of whole sciatic nerve (omitting PPI) was very similar to that reported for isolated chick sciatic nerve myelin thereby confirming the choice of nerve for a study of myelin-associated PPI.

In chick brain, TPI deposition continued long after the most active phase of myelination as did cerebrosides deposition. Since myelin contributes a major portion of the lipids in adult brain, this increasing proportion of TPI could reflect a long-term increase in the concentration of this lipid in myelin after it has been formed. A similar proposal has been made for cerebrosides based on analyses of isolated myelin, a type of experiment which has not been possible for TPI due to post-mortem catabolism during preparation of myelin. Lipid composition of sciatic nerve more closely resembled that of the myelin. A similar increase in the concentration of TPI in older chick sciatic nerve provided better evidence for the long increase in the proportion of TPI in myelin.

In order to correlate lipid changes with morphological changes, the periods of most active myelination were defined in both tissues by galactolipid deposition. The periods defined by this criterion were consistent with earlier

anatomical, histological and biochemical studies.

The developmental patterns for the lipids in chick brain were similar to that observed earlier for both chick and rat brains. The developmental changes for PPI were much greater than for other lipids with the exception of cerebrosides and plasmalogens, both of which are known to be associated with myelin. PPI were present in appreciable concentrations in pre-myelination embryonic brain but the amount of DPI relative to the adult concentration (31%) was greater than that observed for TPI (11%). DPI and TPI concentrations increased rapidly during the period of active myelination and their deposition coincided with that of cerebrosides. TPI continued to increase after this period while DPI remained about the same.

Additional support for the presence of a separate myelin and non-myelin pool of PPI in brain was obtained. The presence of appreciable quantities of both DPI and TPI in unmyelinated embryonic brain followed by much greater increases in TPI concentrations is consistent with the presence of both DPI and TPI in non-myelin structures and a myelin compartment containing mainly TPI.

Both DPI and TPI were absent in unmyelinated embryonic nerve (15 days). Their concentrations increased more rapidly than any other lipid in nerve and faster than that observed for PPI in developing brain. The developmental

pattern for these lipids was correlated more closely with galactolipid deposition. Furthermore, the ratios of TPI/DPI were much higher in peripheral nerve as compared to brain. These data suggest that PPI in peripheral nerve are associated with myelin and that the non-myelin pool of PPI is probably absent or very small in this tissue.

The PPI are synthesized by the sequential phosphorylation of PI. The reaction conditions employed to measure the PIK and DPIK activities were similar to those used in other laboratories. The procedure for isolating reaction products was altered to improve the recovery. This permitted a better estimate of initial reaction rates, an important consideration since the reactions proceed for only a very short time. The characteristics of PIK and DPIK were the same in chick brain and sciatic nerve and did not change with age. High concentrations of ATP (> 6 mM) strongly inhibited the DPIK activities but the effect on PIK was much smaller. Reports of ATP inhibition were not reported previously for brain. DPIK was greatly inhibited by very low concentrations of TPI. This finding emphasized the necessity of using pure DPI as the substrate (TPI is the most common contaminant of DPI preparations), a condition not always met in other studies. The effect of Cutscum, a frequently used detergent, proved to be more complex than previously appreciated. The detergent/protein ratio was critical. The optimum ratios were different for the two enzymes and were different in brain and nerve for DPIK. Cutscum also altered the "apparent" affinity of

the sciatic nerve DPIK for its substrate.

PPI are degraded by a phosphatase and a diesterase. The characteristics of both enzymes were the same in homogenates of chick brain and sciatic nerve of all ages. TPI phosphodiesterase was inhibited by EGTA, a compound which selectively binds Ca^{2+}. This provides the first evidence that the TPI phosphodiesterase of chick neural tissues has an absolute requirement for Ca^{2+}. It also permitted the measurement of TPI phosphatase, a Mg^{2+} requiring enzyme, without competition for the same substrate by the diesterase. Contrary to some earlier reports, supplementation of the homogenates with the appropriate divalent cation was necessary for full activity. Furthermore, the phosphatase was inhibited by Ca^{2+} thereby permitting the measurement of the diesterase without any competition from the phosphatase.

As reported earlier the cationic detergent CETAB stimulated both activities by reducing the negative charge on the substrate. However, the effect of CETAB was found to be complex. At the optimum CETAB/TPI ratio (2.0 for both activities in the two tissues), the concentration of substrate required to saturate the TPI phosphatase was increased from about 0.5 mM to over 2 mM. The effect was greater for TPI phosphodiesterase making it difficult to fully saturate this enzyme at any practical substrate-detergent concentrations and a short incubation time was required. Under the standard assay conditions, the effects of non-specific phosphatases

were not significant.

In chick brain, both kinase activities increased rapidly during the period of active myelination and correlated well with the concentration of TPI in this tissue. This report contradicts earlier studies in rat brain where PIK activity was shown to increase prior to the onset of myelination and then decrease with age. Both TPI phosphatase and phosphodiesterase activities in chick brain also increased at the time of rapid myelin deposition as has been reported for postnatal rat brain. This correlation with myelination is much better for the phosphatase. The increase in the phosphodiesterase activity occurred over a much longer time period with no abrupt rise during myelination. All four activities remained high in adult brain. These data were interpreted as reflecting both the synthesis of PPI for deposition and the presence in adult brain of PPI having a high rate of turnover (presumably the non-myelin pool).

The developmental patterns for synthetic and catabolic enzymes are quite different in sciatic nerve. Both kinases showed a peak of activity during active myelination. The activities then are correlated with the rate of PPI deposition rather than with the concentration of PPI as was observed in brain. The TPI phosphodiesterase activity increased just prior to myelination and thereafter declined to very low values which are characteristics of adult nerves. The TPI phosphatase activity exhibited little change with

age. Both activities were similar to those found in embryonic brain. All the four activities were low in adult nerve. These data were interpreted as reflecting the synthesis of PPI as relatively inert myelin components which are not subject to rapid metabolic turnover in adult nerve. This is consistent with the previous metabolic studies of this tissue.

ORIGINAL CONTRIBUTIONS

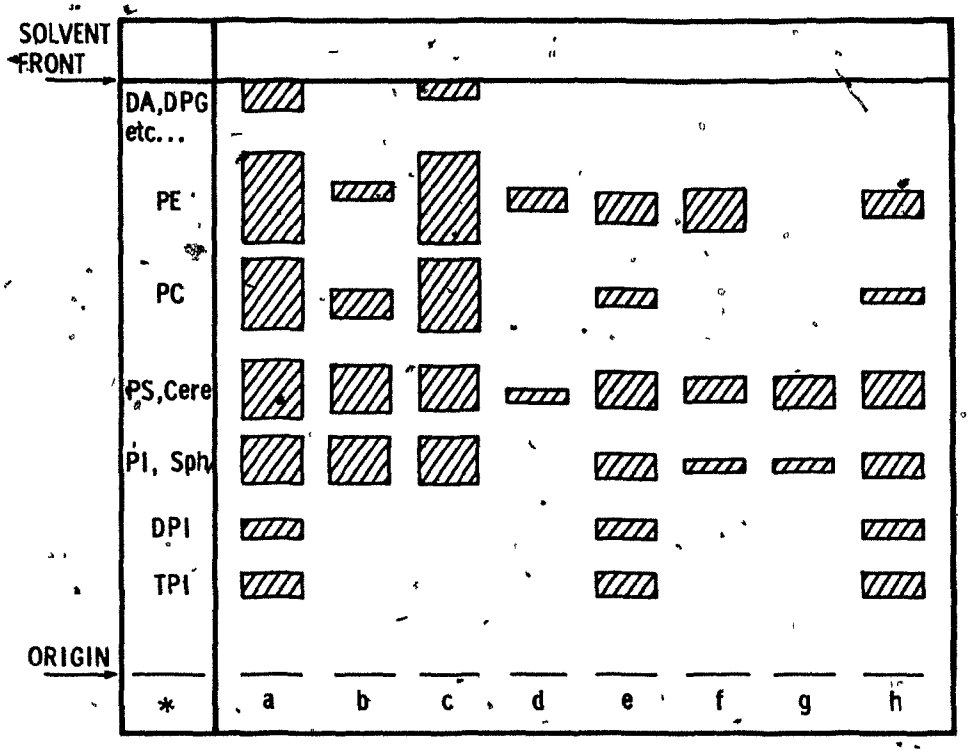
(1) This thesis represents the first comprehensive analysis of phospholipids in developing chicken brain and sciatic nerve to include the values for phosphoinositides. Evidence from brain supported the concept that polyphosphoinositides exist in both myelin and non-myelin pools which are metabolically different. Data from sciatic nerve suggested that only the myelin pool of these lipids is present in this tissue. The first evidence was obtained that subsequent maturation of the deposited myelin involves changes in the polyphosphoinositides concentrations in both tissues similar to that noted earlier for certain other lipids (e.g. cerebrosides, phosphatidylcholines, ethanolamine plasmalogens)

(2) This thesis represents the first description of PI kinase, TPI phosphatase and TPI phosphodiesterase activities in peripheral nerve and the first developmental study of the enzymes of polyphosphoinositides metabolism (including DPI kinase) in chick brain and in the peripheral nerve of any species.

(3) Using improved procedures for measurements of these enzymatic activities, the developmental pattern of PI and DPI kinases in chick brain showed a correlation to the deposition of myelin and clarified the previous conflicting reports in the literature based on studies in postnatal rat brain. In sciatic nerve, the synthetic enzymes were correlated with the rate of myelin deposition. Low activities of all four enzymes in adult nerve were consistent with reported low metabolic activities in this tissue. Data was interpreted to suggest that polyphosphoinositides in nerve are synthesized as relatively inert myelin components; whereas in brain, the enzymatic activities remained high in adult tissue to participate in the rapid turnover of the non-myelin pool of polyphosphoinositides.

VI. APPENDICES

APPENDIX I. PREPARATION OF PURE DI- AND TRIPHOSPHOINOSITIDES

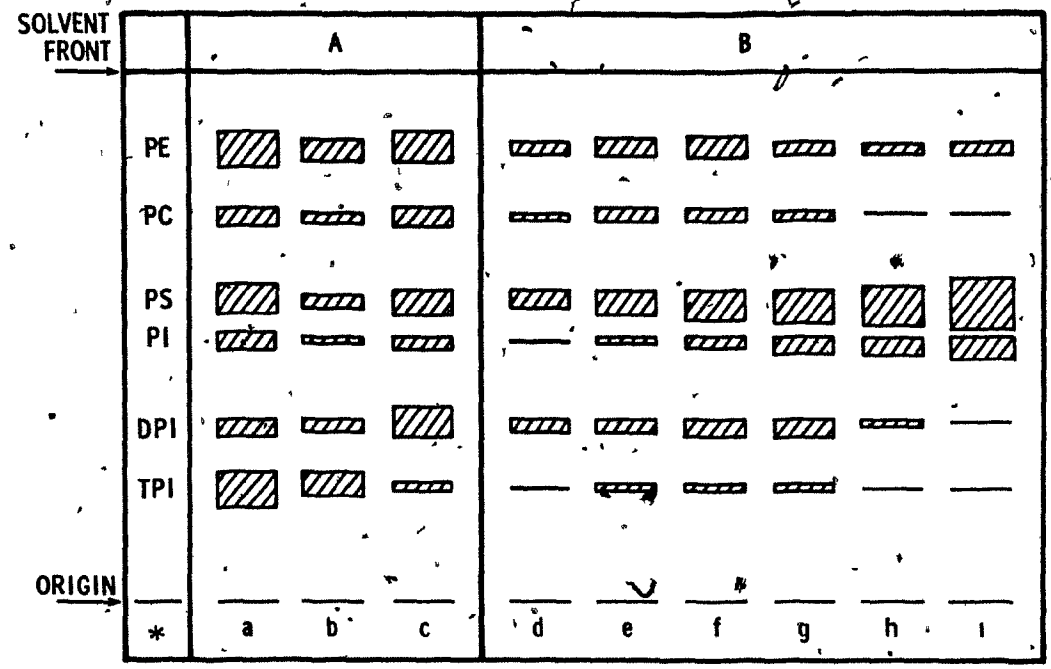


(a) Qualitative Thin-Layer Chromatograms of Various Solvent Washes and Fractions Obtained During Preparations of Crude "Inositol Phosphatide" Fraction. Silica Gel HR plate was developed in C-A-M-AA-W (40:15:13:12:8, by vol.). Figure shows relative proportion of lipid component in each fraction.

Column

* Reference phospholipids (diacyl form but the bands corresponding PS, PC and PE may represent SPG, CPG and EPG forms respectively).
a. Combined diethyl ether extract.
b. Diethyl ether insoluble material.
c. Diethyl ether - ethanol supernatant.
d. Acetone washes of diethyl ether-ethanol precipitate (cephalin).
e. Cephalin fraction.
f. Chloroform-ethanol, supernatant.
g. Ethanol wash of viscous underlayer.
h. Crude "inositol phosphatide" fraction.

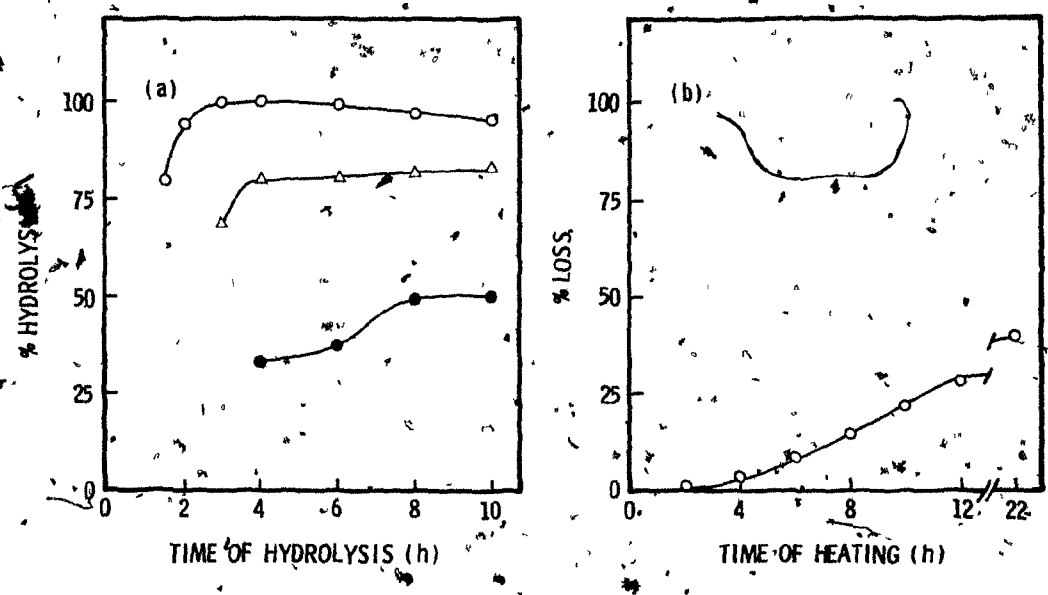
APPENDIX I. Cont.



(b) Qualitative Thin-Layer Chromatograms of Various Fractions Isolated from "Washed Crude PPI" Fraction in the Chloroform-Methanolic NaOH-Acetone Procedure: Chromatograms drawn as they appeared on t.l.c., developed in C-A-M-AA-W (40:15:13:10:7, by vol.). Various volumes of acetone (ml of acetone/total volume of C-M) were added to successive supernatants and the precipitate collected. Figure shows relative proportions of lipid components in individual fraction.
A, The final adopted method profile; B, successive precipitation profile.

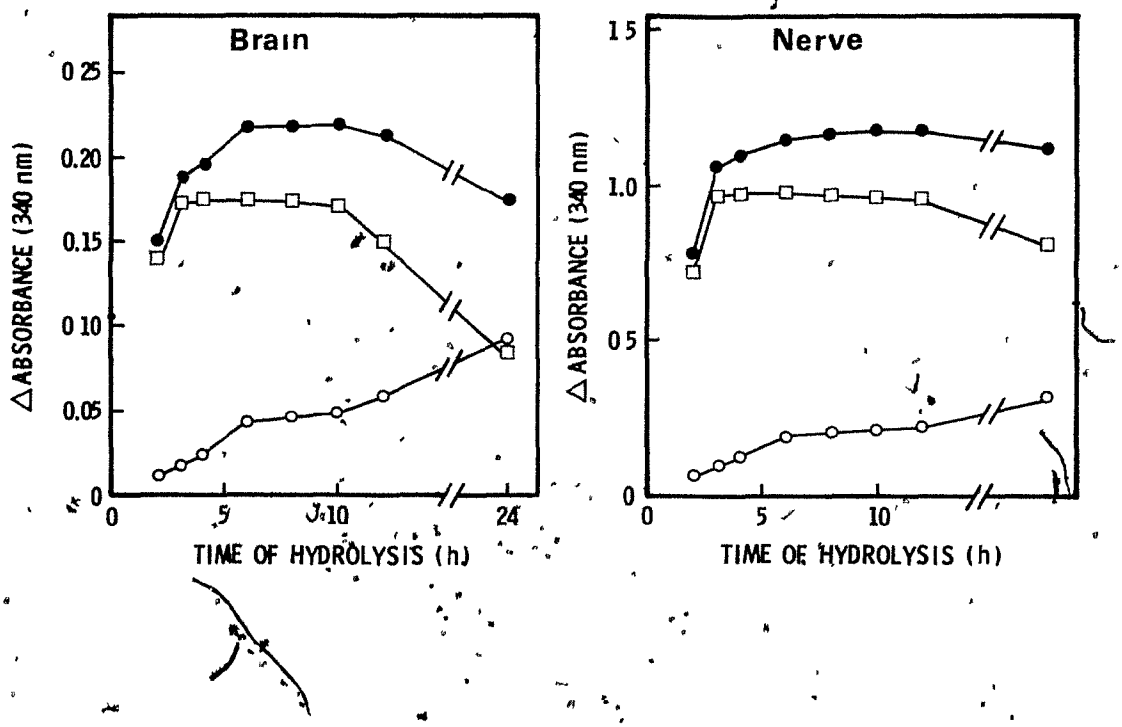
Column
* Reference phospholipids (for detail see Appendix I.a).
a. "Washed PPI" fraction.
b. Methanol insoluble sodium PPI.
c. Methanol-acetone insoluble sodium PPI.
d. 0.25 volume acetone insoluble sodium PPI.
e. 0.50 volume acetone insoluble sodium PPI.
f. 0.75 volume acetone insoluble sodium PPI.
g. 1.0 volume acetone insoluble sodium PPI.
h. 1.5 volume acetone insoluble sodium PPI.
i. 2.0 volume acetone insoluble sodium PPI.

APPENDIX II. CHARACTERIZATION OF PARAMETERS FOR GALACTOLIPID DETERMINATION



(a) Hydrolysis of Cerebroside Standard as a Function of Temperature and Acid Concentration. 1 µmol of cerebroside standard was hydrolyzed with 2 ml each of 3N-H_2SO_4 at 80°C, ●; 3N-H_2SO_4 at 100°C, ○; 2N-H_2SO_4 at 100°C, △. After neutralization, galactose release was measured in the hydrolysate (0.5/2.65 ml) using standard assay conditions. (b) Effect of Heat on Galactose in Acid Solution. Galactose (0.2 µmol) was mixed with 2 ml 3N-H_2SO_4 and was heated at 100°C. After neutralizing the hydrolysate, galactose in 0.5/2.65 ml was assayed using standard conditions.

APPENDIX II. Cont.



(c) Hydrolysis of Galactolipids in H_2SO_4. Lipid extracts from 17 day embryonic nerve and 21 day embryonic brain (containing about 0.17 and 0.85 μmol galactolipid respectively) were hydrolyzed in 2 ml 3N-H_2SO_4. Hydrolysates were neutralized by conc. NH_4OH and 0.5/2.65 ml sample was taken for galactose assay (standard Method: ●, Sample; ○, protein blank; □, Sample when respective protein blank is subtracted.

APPENDIX III. THIN-LAYER CHROMATOGRAPHY OF LIPIDS

(a) Thin-Layer Chromatography of Lipids Extracted with Acidfied Chloroform-Methanol: Rf values for Phospholipids in Various Solvents

Solvent System →	I			II	III
Phospho-lipids ↓	500 µm layer	250 µm layer			
TPI	0.27	0.29*	0.29	0.16	0.22
DPI	0.37	0.43	0.42	0.24	0.34
PI	0.51	0.55	0.53	0.35	0.48
PS	0.62	-	0.64	0.42	0.58

* Literature values (Gonzalez-Sastre and Folch PI, 1968). Individual phospholipids (3 - 4 µg P) were spotted on Silica Gel H plates (250 and 500 µm thick layers) containing oxalate. The plates were developed in solvents of different composition. I, n-propanol-4N-NH_4OH (2:1, v/v); II, C-A-M-AA-W (40:15:13:10:7, by vol.); III, C-A-M-AA-W (40:15:13:12:8, by vol.).

APPENDIX III. Cont.

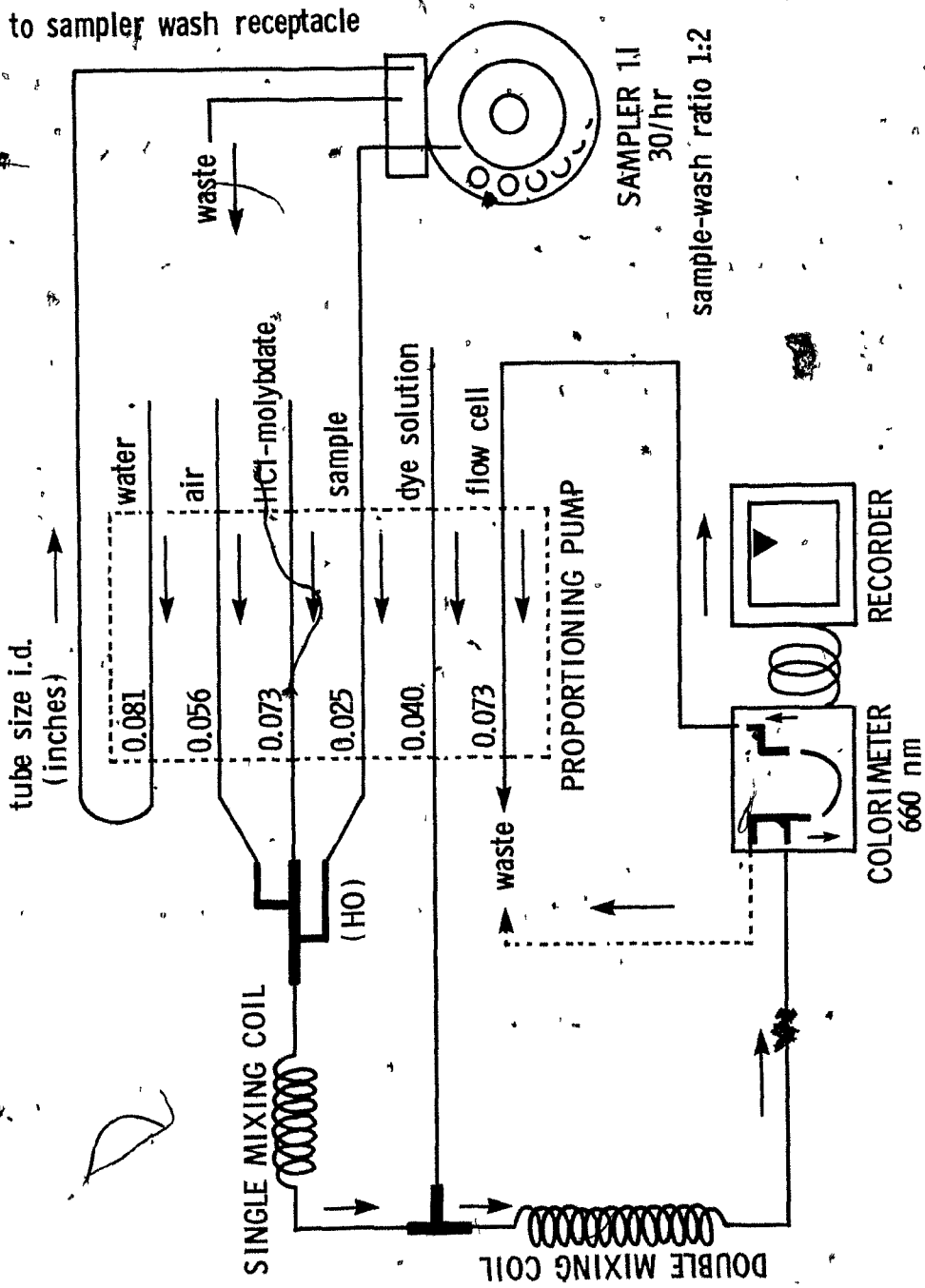
(b) Recovery of Reference Phospholipids After Thin-Layer Chromatography

Compound	Reference Solution*	Recovery Procedure**		
		A	B	C
	µmol P	%		
Lyso-PC	0.190	99.8	98.5	98.4
Sph	0.152	99.5	97.3	98.0
PC	0.161	100.3	99.2	99.4
PI	0.155	100.0	99.0	98.4
PS	0.168	99.5	97.6	97.2
PE	0.181	99.6	98.1	98.2
DPI	0.161	98.9	99.2	-
TPI	0.178	99.1	98.4	-

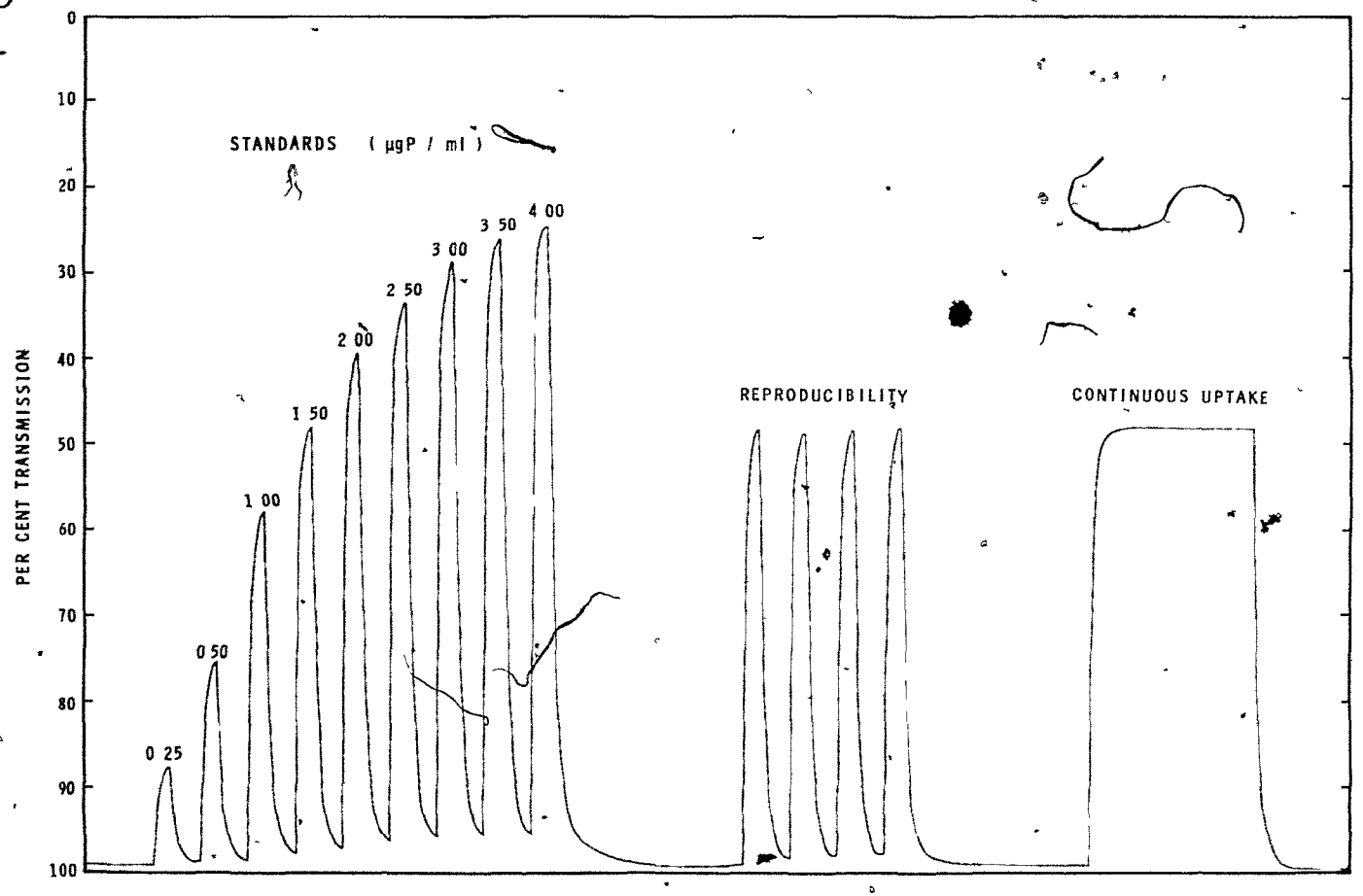
* µmol of lipid-P in 25 µl aliquot of reference solution.

** A: 25 µl of each reference solution (duplicate) applied to t.l.c. plates, then the spots were scraped off and analyzed. B: Similar to A except plate was developed. C: Six phospholipid reference solutions (no DPI & TPI) were pooled and applied as 2.5 cm band; plate developed and analyzed. All values were corrected for Silica Gel blank P content. Oxalate impregnated Silica Gel HR (for pure DPI & TPI) and Silica Gel HR plates (for other phospholipids) were used and developed in C-A-M-AA-W (40:15:13:12:8, by vol.) at room temperature and in C-M-AA-W (100:45:20:7, by vol.) at 21 - 22°C, respectively.

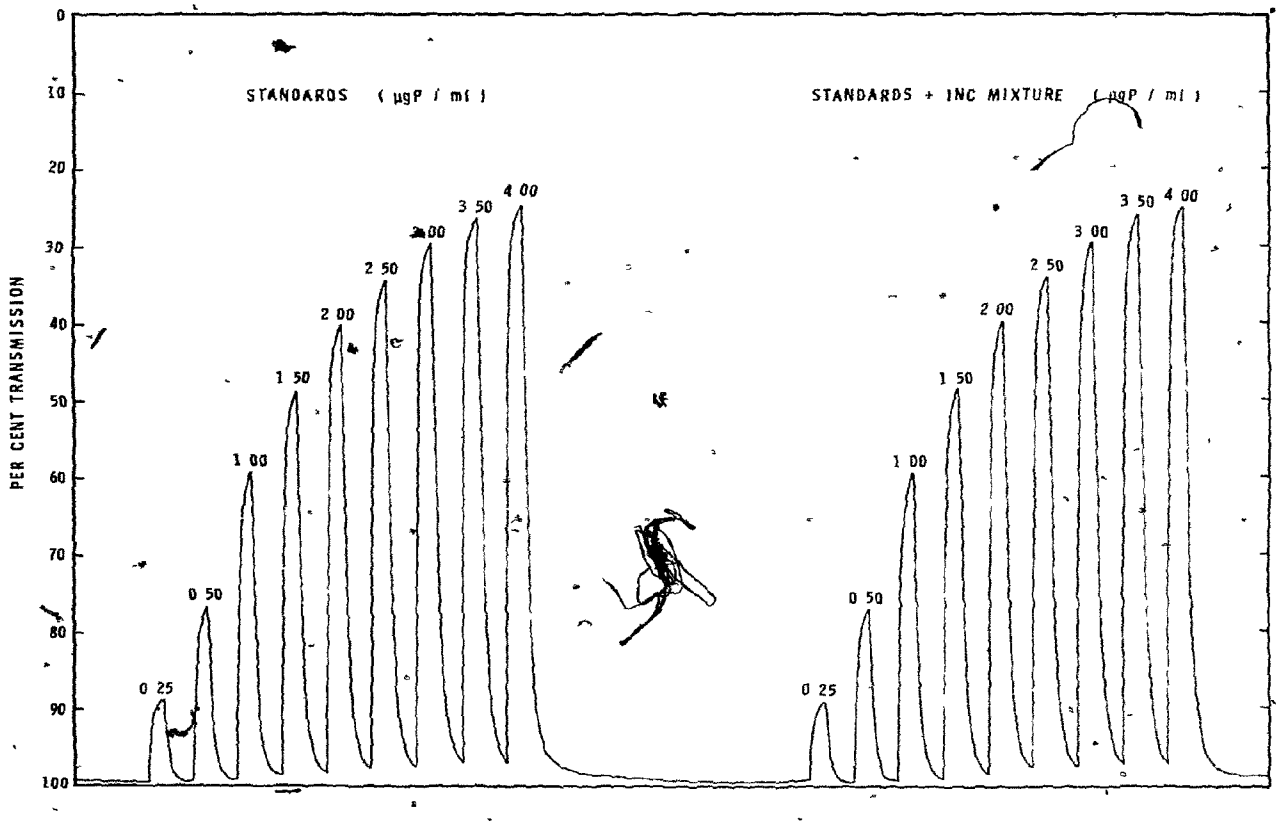
APPENDIX IV. METHODS FOR DETERMINATION OF INORGANIC AND TOTAL PHOSPHATE USING AUTOANALYZER



(a) Continuous Flow Manifold for Determination of Pi.



(b) Actual Tracings of Pi Determination Showing Standards, Reproducibility, Peak Separation and Continuous Uptake.



(c) Effect of Incubation Mixture Constituent on Pi Determination (see text for detail).

APPENDIX IV. Cont.

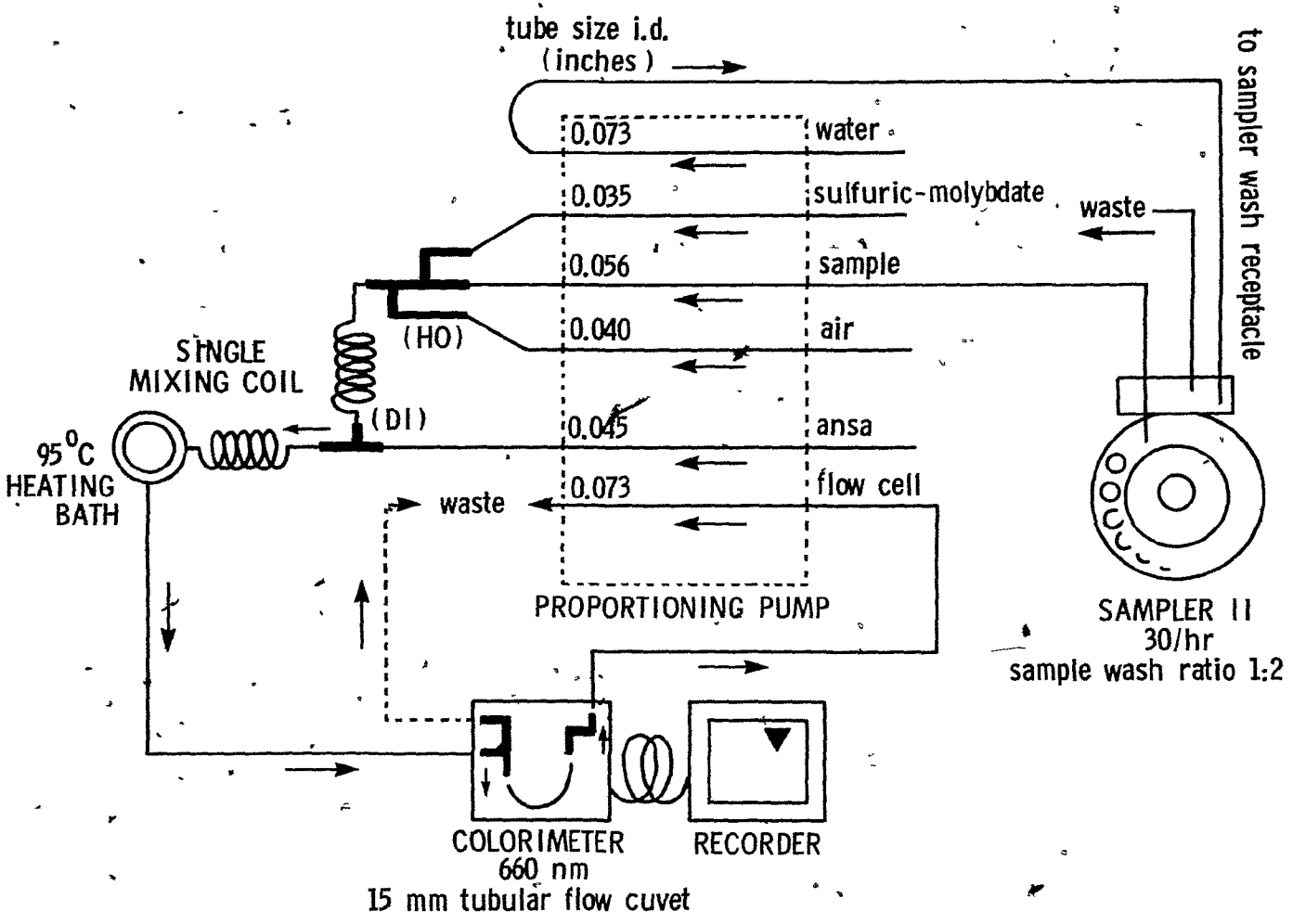
(d) Effect of TPI, CETAB and TPI-CETAB Mixture on Inorganic Phosphorus Determination

	mg P/ml			
Additions*	Pi Added	Pi Estimated	Inc. Bl**	Final "P" Estimated
None	0.8	0.85	0.05	0.80
2.8 mM-TPI	0.8	0.85	0.05	0.80
5.6 mM-CETAB	0.8	0.85	0.05	0.80
2.8 mM-TPI + 5.6 mM-CETAB	0.8	0.85	0.08	0.80

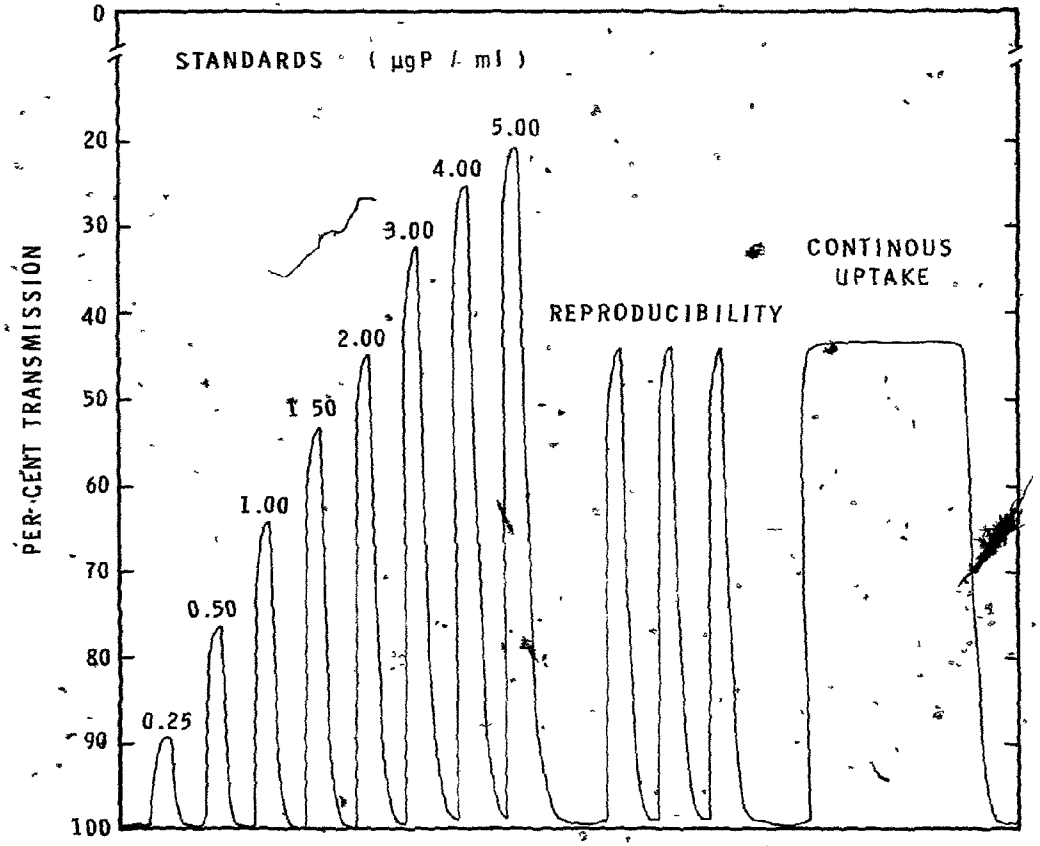
Each value is a mean of duplicate analyses.

* Incubation mixture contained: Tris-HCl, 45 mM; KCl, 0.02 mM; EGTA, 2 mM; $MgCl_2$, 1.0 mM in a total volume of 0.25 ml. Tubes were kept on ice all the time. 0.05 ml of 10% (w/v) BSA containing 10 mM-EDTA was added, followed by 0.2 ml of 10% (v/v) $HClO_4$. Additions of TPI, CETAB and TPI-CETAB mixtures were done after BSA addition. 0.2 ml aliquots of each supernatant was diluted to 1 ml with water containing 0.8 µg inorganic phosphate, and inorganic phosphate measured with autoanalyzer.

** Incubation blank represents phosphate content of BSA.

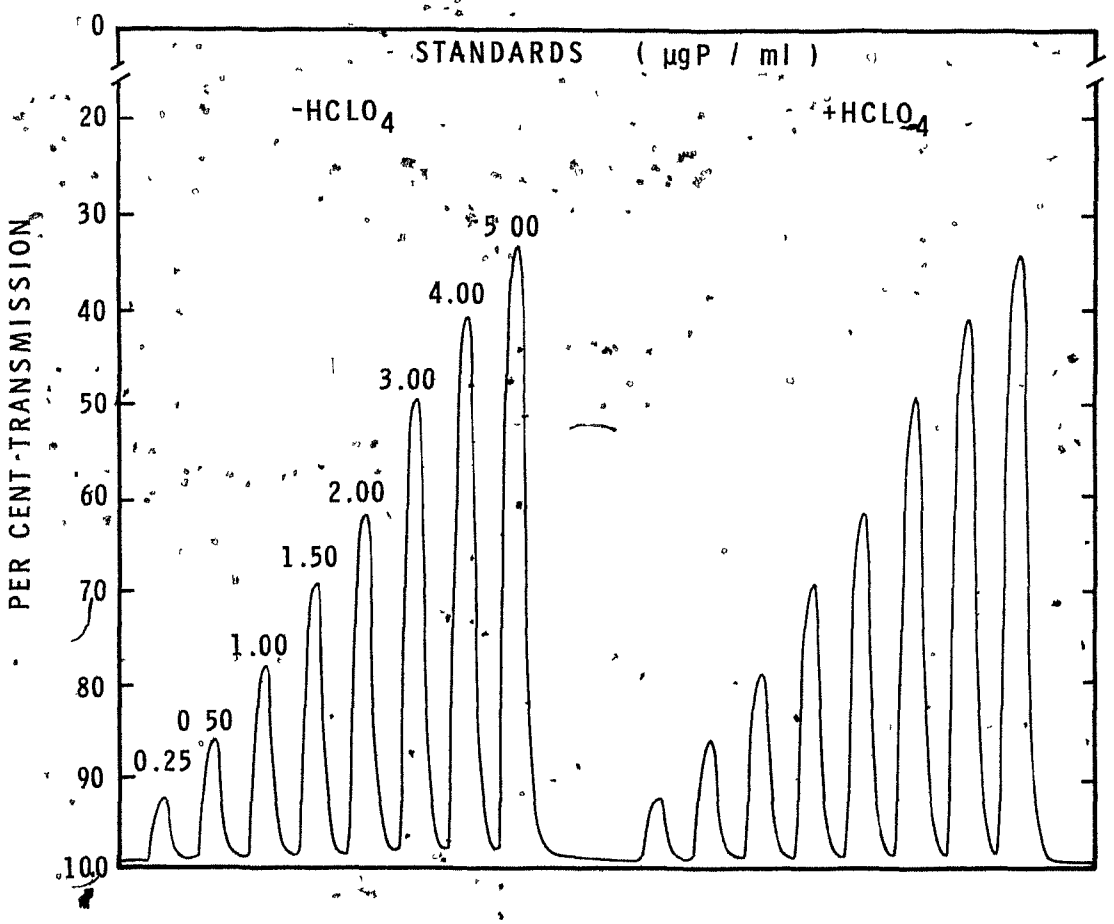


(e). Continuous Flow Manifold for Determination of Total Phosphate.



(f) Actual Tracings of Total Phosphate Determination Showing Standards, Reproducibility, Peak Separation and Continuous Uptake.

APPENDIX IV.Cont.



(g) Effect of Acid on Profile of Total Phosphate Estimation (see text for detail).

APPENDIX IV. Cont.

(h) Effect of TPI, CETAB and TPI-CETAB on Total Phosphate Determination

Addition*	System** (mg P/assay) I	II	III	IV	V
None$^{\nabla}$	0.07	0.12	0.15	0.17	0.10
2.8 mM-TPI	0.26	0.21	0.07	0.08	0.19
5.6 mM-CETAB	-	-	-	-	-
2.8 mM-TPI + 5.6 mM-CETAB	0.86	0.53	0.30	0.35	0.70

Each value is the mean of duplicate analyses.

∇ Represents P content in BSA solutions; these values and the P content of the homogenates were subtracted from other values.

* Incubation mixture contained: 45 mM - Tris-HCl (pH 7.2), 0.2 M-KCl, 0.2 mM-$CaCl_2$ in a total volume of 0.25 ml. Tubes were kept on ice all the time. 0.05 ml of 2% (w/v) chick brain homogenate was added followed by various amounts of BSA and $HClO_4$. Additions of TPI (2.8 mM), CETAB (5.6 mM) and TPI-CETAB (2.8/5.6 mM) mixture were done after BSA addition.

** System I designates addition of 0.05 ml 10% (w/v) BSA containing 10 mM-EDTA; II, 0.15 ml of 10% (w/v) BSA with 10 mM-EDTA; III, 0.15 ml of 15% (w/v) BSA; IV, 0.15 ml of

continued on next page

Appendix IV.h cont.

25% (w/v) BSA with 25 mM-$MgCl_2$; V, 0.15 ml of 10% (w/v) BSA with 25 mM-$MgCl_2$. Following BSA and substrate additions, 0.2 ml 10% (w/v) $HClO_4$ in case of system I and 0.1 ml of 20% (w/v) $HClO_4$ for the other systems was added. Aliquots (0.2 ml) of each supernatant were analyzed for total phosphate as described in the text.

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