PHOSPHOLIPID METABOLISM IN BRAIN

by

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CMP	Cytidine monophosphate
CDP	Cytidine diphosphate
CTP	Cytidine triphosphate
DNP	2:4-dinitrophenol
DPNH	Reduced diphospho-pyridine nucleotide
GABA	Y-aminobutyric acid
Pi	Inorganic phosphate
P-P	Pyrophosphate
α-GP	α-glycerophosphate
7mnp.	7-minute acid hydrolyzable nucleotide phosphate

CHAPTER 1

GENERAL INTRODUCTION

For many years, phospholipid has been considered
as perhaps one of the most obscure fields in biochemistry.
Indeed, although the history of the study of phosphorus
associated with lipid dates back to at least the 1880's
(1, 2), subsequent investigations in this area have been
characterised by the almost complete inability to penetrate
the enigma surrounding the biological synthesis of the
metabolic function of phospholipid.

During the last two decades, with the development
of refined methods for the separation and isolation of these
compounds, as well as the introduction of isotope tracer
techniques, the elucidation of the mechanisms concerned with
the formation of phospholipids has progressed rapidly. In a
large part due to the efforts of Kennedy (3, 4, 5) and
Rossiter (6), several pathways of phospholipid metabolism
occurring in liver and brain have been elucidated. Moreover,
with the advance in our knowledge of the biochemistry and
physiology of living organisms, phospholipids appear to be
involved in a multiplicity of functions of the greatest im-
portance. It has become obvious that they are associated with
other cell constituents in a wide variety of manners. The
point has now been reached where the full significance of the
role of phospholipid in biology is becoming more and more
apparent.

PHOSPHOLIPIDS AND BRAIN METABOLISM

1.   OCCURRENCE AND DISTRIBUTION OF PHOSPHOLIPIDS

The brain is a rich source of phospholipids and
together with the spinal cord probably possesses the highest
phospholipid content of all the organs.   There is approx-
imately twice as much phospholipid in brain as in liver and
kidney and about three times as much as in heart muscle (7).
Some of the earliest evidence for the occurrence of phospho-
lipids in the brain was demonstrated by Thudichum (1, 2).
During the course of an extensive investigation on the
chemical constituents of brain, this worker isolated lecithin,
the "cephalin fraction" and sphingomyelin.   In 1942, Folch
and Woolley (8) showed that the classically termed "cephalin
fraction" was a mixture of phosphatidyl ethanolamine,
phosphatidyl serine, as well as an inositol-containing
compound for which they proposed the name diphosphoinositide.

Although chemical investigations are still in
progress, many details of the structure of the diacyl
glycerophosphatides and of sphingomyelin have been well
established (9).   In addition to the three glycerophosphatides
mentioned above, there are others in brain which contain
fatty aldehydes in place of one or perhaps both fatty acids
(10, 11).   These have been termed plasmalogen and Rapport

et al. (12) and Debuch (13) have shown that the aldehydogenic groups in these compounds are attached by a vinyl ether bond to the α-position of the glycerol moiety.

Several inositol-containing phospholipids seem to exist in brain. Folch (14) isolated and partially characterised a diphosphoinositide which on hydrolysis yielded fatty acid, glycerol and inositol metadiphosphate. Hawthorne and Chargaff (15) reported the presence of inositol monophosphate in hydrolysates of brain lipids. Horhammer et al. (16) succeeded in separating by countercurrent distribution, the lipids of ox brain into two inositol-containing fractions. The first of these fractions, when chromatographed on paper, exhibited the same behaviour as liver monophosphoinositide. The second inositol-containing fraction was identified as the same brain diphosphoinositide previously isolated by Folch (14). Hokin and Hokin (17), while studying the effect of acetylcholine on the incorporation of inositol-2-H^3 into the phosphoinositides of guinea-pig brain cortex slices, found that the radioactive phospho-inositide isolated by chromatography, after incubation in vitro, was a monophosphoinositide. Dittmer and Dawson (18) have reported recently that brain tissue also contains triphosphoinositides which are tightly attached to brain protein.

Almost half the dry matter of the brain consists

of lipid.   Rossiter (19) and Lebaron et al. (9) collected
data from previous investigations on the lipid content of
mammalian brain, and showed that phospholipids were the
major component of cerebral lipids, with white matter being
richer in phospholipid than the grey matter.   Lecithin was
shown to be the most important phospholipid of grey matter
and the different brain cortex regions did not differ
significantly in phospholipid content (20, 21).   On the
other hand, sphingomyelin was the chief phospholipid component
of white matter which is rich in myelinated fibres.   Johnson
et al. (22, 23) observed that the lipids in the peripheral
nerves of several animals and man resemble that contained in
the white matter of brain rather than that in the grey matter,
in that they are richer in cholesterol, cerebrosides and
sphingomyelin.   Johnson et al. (24) subsequently reported
that these three lipids distinguish the white matter of the
adult brain from that of the brain of the new born infant
where myelination is incomplete.   The concentration of these
lipids is also found to decrease in peripheral nerves during
Wallerian degeneration (25).   Folch-Pi (26) showed that
during development of the nervous system the concentration
of the various phospholipids increases with age until adult-
hood.   Lecithin and phosphatidyl serine increase gradually
in the order to be expected from the gradual increment with
age in the percentage of total solids in nervous tissue.

However sphingomyelin, plasmalogen and probably phosphatidyl ethanolamine increase at a much higher rate than expected. Therefore it has been suggested that these three phospholipids and possibly inositol phosphatide (27) are among the principal components of the myelin sheath.

The intracellular localisation of brain phospholipids has also been determined (28). When rat brain homogenate is submitted to centrifugal fractionation in isotonic sucrose, phospholipids are found in each of the subcellular fractions. Lecithin, cephalin and sphingomyelin constitute the major lipids of the nuclear, mitochondrial and microsomal fractions, whereas their concentration is considerably lower in the supernatant fluid. Quantitatively, lecithin is the most prominent phospholipid in each of the cell fractions.

2. ROLE AND FUNCTION OF PHOSPHOLIPIDS IN BRAIN

The demonstration that phospholipids are widespread in brain has aroused considerable speculation on the possible role and metabolic function of these substances in brain metabolism. It has long been thought that phospholipids are essentially structural components of the cell. However, contrary to older ideas of their metabolic inertness, there is at present a general inclination to the view that other

possible functions of the phospholipids may exist. This has led Wittcoff (7) to postulate that "the concept of a structural function of the phospholipid is acceptable only when it is understood that a phospholipid molecule which has a structural function at one moment may have a metabolic function the next."

a)   Phospholipids and oxidative metabolism

The enzymes catalysing the oxidation of the tricarboxylic acid cycle intermediates as well as those responsible for oxidative phosphorylation are known to be located in the intracellular mitochondrial particles which are particularly rich in phospholipids (20). Braganca and Quastel (29) showed that when small quantities of venom lecithinase are added to a brain homogenate, a number of mitochondrial enzymes (e.g. pyruvic oxidase, succinic oxidase, cytochrome oxidase) are inhibited, whereas the enzymes responsible for glycolysis in brain or for fermentation in yeast are not affected. Subsequently Petrushka et al. (30, 31) observed that when rat brain cortex slices, but not rat kidney or liver slices, are incubated with heated snake venom, an initial rise in the rate of oxygen uptake results, followed by a sharp decline if the action of phospholipase A is prolonged. This result implies that brain cell membranes contain phospholipid groups that may be attacked by phospholipase A and that the metabolic activities associated with

the membranes are thus affected. Essentially identical results are obtained with mitochondria of brain, liver and kidney where decreases in the P/O ratio are also observed (31). It was therefore concluded by the authors that the destruction of phospholipid groups in the mitochondrial structure by phospholipase A results in a complete rupture of the mitochondria, releasing protein and demonstrating a concomitant fall in the rate of oxidative phosphorylation. Other reports (32, 33, 34) that lipids, including in large part phospholipids, are associated with the electron transport system, substantiate this hypothesis and make it very probable that phospholipids are not only essential as structural elements of the cell but are also important for the maintenance of normal oxidative metabolism (35).

b) Phospholipids and functional activity

Although glucose has always been considered to be the main source of energy to satisfy the brain requirements during both resting and activity conditions (20), the evidence obtained by Geiger and his colleagues with brain perfusion experiments suggest that phospholipid may play an important role in maintaining functional activity in brain.

Geiger et al. (36) have shown that in brain, a number of physiological functions including respiration can be maintained for over one hour in the complete absence of glucose during perfusion experiments. The electrical

activity, oxygen consumption and endogenous carbohydrate stores are only slightly below those obtained when glucose is present in the perfusing fluid. In subsequent findings, Abood and Geiger (34) reported that during glucose-free perfusion of cat cerebral cortex, there is an appreciable loss of phospholipids, whereas no change occurs in the presence of glucose. Geiger et al. (38) also observed that when the brain cortex of narcotized cats is stimulated by means of the brachial plexus, a decrease in the concentration of lipid nitrogen occurs which is proportional to the magnitude of the stimulation. These results led Geiger to postulate that the breakdown of phospholipids is occurring in order to provide endogenous material for oxidative and glycolytic metabolism in the absence of exogenous substrates, and that lipid metabolism is involved in the process of activity evoked by stimulation (39, 40).

c)   Phospholipids and ion transport

There are a number of observations which suggest that phospholipids may play some role in ion transport in cerebral tissues. Large amounts of sodium and potassium are found associated with the lecithin, cephalin and sulphatides isolated from brain (41, 42). Moreover the cations Na^+, K^+, Ca^{++} and Mg^{++} compete with each other and can be reversibly displaced from combination with phospholipids (43). This property of phospholipids to form

lipoid-soluble complexes with cations has prompted the suggestion that they may be involved as cation carriers at the nerve cell membrane (44, 45, 46).

Hokin and Hokin in a series of experiments on the effect of cholinergic drugs on the rate of turnover of phospholipids in vitro have shown that acetylcholine stimulates the turnover of phospholipids, in particular phosphoinositide and phosphatidic acid, in slices of guinea pig cerebral cortex (44, 17). A similar phenomenon was observed in slices of adrenal medula (48) and in slices of various glandular tissues on stimulation of secretion (49, 50). It was suggested that in glandular tissues the increment in the exchange of phosphate in phosphatidic acid and in inositol and phosphate in phosphoinositide is associated with secretory activity and with the transport of the secretory products out of the cell. In subsequent experiments with the avian salt gland, Hokin and Hokin (51) found that acetylcholine, which is the normal secretogogue of this tissue, stimulates the incorporation of P^{32} into phosphatidic acid and phosphoinositide. Since the effect observed occurs in the microsomal fraction where diglyceride kinase and phosphatidic acid phosphatase are present in high concentration, a hypothetical phosphatidic acid cycle was suggested by the authors in which phosphatidic acid acts as a carrier for the active transport of sodium ions out of the salt-secreting cell (51).

Hokin and Hokin (52), working with slices of sympathetic ganglia, nerves and various parts of the cat and guinea pig brain, recently showed that acetylcholine stimulates the incorporation of P^{32} into phosphatidic acid and phosphoinositide in those areas of the brain which contain cholinergic synapses. It had been previously postulated by these authors, in view of the similarity of the effect observed in adrenal medula and different exocrine and endocrine glands, that the phospholipid effect in brain is concerned with the secretion of organic molecules by acetylcholine (53) and thus with the transport of these molecules (54). However, no evidence has yet been given for such secretion in those areas of the brain where the phospholipid effect is observed, although this hypothesis is not precluded (52). On the other hand, acetylcholine, which is the synaptic transmitter in sympathetic ganglia, has been shown to cause depolarization of the post-synaptic membrane by an increase of membrane permeability to sodium and potassium ions (55). The process consists of an inward movement of sodium across the membrane with an outward movement of potassium during repolarization, and restoration of the original membrane impermeability to sodium. This is followed by the active extrusion of sodium from the cell and by an inward movement of potassium. In view of this property of acetylcholine and by analogy with the salt gland results,

Hokin and Hokin (52) have suggested that the phospholipid
effect in brain is concerned with the active transport of
sodium ions out of the cell across the post-synaptic membrane
of cholinergic neurons during the recovery process.

3.   BIOSYNTHESIS OF PHOSPHOLIPIDS IN BRAIN

        In recent years, considerable progress has been
made in working out the network of enzymatic reactions
leading to the biosynthesis of phospholipids from simple
precursors.   Largely as a result of the work of Kennedy
(4), a rather complete picture of the metabolic pathways
whereby phospholipids are formed has been developed and is
shown in Figure 1.   These reactions were worked out for
the most part using liver enzymes.   However, Rossiter and
his colleagues (6) have obtained evidence that the biosynthesis
of phospholipids in brain tissue follows the same pattern.

a)   Glycerophosphatides

        Formation of phosphatidic acid.   Early experiments
with brain mitochondria (56) and brain homogenates (56, 57)
incubated with inorganic P^{32}, suggested that phosphatidic
acid is an intermediate in the synthesis of glycerophosphatides.
Thus a large percentage of the radioactivity was recovered
from a fraction identified as   phosphatidic acid. McMurray
et al. (56) also reported that the incorporation of P^{32}
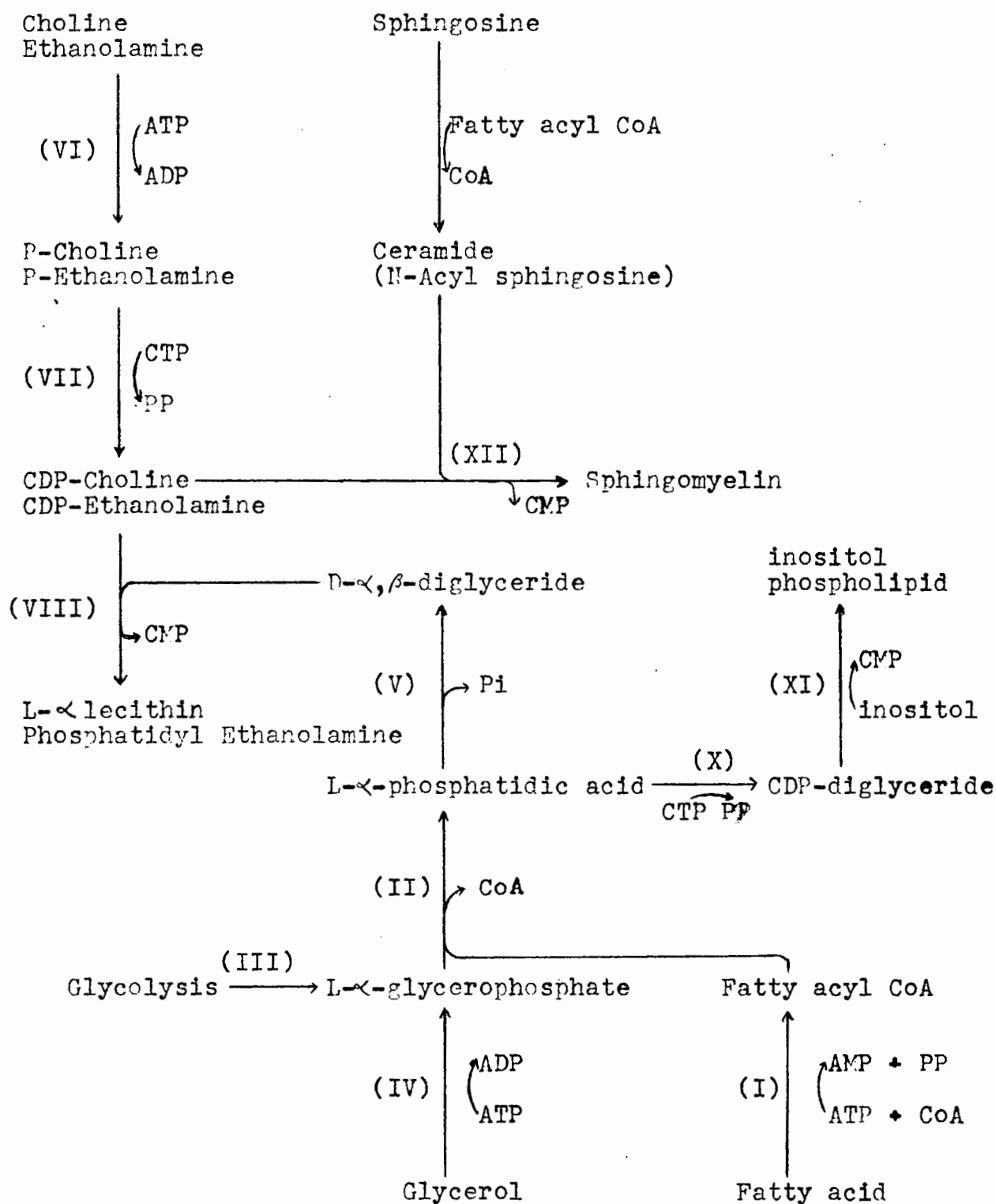
Figure 1.   BIOSYNTHESIS   OF   PHOSPHOLIPIDS
(After Kennedy (3) and Rossiter and Strickland (6))
Figures in parentheses refer to reactions in text

labelled α-glycerophosphate (α-GP) into the phospholipids
of a brain preparation occurs mainly into phosphatidic acid.
The addition of coenzyme A stimulates this incorporation
whereas conditions which prevent the production of ATP
inhibit the labelling of the phosphatidic acid.   These
findings prompted the suggestion that phosphatidic acid
is synthesized in brain from α-glycerophosphate and
coenzyme A thiol esters of fatty acid as previously
described in liver by Kornberg and Pricer (58, 59).

$$\text{Fatty acid} + \text{ATP} + \text{CoA} \rightleftharpoons \text{acyl-CoA} + \text{AMP} + \text{P-P} \qquad \text{(I)}$$
$$\text{L-}\alpha\text{-GP} + 2 \text{ fatty acyl-CoA} \longrightarrow \text{L-}\alpha\text{-phosphatidic acid} + 2\text{CoA} \quad \text{(II)}$$
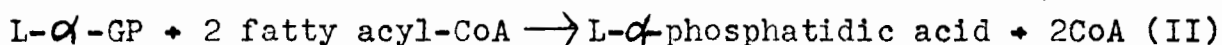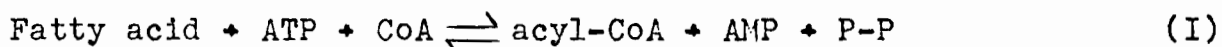
Additional evidence for reaction II has been
provided by Jedeikin and Weinhouse (60) who found that
palmitate-1-C^{14} is incorporated into the phospholipids of
both slices and homogenates of rat brain.   The homogenate
system requires the addition of CoA for optimal activity.

A second pathway for the biosynthesis of phospha-
tidic acid appears to be operative in brain.   Hokin and
Hokin (61) have reported that phosphatidic acid can also be
formed from α,β-diglyceride and ATP, presumably by the
action of a diglyceride kinase.   These authors found that
the incorporation of P^{32} from ATP^{32} into phosphatidic acid
of brain microsomes is highly dependent upon the presence of
α,β-diglyceride, a phenomenon very similar to the one pre-
viously observed by Strickland (62).

The observation that α-glycerophosphate is a precursor of phosphatidic acid has caused speculation on its pathway of formation in brain.   It is well established that α-glycerophosphate can arise from dihydroxyacetone phosphate formed in glycolysis (reaction III).   The enzyme glycerophosphate dehydrogenase, which catalyses the reduction of dihydroxyacetone phosphate to α-glycerophosphate is present in brain tissue (63, 64).   On the other hand, Bublitz and Kennedy (65) have isolated from rat liver a glycerokinase which catalyses the transfer of phosphate from ATP to glycerol (66) with the formation of α-glycerophosphate (reaction IV).   Whether this enzyme is present in brain is still unknown.   The only indication that this reaction may be operative in brain arises from the finding that glycerol-1-C^{14} is incorporated into the phospholipids of rat brain slices (67, 68).

Dephosphorylation of phosphatidic acid.   Following the observation that the radioactivity of α-GP^{32} is incorporated into phosphatidic acid but not into the glycerophosphatides (56), Rossiter and Strickland (69) have shown the existence of a phosphatidic acid phosphatase in brain. This enzyme removes the phosphate moiety of phosphatidic acid to form α, β-diglyceride a precursor of glycerophosphatides (70):

$$\text{L-}\alpha\text{-phosphatidic acid} \longrightarrow \text{D-}\alpha,\beta\text{-diglyceride} + Pi \qquad (V)$$
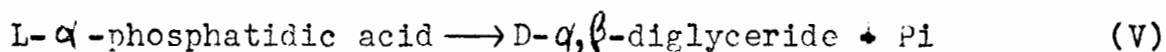
This enzyme is therefore responsible for the lack of labelling of glycerophosphatides from α-GP^{32} by causing the liberation of radioactive Pi, a phenomenon not observed when C^{14} labelled α-glycerophosphate is used (69).

Formation of lecithin.   In 1958, Berry et al. (71) found that extracts of acetone-dried powders from brain and peripheral nerves contain an enzyme similar to the one described by Wittenberg and Kornberg (72), which is capable of catalysing the phosphorylation of choline from ATP:

Choline + ATP $\longrightarrow$ ADP + phosphorylcholine   (VI)
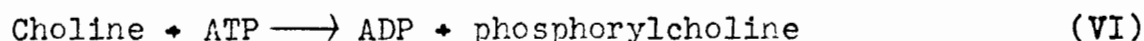
This evidence that phosphorylcholine could be formed in brain led to the suggestion that lecithin is synthesised in brain via reaction VII and VIII as observed in liver tissue (70):

Phosphorylcholine + CTP $\longleftrightarrow$ CDP-choline + P-P   (VII)

CDP-choline + α,β-diglyceride $\longleftrightarrow$ L-α-lecithin + CMP (VIII)
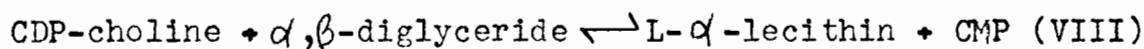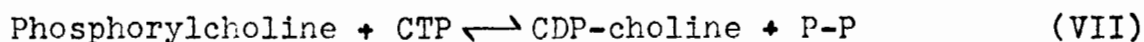
Thus McMurray et al. (56) and Rossiter and Strickland (69) showed that P^{32} or C^{14} labelled phosphorylcholine is incorporated into α-lecithin and that the incorporation is highly dependent upon the presence of CTP.   Furthermore, P^{32} or C^{14} labelled CDP-choline is also incorporated into the α-lecithin fraction of brain preparation and the incorporation is stimulated by the addition of α,β-diglyceride (69).   It was therefore concluded that the presence in brain of

phosphorylcholine-citidyl and phosphorylcholine-glyceride
transferases is responsible for the effect observed and that
both reactions VII and VIII are operative in brain.

Phosphatidyl ethanolamine and phosphatidyl serine.
The pathway for the biological synthesis of phosphatidyl
ethanolamine appears to be analogous to that described for
α-lecithin.   Thus liver and brain contain enzymes which are
responsible for the formation of phosphoryl ethanolamine
(73, 74), CDP-ethanolamine (70) and phosphatidyl ethanolamine
(69, 70).   On the other hand, virtually nothing is known
at present concerning the synthesis of phosphatidyl serine,
either in brain or in other tissues.   No evidence has yet
been presented for the occurrence of phosphatidyl serine
intermediates similar to those described above for α-lecithin
and phosphatidyl ethanolamine.   It would therefore appear
that a different sequence of reactions is involved in the
synthesis of phosphatidyl serine.

Plasmalogen.   The enzymatic synthesis of plasmalogen
involves very similar reactions to those of the conventional
glycerophosphatides.   Gambal and Monty (75) have demonstrated
that a cell-free preparation from rat brain can incorporate
palmitate-1-C^{14} into the aldehydogenic moiety of the
plasmalogen;   the system requires CoA, ATP, Mg^{++}, α-glycero-
phosphate, CTP and ethanolamine, all of which are well known
co-factors necessary for the synthesis of phosphatidyl

ethanolamine. Evidence has also been obtained by Kiyasu
et al. (76, 77) suggesting the presence in liver of phosphoryl-
choline and phosphorylethanolamine plasmalogenic glyceride
transferases catalysing the following reactions:

CDP-choline
$\qquad$ + "Plasmalogenic diglyceride" $\rightleftharpoons$
CDP-ethanolamine

"Plasmalogenic phosphatidyl choline"
+ CMP (IX)
"Plasmalogenic phosphatidyl ethanolamine"
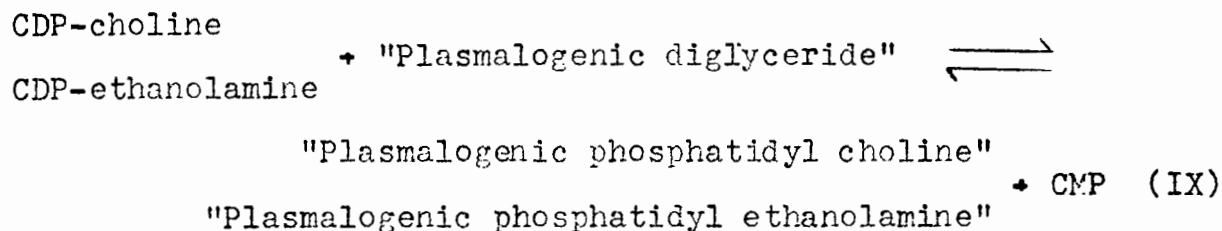
Thus, these authors showed that when "plasmalogenic diglyceride"
is incubated with rat liver particles and CDP-choline or
CDP-ethanolamine, plasmalogen is formed (77).

b)   Inositol phospholipid

The failure to show the phosphorylation of inositol
by tissue extracts in the presence of ATP (78), and the
observation by Dawson (57) and others (56, 67, 79) that the
incorporation of P^{32} into brain phosphoinositide and phospha-
tidic acid is greater than that into the other phospholipids,
prompted the suggestion that inositol phospholipid is
synthesized by a pathway different from that postulated for
the classical glycerophosphatides.

Studies by Agranoff et al. (80) indicated that
phosphatidyl inositol is formed by the reaction of a
diglyceride derivative with free inositol. The incorporation
of tritium-labelled inositol into the inositol phospholipid

of guinea pig kidney preparations is stimulated by the presence of cytidine nucleotides, particularly CDP-choline, as well as by the addition of phosphatidic acid but not by the addition of D-α,β-diglyceride. When tritium-labelled CMP was incubated in this cell-free particulate preparation, in the absence of inositol, a chloroform-methanol soluble radioactive product is formed which yielded CMP on alkaline hydrolysis. It was therefore postulated that a lipid-soluble cytidine nucleotide compound, probably CDP-diglyceride, is involved in this reaction and acts as an intermediate in the synthesis of inositol phospholipid (80).

Paulus and Kennedy (81, 82, 83), in a series of experiments with liver tissues, observed that there are two possible mechanisms by which inositol may be incorporated into phospholipid molecule. The first is by an enzymatic exchange reaction with inositol phospholipid present in the enzyme preparation. The reaction is stimulated by the presence of cytidine nucleotides which Agranoff et al. mentioned earlier (80). The second mechanism is by a true de novo synthesis of inositol phospholipid stimulated by CTP. The authors showed that their system catalyses the conversion of α-glycerophosphate and phosphatidic acid to phosphatidyl inositol in the presence of free inositol and CTP but not in the presence of CDP-choline or other nucleotide triphosphates. When α-glycerophosphate and CTP are incubated in the absence

of inositol, an ether-soluble compound accumulates which appears to be identical with CDP-diglyceride. Finally, Paulus and Kennedy demonstrated the synthesis of inositol phospholipid from synthetic CDP-diglyceride and inositol. The following reactions were therefore proposed for the synthesis of inositol phospholipid (83):

Phosphatidic acid + CTP $\longrightarrow$ CDP-diglyceride + P-P   (X)

CDP-diglyceride + Inositol $\longrightarrow$ Inositol phospholipid + CMP (XI)
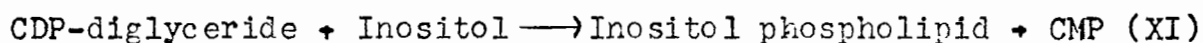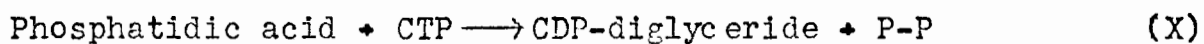
These reactions are analogous to those proposed by Agranoff et al. (80) except that Paulus and Kennedy consider that CTP, rather than CDP-choline is involved in the formation of CDP-diglyceride.

Evidence has been obtained by Rossiter and his colleagues (6) to suggest that a similar pathway is operative in brain. McMurray et al. (56) found that CTP stimulates the incorporation of inorganic P^{32} into the inositol phospholipid of brain glycolyzing homogenate and phosphorylating mitochondria. Thompson et al. (84) showed that CTP and CDP are more effective than CMP and CDP-choline in stimulating the incorporation of tritium-labelled inositol into the lipids of such systems and that phosphatidic acid increases the incorporation in the glycolyzing homogenate. The C^{14} label from the glycerol moiety of C^{14} phosphatidic acid is also found to be incorporated into brain inositol

phospholipid and the incorporation is stimulated by the addition of CTP (85).

Thus phosphatidic acid has been shown to participate directly in the formation of inositol phospholipid without prior dephosphorylation by phosphatidic acid phosphatase to diglyceride and inorganic phosphate. This observation helps to explain the similarity of behaviour of inositol phospholipid and phosphatidic acid in turnover studies (67).

c) Sphingomyelin

The isolation of phosphorylcholine and sphingosine phosphate as hydrolysis products of sphingomyelin has helped to establish that the choline moiety is attached through a phosphate ester bond to one of the hydroxyl groups of sphingomyelin (86). The similarity of this phosphorylcholine moiety with the one found in lecithin prompted Sribney and Kennedy (87) to propose that sphingomyelin is synthesized enzymatically by an analogous reaction to that of lecithin.

Sribney and Kennedy (87, 88) found that an enzyme, phosphorylcholine ceramide transferase, which is present in various tissues including brain homogenate, catalyses the formation of sphingomyelin by transferring phosphorylcholine from CDP-choline to the hydroxyl group of a ceramide (N-acyl sphingosine)

$$\text{CDP-choline} + \text{ceramide} \rightleftharpoons \text{sphingomyelin} + \text{CMP} \qquad \text{(XII)}$$
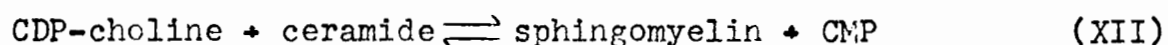
This reaction is similar to the phosphorylcholine glyceride transferase (see reaction VIII), except that ceramide rather than α,β-diglyceride is the acceptor of the phosphorylcholine moiety, and that manganese ions instead of magnesium ions are required for optimal activity of the enzyme. Rossiter et al. (89, 90) have also obtained evidence that CDP^{32}-choline is incorporated into the spingomyelin fraction of rat brain homogenate; thus suggesting that the labelling occurs via the above mechanism.

Brady and his colleagues (91, 92) and Zabin (93) have shown that cell-free preparations from brain can catalyse the formation of sphingosine and ceramide the immediate precursors of sphingomyelin. According to Sribney, and Kennedy (88) the phosphorylcholine acceptor, ceramide, in order to be active, must possess a sphingosine moiety that has the threo (N-acyl threo sphingosine) and not the erythro configuration.

As a result of the elucidation of the various phospholipid pathways mentioned above, two striking points of interest have arisen worth emphasising in that they are common to each pathway (see Figure 1). It is a characteristic of all phospholipid syntheses to utilize CTP at one stage or another of the biosynthesis. Moreover, ATP plays a central role in the formation of these compounds by controlling the rates of synthesis of the different intermediates leading to

phospholipid formation, either directly by means of the
kinase reactions or indirectly by activation of fatty acids
or regeneration of CTP.   It is therefore evident that any
factors in brain which influence the energy supply may affect
phospholipid synthesis in a parallel manner.

4.   LABELLING OF PHOSPHOLIPID PHOSPHORUS

Early workers, concerned with the elucidation of
phospholipid metabolism in brain, made extensive use of
radioactive isotopes.   One of the most fruitful of these
substances has been radioactive phosphorus with which much
of the behaviour of phospholipids in body tissues has been
determined.

Incorporation of P^{32} in vivo.   Hahn and Hevesy
(94) observed that one hour after subcutaneous injection,
P^{32} is found in the brains of adult rats, mice and rabbits.
However, despite the rapid appearance of the isotope, the
maximum amount of P^{32} is not deposited in the brain phospho-
lipid until 200 to 300 hours after administration, and its
loss from these compounds is found to be very slow (95, 96).
When this incorporation in the brain is compared with that
found in other organs, it proves to be much lower (97, 98).
However, if the inorganic P^{32} is injected into the cerebro-
spinal fluid, the isotope uptake in the brain is more rapid

(99, 100) suggesting therefore that the slow rate of incorporation of P^{32} in vivo is attributed to the properties of the blood-brain barrier which controls its rate of penetration (101).

Incorporation of P^{32} in vitro.   The in vitro incorporation of radioactive inorganic phosphate into the phospholipids of brain was first observed by Chaikoff and his colleagues (102, 103).   These workers showed that slices of rat brain, respiring in a physiological medium, incorporate P^{32} into the phospholipids.   Addition of hexoses such as glucose, galactose, mannose or fructose stimulates the incorporation as much as five-fold.   Under anaerobic conditions, the incorporation of the isotope is negligible. Strickland (104) confirmed these observations and showed that the presence of pyruvate and lactate increases the incorporation of P^{32} whereas with glutamate, succinate and certain others tricarboxylic acid cycle intermediates this effect is not observed.   Strickland also found that a wide range of metabolic inhibitors, in concentrations that inhibit the oxygen uptake of brain slices, also inhibit the incorporation of P^{32}.   This author therefore suggested that the incorporation of P^{32} is "a metabolic phenomenon and is dependent upon the maintenance of an adequate phosphorylating mechanism within the slices" (104).   Schachner et al. (103) and Strickland (104) observed that if the brain tissue is

homogenized before incubation, the incorporation of P^{32} into phospholipid is reduced to very low levels. However, if the homogenates are suitably "reinforced" in order to have normal respiratory activity, good labelling is obtained (105, 106, 107). When the various phospholipids of brain homogenates are separated by chromatography, the highest specific activity is found in the phosphatidic acid and inositol phospholipid fractions (57). Hokin and Hokin (67) and Magee et al. (108) have also reported similar findings with brain slices.

ATP^{32} as a prerequisite in the labelling of the phospholipid phosphorus. The labelling of ATP from inorganic P^{32} either by glycolytic or oxidative phosphorylation, appears to be the first step in the labelling of the immediate precursors of phospholipids (3). As a result of the experiments performed by McMurray et al. (56, 107) and by Berry and McMurray (109), evidence has been obtained showing that the labelling of the lipid phosphorus is closely associated with ATP. These workers observed that in brain preparations which demonstrate either glycolytic or oxidative phosphorylation, the well known metabolic inhibitors iodoacetate or 2-4 dinitrophenol decrease the incorporation of P^{32} into phospholipids and in a parallel manner the concentration and the labelling of ATP. Moreover, ATP^{32} was shown to be, on a relative basis, more

readily incorporated into the phospholipids than inorganic P^{32} (56). In view of the well established requirement of ATP for the biological synthesis of phospholipid, it was therefore concluded that ATP plays a dual role in the labelling of these compounds in providing both the labelled phosphorus and the energy supply (56).

5. FACTORS AFFECTING METABOLIC PROCESSES IN THE BRAIN

a) Effect of potassium and calcium ions

It is well known that brain cortex contains on the average 100 meq. of potassium per kilogram of tissue. Terner, Eggleston and Krebs (110) found that when slices of brain cortex are incubated in a normal saline medium, under anaerobic conditions, there is a loss of potassium ions by diffusion into the medium and a gain of sodium ions. Under aerobic conditions, when glucose was provided as substrate and in the presence of L-glutamate, this phenomenon could be reversed; after an initial loss, brain slices accumulate potassium ions as well as L-glutamate against the concentration gradient (110, 111, 112). Takagaki et al. (113) confirmed these observations and reported that the ionic composition of the incubation medium greatly influences the uptake of both potassium and glutamate. Thus the content of potassium ions in slices decreases during incubation even in the

presence of glucose and L-glutamate when the incubation medium is potassium-free. However, the potassium content in the slices is very much increased and the addition of glutamate further accelerates this accumulation when the incubation is carried out in a high potassium medium. The presence of sodium ions in the medium is indispensable for potassium and glutamate accumulation. In the absence of calcium ions, the potassium content of brain slices slightly decreases on incubation.

Ashford and Dixon (114) were the first to show that the addition of a high concentration of potassium chloride (100 mM) to the ordinary incubation medium enhances the oxygen uptake of rabbit brain cortex slices. This effect was confirmed by Dickens and Greville (115) who reported a 50 - 100% increase in the respiration of rat brain cortex slices incubated with either glucose, fructose, lactate or pyruvate and high potassium concentration, whereas no increase is observed in the absence of substrate. Both groups of investigators also noted an increase in the aerobic glycolysis and a decrease in the anaerobic glycolysis. The latter authors and subsequently Canzanelli et al. (116) observed that lowering the calcium concentration has the same effect as an increase in potassium, suggesting that calcium ions are antagonistic to the stimulatory effect of potassium ions. These workers also reported that the

stimulatory effect is not obtained if the potassium is
increased at the expense of sodium in the medium.   On the
other hand, Dixon (117) observed that the potassium effect
can be obtained in isotonic medium if a lower potassium
concentration (40 mM) is used so that the sodium concentration
can be kept relatively high.   Lipsett and Crescitelli (118),
in addition to confirming the increase in respiration, found
that the stimulation of respiration is inhibited by the
addition of glutamate, succinate, citrate or α-ketoglutarate.
These authors suggested that potassium may increase the
oxygen consumption of brain slices by stimulating the
phosphoenolpyruvate-adenosinediphosphate transphosphorylase
reaction.

Kimura and Niwa (119) showed that malonate, at a
concentration which is slightly inhibitory to normal
respiration in the presence of glucose, completely inhibits
the potassium stimulated respiration.   Subsequently, Tsukada
and Takagaki (120) and Parmar and Quastel (121), while
confirming this result, observed that the malonate inhibition
of the potassium-stimulated respiration can be reversed by
the addition of fumarate or oxalacetate.   These observations
indicate that the presence of excess potassium ions in the
medium activates the citric acid cycle of operations in
brain respiration or some rate limiting step associated
with it (35).

The phenomenon that the potassium-stimulated brain cortex respiration is of the same order as that occurring in normal brain _in vivo_, prompted the suggestion that a metabolic and physiological parallel exists between the stimulated tissue and the brain _in vivo_. McIlwain (122) and Ghosh and Quastel (123) found that narcotics and anaesthetics, at pharmacologically active concentrations, suppress the respiration of the stimulated tissue, whereas that of unstimulated tissue is not affected and presumably represents conditions more remote from those prevailing in living intact brain.

In addition to the phenomena mentioned above on respiration and glycolysis, several other metabolic processes are influenced by the change of potassium and calcium concentration in the medium. Quastel and his colleagues (124) reported that acetylcholine synthesis by brain slices is increased about ten-fold by raising the potassium concentration of the medium from 4 mM to 30 mM, and that the addition of calcium ions prevents this increase. The effect of the potassium salt was seen primarily in brain cortex slices and not in finely minced tissue which could nevertheless synthesize acetylcholine (124). McLennan and Elliott (125) observed that for maximal synthesis of acetylcholine by brain slices, the presence of 1.3 mM calcium is necessary and that higher or lower concentrations inhibit the synthesis.

McIlwain (126, 127) showed that either the omission of calcium ions or the increase in the concentration of potassium ions, causes a decrease in the ability of the slices to maintain their level of phosphocreatine, as well as occasionally producing an increase in inorganic phosphate. It has also been reported that under these conditions, the amount of ADP is higher (128) whereas the concentration of ATP is only slightly affected (129, 131). Findlay et al. (130) found that an increase of extracellular potassium or ammonium ions or a decrease of calcium ions inhibits the incorporation of inorganic P^{32} into phosphorus containing compounds such as phospholipid, ribonucleic acid and phospho-protein. Rossiter (131) subsequently reported that under these conditions the incorporation of P^{32} into ATP decreases to the same relative extent as the labelling of the phospho-lipid. Pritchard (132) also observed that the incorporation of C^{14} labelled precursors, such as acetate, glycerol and choline, into rat brain phospholipids is decreased by the addition of a high concentration of potassium. It was therefore suggested that the lower specific activity of the organic phosphorus compounds is possibly the result of the effect of potassium ions on the concentration of phospho-creatine (132). On the other hand, in contrast to the above observations, Tsukada et al. (133) found that the incorporation of P^{32} into phosphoproteins of guinea pig

brain slices is increased markedly upon addition of 0.1 M potassium. When these experiments are carried out in an isotonic medium where most of the sodium ions are omitted, this increase in P^{32} incorporation is not observed. These authors concluded that the augmentation of glucose metabolism in the presence of potassium is closely coupled to the phosphorylation mechanism, especially as regards the turn-over of phosphoprotein within the slices. Yoshida and Nukada (134) recently reported similar results with phospho-lipids showing that the greatest increase in P^{32} incorporation caused by potassium occurs in the phosphatidic acid fraction.

An increased concentration of potassium or omission of calcium inhibits glycogen synthesis in brain cortex slices (135). Vrba et al. (136) studied the effect of potassium ions on ammonia formation in brain slices using no substrate in the incubating media, and found that potassium at higher concentration brings about a decrease in the level of ammonia. Rybova (139) subsequently reported that in the presence of glucose as substrate, the action of potassium ions is of a different character; at moderately increased potassium concentrations, there is a decrease in ammonium accumulation, whereas the ammonia level is raised on further increase in potassium concentration. In a calcium-free medium, the release of ammonia is greatly increased.

Kini and Quastel (138, 139) have found that the

addition of potassium or the omission of calcium in the
medium brings about a large increase in the labelling of
glutamine and y-aminobutyric acid from glucose uniformly
labelled with carbon-14.   The stimulation of C^{14} incor-
poration may be caused by an acceleration of the citric acid
cycle resulting in an increased turnover of α-ketoglutarate,
a well known precursor of these two amino-acids (138).
Kini and Quastel also observed that the presence of 105 mM
potassium greatly increases the rate of oxidation in brain
cortex of both pyruvate-1-C^{14} and pyruvate-2-C^{14} to carbon-14
dioxide, the latter process being much more inhibited by the
presence of malonate than the former.   These results led
to the conclusion "that the stimulating effect of addition
of potassium ions on the respiration of brain cortex is
largely directed to an acceleration of a pace-making step,
the conversion of pyruvate to acetyl-coenzyme A" (138).

A very close parallel exists between the metabolism
of brain slices in the presence of 0.1 M potassium chloride
and brain tissue stimulated by electrical impulses.
McIlwain and his group have reported an increase in both
oxygen consumption (140) and aerobic glycolysis (141) when
electrical impulses are applied to isolated brain tissue.
This effect is observed when glucose, lactate or pyruvate
are present as substrate, but not with succinate or α-keto-
glutarate.   Under these conditions, the phosphocreatine

level is decreased (142), ATP concentration is not very much
affected (129), the specific radioactivity of phospholipid
(143), phosphocreatine and ATP (144) is decreased whereas
that of the phosphoprotein phosphorus (145) is increased.
The electrical stimulation is highly sensitive to malonate
(146) and narcotics (122) and also causes an increase in the
rate of acetylcholine synthesis in brain slices (147).

The effects produced by cations and electrical
impulses on cerebral tissue metabolism have their parallel
in some muscular tissues but not in non-excitable tissues
such as liver, kidney or spleen (20). As a result of the
evidence obtained that cationic or electrical stimulation
does not occur in brain homogenate or minced tissue (20,
148), it has been suggested that this effect is linked with
the structural integrity of the cell membranes. Changes in
cation concentration or application of electrical impulses
would cause ionic displacements at the nerve cell membrane
resulting in depolarization and metabolic alterations (20, 35).

b)   Effect of acetylcholine

Since the demonstration by Quastel et al. (149)
that acetylcholine synthesis occurs in brain cortex slices
under physiological conditions, much has been written about
the function of this substance in brain. It is well known
that acetylcholine acts as a central synaptic transmitter (150)
and that it may perhaps be involved in conduction of the

nerve impulses along the axon (151). In addition, Hokin
and Hokin have observed a very striking effect of acetyl-
choline on phospholipid metabolism which may further
elucidate the biochemical role of this amine in brain.

These authors, whilst investigating the effect
of cholinergic drugs on the secretion of amylase in
respiring pancreas slices in vitro, found that the stimu-
lation of amylase secretion by acetylcholine is accompanied
by a large increase in the specific activity of the phospho-
lipid (152). Subsequently, an analogous phospholipid
effect was observed with slices of guinea pig cerebral
cortex (47). Thus acetylcholine in presence of eserine
stimulates the incorporation of P^{32} into the phospholipids
of brain cortex slices but has no effect on the oxygen
uptake or the specific activities of the acid-soluble
phosphate ester fractions (152). These results indicate
that the effect on phospholipids is not secondary to a
general increase in the rate of phosphorylation. It was
also reported by these workers that the level of phospholipid
in the stimulated tissue does not increase and that the in-
corporation of glycerol-1-C^{14} into the phospholipid is
slightly inhibited in the presence of acetylcholine, although
the usual stimulatory effect on P^{32} incorporation occurs (47).
As a result of these observations, Hokin and Hokin concluded
that the phospholipid effect in brain cortex slices represents

an increased turnover of phosphate in phospholipids rather than a de novo synthesis (47).

The stimulation of P^{32} incorporation by acetylcholine appears mainly in the phosphatidic acid and phosphoinositide and to a lesser extent in the phosphatidyl choline fractions when the different phospholipids of brain slices are separated by chromatography (67). Although a phospholipid effect is also observed in homogenates and various cytoplasmic particulate fractions of guinea pig brain under conditions of oxidative phosphorylation, in contrast with brain cortex slices, the stimulation by acetylcholine in the cell-free system occurs exclusively in phosphatidic acid (153). Moreover, in brain microsomal preparations, the labelling of phosphatidic acid from α-GP^{32} is not stimulated in response to acetylcholine, whereas the incorporation of ATP^{32} into phosphatidic acid, presumably by the action of a diglyceride kinase, is increased by acetylcholine (54).

In subsequent findings, Hokin and Hokin observed that the phospholipid effect occurs only in the nervous structures of the brain which contain cholinergic synapses. In view of the well known property of acetylcholine to cause depolarization of the post-synaptic membrane at cholinergic synapses, it has been suggested that the phospholipid stimulation is concerned with the transport of inorganic ions (52) (see p.10). However, the mechanism of the

acetylcholine phenomenon in brain is not yet clearly elucidated. Thus the stimulation of phosphate turnover in phosphoinositide and phosphatidyl choline has been shown to occur only in preparations such as tissue slices where the cell membrane is intact.   On the other hand, the increment of phosphatidic acid labelling in cell-free systems indicates that this turnover is not regulated by changes in the concentrations of ions on the intracellular side of the membrane. Hokin and Hokin have therefore expressed the opinion that at least in the case of phosphatidic acid, the stimulation of turnover might be brought about by a direct effect of acetylcholine, which by a common mechanism could cause depolarization and increase the turnover of phosphatidic acid (52).

c)   Effect of morphine

One of the characteristic properties of narcotics is to depress cerebral activity by inhibiting the formation of high energy phosphate bonds thereby affecting a variety of metabolic processes controlled by the presence and rate of production of ATP (35, 154).   Paradoxically, although morphine is classified as a narcotic and that its classical effect in vivo is to reduce the respiratory rate (155), the mode of action of this alkaloid on metabolic functions in vitro appears to be different from that of barbiturates and other groups of narcotics (154).

In 1932, Quastel and Wheatley (156) studied the
effect of morphine and other drugs upon oxidative processes
in nervous tissues, in an attempt to determine the mechanism
of narcosis.   These workers found that the increased oxygen
consumption of guinea pig brain mince in the presence of
lactate, pyruvate, and glutamate is inhibited by morphine
whereas no change occurs if succinate is used as a substrate.
Seevers and Shideman (157, 158) in an extensive investigation,
reported that morphine has no significant effect on the
endogenous oxygen uptake of cortex slices, mince or homogenate
or rat brain.   The oxidation of citrate, succinate, fumarate
and malate is unaffected by morphine, while the results
obtained vary when glucose or α-ketoglutarate are used as
substrate.   Elliott et al. (159) showed that morphine at a
concentration of 10 mM fails to depress the oxygen uptake of
rat brain cortex slices respiring in glucose whereas
meperidine strongly inhibits the glucose oxidation.   These
authors concluded that morphine does not affect the mechanisms
of biological oxidation in a manner similar to other narcotics
(159).   Brody (160) also found that morphine does not un-
couple oxidation from phosphorylation.

Achor and Geiling (161) have reported that morphine
inhibits the uptake of C^{14} into the brain of rats following
an intravenous injection of glucose-C^{14}.   This inhibitory
effect is partially antagonized by nalorphine, and the

results are interpreted as evidence that morphine depresses
the uptake of glucose into the brain. Accordingly,
Siminoff and Saunders (162) studied the effect of morphine,
in doses sufficient to depress respiration (20mg/Kg), upon
the glucose concentration in the brain of rabbits in vivo
and the glucose uptake by brain slices in vitro. There
are no significant differences in the results obtained with
normal and morphinized animals. When morphine is injected
into animals prior to incubation of their brain slices, the
stimulation of glucose uptake, oxygen consumption or oxidation
of glucose-C^{14} by the addition of a high potassium concentra-
tion is not affected. The latter observation is analogous
to the one reported by Franklin (163) for potassium
stimulated brain cortex slices incubated with 3 mM morphine.
Siminoff and Saunders concluded that "the depressent effect
of morphine in vivo cannot be explained on the basis of an
inhibition of the metabolism of glucose unless specific areas
of the brain respond differently from the brain as a whole"
(112).

Bell (164) investigated the effect of morphine
on oxygen consumption and lactic acid formation in normal
and electrically-stimulated rat cerebral cortex slices. In
contrast to the results obtained with potassium stimulated
tissue, this worker found that the increased metabolism
associated with electrical stimulation is practically

abolished by concentrations of morphine which have no effect
upon the unstimulated tissue.

In view of the almost complete inability of
morphine to influence oxidative processes in brain, the
observation that morphine affects cholinesterase and acetyl-
choline has aroused particular interest in order to elucidate
the mode of action of this substance in vitro. Bernheim
and Bernheim (165) found that morphine inhibits the
hydrolysis of acetylcholine by brain cholinesterases pre-
pared from rats, guinea pigs and cats. Eadie (166) confirmed
this finding and suggested that the inhibitor competes with
the substrate for cholinesterase instead of destroying the
the activity of the enzyme. Slaughter and his co-workers
(167) reported that morphine behaves like a cholinergic drug
in its action on the stomach and that its effects are enhanced
by prostigmine. Eserine was also found to potentiate the
effect of morphine on the intestine of the dog and blood
pressure in cat (168) while atropine antagonizes the analgesic
action of morphine (169). As a result of the work of Quastel
and Tennenbaum (170), evidence has been obtained showing that
morphine competes with acetylcholine for receptor groups in
leech muscle preparations, the action being reversible and
competitive. Lewis (171) has also observed that morphine
prevents the action of acetylcholine which causes contraction
of the guinea-pig ileum. Quastel has expressed the opinion

that morphine may act in the brain as a competitor to
acetylcholine for some receptor sites whose combination
with acetylcholine is necessary for normal function of the
neurones (154).

THE PURPOSE OF THIS INVESTIGATION

The use of P^{32} has contributed a great deal to the elucidation of the various pathways by which phospholipids are formed. It is now well established that the incorporation of P^{32} is closely linked to the mechanism of oxidative phosphorylation and evidence has been obtained suggesting that P^{32}, after being transported into the cell as inorganic phosphate, appears to be incorporated into ATP prior to its entry into the phosphorus moiety of the phospholipids.

The labelling of phospholipids from inorganic P^{32} made it possible to follow the behaviour of these compounds under normal in vitro conditions as well as their response to various external factors which alter the overall metabolism of the cell. These contributions supplied some valuable insight on the possible role and function of phospholipids in the cell metabolism.

The experiments to be described in this thesis were directed towards a study of the effects of various agents on the labelling of phospholipids from inorganic P^{32}. Investigations have been carried out to ascertain the stimulatory effect of cations, acetylcholine and morphine on the incorporation of P^{32} into the phospholipids of rat brain cortex slices as well as the factors which govern this stimulation. These investigations were carried out not only to determine the mechanism of action of these substances but also as a first approach to elucidate further the role and function of phospholipids in brain.

CHAPTER II

MATERIALS AND METHODS

Animals.   Hooded adult male rats were used in
all the experiments to be described.   The animals were
bred in this Institute and weighed from 150g. to 180g.

Morphine treatment.   Rats undergoing morphine
treatment were administered morphine sulphate by intra-
peritoneal injection twice daily for fifteen days.   On
the first day of treatment two doses of morphine, 20mg. per
Kg. body weight, were injected with an interval of ten hours
between injections.   The amount of morphine administered
in each dose was progressively increased to a maximum of
250mg. per Kg. body weight on the last day of the treatment.
Four or twenty-four hours after the last injection, the
animals were killed by decapitation and the brains were
removed.

Chemicals.   All common chemicals were of "Reagent"
grade purity and used without further purification.   The
following drugs, morphine sulphate, nalorphine hydrobromide,
atropine sulphate, acetylcholine chloride, eserine sulphate
and tofranil were manufactured and supplied by various
pharmaceutical companies.   Y-aminobutyric acid, and
tubocurarine chloride were obtained from the Nutritional
Biochemicals Corporation, Cleveland. P^{32} specified as

Sodium Radiophosphate solution in hydrochloric acid was supplied by Charles E. Frosst and Company, Montreal.

Norit activated charcoal was obtained from Fisher Scientific Ltd. It was found to be impure and to interfere in the colorimetric determination of phosphate. Purification was achieved by boiling the charcoal for 30 minutes in HCl, suspending it in pyridine for two hours and washing it with 2N HCl. Upon drying, blanks performed on the charcoal did not produce any coloration.

Purification of P^{32}. The commercial solution of P^{32} appeared to be contaminated by a slight amount of radioactivity other than orthophosphate. Originally when the radioactive P^{32} solution was used after boiling for one hour with N HCl to decompose any pyrophosphate present, the specific activity of both the phospholipids and the nucleotide phosphates was sometimes much greater than the calculated specific activity of the medium. Purification of the P^{32} solution was therefore carried out by the following procedure, assayed and suggested by Dr. R.M. Johnstone of this Institute.

After boiling for one hour in a glass container with N HCl, the P^{32} solution was cooled and neutralized with a few drops of ammonium hydroxide. 10 μmoles of Na_2HPO_4, 1 ml. of magnesia mixture, 1 ml. of ammonium hydroxide were added and the final volume made up to 10 ml. with distilled

water. The mixture was then left to precipitate at $5^{o}C$ overnight. On the following day, the residue was separated by centrifuging, washed three times with 5 ml. of 1:10 solution of ammonium hydroxide and water, and finally suspended in 4 ml. of distilled water. The suspension was then neutralized by adding 0.1N HCl drop by drop and with continuous stirring after each addition. When the solution was about neutral, it was centrifuged at 2000g for 15 minutes. A small sediment was left which was subsequently discarded. The main precaution was not to let the pH drop below 7 while dissolving the precipitate with 0.1N HCl. After this treatment, the supernatant was transferred to a clean test tube and diluted to give the desired radioactivity. Thus 2 - 3 ml. of the original P^{32} solution containing 1 mc. per ml. were processed.

Preparation of solutions. All solutions were prepared at appropriate concentrations in distilled water. Stock solutions of substrates and inhibitors were prepared at ten times the desired concentration, adjusted to pH 7.4 and stored at $5^{o}C$ for no longer than fifteen days. Solutions of glucose and acetylcholine were freshly made before each experiment. In some cases, attempts to neutralize concentrated solutions of drugs such as morphine, tofranil and others with sodium hydroxide caused precipitation. However, these drugs could be obtained in solution

at pH 6.0 - 6.8 at concentrations of 20mM to 100mM. When added to buffered incubation media, no precipitation of the drugs was observed and the pH remained constant.

Preparation of Rat Brain Cortex Slices. Rats were killed by decapitation. The brains were quickly removed and chilled in a beaker containing ice cold saline. Slices of rat brain cortex were cut from the surface of the cerebral hemispheres with a chilled Stadie-Riggs tissue slicer and placed in a petri dish standing in crushed ice. Usually one dorsal and one lateral slice from two different brains were pooled and used in each vessel. These two slices weighed together approximately 50 - 70 mg. Considerable caution had to be taken in preparing brain slices, since the maximum respiratory response to the addition of excess potassium chloride was critically dependent on the tissue thickness. Slices that were too thin disintegrated during the course of an experiment, while with thick slices little or no potassium effect was observed. Slices of two brains at a time were thus processed and quickly transferred to Warburg vessels containing ice cold incubation media. The interval between sacrificing the animals and commencing an incubation was approximately 40 minutes. The dry weight / wet weight ratio was established by drying corresponding amounts of tissue at 110°C for 4 hours.

Incubation Methods. The basic saline medium used
for the incubation was "modified" Krebs medium III (172, 152)
containing 135mM NaCl; 5.1mM KCl; 2.9mM $CaCl_2$; 1.3mM
KH_2PO_4; 1.3mM $MgSO_4$; 3.9mM $NaHCO_3$ saturated with carbon
dioxide; and 3mM sodium phosphate buffer, pH 7.4. In
experiments where 10mM tris (hydroxymethyl) aminomethane
buffer pH 7.4 was used instead of the sodium phosphate buffer,
the standard Krebs-Ringer medium (173) was employed. To
each vessel was added 20μc. of radioactive phosphate (P^{32}).
The final volume in the vessels was 3 ml. and all the con-
centrations in the text refer to final molarity in the medium.

The high potassium medium was made by tipping the
KCl solution from the side arm after the equilibrium period
at 37°C and without correction of the osmotic pressure.
When a sodium-free medium was employed, sodium ions were
replaced by choline to maintain the isotonicity of the
medium. To obtain a calcium-free medium, $CaCl_2$ was omitted
from the basic saline medium. Eserine sulphate was always
added with acetylcholine and these solutions were also tipped
from the side arm after thermal equilibrium. Experiments
were always done in duplicate.

Rat brain cortex slices were placed in the main
compartments of chilled Warburg vessels containing the above
incubation media, and studies of their respiratory activities
measured in the conventional Warburg apparatus at 37°C.

A small roll of filter paper was placed in the center well
of each vessel together with 0.2 ml. of 20 per cent potassium
hydroxide solution. The vessels were then attached to
the manometers, gassed for 5 minutes with pure oxygen, and
immersed in the bath. Ten minutes were allowed for
thermal equilibration and then the side arm contents were
added to the main compartment. After an additional 5
minutes, manometric measurements of the oxygen uptake were
started and carried on at 10 minute intervals during the
incubation period as indicated in the text. The results
were expressed as microlitres of oxygen consumed per mg.
dry weight of tissue at 37°C.

Incorporation of P^{32} into rat brain cortex phospholipids.

Extraction of phospholipids. The procedure
described below was based on the method of Hokin and Hokin
(174). At the end of the incubation period, the vessels
were removed from the manometers and placed in crushed
ice. 5 ml. of 10 per cent trichloroacetic acid (TCA)
were added immediately to the vessels and the whole content
transferred to centrifuge tubes. The tissues were washed
with 2 ml. of TCA and homogenized in 5 ml. of cold 10 per
cent TCA. After separating the insoluble fraction by
centrifuging, the residues were resuspended in cold 10
per cent TCA and again centrifuged; this step was repeated
once more. The residue was resuspended in 2 ml. of 95 per

cent ethanol. The ethanolic suspension was mixed with 2 ml.
of chloroform, the tubes tightly stoppered and stored over-
night at $5^{\circ}C$. On the following day, 5 ml. of cold 0.1N
HCl were added to the tubes, the mixture emulsified by
vigorously shaking for 2 minutes and centrifuged at 2000g
for 10 minutes. After centrifugation a disc of insoluble
protein separated the heavy chloroform layer, which con-
tained the phospholipids, from the top lighter layer which
consisted of the HCl plus the ethanol of the original
extraction mixture. Aliquots of the chloroform extract
were removed for determination of total counts per minute
and estimation of the total phospholipid phosphorus.

Estimation of total phospholipid phosphorus.
The total phospholipid phosphorus was estimated by digesting
a 1 ml. sample of the chloroform extract with 1 ml. of
10N H_2SO_4 in an oven at $140^{\circ}C$ overnight. On the following
day the solution was cooled and 1 - 2 drops of 30 per cent
hydrogen peroxide added. The solution was returned to
the oven for at least 3 hours to complete the combustion
and to decompose all the peroxide. 1 ml. of H_2O was then
added and a 0.1 ml. aliquot of this solution was assayed
for phosphorus by the method of Bartlett (177) (see page49)
without further addition of H_2SO_4.

Assay of radioactivity. Aliquots (usually 200μl)
of the chloroform extract were plated on aluminium discs

and the radioactivity was counted with a "Tracerlab" gas flow counter of 20 per cent efficiency.

Measurement of Nucleotide Phosphate Levels

The technique used in these experiments was devised by Dr. D. B. Ellis (175) of this Institute, by combining the charcoal method of Crane and Lipmann(176) for the separation of nucleotides from inorganic phosphate with the method of Bartlett (177) for the determination of phosphorus.

Extraction of the TCA soluble fraction. At the end of the incubation, the vessels were quickly chilled in ice, the slices removed with forceps, deposited in 8 ml. of ice cold salt solution, centrifuged, and the supernatant discarded. The slices were resuspended in 8 ml. of ice cold salt solution, and again separated by centrifuging. The supernatant was carefully removed and the insides of the tube dried up with paper tissues. The slices were homogenized in 5 ml. of cold 5 per cent TCA and the homogenate was then allowed to stand for 30 minutes in the cold to completely extract the acid soluble phosphate esters. All operations were performed rapidly at $2^{o}C$.

Separation of the nucleotides from inorganic phosphate. After centrifugation of the homogenate sus-

pension, the TCA supernatant was decanted into a centrifuge
tube containing approximately 250 mg. of purified charcoal
and mixed by stirring with a glass rod. To reduce the
amount of charcoal which floated, 0.3 ml. of 95 per cent
ethanol was layered onto the solution. The charcoal was
sedimented by centrifugation, the supernatant decanted, and
the charcoal residue washed twice with 5 ml. of distilled
water. After the second washing, the tubes were drained
by inversion and the insides dried with paper tissues. To
recover the labile phosphorus of the nucleotide fraction
(7-minute hydrolyzable nucleotide phosphate), the charcoal
residue was suspended in 4 ml. N HCl and the suspension
placed in a boiling water bath for 4 minutes. After cooling,
the suspension was centrifuged and a 2 ml. aliquot assayed
for phosphate.

Estimation of phosphate. Inorganic ortho-
phosphate was determined by the method of Bartlett (177).
The 2 ml. aliquot of the fraction described above was made
up to 3.4 ml. with distilled water and 1 ml. of 5 N sulphuric
acid, 0.4 ml. of 25 per cent ammonium molybdate, and 0.2 ml.
of the Fiske-SubbaRow reagent added in succession and mixed
thoroughly. The solution was heated for 10 minutes in a
boiling water bath and the colour produced was read at
830 mμ using a Beckman model B spectrophotometer with the

red sensitive phototube. In all experiments, blanks were performed on the charcoal and TCA solution. A standard containing 0.1 µmole of phosphate was performed simultaneously.

Assay of radioactivity. A 0.2 ml. aliquot of the hydrolyzed nucleotide phosphate fraction was plated and neutralized on the planchet by adding a sufficient amount of N sodium hydroxide. The mixture was evenly spread out on the planchet by adding one drop of a 2 per cent solution of cetyltrimethylammonium bromide, dried under an infra-red lamp and counted.

It had been suggested that not only the terminal phosphoric acid groups of ATP and ADP were split off by treatment with boiling N HCl, but that the ribose-5-phosphate group may also be split from the adenine moiety. Preliminary experiments were thus performed in order to determine whether the radioactivity of the 7-minute nucleotide phosphate was contaminated by the presence of possibly labelled ribose-5-phosphate. A 0.5 ml. aliquot of the hydrolyzed nucleotide phosphate fraction was mixed with 1.2 ml. of water, 4 ml. of an isobutanol benzene mixture 1:1 and 0.5 ml. of 2.5 per cent ammonium molybdate. The solution was shaken vigorously for 15 seconds and allowed to stand until the organic phase had cleared and for at least 10 minutes.

Samples of the organic and aqueous phases were plated and
counted. The radioactivity of the organic phase, which
consisted of orthophosphate removed as the phosphomolybdate
complex, was identical to that of the original 0.5 ml.
aliquot assayed. On the other hand, the activity of the
aqueous phase was not significantly above background in-
dicating that the ribose-5-phosphate is either not split
from the adenine moiety during treatment with boiling
N HCl, or if it is split, it is not labelled with inorganic
P^{32} during the course of the experiment.

Expression and Calculation of Results

Incorporation of P^{32} into phospholipids. In
early experiments on the incorporation of P^{32} into phospho-
lipids, it was found that the total phospholipid phosphorus
does not vary significantly in the presence of various external
factors, and that only the phospholipid labelling is affected
(Table I). The estimation of the total phospholipid
phosphorus was therefore only carried out in the prelimi-
nary experiments. Hence, the incorporation of P^{32} into
phospholipids was expressed as total counts/min/100 mg. wet
weight of tissue. In order to have comparative values from
one experiment to another the total counts in phospholipids/
min. were corrected for variations in the specific activity

of the inorganic phosphate in the incubation medium.

Thus: incorporation of P^{32} into phospholipids

$$= \frac{\text{total counts in phospholipids/min./100 mg. wet weight tissue}}{\text{Counts/min/}\mu\text{mole of inorganic phosphate in the incubation medium}}$$

Incorporation of P^{32} into 7 minute nucleotide

phosphate.   The radioactivity of the 7 minute nucleotide

fraction was expressed as total counts/min/100 mg. wet

weight of tissue and corrected for variations in the specific

activity of the inorganic phosphate in the incubation medium

as above.   In some experiments the radioactivity of the 7mnp.

fraction was expressed as counts/min/μmole phosphate.

CHAPTER III

STUDIES ON THE INCREMENT OF P^{32} INCORPORATION INTO PHOSPHOLIPIDS OF BRAIN CORTEX SLICES.

Introduction

A considerable amount of evidence has accumulated indicating that the incorporation of P^{32} into phospholipids of respiring brain slices is dependent on oxidative phosphorylation in the slice(102,103,104,130,131,179). The addition of energy-producing substrates increases the incorporation of P^{32} into phosphorus-containing compounds, whereas a decrease is observed during anaerobiosis or in the presence of metabolic inhibitors which block the formation of high energy phosphates (104,179,107,109). It has been suggested that the labelled P^{32} which is incorporated into phospholipids is derived from and depends on ATP (3,56). It would therefore seem logical to suppose that under conditions where the oxidative metabolism of brain slices is enhanced, the labelling of phospholipids would also be increased unless oxidation is uncoupled from phosphorylation.

It is well known that the addition of a high concentration of potassium ions or the omission of calcium ions from the incubation medium increases the oxygen con-

sumption and aerobic glycolysis of brain cortex slices.
These ionic changes also stimulate a number of other meta-
bolic reactions such as the synthesis of acetylcholine (124)
or the labelling of glutamine from radioactive glucose (138)
which are energy-dependent processes.   At present it is
believed that under these conditions the operation of the
citric acid cycle or some rate limiting step associated with
this cycle is activated (35,138).   On the other hand, it
has been shown that an increase in potassium ion concentration
in the incubating medium decreases the level of phospho-
creatine and ATP (126,127,129,131) as well as inhibiting
the incorporation of P^{32} into phosphorus-containing com-
pounds (130,131).   Much of the cellular metabolism of the
brain is directed towards the maintenance of a high gradient
of potassium between the intracellular and extracellular
concentrations of potassium (126).   It has been suggested
(180,132) that an increase of potassium ions in the in-
cubation medium would lower this gradient and in order to
re-establish it the metabolism of the brain slices would
concentrate potassium ions by reactions that are energy
consuming(110).   This would therefore result in a depletion
of energy-rich phosphate compounds necessary for the incor-
poration of P^{32} into phospholipids.

However, a point of interest arises from the

investigations previously reported (130,131) in that the
effect of cations on the incorporation of P^{32} into phospho-
lipids was determined after a relatively long period of
incubation where the enhancement of oxygen consumption and
presumably also the general metabolism of the brain slices
no longer occurs.   It seemed desirable to extend these
studies to determine whether the incorporation of P^{32} into
phospholipids is parallel to the enhancement of the oxid-
ative metabolism when this process is still taking place.
An investigation of the effects of cations and substrates,
which are known to increase cerebral respiration in vitro,
has been made on the incorporation of P^{32} into phospholipids.
In cases where a stimulatory effect was observed, the incre-
ment was compared to that obtained with acetylcholine which
has been shown to produce such stimulation (47).   The
factors that govern this phenomenon have also been examined.
The cationic stimulation of P^{32} incorporation into phos-
pholipids observed in this investigation is in agreement
with the finding of Yoshida and Quastel who first observed
this effect (201,134).

Results

The effect of potassium, calcium and ammonium ions on the incorporation of P^{32} into phospholipids.

The effect of electrolytes on the incorporation of P^{32} into the phospholipids of rat brain cortex slices was first investigated after a 30 minutes incubation period. It is well known that cationic stimulation of cerebral respiration, which is short-lived and decreases rapidly with time, is still marked at this time. The results obtained are presented in Table I. It can be seen that the addition of 100 mM potassium ions, 10 mM ammonium ions or the omission of calcium ions from the medium increases the oxygen consumption of brain slices respiring in glucose by about 70, 35 and 22 per cent respectively. The P^{32} incorporation into phospholipids follows a similar pattern as the increased oxygen uptake except with ammonium ions where a marked decrease in phospholipids labelling occurs. This inhibition by ammonium ions is in agreement with the results of Findlay et al. (130) who first observed the effect. The stimulation of P^{32} incorporation by the addition of potassium or the omission of calcium ions is of the same order as the stimulation of respiration suggesting that these two effects may be closely related. It

TABLE I

EFFECT OF K^+, Ca^{++} AND NH_4^+ IONS ON THE INCORPORATION OF P^{32} INTO

PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

K^+ (mM)	Ca^{++} (mM)	NH_4^+ (mM)	Oxygen uptake (μl/mg. dry wt. tissue/30 min.)	Phospholipid-P (μmoles/g. of wet wt. tissue)	Incorporation of P^{32} into phospholipids*	Per cent of control incorporation
5.2	2.8	0	5.1	32.0	10.5	100
105.2	2.8	0	8.8	30.8	16.9	161
5.2	0	0	6.9	32.9	14.2	135
5.2	2.8	10	6.2	-	6.7	64

Rat brain cortex slices were incubated for 30 minutes at 37°C in modified Krebs medium III with 10 mM glucose and 20 μc. $NaH_2P^{32}O_4$.

The results presented are the average of at least 3 experiments.

*Incorporation of P^{32} is expressed as total counts in phospholipids/min/100 mg. wet wt. of tissue divided by counts/min/μmole of inorganic phosphate in the incubation medium, and results quoted in this thesis will all be expressed in terms of this parameter.

can also be seen that the concentration of phospholipid
phosphorus does not change although the labelling from
inorganic P^{32} is enhanced.

Time course of the ionic effects.

In order to establish the extent of the ionic
effects on the incorporation of P^{32} into phospholipids, a
time course study of this effect was performed.   The values
for oxygen uptake under these experimental conditions are
given in Fig. 2.   In the normal medium, the respiration
of brain slices carries on almost unimpaired for 180 minutes.
When 100 mM of potassium are added to the medium, there is
a marked stimulation of oxygen consumption during the early
stages of incubation which falls off with time.   In fact
the oxygen uptake during periods of longer duration than
170 minutes is less than that of the control.   With a
calcium-free medium a similar pattern is observed but the
decrease in oxygen consumption occurs more gradually and is
less pronounced than in presence of potassium ions.

The incorporation of P^{32} into phospholipids is
shown in Fig. 3.   The rate of P^{32} incorporation in the
normal medium is almost constant for the first 60 minutes
of incubation and then continues at a slower rate as the
incubation is prolonged to 4 hours.   In the presence of

FIGURE 2

TIME COURSE OF THE OXYGEN UPTAKE OF RAT BRAIN CORTEX SLICES IN THE PRESENCE OF 0.1 M KCl OR IN THE ABSENCE OF CaCl2
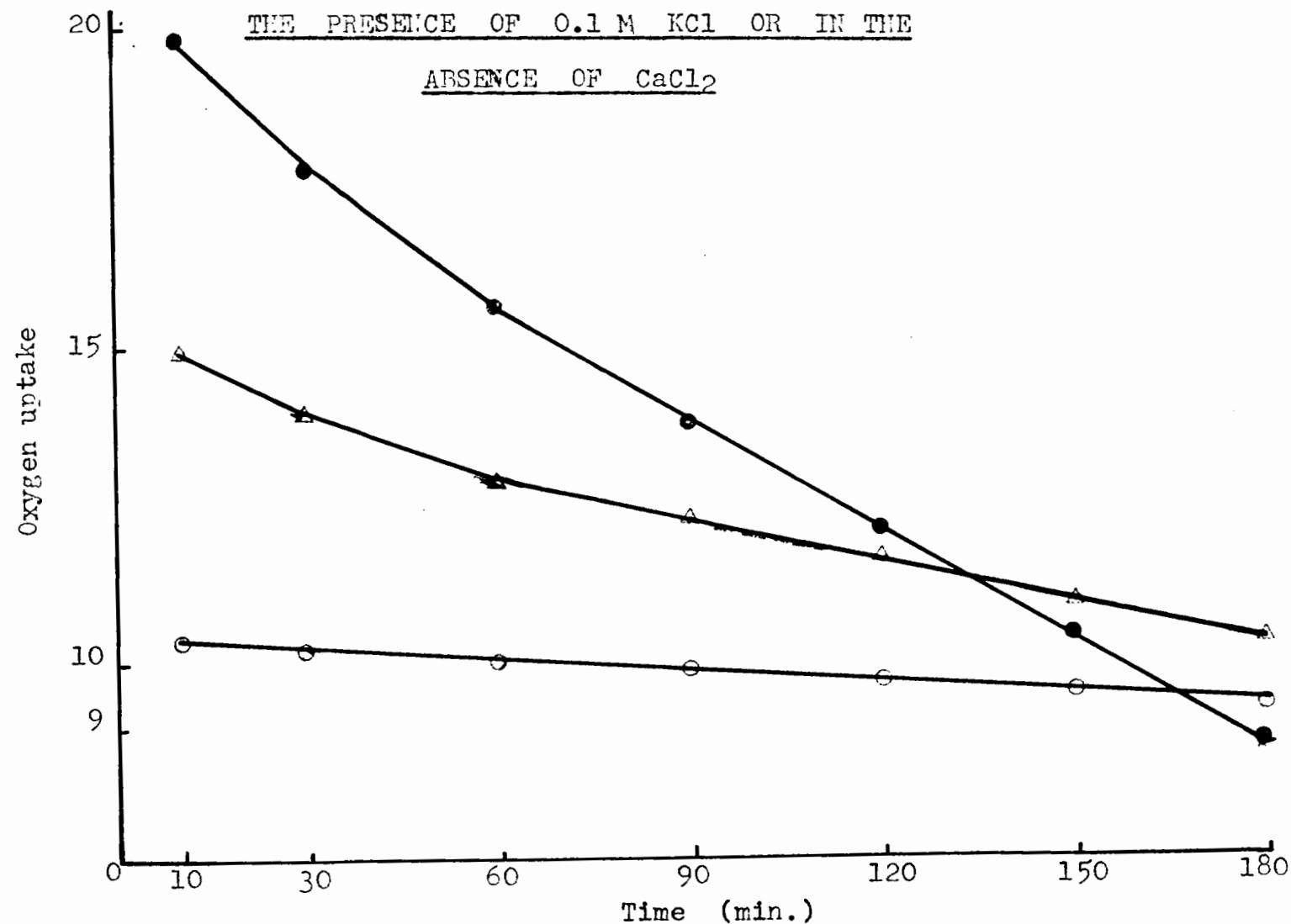
Rat brain cortex slices were incubated at 37°C in modified Krebs medium III with glucose 10mM.

,O Control; ● , + 0.1M KCl; △ , calcium-free.
Oxygen uptake expressed as $-Q_{O_2}$ for the time intervals indicated.

FIGURE 3

TIME COURSE OF THE INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF BRAIN

SLICES IN THE PRESENCE OF 0.1 M KCl OR IN THE ABSENCE OF $CaCl_2$
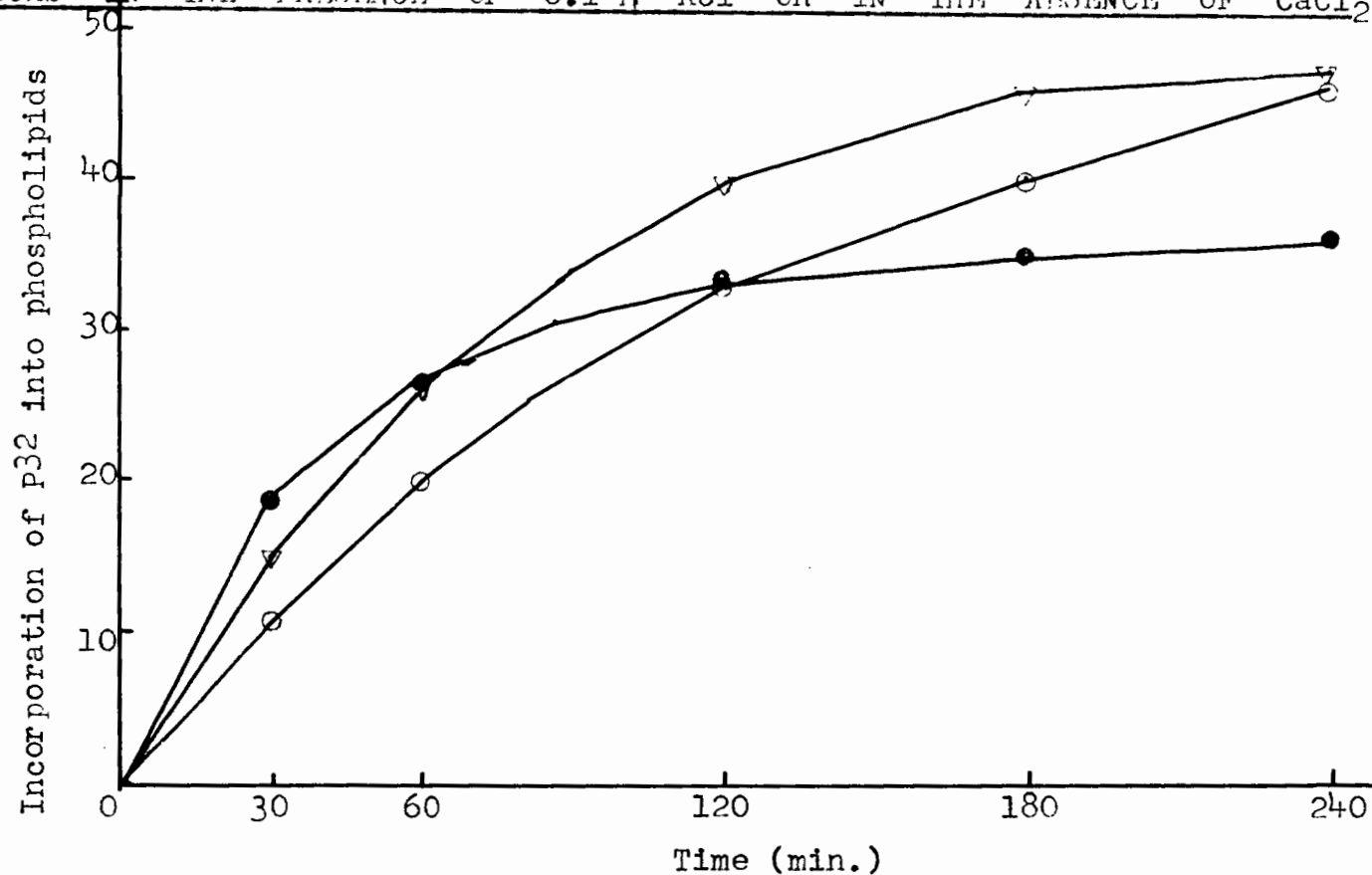
Experimental conditions as described in Table I.
,O Control; ●, ♦ 0.1 M KCl; ∇, calcium-free.
Incorporation of P^{32} expressed as in Table I.

100 $\underline{mM}$ potassium ions, the P^{32} incorporation is markedly
stimulated during the first 30 minutes of incubation.
Subsequent to this, the rate of incorporation decreases
until after 2 hours incubation when the incorporation of
P^{32} almost ceases. In the normal medium, P^{32} incorporation
continues so that after 4 hours incubation the incorporation
in the control medium is approximately 30 per cent greater
than in the medium containing 100 $\underline{mM}$ K^+ ions. In a calcium-
free medium, the initial rate in P^{32} incorporation also
gradually decreases with time, but contrary to the effect
observed with potassium ions the phospholipid labelling in
absence of Ca^{++} does not cease in 2 hours incubation. At
the end of 4 hours the P^{32} incorporation was the same in
normal and Ca^{++} free media.

The effect of 100 $\underline{mM}$ potassium ions and calcium-free medium
on the concentration and P^{32} labelling of nucleotide phosphate.

In view of the above observations as well as the
recognized fact that the degree of incorporation of P^{32} into
phospholipids is dependant not only upon the concentration
of ATP but also on the degree of incorporation of the iso-
tope into ATP, it was of interest to study the effect of
potassium and calcium ions on the level of ATP, and on the
P^{32} incorporation into ATP. The 7-minute acid-hydrolyz-

able nucleotide phosphate (7 mnp.) was taken as a measure
of the ATP in the slices and was defined as the amount of
inorganic orthophosphate released from the charcoal absorbed
nucleotides when they are hydrolyzed in N HCl for 7 minutes
at 100°C.   The inorganic phosphate fraction thus liberated
consists mainly of the acid labile phosphate groups of ATP.
This relatively non-specific method was used because of the
complex determinations required in estimating the concent-
ration of ATP.   A time course study of the effect of 100 $\underline{mM}$
potassium ions on the level of ATP and on the incorporation
of P^{32} into the 7-minute nucleotide phosphate was performed.
The results are illustrated in Fig. 4.   It can be seen that
the level of nucleotide phosphate in slices incubated in the
normal medium remains almost constant for 4 hours.   In the
presence of 100 $\underline{mM}$ potassium, a small decrease in the 7-minute
nucleotide phosphate concentration is found after 30 minutes
incubation and the level of 7mnp. continues to fall at a
constant rate, so that after 4 hours incubation it is only
40 per cent of the 7 mnp. level in the control medium.

The P^{32} incorporation into the nucleotide phos-
phate of tissue incubated in normal medium increases gra-
dually with time period and reaches equilibrium within ap-
proximately 120 minutes.   In the potassium-rich medium,
the incorporation of P^{32} into the nucleotide phosphate is

stimulated during the first 60 minutes incubation and comes
to equilibrium after this time.   The amount of P^{32} incor-
porated in the K^+ stimulated tissue after 60 minutes in-
cubation is identical to that incorporated in the unstimu-
lated tissue after 120 minutes.   It appears, therefore,
that after a certain period of incubation a maximum rate
of P^{32} incorporation is reached, after which a further incr-
ease in nucleotide phosphate labelling does not occur, or
at least is not apparent.   Ahmed and Scholefield (203)
have reported a similar observation with rat-liver slices.
It is possible that the 7mnp. may equilibrate with the
orthophosphate of the tissue, and after equilibrium is
reached it would not be possible to detect any increased turn-
over since the organic phosphate would be exchanging with
orthophosphate of the same specific activity.   The obser-
vation that although the level of nucleotide phosphate is
markedly decreased in the presence of 100 $\underline{mM}$ K^+ ions after
2 - 4 hours incubation, and that the incorporation of P^{32}
into the nucleotide phosphate is almost identical to that
of the unstimulated tissue in which the nucleotide phosphate
level is unimpaired is a paradox.

The results obtained during an incubation period
of 2 - 4 hours throw no light on the relationship between
the labelling of nucleotide phosphate and the labelling of

FIGURE 4

TIME COURSE OF THE EFFECT OF 0.1 M KCl ON THE LEVEL
OF 7MNP (A), AND ON THE INCORPORATION OF P^{32} INTO
7MNP (B), IN RAT BRAIN CORTEX SLICES
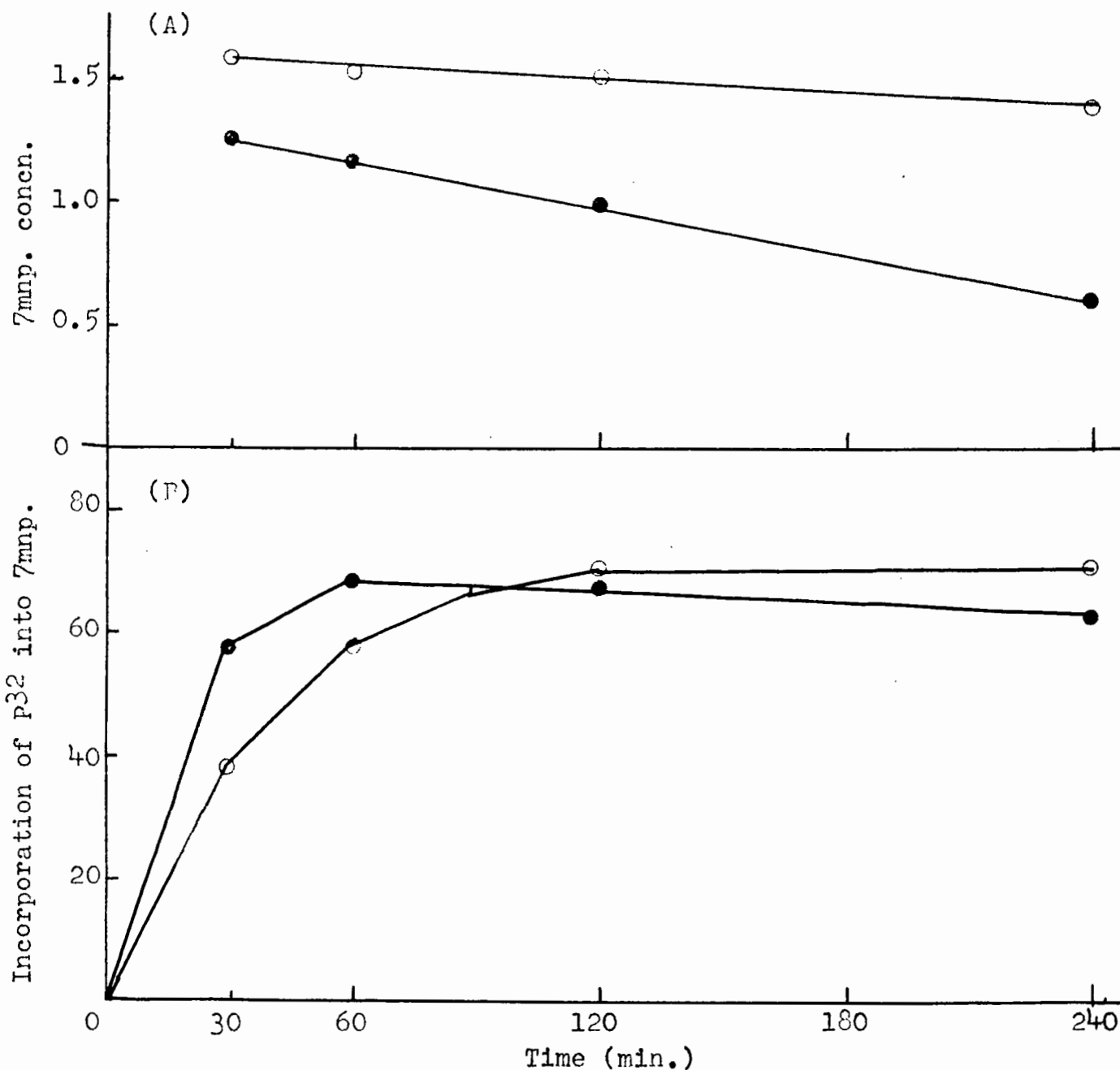
Rat brain cortex slices were incubated at 37°C in modified
Krebs medium III with 10mM glucose, and 20 μc. $NaH_2P^{32}O_4$.
O, control; ● , + 0.1M KCl.
7mnp. concn. expressed as μ moles phosphate/g. wet wt. tissue.
Incorporation of P^{32} into 7mnp. expressed as in Table I.

phospholipids either in unstimulated or stimulated tissue.
However, it is of particular interest to note that after
an incubation period of 30 minutes where the state of equilibrium is not yet attained, a marked stimulation in P^{32}
incorporation (Fig. 4) or specific activity (Table II) of
the nucleotide phosphate is observed in the presence of
100 mM K^+ ions.   An analogous effect is also obtained in
a calcium-free medium and the results are shown in Table II.
Another point worth mentioning is that after a 30 minute
incubation period, the percentage of stimulation of the P^{32}
incorporation into 7mnp. in the presence of 100 mM K^+ or in
the absence of Ca^{++} ions is relatively identical to that of
the phospholipids labelling and of the oxygen consumption
(Table I, II).

The effect of various concentrations of potassium ions.

The results presented in Table I showed that
there is a striking stimulation of P^{32} incorporation into
the phospholipids of brain cortex slices on addition of 100 mM
of potassium ions to the incubation medium.   This effect
was investigated further by studying a range of concentrations
of potassium ions from 25 to 150 mM.   The results which are
presented in Table III, indicate that the addition of potassium ions at concentration of 25 and 50 mM have practically

TABLE II

EFFECT OF THE ADDITION OF K^+ (0.1M) OR THE OMISSION OF Ca^{++} ON THE LEVEL AND
SPECIFIC ACTIVITY OF 7 MINUTE NUCLEOTIDE PHOSPHATE IN RAT BRAIN CORTEX SLICES

Condition	Expt. 1		Expt. 2		Expt. 3	
	7 mnp. Conc.	Specific Activity	7 mnp. Conc.	Specific Activity	7 mnp. Conc.	Specific Activity
Normal medium	1.6 (100)	30.0×10^4(100)	1.52(100)	24.7×10^4(100)	1.7(100)	28.8×10^4(100)
+ KCL (0.1M)	1.25(78)	54.0 (180)	1.17 (76)	39.0 (157)	1.3 (77)	48.0 (167)
Ca^{++} free	1.18 (74)	40.0 (133)	1.25 (82)	34.6 (140)	1.3 (77)	36.0 (125)

Rat brain cortex slices were incubated for 30 minutes as described in Table I.
7 mnp. concentration expressed as μmoles phosphate per g. wet weight tissue.
Specific activity expressed as cts/min/μmole phosphate.
The figures in parentheses refer to percentages of the normal medium values.

TABLE   III

EFFECT   OF   INCREASING   CONCENTRATIONS   OF K^+ IONS   ON   THE   INCORPORATION   OF P^{32}

INTO   THE   PHOSPHOLIPIDS   OF   RAT   BRAIN   CORTEX   SLICES

Addition	Concn. (mM)	Oxygen Uptake (μl/mg. dry wt./30 min.)	Incorporation of P^{32} into phospholipids[*]	Percentage of the control
Nil	-	5.3	10.1	100
KCl	25	6.6	9.9	98
KCl	50	7.4	11.6	115
KCl	75	8.8	15.1	150
KCl	100	9.0	16.4	162
KCl	125	8.9	14.3	143
KCl	150	7.1	13.1	130

Rat brain cortex slices were incubated in modified Krebs medium III with glucose 10 mM for 30 min. at 37°.   All vessels contained 20 μc. $NaH_2P^{32}O_4$, and KCl was added as indicated.

Results represent mean values of two experiments or more.

[*]Incorporation P^{32} expressed as in Table I.

no effect on the labelling of phospholipids, although an increase in oxygen uptake identical to that caused by a calcium-free medium (Table I) is observed. With higher concentrations, the stimulatory effect on P^{32} incorporation increases rapidly reaching a maximum at 100 $\underline{mM}$ of K^+ ions, and subsequently decreases with concentrations of 125 and 150 $\underline{mM}$. Since low concentrations of potassium ions stimulate the oxygen consumption but not the incorporation of P^{32}, it can be suggested that the potassium-stimulated incorporation may be linked to factors other than just the increment of the oxidative metabolism.

The effect of various substrates on the incorporation of P^{32} into brain cortex slices metabolizing glucose.

It is well known that the addition of tricarboxylic acid cycle intermediates or various substrates such as glutamate and Y-aminobutyric acid increases the oxygen consumption of brain slices metabolizing glucose (181, 118, 182, 133,183). The effect of these substances on the glucose stimulated incorporation of P^{32} into phospholipids was investigated and the results are shown in Table IV. It can be seen that the oxygen uptake is stimulated after an incubation period of 30 minutes, but practically no effect is observed on the incorporation of P32. However, if the incubation period is prolonged to 2 hours, a marked in-

TABLE IV

EFFECT OF THE ADDITION OF VARIOUS SUBSTRATES ON THE INCORPORATION OF P^{32}
INTO THE PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES METABOLIZING GLUCOSE

Addition (mM)	Incubation time (30 minutes)		Incubation time (120 minutes)	
	Oxygen uptake (µl/mg. dry wt.)	Incorporation of P^{32} into phospholipids*	Oxygen uptake (µl/mg. dry wt.)	Incorporation of P^{32} into phospholipids*
Nil	5.2	10.2 (100)	19.5	31.0 (100)
Succinate, 10	7.0	11.4 (112)	21.2	16.0 (51)
α-Keto-glutarate, 10	6.2	10.3 (100)	-	-
L-glutamate, 10	6.9	9.0 (88)	22.5	12.7 (41)
Y-amino-butyric acid, 5	6.2	9.9 (97)	20.6	19.8 (64)
Y-amino-butyric acid, 10	5.9	7.5 (73)	-	-

Rat brain cortex slices were incubated at 37° in modified Krebs medium III with glucose, 10 mM and $NaH_2P^{32}O_4$, 20 µc. Substrates were added as indicated.

The figures in parentheses refer to percentages of the normal medium values.

Results presented are the average of 3 experiments.

*Incorporation of P^{32} expressed as in Table I.

hibition of P^{32} incorporation is obtained in the presence of succinate, glutamate and Y-aminobutyric acid although the respiratory values at this period of time are still at a maximum. The inhibitory effect on P^{32} incorporation observed with glutamate is in agreement with earlier studies (130). The fact that the inhibitory effects were mostly observed after long period of incubation (2 hours) as observed above with 100 mM K^+ ions, emphasizes that although the oxidative metabolism is enhanced under these conditions, other metabolic phenomena are taking place which are counter-acting the stimulatory effect. Therefore, subsequent studies on the effect of 100 mM of potassium ions or of cal-cium-free medium were always determined after a 30 minute incubation period.

The effect of various substrates on the stimulated-incorporation of P^{32} into phospholipids

a)   Stimulation by potassium-rich or calcium-free media.

The cationic stimulation of brain cortex res-piration (115,123) and the incorporation of P^{32} into phos-pholipids of unstimulated slices (103,104,179) take place only with energy-producing substrates. It seemed desirable to examine the effect of various substrates on the stimulated P^{32} incorporation in brain slices in the presence of 100 mM

K^+ ions or in the absence of Ca^{++} ions.   The results are
shown in Tables V and VI.   In the normal incubation medium,
the addition of glucose or mannose causes the maximal in-
corporation of P^{32} into the phospholipids.   Fructose or
pyruvate also increases the P^{32} labelling, but only to a
limited extent as compared to the former substrates, whereas
glutamate or succinate are almost ineffective.   When 100 $\underline{mM}$
of K^+ ions are added to the incubation medium or Ca^{++} ions
omitted, the increment of P^{32} incorporation occurs only with
the addition of glucose, mannose, fructose or pyruvate as
substrates.   In the absence of added substrate or in the
presence of glutamate or succinate there is no cationic
stimulation of P^{32} incorporation.   On the contrary, with
the endogenous or the succinate-containing medium, a small
but consistent inhibition of P^{32} incorporation occurs when
the slices are stimulated.   These results emphasize once
more the close parallel that exists between the stimulated
respiration and the stimulated incorporation of P^{32} into
phospholipids.

B)   Acetylcholine-stimulated incorporation of P^{32} into
phospholipids.

It has been previously shown by Hokin and Hokin
(47) that acetylcholine stimulates the incorporation of P^{32}

TABLE V

EFFECT OF VARIOUS SUBSTRATES ON THE POTASSIUM-STIMULATED INCORPORATION OF P^{32} INTO THE PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

Substrate (10mM)	Incorporation of P^{32} into phospholipids*			Oxygen uptake (µl/mg. dry wt./30 min.)		
	Control	+KCl(0.1M)	% of control	Control	+KCl(0.1M)	% of control
Nil	3.3	3.0	91	3.0	2.6	87
Glucose	10.1	16.0	158	5.0	8.8	176
Mannose	10.8	16.2	150	4.8	8.0	166
Fructose	5.0	6.6	132	4.7	8.1	172
Pyruvate	4.6	7.2	157	5.4	9.2	170
L-glutamate	3.8	3.6	95	5.4	5.0	92
Succinate	3.4	2.9	85	4.6	3.7	81

Rat brain cortex slices were incubated at 37°C for 30 minutes in modified Krebs medium III with $NaH_2P^{32}O_4$, 20 µc. Substrates added as indicated.

The results presented are the average of at least 3 experiments.

*Incorporation of P^{32} expressed as in Table I.

TABLE VI

EFFECT OF VARIOUS SUBSTRATES ON THE CALCIUM-FREE -STIMULATED INCORPORATION OF P^{32} INTO THE PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

Substrate (10 $\underline{mM}$)	Incorporation of P^{32} into phospholipids*			Oxygen uptake (μl/mg. dry wt./30 min.)		
	Control	Ca^{++} free	% of control	Control	Ca^{++} free	% of control
Nil	3.0	2.8	94	3.0	2.7	90
Glucose	10.2	13.9	136	5.1	6.9	135
Mannose	10.8	14.6	135	4.8	6.4	134
Fructose	5.0	6.1	122	4.7	6.1	130
Pyruvate	4.6	6.5	141	5.4	7.6	141
L-glutamate	3.8	3.7	101	5.4	5.3	98
Succinate	3.4	3.2	95	4.6	4.2	91

The experimental conditions were as described in Table V.

*Incorporation of P^{32} expressed as in Table I.

into phospholipids of brain cortex slices respiring in glucose. Since an analogous effect was obtained with potassium-rich or calcium-free medium, it seemed of interest to undertake a comparative study of these effects and to examine the factors which also govern the acetylcholine stimulation.

Preliminary investigations under normal experimental conditions showed that acetylcholine at a concentration of 2 $\underline{mM}$ stimulated the incorporation of P^{32} by approximately 50% after an incubation period of 2 hours. Although an effect was obtained with smaller concentrations of acetylcholine, along with a shorter incubation period, the stimulation was too small and inconsistent to permit a precise investigation. Subsequent studies with acetylcholine were thus performed with a concentration of 2 $\underline{mM}$ acetylcholine during an incubation period of 2 hours. The substrates that support the stimulatory effect of acetylcholine on the incorporation of P^{32} into the phospholipids were examined under these conditions. The results are presented in Table VII. It can be seen that the acetylcholine stimulation of P^{32} incorporation takes place only in the presence of glucose, mannose or pyruvate to the extent of 54, 49 and 61 per cent respectively. In the presence of fructose and glutamate there is no stimulation by acetylcholine, whereas

TABLE VII

EFFECT OF VARIOUS SUBSTRATES ON THE ACETYLCHOLINE-STIMULATED INCORPORATION OF

P^{32} INTO PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

Substrate (10 mM)	Incorporation of P^{32} into phospholipids*			Oxygen uptake (µl/mg. dry wt./2 hours)		
	Control	+ACh	% of control	Control	+ACh	% of control
Nil	4.8	3.2	66	7.7	7.4	96
Glucose	29.2	45.0	154	19.4	21.7	112
Mannose	27.0	40.1	149	18.7	20.2	108
Fructose	10.3	10.5	102	16.6	16.7	100
Pyruvate	8.6	13.8	161	21.7	23.8	110
L-glutamate	6.4	6.6	103	17.4	17.6	101
Succinate	5.8	4.0	69	13.3	12.5	94

Rat brain cortex slices were incubated at $37^{o}C$ for 2 hours in modified Krebs medium III with 20 µc. $NaH_2P^{32}O_4$, acetylcholine (ACh) $2x10^{-3}M$ and eserine $3x10^{-4}M$. Substrates added as indicated.

The results presented are the average of at least 3 experiments.

*Incorporation of P^{32} expressed as in Table I.

in the absence of added substrate or in succinate-containing
medium the addition of acetylcholine produces a marked in-
hibition of P^{32} incorporation into phospholipids.   A small
but consistent increase in oxygen consumption also occurs
in the presence of acetylcholine with the substrates which
support the stimulation of P^{32} incorporation.

The effect of sodium ions on the increased incorporation of P^{32} into phospholipids

The effect of 100 mM potassium chloride on the
oxygen uptake of brain cortex slices is not observed if the
normal amount of sodium ions in the incubation medium is
removed completely or diminished to a certain extent (115,
116,184).   A study of the effect of sodium ions on the
increase of P^{32} incorporation into phospholipids by 100 mM
K^+, absence of Ca^{++} or acetylcholine was therefore undertaken.
The sodium-free medium was obtained by replacing the sodium
phosphate buffer with tris buffer and the sodium chloride
by an equivalent amount of choline chloride in order to
maintain the correct osmotic pressure.   The experimental
results are shown in Tables VIII and IX.   It can be seen
that in the control medium, the complete removal of sodium
ions causes a marked inhibition of the incorporation of P32
into phospholipids, and this effect is gradually abolished

TABLE   VIII

EFFECT   OF   Na$^+$ IONS   ON   THE   POTASSIUM   AND   CALCIUM-FREE   STIMULATED   INCORPORATION OF   P^{32} INTO   PHOSPHOLIPIDS   OF   RAT   BRAIN   CORTEX   SLICES

Na$^+$ Concn. (mM)	Incorporation of P^{32} into phospholipids*			Oxygen uptake (µl/mg. dry wt./30 min.)		
	Control	+KCl (0.1M)	Ca^{++} free	Control	+KCl (0.1M)	Ca^{++} free
140	9.7	16.3 (167)	13.0 (134)	4.6	7.5 (163)	6.1 (133)
100	9.2	14.5 (158)	12.4 (135)	4.5	7.0 (155)	5.9 (131)
33	7.7	9.0 (117)	10.2 (133)	4.3	4.9 (114)	5.5 (128)
0	6.2	6.1 (100)	8.5 (137)	4.0	3.6 (90)	4.9 (123)

Rat brain cortex slices were incubated at 37°C for 30 minutes in standard Krebs ringer medium with glucose, 10 mM, tris buffer, 10 mM, pH 7.4 and NaH$_2$P^{32}O$_4$, 10 µc.

Na$^+$ was replaced by an equivalent amount of choline to maintain the correct osmotic pressure. The values in parentheses refer to percentages of the control values obtained under the same experimental conditions.

Results presented are the average of 3 experiments.

Incorporation of P^{32} expressed as in Table I.

TABLE   IX

EFFECT   OF   Na$^+$ ON   THE   ACETYLCHOLINE-STIMULATED   INCORPORATION   OF   P^{32} INTO

PHOSPHOLIPIDS   OF   RAT   BRAIN   CORTEX   SLICES

Na$^+$ Concn. (mM)	Incorporation of P^{32} into phospholipids*			Oxygen uptake (µl/mg. dry wt./ 2 hours.)	
	Control	+ ACh	% of control	Control	+ ACh
140	37.1	54.1	146	17.5	18.9
100	39.3	56.0	142	17.3	18.2
66	35.0	48.0	137	16.9	17.4
33	27.8	32.9	119	16.1	15.8
0	22.0	23.1	105	15.5	15.6

Rat brain cortex slices were incubated at 37°C for 2 hours in standard Krebs ringer medium with glucose 10 mM, tris buffer 10 mM, pH 7.4, NaH$_2$P^{32}O$_4$ 10 µc., acetylcholine (ACh) 2x10^{-3}M and eserine 3 x10^{-4}M. Na$^+$ was replaced by choline.

Results presented are the average of 3 experiments.

Incorporation of P^{32} expressed as in Table I.

by the presence of minimum amounts of sodium ions. The in-
hibitory effect on P32 incorporation is not paralleled by an
equivalent inhibition of oxygen uptake. When the effects
of 100 mM K+ ions or acetylcholine were studied under these
experimental conditions, the stimulation of P^{32} incorporation
is not observed unless a minimum amount of sodium ions are
present. The omission of calcium ions in a sodium-free
medium produces an effect different from that observed with
the addition of 100 mM K+ or acetylcholine in a sodium-free
medium. The percentage stimulation of P^{32} incorporation is
always observed with decreasing amounts of sodium ions in the
medium, although the level of P^{32} incorporated is lower than
that obtained under normal stimulatory conditions.

DISCUSSION

It has been well established that the incorporation of
P^{32} into the phospholipids of rat brain preparations is de-
pendent upon the supply of metabolic energy and that the
isotope first appears to be incorporated into ATP prior to
its entry into phospholipids (3,56,104,107,109,179). These
conclusions have been confirmed in the present study and have
been extended to cationic stimulated brain cortex slices.

The results presented in this chapter show that
the potassium-stimulated oxygen consumption of brain slices

after 30 minutes incubation is accompanied by a stimulation of P^{32} incorporation into phospholipids and ATP (7 -minute nucleotide phosphate) although the level of ATP is slightly decreased. If incubation with 100 mM potassium ions is prolonged for 4 hours, an inhibitory effect is observed on the oxygen uptake, the incorporation of P^{32} into phospholipids, as well as on the concentration of ATP. These results, obtained after a long period of incubation, are in agreement with earlier studies demonstrating that the addition of potassium ions has an inhibitory effect on several metabolic processes of the central nervous system in vitro (126, 130,131,132,135). As a result of the present and of earlier investigations, it seems likely that two metabolic phenomena take place in brain slices incubated in a potassium-rich medium, which are concomitantly influencing the incorporation of P^{32} into the phospholipids.

The first of these phenomena, which results in the enhancement of P^{32} incorporation, would be a direct consequence of the stimulated oxidative mechanism initiated by the presence of excess potassium ions. Quastel (35) has suggested that the ionic movements at the nerve cell membrane in the presence of 100 mM K^{+} ions may cause a series of reactions involving the accumulation of ADP since it has been shown that excitation of the brain results in a drop

in the content of ATP (185) and an increase in the ADP/ATP
ratio (186). The key role played by ADP in the regulation
of respiratory and glycolytic processes is well known.
Therefore, such reactions by accelerating rate regulating
steps would cause the stimulation of respiration of iso-
lated intact brain tissue, and thus affect the catabolism
of hexosephosphate to pyruvate. Kini and Quastel (138)
have also concluded that the stimulatory effect caused by
the addition of potassium ions is largely directed towards
the acceleration of a pace-making step, that is, the con-
version of pyruvate to acetyl-CoA. It is possible there-
fore to interpret the stimulation of P^{32} incorporation into
the phospholipids in the presence of 100 $\underline{mM}$ K+ ions. The
increased glucose oxidation (187,121) and increased rate
of acetyl-CoA formation lead to a higher level of the avail-
able intermediate compounds, resulting in an acceleration
of the citric acid cycle and therefore in an acceleration
of the incorporation of P^{32} into ATP. Since phospholipids
are labelled from ATP^{32}, an increase P^{32} incorporation should
result, and this was indeed observed after a 30 minute in-
cubation. In relation to the acceleration of the citric
acid cycle, Kini and Quastel (138) found that 100 $\underline{mM}$ K+ ions
stimulate the labelling from glucose-U-C^{14} of various amino-
acids derived from the citric acid cycle intermediates.

The second phenomenon which results in an inhibition of the incorporation of P^{32} into the phospholipids, may be a consequence of the depletion of high energy phosphate compounds in the presence of excess potassium (126,131). In order to explain this phenomenon it has been suggested (180,132) that since much of the cellular metabolism is directed towards the maintenance of a high gradient of potassium ions between the intracellular and extracellular compartments of the tissue (126), addition of potassium ions to the incubation medium would lower this gradient and in an attempt to re-establish it the metabolism of the brain slices would be directed towards the concentration of potassium ions by reactions that are energy consuming (110). This would result in the lowered availability of energy for energy-consuming reactions such as the incorporation of P^{32} into phospholipids. An alternative explanation closely related to the above arises from the suggestion of McIlwain (20) that the addition of potassium ions causes depolarization of the nerve cells resulting in the entry of sodium ions in the cells. Keynes (188) has concluded that metabolic energy is required to drive the recovery process responsible for maintaining the low internal sodium and high internal potassium concentrations in the nerve cell.

The present results therefore illustrate the
above two phenomena. In the early stages of the incubation
in the presence of 100 m$\underline{M}$ potassium ions, the oxygen con-
sumption as well as the incorporation of P^{32} into ATP (7 mnp.)
and phospholipids are stimulated although the amount of ATP
(7 mnp.) is slightly decreased, caused by the energy re-
quirement, but is still high enough to support the stimu-
lation of substrates oxidation. As the duration of the
incubation period is extended, the depletion of ATP (7 mnp.)
increases and becomes a limiting factor for the processes
dependent upon it, resulting in a gradual decrease of the
stimulatory effect, and finally leading to an inhibition
when the amount of ATP is very low, which is the case after
4 hours incubation.

The experiments with a Ca^{++} free medium indic-
ate that similar effects to those observed with a potassium
rich medium take place. Perhaps the calcium lack exerts
its effect by a mechanism similar to that of high potassium
ions concentration. Indeed calcium ions appear to affect
primarily the constraint imposed upon movements of Na^{+} and
K^{+} across the membrane; they react with and become part of
the surface structure of the cell (18,9). In this connection
McIlwain (20) has suggested that the omission of calcium
ions in the medium causes depolarization of nerve cells.

The findings that ammonium ions or glutamate
inhibit the incorporation of P^{32} into the phospholipids of
brain slices metabolizing glucose are in agreement with the
earlier studies of Findlay et al. (130) and of Rossiter (131).
Ammonium ions and glutamate participate actively in glut-
amine synthesis, an energy dependent process, and in the
presence of these substances the level of ATP (190,204,205)
and of phosphocreatine (200,126,193) is depleted in brain
slices.   Therefore, reactions that are energy requiring,
such as the incorporation of P^{32} into phospholipids or the
synthesis of acetylcholine (191), are bound to be inhibited
under these conditions.   Branganca et al. (192) showed that
inhibitors of glutamine synthesis overcame the inhibition
of acetylcholine synthesis by ammonium ions.   Recently
Woodman and McIlwain (193) found that the rate of break-
down of phosphocreatine in cerebral tissue on the addition
of glutamate or ammonium ions is a very rapid process.

The inhibitory effects observed on the incor-
poration of P^{32} with the addition of succinate or Y-amino-
butyric acid (GABA) parallel the above phenomenon.   It has
been shown that the addition of these substrates to brain
slices metabolizing glucose decreases the ability of the
tissues to maintain their level of ATP (190,204) and of
phosphocreatine (193).   This inhibitory effect with GABA

does not concur with the finding of Tsukada et al. (194)
who reported that GABA stimulates the incorporation of P^{32}
into the phospholipid fractions of guinea pig brain cyto-
plasmic particulates.   The similarity of the inhibitory
effect of GABA with that of glutamate or succinate is a
point of interest.   It has been shown that GABA may be
metabolized in brain via transamination with α-ketoglutarate
to yield glutamic acid and succinic semi-aldehyde which is
then further metabolized by oxidation to succinic acid (195,
196).   In this connection it appears that the inhibitory
effect of GABA on the incorporation of P^{32} may be due to its
oxidation to glutamate and succinate which then cause a
depletion of high energy phosphate compounds.   Another pos-
sible explanation for the depletion of the labile-P in the
tissue by glutamate has been suggested by Rossiter (180).
Brain cortex slices in the presence of glucose and L-glut-
amate are able to concentrate potassium ions and L-glutamate
within the tissue against a concentration gradient (111,112,
113,), and this process would be expected to deplete the
tissue of high energy phosphates. Elliott and Van Gelder
(197) also reported that the uptake of GABA can occur against
a concentration gradient.

The stimulation of P^{32} incorporation into phos-
pholipids in the presence of 100 mM K^{+} or in the absence of

Ca^{++} was found to take place with glucose, fructose, man-
nose or pyruvate but not with glutamate or succinate as
substrate. This stimulation parallels the enhancement of
oxygen uptake which occurs with the identical substrates as
previously observed for potassium (115,123) or electrical
stimulation (140) of brain cortex slices. The postulation
by Kini and Quastel (138) that the stimulatory effect of
100 mM K^+ ions is largely directed towards the conversion
of pyruvate to acetyl-CoA makes it possible to explain the
above results. The substrates that increase the avail-
ability of pyruvate will support the stimulation of the
pyruvate-acetyl-CoA step and therefore of the incorpor-
ation of P^{32} into phospholipids as explained above. It
may be mentioned that the synthesis of acetylcholine, which
is synthesized from acetyl-CoA, is stimulated by increased
K^+ ions in the medium (124).

It was found that with the exception of fruc-
tose, the substrates that support the incorporation of P^{32}
into phospholipids in the presence of acetylcholine are the
same as those that support the cationic stimulation. In
this connection, it is of interest to recall the results of
Mann et al. (198) who found that the synthesis of acetyl-
choline by brain slices occurs only in the presence of
glucose, mannose, lactate or pyruvate. There is thus a

similarity between the substrates that will support the normal incorporation of P^{32} into phospholipids, (104,179) the formation of acetylcholine (198) and the stimulation of P^{32} incorporation into phospholipids by cations or acetylcholine, in brain slices. These results lead to the conclusion that the stimulation of P^{32} incorporation by cations or acetylcholine is a metabolic phenomenon and is dependent upon an adequate phosphorylating mechanism within the slices, since the so-called "non energy-yielding" substrates glutamate and succinate fail to support the stimulation.

The experiments with sodium-free medium indicate that the presence of a certain concentration of Na^+ ions is required to support the normal incorporation of P^{32} into phospholipids as well as the stimulation of P^{32} incorporation by 100 $\underline{mM}$ K^+ ions or by acetylcholine, in agreement with earlier studies (133,134,202). This specific requirement of Na^+ ions is obscure and difficult to explain, although it seems likely that the stimulatory effects observed above are closely linked to the ionic movement of Na^+ and K^+ ions at the cell membrane (20,35). A number of observations support this hypothesis. It is known that brain slices which are actively producing energy, contain a high concentration of potassium after incubation in a normal saline medium (110,112,113). This concentration of

potassium in the tissue is increased further in the presence
of 100 mM K⁺ ions (112,113). Pappius et al. (199) showed
that the increased potassium concentration in the incubated
slices occurs only when sodium ions are present in the medium.
Gore and McIlwain (200) also reported that the omission of
sodium ions from the normal medium results in lowering the
phosphocreatine level in the slices. This latter obser-
vation would help to explain the inhibitory effect of a
sodium-free medium on the incorporation of P^{32} into phospho-
lipids (Table VIII) and in phosphoproteins (133) which are
ATP dependent processes. The results of Gore and McIlwain
(2 00) also lead to the suggestion that the accumulation of
potassium in the slices is dependent upon the provision of
high energy phosphate which would be inadequate when Na⁺ ions
are absent as proposed by Pappius et al. (199). It has
also been suggested that the accumulation of the potassium
in the tissue is a result of the activity of a mechanism
which extrudes sodium and accumulates potassium within the
cells (199). It would therefore seem likely that the con-
centration of potassium ions in the tissue is specifically
dependent on the presence of Na⁺ ions, and in its absence,
the stimulatory effect of 100 mM K⁺ ions would not occur
since the amount of potassium in the slices would be at a
minimum. In the case of the acetylcholine stimulation of

P^{32} incorporation which has been linked to the depolarization property of this substance (52), the lack of sodium would thus cancel this phenomenon and the effect associated with it.

In contrast to the potassium and acetylcholine results, it was somewhat surprising to find that in a calcium-free medium a stimulation of oxygen uptake and P^{32} incorporation occurs when Na^+ is omitted from the medium (Table VIII). The explanation for this stimulatory effect can only be a point of speculation at present. It may be due to the fact that the inhibition of oxygen uptake or P^{32} incorporation observed in a Na^+ free medium does not take place when calcium ions are also omitted from the medium.

SUMMARY

1.   The addition of 100 mM KCl or the omission of
        $CaCl_2$ from the normal incubating medium causes a
        marked stimulation of oxygen uptake and a parallel
        stimulation of P^{32} incorporation into phospholipids
        of brain cortex slices during the early phases of
        incubation. The potassium-stimulated P^{32} incorpor-
        ation is not observed after an incubation period of
        2 hours. Longer periods of incubation caused a
        progressive inhibition in the labelling of phospho-

lipids.   The calcium-free stimulated P^{32} incorporation progressively decreases with incubation time, but does not result in an inhibition after 4 hours incubation.

2.   The level of 7-minute hydrolyzable nucleotide phosphate is slightly decreased after 30 minutes incubation by the addition of 100 $\underline{mM}$ KCl or by the omission of $CaCl_2$ from the medium.   This decrease becomes more marked with incubation periods of longer duration.   The stimulated incorporation of P32 into the 7-minute nucleotide phosphate gives approximately the same quantitative picture as the stimulated incorporation into phospholipids by the addition of 100 $\underline{mM}$ KCl or by the omission of $CaCl_2$ after 30 minutes incubation.

3.   When brain cortex slices respiring in glucose are incubated in the presence of NH_4^+ ions, succinate, glutamate or Y-aminobutyric acid, a marked inhibition of P32 incorporation into phospholipids occurs.

4.   The stimulated P32 incorporation into phospholipids by the addition of 100 $\underline{mM}$ K^+ ions, acetylcholine or by the omission of Ca^{++} ions from the medium takes place only in the presence of high

energy producing substrates.

5.   When sodium ions are omitted from the normal incubation medium, the incorporation of P^{32} into phospholipids is inhibited. The stimulatory effect of 100 $\underline{mM}$ K^+ ions or acetylcholine does not occur in a sodium-free medium. In contrast, a stimulation of P^{32} incorporation is observed in a sodium-free medium when Ca^{++} ions are absent.

CHAPTER IV

EFFECTS OF METABOLIC INHIBITORS ON THE STIMULATED INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

Introduction

A striking feature of the cationic stimulation of brain cortex slices is to increase respiratory rates to approximately those found in vivo. Moreover, the cationic-stimulated slices possess many of the metabolic characteristics of brain tissue in the excited state as well as those observed during increased activity in the brain in vivo (35,20). These metabolic and physiological parallels have focused attention on the mechanism of the potassium-stimulated neuronal respiration and have been of particular interest in regard to the effect produced by metabolic inhibitors and neurotropic drugs on the metabolism of the brain in vitro. It is now well established that potassium-stimulated brain cortex respiration is very sensitive and is easily suppressed by metabolic inhibitors or low pharmacologically active concentrations of narcotics which have no demonstrable effects on the unstimulated respiration in the presence of glucose (119,122,123,210,35).

In the previous chapter, the stimulatory effect
of cations or acetylcholine on the incorporation of P^{32} into
phospholipids of brain cortex slices was described.   It was
postulated, in agreement with earlier studies (201,152,104,
179,131), that this effect is an energy-dependent phenomenon.
The cationic-stimulated P^{32} incorporation into phospholipids
was considered to be a consequence of an increased rate of
synthesis or turnover of ATP due to the activation of the
citric acid cycle involved in glucose oxidation, or of a
pace-making step closely associated with it.   Since meta-
bolic inhibitors are known to inhibit the incorporation of
P^{32} into phospholipids (104,56,107,131,109,179) and ATP (107,
131,179,109), it was of interest therefore to investigate
the effect of these agents on the stimulated incorporation
of P^{32} into phospholipids.

In the present chapter, experiments on the effects
of some metabolic inhibitors on the cationic or acetylcholine-
stimulated incorporation of P^{32} into phospholipids are re-
ported.   Typical respiratory and glycolytic inhibitors such
as malonate, iodoacetate and fluoride have been studied.   The
effect of some typical central nervous system depressants
like amytal, chloretone and ethanol at concentrations ap-
proximately equal to the narcotizing concentrations have
been examined.   Finally, the effect of atropine and tubo-

curarine, well known acetylcholine antagonists, have also
been studied.

Results

The effect of sodium malonate on the stimulated incorporation
of P^{32} into phospholipids of brain slices

The addition of 2 $\underline{mM}$ malonate slightly decreases
the oxygen uptake of brain slices incubated with glucose but
completely abolishes the potassium-stimulated oxygen uptake
as well as that of the calcium-free stimulation, although
to a lesser extent (Table X).   This is in agreement with
previous reports in the literature (119,120,121).   The in-
corporation of P^{32} into phospholipids is not altered signi-
ficantly at this concentration but contrary to a previous
finding (104) there is a definite inhibition when higher
concentrations such as 5 and 10 $\underline{mM}$ malonate are used.   In
the presence of 100 $\underline{mM}$ K^+ or in the absence of Ca^{++} ions
there is a greater inhibition of the incorporation of P^{32}
by malonate than is demonstrated in the unstimulated tissue.
In fact, not only is the cationic stimulation abolished by
malonate, but the total amount of P^{32} incorporated is re-
duced to lower values than those of the unstimulated slices.
Since malonate inhibits the oxidation of succinate in brain

TABLE X

EFFECT OF MALONATE ON THE STIMULATED INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS
OF RAT BRAIN SLICES IN RESPONSE TO 0.1M K^+, Ca^{++}FREE OR ACETYLCHOLINE

Malonate concn. (mM)	Incorporation of P^{32} into phospholipids*		Per cent of control	Oxygen uptake (µl/mg. dry weight)		Per cent of control	Incubation time (min.)
	Control	+KCl(0.1M)		Control	+KCl(0.1M)		
0	10.8	16.7	155	5.0	8.5	170	
2	10.5	11.1	105	4.8	5.0	104	30
5	6.5	4.4	68	3.4	2.9	86	
	Control	Ca^{++}free	%	Control	Ca^{++}free	%	
0	12.3	16.4	133	4.8	6.5	135	
2	11.3	13.0	115	4.5	5.6	124	30
5	7.8	7.1	91	3.4	3.5	103	
	Control	+ACh	%	Control	+ACh	-	
0	30.0	51.0	170	20.2	22.0	-	
2	28.4	39.6	139	18.6	18.9	-	120
5	19.5	24.9	127	12.2	12.5	-	
10	8.9	9.3	104	10.0	9.9	-	

Experimental conditions as in Table I.   Acetylcholine (ACh) 2 mM was added with eserine 3×10^{-4}M.
Results represent mean values obtained from 3 experiments.
*Incorporation of P^{32} expressed as in Table I.

slices by competing with succinate for the enzyme succinic dehydrogenase (206), it makes it a potent inhibitor of the citric acid cycle. These results indicate, therefore, the importance of the role played by the citric acid cycle in the stimulated incorporation of P^{32} into phospholipids.

The inhibitory effect of malonate on the stimulated P^{32} incorporation by acetylcholine follows a similar pattern to the results obtained in the presence of the cationic stimulation although the inhibitory effect is less marked. The presence of 10 mM malonate completely blocks the acetylcholine stimulation. However, even at this high malonate concentration, the amount of P^{32} incorporated into phospholipids in the presence of acetylcholine is not lower than that of the control value, contrary to what is observed with the cationic-stimulated tissue. The inhibition of oxygen consumption by malonate is the same in the presence or the absence of acetylcholine.

The effect of iodoacetate and fluoride on the stimulated incorporation of P^{32} into phospholipids of brain slices

The effect of iodoacetate and fluoride, two strong glycolytic inhibitors, was then investigated with special emphasis being placed on the stimulatory effect of acetylcholine. The results are shown in Table XI. In

TABLE XI

EFFECT OF IODOACETATE AND FLUORIDE ON THE STIMULATED INCORPORATION OF P^{32} INTO
PHOSPHOLIPIDS OF BRAIN SLICES IN RESPONSE TO 0.1M K^+ OR ACETYLCHOLINE

Inhibitors Conc. (M)	Incorporation of P^{32} into phospholipids*		Per cent of control	Oxygen uptake (µl/mg. dry weight)		Per cent of control	Incubation time (min.)
	Control	+KCl(0.1M)		Control	+KCl(0.1M)		
None —	10.7	17.1	160	4.9	8.3	170	
Iodoacetate 10^{-5}	9.0	12.3	136	4.8	7.7	160	30
Iodoacetate 10^{-4}	5.2	2.5	48	4.3	3.6	83	
	Control	+ACh	%	Control	+ACh	—	
None —	31.5	51.6	164	18.8	19.8	—	
Iodoacetate $5x10^{-5}$	20.0	28.8	144	15.3	14.6	—	
Iodoacetate 10^{-4}	12.7	13.4	105	9.8	9.4	—	120
Na fluoride 10^{-3}	23.6	33.5	142	17.3	17.5	—	
Na fluoride $5x10^{-3}$	11.0	12.7	114	10.1	12.2	—	

Experimental conditions as in Table I.   Acetylcholine (ACh) 2 mM was added with eserine $3x10^{-4}$M.
Results represent mean values obtained from 3 experiments.
*Incorporation of P^{32} expressed as in Table I.

agreement with the observation of Strickland (104), iodo-
acetate and fluoride exert a marked inhibitory effect on the
incorporation of P^{32} into the phospholipids of unstimulated
brain slices.   Iodoacetate (10^{-4}M) inhibits the oxygen up-
take by about 12 per cent when the slices are incubated for
30 minutes (experiments with 100 $\underline{m}$M K^+) whereas the incor-
poration of P^{32} into phospholipids is decreased by 52 per
cent.   After a two hour incubation period (experiments with
ACh) the rate of oxygen uptake, as well as the incorporation
of P^{32} is markedly decreased.   These results illustrate
the well known uncoupling property of iodoacetate.   Simi-
larly to the stimulated oxygen consumption, the potassium-
stimulated incorporation of P^{32} is much more sensitive to
iodoacetate than the unstimulated P^{32} incorporation resulting
in a complete blocking of the potassium stimulatory effect.
Moreover, the amount of P^{32} incorporated into the phospho-
lipids is decreased to the endogenous level in the stimu-
lated tissue when 10^{-4}M iodoacetate is used.   The acetyl-
choline stimulatory effect is less sensitive to iodoacetate
than the cationic stimulation, a result similar to that ob-
tained with malonate.   Nevertheless, the acetylcholine
stimulation of P^{32} incorporation is completely blocked by
10^{-4}M iodoacetate.   It can also be seen that fluoride
(10^{-3} or 5×10^{-3}M) exercises a profound inhibitory effect on

the acetylcholine stimulation of P32 incorporation into phospholipids.

The effect of amytal and chloretone on the stimulated incorporation of P32 into phospholipids of brain cortex slices.

It is well known that narcotics cause reduced respiration in the central nervous system *in vivo* (207, 208). Similarly, pharmacological concentrations of narcotics produce an analogous effect *in vitro* by uncoupling phosphorylation from oxidation (209), resulting in suppression of the stimulation of neuronal respiration which is brought about by the addition of 100 mM K$^+$ ions or the omission of Ca^{++} ions from the incubation medium (122,123). In view of these considerations, the effect of amytal and chloretone on the cationic and acetylcholine-stimulated P32 incorporation into phospholipids was examined.

In agreement with earlier studies (104,201), the results demonstrate (Table XII) that at the concentration used, amytal and chloretone have only a slight inhibitory effect on the labelling of phospholipids in unstimulated slices. In the presence of 100 mM K$^+$ ions (or omission of Ca^{++}) the addition of either of these narcotics exerts a marked inhibition of the phospholipid labelling, and the cationic stimulation is almost completely abolished in a

TABLE XII

EFFECT OF AMYTAL AND CHLORETONE ON THE STIMULATED INCORPORATION OF P^{32} INTO PHOS-PHOLIPIDS OF BRAIN SLICES IN RESPONSE TO 0.1M K^+, Ca^{++} FREE OR ACETYLCHOLINE

Inhibitors	Incub-ation time (min)	Incorporation of P^{32} into phospholipids*		Per cent inhibition		Oxygen uptake ($\mu l/mg.$ dry wt.)	
		Control	+KCl(0.1M)	Control	+KCl(0.1M)	Control	+KCl(0.1M)
None		10.8	17.2	-	-	5.2	9.1
Amytal 0.5 mM	30	9.2	10.3	15	40	3.7	4.0
Chloretone 2 mM		8.6	9.0	20	48	3.1	3.0
		Control	Ca^{++}free	Control	Ca^{++}free	Control	Ca^{++}free
None		10.1	13.5	-	-	5.0	7.1
Amytal 0.5 mM	30	8.2	9.6	19	29	3.8	4.3
Chloretone 2 mM		8.0	8.3	21	38	3.0	3.2
		Control	+ACh	Control	+ACh	Control	+ACh
None		30.9	50.7	-	-	19.8	22.0
Amytal 0.5 mM	120	24.2	31.6	21	38	14.4	14.0
Chloretone 2 mM		23.4	25.9	24	49	11.1	11.3

Experimental conditions as in Table I.   Acetylcholine (ACh) 2 mM was added with eserine 3×10^{-4}M. Results represent mean values obtained from 2 experiments.

*Incorporation of P^{32} expressed as in Table I.

manner parallel to the inhibition of oxygen uptake. Similarly, the acetylcholine-stimulated P^{32} incorporation is completely suppressed by the addition of 0.5 $\underline{mM}$ amytal or 2 $\underline{mM}$ chloretone.

The effect of ethanol on the stimulated incorporation of P^{32} into phospholipids of brain slices

Recent investigations have shown that the addition of ethanol at small concentrations diminishes the oxygen uptake of rat brain cortex slices respiring in glucose when they have been stimulated by the addition of 100 $\underline{mM}$ K^+ ions (123,210) or by electrical impulses (211,212). In this respect the inhibition produced by ethanol is similar to that brought about by other narcotics. However, contrary to the action of other narcotics, ethanol slightly stimulates the normal respiration of brain slices at a concentration of the same order as that necessary to cause narcosis in the rat (123,210). Moreover, ethanol has not been observed to act as an uncoupling agent of oxidative phosphorylation (213) and it does not affect mitochondrial respiration in pharmacologically active concentrations (210). Nevertheless, it has been suggested that ethanol may interfere with ATP formation in brain cortex slices since it inhibits glycine incorporation into brain proteins (217). It was

of interest, therefore, to study the effect of ethanol on
the incorporation of P^{32} into phospholipids since this bio-
chemical process is very sensitive to agents which interfere
with the metabolism of energy-rich phosphates.

The results are presented in Tables XIII and XIV.
It can be seen that at the concentrations tested (0.2 - 0.8M),
ethanol has no inhibitory effect upon the unstimulated oxygen
uptake.   However, when 0.6 and 0.8M ethanol are employed,
a small but consistent inhibition of P^{32} incorporation oc-
curs with the unstimulated tissue.   In the presence of 100 mM
potassium ions, an inhibition of the stimulated oxygen con-
sumption as well as P^{32} incorporation is obtained with 0.2M
ethanol.   The inhibitory effect under these conditions in-
creases markedly with increasing concentrations of ethanol,
and the potassium stimulation is almost abolished at 0.8M
ethanol.   In the presence of 2 mM acetylcholine, the ad-
dition of ethanol exerts an inhibitory effect on the sti-
mulated incorporation of P^{32} even at low concentrations
(Table XIV).   Here again the acetylcholine effect is not
as sensitive as the potassium effect.

TABLE XIII

EFFECT OF ETHANOL ON THE INCORPORATION OF P^{32} INTO THE PHOSPHOLIPIDS OF BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF KCl (0.1M)

Ethanol concn. (M)	Incorporation of P^{32} into phospholipids*		Per cent inhibition of K^+ stimulation	Oxygen uptake (μl/mg. dry wt/30 min)	
	Control	+KCl(0.1M)		Control	+KCl(0.1M)
0	11.8	19.5	-	5.2	9.0
0.2	12.6	17.9	31	5.7	8.6
0.4	12.0	15.7	52	5.3	7.8
0.6	10.9	13.6	65	5.2	7.0
0.8	9.9	10.9	87	5.0	5.9

Rat brain cortex slices were incubated for 30 minutes in modified Krebs medium III with glucose 10 mM, $NaH_2P^{32}O_4$ 20 μc. and ethanol as indicated.

Results represent mean values obtained from 3 experiments.

*Incorporation of P^{32} expressed as in Table I.

<center>TABLE XIV</center>

EFFECT OF ETHANOL ON THE INCORPORATION OF P^{32} INTO THE PHOSPHOLIPIDS

OF BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF ACETYLCHOLINE

Ethanol conc. (M)	Incorporation of P^{32} into phospholipids*		Per cent of inhibition of ACh stimulation	Oxygen uptake (μl/mg./ dry wt/30 min)	
	Control	+ACh		Control	+ACh
0	36.8	57.0	-	19.1	21.5
0.2	36.9	54.8	11	19.8	20.5
0.4	35.0	49.3	30	19.5	19.6
0.6	31.5	42.0	48	18.6	19.0
0.8	29.4	37.1	62	18.4	18.8

Rat brain cortex slices were incubated for 2 hours in modified Krebs medium III with glucose 10 mM, $NaH_2P^{32}O_4$ 20 μc. and ethanol as indicated.

Acetylcholine (ACh) 2 mM was added with eserine 3×10^{-4}M.

Results represent mean values obtained from 4 experiments.

*Incorporation of P^{32} expressed as in Table I.

The effect of atropine, hyoscine and tubocurarine on the
stimulated incorporation of P32 into phospholipids of brain
slices.

Hokin and Hokin (47) demonstrated that atropine
abolishes the stimulatory effect of acetylcholine on the
incorporation of P^{32} into the phospholipids of guinea-pig
brain slices. In view of the similarity observed between
the cationic and acetylcholine stimulation of P^{32} incor-
poration, a study of the effects of atropine and some re-
lated compounds on the cationic stimulation of P^{32} incor-
poration was undertaken. The results given in Table XV
corroborate the findings of Hokin and Hokin. They indicate
that low concentrations of atropine have no effect on the
incorporation of P^{32} into phospholipids whereas high con-
centrations stimulate P^{32} incorporation. It can also be
seen that the stimulatory effect of acetylcholine is in-
hibited by atropine, although the inhibition is not as
marked as reported by the above authors. A possible explan-
ation for the lowered sensitivity to atropine could be that
the concentration of acetylcholine used in the present ex-
periments is 20 times greater than that used by Hokin and
Hokin (47). As might be expected, hyoscine exerts an
effect similar to that produced by atropine.

In the presence of 100 mM potassium or in the

TABLE   XV

EFFECT   OF   ATROPINE   AND   HYOSCINE   ON   THE   STIMULATED   INCORPORATION   OF P^{32}

INTO   PHOSPHOLIPIDS   OF   BRAIN   SLICES   IN   RESPONSE   TO   ACETYLCHOLINE

Additions	Concn. (M)	Incorporation of P^{32} into phospholipids*		Per cent inhibition of ACh stimulation	Oxygen uptake (µl/mg. dry wt./ 120 min)	
		Control	+ ACh		Control	+ ACh
None	–	29.0	46.1	–	19.4	20.5
Atropine	2×10^{-5}	28.1	38.5	40	19.0	20.3
Atropine	10^{-4}	34.2	41.8	55	19.6	19.4
Atropine	2×10^{-3}	43.0	48.5	68	18.8	19.0
Hyoscine	2×10^{-5}	29.5	41.1	32	19.5	19.5
Hyoscine	10^{-4}	33.8	43.2	45	19.1	20.0

Experimental conditions as in Table I.   Incubation time 2 hours.

Acetylcholine 2 mM was added with eserine 3×10^{-4}M.

Results represent mean values obtained from 2 experiments.

*Incorporation of P^{32} expressed as in Table I.

absence of calcium ions (Table XVI), atropine and hyoscine exert no inhibitory effect on either the stimulated oxygen uptake or incorporation of P^{32} into phospholipids. On the contrary, the stimulatory effect produced by $10^{-3}M$ atropine on the incorporation of P^{32} into phospholipids was additive to that produced by 100 mM potassium or calcium-free medium.

Table XVII shows that tubocurarine has no major effect either on the oxygen uptake or the incorporation of P^{32} into phospholipids of unstimulated cerebral cortex in concentrations between 2×10^{-5} and $10^{-3}M$. The effect of this compound on the potassium-stimulated oxygen uptake or on the enhanced P^{32} incorporation was examined under conditions which gave a maximal stimulation. Again no consistent increase or decrease of stimulation in response to 100 mM potassium or to acetylcholine is observed.

DISCUSSION

It was concluded in the previous chapter that the stimulation of P^{32} incorporation into phospholipids is closely related to the stimulation of oxidative metabolism brought about by the addition of 100 mM K^+ ions (or omission of Ca^{++} ions), and that the factors which support this increment resemble those which support the acetylcholine effect. In the present studies, the effects of metabolic

TABLE XVI

EFFECT OF ATROPINE AND HYOSCINE ON THE STIMULATED INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF BRAIN SLICES IN RESPONSE TO K^+ 0.1M OR OMISSION OF CALCIUM

Additions	Concn. M	Incorporation of P^{32} into phospholipids*			Oxygen uptake (µl/mg. dry wt./30 min.)		
		Control	+KCl(0.1M)	Ca^{++}free	Control	+KCl(0.1M)	Ca^{++}free
None	-	10.5	16.0	13.9	5.3	8.8	7.1
Atropine	2×10^{-5}	10.0	16.4	14.3	5.1	8.7	7.1
Atropine	2×10^{-4}	13.1	18.0	16.5	5.4	8.8	6.9
Atropine	10^{-3}	14.3	19.0	17.3	5.0	8.3	6.8
Hyoscine	2×10^{-4}	11.8	16.9	-	5.3	8.6	-
Hyoscine	10^{-3}	13.5	17.5	-	5.1	8.2	-

Experimental conditions as in Table I.   Incubation time, 30 minutes.

Results represent mean values obtained from at least 3 experiments.

*Incorporation of P^{32} expressed as in Table I.

TABLE   XVII

EFFECT   OF   TUBOCURARINE   ON   THE   STIMULATED   INCORPORATION   OF P^{32} INTO   PHOSPHOLIPIDS   OR   BRAIN   SLICES   IN   RESPONSE   TO   0.1M K^+ OR   ACETYLCHOLINE

Tubocurarine concn. (M)	Incubation time (min.)	Incorporation of P^{32} into phospholipids[*]			Oxygen uptake (µl/mg. dry wt.)	
		Control	+ACh	% stimulation	Control	+ACh
0		29.1	44.0	51	19.2	19.9
2×10^{-5}		30.4	43.6	44	19.6	19.3
10^{-4}	120	27.0	41.5	54	19.4	19.9
5×10^{-4}		30.9	44.8	45	19.1	20.7
10^{-3}		29.4	42.0	43	19.4	18.6
		Control	+KCl(0.1M)	%	Control	+KCl(0.1M)
0		13.0	20.7	59	4.9	8.7
10^{-4}	30	13.4	21.0	57	5.0	8.9
10^{-3}		14.5	21.8	50	4.6	8.4

Experimental conditions as in Table I.   Acetylcholine 2 mM was added with eserine 3×10^{-4}M.
Results represent mean values obtained from at least 2 experiments.
[*]Incorporation of P^{32} expressed as in Table I.

inhibitors on the stimulated P^{32} incorporation have been investigated. The results definitely indicate that the stimulations produced by cations or acetylcholine are much more sensitive to the presence of metabolic inhibitors than are the unstimulated phenomena. The present results on the inhibition of the potassium-stimulated respiration and of the labelling of phospholipids in unstimulated slices are in general agreement with earlier studies (119,121,122,123, 210,104,179).

In the interpretation of the results obtained in studies of the effects of malonate on the normal and potassium (or calcium-free) stimulated incorporation of P^{32}, the finding of Quastel et al. (206) that malonate is a highly effective inhibitor of the oxidation of succinate in brain slices must be taken into consideration. This ability of malonate to compete with succinate for the enzyme succinic dehydrogenase makes it an effective inhibitor of the citric acid cycle. The inhibition of the incorporation of P^{32} into phospholipids in the normal and potassium-stimulated brain cortex slices by 5 mM malonate is therefore due to the lowered availability of oxaloacetate required for the metabolism of pyruvate through the citric acid cycle. This results in a decreased formation of the energy-rich phosphate compounds essential for the labelling and synthesis

of phospholipids. This point is illustrated by the ob-
servation of Parmar and Quastel (121) that the inhibition
by malonate of the potassium-stimulated respiration could
be reversed by the addition of oxaloacetate. The finding
that a low concentration of malonate (2 mM) does not signi-
ficantly affect the labelling of phospholipids in unsti-
mulated tissue implies that succinic dehydrogenase is not
greatly inhibited at this concentration.

However, the larger inhibition of P^{32} incorpor-
ation in K^+ - stimulated tissue caused by low and high con-
centration of malonate may not only be the result of the
suppression of oxaloacetate formation; the condensation of
oxaloacetate with acetyl-CoA being probably increased in the
K^+ - stimulated tissue due to the increased formation of
acetyl-CoA (138). It is not unreasonable to believe that
the increased inhibitory effect of malonate in stimulated
tissue may equally well be the consequence of an increased
depletion of ATP caused by the additive effects of 100 mM
K^+ ions (or Ca^{++}-free) and by malonate (or other inhibitors).
It was shown in the first chapter that the addition of
100 mM K^+ ions (or omission of Ca^{++}) brings about a 20 per
cent decrease in the level of 7 mnp. (ATP). It seems
likely, therefore, that a tissue which is already being
depleted of its energy-rich phosphate compounds to a certain

extent will respond more drastically to a concomittant blocking of the formation of these compounds. The additive effect of the cationic depletion of ATP and the inhibition of ATP synthesis by malonate would result in a very low level of ATP in the tissue, resulting in a striking inhibition of P^{32} incorporation into phospholipids as well as of oxygen consumption. In fact, the P^{32} incorporation in the cationic-stimulated slices in the presence of 5 mM malonate was of the same order as that obtained in the complete absence of energy producing substrates (Table V). This hypothesis is further supported by the observation of Kini and Quastel (138) that the increased rate of formation of radioactive glutamine from glucose-U-C^{14}, due to added 100 mM K^+ ions, is much more inhibited by malonate than the normal rate.

The decrease of the acetylcholine-stimulated P^{32} incorporation by the addition of malonate illustrates the ATP requirement of the acetylcholine effect. If the hypothesis of Hokin and Hokin (52) is correct that the phospholipid effect in response to acetylcholine is connected with the transport of Na^+ ions during the recovery process following depolarization, although the mechanism by which this phenomenon is occurring is still at present obscure, the malonate inhibitory effect can be tentatively

explained. According to Hokin and Hokin (51), phosphatidic acid carries Na^+ ions across the membrane and is involved in a cycle where it is hydrolized to diglyceride by phosphatidic acid phosphatase and subsequently resynthesized through a diglyceride kinase which catalyses the reaction between ATP and the diglyceride. An increased activity of this cycle results in an increased turnover of p^{32} into phosphatidic acid. Therefore, an inhibition of ATP formation by malonate would result in an inhibition of the above cycle with a concomittant decrease in the acetylcholine stimulatory effect. Another possible explanation for the inhibitory effect of malonate arises from the conclusion of Keynes (188) that metabolic energy is necessary to drive the recovery process and therefore to maintain a high potassium low sodium level inside the cell. The depletion of energy-rich phosphates would thus cause an inhibition of this phenomenon with a concomittant decrease in the acetylcholine-effect due to its depolarizing property. The finding that the acetylcholine-stimulated incorporation of p^{32} is less sensitive to the inhibitory effect of malonate (or other inhibitors) than the cationic stimulation could be explained by the fact that the acetylcholine effect is not accompanied by a concomittant fall in the level of 7mnp. (ATP) (Table XXI), contrary to what is observed with

the addition of 100 $\underline{m}\underline{M}$ K$^+$ or the omission of Ca^{++} ions
(Table II).

The presence of iodoacetate or fluoride exercises
a considerable inhibitory effect on the incorporation of
P^{32} into phospholipids in the normal brain cortex slices,
while in the stimulated slices these compounds cause an
even greater inhibition. They also abolish the response
of brain slice to acetylcholine. These effects are pro-
bably due to the inhibitory action of iodoacetate and fluo-
ride on the glycolytic cycle causing a depletion of high-
energy phosphate compounds, resulting in effects similar to
those observed with malonate.

The results obtained with amytal and chloretone
illustrate the interference of these narcotics with ATP
synthesis in the brain. Earlier studies demonstrated the
inhibitory effect of these narcotics on ATP dependent pro-
cesses such as acetylcholine synthesis (214), P^{32} incor-
poration into phospholipids (104) or phosphoproteins (215)
and glutamine synthesis (136). It has been well estab-
lished that amytal is a highly effective inhibitor of the
oxidation of DPNH and its associated phosphorylation (216).
It therefore appears likely that in the potassium-stimulated
tissue, where the operation of the citric acid cycle is
enhanced and the level of ATP (7mnp.) diminished, the pre-

sence of narcotics is bound to exercise larger inhibition of phospholipid labelling than in the unstimulated tissue.

The inhibitory effect of high concentrations of ethanol on the unstimulated P^{32} incorporation and of low concentrations on the stimulated P^{32} incorporation indicate the interference of this compound with the metabolism of energy-rich phosphates. This observation is in agreement with the finding of Lindan et al. (217) that ethanol at narcotic concentrations inhibits glycine incorporation into brain proteins. Ethanol has also been shown to inhibit the K^+-stimulated formation of radioactive glutamine from glucose $-U-C^{14}$ (128,218).

It was found that atropine abolishes the acetylcholine-stimulated P^{32} incorporation into phospholipids, whereas the potassium-stimulated incorporation or oxygen uptake was not affected. The lack of effect of atropine on the potassium-stimulated respiration is in agreement with the earlier finding of McIlwain (219) who also reported that the electrical stimulation of oxygen uptake is almost completely abolished by atropine. The results with atropine suggest that although 100 $\underline{mM}$ K^+ ions (or Ca^{++}-free) and acetylcholine produce an analogous effect on the incorporation of P^{32} into the phospholipids, the cationic stimulatory effect is not a consequence of the participation of

the acetylcholine system.   This latter hypothesis had
arisen from the observations that the addition of potassium
ions or the omission of calcium ions from the incubation
medium stimulates the acetylcholine synthesis in brain
slices (124,125).   Therefore, atropine (or hyoscine) a
highly specific pharmacological antagonist of acetylcholine,
should have abolished not only the acetylcholine-stimulated
incorporation of P^{32} into phospholipids but also the cationic
stimulated incorporation.   A further point of interest
arises from the results obtained with atropine.   They con-
firm and emphasize the existence of an uncommon dissimil-
arity between the cationic and electrical impulses properties.

The lack of effect of tubocurarine on the acetyl-
choline stimulation was somewhat surprising and is dif-
ficult to explain in view of the well known antagonism
between these two compounds.   Perhaps this may be due to
low permeability of the slices to tubocurarine.

Summary

1.   The action of some typical inhibitors of the
          respiratory and glycolytic cycles have been investi-
          gated.   Addition of malonate, iodoacetate or fluoride
          brings about decreases in the labelling of phospho-
          lipids.   In the presence of 100 mM KCl or omission

of $CaCl_2$, the inhibitory effect of these agents is larger and brings the rate of P^{32} incorporation to the endogenous level. The acetylcholine stimulation of P^{32} incorporation is abolished in the presence of these metabolic inhibitors. However, this inhibitory effect is less accentuated than the one observed with the cationic stimulation.

2.   The addition of the narcotics, amytal or chloretone, at concentrations of 0.5 mM and 2 mM respectively, produces a slight decrease in the labelling of phospholipids from inorganic P^{32}. The enhanced P^{32} incorporation caused by 100 mM K^+ ions or acetylcholine is much more sensitive to these narcotics.

3.   The presence of narcotizing concentrations of ethanol has no effect on the phospholipid labelling of unstimulated tissue whereas it inhibits the potassium or acetylcholine-stimulated incorporation.

4.   Atropine abolishes the stimulatory effect of acetylcholine on the incorporation of P^{32} into the phospholipids, but does not affect the cationic stimulatory effect.

CHAPTER V

EFFECT OF MORPHINE AND OTHER DRUGS ON THE INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF BRAIN CORTEX SLICES

Introduction

The biochemical mechanism of action of morphine on brain cortex slices has been the subject of a considerable amount of study (220, 163). A striking feature that arises from these earlier studies is the almost complete inability of morphine to affect oxidative processes in brain in vitro. Morphine has been shown not to influence the oxidation of a number of respiratory substrates (157, 159), it does not uncouple oxidation from phosphorylation (160, 213), and it has no effect on the potassium-stimulated oxygen consumption of brain cortex slices (162, 163). In this connection, the effect of morphine differs from that of other narcotic agents which have a marked inhibitory effect on energy-dependent processes in vitro. It is believed, therefore, that the mode of action of morphine in vitro is different from that of barbiturates and other groups of narcotics (154).

A number of studies on the incorporation of P^{32} into the phospholipids of brain slices have revealed a very interesting phenomenon. Drugs like acetylcholine or atro-

pine (47), which have no particular effect on tissue
oxidative metabolism, or tranquilizers (108), at concent-
rations which do not affect the oxidative metabolism of
brain, have been shown to stimulate the labelling of phos-
pholipids from inorganic P^{32} in brain slices. Moreover,
a number of observations (165,166,167,170,171) suggest that
morphine may interact with the acetylcholine system in brain.
It was therefore decided to investigate the effect of mor-
phine on the incorporation of P^{32} into phospholipids of
brain slices in order to find a biochemical process which
would be influenced by morphine and which could be related
to the pharmacological properties of this narcotic.

Results

The effect of morphine on the incorporation of P^{32} into phospholipids of brain cortex slices in the presence and absence of acetylcholine

Quastel and Tennenbaum (170) have demonstrated
that morphine competes with acetylcholine for receptor groups
in leech muscle preparations.   It was shown in the previous
chapters, in agreement with the findings of Hokin and Hokin
(47), that acetylcholine stimulates the incorporation of P^{32}
into phospholipids of brain cortex slices.   It seemed of

interest to examine the action of morphine on the acetyl-
choline stimulatory effect, since this phenomenon appears
to be a direct consequence of acetylcholine action (52).

The effect of morphine on the incorporation of
P^{32} into phospholipids and on the acetylcholine-stimulated
P^{32} incorporation are illustrated in Table XVIII.   The
concentration range of morphine is from 0.2 to 10 mM.   It
can be seen that morphine has no effect on the oxygen uptake
of brain slices; this observation is in agreement with the
results of Elliott et al. (159).   At low concentrations
(0.2, 2 mM), no effect is observed on the incorporation of
P^{32} into phospholipids. At a higher concentration (5 mM),
morphine causes a stimulation of P^{32} incorporation of ap-
proximately 30 per cent and this enhancement of phospho-
lipid labelling is increased to approximately 48 per cent
in the presence of 10 mM morphine.   In fact the stimulation
of P^{32} incorporation at this latter concentration is of the
same order as that obtained with 2 mM acetylcholine.   It
can also be seen that in the presence of 0.2 and 2 mM mor-
phine, the observed stimulation of P^{32} incorporation by
acetylcholine is not affected.   At higher concentrations
(5 or 10 mM) the stimulation produced by morphine is ad-
ditive to that produced by acetylcholine resulting in a
very marked increase in the incorporation of P32 into

TABLE XVIII

EFFECT OF MORPHINE ON THE INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES IN THE ABSENCE AND PRESENCE OF ACETYLCHOLINE

Morphine concn. (mM)	No. of expts.	Oxygen uptake (µl/mg. dry wt./2 hrs.) (Control)	Incorporation of P^{32} into phospholipids*	
			Control	+ ACh
0	6	19.5	31.3 (100)	47.0 (150)
0.2	2	19.1	30.1 (97)	48.1 (154)
2	5	19.8	32.5 (104)	45.9 (146)
5	2	19.4	40.8 (130)	53.6 (171)
10	4	18.0	46.5 (148)	60.4 (193)

Rat brain cortex slices were incubated at 37° in modified Krebs medium III with 10 mM glucose for 2 hours. All vessels contained 20 µc $NaH_2P^{32}O_4$. Acetylcholine (ACh) 2×10^{-3}M was added with eserine 3×10^{-4}M.

The figures in parentheses refer to percentages of the normal control value without morphine or acetylcholine.

*Incorporation of P^{32} expressed as in Table I.

phospholipids in the presence of these two compounds.

These results indicate that morphine exerts a similar effect as acetylcholine on the labelling of phospholipids from inorganic P^{32} and that it does not antagonize the stimulatory effect of acetylcholine on this labelling.

Effect of morphine on the potassium-stimulated incorporation of P^{32} into phospholipids

It has been shown previously that morphine has no effect on the uptake of glucose and on the oxygen consumption in potassium-stimulated brain cortex slices (162, 163). However, because of the great sensitivity of the phospholipid labelling process as observed with metabolic inhibitors (Chapter IV), and because of the stimulatory effect produced by morphine on the incorporation of P^{32} as observed above, preliminary studies of the effect of morphine on the K^{+}-stimulated incorporation of P^{32} into phospholipids were undertaken. It was observed that 2 or 10 mM morphine does not inhibit the K^{+}-stimulated incorporation of P^{32} into phospholipids. On the contrary, the K^{+}-stimulated P^{32} incorporation was always further increased by the addition of morphine, even at a low concentration such as 2 mM, which has no effect on the incorporation of P^{32} in unstimulated tissue. Moreover, this stimulatory effect of morphine

appeared to increase with longer periods of incubation.
These observations prompted further investigations regarding
the stimulatory effect of morphine on the incorporation of
P^{32} into phospholipids of K^+-stimulated slices.

A time course of the effect of 2 $\underline{mM}$ morphine on
the incorporation of P^{32} into phospholipids of K^+-stimulated
slices was performed. The results are illustrated in
Figure 5. It can be seen, as previously shown (Fig. 3),
that the incorporation of P^{32} in the unstimulated tissue
increases with the duration of the incubation period. With
the addition of 2 $\underline{mM}$ morphine, the incorporation of P^{32} is
not affected. In the K^+-stimulated slices, a marked incr-
ease of approximately 60 per cent is obtained after a 30
minute incubation. The K^+-stimulated incorporation of P^{32}
subsequently decreases with time, and after 2 hours incub-
ation it is negligible. After 3 hours incubation, the P^{32}
incorporation in the presence of 100 $\underline{mM}$ K^+ ions is inhibited
as compared to the normal P^{32} incorporation in the control
medium.

When 2 $\underline{mM}$ morphine is added to the potassium-rich
medium a striking effect is observed. It appears as if the
decrease of the K^+-stimulated incorporation of P^{32}, which
occurs with the duration of the incubation period, is in-
hibited by morphine. Indeed, after a 30 minute incubation

FIGURE 5

TIME COURSE OF THE EFFECT OF 2mM MORPHINE ON THE
INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF BRAIN
SLICES IN THE PRESENCE AND ABSENCE OF 0.1 M KCl
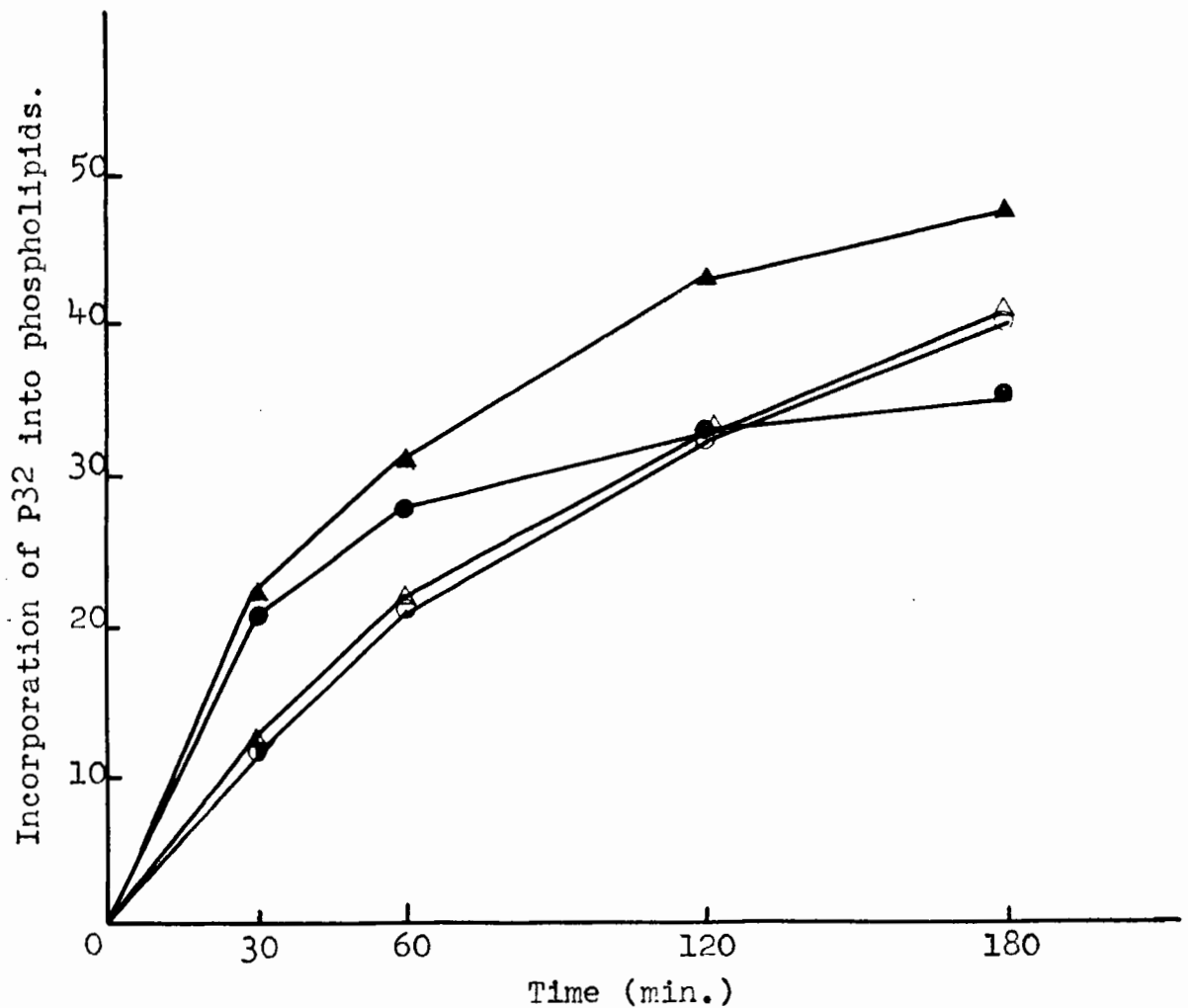
Rat brain cortex slices were incubated at 37°C in modified
Krebs medium III with 10mM glucose and 20 μc. NaH$_2$P^{32}O$_4$.
Additions: O , nil; △ , 2mM morphine;
●, 0.1M KCl; ▲, 2mM morphine and 0.1M KCl.
Incorporation of P^{32} into phospholipids expressed as in
Table I.

when the K^+-stimulated P^{32} incorporation is at its maximum
(60%), the addition of morphine only produces a slight
stimulation (10%). After 60 minutes incubation, when the
K^+-stimulated P^{32} incorporation is decreased to approxi-
mately 30 per cent, the presence of morphine produces a
total stimulation of 50 per cent. After 2 hours incub-
ation, the K^+-stimulated incorporation is negligible. How-
ever, in the presence of morphine a stimulation of approxi-
mately 40 per cent is observed in the labelling of phos-
pholipids.

The stimulatory effect produced by the addition
of morphine to K^+-stimulated slices was further tested
through a concentration range of 0.2 to 10 mM and the re-
sults are shown in Table XIX. The emphasis was placed on
experiments performed after a 2 hour incubation since at
this time the stimulation of P^{32} incorporation produced
by 100 mM K^+ ions is not occurring and therefore does not
interfere with the stimulation produced by morphine. It
can be seen (Table XIX) that morphine at concentrations of
0.2 and 2 mM exerts no effect on the incorporation of P^{32}
in the unstimulated tissue. In the presence of 100 mM
potassium ions, the addition of 0.2 and 2 mM morphine
causes stimulations of 17 and 40 per cent respectively.
With 5 and 10 mM morphine, stimulations of P^{32} incorporation

TABLE XIX

EFFECT OF MORPHINE ON THE INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF KCl (0.1M)

Morphine concn. (mM)	Incubation time 60 minutes			Incubation time 120 minutes		
	Incorporation of P^{32} into phospholipids[*]			Incorporation of P^{32} into phospholipids[*]		
	No. expt.	Control	+KCl (0.1M)	No. expt.	Control	+KCL (0.1M)
0	4	21.0 (100)	27.3 (130)	16	30.5 (100)	29.4 (96)
0.2	2	20.1 (96)	27.8 (132)	4	30.8 (101)	35.6 (117)
2.0	4	22.8 (108)	32.0 (152)	13	31.7 (105)	42.5 (140)
5.0		-	-	2	39.9 (131)	48.5 (159)
10.0	2	28.5 (136)	38.7 (184)	4	45.0 (147)	54.0 (177)

Rat brain cortex slices were incubated at 37° C in modified Krebs medium III with glucose 10 mM for the period of time indicated.

All vessels contained 20 µc. $NaH_2P^{32}O_4$.   Morphine and KCl 0.1M were added as indicated.

The figures in parentheses refer to percentages of the normal control value without morphine and KCl.

[*]Incorporation of P^{32} expressed as in Table I.

of 31 and 47 per cent respectively are obtained in the unstimulated tissue and these increments are further increased to 59 and 77 per cent respectively in the K^+-stimulated tissue.

It can also be seen that in experiments carried out during a 60 minute incubation period, where the presence of 100 mM K^+ ions produces a 30 per cent stimulation of the incorporation of P^{32} into phospholipids, the addition of 2 and 10 mM morphine further increases this stimulation caused by 100 mM K^+ ions. However, after a 60 minute incubation period, the stimulatory effect produced by morphine is smaller than that observed after 120 minutes of incubation.

The effect of various concentrations of morphine on the K^+-stimulated oxygen consumption during the experiments described above is shown in Fig. 6. The inability of morphine to markedly influence respiratory process even at a high concentration (10 mM) is clearly illustrated.

The above results indicate that morphine exerts a stimulatory effect on the incorporation of P^{32} into the phospholipids of K^+ stimulated slices. Two possible interpretations of this morphine effect arise from the above experiments; morphine is either acting directly on the labelling of phospholipids in K^+ stimulated tissue and the

FIGURE 6

TIME COURSE OF THE EFFECT OF MORPHINE ON THE
OXYGEN UPTAKE OF BRAIN CORTEX SLICES IN THE
PRESENCE AND ABSENCE OF 0.1M KCl
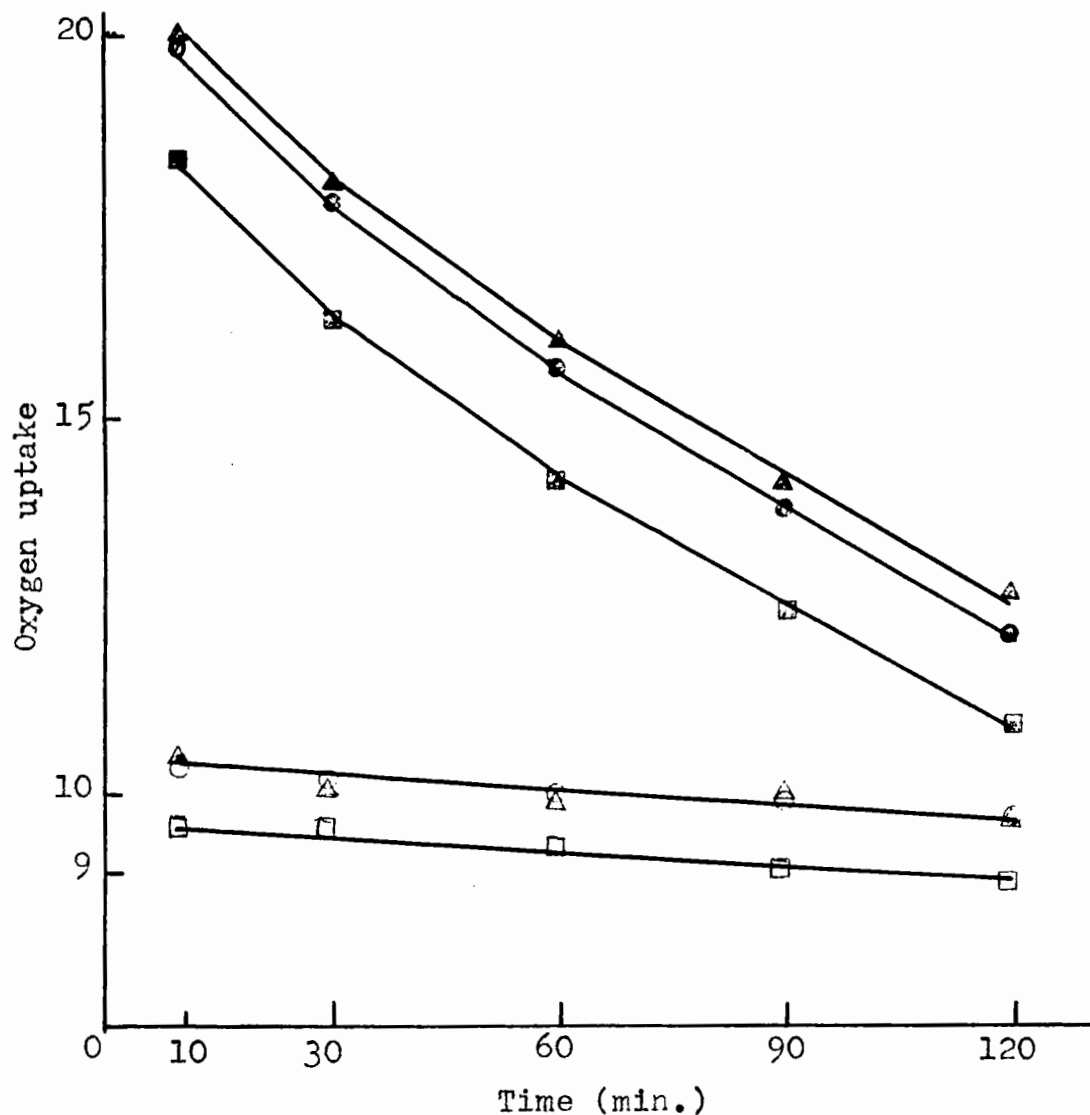
Rat brain cortex slices were incubated at 37°C in modified
Krebs medium III with 10mM glucose.
Additions: o, nil; △, 2mM morphine; □ , 10mM morphine;
●, 100mM KCl; ▲ , 100mM KCl + 2mM morphine; ■ , 100mM KCl +
10mM morphine.
Oxygen uptake expressed as $-QO_2$ for the time intervals
indicated.

magnitude of the effect is increasing with time, or morphine is acting indirectly, i.e. by impeding the decrease of the K^+-stimulated P^{32} incorporation itself which occurs with time (Fig. 5).   Subsequent studies were undertaken in order to elucidate this effect of morphine in K^+-stimulated slices.

The effect of eserine on the incorporation of P^{32} into phospholipids in the presence and absence of 100 mM potassium.

Mann et al. (124) have shown that increasing the potassium ion concentration in the medium surrounding brain cortex slices stimulates the rate of acetylcholine synthesis.   It is also well established that morphine inhibits the hydrolysis of acetylcholine by brain cholinesterase (163, 165,166).   It was thought that the stimulatory effect of morphine in the presence of 100 mM potassium ions could be the result of an acetylcholine effect, resulting from an increased synthesis of acetylcholine due to potassium ions and from the inhibition of cholinesterase by morphine.   In fact, the morphine stimulatory phenomenon in K^+-stimulated tissue possesses some characteristics of the acetylcholine effect.   2 mM morphine, like acetylcholine, causes a small but consistent increase in oxygen uptake in K^+-stimulated tissue (Fig. 6) and the magnitude of the morphine stimulatory effect on P^{32} incorporation gradually increases with

time in a manner similar to the stimulation produced by acetylcholine (47).

In view of these considerations, the effect of eserine, a typical anticholinesterase drug, on the incorporation of P^{32} into phospholipids of K^+-stimulated slices was studied. The results are shown in Table XX. It can be seen that eserine (0.15 to 5 $\underline{mM}$) has no effect either on the unstimulated or stimulated oxygen uptake of brain cortex slices. At concentrations of 1.5 and 5 $\underline{mM}$, eserine stimulates the incorporation of P^{32} into phospholipids by approximately 20 per cent. In the presence of 100 $\underline{mM}$ K^+ ions, contrary to what is observed with morphine, eserine does not produce a further increase in phospholipid labelling. These results suggest that the stimulatory effect of morphine in the presence of 100 $\underline{mM}$ K^+ ions does not occur according to the hypothesis postulated above in the first paragraph.

The effect of morphine on the concentration of 7-minute nucleotide phosphate

In view of the negative results obtained with eserine, it was decided to determine if the stimulatory effect of morphine in the presence of 100 $\underline{mM}$ K^+ ions might not be the result of an inhibition of the decrease of the

TABLE XX

EFFECT OF ESERINE ON THE INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF

BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF KCl (0.1M)

Expt. No.	Eserine concn. (mM)	Incorporation of P^{32} into phospholipids*		Oxygen uptake (µl/mg. dry weight/2 hours)	
		Control	+ KCl (0.1M)	Control	+ KCl (0.1M)
1	-	32.4 (100)	34.0 (105)	19.8	25.1
	0.15	34.0 (105)	33.5 (103)	19.5	24.3
	1.5	40.1 (124)	38.7 (120)	18.6	26.0
	5.0	39.3 (121)	41.0 (126)	18.0	27.3
2	-	29.1 (100)	27.8 (95)	19.4	24.5
	0.15	30.4 (104)	29.3 (101)	19.6	25.3
	1.5	34.6 (119)	32.0 (110)	19.0	25.4
	5.0	36.1 (124)	33.9 (116)	18.4	26.7

Rat brain cortex slices were incubated for 2 hours as described in Table XIX.

Eserine and KCl 0.1M were added as indicated.

The figures in parentheses refer to percentages of the normal control value without eserine and KCl.

*Incorporation of P^{32} expressed as in Table I.

potassium-stimulated P^{32} incorporation, since the morphine
stimulation increases when the potassium stimulation de-
creases (Fig. 5). It was shown in the first chapter that
the decrease in the potassium-stimulated P^{32} incorporation,
which occurs with the duration of the incubation period,
is accompanied by a concomitant decrease in the concentration
of 7-minute nucleotide phosphate. It was, therefore, of
interest to study the effect of morphine on the level of
7-minute nucleotide phosphate in order to see if the mor-
phine stimulatory effect in K^+-stimulated slices was the
result of an increased level of 7mnp.

The results in Table XXI show that 2 mM morphine
has no effect on the concentration of 7mnp. in the unsti-
mulated tissue. When the 7mnp. level is decreased by ap-
proximately 35 per cent in the presence of 100 mM K^+ ions
after 2 hours incubation, the addition of morphine does not
produce any change in the level of 7-minute nucleotide
phosphate. Table XXI also shows the effect of 2 mM acetyl-
choline on the level of 7-minute nucleotide phosphate.
This compound has no effect.

P^{32} incorporation into phospholipids of brain slices from morphine-treated rats

The effect of morphine on the incorporation of

TABLE XXI

EFFECT OF MORPHINE ON THE LEVEL OF 7 MINUTE NUCLEOTIDE PHOSPHATE IN

RAT BRAIN CORTEX SLICES

Additions	7-minute nucleotide phosphate (μmoles/g. wet weight tissue)			
	Expt. 1	Expt. 2	Expt. 3	MEAN
Nil	1.5	1.68	1.55	1.58
Morphine 2 mM	1.64	1.5	1.73	1.62
Morphine 5 mM	1.48	-	-	-
KCl 100 mM	1.0	1.2	1.04	1.08
KCl 100 mM + Morphine 2 mM	1.12	1.21	1.15	1.16
KCl 100 mM + Morphine 5 mM	0.95	-	-	-
Acetylcholine 2 mM	1.52	1.57	1.68	1.59

Experimental conditions as described in Table XIX.

Incubation time 2 hours.

133

P^{32} into phospholipids of K$^+$-stimulated tissue was also
investigated with brain cortex slices from male rats which
had been treated with progressively increasing doses of
morphine for 10 days.   The results of three experiments
are shown in Table XXII.   There is no significant dif-
ference between the P^{32} incorporation into phospholipids in
either unstimulated or K$^+$-stimulated slices of morphine
treated rats and that of control rats.

The effect of nalorphine on the incorporation of P^{32} into phospholipids of brain slices in the presence and absence of 0.1 M KCl.

Nalorphine, whose chemical structure is closely
akin to that of morphine, has the ability of antagonizing
many of the pharmacological properties of morphine (220,
221).   It was of interest to investigate the action of
nalorphine on the morphine stimulatory effect in K$^+$-stimulated
tissue.

Results given in Table XXIII show that the ad-
dition of nalorphine at concentrations of 0.5 and 2 mM
exerts no effect on the incorporation of P^{32} into phospho-
lipids of normal brain slices.   With the K$^+$-stimulated
slices, after 2 hours incubation, nalorphine (0.5, 2mM)
stimulates the incorporation of P^{32} by approximately 15 and

TABLE XXII

THE EFFECT OF MORPHINE ON THE INCORPORATION OF P^{32}
INTO PHOSPHOLIPIDS OF K^+-STIMULATED BRAIN SLICES FROM
NORMAL AND MORPHINE-TREATED RATS

Expt.		Morphine added to vessels (mM)	Incorporation of P^{32} into phospholipids*	
			Control	+KCl 100 mM
1	Normal rats	-	28.1	26.2
		2	29.8	40.6
	Morphine-treated rats	-	31.4	31.6
		2	30.2	43.0
2	Normal rats	-	34.6	35.4
		2	37.0	46.4
	Morphine-treated rats	-	32.8	33.5
		2	35.7	47.3
3	Normal rats	-	30.5	29.3
		2	31.9	45.3
	Morphine-treated rats	-	31.2	32.8
		2	33.6	41.0
Mean Normal rats		-	31.1 (100)	30.3 (97)
		2	32.9 (106)	44.1 (142)
	Morphine-treated rats	-	31.8 (102)	32.6 (105)
		2	33.8 (108)	43.8 (140)

Brain cortex slices from normal or morphine treated rats were
incubated in modified Krebs medium III with glucose 10 mM,
$NaH_2P^{32}O_4$ 20µc, KCl 100 mM, and morphine 2 mM as indicated
for 2 hours.

Figures in parentheses refer to per cent of the control medium
(normal rats without added KCl or morphine).

*Incorporation of P^{32} expressed as in Table I.

TABLE XXIII

EFFECT OF NALORPHINE ON THE INCORPORATION OF P^{32} INTO PHOSPHO-
LIPIDS OF BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF
0.1M KCl AND 2 mM MORPHINE

Expt.	Additions		Incorporation of P^{32} into phospholipids[*]	
	Morphine (mM)	Nalorphine (mM)	Control	+ KCl (100 mM)
I	–	–	33.1 (100)	30.5 (92)
	2	–	34.2 (103)	44.5 (134)
	–	0.5	30.0 (91)	37.4 (113)
	–	2	31.1 (94)	47.0 (142)
	2	2	36.0 (109)	52.9 (160)
II	–	–	35.4 (100)	34.9 (98)
	2	–	34.3 (97)	48.8 (138)
	–	0.5	37.0 (105)	42.5 (120)
	–	2	35.6 (100)	51.6 (146)
	2	2	39.8 (113)	58.4 (165)

Rat brain cortex slices were incubated for 2 hours as indicated in
Table XIX. Morphine, nalorphine and KCl were added as indicated.

The figures in parentheses refer to percentages of the normal control
value without any additions.

[*]Incorporation of P^{32} expressed as in Table I.

45 per cent in a manner parallel to the stimulation brought
about by the addition of morphine.   When 2 mM morphine
and 2 mM nalorphine are added together a small increase in
the incorporation of P^{32} into phospholipids of unstimulated
tissue is observed.   In the presence of 100 mM K^+ ions,
the addition of 2 mM morphine and 2 mM nalorphine produces
a marked increase in the labelling of the phospholipids.
This increase is greater than the individual increase due
to morphine or nalorphine alone.   These results indicate
that nalorphine produces an effect similar to that of mor-
phine on the incorporation of P^{32} in K^+-stimulated tissue,
and that it does not have an antagonistic effect on the
latter stimulation.

The effect of tofranil on the incorporation of P^{32} into phospholipids

Abadom et al (222) demonstrated that the bio-
chemical effects of tofranil on mitochondrial and tissue
metabolism are similar to those produced by chlorpromazine
although the pharmacological properties of these drugs are
different.   It has been shown by Magee et al (108) that
the addition of chlorpromazine, at a concentration of 10^{-4}M,
to slices of guinea-pig brain produces an increased label-
ling of phospholipids from inorganic P^{32}.   It was of

interest to investigate in a parallel manner the effect of tofranil on the incorporation of P^{32} into phospholipids.

Table XXIV shows the results of two experiments performed with various concentrations of tofranil. It can be seen that at low concentrations (0.05, 0.1 $\underline{mM}$) tofranil has only a slight inhibitory effect on the oxygen consumption of slices whereas 0.5 $\underline{mM}$ tofranil strongly inhibits the oxygen uptake, in agreement with the results of Abadom et al (222). However, although the addition of 0.05 or 0.1 $\underline{mM}$ tofranil does not affect significantly the respiration of brain slices, increases of 39 and 70 per cent respectively on the incorporation of P^{32} are observed at these concentrations. Along with the inhibition of oxygen uptake, 0.5 $\underline{mM}$ tofranil exerts a marked inhibitory effect on the labelling of phospholipids.

DISCUSSION

The results described in this chapter definitely indicate, in accordance with previous conclusions (157,159, 160,162,163,213), that morphine does not affect the oxidative metabolism of brain cortex slices. The incorporation of P^{32} into phospholipids, which depends on an adequate phosphorylating mechanism within the slices (104) is not inhibited in the presence of high concentrations of morphine.

TABLE XXIV

EFFECT OF TOFRANIL ON THE INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS OF RAT BRAIN

CORTEX SLICES

Tofranil concn. (mM)	Experiment 1			Experiment 2			% Mean of tofranil effect
	Oxygen uptake	Incorporation of P^{32} into phospholipids[*]	% of control	Oxygen uptake	Incorporation of P^{32} into phospholipids[*]	% of control	
0	19.8	28.4	100	19.2	30.1	100	100
0.05	19.1	40.0	141	19.0	40.9	136	139
0.1	18.3	46.9	165	18.6	52.5	175	170
0.5	12.1	11.3	40	10.7	10.6	35	37

Rat brain cortex slices were incubated in modified Krebs medium III with glucose 10 mM, $NaH_2P^{32}O_4$, 20 µc. and tofranil for 2 hours.

Oxygen uptake expressed as µl/mg. dry wt./2 hours.

[*]Incorporation of P^{32} expressed as in Table I.

With the potassium-stimulated brain slices, which were shown

in the previous chapter to be much more sensitive to narco-

tics than the unstimulated tissue, the addition of morphine

does not exert any inhibitory effect.   This inability of

morphine, even at a concentration of 10 $\underline{mM}$, to inhibit the

potassium-stimulated oxygen consumption and the incorpor-

ation of P^{32} into phospholipids is in sharp contrast to its

effect on the electrically stimulated oxygen uptake.   Bell

(164) has reported that the increased oxygen consumption

and lactic acid formation associated with electrical stimu-

lation is inhibited by 1 $\underline{mM}$ morphine, a concentration which

is without effect upon unstimulated tissue.   In this con-

nection, the results obtained above with morphine are of

particular interest in that they illustrate a marked dif-

ference between the potassium and electrical stimulation

phenomena which have always been closely associated (35,20).

A striking feature which arises from the present

results is the stimulatory property of morphine on the in-

corporation of P^{32} into phospholipids of unstimulated and

potassium-stimulated slices although the concentration of

morphine required to produce such an effect is quite high.

The stimulatory effect of 10 $\underline{mM}$ morphine on the phospho-

lipid labelling is somewhat similar to that of acetylcholine,

and its effect is additive to that produced by acetylcholine.

This morphine behavior resembles that of high concentrations of atropine (10^{-3} - 10^{-2}M) which stimulate the incorporation of P^{32} and do not completely abolish the acetylcholine stimulatory effect on phospholipid labelling (47). However, in contrast with atropine, low concentrations of morphine have no inhibitory effect on the acetylcholine stimulation.

The results on the effect of morphine on the incorporation of P^{32} into phospholipids in potassium-stimulated slices reveal the peculiarity of the drug in being much more sensitive under these conditions. Moreover, the stimulatory effect of morphine appears to increase concomitantly with the decrease of the potassium stimulation with time. It seems likely that the morphine effect is gradually increasing with time and is not the result of an inhibition of the decrease of the potassium-stimulated P^{32} incorporation, since the oxygen consumption and 7-minute nucleotide phosphate concentration are not significantly affected by morphine in K^+-stimulated tissue. The acetylcholine stimulation of P^{32} incorporation has been reported to increase by only 12 per cent during one hour incubation as compared to 68 and 82 per cent during two and three hour incubations, respectively (47). The fact that the 7-minute nucleotide phosphate concentration as well as the

oxygen uptake are not affected by either morphine or ace-
tylcholine, suggests that the mechanism of action of these
two compounds on the labelling of phospholipids from in-
organic P^{32} may be analogous.

The results obtained with eserine on the incor-
poration of P^{32} into phospholipids with potassium-stimulated
slices are in good agreement with the previous findings on
the effect of atropine on the incorporation of P^{32} into
phospholipids of K^+-stimulated tissue (Table XVI). These
results clearly suggest that the effect of 100 mM K^+ ions
on the incorporation of P^{32} into phospholipids is not secon-
dary to an acetylcholine effect following the stimulation
of acetylcholine synthesis by potassium ions (124). If
this had been the case, eserine and atropine should have
activated and inhibited respectively the incorporation of
P^{32} into phospholipids in the presence of 100 mM K^+ ions,
since they respectively support and inhibit the stimulation
of P^{32} incorporation produced by acetylcholine in brain
slices (47). This does not mean that the addition of
potassium ions to the medium does not stimulate the acetyl-
choline synthesis in brain slices, but that the stimulation
of P^{32} incorporation into phospholipids by 100 mM K^+ ions
is caused by a different factor and not by acetylcholine.

The results obtained with nalorphine indicate

that this compound is producing an effect similar to that
of morphine on the incorporation of P^{32} into phospholipids
and that it is not antagonizing the stimulatory effect of
morphine but together with morphine produces an additive
effect.   This analogous and additive effect of nalorphine
and of morphine in vitro is in agreement with earlier re-
sults (163) showing that morphine and nalorphine respectively
inhibit cholinesterase and that the combined effects of
these two drugs are additive.   The above findings with
nalorphine suggest that the morphine effect on P^{32} incor-
poration has no pharmacological significance.   This con-
clusion is further supported by the experiments performed
with morphine-treated rats in which no effect is observed
on the incorporation of P^{32} into phospholipids.   These
negative results with morphine-treated rats are not sur-
prising if one considers the concentration of morphine found
in the brains of animals following administration of non-
fatal doses of the drug.   Miller and Elliott (223) found
a concentration of approximately 0.001 - 0.002 mM morphine
in rat brain after a dose of 5 mg./kg.   In the present
study, concentrations of morphine larger than 0.2 mM are
necessary to produce an effect on the incorporation of P^{32}
into K^+-stimulated brain slices.

The stimulatory effect of tofranil on the in-

corporation of P^{32} into phospholipids at low concentrations
is analogous to that reported for chlorpromazine (108).
These results are in agreement with the findings of Abadom
et al (222) who demonstrated that tofranil exerts similar
effects to those of chlorpromazine in inhibiting oxygen
consumption, the uptake and incorporation of glycine-1-C^{14}
in brain cortex slices as well as in uncoupling oxidation
from phosphorylation.. The results obtained with tofranil
are of particular interest since they illustrate the sti-
mulatory effect of another compound on the incorporation of
P^{32} into phospholipids without stimulating the oxidative
metabolism of the brain slices. As previously mentioned,
acetylcholine and atropine (47), chlorpromazine and azacy-
clonol (108), ouabain (224), digitoxin (225), morphine and
nalorphine, all produce similar effects.

It may be concluded from the present study that
morphine, nalorphine and tofranil exert a strong stimul-
atory effect on the incorporation of P^{32} into phospholipids
of brain cortex slices without affecting the factors which
usually govern the incorporation of P^{32} into phospholipids.
In this connection, their effect on the phospholipid label-
ling is very similar to that produced by acetylcholine which
is being thoroughly investigated at the present time and
which appears to be involved in ion transport (51.52).

SUMMARY

1.   Morphine does not significantly affect the
        normal or the potassium-stimulated oxygen uptake of
        rat brain cortex slices at concentrations ranging
        from 0.2 to 10 $\underline{mM}$.

2.   Morphine does not affect the incorporation of
        P^{32} into phospholipids of brain slices at low con-
        centrations (0.2 - 2 $\underline{mM}$).   There is, however, a
        marked stimulation of P^{32} incorporation produced by
        morphine at concentrations of 5 and 10 $\underline{mM}$.

3.   Morphine does not significantly affect the sti-
        mulation of P^{32} incorporation into phospholipids
        caused by 2 $\underline{mM}$ acetylcholine.

4.   In the presence of 100 $\underline{mM}$ potassium ions, mor-
        phine does not inhibit the stimulated P^{32} incorpor-
        ation.   On the contrary, it exerts a slight stimu-
        latory effect which is additive to the potassium
        stimulation.   The stimulatory effect of morphine
        increases with the duration of the incubation period,
        while the stimulatory effect produced by 100 $\underline{mM}$
        potassium ions decreases with time.

5.   Eserine exerts a small stimulation on the in-
        corporation of P^{32} into phospholipids of unstimulated
        slices.   In the presence of 100 $\underline{mM}$ potassium ions,

after 2 hours incubation, eserine does not produce
a different effect, contrary to what is observed
with morphine.

6.   Morphine has no effect on the level of 7-minute
nucleotide phosphate either in unstimulated or in
potassium-stimulated brain slices.

7.   Nalorphine (2 $\underline{mM}$) has no effect on the incor-
poration of P^{32} into phospholipids of unstimulated
slices.   In the presence of 100 $\underline{mM}$ potassium ions,
nalorphine exerts a stimulatory effect on the in-
corporation of P^{32} similar to that produced by
morphine.   However, nalorphine does not antagonize
the stimulatory effect produced by morphine.

8.   Tofranil at low concentrations exerts a
stimulatory effect on the incorporation of P^{32} into
phospholipids of brain slices whereas at higher
concentrations it causes an inhibition.

CHAPTER VI

GENERAL DISCUSSION

Since the establishment of the various pathways
of phospholipid synthesis in brain, one of the principal
fields of interest has been the elucidation of the functions
of phospholipids in living organisms.   There are an in-
creasing number of observations which suggest that phospho-
lipids may play some role in ion transport (41 - 46, 51, 52,
6).   The cationic stimulation of P^{32} incorporation into
phospholipids, observed in the present and previous studies
(201, 134), may simply be looked upon as a relationship
between alterations of the general oxidative metabolism of
the brain slices and the incorporation of P^{32} into phospho-
lipids, which is closely linked to the former mechanism.
However, a number of facts suggest that it is not unreasonable
to think that the cationic stimulated P^{32} incorporation into
phospholipids is concerned with the ionic movement at the
cell membrane and implicated in ion transport.

The main features which arise from the present
work is the similarity between the effects of 100 mM potassium
ions (or Ca^{++} free medium) and acetylcholine on the in-
corporation of P^{32} into phospholipids (Table XXV). Although
the above substances which stimulate P^{32} incorporation are
different, they appear to be producing their stimulatory

<u>TABLE XXV</u>

<u>BIOCHEMICAL EFFECTS OF 0.1M KCl AND ACETYLCHOLINE</u>

<u>ON BRAIN CORTEX METABOLISM</u>

	Metabolic Process	KCl, 0.1M	ACh, 2mM
1.	Oxygen uptake with glucose or pyruvate as the substrate	Increased	No effect
2.	Oxygen uptake with succinate or glutamate as the substrate	No effect	No effect
3.	Incorporation of P^{32} into phospholipids in presence of glucose or pyruvate	Increased	Increased
4.	Incorporation of P^{32} into phospholipids in presence of succinate or glutamate	No effect	No effect
5.	Incorporation of P^{32} into phospholipids in presence of glucose and malonate or iodoacetate	Decreased	Decreased
6.	Incorporation of P^{32} into phospholipids in presence of glucose and narcotics	Decreased	Decreased
7.	Incorporation of P^{32} into phospholipids in presence of glucose and atropine	No effect	Decreased
8.	Concentration of 7-minute nucleotide phosphate	Decreased	No effect
9.	Incorporation of P^{32} into 7-minute nucleotide phosphate	Increased	--
10.	Incorporation of P^{32} into phosphatidic acid	Increased (134)	Increased (67)
11.	Incorporation of P^{32} into phospholipids in presence of glucose and sodium-free medium	No effect	No effect

effect through a common biochemical mechanism.

It was shown that the addition of 100 $\underline{mM}$ potassium ions or the omission of calcium ions from the incubation medium causes an increase in the oxygen uptake and an increase in the incorporation of P^{32} into phospholipids and 7-minute nucleotide phosphate (ATP) after a 30 minute incubation period. In view of the general belief that the incorporation of P^{32} into the phospholipids is dependent upon the supply of energy and is derived from ATP^{32}, it appears as logical that the incorporation of P^{32} into phospholipids should be increased when the incorporation of P^{32} into ATP is also increased, as observed above.

In this respect, the stimulatory effect produced by 100 $\underline{mM}$ K^+ ions (or omission of Ca^{++} ions) differs from that produced by dinitrophenol (DNP). On the basis of the similarity of the effects of excess potassium and DNP in increasing the rates of oxygen consumption and glycolysis and decreasing the incorporation of P^{32} into phospholipids (after long periods of incubation) of brain slices, the suggestion had arisen that potassium ions might uncouple oxidation from phosphorylation like DNP. Yoshida and Nukada (134) demonstrated that DNP, although it stimulates the oxygen uptake, inhibits the incorporation of P^{32} into phospholipids even after a 30 minute incubation. Kini (218) has also reported that 100 $\underline{mM}$ potassium ions

stimulate the synthesis of glutamine from glucose-U-C^{14}
while DNP exercises a definite inhibition on the formation
of glutamine.

The depletion of high energy phosphates by the
addition of 100 $\underline{mM}$ potassium ions does not appear to be a
consequence of an uncoupling effect of potassium, but as
previously postulated (180, 132) may be the result of a
direct effect of potassium at the cell membrane where it
is accumulated against a concentration gradient.

Cationic or acetylcholine stimulation of P^{32}
incorporation into phospholipids was shown to occur only
in the presence of high energy substrates or under conditions
where the oxidative phosphorylation is maximum since, under
circumstances where the formation of energy-rich bonds is
inhibited, the stimulation is also inhibited. Moreover,
these two stimulatory phenomena were also shown to be
closely linked to the presence of sodium ions in the medium.

Yoshida and Nukada (134) have reported that the
potassium stimulation of P^{32} incorporation into phospho-
lipids occurs mainly in the phosphatidic acid fraction.
Similarly, Hokin and Hokin (67) have also shown that the
acetylcholine stimulation of P^{32} incorporation into phos-
pholipids takes place in phosphatidic acid and phospho-
inositide.

Two possible interpretations may be offered for

the increased incorporation of P^{32} into phospholipids in
response to 100 mM potassium ions.   First, it is possible
that the increased labelling of phosphatidic acid by the
addition of 100 mM K^+ ions is the result of an increased
de novo synthesis of phosphatidic acid through dihydroxy-
acetone phosphate and ∝-glycerol phosphate, due to in-
creased glucose oxidation caused by K^+ ions.   However, the
fact that pyruvate supports the stimulation of P^{32} incor-
poration to the same extent as glucose may be cited as
evidence against the above possibility, since this substrate
does not lead directly to the synthesis of phosphatidic acid
through dihydroxyacetone phosphate.   The same objection
could be applied to an alternative route of phosphatidic
acid formation, previously postulated (65, 67, 68, 226, 6)
that is:

glucose ⟵⟶ glycerol + ATP ⟶ ∝-glycerol phosphate

⟶ phosphatidic acid
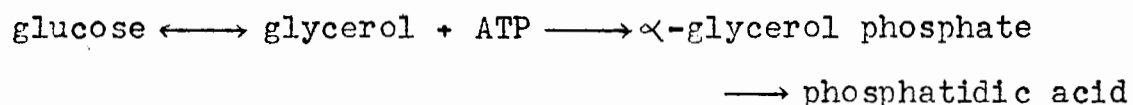
Pritchard (132) has also demonstrated that the incorporation
of glycerol-1-C^{14} into phospholipids of brain slices is
markedly inhibited in the presence of 65 or 124 mM
potassium ions.   Although Pritchard's experiments were
carried out for two hours and cannot be compared with 30-
minute experiments, his finding that the incorporation of
glycerol-1-C^{14} is inhibited approximately 73 per cent in
the presence of 65 mM K^+ ions is of interest, since the

incorporation of P^{32} under similar conditions is practically not affected (131). Nevertheless, subsequent experiments are necessary to solve this question unequivocally.

The second possible interpretation for the stimulation of P^{32} incorporation in response to potassium ions is that the phospholipid effect represents an increased turnover of phosphate in preformed phospholipids (or phosphatidic acid) and not an increased net synthesis. In this respect the glycerol-phosphate bond would be cleaved and resynthesized.

It has been suggested (20, 35) that the exposure of the cells to an environment with a cationic balance different from the normal presumably affects ionic movements at the nerve cell membrane resulting in depolarization. McIlwain (126) has suggested that much of the cellular metabolism is directed towards the maintenance of a high gradient of potassium between the intracellular and extracellular compartments of the tissue. The presence of 100 mM potassium ions in the incubation medium would lower this gradient and cause depolarization resulting in the entry of sodium in the nerve cell. The metabolism of the cell would be directed to re-establish this gradient by extrusion of sodium ions from the intracellular fluid during the recovery process and presumably by concentrating potassium ions.

On the basis of these considerations and by
analogy with the acetylcholine effect, which has been linked
with the active transport of sodium ions out of the cell
during the recovery process (51, 52), it is not unreasonable
to believe that the potassium stimulatory effect on phos-
phatidic acid labelling from inorganic P^{32} may be the
result of a similar mechanism. This mechanism would
therefore imply, as illustrated by Hokin and Hokin (51) in
their scheme, that phosphatidic acid is hydrolyzed by
phosphatidic acid phosphatase and resynthesized through a
diglyceride kinase, resulting in an increased turnover of
phosphate. In this connection, it is noteworthy to mention
that protoveratrine, which produces similar effects as those
produced by 100 $\underline{mM}$ potassium ions on brain cortex metabolism
(227, 139, 218), also stimulates the incorporation of P^{32}
into phospholipids (218) . It is well known (228) that
the veratrine alkaloids bring about marked changes in ion
permeability.

The stimulatory effects of morphine and tofranil,
as well as that of tranquilizers (108), ouabain (224),
digitoxin (225), and atropine (47) on the incorporation of
P^{32} into phospholipids are characterized by a common factor;
i.e., the inability of these compounds while producing
their stimulatory effect, to affect the oxidative metabolism
of the brain slices which governs the incorporation of P^{32}

into phospholipids under normal conditions. Quoting
Rossiter and Strickland (6), the stimulatory effects produced
by the above drugs may "reflect actions on the enzymes or
intermediates concerned with the synthesis of phospholipids."
The stimulation of P^{32} incorporation may also be considered
to involve membrane phospholipids and thus a permeability
process might be implicated. By analogy with acetylcholine,
the increased incorporation of P^{32} may be associated with
the transport of a specific cation or substrate across the
cell membrane.

In conclusion it may be said that although the
mechanism of the stimulation of P^{32} incorporation into
phospholipids by various agents is still at present obscure,
the elucidation of this stimulatory phenomenon may well
elucidate the function of phospholipids in ion transport.

CLAIMS TO ORIGINAL RESEARCH

1. The addition of 100 $m\underline{M}$ potassium or the omission of
   calcium ions from the incubation medium stimulates the
   incorporation of P^{32} into 7-minute nucleotide phosphate
   of rat brain cortex slices in a manner similar to the
   effect produced on oxygen consumption and phospholipid
   labelling, after a 30 minute incubation period.

2. The addition of 10 $m\underline{M}$ succinate or 5 $m\underline{M}$ Y-aminobutyric
   acid to rat brain cortex slices metabolizing glucose
   does not significantly affect the incorporation of
   P^{32} into phospholipids after an incubation period of
   30 minutes.   A marked inhibition of phospholipid
   labelling is observed in the presence of these substrates
   after an incubation period of 2 hours.

3. The stimulation of P^{32} incorporation into phospholipids
   of brain slices by a calcium-free medium is found to
   occur only with glucose, mannose, fructose or pyruvate
   as substrates, but not in the presence of glutamate or
   succinate.

4. The removal of sodium chloride from the incubation
   medium and its replacement by choline chloride exerts
   an inhibitory effect on the incorporation of P^{32}
   into phospholipids of brain cortex slices.   The
   stimulation of P^{32} incorporation produced by 2 $m\underline{M}$
   acetylcholine is not observed under these incubation
   conditions unless a minimum amount of sodium

chloride is present. In a sodium-free medium, the omission of calcium ions produces a stimulation of P^{32} incorporation into phospholipids.

5. The addition of iodoacetate and ethanol blocks the stimulation of P^{32} incorporation into phospholipids brought about by 100 mM KCl. Similarly, addition of malonate, amytal and chloretone inhibits the stimulation caused by the omission of calcium ions in the incubation medium. In the presence of malonate, iodoacetate, fluoride and ethanol, the stimulation by acetylcholine of P^{32} incorporation into phospholipids of brain slices is abolished. The inhibition produced by these substances is greater in the cationic-stimulated than in the acetylcholine-stimulated P^{32} incorporation.

6. The addition of various concentrations of atropine or hyoscine does not inhibit the cationic-stimulated incorporation of P^{32} into phospholipids.

7. Tubocurarine, at various concentrations, has no effect on the stimulated incorporation of P^{32} into phospholipids of brain slices produced by 100 mM KCl or 2 mM acetylcholine.

8. The addition of morphine at concentrations of 5 and 10 mM to brain slices stimulates the incorporation of P^{32} into phospholipids.

9. Morphine has no inhibitory effect on the potassium-

stimulated incorporation of P^{32} into phospholipids. On the contrary, in the presence of 100 m$\underline{M}$ potassium ions, 2 m$\underline{M}$ morphine exerts a small stimulation which is additive to that produced by 100 m$\underline{M}$ potassium ions. This stimulatory effect produced by morphine increases with the duration of the incubation period, whereas the stimulation produced by 100 m$\underline{M}$ potassium ions decreases with time.

10. Eserine has no effect on the incorporation of P^{32} into phospholipids of K^+-stimulated brain cortex slices after an incubation period of 2 hours, contrary to what is observed with morphine.

11. Morphine has no effect on the decreased level of 7-minute nucleotide phosphate of brain cortex slices caused by the addition of 100 m$\underline{M}$ potassium ions during an incubation period of 2 hours.

12. There is no significant difference in the incorporation of P^{32} into phospholipids between slices from morphine-treated rats and control rats, irrespective of the potassium concentration in the incubation medium.

13. Nalorphine stimulates the incorporation of P^{32} into phospholipids of potassium stimulated brain slices after an incubation period of 2 hours. Nalorphine does not antagonize the stimulatory effect of morphine under

analogous experimental conditions.

14. Tofranil at low concentrations exerts a stimulatory
    effect on the incorporation of P^{32} into phospholipids
    of brain cortex slices, while at high concentration
    it produces an inhibitory effect.

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