PHOSPHOLIPID METABOLISM IN BRAIN

by

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<th>Full Form</th>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CMP</td>
<td>Cytidine monophosphate</td>
</tr>
<tr>
<td>CDP</td>
<td>Cytidine diphosphate</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine triphosphate</td>
</tr>
<tr>
<td>DNP</td>
<td>2:4-dinitrophenol</td>
</tr>
<tr>
<td>DPNH</td>
<td>Reduced diphospho-pyridine nucleotide</td>
</tr>
<tr>
<td>GABA</td>
<td>Y-aminobutyric acid</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>P-P</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>κ-GP</td>
<td>κ-glycerophosphate</td>
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<tr>
<td>7mnp.</td>
<td>7-minute acid hydrolyzable nucleotide phosphate</td>
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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 importance. 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.
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 approximately 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 phospholipids 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 \( \alpha \)-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 metaphosphohosphate. 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-\( \text{H}3 \) into the phosphoinositides of guinea-pig brain cortex slices, found that the radioactive phosphoinositide 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 newborn 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 adulthood. 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⁺, Ca²⁺ and Mg²⁺ 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 secretagogue of this tissue, stimulates the incorporation of P\textsuperscript{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 P³², 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. Mc'Nurray et al. (56) also reported that the incorporation of P³²
Figure 1. BIOSYNTHESIS OF PHOSPHOLIPIDS
( After Kennedy (3) and Rossiter and Strickland (6) )
Figures in parentheses refer to reactions in text
labelled \( \alpha \)-glycerophosphate \((\alpha -GP)\) 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 \( \alpha \)-glycerophosphate and coenzyme A thiol esters of fatty acid as previously described in liver by Kornberg and Pricer (58, 59).

\[
\text{Fatty acid} + \text{ATP} + \text{CoA} \xrightarrow{\text{acyl-CoA}} \text{AMP} + \text{P-P} \quad \text{(I)}
\]

\[
\text{L-}\alpha\text{-GP} + 2 \text{fatty acyl-CoA} \rightarrow \text{L-}\alpha\text{-phosphatidic acid} + 2\text{CoA} \quad \text{(II)}
\]

Additional evidence for reaction II has been provided by Jedeikin and Weinhouse (60) who found that palmitate-1-\( ^{14} \)C 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 phosphatidic acid appears to be operative in brain. Hokin and Hokin (61) have reported that phosphatidic acid can also be formed from \( \alpha,\beta \)-diglyceride and ATP, presumably by the action of a diglyceride kinase. These authors found that the incorporation of \( ^{32} \)P from ATP\(^{32} \) into phosphatidic acid of brain microsomes is highly dependent upon the presence of \( \alpha,\beta \)-diglyceride, a phenomenon very similar to the one previously 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, Rublitz 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):

\[
\text{L-α'-phosphatidic acid} \rightarrow \text{D-α',β'-diglyceride + Pi} \quad (V)
\]
This enzyme is therefore responsible for the lack of labelling of glycerophosphatides from $\alpha'$-GP$^{32}$ by causing the liberation of radioactive Pi, a phenomenon not observed when C$^{14}$ labelled $\alpha'$-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:

$$\text{Choline} + \text{ATP} \rightarrow \text{ADP} + \text{phosphorylcholine} \quad \text{(VI)}$$

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):

$$\text{Phosphorylcholine} + \text{CTP} \rightarrow \text{CDP-choline} + \text{P-P} \quad \text{(VII)}$$
$$\text{CDP-choline} + \alpha',\beta\text{-diglyceride} \rightarrow \text{L-}$${$\alpha'$}-lecithin + CMP \quad \text{(VIII)}$$

Thus McMurray et al. (56) and Rossiter and Strickland (69) showed that P$^{32}$ or C$^{14}$ labelled phosphorylcholine is incorporated into $\alpha'$-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 $\alpha'$-lecithin fraction of brain preparation and the incorporation is stimulated by the addition of $\alpha',\beta\text{-diglyceride}$ (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 \( \alpha \)-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 \( \alpha \)-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-\(^{14}\)C into the aldehydogenic moiety of the plasmalogen; the system requires CoA, ATP, Mg\(^{++}\), \( \alpha \)-glycerophosphate, 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 phosphorylcholine and phosphorylethanolamine plasmalogenic glyceride transferases catalysing the following reactions:

\[
\begin{align*}
\text{CDP-choline} & \xrightarrow{\text{"Plasmalogenic diglyceride"}} \text{CDP-ethanolamine} \\
\text{"Plasmalogenic phosphatidyl choline"} & \xleftarrow{\text{CMP (IX)}} \text{"Plasmalogenic phosphatidyl ethanolamine"}
\end{align*}
\]

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 (73), and the observation by Dawson (57) and others (56, 67, 79) that the incorporation of P\textsuperscript{32} into brain phosphoinositide and phosphatidic 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 CDP 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):

$$\text{Phosphatidic acid } + \text{CTP} \rightarrow \text{CDP-diglyceride } + \text{P-P} \quad (X)$$
$$\text{CDP-diglyceride } + \text{Inositol} \rightarrow \text{Inositol phospholipid } + \text{CMP} \quad (XI)$$

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 $^{32}$P 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 $^{14}$C label from the glycerol moiety of $^{14}$C 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)

\[ \text{CDP-choline} + \text{ceramide} \rightarrow \text{sphingomyelin} + \text{CMP} \] (XII)
This reaction is similar to the phosphorylcholine glyceride transferase (see reaction VIII), except that ceramide rather than $\alpha,\beta$-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 ($\beta$-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 phospholipid 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 cerebrospinal fluid, the isotope uptake in the brain is more rapid.
suggesting therefore that the slow rate of incorporation of p32 \textit{in vivo} is attributed to the properties of the blood-brain barrier which controls its rate of penetration (101).

**Incorporation of p32 \textit{in vitro}**. The \textit{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 P32 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 P32 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 P32. This author therefore suggested that the incorporation of P32 is "a metabolic phenomenon and is dependent upon the maintenance of an adequate phosphorylating mechanism within the slices" (104). Schachner \textit{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 \textsuperscript{p32} (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 II: 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 \textit{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 $\alpha$-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 un-stimulated 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\textsuperscript{32} into phosphorus containing compounds such as phospholipid, ribonucleic acid and phosphoprotein. Rossiter (131) subsequently reported that under these conditions the incorporation of P\textsuperscript{32} into ATP decreases to the same relative extent as the labelling of the phospholipid. Pritchard (132) also observed that the incorporation of C\textsuperscript{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 phosphocreatine (132). On the other hand, in contrast to the above observations, Tsukada et al. (133) found that the incorporation of P\textsuperscript{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 P32 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 turnover of phosphoprotein within the slices. Yoshida and Kukada (134) recently reported similar results with phospholipids showing that the greatest increase in P32 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 \( \gamma \)-aminobutyric acid from glucose uniformly labelled with carbon-14. The stimulation of \( ^{14} \text{C} \) incorporation may be caused by an acceleration of the citric acid cycle resulting in an increased turnover of \( \alpha \)-ketoglutarate, a well known precursor of these two amino-acids (138). Kini and Quastel also observed that the presence of 105 m\( \text{M} \) potassium greatly increases the rate of oxidation in brain cortex of both pyruvate-1-\( ^{14} \text{C} \) and pyruvate-2-\( ^{14} \text{C} \) 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 \( \alpha \)-ketoglutarate. 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-excit able 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 acetylcholine 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 stimulation of amylase secretion by acetylcholine is accompanied by a large increase in the specific activity of the phospholipid (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 p32 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 incorporation of glycerol-1-C\textsuperscript{14} into the phospholipid is slightly inhibited in the presence of acetylcholine, although the usual stimulatory effect on p32 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 \( d^{-}GP^{32} \) 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 uncouple oxidation from phosphorylation.

Achar and Geiling (161) have reported that morphine inhibits the uptake of C\textsuperscript{14} into the brain of rats following an intravenous injection of glucose-C\textsuperscript{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\textsuperscript{14} by the addition of a high potassium concentration is not affected. The latter observation is analogous to the one reported by Franklin (163) for potassium stimulated brain cortex slices incubated with 3 mV 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 acetylcholine 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 prepared 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 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 $^{32}$P 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 $^{32}$P is closely linked to the mechanism of oxidative phosphorylation and evidence has been obtained suggesting that $^{32}$P, 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 $^{32}$P 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 $^{32}$P. Investigations have been carried out to ascertain the stimulatory effect of cations, acetylcholine and morphine on the incorporation of $^{32}$P 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.
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 intraperitoneal 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. P32 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 P32. The commercial solution of P32 appeared to be contaminated by a slight amount of radioactive other than orthophosphate. Originally when the radioactive P32 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 P32 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 P32 solution was cooled and neutralized with a few drops of ammonium hydroxide. 10 μmoles of Na2HPO4, 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°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 P32 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°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$_2$PO$_4$; 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 concentrations 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 \(^{32}\)P 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 overnight at 5°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 contained 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 H₂SO₄ in an oven at 140°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 H₂O was then added and a 0.1 ml. aliquot of this solution was assayed for phosphorus by the method of Bartlett (177) (see page 49) without further addition of H₂SO₄.

**Assay of radioactivity.** Aliquots (usually 200 µl) of the chloroform extract were plated on aluminium discs
and the radioactivity was counted with a "Tracelab" gas flow counter of 20 per cent efficiency.

Measurement of Nucleotide Phosphate Levels

The technique used in these experiments was devised by Dr. D. R. 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°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. of 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 orthophosphate 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 630 mp 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 indicating 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 phospholipids, 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 preliminary 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 P32 into phospholipids

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

Incorporation of P32 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/\mu mole 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-
assumption and aerobic glycolysis of brain cortex slices. These ionic changes also stimulate a number of other metabolic 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 phosphocreatine and ATP (126,127,129,131) as well as inhibiting the incorporation of p$^{32}$ into phosphorus-containing compounds (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 (130,132) that an increase of potassium ions in the incubation 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 incorporation 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 p³² into phospholipids 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 p³² into phospholipids is parallel to the enhancement of the oxidative 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 p³² into phospholipids. In cases where a stimulatory effect was observed, the increment 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 p³² incorporation into phospholipids 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 $^{32}P$ into phospholipids.

The effect of electrolytes on the incorporation of $^{32}P$ 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 $^{32}P$ 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 $^{32}P$ 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 NH₄⁺ IONS ON THE INCORPORATION OF p³² INTO PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

<table>
<thead>
<tr>
<th>K⁺ (mM)</th>
<th>Ca⁺⁺ (mM)</th>
<th>NH₄⁺ (mM)</th>
<th>Oxygen uptake (μl/mg. dry wt. tissue/30 min.)</th>
<th>Phospholipid-P (μmoles/g. of wet wt. tissue)</th>
<th>Incorporation of p³² into phospholipids*</th>
<th>Per cent of control incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2</td>
<td>2.8</td>
<td>0</td>
<td>5.1</td>
<td>32.0</td>
<td>10.5</td>
<td>100</td>
</tr>
<tr>
<td>10.5 2.8</td>
<td>0</td>
<td>8.8</td>
<td>30.8</td>
<td>16.9</td>
<td>135</td>
<td>135</td>
</tr>
<tr>
<td>5.2</td>
<td>0</td>
<td>0</td>
<td>6.9</td>
<td>32.9</td>
<td>14.2</td>
<td>135</td>
</tr>
<tr>
<td>5.2</td>
<td>2.8</td>
<td>10</td>
<td>6.2</td>
<td>-</td>
<td>6.7</td>
<td>64</td>
</tr>
</tbody>
</table>

Rat brain cortex slices were incubated for 30 minutes at 37°C in modified Krebs medium III with 10 mM glucose and 20 μc. NaH₂P³²O₄₄.

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

*Incorporation of p³² 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 $^{32}$P is enhanced.

**Time course of the ionic effects.**

In order to establish the extent of the ionic effects on the incorporation of $^{32}$P 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 $^{32}$P into phospholipids is shown in Fig. 3. The rate of $^{32}$P 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
Rat brain cortex slices were incubated at 37°C in modified Krebs medium III with glucose 10mM.

○ Control; ● + 0.1M KCl; △ calcium-free.

Oxygen uptake expressed as -\text{\textsuperscript{0}}\text{O}_2\text{ for the time intervals indicated.}
FIGURE 3
TIME COURSE OF THE INCORPORATION OF P32 INTO PHOSPHOLIPIDS OF BRAIN
SLICES IN THE PRESENCE OF 0.1 M KCl OR IN THE ABSENCE OF CaCl2

Experimental conditions as described in Table I.
- Control; •, + 0.1 M KCl; ▼, calcium-free.
Incorporation of P32 expressed as in Table I.
100 mM potassium ions, the $\text{P}^{32}$ 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 $\text{P}^{32}$ almost ceases. In the normal medium, $\text{P}^{32}$ 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 mM K+ ions. In a calcium-free medium, the initial rate in $\text{P}^{32}$ 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 $\text{P}^{32}$ incorporation was the same in normal and Ca++ free media.

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

In view of the above observations as well as the recognized fact that the degree of incorporation of $\text{P}^{32}$ into phospholipids is dependant not only upon the concentration of ATP but also on the degree of incorporation of the isotope into ATP, it was of interest to study the effect of potassium and calcium ions on the level of ATP, and on the $\text{P}^{32}$ 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 concentration of ATP. A time course study of the effect of 100 mM potassium ions on the level of ATP and on the incorporation of p32 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 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 p32 incorporation into the nucleotide phosphate of tissue incubated in normal medium increases gradually with time period and reaches equilibrium within approximately 120 minutes. In the potassium-rich medium, the incorporation of p32 into the nucleotide phosphate is
stimulated during the first 60 minutes incubation and comes to equilibrium after this time. The amount of P32 incorporated in the K\(^+\) stimulated tissue after 60 minutes incubation is identical to that incorporated in the unstimulated tissue after 120 minutes. It appears, therefore, that after a certain period of incubation a maximum rate of P32 incorporation is reached, after which a further increase 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 turnover since the organic phosphate would be exchanging with orthophosphate of the same specific activity. The observation that although the level of nucleotide phosphate is markedly decreased in the presence of 100 m\(\uparrow\) K\(^+\) ions after 2 - 4 hours incubation, and that the incorporation of P32 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 P32 INTO 7MnP (B), IN RAT BRAIN CORTEX SLICES

(A)

7MnP conc.

(B)

Incorporation of P32 into 7MnP.

Rat brain cortex slices were incubated at 37°C in modified Krebs medium III with 10mM glucose, and 20 μc NaH2P32O4.

O, control; ●, + 0.1M KCl.
7MnP conc. expressed as μ moles phosphate/g, wet wt. tissue.
Incorporation of P32 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 7mm.p. 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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Expt. 1</th>
<th></th>
<th>Expt. 2</th>
<th></th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 mnp.</td>
<td>Specific Activity</td>
<td>7 mnp.</td>
<td>Specific Activity</td>
<td>7 mnp.</td>
</tr>
<tr>
<td>Normal medium</td>
<td>1.6 (100)</td>
<td>30.0x10⁴ (100)</td>
<td>1.52 (100)</td>
<td>24.7x10⁴ (100)</td>
<td>1.7 (100)</td>
</tr>
<tr>
<td>+ KCL (0.1M)</td>
<td>1.25 (78)</td>
<td>54.0 (180)</td>
<td>1.17 (76)</td>
<td>39.0 (157)</td>
<td>1.3 (77)</td>
</tr>
<tr>
<td>Ca⁺⁺ free</td>
<td>1.18 (74)</td>
<td>40.0 (133)</td>
<td>1.25 (82)</td>
<td>34.6 (140)</td>
<td>1.3 (77)</td>
</tr>
</tbody>
</table>

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 P³² INTO THE PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conc. (mM)</th>
<th>Oxygen Uptake (μl/mg. dry wt./30 min.)</th>
<th>Incorporation of P³² into phospholipids*</th>
<th>Percentage of the control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>-</td>
<td>5.3</td>
<td>10.1</td>
<td>100</td>
</tr>
<tr>
<td>KCl</td>
<td>25</td>
<td>6.6</td>
<td>9.9</td>
<td>98</td>
</tr>
<tr>
<td>KCl</td>
<td>50</td>
<td>7.4</td>
<td>11.6</td>
<td>115</td>
</tr>
<tr>
<td>KCl</td>
<td>75</td>
<td>8.8</td>
<td>15.1</td>
<td>150</td>
</tr>
<tr>
<td>KCl</td>
<td>100</td>
<td>9.0</td>
<td>16.4</td>
<td>162</td>
</tr>
<tr>
<td>KCl</td>
<td>125</td>
<td>8.9</td>
<td>14.3</td>
<td>143</td>
</tr>
<tr>
<td>KCl</td>
<td>150</td>
<td>7.1</td>
<td>13.1</td>
<td>130</td>
</tr>
</tbody>
</table>

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. NaH₂P³²O₄, and KCl was added as indicated.

Results represent mean values of two experiments or more.

*Incorporation P³² 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 p32 incorporation increases rapidly reaching a maximum at 100 mM of K+ ions, and subsequently decreases with concentrations of 125 and 150 mM. Since low concentrations of potassium ions stimulate the oxygen consumption but not the incorporation of p32, 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 p32 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 γ-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 p32 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**

<table>
<thead>
<tr>
<th>Addition (mM)</th>
<th>Incubation time (30 minutes)</th>
<th>Incubation time (120 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxygen uptake (µl/mg. dry wt.)</td>
<td>Incorporation of P(^{32}) into phospholipids*</td>
</tr>
<tr>
<td>Nil</td>
<td>5.2</td>
<td>10.2 (100)</td>
</tr>
<tr>
<td>Succinate, 10</td>
<td>7.0</td>
<td>11.4 (112)</td>
</tr>
<tr>
<td>α-Keto-glutarate, 10</td>
<td>6.2</td>
<td>10.3 (100)</td>
</tr>
<tr>
<td>L-glutamate, 10</td>
<td>6.9</td>
<td>9.0 (88)</td>
</tr>
<tr>
<td>Y-amino-butyric acid, 5</td>
<td>6.2</td>
<td>9.9 (97)</td>
</tr>
<tr>
<td>Y-amino-butyric acid, 10</td>
<td>5.9</td>
<td>7.5 (73)</td>
</tr>
</tbody>
</table>

Rat brain cortex slices were incubated at 37° C in modified Krebs medium III with glucose, 10 mM and NaH\(_2\)P\(^{32}\)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 p32 incorporation is obtained in the presence of succinate, glutamate and γ-aminobutyric acid although the respiratory values at this period of time are still at a maximum. The inhibitory effect on p32 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 counteracting the stimulatory effect. Therefore, subsequent studies on the effect of 100 mM of potassium ions or of calcium-free medium were always determined after a 30 minute incubation period.

The effect of various substrates on the stimulated-incorporation of p32 into phospholipids

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

The cationic stimulation of brain cortex respiration (115,123) and the incorporation of p32 into phospholipids 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 p32 incorporation in brain slices in the presence of 100 mM
K⁺ ions or in the absence of Ca²⁺ ions. The results are shown in Tables V and VI. In the normal incubation medium, the addition of glucose or mannose causes the maximal incorporation of p³² into the phospholipids. Fructose or pyruvate also increases the p³² labelling, but only to a limited extent as compared to the former substrates, whereas glutamate or succinate are almost ineffective. When 100 mM of K⁺ ions are added to the incubation medium or Ca²⁺ ions omitted, the increment of p³² 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 p³² incorporation. On the contrary, with the endogenous or the succinate-containing medium, a small but consistent inhibition of p³² 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 p³² into phospholipids.

B) **Acetylcholine-stimulated incorporation of p³² into phospholipids.**

It has been previously shown by Hokin and Hokin (47) that acetylcholine stimulates the incorporation of p³²
### TABLE V

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

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

Rat brain cortex slices were incubated at 37°C for 30 minutes in modified Krebs medium III with NaH\(_2\)P\(^{32}\)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\textsuperscript{32} INTO THE PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

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

The experimental conditions were as described in Table V.

*Incorporation of p\textsuperscript{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 mM stimulated the incorporation of $^{32}$P 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 mM acetylcholine during an incubation period of 2 hours. The substrates that support the stimulatory effect of acetylcholine on the incorporation of $^{32}$P into the phospholipids were examined under these conditions. The results are presented in Table VII. It can be seen that the acetylcholine stimulation of $^{32}$P 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**

<table>
<thead>
<tr>
<th>Substrate (10 mM)</th>
<th>Incorporation of P(^{32}) into phospholipids</th>
<th>Oxygen uptake (µl/mg. dry wt./2 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+ACh</td>
</tr>
<tr>
<td>Nil</td>
<td>4.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>29.2</td>
<td>45.0</td>
</tr>
<tr>
<td>Mannose</td>
<td>27.0</td>
<td>40.1</td>
</tr>
<tr>
<td>Fructose</td>
<td>10.3</td>
<td>10.5</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>8.6</td>
<td>13.8</td>
</tr>
<tr>
<td>L-glutamate</td>
<td>6.4</td>
<td>6.6</td>
</tr>
<tr>
<td>Succinate</td>
<td>5.8</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Rat brain cortex slices were incubated at 37°C for 2 hours in modified Krebs medium III with 20 µc. NaH\(_2\)P\(^{32}\)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 inhibition of \( \text{p}^{32} \) 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 \( \text{p}^{32} \) incorporation.

**The effect of sodium ions on the increased incorporation of \( \text{p}^{32} \) 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 \( \text{p}^{32} \) 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 \( \text{p}^{32} \) into phospholipids, and this effect is gradually abolished
TABLE VIII

EFFECT OF Na⁺ IONS ON THE POTASSIUM AND CALCIUM-FREE STIMULATED INCORPORATION OF P⁳² INTO PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

<table>
<thead>
<tr>
<th>Na⁺ Conc. (mM)</th>
<th>Incorporation of P³² into phospholipids*</th>
<th>Oxygen uptake (µl/mg. dry wt./30 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control +KCl (0.1M) Ca⁺⁺ free</td>
<td>Control +KCl (0.1M) Ca⁺⁺ free</td>
</tr>
<tr>
<td>140</td>
<td>9.7</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>16.3 (167)</td>
<td>7.5 (163)</td>
</tr>
<tr>
<td></td>
<td>13.0 (134)</td>
<td>6.1 (133)</td>
</tr>
<tr>
<td>100</td>
<td>9.2</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>14.5 (158)</td>
<td>7.0 (155)</td>
</tr>
<tr>
<td></td>
<td>12.4 (135)</td>
<td>5.9 (131)</td>
</tr>
<tr>
<td>33</td>
<td>7.7</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>9.0 (117)</td>
<td>4.9 (114)</td>
</tr>
<tr>
<td></td>
<td>10.2 (133)</td>
<td>5.5 (128)</td>
</tr>
<tr>
<td>0</td>
<td>6.2</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>6.1 (100)</td>
<td>3.6 (90)</td>
</tr>
<tr>
<td></td>
<td>8.5 (137)</td>
<td>4.9 (123)</td>
</tr>
</tbody>
</table>

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 NaH₂P³²O₄, 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 P³² expressed as in Table I.
TABLE IX

EFFECT OF Na⁺ ON THE ACETYLCHOLINE-STIMULATED INCORPORATION OF P₃² INTO PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

<table>
<thead>
<tr>
<th>Na⁺ Concen. (mM)</th>
<th>Incorporation of P₃² into phospholipids*</th>
<th>Oxygen uptake (µl/mg. dry wt./2 hours.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + ACh % of control</td>
<td>Control + ACh</td>
</tr>
<tr>
<td>140</td>
<td>37.1 54.1 146</td>
<td>17.5 18.9</td>
</tr>
<tr>
<td>100</td>
<td>39.3 56.0 142</td>
<td>17.3 18.2</td>
</tr>
<tr>
<td>66</td>
<td>35.0 48.0 137</td>
<td>16.9 17.4</td>
</tr>
<tr>
<td>33</td>
<td>27.8 32.9 119</td>
<td>16.1 15.8</td>
</tr>
<tr>
<td>0</td>
<td>22.0 23.1 105</td>
<td>15.5 15.6</td>
</tr>
</tbody>
</table>

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, NaH₂P₃²O₄ 10 µc., acetylcholine (ACh) 2x10⁻³ M and eserine 3x10⁻⁴ M. Na⁺ was replaced by choline.

Results presented are the average of 3 experiments.

Incorporation of P₃² expressed as in Table I.
by the presence of minimum amounts of sodium ions. The inhibitory 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 P32 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 P32 incorporation is always observed with decreasing amounts of sodium ions in the medium, although the level of P32 incorporated is lower than that obtained under normal stimulatory conditions.

DISCUSSION

It has been well established that the incorporation of P32 into the phospholipids of rat brain preparations is dependent 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 P32 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 P32 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 P32 into the phospholipids.

The first of these phenomena, which results in the enhancement of P32 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 isolated 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 conversion of pyruvate to acetyl-CoA. It is possible therefore to interpret the stimulation of P32 incorporation into the phospholipids in the presence of 100 mM K+ ions. The increased glucose oxidation (187,121) and increased rate of acetyl-CoA formation lead to a higher level of the available intermediate compounds, resulting in an acceleration of the citric acid cycle and therefore in an acceleration of the incorporation of P32 into ATP. Since phospholipids are labelled from ATP32, an increase P32 incorporation should result, and this was indeed observed after a 30 minute incubation. In relation to the acceleration of the citric acid cycle, Kini and Quastel (138) found that 100 mM K+ ions stimulate the labelling from glucose-1-14C 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 mM potassium ions, the oxygen consumption 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 requirement, but is still high enough to support the stimulation 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 indicate 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 (189). 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 $^{32}$P 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 glutamine 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 $^{32}$P 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 breakdown of phosphocreatine in cerebral tissue on the addition of glutamate or ammonium ions is a very rapid process.

The inhibitory effects observed on the incorporation of $^{32}$P with the addition of succinate or γ-aminobutyric 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 cytoplasmic 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 $\alpha$-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 possible 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-glutamate 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 phospholipids in the presence of 100 mM K$^+$ or in the absence of
Ca** was found to take place with glucose, fructose, mannose 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 availability of pyruvate will support the stimulation of the pyruvate-acetyl-CoA step and therefore of the incorporation of P32 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 fructose, the substrates that support the incorporation of P32 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 acetylcholine 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 P₃² into phospholipids, (10⁴, 179) the formation of acetylcholine (198) and the stimulation of P₃² incorporation into phospholipids by cations or acetylcholine, in brain slices. These results lead to the conclusion that the stimulation of P₃² 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 P₃² into phospholipids as well as the stimulation of P₃² incorporation by 100 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 m\textsuperscript{M} K\textsuperscript{+} 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 observation would help to explain the inhibitory effect of a sodium-free medium on the incorporation of P\textsuperscript{32} into phospholipids (Table VIII) and in phosphoproteins (133) which are ATP dependent processes. The results of Gore and McIlwain (200) 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\textsuperscript{+} 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 concentration of potassium ions in the tissue is specifically dependent on the presence of Na\textsuperscript{+} ions, and in its absence, the stimulatory effect of 100 m\textsuperscript{M} K\textsuperscript{+} 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
P32 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 P32 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 P32 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 CaCl2 from the normal incubating medium causes a marked stimulation of oxygen uptake and a parallel stimulation of P32 incorporation into phospholipids of brain cortex slices during the early phases of incubation. The potassium-stimulated P32 incorporation 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 P32 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 mM KCl or by the omission of CaCl₂ 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 mM KCl or by the omission of CaCl₂ after 30 minutes incubation.

3. When brain cortex slices respiring in glucose are incubated in the presence of NH₄⁺ ions, succinate, glutamate or γ-aminobutyric acid, a marked inhibition of P32 incorporation into phospholipids occurs.

4. The stimulated P32 incorporation into phospholipids by the addition of 100 mM K⁺ ions, acetylcholine or by the omission of Ca²⁺ ions from the medium takes place only in the presence of high
When sodium ions are omitted from the normal incubation medium, the incorporation of $\text{p}^{32}$ into phospholipids is inhibited. The stimulatory effect of 100 mM $\text{K}^+$ ions or acetylcholine does not occur in a sodium-free medium. In contrast, a stimulation of $\text{p}^{32}$ incorporation is observed in a sodium-free medium when $\text{Ca}^{++}$ ions are absent.
CHAPTER IV

EFFECTS OF METABOLIC INHIBITORS ON THE STIMULATED INCORPORATION OF $^{32}P$ 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\textsuperscript{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\textsuperscript{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 metabolic inhibitors are known to inhibit the incorporation of P\textsuperscript{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\textsuperscript{32} into phospholipids.

In the present chapter, experiments on the effects of some metabolic inhibitors on the cationic or acetylcholine-stimulated incorporation of P\textsuperscript{32} into phospholipids are reported. 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 approximately 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 P32 into phospholipids of brain slices

The addition of 2 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 incorporation of P32 into phospholipids is not altered significantly at this concentration but contrary to a previous finding (104) there is a definite inhibition when higher concentrations such as 5 and 10 mM malonate are used. In the presence of 100 mM K+ or in the absence of Ca** ions there is a greater inhibition of the incorporation of P32 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 P32 incorporated is reduced 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**

<table>
<thead>
<tr>
<th>Malonate concn. (mM)</th>
<th>Incorporation of (P^{32}) into phospholipids*</th>
<th>Per cent of control</th>
<th>Oxygen uptake ((\mu l/mg.) dry weight)</th>
<th>Per cent of control</th>
<th>Incubation time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>16.7</td>
<td>5.0</td>
<td>8.5</td>
<td>170</td>
</tr>
<tr>
<td>2</td>
<td>10.5</td>
<td>11.1</td>
<td>4.8</td>
<td>5.0</td>
<td>104</td>
</tr>
<tr>
<td>5</td>
<td>6.5</td>
<td>4.4</td>
<td>3.4</td>
<td>2.9</td>
<td>86</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ca(^{++})free</th>
<th>%</th>
<th>Control</th>
<th>Ca(^{++})free</th>
<th>%</th>
<th>Incubation time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.3</td>
<td>16.4</td>
<td>133</td>
<td>4.8</td>
<td>6.5</td>
<td>135</td>
<td>30</td>
</tr>
<tr>
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<td>11.3</td>
<td>13.0</td>
<td>115</td>
<td>4.5</td>
<td>5.6</td>
<td>124</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>7.8</td>
<td>7.1</td>
<td>91</td>
<td>3.4</td>
<td>3.5</td>
<td>103</td>
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<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ACh</th>
<th>%</th>
<th>Control</th>
<th>+ACh</th>
<th>-</th>
<th>120</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>30.0</td>
<td>51.0</td>
<td>170</td>
<td>20.2</td>
<td>22.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>28.4</td>
<td>39.6</td>
<td>139</td>
<td>18.6</td>
<td>18.9</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>19.5</td>
<td>21.9</td>
<td>127</td>
<td>12.2</td>
<td>12.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8.9</td>
<td>9.3</td>
<td>104</td>
<td>10.0</td>
<td>9.9</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

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.
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 P32 into phospholipids.

The inhibitory effect of malonate on the stimulated P32 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 P32 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 P32 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 $^{32}P$ INTO PHOSPHOLIPIDS OF BRAIN SLICES IN RESPONSE TO 0.1M $K^+$ OR ACETYLCHOLINE

<table>
<thead>
<tr>
<th>Inhibitors Conc. (M)</th>
<th>Incorporation of $^{32}P$ into phospholipids</th>
<th>Oxygen uptake (µl/mg. dry weight)</th>
<th>Incubation time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control $+KCl$</td>
<td>$+KCl$ (0.1M)</td>
<td>% of control</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodoacetate $10^{-5}$</td>
<td>10.7</td>
<td>17.1</td>
<td>160</td>
</tr>
<tr>
<td>Iodoacetate $10^{-4}$</td>
<td>5.2</td>
<td>2.5</td>
<td>48</td>
</tr>
<tr>
<td>Iodoacetate $10^{-4}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na fluoride $10^{-3}$</td>
<td>23.6</td>
<td>33.5</td>
<td>142</td>
</tr>
<tr>
<td>Na fluoride $5x10^{-3}$</td>
<td>11.0</td>
<td>12.7</td>
<td>114</td>
</tr>
</tbody>
</table>

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 $^{32}P$ expressed as in Table I.
agreement with the observation of Strickland (104), iodoacetate and fluoride exert a marked inhibitory effect on the incorporation of P³² into the phospholipids of unstimulated brain slices. Iodoacetate (10⁻⁴M) inhibits the oxygen uptake by about 12 per cent when the slices are incubated for 30 minutes (experiments with 100 mM K⁺) whereas the incorporation of P³² 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 P³² is markedly decreased. These results illustrate the well known uncoupling property of iodoacetate. Similarly to the stimulated oxygen consumption, the potassium-stimulated incorporation of P³² is much more sensitive to iodoacetate than the unstimulated P³² incorporation resulting in a complete blocking of the potassium stimulatory effect. Moreover, the amount of P³² incorporated into the phospholipids is decreased to the endogenous level in the stimulated tissue when 10⁻⁴M iodoacetate is used. The acetylcholine stimulatory effect is less sensitive to iodoacetate than the cationic stimulation, a result similar to that obtained with malonate. Nevertheless, the acetylcholine stimulation of P³² incorporation is completely blocked by 10⁻⁴M iodoacetate. It can also be seen that fluoride (10⁻³ or 5×10⁻³M) exercises a profound inhibitory effect on
the acetylcholine stimulation of $\text{P}^{32}$ incorporation into phospholipids.

The effect of amytal and chloretone on the stimulated incorporation of $\text{P}^{32}$ 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 Ca²⁺ ions from the incubation medium (122, 123). In view of these considerations, the effect of amytal and chloretone on the cationic and acetylcholine-stimulated $\text{P}^{32}$ 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 Ca²⁺) 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 P³² INTO PHOSPHOLIPIDS OF BRAIN SLICES IN RESPONSE TO 0.1M K⁺, Ca⁺⁺ FREE OR ACETYLCHOLINE**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Incubation time (min)</th>
<th>Incorporation of P³² into phospholipids</th>
<th>Per cent inhibition</th>
<th>Oxygen uptake (µl/mg. dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control +KCl(0.1M)</td>
<td>Control +KCl(0.1M)</td>
<td>Control +KCl(0.1M)</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>10.8</td>
<td>17.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Amytal 0.5 mM</td>
<td></td>
<td>9.2</td>
<td>10.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Chloretone 2 mM</td>
<td></td>
<td>8.6</td>
<td>9.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Amytal 0.5 mM</td>
<td>30</td>
<td>10.1</td>
<td>13.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Chloretone 2 mM</td>
<td></td>
<td>8.2</td>
<td>9.6</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.0</td>
<td>8.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Amytal 0.5 mM</td>
<td>120</td>
<td>30.9</td>
<td>50.7</td>
<td>19.8</td>
</tr>
<tr>
<td>Chloretone 2 mM</td>
<td></td>
<td>24.2</td>
<td>31.6</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.4</td>
<td>25.9</td>
<td>11.1</td>
</tr>
</tbody>
</table>

Experimental conditions as in Table I. Acetylcholine (ACh) 2 mM was added with eserine 3x10⁻⁴ M. Results represent mean values obtained from 2 experiments.

*Incorporation of P³² 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 mM amytal or 2 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 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\textsuperscript{32} into phospholipids since this biochemical 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\textsuperscript{32} incorporation occurs with the unstimulated tissue. In the presence of 100 mM potassium ions, an inhibition of the stimulated oxygen consumption as well as P\textsuperscript{32} incorporation is obtained with 0.2M ethanol. The inhibitory effect under these conditions increases 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 addition of ethanol exerts an inhibitory effect on the stimulated incorporation of P\textsuperscript{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)**

<table>
<thead>
<tr>
<th>Ethanol concn. (M)</th>
<th>Incorporation of (\text{P}^{32}) into phospholipids (^*)</th>
<th>Per cent inhibition of K(^+) stimulation</th>
<th>Oxygen uptake ((\mu l/\text{mg. dry wt}/30) min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control +KCl (0.1M)</td>
<td></td>
<td>Control +KCl (0.1M)</td>
</tr>
<tr>
<td>0</td>
<td>11.8</td>
<td>19.5</td>
<td>5.2</td>
</tr>
<tr>
<td>0.2</td>
<td>12.6</td>
<td>17.9</td>
<td>5.7</td>
</tr>
<tr>
<td>0.4</td>
<td>12.0</td>
<td>15.7</td>
<td>5.3</td>
</tr>
<tr>
<td>0.6</td>
<td>10.9</td>
<td>13.6</td>
<td>5.2</td>
</tr>
<tr>
<td>0.8</td>
<td>9.9</td>
<td>10.9</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Rat brain cortex slices were incubated for 30 minutes in modified Krebs medium III with glucose 10 mM, NaH\(_2\)P\(^{32}\)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.
TABLE XIV

EFFECT OF ETHANOL ON THE INCORPORATION OF P\textsuperscript{32} INTO THE PHOSPHOLIPIDS OF BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF ACETYLCHOLINE

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

Rat brain cortex slices were incubated for 2 hours in modified Krebs medium III with glucose 10 mM, NaH\textsubscript{2}P\textsuperscript{32}O\textsubscript{4} 20 \textmu c. and ethanol as indicated.

Acetylcholine (ACh) 2 mM was added with eserine 3x10\textsuperscript{-4}M.

Results represent mean values obtained from 4 experiments.

\*Incorporation of \textsuperscript{32}P 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 P32 into the phospholipids of guinea-pig brain slices. In view of the similarity observed between the cationic and acetylcholine stimulation of P32 incorporation, a study of the effects of atropine and some related compounds on the cationic stimulation of P32 incorporation 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 P32 into phospholipids whereas high concentrations stimulate P32 incorporation. It can also be seen that the stimulatory effect of acetylcholine is inhibited by atropine, although the inhibition is not as marked as reported by the above authors. A possible explanation for the lowered sensitivity to atropine could be that the concentration of acetylcholine used in the present experiments 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 P³²
INTO PHOSPHOLIPIDS OF BRAIN SLICES IN RESPONSE TO ACETYLCHOLINE

<table>
<thead>
<tr>
<th>Additions</th>
<th>Conc. (M)</th>
<th>Incorporation of P³² into phospholipids</th>
<th>Per cent inhibition of Ach stimulation</th>
<th>Oxygen uptake (μl/mg. dry wt./120 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>+ ACh</td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>29.0</td>
<td>46.1</td>
<td>-</td>
</tr>
<tr>
<td>Atropine</td>
<td>2x10⁻⁵</td>
<td>28.1</td>
<td>38.5</td>
<td>40</td>
</tr>
<tr>
<td>Atropine</td>
<td>10⁻⁴</td>
<td>34.2</td>
<td>41.8</td>
<td>55</td>
</tr>
<tr>
<td>Atropine</td>
<td>2x10⁻³</td>
<td>43.0</td>
<td>48.5</td>
<td>68</td>
</tr>
<tr>
<td>Hyoscine</td>
<td>2x10⁻⁵</td>
<td>29.5</td>
<td>41.1</td>
<td>32</td>
</tr>
<tr>
<td>Hyoscine</td>
<td>10⁻⁴</td>
<td>33.8</td>
<td>43.2</td>
<td>45</td>
</tr>
</tbody>
</table>

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

Acetylcholine 2 mM was added with eserine 3x10⁻⁴M.

Results represent mean values obtained from 2 experiments.

*Incorporation of P³² 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 \( 2 \times 10^{-5} \) 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\(^{2+}\) 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 OMSSION OF CALCIUM**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concen. M</th>
<th>Incorporation of P(^{32}) into phospholipids</th>
<th>Oxygen uptake ((\mu)l/mg. dry wt./30 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control +KCl(0.1M) Ca(^{++})free</td>
<td>Control +KCl(0.1M) Ca(^{++})free</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>10.5 16.0 13.9</td>
<td>5.3 8.8 7.1</td>
</tr>
<tr>
<td>Atropine</td>
<td>2x10(^{-5})</td>
<td>10.0 16.4 14.3</td>
<td>5.1 8.7 7.1</td>
</tr>
<tr>
<td>Atropine</td>
<td>2x10(^{-4})</td>
<td>13.1 18.0 16.5</td>
<td>5.4 8.8 6.9</td>
</tr>
<tr>
<td>Atropine</td>
<td>10(^{-3})</td>
<td>14.3 19.0 17.3</td>
<td>5.0 8.3 6.8</td>
</tr>
<tr>
<td>Hyoscine</td>
<td>2x10(^{-4})</td>
<td>11.8 16.9 -</td>
<td>5.3 8.6 -</td>
</tr>
<tr>
<td>Hyoscine</td>
<td>10(^{-3})</td>
<td>13.5 17.5 -</td>
<td>5.1 8.2 -</td>
</tr>
</tbody>
</table>

*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\textsuperscript{32} INTO PHOSPHOLIPIDS OR BRAIN SLICES IN RESPONSE TO 0.1M K\textsuperscript{+} OR ACETYLCHOLINE

<table>
<thead>
<tr>
<th>Tubocurarine concn. (M)</th>
<th>Incubation time (min.)</th>
<th>Incorporation of P\textsuperscript{32} into phospholipids*</th>
<th>Oxygen uptake (µl/mg. dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>+ACh</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>29.1</td>
<td>44.0</td>
</tr>
<tr>
<td>2x10\textsuperscript{-5}</td>
<td>120</td>
<td>30.4</td>
<td>43.6</td>
</tr>
<tr>
<td>10\textsuperscript{-4}</td>
<td></td>
<td>27.0</td>
<td>41.5</td>
</tr>
<tr>
<td>5x10\textsuperscript{-4}</td>
<td>30</td>
<td>30.9</td>
<td>44.8</td>
</tr>
<tr>
<td>10\textsuperscript{-3}</td>
<td></td>
<td>29.4</td>
<td>42.0</td>
</tr>
<tr>
<td>0</td>
<td>30</td>
<td>13.0</td>
<td>20.7</td>
</tr>
<tr>
<td>10\textsuperscript{-4}</td>
<td></td>
<td>13.4</td>
<td>21.0</td>
</tr>
<tr>
<td>10\textsuperscript{-3}</td>
<td></td>
<td>14.5</td>
<td>21.8</td>
</tr>
</tbody>
</table>

Experimental conditions as in Table I. Acetylcholine 2 mM was added with eserine 3x10\textsuperscript{-4} M. Results represent mean values obtained from at least 2 experiments.

*Incorporation of P\textsuperscript{32} expressed as in Table I.
Inhibitors on the stimulated $^{32}$P 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 $^{32}$P, 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 $^{32}$P 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 observation 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 significantly affect the labelling of phospholipids in unstimulated tissue implies that succinic dehydrogenase is not greatly inhibited at this concentration.

However, the larger inhibition of P32 incorporation in K⁺-stimulated tissue caused by low and high concentration 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 percent 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 mM K+ or the omission of Ca++ ions (Table II).

The presence of iodoacetate or fluoride exercises a considerable inhibitory effect on the incorporation of $^{32}P$ 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 probably due to the inhibitory action of iodoacetate and fluoride 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 processes such as acetylcholine synthesis (214), $^{32}P$ incorporation into phospholipids (104) or phosphoproteins (215) and glutamine synthesis (135). It has been well established 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 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 P32 into phospholipids but also the cationic stimulated incorporation. A further point of interest arises from the results obtained with atropine. They confirm and emphasize the existence of an uncommon dissimilarity between the cationic and electrical impulse properties.

The lack of effect of tubocurarine on the acetylcholine stimulation was somewhat surprising and is difficult 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 investigated. Addition of malonate, iodoacetate or fluoride brings about decreases in the labelling of phospholipids. In the presence of 100 mM KCl or omission
of CaCl₂, the inhibitory effect of these agents is larger and brings the rate of P³² incorporation to the endogenous level. The acetylcholine stimulation of P³² 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 chloralose, at concentrations of 0.5 mM and 2 mM respectively, produces a slight decrease in the labelling of phospholipids from inorganic P³². The enhanced P³² 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 P³² into the phospholipids, but does not affect the cationic stimulatory effect.
CHAPTER V

EFFECT OF MORPHINE AND OTHER DRUGS ON THE INCORPORATION OF P32 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 P32 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 concentrations which do not affect the oxidative metabolism of brain, have been shown to stimulate the labelling of phospholipids from inorganic P\textsuperscript{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 morphine on the incorporation of P\textsuperscript{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\textsuperscript{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\textsuperscript{32} into phospholipids of brain cortex slices. It seemed of
interest to examine the action of morphine on the acetylcholine stimulatory effect, since this phenomenon appears to be a direct consequence of acetylcholine action (52).

The effect of morphine on the incorporation of $^{32}P$ into phospholipids and on the acetylcholine-stimulated $^{32}P$ 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 $^{32}P$ into phospholipids. At a higher concentration (5 mM), morphine causes a stimulation of $^{32}P$ incorporation of approximately 30 per cent and this enhancement of phospholipid labelling is increased to approximately 48 per cent in the presence of 10 mM morphine. In fact the stimulation of $^{32}P$ 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 morphine, the observed stimulation of $^{32}P$ incorporation by acetylcholine is not affected. At higher concentrations (5 or 10 mM) the stimulation produced by morphine is additive to that produced by acetylcholine resulting in a very marked increase in the incorporation of $^{32}P$ into
TABLE XVIII

EFFECT OF MORPHINE ON THE INCORPORATION OF P₃² INTO PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES IN THE ABSENCE AND PRESENCE OF ACETYLCHOLINE

<table>
<thead>
<tr>
<th>Morphine concn. (mM)</th>
<th>No. of expts.</th>
<th>Oxygen uptake (µl/mg. dry wt./2 hrs.)</th>
<th>Incorporation of P₃² into phospholipids*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Control)</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>19.5</td>
<td>31.3 (100)</td>
</tr>
<tr>
<td>0.2</td>
<td>2</td>
<td>19.1</td>
<td>30.1 (97)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>19.8</td>
<td>32.5 (104)</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>19.4</td>
<td>40.8 (130)</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>18.0</td>
<td>46.5 (148)</td>
</tr>
</tbody>
</table>

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 NaH₂P₃²O₄. Acetylcholine (ACh) 2x10⁻³M was added with eserine 3x10⁻⁴M. The figures in parentheses refer to percentages of the normal control value without morphine or acetylcholine.

*Incorporation of P₃² 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\textsuperscript{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\textsuperscript{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\textsuperscript{32} as observed above, preliminary studies of the effect of morphine on the K\textsuperscript{+}-stimulated incorporation of P\textsuperscript{32} into phospholipids were undertaken. It was observed that 2 or 10 mM morphine does not inhibit the K\textsuperscript{+}-stimulated incorporation of P\textsuperscript{32} into phospholipids. On the contrary, the K\textsuperscript{+}-stimulated P\textsuperscript{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\textsuperscript{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 $^{32}P$ into phospholipids of $K^+$-stimulated slices.

A time course of the effect of 2 mM morphine on the incorporation of $^{32}P$ 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 $^{32}P$ in the unstimulated tissue increases with the duration of the incubation period. With the addition of 2 mM morphine, the incorporation of $^{32}P$ is not affected. In the $K^+$-stimulated slices, a marked increase of approximately 60 per cent is obtained after a 30 minute incubation. The $K^+$-stimulated incorporation of $^{32}P$ subsequently decreases with time, and after 2 hours incubation it is negligible. After 3 hours incubation, the $^{32}P$ incorporation in the presence of 100 mM $K^+$ ions is inhibited as compared to the normal $^{32}P$ incorporation in the control medium.

When 2 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 $^{32}P$, which occurs with the duration of the incubation period, is inhibited by morphine. Indeed, after a 30 minute incubation
Rat brain cortex slices were incubated at 37°C in modified Krebs medium III with 10mM glucose and 20μc NaH₂P³₂O₄. Additions: O, nil; Δ, 2mM morphine; •, 0.1M KCl; ▲, 2mM morphine and 0.1M KCl. Incorporation of P³₂ into phospholipids expressed as in Table I.
when the K⁺-stimulated P³² incorporation is at its maximum (60%), the addition of morphine only produces a slight stimulation (10%). After 60 minutes incubation, when the K⁺-stimulated P³² incorporation is decreased to approximately 30 per cent, the presence of morphine produces a total stimulation of 50 per cent. After 2 hours incubation, the K⁺-stimulated incorporation is negligible. However, in the presence of morphine a stimulation of approximately 40 per cent is observed in the labelling of phospholipids.

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 results are shown in Table XIX. The emphasis was placed on experiments performed after a 2 hour incubation since at this time the stimulation of P³² 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 P³² 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 P³² incorporation
### TABLE XIX

**EFFECT OF MORPHINE ON THE INCORPORATION OF P³² INTO PHOSPHOLIPIDS OF BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF KCl (0.1M)**

<table>
<thead>
<tr>
<th>Morphine concn. (mM)</th>
<th>Incubation time 60 minutes</th>
<th>Incubation time 120 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incorporation of P³² into phospholipids*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No. exp.</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>21.0 (100)</td>
</tr>
<tr>
<td>0.2</td>
<td>2</td>
<td>20.1 (96)</td>
</tr>
<tr>
<td>2.0</td>
<td>4</td>
<td>22.8 (108)</td>
</tr>
<tr>
<td>5.0</td>
<td>4</td>
<td>28.5 (136)</td>
</tr>
<tr>
<td>10.0</td>
<td>4</td>
<td>28.5 (136)</td>
</tr>
</tbody>
</table>

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 NaH₂P³²O₄. 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 P³² 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\textsuperscript+ 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\textsuperscript+ ions produces a 30 per cent stimulation of the incorporation of p\textsuperscript{32} into phospholipids, the addition of 2 and 10 mM morphine further increases this stimulation caused by 100 mM K\textsuperscript+ 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\textsuperscript+ 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\textsuperscript{32} into the phospholipids of K\textsuperscript+ 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\textsuperscript+ 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; Δ, 2mM morphine; □, 10mM morphine; ●, 100mM KCl; ▲, 100mM KCl + 2mM morphine; ■, 100mM KCl + 10mM morphine.
Oxygen uptake expressed as -QO2 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\textsuperscript{+}-stimulated P\textsuperscript{32} incorporation itself which occurs with time (Fig. 5). Subsequent studies were undertaken in order to elucidate this effect of morphine in K\textsuperscript{+}-stimulated slices.

The effect of eserine on the incorporation of P\textsuperscript{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\textsuperscript{+}-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\textsuperscript{+}-stimulated tissue (Fig. 6) and the magnitude of the morphine stimulatory effect on P\textsuperscript{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 incorpo-
ration of $^{32}P$ 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 mM) has no effect either
on the unstimulated or stimulated oxygen uptake of brain
cortex slices. At concentrations of 1.5 and 5 mM, eserine
stimulates the incorporation of $^{32}P$ into phospholipids by
approximately 20 per cent. In the presence of 100 mM $K^+$
ions, contrary to what is observed with morphine, eserine
does not produce a further increase in phospholipid label-
ing. These results suggest that the stimulatory effect
of morphine in the presence of 100 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 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 $^{32}P$ INTO PHOSPHOLIPIDS OF BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF KCl (0.1M)

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

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 $^{32}P$ expressed as in Table I.
potassium-stimulated P\textsuperscript{32} incorporation, since the morphine stimulation increases when the potassium stimulation decreases (Fig. 5). It was shown in the first chapter that the decrease in the potassium-stimulated P\textsuperscript{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 morphine stimulatory effect in K\textsuperscript{+}-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 unstimulated tissue. When the 7mnp. level is decreased by approximately 35 per cent in the presence of 100 mM K\textsuperscript{+} 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 acetylcholine on the level of 7-minute nucleotide phosphate. This compound has no effect.

P\textsuperscript{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**

<table>
<thead>
<tr>
<th>Additions</th>
<th>7-minute nucleotide phosphate (µmoles/g. wet weight tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>Nil</td>
<td>1.5</td>
</tr>
<tr>
<td>Morphine 2 mM</td>
<td>1.64</td>
</tr>
<tr>
<td>Morphine 5 mM</td>
<td>1.48</td>
</tr>
<tr>
<td>KCl 100 mM</td>
<td>1.0</td>
</tr>
<tr>
<td>KCl 100 mM + Morphine 2 mM</td>
<td>1.12</td>
</tr>
<tr>
<td>KCl 100 mM + Morphine 5 mM</td>
<td>0.95</td>
</tr>
<tr>
<td>Acetylcholine 2 mM</td>
<td>1.52</td>
</tr>
</tbody>
</table>

Experimental conditions as described in Table XIX.
Incubation time 2 hours.
p\textsuperscript{32} into phospholipids of K\textsuperscript{+}-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 difference between the p\textsuperscript{32} incorporation into phospholipids in either unstimulated or K\textsuperscript{+}-stimulated slices of morphine treated rats and that of control rats.

The effect of nalorphine on the incorporation of p\textsuperscript{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\textsuperscript{+}-stimulated tissue.

Results given in Table XXIII show that the addition of nalorphine at concentrations of 0.5 and 2 mM exerts no effect on the incorporation of p\textsuperscript{32} into phospholipids of normal brain slices. With the K\textsuperscript{+}-stimulated slices, after 2 hours incubation, nalorphine (0.5, 2 mM) stimulates the incorporation of p\textsuperscript{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**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Morphine added to vessels (mM)</th>
<th>Incorporation of p_{32}^{32} into phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control +KCl 100 mM</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal rats</td>
<td>-</td>
<td>28.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>29.8</td>
</tr>
<tr>
<td>Morphine-treated rats</td>
<td>-</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30.2</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal rats</td>
<td>-</td>
<td>34.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37.0</td>
</tr>
<tr>
<td>Morphine-treated rats</td>
<td>-</td>
<td>32.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal rats</td>
<td>-</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>31.9</td>
</tr>
<tr>
<td>Morphine-treated rats</td>
<td>-</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>33.6</td>
</tr>
<tr>
<td>Mean Normal rats</td>
<td>-</td>
<td>31.1 (100)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32.9 (106)</td>
</tr>
<tr>
<td>Morphine-treated rats</td>
<td>-</td>
<td>31.8 (102)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>33.8 (108)</td>
</tr>
</tbody>
</table>

Brain cortex slices from normal or morphine treated rats were incubated in modified Krebs medium III with glucose 10 mM, NaH₂P³₂O₄ 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 PHOSPHOLIPIDS OF BRAIN CORTEX SLICES IN THE PRESENCE AND ABSENCE OF 0.1M KCl AND 2 mM MORPHINE**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Morphine (mM)</th>
<th>Nalorphine (mM)</th>
<th>Incorporation of P(^{32}) into phospholipids*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control + KCl (100 mM)</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>0.5</td>
<td>33.1 (100)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>34.2 (103)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>30.0 (91)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>31.1 (94)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>36.0 (109)</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>0.5</td>
<td>35.4 (100)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>34.3 (97)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>37.0 (105)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>35.6 (100)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>39.8 (113)</td>
</tr>
</tbody>
</table>

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 $\text{P}^{32}$ 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 morphine on the incorporation of $\text{P}^{32}$ 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 $\text{P}^{32}$ into phospholipids

Abadom et al (222) demonstrated that the biochemical 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 $10^{-4} \text{M}$, to slices of guinea-pig brain produces an increased labelling of phospholipids from inorganic $\text{P}^{32}$. It was of
interest to investigate in a parallel manner the effect of tofranil on the incorporation of $^{32}P$ 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 mM) tofranil has only a slight inhibitory effect on the oxygen consumption of slices whereas 0.5 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 mM tofranil does not affect significantly the respiration of brain slices, increases of 39 and 70 per cent respectively on the incorporation of $^{32}P$ are observed at these concentrations. Along with the inhibition of oxygen uptake, 0.5 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 $^{32}P$ 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 $^{32}$P INTO PHOSPHOLIPIDS OF RAT BRAIN CORTEX SLICES

<table>
<thead>
<tr>
<th>Tofranil concn. (mM)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>% Mean of tofranil effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxygen uptake</td>
<td>Incorporation % of $^{32}P$ into phospholipids*</td>
<td>Oxygen uptake</td>
</tr>
<tr>
<td>0</td>
<td>19.8</td>
<td>28.4</td>
<td>100</td>
</tr>
<tr>
<td>0.05</td>
<td>19.1</td>
<td>40.0</td>
<td>141</td>
</tr>
<tr>
<td>0.1</td>
<td>18.3</td>
<td>46.9</td>
<td>165</td>
</tr>
<tr>
<td>0.5</td>
<td>12.1</td>
<td>11.3</td>
<td>40</td>
</tr>
</tbody>
</table>

Rat brain cortex slices were incubated in modified Krebs medium III with glucose 10 mM, NaH$_2$P$_{32}$O$_4$, 20 μc. and tofranil for 2 hours.

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

*Incorporation of $^{32}P$ expressed as in Table I.
With the potassium-stimulated brain slices, which were shown in the previous chapter to be much more sensitive to narcotics than the unstimulated tissue, the addition of morphine does not exert any inhibitory effect. This inability of morphine, even at a concentration of $10 \text{ mM}$, to inhibit the potassium-stimulated oxygen consumption and the incorporation of $\text{P}^{32}$ 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 stimulation is inhibited by $1 \text{ mM}$ morphine, a concentration which is without effect upon unstimulated tissue. In this connection, the results obtained above with morphine are of particular interest in that they illustrate a marked difference 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 incorporation of $\text{P}^{32}$ 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 $10 \text{ mM}$ morphine on the phospholipid 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 ($10^{-3} - 10^{-2} \text{M}$) which stimulate the incorporation of $\text{P}_3^2$ 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 $\text{P}_3^2$ 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 $\text{P}_3^2$ 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 $\text{P}_3^2$ 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 acetylcholine, suggests that the mechanism of action of these two compounds on the labelling of phospholipids from inorganic p³² may be analogous.

The results obtained with eserine on the incorporation of p³² into phospholipids with potassium-stimulated slices are in good agreement with the previous findings on the effect of atropine on the incorporation of p³² into phospholipids of K⁺-stimulated tissue (Table XVI). These results clearly suggest that the effect of 100 mM K⁺ ions on the incorporation of p³² into phospholipids is not secondary 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 p³² into phospholipids in the presence of 100 mM K⁺ ions, since they respectively support and inhibit the stimulation of p³² 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 acetylcholine synthesis in brain slices, but that the stimulation of p³² 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 results (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 $p^{32}$ incorporation has no pharmacological significance. This conclusion 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 surprising 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-
The incorporation of $^{32}$P 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-$^{14}$C 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 stimulatory effect of another compound on the incorporation of $^{32}$P into phospholipids without stimulating the oxidative metabolism of the brain slices. As previously mentioned, acetylcholine and atropine (147), chlorpromazine and azacyclonol (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 stimulatory effect on the incorporation of $^{32}$P into phospholipids of brain cortex slices without affecting the factors which usually govern the incorporation of $^{32}$P into phospholipids. In this connection, their effect on the phospholipid labelling 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 mM.

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

3. Morphine does not significantly affect the stimulation of $^{32}$P incorporation into phospholipids caused by 2 mM acetylcholine.

4. In the presence of 100 mM potassium ions, morphine does not inhibit the stimulated $^{32}$P incorporation. On the contrary, it exerts a slight stimulatory 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 mM potassium ions decreases with time.

5. Eserine exerts a small stimulation on the incorporation of $^{32}$P into phospholipids of unstimulated slices. In the presence of 100 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 mM) has no effect on the incorporation of P32 into phospholipids of unstimulated slices. In the presence of 100 mM potassium ions, nalorphine exerts a stimulatory effect on the incorporation of P32 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 P32 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 increasing number of observations which suggest that phospholipids may play some role in ion transport (41 - 46, 51, 52, 6). The cationic stimulation of P32 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 P32 into phospholipids, 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 P32 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 incorporation of P32 into phospholipids (Table XXV). Although the above substances which stimulate P32 incorporation are different, they appear to be producing their stimulatory
### TABLE XXV

**BIOCHEMICAL EFFECTS OF 0.1M KCl AND ACETYLCHOLINE ON BRAIN CORTEX METABOLISM**

<table>
<thead>
<tr>
<th>Metabolic Process</th>
<th>KCl, 0.1M</th>
<th>ACh, 2mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oxygen uptake with glucose or pyruvate as the substrate</td>
<td>Increased</td>
<td>No effect</td>
</tr>
<tr>
<td>2. Oxygen uptake with succinate or glutamate as the substrate</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>3. Incorporation of P32 into phospholipids in presence of glucose or pyruvate</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>4. Incorporation of P32 into phospholipids in presence of succinate or glutamate</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>5. Incorporation of P32 into phospholipids in presence of glucose and malonate or iodoacetate</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>6. Incorporation of P32 into phospholipids in presence of glucose and narcotics</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>7. Incorporation of P32 into phospholipids in presence of glucose and atropine</td>
<td>No effect</td>
<td>Decreased</td>
</tr>
<tr>
<td>8. Concentration of 7-minute nucleotide phosphate</td>
<td>Decreased</td>
<td>No effect</td>
</tr>
<tr>
<td>9. Incorporation of P32 into 7-minute nucleotide phosphate</td>
<td>Increased</td>
<td>--</td>
</tr>
<tr>
<td>10. Incorporation of P32 into phosphatidic acid</td>
<td>Increased</td>
<td>Increased (134)</td>
</tr>
<tr>
<td>11. Incorporation of P32 into phospholipids in presence of glucose and sodium-free medium</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>
effect through a common biochemical mechanism.

It was shown that the addition of 100 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 $^{32}$P into phospholipids and 7-minute nucleotide phosphate (ATP) after a 30 minute incubation period. In view of the general belief that the incorporation of $^{32}$P into the phospholipids is dependent upon the supply of energy and is derived from ATP$^{32}$, it appears as logical that the incorporation of $^{32}$P into phospholipids should be increased when the incorporation of $^{32}$P into ATP is also increased, as observed above.

In this respect, the stimulatory effect produced by 100 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 $^{32}$P 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 $^{32}$P into phospholipids even after a 30 minute incubation. Kini (218) has also reported that 100 mM potassium ions
stimulate the synthesis of glutamine from glucose-U-C\text{14}\text{4} while DNP exercises a definite inhibition on the formation of glutamine.

The depletion of high energy phosphates by the addition of 100 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\text{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\text{32} incorporation into phospholipids occurs mainly in the phosphatidic acid fraction. Similarly, Hokin and Hokin (67) have also shown that the acetylcholine stimulation of P\text{32} incorporation into phospholipids takes place in phosphatidic acid and phosphoinositide.

Two possible interpretations may be offered for
the increased incorporation of $\text{p}^{32}$ 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 dihydroxyacetone phosphate and $\alpha$-glycerol phosphate, due to increased glucose oxidation caused by K$^+$ ions. However, the fact that pyruvate supports the stimulation of $\text{p}^{32}$ incorporation 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:

$$\text{glucose} \leftrightarrow \text{glycerol} + \text{ATP} \rightarrow \alpha\text{-glycerol phosphate} \rightarrow \text{phosphatidic acid}$$

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 phosphatidic acid labelling from inorganic P³² 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 mM potassium ions on brain cortex metabolism (227, 139, 218), also stimulates the incorporation of P³² 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 P³² 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 P³².
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 mM potassium or the omission of calcium ions from the incubation medium stimulates the incorporation of P32 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 mM succinate or 5 mM Y-aminobutyric acid to rat brain cortex slices metabolizing glucose does not significantly affect the incorporation of P32 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 P32 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 P32 into phospholipids of brain cortex slices. The stimulation of P32 incorporation produced by 2 mM 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 $^{32}P$ into phospholipids. On the contrary, in the presence of 100 mM potassium ions, 2 mM morphine exerts a small stimulation which is additive to that produced by 100 mM potassium ions. This stimulatory effect produced by morphine increases with the duration of the incubation period, whereas the stimulation produced by 100 mM potassium ions decreases with time.

10. Eserine has no effect on the incorporation of $^{32}P$ 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 mM potassium ions during an incubation period of 2 hours.

12. There is no significant difference in the incorporation of $^{32}P$ 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 $^{32}P$ into phospholipids of potassium stimulated brain slices after an incubation period of 2 hours. Nalorphine does not antagonize the stimulatory effect of morphine under
14. Tofranil at low concentrations exerts a stimulatory effect on the incorporation of $\text{p}^{32}$ into phospholipids of brain cortex slices, while at high concentration it produces an inhibitory effect.


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