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Commentary by

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on 'Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices'
by L.E. Hokin and M.R. Hokin
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The paper which is reproduced here [1] provided the first indication that phosphoinositide metabolism was implicated in the response of cells to receptor occupation by agonists. As such, it could be said to be the progenitor of all the work which has been done since then on the phosphoinositide second messenger system. Several observations which were to become a part of the 'phosphoinositide cycle', which we proposed some years later, were foreshadowed in this paper. These findings were that stimulation of pancreas and brain cortex slices by acetylcholine resulted in increased incorporation of ^{32}P into both phosphoinositide and phosphatidic acid and that the incorporation appeared to be due to turnover of phosphate or phosphoryl-units on preformed diacylglycerol (DG) rather than synthesis de novo. The follow-up on these observations was to keep us busy for some time.

In work which led up to this paper, between 1952 and 1954, we had found that exposure of pancreas slices and brain cortex slices to acetylcholine greatly stimulated the incorporation of ^{32}P into phospholipids. This was initially a serendipitous observation which arose out of experiments which Lowell performed in H.A. Krebs' laboratory at Sheffield University. Lowell was studying protein secretion and synthesis in pigeon pancreas slices and at that time was interested in the possible involvement of RNA in protein synthesis. Some cytological studies in the late 1940s by Caspersson and Brachet had shown that RNA levels in a variety of dividing and non-dividing cells were correlated with their protein synthesis activity; RNA levels are particularly high in the pancreas, which has a high rate of synthesis of digestive enzymes. An experiment was designed to follow up on an abstract which had just been published by the Russian workers, M.A. Guberniev and L.I. Il'ina. These workers reported that, on cholinergic stimulation of secretion in pancreas, stomach, and salivary glands in anesthetized dogs, there were marked

Lowell and Mabel Hokin in the early 1950s at a meeting in Copenhagen

increases in the incorporation of ^{32}P into 'nucleoproteins' isolated by the method of Schmidt and Thannhauser. This method was really designed to isolate phosphorus-containing fractions, including DNA and RNA (separated by alkaline hydrolysis), and not nucleoproteins, but no further details were given. Lowell found that on stimulation of the pancreas slices with a cholinergic agent, carbachol, the incorporation of ^{32}P into the RNA fraction was increased by about 100%.

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The Schmidt and Thannhauser separation technique was, however, rather primitive, especially when applied to small tissue samples, and Lowell began to suspect that an alkaline hydrolysis product of phospholipids might be contaminating the RNA fraction. At this stage, in 1952, we both transferred to J.H. Quastel's laboratory at the Montreal General Hospital Research Institute, McGill University, taking with us samples from an experiment which was in progress. (Time did not permit its completion before we embarked for Canada.) When we arrived in Montreal we purified the total lipid fraction from these samples and found a very large increase in the ^{32}P specific radioactivity in the lipids from pancreas slices that had been stimulated with carbachol. Almost immediately we looked to see whether acetylcholine would produce the 'phospholipid effect' in brain slices. We were very excited to find that it did, and we indulged in considerable speculation as to how this might relate to synaptic transmission. With more rigorous methods for the isolation and purification of nucleic acids from small samples of tissue, developed by others in 1952, we were able to show that there was no stimulation of ^{32}P incorporation into RNA. Our first paper on this 'phospholipid effect' was published in 1953 and it is often incorrectly cited as demonstrating the stimulated turnover of phosphatidylinositol (PI) [2]. Stimulated turnover of phosphoinositide was not actually shown until the BBA paper which is featured here [1].

In 1954, R.M.C. Dawson published in this journal the first method which could be used to investigate the incorporation of ^{32}P into individual phospholipids [3]. By this method, the total lipid fraction is submitted to mild alkaline hydrolysis and the water-soluble products of the individual phospholipids are separated by two-dimensional chromatography. Losing no time, we put this method to work, to produce the results given in the paper which is reprinted here. It was very exciting to find for the first time that, in both pancreas slices and brain cortex slices, phosphoinositide, which is a quantitatively minor lipid component of cell membranes, had the most markedly stimulated incorporation of ^{32}P in response to acetylcholine! At this time the chemical nature of the inositol-containing lipids was very obscure. For example, in a paper in the same journal issue as our cited paper, J.N. Hawthorne starts out with the observation that, "Several lipids containing inositol have been shown to occur in plant or animal tissues, but the chemical structure of none is completely known." [4]. Our hydrolysis product from brain was presumed to be from diphosphoinositide, although the actual structure of this lipid was not known. This presumption was based on reports from J. Folch's laboratory in the 1940s that the only phosphoinositide fraction found in brain showed a phosphorus : inositol ratio of 2; this he called diphosphoinositide. When more sophisticated methodology became available, three phosphoinositides, PI, phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2) were identified in animal tissues, including brain. The structures of the latter two phosphoinositides were determined in the early 1960s in the laboratories of R.M.C. Dawson and C.E. Ballou. The development in G.V. Marinetti's laboratory of methods for the chromatography of the intact phospholipids on silicic acid-impregnated paper [5] greatly facilitated our phosphoinositide work. In a paper later than the one cited here [6], we were able to show that the increase in phosphoinositide ^{32}P in pancreas slices was primarily in a lipid, separated by chromatography, that gave inositol monophosphate after acid hydrolysis – a predicted property of PI. We also synthesized *myo*-[2-^{3}H]inositol by catalytic reduction of *myo*-inosose-2 in the presence of tritium and found that there was an increased incorporation of inositol into phosphoinositide during stimulation with acetylcholine. This also occurred in brain cortex slices [7].

In the cited paper we observed that acetylcholine stimulated the incorporation of ^{32}P not only into a phosphoinositide, but also into a highly labeled lipid in pancreas and brain slices which was presumed to be phosphatidic acid (PA) since it yielded glycerophosphate after mild alkaline hydrolysis. This foreshadowed a place for PA in the overall reactions of what eventually has become known as the phosphoinositide cycle. There was considerable uncertainty at this time as to the presence of PA in animal tissues in vivo, so we felt compelled to establish this. We obtained several lines of evidence which indicated that PAs occurred in animal tissues both in vivo and in vitro, and that they contained unsaturated fatty acids [8].

While we were still in Montreal, at McGill University, we showed that increased turnover of phosphoinositides occurred in several different tissues in response to several agents. Even at that early time it seemed that the phenomenon was eventually going to be far reaching and of wide importance. Currently, very many cells and tissues have been shown to give a phosphoinositide response to a multitude of agents.

The results in the accompanying paper led us, by a comparison of the incorporation of [1-^{14}C]glycerol and ^{32}P into the phospholipids, to suggest that the bulk of the increased incorporation of ^{32}P into the phospholipids was due to a turnover of either phosphoryl units or phosphate on preformed DG. This implied that the effect did not involve the synthesis of PA by the known pathway of acylation of glycerophosphate. Accordingly, one of the first things that we did after moving to the University of Wisconsin, Madison, in 1957, was to look for an enzyme which would form PA by phosphorylation of DG. We soon found DG kinase activity in brain microsomes [9]. At about this time, the pathway for PI synthesis via PA and CDP-DG was being reported from

the laboratories of Agranoff and of Kennedy. This provided a pathway whereby DG could be phosphorylated by DG kinase and then converted to PI. DG kinase was destined to become one of the enzymes in the overall phosphoinositide cycle.

Shortly after our transfer to Madison, an event occurred which was to have a very significant effect on the direction of much of our research during the next 15 years. Schmidt-Nielsen and his co-workers published a series of papers in which they reported that the function of the supraorbital glands of marine birds was to secrete a hypertonic solution containing up to 1 M NaCl after ingestion of excessive NaCl, and that the secretory activity of these salt glands was under parasympathetic control and could be stimulated by the injection of acetylcholine. We thought that this tissue would be well suited for an attack on the possible role of phosphatides in ion transport – an area which was receiving much attention from people in the membrane field at the time. The original report from Schmidt-Nielsen mentioned only the salt gland of the albatross. Following up on it, we contacted the U.S. Navy, which kindly provided us with laysan and black-footed albatrosses from the Midway Islands in the spring and summer of 1959. These 'gooney birds' were delightful, friendly creatures and we were sad to have to use them. Our two small daughters, Linda and Cathy, born in 1953 and 1957, respectively, enjoyed feeding them squid; our son, Sam, was born in 1960, so he missed out on this! Later, we obtained permission to use sea gulls from the Great Lakes. Then it became apparent that water birds such as domestic geese and domestic ducks would develop hypertrophied salt glands if given a saline solution as their drinking water. These domestic species are used now mainly by workers who wish to study salt gland function.

In our first work with the albatross salt gland we found that incubation of tissue slices with acetylcholine led to a 13-fold increase in the rate of incorporation of ^{32}P into PA and a 3-fold increase in the incorporation of ^{32}P and [2-^{3}H]inositol into phosphoinositide. Incorporation of ^{32}P into PC and PE was increased relatively slightly or not at all [10]. From this very big effect of acetylcholine on PA metabolism we were tempted to speculate that PA might be a component of the much sought after sodium pump. We proposed a scheme whereby PA functioned as a sodium carrier in the extrusion of Na^+ ions. This scheme involved a PA-DG cycle in the plasma membrane that was catalyzed by the enzymes PA phosphatase and DG kinase; both of these enzymes were present in the membrane fraction from the tissue. After the discovery by J.C. Skou of an Na^+, K^+-dependent ATPase activity in crab nerve [11], evidence mounted over the next several years (mainly, at that time, from the laboratories of R.L. Post, I.M. Glynn and R.W. Albers) that this enzyme was the molecular machine which effected Na^+ and K^+ transport across the plasma membrane. In order to test whether phosphorylation of DG to form PA might be a component of this enzyme activity, we compared the turnover of PA in salt gland homogenates with the catalytic activity of the Na^+/K^+-ATPase; the latter was orders of magnitude higher [12]. Thus, PA could not be an intermediate in the activity of Na^+/K^+-ATPase and the hypothesis was abandoned. The work had a far-reaching effect on our future, however. We became primarily identified in the field of biological transport, perhaps partly because there was still very little general interest in the phosphoinositide story. The 1960s saw the discovery of the cyclic-AMP/adenyl cyclase second messenger system for cell regulation by hormones and neurotransmitters. The system was to dominate the second messenger field for almost two decades. From the latter half of the 1960s until the 1980s, Lowell devoted most of his attention to the isolation and characterization of Na^+/K^+-ATPase.

Before that, however, a dramatic moment occurred in the salt gland work. During the development of an autoradiogram of lipids from a rather complicated salt gland kinetic experiment, the ^{32}P-labeled PI and PA spots darkened up, and it became clear from the pattern of their labeling that, on stimulation with acetylcholine, PI breakdown occurred and this was followed by PA synthesis. Stimulation of hydrolysis of PI which had been prelabeled with [2-^{3}H]inositol confirmed this result. Conversely, when stimulation was quenched by atropine, the stimulated [^{32}P]PA disappeared and a virtually stoichiometric amount of [^{32}P]PI was formed. We had a cycle!

We published these studies and proposed the PI-PA cycle in 1964 in a symposium edited by R.M.C. Dawson and D.N. Rhodes [13] (Fig. 1). (The same PA-PI cycle was also included in an article by us on the Chemistry of Cell Membranes in 'Scientific American', October, 1965.) The main points of this phosphoinositide cycle were that, on cholinergic stimulation of salt gland slices, phospholipase C, which had been reported from the laboratories on J.N. Hawthorne and R.M.C. Dawson, catalyzed the hydrolysis of PI to DG and inositol 1-monophosphate (IP). DG kinase then phosphorylated DG to form PA. On quenching with atropine, the resting steady-state level of PI was restored at the expense of PA by the sequential actions of PA-cytidyltransferase and PI synthase. This cycle was confirmed in pancreas tissue using sequential stimulation by acetylcholine, quenching by atropine, and restimulation with cholecystokinin-pancreozymin. It was supported by the finding that, during stimulation, there was a net loss of the mass in PI and a net rise in the mass of PA [14]. The newly formed PA had the same stearoyl, arachidonoyl composition as that of PI [15].

In 1973, Mabel attended the 16th International Con-

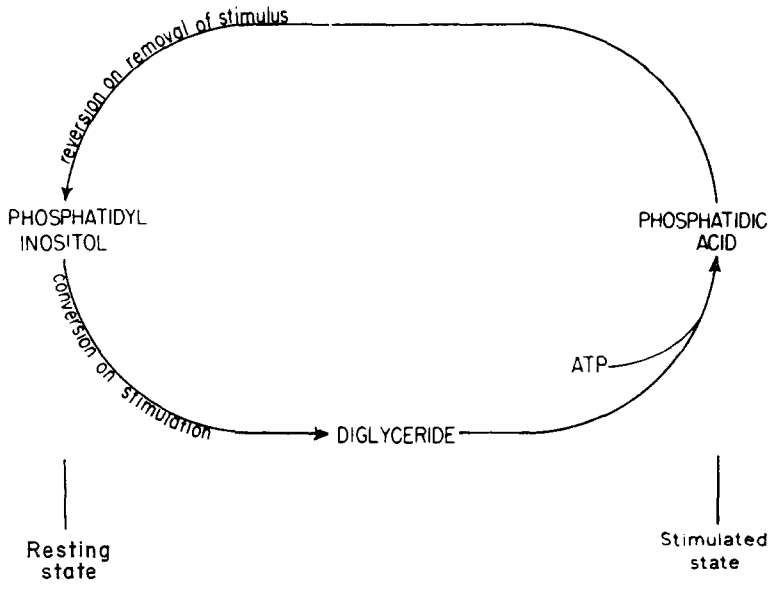

Fig. 1. The original version of the phosphoinositide cycle. Ref. 13.

ference on the Biochemistry of Lipids, organized by J.N. Hawthorne and held in Nottingham, England. R.H. Michell and others in the field were there and we all had many fruitful discussions. At that time, only cyclic AMP and Ca^{2+} were known to act like second messengers. (The classic experiments from the laboratories of W.W. Douglas and B. Katz which pointed to the importance of Ca^{2+} in stimulus-response coupling were carried out in the early 1960s.) The only system other than the adenyl cyclase system which was known to show fairly rapid changes in the rate of catalytic activity of existing enzymes (as opposed to the time needed for inductive protein synthesis) was the agonist-receptor-stimulated changes in membrane phosphatides. In 1972, Michell and Lapetina had put forward the provocative idea that inositol cyclic phosphate, formed by hydrolysis of PI, might act as a second messenger [16]. (R.M.C. Dawson and his co-workers had shown that inositol 1-phosphate and inositol 1,2-cyclic phosphate were the products of PI-specific phospholipase C activity.) Several of us tested out this idea in various ways but were unable to find any evidence for it. However, the idea probably influenced the thinking that later led to the search for a second messenger function for inositol 1,4,5-trisphosphate (IP_3) – a product of the breakdown of PIP_2. At the Nottingham meeting we discussed the fact that many of the responses which are evoked in tissues by stimuli which give increased PI turnover appeared to involve Ca^{2+}. Perhaps partly as a result of this meeting, in 1975 Michell published, in this Journal, his monumental review on inositol phospholipids and cell surface receptor function [17]. In this he reviewed the entire field, noted the correlation between the PI turnover and Ca^{2+} mobilization, and put forward his seminal hypothesis of a link between receptor-activated PI turnover and cell Ca^{2+} mobilization, in which PI hydrolysis was linked to and antecedent to Ca^{2+} gating in the plasma membrane. This review stimulated a great deal of interest and activity in the field.

In the early 1980s much activity began to focus on the polyphosphoinositides, PIP and PIP_2, and their possible relationship to Ca^{2+} gating. In our early studies in the 1960s, we showed for the first time the presence of kinases for PI and PIP (in the erythrocyte membrane), as well as the rapid exchange of ^{32}P in the mono-esterified phosphates of PIP and PIP_2 in erythrocyte membranes on incubation with [^{32}P]ATP. We also reported a decrease in the level of ^{32}P-labeled PIP and PIP_2 after stimulation with acetylcholine in the salt gland [18]. Alas, we did not carry this any further! In the late 1960s, J. Durell demonstrated the formation of IP and inositol 1,4-bisphosphate (IP_2) in cholinergically stimulated synaptosomes, and suggested that the primary response might be the phosphodiesteratic cleavage of the polyphosphoinositides, releasing inositol phosphates. A.A. Abdel-Latif pursued this line in the late 1970s and showed that cholinergic or adrenergic stimulation of iris smooth muscle caused a rapid breakdown of PIP_2, which was accompanied by an increase in IP, IP_2 and IP_3. At the time, Abdel-Latif did not believe that polyphosphoinositide breakdown was antecedent to Ca^{2+} gating, for two reasons. The breakdown was dependent on Ca^{2+} (later shown to be at concentrations lower than basal cytosolic levels) and it was stimulated by Ca^{2+} ionophores (this was later found to be due to a direct release by ionophores of norepinephrine, a Ca^{2+}-mobilizing neurotransmitter). In 1983, Michell and co-workers reported a very rapid breakdown of PIP_2 in

response to the Ca^{2+}-mobilizing agonist, vasopressin, in hepatocytes and the effect was independent of, or only partially dependent on, the presence of Ca^{2+} in the incubation medium [19]. Also in 1983, M.J. Berridge showed the rapid accumulation of IP_3 in the blow-fly salivary gland in response to 5-hydroxytryptamine [20]. IP_3 was soon found by H. Streb, R.F. Irvine, M.J. Berridge and I. Schulz to mobilize Ca^{2+} from the endoplasmic reticulum of permeabilized pancreatic cells [21]. A new second messenger was born! At about the same time, Y. Nishizuka in Japan found that protein kinase C could be activated by diacylglycerol and proposed that the diacylglycerol released by hydrolysis of phosphoinositides acted as a second messenger to regulate the activity of protein kinase C [22]. In the overall scheme proposed by these workers, PIP_2 in the plasma membrane is hydrolyzed to DG and IP_3, with each acting as a second messenger. Currently, other inositol phosphates such as inositol 1 : 2 cyclic 4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, and inositol 1,4-bisphosphate are under active investigation as possible second messengers. So, finally, the phosphoinositide effect has come to some fruition. The old phosphoinositide cycle is the basis for a new scheme in which PI kinase and PIP kinase have been included. There is still some controversy as to whether there is also direct phosphodiesteratic cleavage of PI and PIP as well as PIP_2, perhaps as an additional source of DG.

It has been 35 years since we first squinted along the two-dimensional chromatogram and saw the correspondence between the major radioactive spot and the stained phosphate spot from inositol lipid. It is gratifying to see the current explosion of interest in this field and to be finally sure that the phosphoinositide system can take its place alongside the adenyl cyclase system as a fundamental mechanism for the regulation of a multitude of cellular functions.

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EFFECTS OF ACETYLCHOLINE ON THE TURNOVER OF PHOSPHORYL UNITS IN INDIVIDUAL PHOSPHOLIPIDS OF PANCREAS SLICES AND BRAIN CORTEX SLICES*

by

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Earlier studies showed that acetylcholine stimulates the turnover of phosphate in the total phospholipids of slices of pancreas, parotid gland, submaxillary gland and brain cortex[1,2,3,4]. In pancreas slices maximal stimulation (up to ten-fold) of phosphate turnover in phospholipids was achieved with a relatively low concentration of acetylcholine ($10^{-5}M$), whilst in brain cortex slices this stimulation increased with increasing concentrations of acetylcholine, being approximately two to three-fold at $10^{2}M$ acetylcholine. Chemical fractionation procedures showed that in pancreas the turnover of phosphate in the cephalins was stimulated to a much greater extent than in lecithin[2]. This marked difference in the behaviour of the cephalins as compared to lecithin was not found in brain cortex slices. In brain cortex the stimulation of phosphate turnover was greater in the total ether-soluble phospholipids than in the glycerophosphate isolated after strong alkaline hydrolysis of the lipid extract[4]. This indicated that in brain cortex, phosphate turnover in some ether-soluble phospholipid which did not yield glycerophosphate on strong alkaline hydrolysis was particularly responsive to acetylcholine. It was suggested that this phospholipid might be diphosphoinositide.

Naturally, the isolation of the individual phospholipids in pure form would be preferable to the somewhat crude chemical fractionation procedures employed in our earlier studies. Recently, DAWSON[5] described chromatographic procedures for isolating the water-soluble hydrolysis products of the individual phospholipids. The hydrolysis products which were identified on paper were glycerylphosphorylcholine, glycerylphosphorylethanolamine, glycerylphosphorylserine, a phosphorus-containing hydrolysis product of diphosphoinositide, and glycerophosphate. These hydrolysis products were derived from phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, diphosphoinositide and phosphatidic acid, respectively. Quantities of tissue as small as 150 mg were found satisfactory for this procedure. With the use of this method, DAWSON[5] was able to determine the specific activities of the individual phospholipids of brain dispersions after incubation with ^{32}P under appropriate conditions. We have found the procedure of DAWSON[5] to be well suited for studies on the effect of acetylcholine on the turnover of phosphate in the individual phospholipids in small quantities of tissue slices incubated *in vitro*. The data reported below are from such studies.

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MATERIALS AND METHODS

Procedures already described[1,2,4] were used for the preparation and incubation of slices of pigeon pancreas and guinea pig brain cortex. In experiments with brain cortex, equal portions of surface slices from each of two guinea pig brains were added to the control vessel and to the vessel containing acetylcholine (plus eserine). In experiments with pancreas, slices from the pancreas of one pigeon were added to control and to experimental vessels. 50 to 100 mg of tissue were added per ml of incubation medium. The slices were incubated in conical flasks with shaking. The volume of the flask was approximately ten times the volume of the incubation medium.

The following isotopes were used in the various experiments: $NaH_2{}^{32}PO_4$ (obtained from Atomic Energy of Canada, Ltd., Chalk River, Ontario), glycerol-1-^{14}C (obtained from the Radiochemical Centre, Amersham, England), and ethanolamine-2-^{14}C ($HOCH_2{}^{14}CH_2NH_2$) which was synthesised by Dr. D. E. DOUGLAS of the Research Institute, the Montreal General Hospital.

After incubation the tissues were homogenised in 5% trichloroacetic acid and treated according to the method of DAWSON[5], with the following modifications. Instead of ascending chromatography, descending chromatography was used in the second dimension with the *tert*-butanol/trichloroacetic acid solvent. The phosphorus compounds were identified on paper by the method of WADE AND MORGAN[6]. This method does not decompose the compounds, so they can be eluted from the paper. The spots were cut out and eluted with molar ammonia by a previously described technique[7]. Counts and total phosphorus were determined on aliquots of the eluate. Phosphorus determinations on the samples of glycerylphosphorylserine, the hydrolysis product of diphosphoinositide, and glycerophosphate were carried out by the method of BERENBLUM AND CHAIN[8]. Glycerylphosphorylcholine and glycerylphosphorylethanolamine were obtained in larger amounts; the phosphorus in these samples was determined by the method of FISKE AND SUBBAROW[9]. Specific activities after incubation with either ^{32}P or ^{14}C labelled compounds are expressed as counts/min/μg phospholipid P (corrected in the case of ^{32}P to a specific activity of 100,000 counts/min/μg inorganic P in the medium).

RESULTS

Effects of acetylcholine on the incorporation of ^{32}P into the individual phospholipids of pigeon pancreas slices

The paper chromatogram obtained from the lipids of pancreas slices showed phosphorus-containing spots corresponding in R_F values to each of the spots illustrated by DAWSON[5] on a chromatogram obtained from the lipids of a guinea pig brain dispersion. The products of mild alkaline hydrolysis of the lipids of these two tissues would appear therefore to be qualitatively identical. The material from brain tissue which has the lowest R_F in each of the solvents was shown by DAWSON[5] to be hydrolysis product of brain diphosphoinositide. The similarity in R_F values of the corresponding spot on the chromatogram obtained from pancreas material suggests that the inositol-containing phospholipids of pancreas give rise to the same hydrolysis product as that obtained from brain diphosphoinositide. For convenience, we refer to the parent material of this hydrolysis product as pancreas phosphoinositide, although it should be pointed out that there is at this stage no rigorous proof of identification of the material isolated. The parent material of the small quantity of glycerophosphate obtained after mild alkaline hydrolysis of the lipid extracts is referred to as "phosphatidic acid". The existence of phosphatidic acid in the tissue may be an *in vitro* artefact since DAWSON[5] was unable to find free glycerophosphate in alkaline hydrolysates of the lipids extracted from fresh liver or brain.

Of the five phospholipids studied, "phosphatidic acid" and phosphoinositide had the highest ^{32}P specific activity in slices of pigeon pancreas incubated without acetylcholine. The specific activity of phosphatidyl ethanolamine was intermediate; the specific activities of phosphatidyl choline and phosphatidyl serine were by comparison very low. Acetylcholine ($10^{-3}M$) stimulated the incorporation of ^{32}P into each of the five phospho-

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lipids—but to an unequal degree. The average per cent increases in specific activity as compared with the controls in three experiments were as follows: phosphatidyl choline 73%, phosphatidyl ethanolamine 75%, phosphatidyl serine 293%, phosphoinositide 1093%, and "phosphatidic acid" 50%. A representative experiment is shown in Table I.

TABLE I

EFFECTS OF ACETYLCHOLINE ON THE INCORPORATION OF ^{32}P INTO THE PHOSPHOLIPIDS OF PIGEON PANCREAS SLICES

Phospholipid type	*Specific activity (counts/min/μg P)*	
	Control	*With acetylcholine (10^{-3} M)*
Phosphatidyl choline	41	55
Phosphatidyl ethanolamine	236	433
Phosphatidyl serine	9	25
Phosphoinositide	440	7480
"Phosphatidic acid"	754	1250
Calculated overall specific activity*	135	782

* Calculated from the relative quantities of phosphorus in each of the five derivatives isolated (see text).

Approximately 650 mg tissue incubated for 3 hours at 39° C in 14 ml Krebs-Henseleit bicarbonate saline containing 200 mg per cent glucose and $NaH_2{}^{32}PO_4$ (20 μc/ml). Eserine sulphate (final concentration $6 \cdot 10^{-4}$ M) was added with the acetylcholine. All counts are corrected to a specific activity of 100,000 counts/min/μg P for the inorganic P in the medium.

The figures for the specific activity of the "phosphatidic acid" were more variable than for the other derivatives. It is of considerable interest that by far the greatest increase in specific activity after incubation with acetylcholine was found in the hydrolysis product of phosphoinositide. Calculations indicated that the increase in radioactivity of the phosphoinositide accounted on average for about 75% of the increase in the net radioactivity of the "total lipid phosphorus" (calculated by summation of the quantities of phosphorus in the five phospholipid derivatives isolated). It was previously observed that acetylcholine markedly stimulated (850%) the incorporation of ^{32}P into those phospholipids which yield glycerophosphate on strong alkaline hydrolysis[2]. The data in Table I indicate that this could not have been due to the stimulation of ^{32}P-incorporation into phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine. The only substance which shows an increase in specific activity comparable to that of the glycerophosphate obtained on strong alkaline hydrolysis is phosphoinositide. It seems likely therefore that under the conditions used previously (strong alkaline hydrolysis) a large part of the phosphoinositide was hydrolysed to give glycerophosphate. This does not apparently occur to the same extent with brain diphosphoinositide (see below), suggesting that the properties of pancreas phosphoinositide are somewhat different from those of brain diphosphoinositide. The marked stimulation by acetylcholine of ^{32}P incorporation into pancreas phosphoinositide can account for the much greater stimulation of ^{32}P incorporation into the total ether-soluble phospholipids of pancreas as compared with brain cortex slices (compare Tables I and III). This suggests that phosphoinositide may play an important role in pancreatic function; the suggestion is supported by the fact that pancreas has an unusually high inositol content (TAYLOR AND MCKIBBEN[10]). The marked stimulation of ^{32}P incorporation into pancreas phosphoinositide can also

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account for our earlier observation[2] that acetylcholine stimulates the incorporation of ^{32}P into the cephalins three to five times more than into the lecithins.

Although the specific activity of phosphatidyl choline was not high in pancreas slices, its total radioactivity was appreciable, since this substance accounted for 54% of the "total lipid phosphorus". The total activity of phosphatidyl ethanolamine was even greater; this substance had a high specific activity and accounted for 26% of the "total lipid phosphorus". It would be of interest to know whether the relatively high incorporation of ^{32}P into phosphatidyl ethanolamine is related to the high content of phosphorylethanolamine in the pancreas (AWAPARA, LANDUA AND FUERST[11]; TALLEN, MOORE AND STEIN[12].) Phosphatidyl serine, phosphoinositide and "phosphatidic acid" accounted for 10, 8 and 2%, respectively, of the "total lipid phosphorus".

Effect of acetylcholine on the incorporation of ethanolamine-2-^{14}C into phosphatidyl ethanolamine and phosphatidyl choline in pigeon pancreas slices

Earlier studies[2] showed that glycerol-1-^{14}C incorporation into glycerophosphatides in pancreas slices was not stimulated by acetylcholine, indicating that the increased ^{32}P incorporation was due to an accelerated rate of independent turnover of phosphate in the phospholipids. Since increased ^{32}P incorporation in response to acetylcholine in the pancreas takes place in glycerophosphatides in which the phosphate is esterified both to glycerol and to a base or amino acid (choline, ethanolamine or serine) it would be expected that in these phospholipid types the incorporation of the base or amino acid would be increased to the same extent as that of phosphate. In liver, radioactive ethanolamine has been shown to be incorporated into phosphatidyl ethanolamine and, by conversion to choline, into phosphatidyl choline (PILGERAM, GAL, SASSENRATH AND GREENBERG[13]). To test whether acetylcholine stimulates the turnover of the base, pancreas slices were incubated with ethanolamine-2-^{14}C in the presence and absence of acetylcholine ($10^{-3}M$). Table II shows that the ^{14}C was readily incorporated into phosphatidyl ethanolamine, and to a lesser extent into phosphatidyl choline; in slices incubated in the presence of acetylcholine the specific activities of both phosphatidyl ethanolamine and phosphatidyl choline were approximately doubled. These increases in specific activity were of the same order as found with ^{32}P—the average increases in specific activities in three experiments with ^{32}P were 75% for phosphatidyl ethanolamine and 73% for phosphatidyl choline. In view of this it seems likely that acetylcholine stimulates the turnover of phosphorylcholine and phosphorylethanolamine as units in their respective

TABLE II

EFFECT OF ACETYLCHOLINE ON THE INCORPORATION OF ETHANOLAMINE-2-^{14}C INTO THE PHOSPHOLIPIDS OF PIGEON PANCREAS SLICES

Phospholipid type	*Specific activity (counts/min/μg P)*	
	Control	*With acetylcholine (10^{-3} M)*
Phosphatidyl choline	0.9	2.2
Phosphatidyl ethanolamine	79.5	151

Approximately 300 mg tissue incubated for 3 hours at 39° C in 6 ml Krebs-Henseleit bicarbonate saline containing 200 mg per cent glucose and 2.5 μmol/ml ethanolamine-2-^{14}C·HCl (35,000 counts/min/μmol). Eserine sulphate (final concentration $6 \cdot 10^{-4}$ M) was added with the acetylcholine.

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phospholipids. The specific activities after incubation with ^{32}P or ethanolamine-2-^{14}C are not, of course, directly comparable since the specific activities of ^{32}P and ^{14}C in the donor moieties are not known.

Effects of acetylcholine on the incorporation of ^{32}P into the individual phospholipids of guinea pig brain cortex slices

In brain cortex slices ^{32}P was incorporated very readily into diphosphoinositide and "phosphatidic acid" and to a lesser extent into phosphatidyl choline, but there was relatively little incorporation into phosphatidyl ethanolamine and phosphatidyl serine (Table III). Similar findings were made by DAWSON[5] in brain dispersions, except that he failed to find any significant incorporation of ^{32}P into phosphatidyl choline. As compared to the atropinised control, the incorporation ^{32}P into diphosphoinositide and into "phosphatidic acid" was increased about 100% in the presence of acetylcholine ($10^{-2}M$); the incorporation into phosphatidyl choline was increased by about 50%. The specific activities of phosphatidyl ethanolamine and phosphatidyl serine in these experiments were too low for the slight increases to be regarded as significant.

TABLE III

EFFECTS OF ACETYLCHOLINE ON THE INCORPORATION OF ^{32}P INTO THE PHOSPHOLIPIDS OF GUINEA PIG BRAIN CORTEX SLICES

Phospholipid type	*Specific activity (counts/min/μg P)*	
	*Control**	*With acetylcholine (10^{-2} M)*
Phosphatidyl choline	76	126
Phosphatidyl ethanolamine	9	12
Phosphatidyl serine	2	3
Diphosphoinositide	710	1650
"Phosphatidic acid"	1020	2180
Calculated overall specific activity**	70	135

* Atropine sulphate (final concentration 10^{-7} M) was added to the control vessel.

** Calculated from the relative quantities of phosphorus in each of the five derivatives isolated (see text).

Approximately 400 mg tissue incubated for 3 hours at 39° C in 4 ml Krebs-Henseleit bicarbonate saline containing 200 mg per cent glucose and $NaH_2{}^{32}PO_4$ (20 μc/ml). Eserine sulphate (final concentration $6 \cdot 10^{-4}$ M) was added with the acetylcholine. All counts are corrected to a specific activity of 100,000 counts/min/μg P for the inorganic P in the medium.

The observation that acetylcholine stimulated the incorporation of ^{32}P into the total ether-soluble phospholipids to a greater extent than into those ether-soluble phospholipids which yield glycerophosphate on strong alkaline hydrolysis[4] indicates that brain diphosphoinositide, which appears to be responsible for a large part of the overall stimulation, does not yield glycerophosphate to any great extent during strong alkaline hydrolysis. The "total lipid phosphorus" (calculated, as in the case of the pancreas, by summation of the quantities of phosphorus in the five phospholipid derivatives isolated) showed the following per cent composition: phosphatidyl choline, 47.4; phosphatidyl ethanolamine 27.8; phosphatidyl serine 19.3; diphosphoinositide 3.4; "phosphatidic acid" 0.7.

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Incorporation of glycerol-1-^{14}C into the individual phospholipids of guinea pig brain cortex slices.

When slices of brain cortex were incubated with glycerol-1-^{14}C and the specific activities of the various phospholipids determined it was found that these specific activities were all of the same order of magnitude (Table IV). KARNOVSKY AND GIDEZ[14] have provided good evidence that glycerol is an obligatory intermediate in the synthesis of phospholipids. The incorporation of glycerol-1-^{14}C should therefore be a good measure of phospholipid synthesis. If this is so it can be concluded from the data in Table IV that the rate of synthesis of each of the five phosphatides examined is approximately the same.

TABLE IV

THE INCORPORATION OF GLYCEROL-1-^{14}C INTO THE PHOSPHOLIPIDS OF GUINEA PIG BRAIN CORTEX SLICES

Phospholipid type	*Specific activity (counts/min/μg P)*
Phosphatidyl choline	41.0
Phosphatidyl ethanolamine	17.7
Phosphatidyl serine	38.8
Diphosphoinositide	28.8
"Phosphatidic acid"	27.0

Approximately 200 mg tissue incubated 3 hours at 37° C in 4 ml Krebs-Henseleit bicarbonate saline containing 200 mg per cent glucose, 2.5 μmol/ml glycerol-1-^{14}C (1 μc/μmol) and 10^{-7} *M* atropine sulphate.

The relatively equal rates of incorporation of glycerol-1-^{14}C into the phospholipids contrast sharply with the very unequal rates of incorporation of ^{32}P into these phospholipids. KORNBERG AND PRICER[15,16], KENNEDY[17,18] and KENNEDY AND WEISS[19] have shown alternative pathways for the incorporation of ^{32}P and choline into phospholipids in cell-free liver preparations—one in which glycerophosphate is an intermediate for phosphatidic acid synthesis[15,17], one in which free choline and presumably phosphatidic acid are the intermediates for lecithin formation[18] and one in which phosphorylcholine is an intermediate for lecithin formation[16,19]. If, in the present work, glycerophosphate was the intermediate for phospholipid synthesis it follows that the amount of ^{32}P incorporated into that phospholipid with the lowest ^{32}P/^{14}C ratio, *i.e.* phosphatidyl serine, would be the maximum amount of ^{32}P which could be incorporated as a result of phospholipid synthesis–unless it is postulated that the glycerophosphate moieties forming the different phospholipid types have different ^{32}P/^{14}C ratios. Thus with glycerophosphate as an intermediate the bulk of the incorporation of ^{32}P into the various phospholipids would be due to turnover of phosphoryl units on preformed diglyceride. The very high ^{32}P/^{14}C ratio of the glycerophosphate isolated argues against "phosphatidic acid" being a precursor for the incorporation of ^{32}P into phosphatidyl choline, phosphatidyl ethanolamine or phosphatidyl serine, since these all have lower ^{32}P/^{14}C ratios.

If the phospholipids were synthesised by esterification of diglycerides with various phosphoryl units such as phosphorylcholine, phosphorylethanolamine and phosphorylcholine or, in the case of "phosphatidic acid", with free phosphate it becomes more difficult to distinguish between synthesis (defined as including the incorporation of

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glycerol) and turnover (defined as the turnover of phosphoryl units in preformed glyceride). It would be possible to obtain the results shown in Tables III and IV if the phospholipids were synthesised by esterification of newly formed diglyceride with phosphoryl units having different individual specific activities (*e.g.* that of phosphorylserine would be the lowest). By this mechanism different $^{32}P/^{14}C$ ratios could be obtained in different glycerophosphatides without any independent turnover of phosphoryl units taking place on preformed diglyceride. However, in view of the fact that the increased incorporation of ^{32}P into the phospholipids of slices incubated in the presence of acetylcholine is exclusively due to an increased turnover of phosphoryl units in preformed lipid rather than to an increased synthesis of lipid[4], it seems rather likely that an appreciable incorporation of ^{32}P into the phospholipids of slices incubated in the absence of acetylcholine may also the due to this type of turnover. This idea is strengthened by the fact that, in general, phosphate turnover was stimulated most in those phospholipids which have the highest unstimulated rate of ^{32}P incorporation.

The exact chemical composition of the alkaline hydrolysis product of diphosphoinositide which is isolated on paper is unknown. DAWSON[5] was of the opinion that it might be inositol metadiphosphate or that it might contain a glycerol residue. The fact that the specific activity (^{14}C counts/min/μg P) of this substance after incubation with glycerol-1-^{14}C was of the same order of magnitude as that of the hydrolysis products of the glycerophosphatides suggests that this hydrolysis product of diphosphoinositide does contain glycerol.

DISCUSSION

It is of interest that in both pancreas slices and brain cortex slices the major stimulation by acetylcholine of phosphate turnover occurs in the inositol-containing phospholipids. FOLCH[20] has shown that all of the phosphoric acid of brain diphosphoinositide is present in the diesterified form. The structure of pancreas phosphoinositide has not yet been defined; however, there is as yet no evidence that the phosphate of phosphoinositides from other tissues is present in the monoesterified form. One can therefore predict that the stimulation by acetylcholine of the turnover of phosphate in the inositol-containing phospholipids of brain and probably of pancreas involves the turnover of phosphorylinositol as a unit in a manner analogous to the turnover of phosphorylethanolamine and phosphorylcholine.

It is apparent from the effects of acetylcholine on ^{32}P incorporation into phospholipids that enzymes exist which are capable of catalysing the turnover of phosphoryl units on preformed diglyceride. In view of this, the widely accepted concept of the measurement of synthesis of phospholipids by the incorporation of ^{32}P should perhaps be revised. If the synthesis of lipids is defined as including the condensation of fatty acids with glycerol, then in the absence of evidence that glycerol is being incorporated to the same extent as ^{32}P in any particular system, it would perhaps be better to described the incorporation of ^{32}P-labelled compounds as turnover rather than synthesis.

The physiological significance of the stimulation by acetylcholine of the turnover of phosphate and phosphoryl units in the phospholipids of pancreas and brain remains unknown, although these and earlier studies do point to the phospholipids as participating in the physiological events thrown into play by acetylcholine.

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SUMMARY

The incorporation of ^{32}P has been followed in five individual phospholipid types in slices of pigeon pancreas and guinea pig brain cortex, incubated in the presence and absence of acetylcholine. In pancreas slices the average ^{32}P-specific activities of the individual phospholipids show the following increases after incubation with acetylcholine (10^{-3} *M*) as compared with the control: phosphatidyl choline 73%, phosphatidyl ethanolamine 75%, phosphatidyl serine 293%, phosphoinositide 1093%, "phosphatidic acid" 50%. The increase in radioactivity of phosphoinositide accounts for about 75% of the increase in radioactivity of the "total phospholipid phosphorus". In brain cortex slices acetylcholine (10^{-2} *M*) stimulates ^{32}P incorporation into diphosphoinositide and "phosphatidic acid" by about 100% and into phosphatidyl choline by about 50%. The incorporation of ^{32}P into phosphatidyl ethanolamine and phosphatidyl serine under these conditions is too low for the slight increases to be regarded as significant.

The incorporation of ethanolamine-2-^{14}C into phosphatidyl ethanolamine and phosphatidyl choline was followed in pancreas slices incubated in the presence and absence of acetylcholine (10^{-3} *M*). Acetylcholine stimulates the incorporation of ^{14}C into these phospholipids to approximately the same extent as it stimulates the incorporation of ^{32}P. This suggests that acetylcholine stimulates the turnover of phosphorylcholine and phosphorylethanolamine as units in their respective phospholipids.

The rates of incorporation of glycerol-1-^{14}C into the various phospholipids of brain cortex slices are approximately the same, indicating that the total synthesis (or total turnover) of each of these phospholipids is of the same order of magnitude. The rates of ^{32}P incorporation into the various phospholipids in this tissue are highly disproportionate, suggesting that most of the incorporation of ^{32}P into the different phospholipids may be due to the same type of independent turnover of phosphoryl moieties as that which is found in the presence of acetylcholine.

RÉSUMÉ

L'incorporation de ^{32}P dans cinq types individuels de phospholipides a été suivie dans des coupes de pancréas de pigeon et de cortex cérébral de cobaye, incubées en présence et en absence d'acétylcholine. Dans des coupes de pancréas, les activités spécifiques moyennes de ^{32}P dans les phospholipides individuels présentent les augmentations suivantes, après incubation avec de l'acétylcholine (10^{-3} *M*), par rapport à un témoin: phosphatidyl choline 73%, phosphatidyl éthanolamine 75%, phosphatidyl sérine 293%, phosphoinositide 1093%, "acide phosphatidique" 50%. L'augmentation de la radioactivité du phosphoinositoside représente environ 75% de l'augmentation de la radioactivité du "phosphore phospholipidique total". Dans les coupes de cortex cérébral, l'acétylcholine (10^{-2} *M*) stimule l'incorporation de ^{32}P dans le phosphoinositoside et dans l'"acide phosphatidique" d'environ 100% et, dans la phosphatidyl choline d'environ 50%. L'incorporation de ^{32}P dans la phosphatidyl éthanolamine et la phosphatidyl sérine dans ces conditions est trop faible pour que les légers accroissements observés soient significatifs. L'incorporation de la 2-^{14}C-éthanolamine dans la phosphatidyl éthanolamine et dans la phosphatidyl choline a été suivie dans les coupes de pancréas incubées en présence et en absence d'acétyl choline (10^{-3} *M*). L'acétyl choline stimule l'incorporation du ^{14}C dans ces phospholipides à peu près dans les mêmes proportions que celle du ^{32}P. Ceci suggère que l'acétylcholine stimule le "turn-over" de la phosphorylcholine et de la phosphoryléthanolamine en tant que telles dans leurs phospholipides respectifs.

Les vitesses d'incorporation du 1-^{14}C-glycérol dans les divers phospholipides du cortex cérébral sont à peu près les mêmes, ce qui indique que la synthèse totale (ou le "turn-over" total) de chaque phospholipide est du même ordre de grandeur. Les vitesses d'incorporation de ^{32}P dans les divers phospholipides de ce tissu sont fortement disproportionnées, ce qui suggère que la majeure partie de l'incorporation de ^{32}P dans les divers phospholipides peut être due au même type de "turn-over" indépendant des moitiés phosphorylées, que celui qui a été trouvé en présence d'acétylcholine.

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ZUSAMMENFASSUNG

Man verfolgte die Einverleibung von ^{32}P in fünf individuelle Phospholipidtypen in Taubenpankreas- und Meerschweinchengehirnrindenschnitten, welche in Gegenwart und in Abwesenheit von Azetylcholin inkubiert wurden. Nach Inkubation mit Azetylcholin (10^{-3} *M*) wurde in Pankreasschnitten, verglichen mit dem Kontrollversuch, folgende Erhöhung der durchschnittlichen ^{32}P-spezifischen Aktivitäten der individuellen Phospholipide festgestellt: Phosphatidylcholin 73%, Phosphatidylethanolamin 75%, Phosphatidylserin 293%, Phosphoinositid 1093%, "Phosphatidsäure" 50%. Die in der Phosphoinositidfraktion gefundene Erhöhung der Radioaktivität stellt ungefähr 75% der Erhöhung dar, welche in der Radioaktivität des "in Phospholipiden enthaltenen totalen Phosphors" gefunden wurde. In Gehirnrindenschnitten wird durch Azetylcholin (10^{-2} *M*) die Einverleibung von ^{32}P in Diphosphoinositid und "Phosphatidsäure" ungefähr 100 prozentig und in Phosphatidylcholin ungefähr 50 prozentig gesteigert. Die Einverleibung von ^{32}P in Phosphatidylethanolamin und Phosphatidylserin ist unter diesen Bedingungen so gering, dass die kleinen Erhöhungen nicht als bedeutungsvoll angesehen werden können.

Die Einverleibung von Ethanolamin-2-^{14}C in Phosphatidylethanolamin und Phosphatidylcholin wurde in Pankreasschnitten verfolgt, welche in Gegenwart und in Abwesenheit von Azetylcholin (10^{-3} *M*) inkubiert worden waren. Azetylcholin steigert die Einverleibung von ^{14}C in diese Phospholipide ungefähr in gleichem Masse, wie die Einverleibung von ^{32}P. Dies führt zu der Annahme, dass Azetylcholin die Umsetzung von Phosphorylcholin und Phosphorylethanolamin als Einheiten innerhalb ihrer betreffenden Phospholipiden steigert.

Die Einverleibungsgeschwindigkeiten von Glyzerin-1-^{14}C in die verschiedenen Phospholipide der Gehirnrindenschnitten sind ungefähr die gleichen; dieses Ergebnis weist darauf hin, dass die gesamte Synthese (oder gesamte Umsetzung) eines jeden dieser Phospholipide von der gleichen Grössenordnung ist. Die Einverleibungsgeschwindigkeiten von ^{32}P in die verschiedenen Phospholipide dieser Gewebe sind sehr ungleichmässig; dies führt zu der Annahme, dass der grösste Teil der Einverleibung von ^{32}P in die verschiedenen Phospholipide durch denselben unabhängigen Umsetzungstypus der Phosphorylhälften verursacht wird, wie derjenige, welcher in Gegenwart von Azetylcholin gefunden wird.

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