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The Biosynthesis of Phosphatidic Acid and Phosphatidylinositol in Mammalian Pancreas

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1. The labelling of guinea-pig pancreas phospholipids *in vivo* after intraperitoneal injection of [^{32}P]orthophosphate is described. 2. Acyl-CoA synthetase activity in pancreas homogenates has been studied. There is no absolute requirement for added fatty acids, indicating an adequate supply of endogenous fatty acids in these preparations. 3. Phosphatidic acid is formed in guinea-pig pancreas preparations by two distinct routes, namely the acylation of L-3-glycerophosphate and the phosphorylation of 1,2-diglyceride. Phosphatidic acid formed by either mechanism is converted into phosphatidylinositol by guinea-pig pancreas *in vitro*. 4. The enzymes of pancreas that convert phosphatidic acid into phosphatidylinositol via CDP-diglyceride have been characterized. 5. Addition of bovine serum albumin is necessary in assaying certain of these enzymes.

Incorporation of [^{32}P]orthophosphate into the phosphatidic acid and phosphatidylinositol of pancreas slices is markedly increased during enzyme secretion (Hokin & Hokin, 1955, 1964). The secretion may be initiated by adding acetylcholine or pancreozymin to the medium in which the slices are incubated. The mechanism of the accompanying phospholipid effect has not been elucidated, nor is it known exactly how phospholipid metabolism is related to the secretory process. Hokin (1966) has suggested that the increased incorporation of ^{32}P into phospholipids may be concerned with intracellular transport of newly synthesized digestive enzymes. Little is known of the enzymes responsible for the synthesis of phosphatidic acid and phosphatidylinositol in pancreas. As a step towards understanding the exact relation between phospholipid metabolism and secretion, these enzymes have been studied and some of their properties are now reported. A previous paper (Prottey & Hawthorne, 1966) described the lipids of pancreas.

MATERIALS AND METHODS

Materials

Special reagents used were obtained as follows: [^{32}P]-orthophosphate (PBS 1) and [U-^{14}C]*myo*inositol (CFB 49) from The Radiochemical Centre (Amersham, Bucks.); 3-phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase and glycerokinase from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); ATP, CTP, UTP, ITP, GTP, CDP-choline, CMP, DL-3-glycerophosphate, bovine serum albumin (fraction V), GSH, cysteine (free base) and tris (type 7–9) from Sigma Chemical Co., London, S.W. 6; *Clostridium perfringens* type A filtrate concentrate from Wellcome Research Laboratories (Beckenham, Kent). All organic solvents were from Fisons Scientific Apparatus Ltd. (Loughborough, Leics.); chloroform of this brand contains about 2% of ethanol, this being taken into account when chloroform–methanol mixtures were used in chromatographic methods.

Analytical methods

Protein. This was determined by the method of Weichselbaum (1946). Pancreas samples produce excessive turbidity on incubation. This was removed by extracting with 2ml. of diethyl ether before measuring colour intensity.

Phosphate. Organic and inorganic phosphate were determined by the method of King (1932).

Paper chromatography. Formaldehyde-impregnated paper chromatography followed the method described by Kai & Hawthorne (1966) with these modifications: the solvent was not equilibrated with ether; the development time was never more than 10hr. since phosphatidic acid moves near the solvent front; when radioactive phosphatidic acid and phosphatidylinositol were to be separated, carrier lipid was added to the samples.

Preparative methods

[γ-^{32}P]*ATP.* This was prepared by the method of Glynn & Chappell (1964). After chromatographic purification the ATP solution was adjusted to pH 9·8 with KOH, frozen and stored at −20° until required.

L-3-*Glycero*[^{32}P]*phosphate.* The glycerokinase method was used. Excess of glycerol was added to achieve maximum conversion of added [^{32}P]ATP.

The incubation mixture contained, in a total volume of 30–50ml.: [γ-^{32}P]ATP (1–2mM; containing 1–5mc of ^{32}P), $MgCl_2$ (20mM), glycerol (0·1ml.) and enzyme (0·2mg. of

protein). No buffer was added, but the pH was readjusted to 9·8 with dilute KOH where necessary. After incubation for 90 min. at 37° the reaction was stopped by adding an equal volume of 5% (w/v) trichloroacetic acid containing Norit A (12·5%, w/v). The mixture was cooled on ice for 5 min. after which the charcoal was removed by filtration. The acidic solution containing L-3-glycero[^{32}P]phosphate was extracted six times with diethyl ether until the pH was about 4. The product, free from nucleotide but containing traces of [^{32}P]orthophosphate, was purified on a column of Dowex 1 (X10, formate form; 100–200 mesh). Gradient elution was effected by means of 1 l. of 1·5 N-formic acid in the reservoir and 1 l. of 0·5 N-formic acid in the mixing vessel. Glycerophosphate was eluted as a sharp peak between 500 and 900 ml. After flash evaporation followed by freeze-drying a standard solution was prepared by adding DL-3-glycerophosphate and adjusting to pH 7·5 with $KHCO_3$. Paper chromatography in propan-1-ol–acetic acid–water (5 : 4 : 1, by vol.) showed one compound, corresponding to 3-glycerophosphate (R_F 0·37); no 2-glycerophosphate (R_F 0·43) was detected by phosphate spray or radioactivity.

Phosphatidic acid. This lipid was prepared from ox-brain lecithin by the procedure of Hübscher & Clarke (1960) with cabbage phospholipase D (Davidson & Long, 1958). In some cases, before chromatography on silicic acid (no Celite was used) the calcium phosphatidate was washed by the method of Folch, Lees & Sloane-Stanley (1957) with 0·2 vol. of saturated sodium EDTA (adjusted to pH 7·5 with $NaHCO_3$) followed by M-NaCl, in an attempt to convert it into sodium phosphatidate. When this product was chromatographed two peaks were obtained, one being eluted with 3·5% (v/v) methanol in chloroform, the other with 6% (v/v) methanol in chloroform. The two components had similar mobilities on silica-gel thin-layer chromatography (chloroform–methanol–water; 19 : 7 : 1, by vol.), but the second peak produced a discrete spot, not streaking like the first. The peak eluted with 3·5% methanol in chloroform may represent unchanged calcium phosphatidate and the peak eluted with 6% methanol in chloroform may be due to the sodium salt. In no instance was it possible to convert more than 50% of the calcium phosphatidate into the alleged sodium phosphatidate. Abramson, Katzman, Wilson & Gregor (1964) have remarked on the vigorous treatment required to remove all the Ca^{2+} from calcium phosphatidate. The salt eluted with 3·5% methanol in chloroform was more difficult to emulsify in water than that obtained by elution with 6% methanol in chloroform.

CDP-diglyceride. The method of Agranoff & Suomi (1963) was followed, with certain modifications. (*a*) A phosphatidate sample (approx. 40 mg. of P, either Ca^{2+} or Na^+ form) was dissolved in 10 ml. of redistilled benzene and rapidly mixed with 50 ml. of water in a high-speed mixer. The thick milky emulsion that was formed was evaporated at reduced pressure until no benzene could be detected by smell. Then N-HCl was added to the emulsion so as to make the final concentration 0·1 N. The emulsion was dialysed overnight at 4° against 5 mN-HCl, with at least four changes of the outer solution. The flocculated emulsion was extracted six times with 50 ml. portions of diethyl ether and the pooled organic layers were evaporated to dryness. The lipid was washed with 0·1 N-HCl (method of Folch *et al.* 1957) and then dried in a stream of N_2. The 'free acid' phosphatidic acid so obtained was freeze-dried from redistilled benzene. The efficiency of the vacuum pump was an important factor. In these preparations a combined rotary–mercury diffusion pump was used and the final vacuum produced was about 0·005 mm. Hg. Unless such low pressures were achieved it was found that the last traces of water were not removed from the phosphatidic acid and the linking with CMP did not occur. (*b*) The phosphatidic acid was linked with CMP-morpholidate by the method of Agranoff & Suomi (1963) and the product purified as far as the second chloroform extraction described by these authors. The water/solvent partition procedure for purification of CDP-diglyceride described by Agranoff & Suomi (1963) was not used, since it was found that serious losses of the product resulted. This was probably due to the solubility of CDP-diglyceride in water, itself dependent on the fatty acids of the diglyceride moiety. In all these experiments ox-brain lecithin was the origin of the diglyceride; Agranoff & Suomi (1963) used the dipalmitoyl compound. The chloroform layers were pooled, evaporated to dryness at low temperature, redissolved in 50 ml. of chloroform–methanol (2 : 1, v/v) and shaken with 0·2 vol. of 0·05 N-HCl. The resulting lower phase was separated, solvent removed *in vacuo* and the residue redissolved in chloroform. Silicic acid was activated by heating overnight at 110°, sieved to remove particles finer than 300 mesh and made into a column of which the length was three times the diameter. The crude CDP-diglyceride in chloroform was applied to this column (0·5 mg. of P/g. of silicic acid). Unchanged phosphatidic acid was removed by stepwise elution with chloroform–methanol mixtures, the highest methanol concentration being 25% (v/v). Elution with 50% (v/v) methanol in chloroform removed CDP-diglyceride as a very sharp peak, which gave one spot on thin-layer chromatography in di-isobutyl ketone–acetic acid–water (40 : 30 : 7, by vol.). One such preparation had phosphorus : cytidine : acyl ester proportions 1·87 : 1·0 : 2·07. The pure product from the column was redissolved in 20 ml. of diethyl ether, 30 ml. of water was added and the ether removed by flash evaporation. As the ether was removed a milky emulsion formed, the pH of the solution being about 4. Solid $KHCO_3$ was added carefully when the pH rose. At about pH 6 the turbidity disappeared quite suddenly, the solution now containing the potassium salt of CDP-diglyceride. This solution was used directly in enzyme studies after neutralization to pH 7·5 with $KHCO_3$.

L-1,2-Diglyceride. This lipid was prepared from egg lecithin and ox-heart mitochondrial lecithin, and purified as described by Gurr, Brindley & Hübscher (1965*a*). Emulsions in water were prepared by removing the benzene by flash evaporation and treating the solution at 0–2° for 2 min. in an MSE 60 w Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London, S.W. 1). All emulsions were prepared immediately before use to minimize conversion of 1,2-diglyceride into its 1,3-isomer.

Incorporation of [^{32}P]*orthophosphate* in vivo

Solutions of [^{32}P]orthophosphate were rendered neutral and iso-osmotic by the addition of calculated amounts of NaCl and $NaHCO_3$. Administration was by means of intraperitoneal injection, the guinea pigs being under light ether anaesthesia. Phosphatidic acid and phosphatidylinositol were separated from the total lipid extract by means of silicic acid chromatography. Total lipid representing about 1 mg. of phospholipid P was applied to a column (1 cm. ×

3cm.) containing 3–5g. of silicic acid. After addition of the sample in pure chloroform the column was washed with 50ml. of chloroform to remove neutral lipids. Elution with 6% methanol in chloroform (about 20ml.) removed phosphatidic acid and a final elution with 50% methanol in chloroform removed most of the phospholipid P. The two phospholipid fractions obtained were then deacylated as described by Prottey & Hawthorne (1966). Since the phosphatidic acid fractions usually contained less than 20μg. of P, the amount of base used was decreased to 0·06ml. of 0·5N-NaOH in methanol. The resulting water-soluble diesters were separated by the paper-chromatographic method of Dawson (1960). The specific activities of the phospholipids were calculated after radioautography as described by Gurr, Prottey & Hawthorne (1965*b*).

Preparation of subcellular fractions

Pancreas homogenates were prepared in a Potter–Elvehjem homogenizer (diam. 0·966in., radial clearance 0·003–0·0045in.) with a Teflon pestle rotating at 1100rev./min. One guinea-pig pancreas, weighing about 1g., was homogenized in 3ml. of 0·3M-sucrose, previously neutralized to pH7·5 with $KHCO_3$. In all studies the homogenates were filtered through nylon cloth to remove cell debris and connective tissue and diluted with an equal volume of sucrose solution. These preparations were used in enzyme studies or treated as follows for the preparation of subcellular fractions. Homogenates were centrifuged at 18000rev./min. for 10min. (375000*g*-min.). The supernatant was retained and the pellet was resuspended in half the original volume of sucrose solution and recentrifuged. This step was repeated a third time. It was observed that a pellicle of fat formed at the surface of the solution in the centrifuge tubes after spinning. By cautious decantation and filtration through nylon cloth this could be removed. The pellet obtained after three such centrifugations at 18000rev./min. was resuspended in 3–4ml. of 0·3M-sucrose and is termed the 'low-speed pellet'. The pooled supernatants were then centrifuged for 1hr. at 40000rev./min. (6300000*g*-min.). The pellet obtained is called the 'microsomal pellet' and the supernatant liquid the 'particulate-free supernatant'. All microsomal pellets were washed by decantation and resuspended in 2–3ml. of 0·3M-sucrose.

Enzyme assay procedures

(i) *Acyl-CoA synthetase.* This enzyme (EC 6.2.1.2) was assayed by the method of Brindley & Hübscher (1966), which is a modification of the procedure of Kornberg & Pricer (1953*a*). Unless stated otherwise, the assay system (final vol. 3ml.) contained: hydroxylamine hydrochloride, adjusted to pH7·4 with KOH before use (0·66M), ATP (10mM), CoA (0·25mM), $MgCl_2$ (10mM), GSH (5·23mM), KF (13·3mM), fatty acids as indicated (these were made up as the potassium soaps in a 50% excess of KOH and were added directly to the assays; when potassium palmitate and potassium stearate were used the solutions were heated to 60° before pipetting to dissolve the soaps) and pancreas homogenate (up to 4mg. of protein).

(ii) *Synthesis of phosphatidic acid and phosphatidylinositol by the acylation of* L-3-*glycero*[^{32}P]*phosphate.* The system (total 3ml.) contained: potassium phosphate buffer, pH7·4 (16·6mM), KF (13·3mM), DL-glycerophosphate (only the L-enantiomorph was labelled, about 50000 disintegrations/min./μmole) as indicated, ATP (6·66mM), CoA (0·083mM), $MgCl_2$ (10mM) and homogenate (up to 9mg. of protein). All other additions are described in the text. The reactions were started by adding the enzyme to the otherwise complete system after incubation of the latter for 1min. at 37° and stopped by adding 11·25ml. of chloroform–methanol (1:2, v/v). The lipids were extracted at room temperature for 20min. and to the monophasic system were added, in this order, 3·75ml. of chloroform and 3·75ml. of 0·5M-potassium phosphate buffer, pH7·4, containing KCl (2M). After vigorous shaking for 30sec. the mixture was centrifuged to separate the phases. The upper (aqueous) phase was removed by aspiration and the bottom phase rewashed with 13·35ml. of 'synthetic upper phase' from a similar mixture of solvents. The bottom phase, containing the radioactive lipid products of the reactions, was then free from water-soluble contaminants. Fractions of the bottom phases so obtained were then taken for radioactive counting and chromatography on formaldehyde-impregnated paper.

(iii) L-1,2-*Diglyceride kinase.* The basic system (total vol. 1ml.) contained: potassium phosphate buffer, pH7·4 (50mM), $MgCl_2$ (10mM), [γ-^{32}P]ATP (5mM; about 200000 disintegrations/min./μmole), KF (10mM), L-1,2-diglyceride as an emulsion in water (4mM), and enzyme protein, bovine serum albumin and other additions as indicated in the Results section. All incubations were for 5·5min. at 37°, the reactions being started by adding enzyme after a 1min. preincubation of the otherwise complete system. The reactions were stopped and kinase activity was measured as in (ii) above, except that the amounts of chloroform, wash solution and synthetic upper phase added were decreased to 1·25ml., 1·25ml. and 4·45ml. respectively.

(iv) *Conversion of phosphatidic acid into phosphatidylinositol.* All reactions were performed in 1ml. volumes. The conditions resembled those in (ii) but 3-glycerophosphate, ATP, CoA or KF were not required since phosphatidic acid was added. The components were potassium phosphate buffer, pH7·4 (16·6mM), $MgCl_2$ (10mM), [U-^{14}C]*myo*inositol (1mM; approx. 200000 disintegrations/min./μmole), phosphatidic acid (0·66mM), and bovine serum albumin and enzyme as indicated below. After a preincubation for 1min. at 37° the reactions were begun by adding the enzyme. Reactions were stopped as for (ii) and (iii), and the labelled lipid products similarly washed and extracted. Phosphatidylinositol production was estimated by the following procedure. Portions (2ml.) of the twice-washed bottom phases were evaporated to dryness in a stream of N_2. The residue was dissolved in 6ml. of xylene phosphor solution [6g. of 2,5-diphenyloxazole and 0·12g. of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/l. of xylene] and a 5ml. portion counted in an automatic liquid-scintillation counter.

(v) *CDP-diglyceride–inositol phosphatidyltransferase.* Conditions of assay were very similar to (iv) but with altered concentrations of metal ions and albumin; CDP-diglyceride replaced phosphatidic acid and CTP. All relevant details are quoted in the Results section.

RESULTS

Incorporation of [^{32}P]*orthophosphate into phospholipids of guinea-pig pancreas* in vivo. Guinea pigs injected with [^{32}P]orthophosphate (0·5–1·0mc)

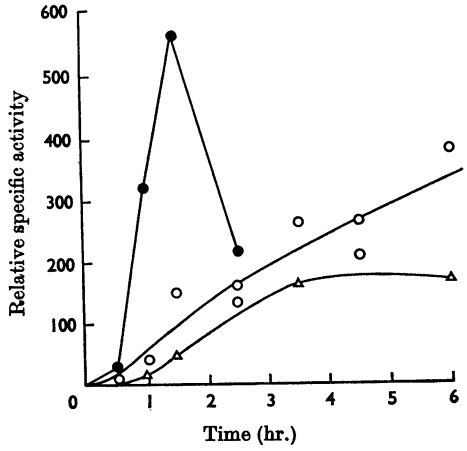

Fig. 1. Incorporation of [^{32}P]orthophosphate into phospholipids of guinea-pig pancreas *in vivo*. ●, Phosphatidic acid; ○, phosphatidylinositol; △, total lipid. Relative specific activity is specific activity of phospholipid/specific activity of acid-soluble pool.

were killed after various times and the pancreases quickly removed for the determination of phospholipid specific activities as described above. To avoid errors due to differences in dose and absorption of ^{32}P, phospholipid specific activity was related to that of the acid-soluble pool (i.e. the 5%-trichloroacetic acid-soluble phosphates) of the whole pancreas at each time-interval. The relative specific activity values so obtained were considered to be independent of both the amount of isotope injected and the weight of the animal.

Fig. 1 shows how the specific activities of phosphatidic acid and phosphatidylinositol varied with time. Both lipids had higher specific activities than the total phospholipid extract. Lecithin was labelled to a smaller extent than the total phospholipids, and phosphatidylethanolamine and phosphatidylserine were barely labelled at all. No specific activities were obtained for phosphatidic acid at incorporation times greater than 2·5hr. This was because both radioautography and the phosphate spray (Dawson, 1960) failed to reveal spots corresponding to 3-glycerophosphate. It is assumed that phosphatidic acid specific activities in these cases were very low. The results shown in Fig. 1 are consistent with the formation of phosphatidylinositol from phosphatidic acid in pancreas, but do not prove this. Since the two specific activity curves do not intersect at the maximum of the phosphatidylinositol curve, phosphatidic acid could not be the immediate precursor (Zilversmit, Entenman & Fishler, 1943).

Acyl-CoA synthetase activity of guinea-pig pancreas homogenates. If phosphatidic acid, the likely intermediate in the synthesis of phosphatidylinositol, is itself produced by the acylation of L-3-glycerophosphate (Kornberg & Pricer, 1953*b*; Paulus & Kennedy, 1960) activated fatty acids are required. For this reason acyl-CoA synthetase was briefly studied. Fig. 2 shows the effects of varying the concentrations of certain cofactors.

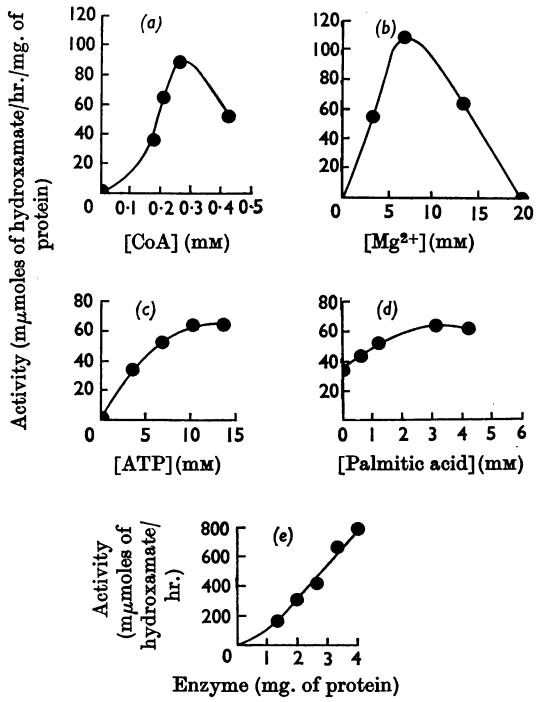

Fig. 2. Effect of cofactor concentrations on acyl-CoA synthetase in guinea-pig pancreas homogenates. The assay conditions are described in the Materials and Methods section. All tubes, except those of curve (*d*), contained potassium palmitate (3·1mM).

With the establishment of correct conditions for the activation of fatty acids in pancreas the replacement of palmitate by potassium soaps of each of the following fatty acids was tested: myristic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and arachidonic acid. The concentrations assayed were 1 and 2mM. For each fatty acid tested there was no difference in hydroxamate formation from that shown in Fig. 2 for palmitate. Significantly, there was always appreciable production of hydroxamate in the absence of added fatty acid, indicating an adequate supply of endogenous free fatty acid in the pancreas homogenates assayed. Since pancreas contains high concentrations of triglyceride (Prottey & Hawthorne, 1966) and the lipase activity of the organ is

Table 1. *Acylation of glycero[^{32}P]phosphate in guinea-pig pancreas homogenates*

In Expt. A the basic conditions were as described in the Materials and Methods section, except that KF was omitted. The concentration of glycero[^{32}P]phosphate was 1·66mM (40000disintegrations/min./μmole) and 6·1mg. of homogenate protein was added. Conditions in Expt. B differed from those in Expt. A in that KF (13·3mM) and GSH (2·66mM) were added to the basic assay medium. The mixture contained 6·0mg. of homogenate protein. All incubations were for 60min. at 37°.

Additions	Phosphatidic acid produced (mμmoles)	Phosphatidylinositol produced (mμmoles)
Expt. A		
None	38·8	0·0
GSH (2·66mM)	37·3	0·0
KF (13·3mM)	45·4	0·0
Inositol (1mM)	38·8	0·0
CTP (1mM)	29·6	11·4
CTP (1mM)+inositol (1mM)	35·3	21·4
CTP (1mM)+inositol (1mM)+KF (13·3mM)	38·5	22·1
CTP (1mM)+inositol (1mM)+KF (13·3mM)+GSH (2·66mM)	29·3	30·4
Complete system, but 30min. incubation	34·6	6·5
Complete system, but CoA and ATP omitted	0·0	0·0
Expt. B		
None	57·0	2·0
$MnCl_2$ (4mM) replacing $MgCl_2$ (10mM)	103·8	10·5
Glycero[^{32}P]phosphate increased to 3·33mM	73·0	5·5
Palmitate (1mM)	71·0	4·0
CTP (1mM)+inositol (1mM)	74·5	8·5
CTP (1mM)+inositol (1mM)+palmitate (1mM)	73·0	4·1

well known (Benzonana *et al.* 1964), this is not surprising.

Acylation of glycero[^{32}P]phosphate. The results quoted above represent minimum rates of phosphatidic acid synthesis, since optimum conditions were not established. Table 1 shows the effect of different substances on the production of phosphatidic acid and phosphatidylinositol from L-3-glycerophosphate in pancreas homogenates.

Table 1 (Expt. A) shows that the addition of potassium fluoride, an inhibitor of certain adenosine triphosphatases (Case & McIlwain, 1951) and of phosphatidate phosphatase (Coleman & Hübscher, 1962), stimulated phosphatidic acid production. No phosphatidylinositol was formed when inositol alone was added to the basic system, but when inositol was added with CTP more phosphatidylinositol was formed than with CTP alone. This indicated an absolute requirement for CTP and only a partial requirement for inositol. More phosphatidylinositol and less phosphatidic acid were formed after 1hr. than after 30min., suggesting that phosphatidylinositol is derived from phosphatidic acid. GSH was without effect on the basic system, but in the complete system it increased the proportion of phosphatidylinositol formed without affecting the total amount of phospholipid. Table 1 (Expt. B) shows that more phosphatidic acid was formed in 1hr. by the second enzyme preparation. Some phosphatidylinositol was formed in the absence of added CTP, suggesting that some endogenous CTP remained after homogenization.

Table 2. *Effect of long-chain fatty acids on the acylation of* L-3-*glycerophosphate by guinea-pig pancreas homogenate*

The basic system is described in the Materials and Methods section. Each tube contained L-3-glycero[^{32}P]-phosphate (1·66mM) and 6·6mg. of homogenate protein. All incubations were for 1hr. at 37°.

Fatty acid (1mM)	Phosphatidic acid synthesized (mμmoles)	% of control
None	27·1	100
$C_{14:0}$	6·9	25
$C_{16:0}$	25·9	96
$C_{18:0}$	38·9	144
$C_{18:2}$	1·4	5
$C_{20:4}$	0·0	0

The effects on the acylation of L-3-glycerophosphate of certain fatty acids generally found in phospholipids were tested. In this case no CTP or inositol was added since only the production of phosphatidic acid was required. Table 2 lists the effects of the acids on phosphatidic acid production.

The addition of palmitate and linoleate did not stimulate phosphatidic acid production. With the different enzyme preparation of Table 1 (Expt. B) palmitate stimulated slightly. Stearate and arachidonate showed a 44% stimulation and complete inhibition respectively. The experiment showed that addition of fatty acids was not obligatory for the acylation of L-3-glycerophosphate, again suggesting that guinea-pig pancreas homogenates have adequate supplies of endogenous free fatty acids. For this reason fatty acids were not added in subsequent acylation studies.

Time-course of the synthesis of phosphatidic acid and phosphatidylinositol. Two large-scale incubations were made, one with the requisite cofactors for the synthesis of phosphatidic acid from L-3-glycerophosphate and the other with CTP and inositol as well (Fig. 3). The formation of phospha-

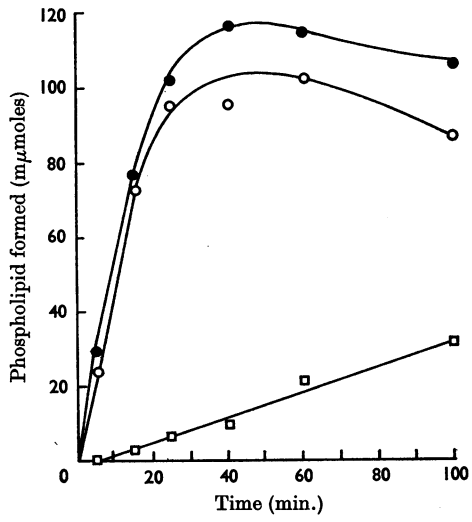

Fig. 3. Time-course of the synthesis of phosphatidic acid and phosphatidylinositol. Tube (A), of total volume 20ml., was as described in the Materials and Methods section, with L-3-glycero[^{32}P]phosphate (3·05mM) and 42·2mg. of protein. Tube (B) was identical with tube (A) except that in addition it contained CTP (1mM) and inositol (1mM). For analysis, 3ml. portions were withdrawn at specific times. ●, Phosphatidic acid synthesized in tube (A); ○, phosphatidic acid in tube (B); □, phosphatidylinositol in tube (B).

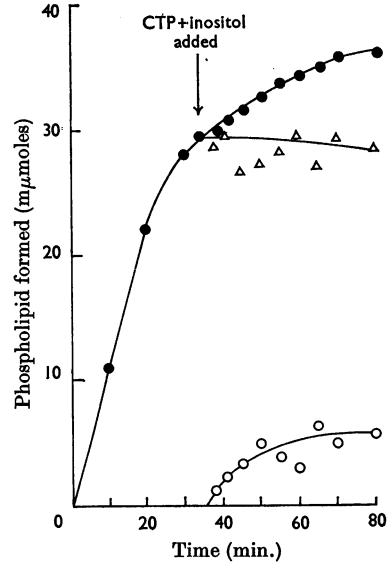

Fig. 4. Production of phosphatidylinositol from phosphatidic acid. The reaction conditions are described in the Materials and Methods section. The original 60ml. of mixture contained 120mg. of homogenate protein and the concentration of glycero[^{32}P]phosphate was 1·66mM. After 35min. the mixture was divided into two. To one half (A) 0·5ml. of water was added, and to the other (B) 0·5ml. of a solution 60mM with respect to CTP and inositol. Each was then incubated for a further 45min. ●, Phosphatidic acid formed in the original mixture and system (A); △, phosphatidic acid produced in system (B); ○, phosphatidylinositol produced in system (B).

tidic acid and phosphatidylinositol was measured at times up to 100min. The same quantity of total labelled lipid was formed in each incubation. As the second incubation proceeded, ^{32}P was increasingly found in phosphatidylinositol rather than phosphatidic acid. The initial rate of phosphatidic acid formation fell off after 20min., probably because of exhaustion of cofactors, and no further synthesis was seen in either incubation after 40min. Phosphatidylinositol was formed at a steady rate throughout the experiment.

In a second experiment (Fig. 4) after 35min. the incubation mixture in which phosphatidic acid was being formed was halved, incubation being continued with added CTP and inositol in one half only. Again the results suggested that phosphatidic acid is the precursor of phosphatidylinositol in pancreas homogenates.

Effect of bovine serum albumin on the acylation of L-3-*glycero*[^{32}P]*phosphate.* Adult guinea-pig pancreas showed no acylation activity. Only when young animals were taken was the synthesis of phosphatidic acid appreciable. It was thought that free fatty acids might be responsible for the lack of activity, especially since the concentration of triglyceride in adult guinea-pig pancreas is high and the organ is rich in lipase. To test the effect of albumin on the acylation of glycerophosphate five experiments were performed with guinea pigs of various weights (Fig. 5). Phospholipid synthesis was more rapid in the younger animals. Addition of albumin affected the rate of acylation in all cases. Each animal had an optimum albumin concentration for the 6·9mg. of homogenate protein assayed. These optima were quite sharp for the younger animals, excess of albumin causing marked inhibition of acylation. The optima increased with the weight of the animals. Clearly, for any activity to be realized with an animal of 900g., very high concentrations of albumin need to be added. This may be why no acylation was detected in the earlier experiments. If free fatty acids were responsible for such failures, and Table 2 suggests this, the albumin probably functions by removing these acids. Fig. 2(*d*) indicates that the concentration of free fatty acids in pancreas can be significant since appreciable hydroxamate formation was observed in the absence of added fatty acids.

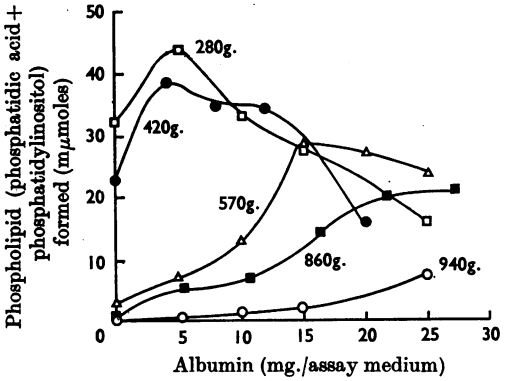

Fig. 5. Acylation of L-3-glycero[^{32}P]phosphate in pancreas homogenates from guinea pigs of various weights. The incubation mixture was that described in the Materials and Methods section, with glycero[^{32}P]phosphate (1·66mM) and 6·9mg. of homogenate protein, together with CTP (1mM) and inositol (1mM). Incubation was for 1hr. with the amounts of albumin indicated.

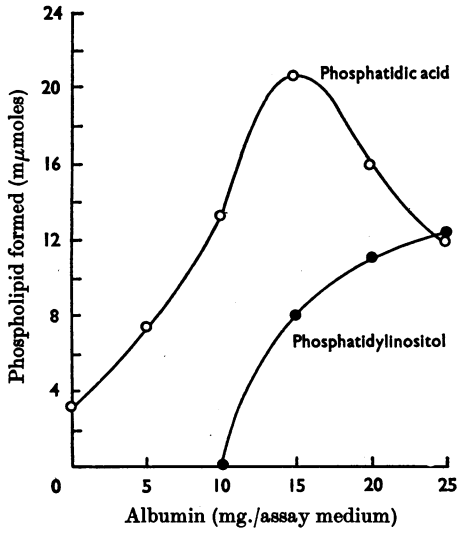

Fig. 6. Effect of albumin on the synthesis of phosphatidic acid (○) and phosphatidylinositol (●) from glycero[^{32}P]-phosphate. Data in this Figure correspond to the phospholipid curve for the 570g. guinea pig of Fig. 5.

With the animal weighing 570g. (Fig. 5), the total lipid, after determination of radioactivity, was analysed further by paper chromatography and radioautography. The amounts of labelled phosphatidic acid and phosphatidylinositol synthesized were then calculated for the different albumin concentration. Fig. 6 shows that for the animal in question phosphatidylinositol was not formed until the albumin concentration had exceeded 10mg./3ml. of assay medium (6·9mg. of homogenate protein), in spite of considerable phosphatidic acid production. Excess of albumin appeared to inhibit phosphatidic acid production while still stimulating the formation of phosphatidylinositol. It would seem that for a guinea pig of this size albumin is required for the formation both of phosphatidic acid from L-3-glycerophosphate and of phosphatidylinositol from phosphatidic acid.

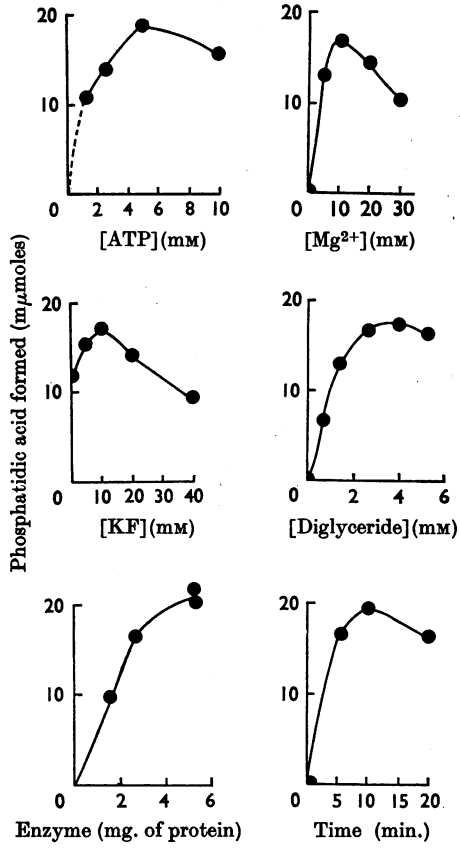

Fig. 7. Properties of pancreatic diglyceride kinase. The assay system, described in the Materials and Methods section, contained 5mg. of albumin and 2·6mg. of homogenate protein from a guinea pig weighing 290g. Chromatography on formaldehyde-impregnated paper showed only one radioactive product, with the properties of phosphatidic acid.

Pancreatic diglyceride kinase. This enzyme has been reported in polymorphonuclear leucocytes (Sastry & Hokin, 1966) and brain (Redman & Hokin, 1964) and provides an alternative route to phosphatidic acid.

Preliminary experiments with pancreas homogenate and L-1,2-diglyceride from egg lecithin showed little diglyceride kinase activity. However, L-1,2-diglyceride prepared from ox-heart mitochondrial lecithin was much more reactive and so the effects of various cofactors were determined with diglyceride from this source (Fig. 7).

It was expected that phosphatidic acid production by this route would occur in the absence of added diglyceride, since diglyceride has been detected in lipid extracts of guinea-pig pancreas (Prottey & Hawthorne, 1966). There was, however, an absolute requirement for added substrate. Phosphate buffer was chosen to minimize the labelling pf phosphatidic acid by reversal of the phosphatidate phosphatase (EC 3.1.3.4) reaction in the presence of labelled inorganic phosphate from ATP hydrolysis. Fluoride stimulated at concentrations below 10mM, probably by inhibiting adenosine triphosphatases. The phosphorylation rate rapidly decreased after 5min. This method of phosphatidic acid production differed from the glycerophosphate pathway in that albumin was not essential; it neither stimulated nor inhibited over the range 0–25mg./ml. Albumin was included, however, so that the conversion of phosphatidic acid into phosphatidylinositol in a similar system could be studied (Table 3). The molar ratio of magnesium chloride to ATP for optimum diglyceride activity was 2:1.

It can be calculated from Fig. 7 that diglyceride kinase activity in guinea-pig pancreas homogenate results in the formation of 73mμmoles of phosphatidic acid/mg. of protein/hr. In the studies on the acylation of L-3-glycerophosphate no attempt was made to assay phosphatidic acid production under zero-order conditions and so a corresponding value for synthesis by this route cannot be obtained. However, from Fig. 3 a value of 48mμmoles/mg. of protein/hr. for the initial rate of phosphatidic acid production can be gained. This must be regarded as a minimum level of activity since cofactor concentrations were not studied in detail.

Fig. 4 demonstrates that phosphatidylinositol can be formed from the phosphatidic acid of the glycerophosphate pathway. The results summarized in Table 3 show that phosphatidylinositol can also be formed from phosphatidic acid synthesized by the diglyceride kinase pathway. The brief incubation time (5·5min.) accounts for the relatively small amount of phosphatidylinositol (compare Fig. 3).

Conversion of phosphatidic acid into phosphatidylinositol in pancreas homogenates, with [^{14}C]*inositol.* The reaction had an absolute requirement for CTP, the optimum concentration being 0·5mM. There was no absolute requirement for phosphatidic acid (prepared from brain lecithin; see the Materials and Methods section); addition of 0·5μmole/ml. increased phosphatidylinositol synthesis by only 40%. Either Mg^{2+} or Mn^{2+} ions were necessary, the optimum concentrations being approx. 15mM and 2mM respectively. At concentrations above 0·5mM, Ca^{2+} ions markedly inhibited phosphatidylinositol synthesis, whether 15mM-magnesium chloride was present or not. The rate of phosphatidylinositol synthesis was independent of inositol concentrations above 0·5mM.

In all experiments of this type young guinea pigs were used, when albumin was found to have marked stimulatory effects. The required concentration was generally estimated from the data of Fig. 5. The rate of phosphatidylinositol synthesis was found to be proportional to homogenate protein concentration over the range examined (0·0–4·0mg.) and when assayed under zero-order conditions with respect to all cofactors the reaction rate was constant for about 25min.

No other nucleoside triphosphate was required in phosphatidylinositol production from phosphatidic acid (Table 4), a result differing from that of Agranoff, Bradley & Brady (1958).

Table 3. *Phosphatidylinositol formed from the phosphatidic acid of the diglyceride kinase pathway in guinea-pig pancreas homogenate*

The conditions of assay are described under Fig. 7. The labelled products were identified by paper chromatography.

Additions	Phosphatidic acid synthesized (mμmoles)	Phosphatidylinositol synthesized (mμmoles)
None	12·4	0·0
CTP (1mM)+inositol (1mM)	10·5	1·4

Table 4. *Effect of various nucleotides on phosphatidylinositol synthesis*

Pancreas homogenate (3·45mg. of protein) from a guinea pig weighing 200g. was assayed under zero-order conditions as described in the Materials and Methods section. Mixtures were incubated for 20min. at 37°; 10mg. of albumin was added to each assay. Nucleotide concentrations were 1mM in each case.

Conditions	Phosphatidylinositol synthesized (mμmoles)
No nucleotide	1·0
CTP	30·6
ATP	1·7
UTP	1·3
ITP	2·3
GTP	1·9
CDP-choline	13·3

Table 5. *Synthesis of phosphatidylinositol from phosphatidic acid by subcellular preparations of guinea-pig pancreas*

Assay conditions are given in the text. No albumin was added.

Enzyme preparation	Protein (mg.)	Phosphatidylinositol synthesis: Sp. activity (mμmoles/mg. of protein/hr.)	Phosphatidylinositol synthesis: Total activity (mμmoles/hr.)
Homogenate	169·5	1·35	229
Low-speed pellet	48·0	0·53	25·6
Microsomal fraction	36·7	2·74	100·5
Particulate-free supernatant	69·3	1·11	77·3
Recovery	91%	—	88·8%

Phosphatidylinositol production from phosphatidic acid was next studied in subcellular fractions of guinea-pig pancreas, with the optimum conditions established above. The fractions were prepared from young guinea pigs weighing about 300g. Each fraction was assayed at two protein concentrations in the absence of added albumin. Comparison assays were made with 7·5mg. and 15mg. of added albumin, but under these conditions the reaction rate was no longer proportional to protein concentration. In addition, not all subcellular fractions showed equal stimulation with albumin. Hence it was decided to compare activities without added albumin. The results are shown in Table 5.

The microsomal fraction contained 22% of the protein of the original homogenate but 44% of the activity, i.e. the enrichment factor was 2. The purity of this fraction was not checked by electron microscopy or marker enzymes (see the Discussion section). For the sake of clarity, however, it was termed 'microsomal' and all subsequent studies were performed on preparations obtained similarly.

Conditions for microsomal synthesis of phosphatidylinositol from phosphatidic acid. Microsomal phosphatidylinositol synthesis required higher concentrations of certain cofactors to give zero-order kinetics than did synthesis in homogenates (Fig. 8). The most striking fact was that there was no requirement for phosphatidic acid, concentrations above 0·75mM being inhibitory. It was thought that the salt form of the added substrate might be causing the inhibition, but the calcium salt, the sodium salt and the free acid all gave identical results. The endogenous phosphatidic acid of the microsomal fraction appears sufficient for the enzyme. Albumin had marked effects. All curves in Fig. 8 except Fig. 8(*c*)(i) and Fig. 8(*f*) were obtained with a microsomal preparation with

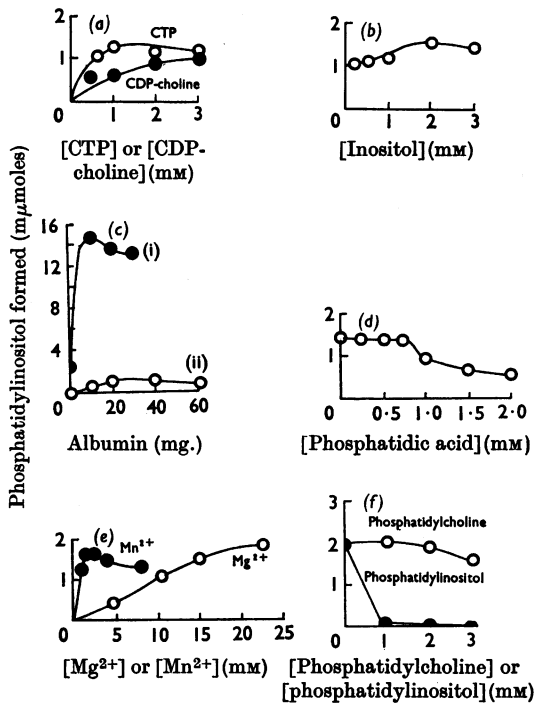

Fig. 8. Microsomal conversion of phosphatidic acid into phosphatidylinositol. Curves (*a*), (*b*), (*c*) (ii), (*d*) and (*e*) were obtained with a pancreas microsomal fraction from two guinea pigs each weighing 280g. Microsomal protein (0·94mg.) was incubated at 37° for 20min. in a basic medium of 1ml. containing phosphate buffer, pH 7·4 (16·6mM), $MgCl_2$ (15mM), CTP (1mM), [^{14}C]inositol (1mM; 230000 disintegrations/min./μmole), phosphatidic acid (0·44mM) and 20mg. of albumin [none in experiment (*c*) (ii)]. Curves (*c*)(i) and (*f*) were obtained with a second microsomal fraction, obtained from two animals of weight 230g. The conditions were as above except that no albumin was added and 1·02mg. of microsomal protein was assayed.

20mg. of albumin/assay medium. Clearly, without albumin, no synthesis would have occurred. This enzyme preparation was not as active as that used for Fig. 8(*c*)(i), which was obtained from somewhat smaller guinea pigs. The curve illustrates the diversity of rates of synthesis in animals of different weights. The absolute albumin requirements/mg. of microsomal protein were variable too.

In multiple-assay experiments of this type the individual incubations were started at 1min. intervals by adding enzyme. Thus in a 30-tube experiment the final tube was started 29min. after the first. Control assay tubes showed that during this time at 0° almost 25% of the activity could be lost. Corrections were made for this.

Conversion of CDP-diglyceride into phosphatidylinositol in pancreas. Initial experiments were

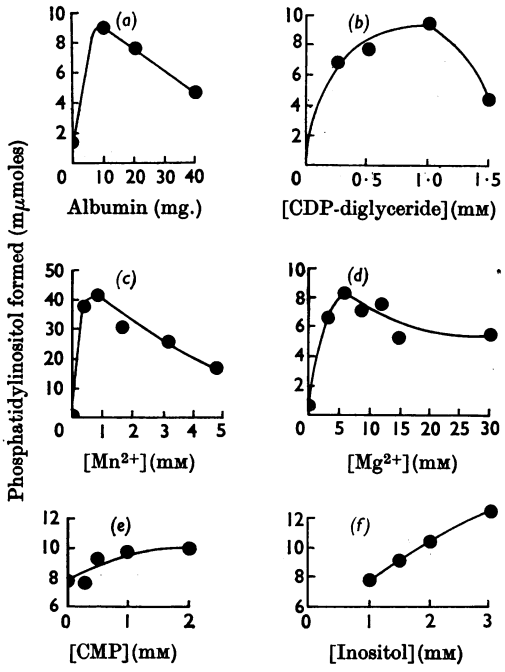

Fig. 9. Microsomal conversion of CDP-diglyceride into phosphatidylinositol. The basic assay conditions are given in the text. The concentration of the labelled inositol was 1 mM; 1·5 mg. of microsomal protein and 20 mg. of albumin were added to each tube. Incubations were for 20 min. at 37°.

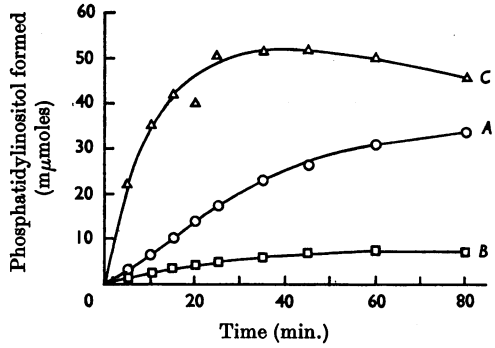

Fig. 10. Rates of phosphatidylinositol synthesis. Curve *A*: conversion of phosphatidic acid into phosphatidylinositol (○); a total volume of 10 ml. contained phosphate buffer, pH 7·4 (16·6 mM), $MgCl_2$ (22·5 mM), [^{14}C]inositol (3·0 mM), CTP (1·5 mM), 12·9 mg. of microsomal protein and 200 mg. of albumin; no phosphatidic acid was added. Curve *B*: conversion of CDP-diglyceride into phosphatidylinositol at optimum Mg^{2+} concentration (□); a total volume of 10 ml. contained the same phosphate buffer and inositol as for curve *A*, Mg^{2+} (7·5 mM), CDP-diglyceride (1·0 mM), 12·9 mg. of enzyme protein and 200 mg. of albumin. Curve *C*: conversion of CDP-diglyceride into phosphatidylinositol at optimum Mn^{2+} concentration (△); the conditions were the same as for curve *B*, except that 1·0 mM-Mn^{2+} replaced 7·5 mM-Mg^{2+}.

performed on pancreas homogenates under conditions very similar to those employed in the study of the overall conversion of phosphatidic acid into phosphatidylinositol. Cofactor concentration curves are not presented but the findings can be summarized as follows. Potassium phosphate buffer, pH 7·4, of concentration 16·6 and 33·3 mM was tested and the same rate of phosphatidylinositol synthesis was observed in each case. The lower concentration was subsequently chosen. The optimum concentration for CDP-diglyceride was about 1·0 mM, variations above or below this resulting in much lower rates of synthesis. Metal ion requirements were different from those for the overall reaction reported above. The optimum Mg^{2+} ion concentration was 7·5 mM. An unexpected result was obtained with Mn^{2+} ions, the rate of phospholipid synthesis at the optimum concentration (0·8 mM-manganese chloride) being twice that at optimum Mg^{2+} concentration. With 2·44 mg. of homogenate protein from the pancreas of a 290 g. guinea pig, the maximum rate of phosphatidylinositol production was obtained with 20 mg. of albumin/assay medium.

The conversion of CDP-diglyceride into phosphatidylinositol was also studied in a microsomal preparation of pancreas (Fig. 9). Conditions were very similar to those established for homogenate, except that Mn^{2+} was not twice but five times as effective as Mg^{2+} in microsomes. The albumin requirement was not the same as for homogenate, presumably because both the nature of the enzyme preparation (homogenate, microsomal etc) and the age of the animal govern the amount of free fatty acid occurring *in vitro*. CMP produced a slight stimulation, probably by decreasing pyrophosphatase breakdown of CDP-diglyceride.

Rate of microsomal phosphatidylinositol synthesis. The rate of the reaction was studied under the optimum conditions for phosphatidylinositol biosynthesis. Fig. 10 shows the conversion of phosphatidic acid into phosphatidylinositol (curve *A*), and the synthesis from CDP-diglyceride at optimum Mg^{2+} (curve *B*) or optimum Mn^{2+} (curve *C*). In the synthesis from CDP-diglyceride Mn^{2+} was far more effective than Mg^{2+}. Although the initial rate of phosphatidylinositol production from CDP-diglyceride was quite high, it rapidly fell off, probably because of breakdown of the liponucleotide. Fig. 10 shows that the conversion of CDP-diglyceride into phosphatidylinositol was far faster than the overall conversion of phosphatidic acid into phospha-

tidylinositol. This is not surprising since in the latter case CDP-diglyceride has to be generated *in situ* before condensing with inositol if the route of Paulus & Kennedy (1960) is operative. From Fig. 10 the initial rates of the reactions concerned were calculated as follows: (*a*) conversion of phosphatidic acid into phosphatidylinositol, 32·7mμmoles of phosphatidylinositol formed/mg. of protein/hr.; (*b*) conversion of CDP-diglyceride into phosphatidylinositol at optimum Mn^{2+} ion concentration: 206mμmoles of phosphatidylinositol formed/mg. of protein/hr.

Care was taken to assay the enzymes under conditions that were first-order with respect to enzyme only. The optimum albumin concentrations were not known for this preparation and so a large amount was added; this may have produced a secondary inhibition. Also, for the CDP-diglyceride reactions the optimum inositol concentration was probably higher than 3mM. Comparison of rates showed that phosphatidylinositol production from CDP-diglyceride is 6·3 times that from phosphatidic acid.

Optimum pH *for CDP-diglyceride–inisotol phosphatidyltransferase.* The pH optima were not measured in the assays involving more than one enzyme, since the results would have been difficult to interpret. The single-enzyme step CDP-diglyceride to phosphatidylinositol was studied and the result is shown in Fig. 11.

The pH optimum was 7·5. This value is considerably lower than that described by Paulus & Kennedy (1960) for the liver system or for *Schizosaccharomyces pombe* (G. L. White, C. Prottey & J. N. Hawthorne, unpublished work).

Products of the reactions. In experiments with glycero[^{32}P]phosphate and [γ-^{32}P]ATP the products were examined by both paper chromatography of the intact lipids and by resin-column chromatography of their deacylation products. Labelled phosphatidic acid and phosphatidylinositol were identified by both methods. In no instance was the formation of lysophosphatidic acid detected, the most plausible explanation being that in the phase separation and washing steps this product would be removed in the aqueous phase. It is highly probable that in the formation of phosphatidic acid from L-3-glycerophosphate some lysophosphatidic acid is formed.

In studies involving [^{14}C]inositol the labelled phospholipid was characterized by paper chromatography on formaldehyde-treated paper. In every case examined phosphatidylinositol was the only labelled lipid formed. No labelling was seen on the chromatograms in the region where diphosphoinositide and triphosphoinositide would be expected to run.

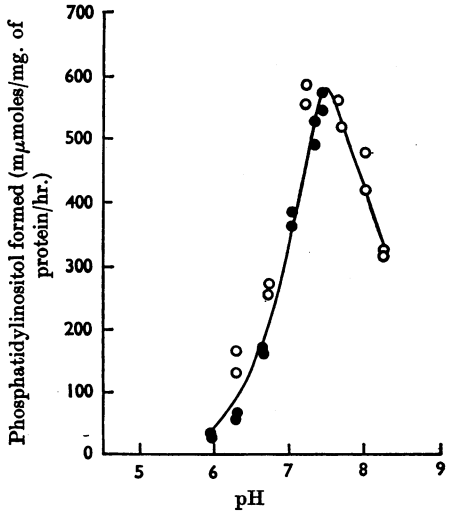

Fig. 11. Optimum pH for CDP-diglyceride–inositol phosphatidyltransferase. The enzyme was assayed with 0·68mg. of microsomal protein from a guinea pig weighing 200g. The conditions resembled those for curve *C* of Fig. 10, except that all buffer concentrations were 25mM. Readings of pH were made on duplicate tubes during 5min. incubations at 37°. ●, 25mM-potassium phosphate buffer; ○, 25mM-tris–HCl buffer.

DISCUSSION

Although the effect of acetylcholine on phospholipid metabolism in pancreas has been known for more than a decade (Hokin & Hokin, 1955), this is the first attempt to study the biosynthesis of phosphatidic acid and phosphatidylinositol in that tissue. It proved difficult to obtain phospholipid synthesis in homogenates and subcellular fractions from pancreas. This was not unexpected, since the organ is rich in lipase and phospholipase. Higher activities were obtained with pancreas from young animals than with tissue from older animals, but the addition of albumin always increased activity and overcame the early difficulty in obtaining reproducible results. The chief inhibitor appeared to be free fatty acid released by the action of lipase on the abundant triglyceride of pancreas (Prottey & Hawthorne, 1966). Serum albumin is well known for its ability both to strongly bind long-chain fatty acids (Goodman, 1957) and to stimulate lipid-synthesizing systems *in vitro* (L. E. Hokin and G. Hübscher, in discussion of Shapiro, 1964). The early failures were first thought to be due to proteolysis by trypsin in the homogenates. Palade & Siekevitz (1956) remarked on the instability of the pancreatic microsomal fraction because of autolysis by ribonuclease and trypsin, but addition of soya-bean trypsin inhibitor in the present work was ineffective.

Brandes, Olley & Shapiro (1963) studied the effects of albumin on the acylation of L-3-glycerophosphate by acyl-CoA in liver particles. They found that in preparations of very low activity addition of 4mg. of albumin/mg. of enzyme protein caused a large increase of transacylase activity, with simultaneous decrease in hydrolysis of acyl-CoA. They suggested that the albumin functioned by removing free fatty acids from the medium. In the present study the acylation of glycerophosphate to phosphatidic acid and the formation of phosphatidylinositol from CDP-diglyceride were particularly stimulated by albumin (Fig. 6), but diglyceride kinase was unaffected.

Acyl-CoA compounds are known to inhibit lipid-synthesizing systems (Lands & Hart, 1965). In addition, albumin has been shown to bind these compounds and so reverse inhibition (Taketa & Pogell, 1966). The steady-state concentrations of acyl-CoA compounds in the pancreas preparations tested were not measured and it was impossible to estimate these since the absolute rates of acylation were not obtained, neither were other reactions studied that consume acyl-CoA, e.g. acylation of glycerides and β-oxidation. Fig. 2 shows that the concentration of endogenous acyl-CoA in pancreas homogenates was very low, since no hydroxamate was formed in the absence of added CoA and ATP. Although one cannot state with certainty whether free fatty acids or fatty acyl-CoA compounds are responsible for the observed inhibitions, Fig. 8(*d*) shows a stimulation of phosphatidylinositol biosynthesis by albumin under conditions where acyl-CoA formation would be very unlikely, but free fatty acid production by lipolysis would be favoured. In addition, it is difficult to see how more mature guinea pigs would possess a higher acyl-CoA synthetase activity, which would be required to explain the results of Fig. 5 if inhibition of acylation were by acyl-CoA compounds.

The pathways established for guinea-pig pancreas in the present work may be summarized as follows:

Fatty acid + ATP + CoASH → acyl-CoA + AMP + pyrophosphate (1)

L-3-Glycerophosphate + 2 acyl-CoA → phosphatidic acid + 2 CoASH (2)

L-1,2-Diglyceride + ATP → phosphatidic acid + ADP (3)

CDP-diglyceride + inositol → phosphatidylinositol + CMP (4)

Though it has not been studied directly, the formation of CDP-diglyceride probably follows reaction (5); Carter & Kennedy (1966) have provided detailed evidence for this reaction in guinea-pig liver:

Phosphatidic acid + CTP → CDP-diglyceride + pyrophosphate (5)

All these steps have been described previously by various workers, but in tissues other than pancreas. The biosynthesis of phosphatidylinositol in pancreas closely resembles that in liver (Paulus & Kennedy, 1960). The acylation of lysophosphatidylinositol in pigeon pancreas has been reported by Keenen & Hokin (1964), but this does not provide a synthetic route for phosphatidylinositol.

The diglyceride kinase reaction (3) may well provide a more effective route to phosphatidic acid in pancreas than the acylation of L-3-glycerophosphate. The relative rates were 73 and 48mμmoles of phosphatidic acid formed/mg. of protein/hr., although the latter value was not necessarily obtained under optimum conditions. Diglyceride kinase is active in brain (Hokin & Hokin, 1959), avian salt gland (Hokin & Hokin, 1960), erythrocytes (Hokin & Hokin, 1961) and polymorphonuclear leucocytes (Sastry & Hokin, 1966).

Fatty acid requirements. Pancreatic acyl-CoA synthetase activity was not dependent on added fatty acid. It is unlikely that the fatty acids tested were added in an unsuitable form since the conditions resembled those of Brindley & Hübscher (1966). Fatty acid synthesis under these conditions can be ruled out, so it would seem that endogenous fatty acids sufficed to saturate the enzyme.

This argument can be extended to the acylation studies. Although Table 1 (Expt. B) shows stimulation by added palmitate, the reverse is true of Table 2. Although Paulus & Kennedy (1960) and Redman & Hokin (1964) in similar types of experiment with other tissues used added oleic acid (0·07 and 3·0mM respectively), it appears that pancreas homogenates supply sufficient endogenous free fatty acid. Sastry & Kates (1966), studying the acylation of L-3-glycerophosphate in cell-free preparations of spinach, added no fatty acid since sufficient was formed by galactolipase and phospholipase breakdown of cell structures during preparation of the enzyme. Certain unsaturated fatty acids appeared to inhibit the acylation (Table 2). This effect has been noticed in glyceride formation in cat intestinal mucosa (Brindley & Hübscher, 1966) and guinea-pig intestinal mucosa (D. N. Brindley, personal communication). The earlier failures of the acylation experiments, before albumin was added as a routine, may have been due to excessive endogenous unsaturated free fatty acid.

Phosphatidic acid requirement. That there was no requirement for phosphatidic acid in the experiment of Fig. 7 was surprising. This might suggest that

phosphatidylinositol could be formed without involving phosphatidic acid, which is very unlikely, especially in the light of the acylation and diglyceride kinase results. An exchange reaction of the type described by Paulus & Kennedy (1960) can be eliminated, since the formation of phosphatidylinositol was dependent on 'high-energy' cytidine derivatives. It may be that the endogenous concentration of phosphatidic acid in the microsomal preparations, though low (Prottey & Hawthorne, 1966), was sufficient for enzyme activity. However, Thompson, Strickland & Rossiter (1963) reported that in rat brain dispersions addition of phosphatidic acid stimulated phosphatidylinositol synthesis.

Subcellular fractionation. The fractionation procedures and the method used to calculate the distribution of the enzymes converting phosphatidic acid into phosphatidylinositol were not ideal. The method of preparing the cell fractions was adapted from techniques employed for other tissues. Marker-enzyme assays, to check purity and degree of cross-contamination, were not performed for the following reasons. In preliminary experiments glucose 6-phosphatase, which is characteristic of microsomal membranes in liver (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) and intestinal mucosa (Ginsburg & Hers, 1960), was not detected in a guinea-pig pancreas homogenate that had been stored at $-20°$ for 3 days. The method of Hübscher & West (1965) was followed. Although the enzyme preparation released considerable quantities of inorganic orthophosphate, this was shown to be due to non-specific phosphatase activity. Secondly, succinate dehydrogenase activity was assayed (Pennington, 1961) in a 3-day-old homogenate that had been stored at $-20°$. Although a slight activity was detected, it was fractional when compared with a similarly stored rat-brain homogenate. Since any marker enzymes to be used in the present study would require to be stable to freezing and thawing, these experiments were not followed up. Most of the earlier work on phospholipid biosynthesis has been carried out on similarly ill-defined subcellular organelles, but Gurr *et al.* (1965*a*), using a well-characterized fractionation procedure, showed that lecithin production in the intestinal mucosa is centred in the endoplasmic reticulum.

Enzyme secretion by pancreas. That enzyme secretion by the pancreas is accompanied by the increased turnover of phosphatidylinositol is well documented (Hokin & Hokin, 1955, 1964). The present paper has described the reactions by which the terminal phosphate group of ATP may be incorporated into phosphatidic acid and phosphatidylinositol. How the enzymes concerned are modified by acetylcholine and pancreozymin remains to be investigated. It is possible that the primary effect either could be a direct action on one or more of the enzymes concerned or could depend on alterations in the permeability of certain intact cell membranes (cf. Durrell & Sodd, 1966).

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