The Metabolism of Phosphatidylinositol in the Thyroid Gland of the Pig

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1. The metabolism of phosphatidylinositol in pig thyroid has been investigated as a basis for understanding the specific stimulation of the synthesis of this phospholipid in the gland by thyrotropin. 2. The gland contained an active Ca^{2+} -dependent phosphatidylinositol-splitting enzyme with an optimum pH of 5.3-5.5. 3. The major water-soluble product (65%) formed by this catabolic enzyme was not phosphorylinositol but a related compound, which may be a cyclic phosphorylinositol. Both this and phosphorylinositol (35%) were released simultaneously from the phosphatidylinositol substrate. 4. The phosphatidylinositol-splitting enzyme was found almost exclusively in the supernatant fraction obtained by homogenization of the gland. It was not present in the acid-phosphatase-containing particulate fraction. 5. The incorporation of $[2\text{-}^3\text{H}_1]\text{inositol}$ into phosphatidylinositol in the presence of either CDP-diglyceride or CTP+ATP was most active in the microsomal fraction. 6. When thyroidal microsomes were labelled with $[^3\text{H}]\text{inositol}$ and ^{32}P , and then incubated with unlabelled inositol, there was a dramatic loss of ^3H labelling from the phosphatidylinositol, which was not accompanied by an equivalent loss of ^{32}P from the phosphate moiety. This turnover of the inositol moiety required nucleotide coenzymes. It is postulated that the phosphatidylinositol is split into inositol and a phosphorus-containing lipid precursor of the phospholipid that remains on the microsomal membrane and is recycled. 7. Isolated thyroidal mitochondria synthesized phosphatidylinositol from $[2\text{-}^3\text{H}_1]\text{inositol}$ only because of their contaminating microsomal component. 8. Some evidence has been obtained of a rapid transfer of phosphatidylinositol molecules from thyroidal microsomes to mitochondria when these were incubated together in the presence of a supernatant fraction. 9. Both phosphatidylinositol breakdown by the supernatant fraction of the gland and synthesis by the microsomes were totally inhibited by 1 mM-chlorpromazine. This drug is known to suppress thyrotrophin-induced stimulation of activity in thyroid slices.

The incorporation *in vitro* of many labelled precursors, e.g. $[^{32}\text{P}]\text{orthophosphate}$, glucose, fatty acid, inositol, into the phospholipids of surviving thyroid tissue is enhanced by the addition of pituitary thyrotrophin to the incubation medium, and this stimulation is known to be largely localized in the small phosphatidylinositol component of the phospholipid fraction (Freinkel, 1957, 1964; Scott, Mills & Freinkel, 1968). The mechanism of this effect of thyrotrophin on phosphatidylinositol metabolism has not yet been elucidated, nor has the relation it bears to the secretory activity of the gland. Clearly, since phosphatidylinositol is an important charged phospholipid component of cellular membranes, localized changes in its concentration or turnover could have a pronounced

influence on the stability of these membranes. Such changes in membrane stability are likely to be involved in the endocytosis of intraluminal thyroglobulin and the formation of 'colloid droplets', the migration of lysosomal granules to the apical portions of the cells and the fusion thereof to 'droplets' and granules (Wollman, Spicer & Burstone, 1964; Wetzel, Spicer & Wollman, 1965; Ekholm & Elmqvist, 1967; Seljelid, 1967).

Little is known about the enzymes concerned in the metabolism of phosphatidylinositol in the thyroid gland although there has been a report on the synthesis of phosphatidic acid (Schneider, 1968). As a step towards understanding the exact relationship between enhanced phosphatidylinositol turnover and glandular secretion, this metabolism has been studied and the location and properties of the enzymes concerned are reported.

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EXPERIMENTAL

Isolation of subcellular fractions. Pig thyroid glands were collected at the local slaughter-house and brought to the laboratory on ice (1 h) (Freinkel, 1957). Capsular material was removed and the glands were cut into smaller wedges and sliced about 0.5 mm thick in a microtome (Stadie & Riggs, 1944). The slices (25–30 g) were washed four times with cold 0.9% NaCl to remove some of the thyroglobulin and adherent extracellular fluid. All subsequent operations were carried out at 0°C. Slices were then cut into small pieces (about 3 mm × 3 mm) in a mechanical chopper (McIlwain & Buddle, 1953). These pieces were homogenized in 8 vol. (original tissue mass) of cold 0.25 M-sucrose–0.1 mM-EDTA in an homogenizer (Aldridge, Emery & Street, 1960; 0.008 in clearance) by using 15–20 strokes over 2–3 min. Subsequent operations were carried out at 0°C. The homogenate was filtered through a double layer of cheese muslin, and centrifuged for 10 min at 2500 rev./min (755 $g_{av.}$) in a Servall refrigerated centrifuge (rotor SS34). The nuclear-fraction pellet was washed once with 5 vol. (original tissue mass) of sucrose–EDTA solution and recovered by centrifuging as described above. The supernatant from the original centrifugation was spun for 10 min at 5500 rev./min (3640 $g_{av.}$) in a Servall refrigerated centrifuge (rotor SS34). The heavy-mitochondrial pellet was washed four times with sucrose–EDTA solution and the mitochondria were again sedimented at 3640 $g_{av.}$ as described above after each resuspension. The supernatant from the original centrifugation yielding the heavy mitochondrial fraction was centrifuged for 20 min at 11 500 rev./min (15 900 $g_{av.}$) in a Servall refrigerated centrifuge (rotor SS34) to give a light-mitochondrial fraction and a supernatant used for obtaining the microsomal fraction. The light-mitochondrial fraction was washed twice with sucrose–EDTA solution, each time being recovered by centrifuging at 15 900 $g_{av.}$ as described above. The supernatant containing the microsomes was spun for 45 min at 50 000 rev./min (165 000 $g_{av.}$) in a MSE 65 ultracentrifuge with aluminium angle rotor (Cat. no. 59594). The microsomal pellet was separated from the final supernatant fraction, washed twice with 5 vol. (original tissue mass) of sucrose–EDTA solution and was recovered by centrifugation at 165 000 $g_{av.}$ as described above. All pellets were suspended in sucrose–EDTA solution and portions were used for the determination of marker enzymes and enzyme systems concerned with the metabolism of phosphatidylinositol.

Marker enzymes. NADPH cytochrome *c* reductase (EC 1.6.2.1) and cytochrome *c* oxidase (EC 1.9.3.1) were assayed as described by McMurray & Dawson (1969). 5'-Nucleotidase (EC 3.1.3.5) was determined by the method of Michell & Hawthorne (1965) by using tartrate to inhibit non-specific phosphatase. Succinate-cytochrome *c* reductase (EC 1.3.99.1) was measured by the method of Tisdale (1967) and acid phosphatase (EC 3.1.3.2) by using β -glycerophosphate as substrate as described by Pollak & Munn (1970). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) by using crystalline serum albumin as standard.

Isolation of thyroidal [32 P]phosphatidylinositol. Slices of thyroid tissue were prepared and incubated as described previously (Scott, Jay & Freinkel, 1966). The incubation medium consisted of Krebs–Ringer bicarbonate medium

modified to contain 0.8 mM- Ca^{2+} , and 100 units of penicillin G and 100 μ g of streptomycin sulphate/ml. Slices (1.5 g) were incubated in 0.5 g lots in 25 ml conical flasks containing 5 ml of the incubation medium to which had been added 0.75 unit of ovine thyrotrophin and 550 μ Ci of [32 P]P_i. After 4 h incubation at 37°C with shaking under a gas phase of O₂+CO₂ (19:1), the slices were washed twice with non-radioactive saline incubation medium and then homogenized in 19 vol. of chloroform–methanol (2:1, v/v). The [32 P]phosphatidylinositol was then isolated as described by Scott & Dawson (1968). Silicic acid was removed from the final product by dissolving it in 4.2 ml of methanol, 4.7 ml of chloroform and 18 ml of light petroleum (b.p. 40–60°C). After addition of 2.8 ml of water and shaking, the upper phase was collected and the lower phase (6.4 ml) was washed twice with a mixture of 0.7 ml of methanol, 4.7 ml of chloroform and 18 ml of light petroleum. The combined upper phases were evaporated to dryness and the [32 P]phosphatidylinositol was stored at –15°C in 10 ml of chloroform–methanol (2:1, v/v). Alkaline ethanolsis (Dawson, Hemington & Davenport, 1962) and ionophoresis of the deacylation products showed that the radioactivity was limited to the glyceryl-phosphorylinositol spot.

Isolation of 32 P- or [3 H]inositol-labelled microsomes. Slices (1.5 g) were incubated with [32 P]P_i (100 μ Ci) or [3 H]inositol (50 μ Ci) as described in the previous section, except that the incubation time was decreased to 1 h. They were thoroughly washed with non-radioactive medium and the microsomes were prepared by the same basic procedure as given above in the section headed 'Isolation of subcellular fractions'.

Phosphatidylinositol degradation. The breakdown of phosphatidylinositol was measured by incubating the enzyme preparation with thyroidal [32 P]phosphatidylinositol. A sample of thyroidal phosphatidylinositol (7.5 μ g of P) was mixed with [32 P]phosphatidylinositol (22 000 c.p.m.) in chloroform solution and the mixture evaporated to dryness. A 0.25 ml portion of 0.1 M-sodium acetate–acetic acid buffer, pH 5.3, was added and the substrate dispersed by shaking. After addition of 0.1 ml of 1 mM- $CaCl_2$ and warming the tube to 38°C, a portion of the enzyme solution and water were introduced to bring the total volume to 0.5 ml. At the end of the incubation period (usually 10–20 min) 0.8 ml of methanol was added, followed after shaking by 1.6 ml of chloroform. After centrifugation, the upper phase was removed, washed again with an equal volume of lower phase (prepared by shaking the solvents and water in the same proportions) and its volume measured. A 0.4 ml portion was diluted to 10 ml with water and the radioactivity was counted directly by Čerenkov radiation in a scintillation counter. Appropriate blanks without enzyme were prepared and a sample of the [32 P]phosphatidylinositol, directly digested with HClO₄, was used as a standard.

Phosphatidylinositol synthetase. Two incubation systems with and without added CDP-diglyceride were used. The phosphatidylinositol synthetase medium contained 40 mM-tris-HCl buffer, pH 7.4 (at 37°C), 18 mM-MgCl₂, 0.25 mM-CTP, 1.7 mM-ATP, 0.133 M-sucrose, 0.83 mM-[3 H]₂inositol (1 μ Ci), and the enzyme preparation, in a total volume of 1.5 ml. CDP-diglyceride–inositol phosphatidyltransferase was assayed in a medium containing 40 mM-tris-HCl buffer, pH 8.4 (at 37°C), 0.36 mM-

potassium CDP-diglyceride, 2.4mg of bovine serum albumin and enzyme. After introducing the enzyme (0.6–1 mg of protein for fractions other than the supernatant and original homogenate, which were 1.2–1.6 mg) and warming to 38°C, 5 mm-MnCl₂ and 1.6 mm-[2-³H₁]inositol (2 μCi) were added finally according to the procedure of Bishop & Strickland (1970) to make a total volume of 0.4 ml.

At the end of the incubation time (60 min) the medium was treated with 20 vol. of chloroform-methanol (2:1, v/v) and left overnight. The supernatant obtained on centrifugation (1200 g) was mixed with that obtained by washing the pellet with a further 5 vol. of the same solvent, and the combined extract shaken with 20% of its own volume of 0.9% NaCl. The lower phase was separated and washed five times by shaking with an equal volume of 0.9% NaCl-chloroform-methanol (47:3:45, by vol.), the first three washes being made 0.25 mm with regard to inositol. The solution was then made up to 20 ml before determination of the radioactivity in the ³H-labelled phosphatidylinositol formed and the lipid P content.

Radioactivity counting. For ³H radioactivity counting 2 ml of the lipid solution was evaporated to dryness under an i.r. lamp in a counting vial (low K, Packard) and then dissolved in 10 ml of scintillation fluid [4 g of 2,5-diphenyl-oxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene]. The radioactivity of the mixture was counted in a Nuclear-Chicago automatic scintillation counter by using channel ratios and the external standard of the instrument to correct for quenching. Double-labelling experiments measuring ³H and ³²P incorporation into the lipid fraction were measured in the same way; ³H gave no counts in the ³²P channel whereas about 5% of the disintegrations of the ³²P were recorded in the ³H channel. The radioactivities of water-soluble ³H-labelled compounds eluted from paper etc. were counted after mixing with Bray's (1960) solution.

Water-soluble ³²P radioactivity was counted directly in 10 ml of water (no scintillator added) in a scintillation counter [Čerenkov radiation giving an efficiency of about 30% (von Haberer, 1965)].

Materials. [2-³H₁]myoInositol was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. Potassium CDP-diglyceride was obtained from Dr J. N. Hawthorne at the University of Birmingham and had been synthesized from a phosphatidic acid prepared from ox brain phosphatidylcholine. AMP, ATP, CTP, cytochrome c and NADPH were from Sigma Chemical Co., St Louis, Mo., U.S.A. Thyrotropin was obtained from the Endocrinology Study Section, National Institutes of Health, Bethesda, Md., U.S.A.

RESULTS

Distribution of 'marker' enzymes in subcellular fractions. Thyroid gland is not an easy organ to homogenize: the collagen and fibrous tissue present prevent the shearing of the tissue segments. Consequently it is necessary to homogenize for a longer time than is required for a readily dispersible tissue such as liver. In addition, some stickiness is conferred by the rich abundance of thyroglobulin. It is not surprising therefore that the degree of cross-

Table 1. Distribution of phosphatidylinositol-splitting enzyme in subcellular fractions of thyroid gland

Fraction	Phosphatidylinositol-splitting enzymes			NADPH-cytochrome c reductase			Cytochrome c oxidase			Acid phosphatase		5'-Nucleotidase	
	Total protein (mg)	Total activity (10 ⁻³ × c.p.m. liberated/h)	Sp. activity (10 ⁻³ × c.p.m. per mg of protein)	Total activity (μmol of cytochrome c reduced/min)	Sp. activity (μmol of cytochrome c reduced per μg of protein)	Total activity (μmol of cytochrome c oxidized/min)	Sp. activity (μmol of cytochrome c oxidized per mg of protein)	Total activity (μg of P/h)	Sp. activity (μg of P/h per mg of protein)	Total activity (μg of P/h)	Sp. activity (μg of P/h per mg of protein)	Total activity (μg of P/h)	Sp. activity (μg of P/h per mg of protein)
Original homogenate	1018	14800	14.6	642	0.63	3500	3.5	4536	4.5	2760	2.7	—	—
Nuclear	104	601	5.8	151	1.45	1180	11.4	1452	14.0	840	8.1	—	—
Heavy mitochondrial	3.6	21	5.8	7	1.94	135	37.6	555	154.0	64	17.8	—	—
Light mitochondrial	21.6	126	5.8	215	9.9	1940	89.9	1386	64.0	555	25.6	—	—
Microsomal	10.2	38	3.7	173	17.0	173	17.0	456	44.8	408	40.0	—	—
Supernatant	450.0	13100	29.2	0	0	780	—	780	1.7	261	0.6	—	—
Recovery	58%	94%	—	85%	—	97%	—	102%	—	77%	—	—	—

Table 2. *Distribution of phosphatidylinositol synthetase and 'marker' enzymes in subcellular fractions of thyroid gland*

Fraction	Phosphatidylinositol synthetase			NADPH ₂ -cytochrome <i>c</i> reductase		Succinate-cytochrome <i>c</i> reductase		5'-Nucleotidase	
	Total protein (mg)	Total activity (μg of inositol incorporated/h)	Sp. activity (μg of inositol per mg of protein)	Total activity (μmol of cytochrome <i>c</i> reduced/min)	Sp. activity (μmol of cytochrome <i>c</i> reduced/min per mg of protein)	Total activity (μmol of cytochrome <i>c</i> reduced/min)	Sp. activity (μmol of cytochrome <i>c</i> reduced/min per mg of protein)	Total activity (μg of P/h)	Sp. activity (μg of P/h per mg of protein)
Original homogenate	752	114*	(0.15)*	130.00	0.17	5510	7.3	6320	8.4
Nuclear	48.0	186	3.9	27.00	0.56	—	—	1680	35.0
Heavy mitochondrial	3.4	39	11.4	0.93	0.27	590	173	147	43.0
Light mitochondrial	21.2	256	12.1	20.5	0.97	2360	112	740	35.0
Microsomal	9.1	442	48.7	22.7	2.50	138	15	576	64.0
Supernatant	0	0	0	0	0	—	—	1070†	1.6
Recovery	—	—	—	55%	—	56%	—	70%	—

* Free inositol in homogenate decreases incorporation of [³H]inositol.

† Calculated value.

Table 3. *Distribution of CDP-diglyceride-inositol phosphatidyltransferase and 'marker' enzymes in subcellular fractions of thyroid gland*

Fraction	CDP-diglyceride-inositol phosphatidyltransferase			NADPH-cytochrome <i>c</i> reductase	
	Protein (mg)	Total activity (μg of inositol incorporated/h)	Sp. activity (μg of inositol incorporated/h per mg of protein)	Total activity (μmol of cytochrome <i>c</i> reduced/min)	Sp. activity (μmol of cytochrome <i>c</i> reduced/min per mg of protein)
Homogenate	2680	(701)*	(0.26)*	—	—
Nuclear	73	466	6.4	321	4.4
Heavy mitochondrial	6.8	5.5	0.81	3.4	0.5
Light mitochondrial	15.2	130	8.55	161	10.6
Microsomal	18.9	193	10.2	170	9.0
Supernatant	1638	(91)*	(0.56)*	0	—
Recovery	65%	—	—	—	—

* Free inositol in homogenate and supernatant decreases incorporation of [³H]inositol.

contamination suggested by marker-enzyme assays (Tables 1, 2 and 3) was greater than found in typical liver subcellular fractionations (e.g. McMurray & Dawson, 1969) and that the yield of fractions such as the mitochondrial and microsomal was poor.

NADPH cytochrome *c* reductase assays indicated that the heavy-mitochondrial fraction was contaminated with about 6–15% of microsomal protein, and the light-mitochondrial fraction was much more heavily contaminated (38–56%). An attempt to further purify the light-mitochondrial fraction by the density-gradient procedure of Fischer, Schulz & Oliner (1968) resulted in no significant success; although four bands were obtained all of these showed appreciable NADPH cytochrome *c* reductase activities. Ekholm (1961) has also reported that the mitochondria prepared from guinea-pig thyroid glands by two centrifugal methods were appreciably contaminated with microsomes.

A difficulty with cytochrome *c* oxidase assays was that the enzyme showed appreciable instability on storage at 0°C. This was much more apparent in the mitochondrial fraction itself than in the mitochondrial fragments contaminating the microsomal fraction (Fig. 1). Consequently the enzyme was assayed as soon as possible after the subcellular fractionation to obtain the best possible estimate of its distribution (Table 1). In one experiment cardiolipin assays (Dawson *et al.* 1962) were performed, giving an 11% contamination of the microsomal protein with mitochondrial fragments compared with 9% by the marker-enzyme determination. Succinate cytochrome *c* reductase assays, although showing the highest specific activity in the mitochondrial fractions (Table 2), suffered a similar loss on storage as cytochrome *c* oxidase. Since instability of these enzymes is not usually seen in other tissues it may be connected with the pronounced cathepsin-like activity present in the mitochondrial fraction due to lysosomal contamination (Balasubramaniam & Deiss, 1965).

In the present experiments the lysosomal marker, acid phosphatase, was present in highest specific activity in the mitochondrial fractions. Likewise in homogenates of bovine thyroid gland differential centrifugation failed to separate cytochrome *c* oxidase-containing particles (mitochondrial) from those containing β -glucuronidase (lysosomal) (Dierick & Hilderson, 1967). Attempts to prepare a lysosomal fraction from the light-mitochondrial fraction by the principles worked out by Ragab, Beck, Dillard & Tappel (1967) for liver resulted in negligible yields of material with no particular enrichment of acid phosphatase activity.

The distribution of 5'-nucleotidase (Tables 1 and 2) suggested that the plasma membranes were significantly enriched in the microsomal fraction.

Fig. 1. Loss of cytochrome *c* oxidase activity on storage of heavy-mitochondrial (○) and microsomal (●) fractions of thyroid at 0°C.

Nevertheless the large amount of this enzyme in the heavy-mitochondrial plus nuclear fractions indicates a greater contamination by plasma membrane than by endoplasmic-reticulum fragments (NADH cytochrome *c* reductase assays) and presumably means that most of the plasma membranes were still intact. A surprising feature was the large amount of 5'-nucleotidase in the supernatant fraction (e.g. Table 2). Its specific activity in this fraction was low because of the large content of soluble protein (thyroglobulin) in the tissue. Although the zero-time control for the enzymic assay was large because of the P_i contained in the supernatant fraction, the activity was shown to be proportional to the amount of supernatant added. Under the conditions used for 5'-nucleotidase assay none of the cellular fractions from thyroid showed any ability to dephosphorylate 2-phosphoglycerol.

Properties and cellular distribution of phosphatidylinositol-degrading enzyme in thyroid. Preliminary investigations showed that thyroid tissue contained an active degradative enzyme that split off water-soluble phosphorus-containing compounds from a phosphatidylinositol substrate. The enzyme

Fig. 2. pH-activity profile of phosphatidylinositol-degrading enzyme of thyroid. The medium contains: phosphatidylinositol, $0.24\mu\text{mol}$; CaCl_2 , $0.5\mu\text{mol}$; thyroidal supernatant fraction, 0.1ml in a total volume of 0.5ml . The incubation time was 10min . ○, 0.05M - NaOH - $\beta\beta'$ -dimethylglutaric acid buffer; ●, 0.05M -sodium acetate-acetic acid buffer.

Fig. 3. Inhibition of thyroidal phosphatidylinositol breakdown by EDTA and stimulation by Ca^{2+} . ○, Inhibition of basic phosphatidylinositol breakdown by EDTA; ●, stimulation of breakdown by CaCl_2 in the presence of 0.125mM -EDTA.

had a pH optimum of 5.3 – 5.5 when assayed in acetate or $\beta\beta'$ -dimethylglutarate buffers (Fig. 2). The activity was stimulated by a low concentration of Ca^{2+} and the stimulated activity was completely inhibited by 1mM -EDTA but not by citrate, and only 32% by ATP at the same concentration. The inhibition of the basic activity by EDTA was completely reversed and the activity further enhanced by the addition of Ca^{2+} (Fig. 3). This effect of Ca^{2+} could not be duplicated by the addition of Mg^{2+} or Mn^{2+} at similar concentrations.

Fig. 4. Inhibition of the phosphatidylinositol-degrading enzyme in thyroidal supernatant fraction by chlorpromazine: ○, 0.2mM - CaCl_2 added; ●, 0.5mM - CaCl_2 added.

When the distribution of the activity in the subcellular fractions of thyroid tissue was assayed under appropriate conditions it was found to be heavily concentrated in the supernatant fraction (Table 1). A high percentage of the activity was recovered in this fraction and its specific activity was by far the highest of all those isolated, in spite of the heavy concentration of thyroglobulin. The activity of the enzyme was not significantly enhanced in any fraction, apart from the nuclear fraction, by freezing and thawing. However, the small increase in the nuclear fraction (42%) can probably be attributed to this fraction still containing whole cells.

The phosphatidylinositol-degrading activity was very sensitive to addition of chlorpromazine to the incubation medium: 1mM -chlorpromazine caused almost complete inhibition of the breakdown (Fig. 4). The degree of the inhibition was not affected by increasing the Ca^{2+} concentration to 2.5 -fold the optimum concentration (0.2mM) required for full activation under these conditions (Fig. 4).

Water-soluble products formed by phosphatidylinositol breakdown. Since a phosphatidylinositol hydrolase active at acid pH and splitting off mainly phosphorylinositol has been extensively described in other tissues (Dawson, 1959; Kemp, Hübscher & Hawthorne, 1961; Friedel, Brown & Durell, 1969) it was expected that this would be the sole or major water-soluble product formed after degradation of $[^{32}\text{P}]$ phosphatidylinositol. In fact paper ionophoresis at pH 3.6 (pyridine-acetic acid buffer) and radioautography showed only a minor

Fig. 5. Release of water-soluble ^{32}P -labelled products from phosphatidylinositol on incubation with thyroidal supernatant. $\blacktriangle$, Total c.p.m. liberated; $\circ$, c.p.m. in phosphorylinositol; $\bullet$, c.p.m. in fast-running spot (ionophoresis); Δ , sum of c.p.m. in $\circ$ and $\bullet$.

radioactive spot moving to the same position as phosphorylinositol. The major product was not glycerylphosphorylinositol but an unknown spot moving slightly more rapidly than phosphorylinositol. After a brief acid hydrolysis (10 min; 1 M-HCl; 100°C) the fast-moving spot was quantitatively converted into a spot moving with phosphorylinositol. On isolation of the two compounds from the paper by elution and reincubation of them with thyroidal supernatant under the same conditions there was no interconversion between the two. Fig. 5 shows that the liberation of the two products was not linear and that there was no differential release of the two compounds with time: the concentration relationship between them remained remarkably constant with the major product containing 62–65% of the organic phosphorus liberated at all time-points. This suggests that both compounds are produced simultaneously from the phosphatidylinositol or perhaps from another phospholipid formed from it. With longer periods of incubation the sum of the water-soluble radioactivity recovered in the two compounds (Fig. 5) became less than the total radioactivity released. This is probably due to the slow enzymic conversion of the products into P_i , which had migrated beyond the limits of the paper: it was often seen in similar experiments with shorter periods of ionophoresis.

The same two initial products in similar pro-

portions were released by the thyroid enzyme from ^{32}P phosphatidylinositol prepared from liver slices and, further, the phosphatidylinositol-degrading enzyme from liver, active at an acid pH, also liberated them. Scott & Dawson (1968) noted release of the same two products on incubation of ram spermatozoa with ^{32}P phosphatidylinositol.

Synthesis of phosphatidylinositol. The subcellular distribution of the system responsible for phosphatidylinositol synthesis in pig thyroid gland was assessed in two ways. The first method in the absence of any added immediate lipid precursor, CDP-diglyceride, represents the incorporation of ^3H -labelled inositol into the fractions in the presence of energy-providing coenzymes (ATP and CTP). Incorporation of ^3H inositol in the absence of these coenzymes was low and presumably they are required for the synthesis of CDP-diglyceride and possibly phosphatidic acid needed for the synthesis of phosphatidylinositol. There is, of course, a possibility that part of the incorporation could be due to the enzymically catalysed, Mn^{2+} -requiring direct-exchange reaction between ^3H inositol and phosphatidylinositol reported in microsomes by Paulus & Kennedy (1960). However, although Mn^{2+} (20 mM) added to the synthetase system in the absence of CTP and ATP did allow some incorporation, this was only 19% of that seen in the full system containing Mg^{2+} and the nucleotide coenzymes.

The distribution of the synthetic activity is shown in Table 2. Most activity was recovered in the microsomal fraction and this had by far the highest specific activity on a protein basis. The apparent activity in the original homogenate was lower than that recovered in the individual fractions and this is probably due to the free inositol present in the thyroid decreasing the specific radioactivity of the inositol available for incorporation. The thyroid gland is very rich in free inositol (Freinkel, Dawson, Ingbar & White, 1959; Dawson & Freinkel, 1961). The incorporation of ^3H inositol into the microsome fraction was depressed by addition of a portion of the supernatant fraction, but this did not happen to any significant extent if the supernatant was dialysed beforehand, presumably because the free inositol had been removed. After incorporation of ^3H inositol into microsomes by this method, t.l.c. (silica gel H; chloroform-methanol-water-acetic acid, 65:50:4:1, by vol.) showed that the ^3H -labelled lipid recovered from the chromatogram ran in the same position as authentic phosphatidylinositol. Both calcium chloride and chlorpromazine at low concentrations (1.0 mM) completely inhibited the synthesis of phosphatidylinositol (Fig. 6).

In the second method ^3H inositol and CDP-diglyceride were added to the incubation medium

Fig. 6. Inhibition by CaCl_2 (●) and chlorpromazine (○) of phosphatidylinositol synthesis by thyroidal microsomes. Phosphatidylinositol synthesis was measured by the incorporation of ^3H inositol into lipids in 1 h in a medium containing CTP, ATP and Mg^{2+} .

and energy-providing coenzymes (CTP, ATP) were omitted. The highest specific activity of the CDP-diglyceride-inositol phosphatidyltransferase on a protein basis was again in the microsome fraction and to a lesser extent in the light-mitochondrial fraction, which was heavily contaminated with microsomes (Table 3). Surprisingly the supernatant fraction contained some activity and it is to be expected that this would be a minimal value owing to the presence of free inositol in the fraction decreasing the specific radioactivity of the precursor ^3H inositol. Incorporation into the microsome fraction was very limited in the absence of added CDP-diglyceride, so presumably insufficient of the latter is present in microsomal membranes for the transfer reaction to proceed unless CTP and ATP are added to ensure its continual synthesis.

Relative turnover of inositol and phosphate moieties of microsomal phosphatidylinositol. Fig. 7 shows the time-course of inositol incorporation into microsomal lipids in the presence of CTP and ATP. If, after 1 h, the incubation system was flooded with unlabelled inositol to decrease drastically the specific radioactivity of the precursor ^3H inositol, the incorporation of ^3H was not only depressed but there was a dramatic decrease in the amount of isotope already incorporated (Fig. 7). Calculation showed that the absolute rate of coenzyme-dependent inositol incorporation shown in Fig. 7 would itself result in several turnovers of the microsomal phosphatidylinositol pool in the course of 1 h. Evidence to substantiate this was obtained by preparing ^3H inositol-labelled microsomes from thyroid slices and incubating these with and without nucleotide coenzymes either in the presence or absence of a high concentration of unlabelled inositol (Fig. 8). It was only in the presence of the coenzymes that the addition of unlabelled inositol resulted in a loss of ^3H inositol from the micro-

Fig. 7. Synthesis of phosphatidylinositol in thyroidal microsomes (○). The microsomes (0.64 mg of protein) were incubated with ^3H inositol in a medium containing CTP, ATP and Mg^{2+} . The effect of adding unlabelled inositol (47 mM) to the system after 60 min incubation is shown (●).

somal lipid. The small increase in ^3H inositol labelling of the microsomal lipids seen in the complete medium (Fig. 8) is probably due to the incorporation of label into the lipid from a non-lipid pool (inositol?) not removed by washing the ^3H -labelled microsomes. The increase was not seen when inactive inositol was added or in the absence of nucleotide coenzymes (Fig. 8).

Attempts to demonstrate the liberation of $^3\text{H}_2\text{O}$ (tritium-hydrogen exchange reaction) or the release of ^3H inositol monophosphate or glycerylphosphoryl ^3H inositol, which might be expected to arise through complete metabolic turnover of the phosphatidylinositol in the ^3H inositol-labelled microsomes, were unsuccessful. In confirmation of this result, experiments with ^{32}P -labelled microsomes showed no loss of ^{32}P from the phospholipid fraction in the presence of unlabelled inositol (47 mM) and the nucleotide coenzymes even though the conditions used for ^{32}P -labelling of the slices (with thyrotropin) resulted in 65% of the phospholipid label being in phosphatidylinositol. A double-labelling experiment in which ^{32}P -labelled microsomes (prepared from ^{32}P -labelled slices) were incubated with ^3H inositol and coenzymes, and then after 1 h either with or without unlabelled inositol, shows this unambiguously (Fig. 9a). The unlabelled inositol resulted in a large loss of ^3H -labelled inositol from the microsomal total phospholipids but little significant loss of the ^{32}P -labelled phospholipids. The same was true of

Fig. 8. Incubation of [^3H]inositol-labelled thyroidal microsomes with unlabelled inositol in the presence and absence of nucleotide coenzymes. The microsomes (0.44 mg of protein) were isolated after incubating slices with [^3H]inositol: they were then incubated in the medium used for assaying phosphatidylinositol synthetase (see the Experimental section). ○, Medium alone; ●, medium plus 42mM-inositol; △, medium with CTP and ATP omitted; ▲, medium plus 42mM-inositol and with CTP and ATP omitted.

the labelling of the phosphatidylinositol itself as measured after the ionophoretic separation of the glycerylphosphorylinositol produced by alkaline deacylation of the phospholipids (Dawson *et al.* 1962) (Fig. 9b).

Phosphatidylinositol labelling by thyroidal mitochondria. Although the heavy-mitochondrial fraction showed some ability to incorporate ^3H -labelled inositol into microsomal phospholipids it seemed likely that, as in liver (McMurray & Dawson, 1969), this could be due to microsomal contamination. The presence of NADPH cytochrome *c* reductase in the heavy-mitochondrial fraction suggested such a contamination although it was clearly less than that in the light-mitochondrial fraction. Fig. 10 shows the results of two experiments in which increasing concentrations of microsomes were added to a constant amount of mitochondria and incubated with [^3H]inositol and either ATP+CTP or CDP-diglyceride. The curve expressing the relationship between activity and microsomal concentration extrapolated either to zero or a

Fig. 9. Effect of incubation with unlabelled inositol on the [^3H]inositol and ^{32}P -labelled phospholipids of thyroidal microsomes. ^{32}P -labelled microsomes were prepared as described in the Experimental section and were labelled with [^3H]inositol as described under phosphatidylinositol synthetase. After 1 h incubation of the ^{32}P -labelled microsomes (588 μg of protein) in the [^3H]inositol-containing medium (2 $\mu\text{Ci}/\text{tube}$) the incubation was either continued (open symbols) or flooded with unlabelled 47mM-inositol (filled-in symbols). (a) Labelling of total phospholipid extract. (b) Labelling of phosphatidylinositol detected by ionophoresis of the deacylation products (Dawson *et al.* 1962). □, ^{32}P labelling; ■, ^{32}P labelling with unlabelled 47mM-inositol added at 1 h; ○, ^3H labelling; ●, ^3H labelling with unlabelled 47mM-inositol added at 1 h.

Fig. 10. Biosynthesis of phosphatidylinositol on addition of various amounts of thyroidal microsomes to a constant amount of heavy-mitochondrial fraction. (a) Incorporation of [³H]inositol in a medium containing CTP, ATP and Mg²⁺ for 60 min: ●, microsomes alone; ○, mitochondria (0.63 mg of protein)+microsomes. (b) Incorporation of [³H]inositol in a medium containing CDP-diglyceride and Mg²⁺ for 30 min: ○, mitochondria (0.46 mg of protein)+microsomes. The microsomal-protein values include that contaminating the mitochondrial fraction as measured by NADPH cytochrome *c* reductase assay: the first point represents mitochondria with no added microsomes.

negative value, thus indicating that the activity contained in mitochondria can be adequately accounted for by microsomal contamination. The first open point in each curve represents the mitochondrial fraction on its own, the microsomal protein contaminant having been computed from the marker-enzyme assay. The presence of mitochondria appears to have no significant effect on the incorporation of [³H]inositol by the microsomes (Fig. 10).

Transfer of phosphatidylinositol from thyroidal microsomes to mitochondria. Because of the inability of thyroidal mitochondria to synthesize phosphatidylinositol, experiments were performed to ascertain whether a transfer of phosphatidylinositol from isolated microsomes to mitochondria could be demonstrated. ³²P-labelled microsomes were chosen because the ³²P-labelling of their phospholipids appears to be reasonably stable on incubation (Fig. 9) and, further, any transfer of label is unlikely to involve a metabolic inter-conversion. The microsomes were incubated with unlabelled mitochondria in the presence of thyroidal supernatant under conditions where it has previously been possible to demonstrate a transfer of labelled phospholipid molecules from liver and brain microsomes to their respective mitochondria (McMurray & Dawson, 1969; E. Miller & R. M. C. Dawson, unpublished work). The results of such experiments (Table 4) suggest that there is a substantial transfer of labelled phosphatidylinositol from the microsomes to the mitochondria within the first 15 min of incubation, i.e. a loss of total counts and specific radioactivity in the microsomal phosphatidylinositol and a gain in the mitochon-

drial fraction. Complete confidence in this interpretation is, however, hampered by the phosphatidylinositol phosphorus determinations being at the limits of the method of determination. The results suggest that the microsomal phosphatidylinositol pool increases during incubation (the phosphatidylinositol/protein ratio rises progressively from 0.3 to 0.81 μg of lipid P/ml) whereas that in the mitochondria decreases (the corresponding ratio falls from 0.53 to 0.13). Phosphatidylethanolamine concentrations in microsomes and mitochondria show similar but less-marked changes on incubation but with phosphatidylcholine changes are minimal. Changes in the labelling or pool size of phosphatidylinositol or other phospholipids in the supernatant fraction were not determined.

DISCUSSION

The phospholipid composition of the pig thyroid gland and the responsiveness of the phosphoinositide to thyrotrophin conforms to that described for the thyroid glands of other species (Scott, Freinkel, Klein & Nitzan, 1970). The subcellular fractionation employed in the present studies gave a reasonable degree of enrichment of the membrane and organelle fractions of this thyroid gland. The separations were not as good as those usually obtained from liver tissue but this is to be expected because of the greater cellular heterogeneity of the glands and their resistance to homogenization. It also has to be appreciated that the use of 'marker' enzymes for assessing cross-contamination may probably paint too gloomy a picture. Unless the localization of the enzyme is absolute in a particular

Table 4. *Transfer of [³²P]phospholipids on incubating ³²P-labelled thyroidal microsomes with the unlabelled heavy-mitochondrial fraction*

³²P-labelled microsomes were prepared from 2.5 g of thyroid slices incubated with [³²P]orthophosphate, and the unlabelled heavy-mitochondrial and supernatant fraction from 55 g of sliced thyroid tissue. The ³²P-labelled microsomes and unlabelled mitochondria (protein values in the table below) were incubated with 0.85 ml of supernatant fraction in 10 mM-K₂HPO₄-HCl buffer, pH 7.4, at 37°C for various times (final volume 1.5 ml). After incubation 8 ml of ice-cold 0.25 M-sucrose + 0.1 mM-EDTA was added. The mitochondrial and microsomal fractions were reisolated by centrifugation (see the Experimental section): the former was washed three times, the latter once. The phospholipids were extracted and labelling of the individual components was measured by the method of Dawson *et al.* (1962), phosphorus being determined as described by Bartlett (1959).

Incubation time (min)	Concn. of protein in reisolated fractions (mg)		Total radioactivity (c.p.m.)	Lipid P (μg)	Sp. radioactivity (c.p.m./μg of P)
	Microsomes	Mitochondria			
0	0.61	1.29			
15	0.67	1.08			
30	0.66	1.03			
60	0.65	0.74			
Phospholipid	Subcellular fraction	Incubation time (min)	Total radioactivity (c.p.m.)	Lipid P (μg)	Sp. radioactivity (c.p.m./μg of P)
Phosphatidylcholine	Microsomes	0	214	2.21	97
		15	212	2.18	97
		30	194	2.26	86
		60	156	2.54	61
	Mitochondria	0	14	2.80	5
		15	28	2.08	14
		30	23	1.50	15
		60	35	1.71	21
Phosphatidylethanolamine	Microsomes	0	90	0.43	209
		15	138	0.70	197
		30	106	—	—
		60	130	0.85	153
	Mitochondria	0	19	1.83	10
		15	16	0.72	22
		30	16	0.56	28
		60	10	0.72	14
Phosphatidylinositol	Microsomes	0	1464	0.18	8160
		15	1430	0.32	4470
		30	1225	0.37	3230
		60	925	0.53	1740
	Mitochondria	0	120	0.68	177
		15	550	0.17	3240
		30	531	<0.10	>5310
		60	491	0.10	4920
Phosphatidylserine	Microsomes	0	—	—	—
		15	8	—	—
		30	9	—	—
		60	9	—	—
	Mitochondria	0	0	—	—
		15	1	—	—
		30	2	—	—
		60	3	—	—
Phosphatidic acid	Microsomes	0	268	—	—
		15	222	—	—
		30	193	—	—
		60	132	—	—
	Mitochondria	0	7	—	—
		15	14	—	—
		30	14	—	—
		60	21	—	—

membrane or organelle, the degree of contamination indicated will be maximal rather than actual. Electron microscopy with negative-staining techniques did in fact give an impression of greater homogeneity than suggested by marker-enzyme assays, but with this method it is difficult to reach a quantitative conclusion and avoid bias in field selection.

The active phosphatidylinositol-degrading enzyme present in the pig thyroid gland appears to be similar in properties to those previously described in pancreas, liver, intestinal mucosa and brain (Dawson, 1959; Kemp *et al.* 1961; Friedel, Brown & Durell, 1967; Thompson, 1967; Atherton & Hawthorne, 1968), which also have a pH optimum reported to be between 5.3 and 5.8, by using acetate or maleate buffers, and an absolute requirement for calcium ions. Again the present result indicating that the enzyme is almost exclusively located in the soluble-protein fraction of the gland is similar to findings for its location in homogenates of intestinal mucosa (Atherton & Hawthorne, 1968) and brain (Friedel *et al.* 1969). The thyroidal microsomal fraction contained negligible activity, in contrast with the brain where 16% seems to be firmly bound to microsomes (Friedel *et al.* 1969). The finding of an enzyme predominantly in the soluble fraction is rather unsatisfactory in that the result may be derived by an isolation artifact. There are now abundant examples of enzymes that have previously been described as soluble and which are now known to be associated with membranes (Margolis & Baum, 1966; Lenaz, 1968). There could possibly be a delicate equilibrium between the enzyme in solution and the same enzyme integrated in or adsorbed on a membrane structure depending on the functional state of the tissue and the concentrations of bivalent ions and substrates etc.

An acid 'hydrolase' of this type might be expected to be associated with lysosomes. However, none of the fractions showed any significant enhancement of activity on freezing and thawing to disrupt lysosomal structures, nor was there any correlation of the distribution of the phosphatidylinositol-degrading enzyme with acid phosphatase. The latter, although present in the supernatant fraction, showed the highest specific activity in the mitochondrial fractions. It has been shown that the acid ribonuclease of liver can be sedimented in particles of different size from those containing acid phosphatase (Rahman & Cerny, 1969). It is not impossible that the phosphatidylinositol-splitting enzyme could itself be associated with small vesicles, e.g. those budding off from the Golgi apparatus (Novikoff, 1963), which then disrupt under the conditions used in the homogenization.

It is clear from the present experiments that the phosphatidylinositol-degrading system does not

cause a simple hydrolysis of phosphatidylinositol into diglyceride and phosphorylinositol. Although thin-layer chromatography showed that a diglyceride was the initial lipoidal product formed with the thyroid and liver enzymes the primary major water-soluble product was not phosphorylinositol but rather a derivative of this phosphate ester. Its properties are quite different from that of glycerylphosphorylinositol, which can be formed as a minor product of the enzymic breakdown of phosphatidylinositol (Kemp *et al.* 1961; Friedel *et al.* 1967) and which may result from a non-specific deacylating phospholipase also being present in the supernatant (Atherton & Hawthorne, 1968). The precise nature of the reaction is being investigated, but meanwhile it is not justifiable to describe the phosphatidylinositol-splitting enzyme of tissues, active at an acid pH, as phosphatidylinositol inositolphosphohydrolase.

Both the complete system responsible for phosphatidylinositol assembly from simple moieties and the final enzymic reaction catalysed by CDP-diglyceride-inositol phosphatidyltransferase appear to be concentrated in the microsomal fraction prepared from pig thyroid gland. The finding of appreciable amounts of the latter enzyme and 5'-nucleotidase but not NADPH cytochrome *c* reductase in the supernatant fraction (Table 3), as well as them both being concentrated (specific activity) in the microsomal fraction, raises the question whether the CDP-diglyceride-inositol phosphatidyltransferase is enriched in plasma membranes. It can be noted in this respect that most of the total activity of both enzymes (5'-nucleotidase and CDP-diglyceride-inositol phosphatidyltransferase) is recovered in the nuclear fraction. However, 5'-nucleotidase was also found in fairly high specific activity in the heavy-mitochondrial fraction (large sheets of plasma membranes?) whereas synthesis of phosphatidylinositol and activity of the endoplasmic reticulum marker-enzyme NADPH cytochrome *c* reductase were rather low in this fraction. Phosphatidylinositol synthesis in the pancreas is also concentrated in the 'microsomal' fraction, with some in the particle-free supernatant (Prottey & Hawthorne, 1967), but marker-enzyme assays to check purity and cross-contamination of fractions were not performed in these investigations. Benjamins & Agranoff (1969) and Bishop & Strickland (1970) have reported that CDP-diglyceride-inositol phosphatidyltransferase is found at highest specific activity in the crude microsomal pellet obtained from guinea-pig and rat brain homogenates.

Calculation shows that the inositol moiety of the microsomal phosphatidylinositol pool is turning over several times per hour on incubation under the present conditions. However, there is no evidence

Scheme 1.

that this is associated with a corresponding catabolism of the molecule by the action of phospholipases that split it into glycerylphosphorylinositol or phosphorylinositol. Thus the last-named are not found in the medium in a labelled form when [^3H]-inositol in the microsomal lipid is removed by addition of unlabelled inositol. Moreover, under the same circumstances of addition of unlabelled inositol the ^{32}P -labelling of the phosphatidylinositol is retained in the molecule and does not become water-soluble. Clearly therefore the inositol moiety may be turning over and leaving the phosphate moiety attached to some lipid residue, which remains in the membrane and is resynthesized into phosphatidylinositol. An alternative explanation that seems less likely is that the inositol labelling is confined to some small rapidly turning-over fraction of the microsomal phosphatidylinositol pool whereas the phosphate label is primarily associated with a much more stable part of the same pool.

Whether the preferential exchange of the inositol moiety can be distinguished from the bivalent-cation-exchange reaction described by Paulus & Kennedy (1960) is not certain. Both the incorporation of [^3H]inositol into the fraction and its removal are dependent on nucleotide coenzymes. Further, the rates of these reactions seem much faster than the exchange reaction catalysed by a high concentration of Mn^{2+} . Probably the metabolic pathway (Scheme 1) by which phosphatidylinositol is synthesized can effect an exchange between the [^3H]inositol of phosphatidylinositol and unlabelled inositol by the reversibility of the last step, the nucleotide coenzymes being used to maintain a catalytic concentration of CDP-diglyceride. The ^{32}P label would be retained in the membrane as CDP-diglyceride or possibly phosphatidic acid and recycled, or it might not be liberated from the enzyme complex (Paulus & Kennedy, 1960) (Scheme 2). Alternatively there could be an enzyme that directly converts phosphatidylinositol into phosphatidic acid, which is recycled.

Since some membrane in the microsomal fraction is certainly the most active site of biosynthesis of phosphatidylinositol it becomes important to know

whether other membranes of the cell can synthesize this phospholipid. With liver, evidence has been presented that isolated mitochondria are incapable of the new synthesis of phospholipids, apart from phosphatidic acid, provided that correction is made for the contaminating microsomes (McMurray & Dawson, 1969; Jungalwala & Dawson, 1970a). The same seems to be true for the phosphatidylinositol fraction of thyroidal mitochondria, for no incorporation of [^3H]inositol occurs above that expected from the microsomes present in the fraction as judged by marker-enzyme assays. Again evidence seems now to be accumulating that the phospholipids in the mitochondrial membranes of liver cells are being renewed by a process of exchange diffusion of whole lipid molecules from the synthetic site (endoplasmic reticulum) within the cell (Dawson, 1966; Wirtz & Zilversmit, 1968; McMurray & Dawson, 1969; Jungalwala & Dawson, 1970b). The present results suggest that the same may be true of the phosphatidylinositol of thyroidal mitochondria. On incubation of these with ^{32}P -labelled microsomes there appears to be a very rapid transfer of radioactivity from the microsomal phosphatidylinositol to the mitochondrial pool. This seems to be far in excess of the amount to be expected if the exchange occurs merely with microsomes contaminating the mitochondrial fraction. It is far more rapid and greater in extent than the transfer of other phospholipids from the microsomes to the mitochondria, and it occurs in the face of a net selective loss of phosphatidylinositol from the mitochondrial fraction and a gain by the microsomal fraction.

Chlorpromazine inhibits both the hydrolysis and synthesis of phosphatidylinositol, and the curves presented in Figs. 4 and 6 show that the effective concentrations are remarkably similar. It seems likely that the inhibition of the catabolic enzyme is due to a displacement of Ca^{2+} from the site of enzymic attack by the drug. The Ca^{2+} required for the enzymic activity is likely to combine readily with the anionic site on the substrate phosphatidylinositol (Hauser & Dawson, 1967), and it is noteworthy that as far as can be judged in experiments without EDTA the maximal Ca^{2+} requirement approximated to that required to neutralize these anionic sites (1 calcium atom/2 phosphatidylinositol molecules). Hauser & Dawson (1968) found that chlorpromazine was remarkably effective in removing $^{45}\text{Ca}^{2+}$ from a phosphatidylinositol-water

Scheme 2.

interface, its hydrophobic region penetrating into the phospholipid so that its positive charge was specifically located in a position where it could very effectively compete with Ca^{2+} for the anionic site of the phospholipid. The overwhelming competitiveness compared with Ca^{2+} may be the reason why the inhibition caused by the drug is not reversed by increasing Ca^{2+} 2.5-fold in the system. If the drug has penetrated the substrate particles it automatically means that the calcium salt form of the substrate is either not available or lessened, so that the reaction is inhibited.

Chlorpromazine is known to inhibit thyrotrophin-induced stimulation of thyroid activity in thyroid slices (Onaya, Solomon & Davidson, 1969). It is reasonable to assume that thyrotrophin activates endocytosis by means of the labilization of certain membranes, and it is tempting to speculate that the active phosphatidylinositol-splitting enzyme plays an important part in this process by decreasing the stability of the membrane. In this connexion the rapid liberation of diglyceride in thyroid slices on treatment with thyrotrophin (Scott *et al.* 1968) may be relevant. Ca^{2+} seems to play a necessary role in the secretory activity of the thyroid gland (Zor, Lowe, Bloom & Field, 1968) and it is conceivable that this could be related to the essential coenzyme role it plays in the enzymic activity rather than to the adenylate cyclase system as suggested by Zor *et al.* (1968). Chlorpromazine would interrupt this process by displacing Ca^{2+} from the substrate sites, resulting in the suppression of enzymic attack and stabilization of the membrane.

Chlorpromazine also inhibits the thyrotrophin-induced phospholipogenesis in thyroid slices (Onaya & Solomon, 1969). The present results show that the drug effectively inhibits the synthesis of phosphatidylinositol in thyroidal microsomes, and since the enhanced phospholipid synthesis in slices induced by thyrotrophin is mainly confined to this specific phospholipid it is perhaps not surprising that it is inhibited by chlorpromazine. Ca^{2+} is not recognized to play any part in the enzymic mechanisms by which phosphatidylinositol is synthesized. It is reasonable to suppose, however, that chlorpromazine would penetrate into the microsomal membrane (Kwant & Seeman, 1969) and effectively compete for the other bivalent ions (Mg^{2+} , Mn^{2+}) known to be vital to the synthetic process. In a similar way it is probable that Ca^{2+} may inhibit the synthesis (see Fig. 6) by displacing these bivalent ions from their reaction sites. Although the synthesis of phosphatidic acid in the thyroid is not inhibited by Ca^{2+} , even though it has an absolute Mg^{2+} requirement (Schneider, 1968), Prottey & Hawthorne (1967) have described the inhibition of phosphatidylinositol synthesis in pancreas homogenates by Ca^{2+} .

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