Pathways of Thyroid Phosphorus Metabolism

THE PHOSPHOLIPIDS OF SHEEP THYROID

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There is much evidence for the importance of phosphorus in the metabolism and function of the thyroid gland. Kocher (1902) first described an inverse relationship between the contents of iodine and phosphorus in this tissue. More recently, Dempsey & Singer (1946) have presented histochemical evidence for marked fluctuations in the activities of acid and alkaline phosphatases and of hexose phosphatases in the gland during periods of varying thyroid activity, and Borell & Holmgren (1949) have demonstrated that the uptake of radioactive phosphorus by the thyroid *in vivo* is increased by pituitary thyrotropin. With preparations *in vitro*, Morton & Schwartz (1953) have reported that pituitary thyrotropin selectively increases the incorporation of ^{32}P into the phospholipids of ox-thyroid slices, and Freinkel & Ingbar (1955*a*) have shown that the first step in the synthesis of thyroid hormone, namely the accumulation of I^- ion, is dependent upon energy derived from oxidative phosphorylation. Despite these data, detailed inquiry into the composition and metabolic pathways of thyroid tissue has not hitherto been made.

The present paper deals with phosphoglyceride metabolism. The phospholipids and related compounds which occur in sheep thyroid have been characterized by recently developed chromatographic techniques (Dawson, 1954, 1955*a*). Moreover, these methods have been used to study the incorporation *in vitro* of both ^{32}P phosphate and ^{14}C glycerol into the individual phosphoglycerides and their metabolic intermediates in sheep-thyroid slices. The studies have yielded information about the metabolic pathways of the phospholipids, and the active turnover of these components suggests that the phospholipids may occupy a central role in the function of this gland.

EXPERIMENTAL

Materials

^{32}P Orthophosphate and ^{14}C glycerol were obtained from the Radiochemical Centre, Amersham, Bucks. The

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glycerol is prepared substantially by the method of Karnovsky & Gidez (1952) and thus consists of DL-[1- ^{14}C]-glycerol (Karnovsky, Hauser & Elwyn, 1956). On paper chromatography, in the solvents described below, the glycerol ran as a single radioactive spot.

Thyroid tissue was obtained from sheep which were 12 months of age or older.

Methods

Fractionation experiments. Intact thyroid glands were excised from sheep within 5 min. of killing and plunged directly into liquid O_2 . For each analysis a weighed portion (0.7-1 g.) of frozen thyroid was added to 4 ml. of water, contained in a graduated centrifuge tube in a boiling-water bath. After 2 min., the boiled tissue and water were homogenized in a Potter-Elvehjem ground-glass homogenizer and returned to the original centrifuge tube. The homogenizer was washed with 7 ml. of ethanol and the washings were combined with the aqueous homogenate and dispersed with a glass rod. After centrifuging, the turbid supernatant solution was shaken for 5 min. with 40 ml. of CHCl_3 ; the mixture then separated into water- and CHCl_3 -soluble phases.

The water-soluble phase was passed through a column of Amberlite IRC 50 (H^+ form) resin to remove cations which would otherwise interfere with the subsequent paper chromatography. The column was washed twice with 10 ml. portions of water and the total effluent was cleared of turbidity and concentrated by shaking with 3 vol. of isobutanol. The aqueous phase was neutralized to pH 7 with aq. NH_3 soln., concentrated *in vacuo* below 50° and quantitatively applied to washed filter paper for two-dimensional chromatography in phenol-aq. NH_3 soln. (descending) and ethanol-aq. NH_3 soln. (ascending) (Dawson, 1955*a*, *b*).

The CHCl_3 -soluble phase was combined with the insoluble residue which had been suspended in 10 ml. of ethanol and transferred quantitatively from the centrifuge tube to an Erlenmeyer flask. The suspension was boiled for 20 min., cooled to 0° and filtered through glass wool. The filtrate was shaken with 2 vol. of 0.1N-HCl in order to remove any acid-soluble, phosphorus-containing contaminants (Dawson, 1955*a*). The purified lipid extract was taken to dryness *in vacuo* and the phospholipids were examined by two-dimensional chromatography after mild alkaline hydrolysis (Dawson, 1954). This mild hydrolysis of the phospholipids selectively removes the fatty acid residues from the phosphoglycerides and leaves the individual water-soluble glycerylphosphoryl-base units intact.

Incubation experiments. Thyroid glands were excised from sheep within 5 min. of killing, and wrapped in cellophane bags, placed on cracked ice and transported to the

laboratory in Dewar flasks. Thyroid slices, 0.3–0.4 mm. thick, were prepared by the method of Deutsch (1936), and placed in Petri dishes containing ice-cold 0.15M-NaCl.

For incubation, two types of suspending media were employed: (a) phosphate medium, composed of 0.131M-NaCl, 5 mM-KCl, 1.2 mM-MgSO₄, 0.8 mM-CaCl₂ and 10 mM-sodium phosphate buffer (pH 7.4), and (b) phosphate-free medium composed of the same constituents except for the substitution of 10 mM-2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer (pH 7.4) for the sodium phosphate buffer.

Thyroid slices were quickly blotted and introduced in 320–350 mg. amounts into 2.7 ml. of phosphate-free medium contained within the main compartment of chilled Warburg vessels. Side arms of the Warburg vessels held 0.3 ml. of the phosphate medium, containing 25–35 μ C of inorganic [³²P] phosphate or 15 μ C of [1-¹⁴C]glycerol (1 μ mole of glycerol/ μ C).

Details of the incubation and manometric techniques have been described previously (Freinkel & Ingbar, 1955b). All incubations were started within 90 min. of killing the animal. The slices were shaken at 38° with pure O₂ in the gas phase. After gassing and temperature equilibration under these conditions for 10 min., the phosphate medium in the side arm was tipped into the phosphate-free medium contained within the main compartment. This facilitated the introduction of phosphorus of known specific radioactivity into the system at a desired time. At intervals during the subsequent 4 hr., slices were removed from the flasks, quickly blotted, weighed, frozen in liquid O₂ and stored to await fractionation as described previously. Suspending media were centrifuged immediately at 4° and the supernatant solutions were stored at –18°.

Chromatography. Sheets of Whatman no. 1 filter paper were well washed with 2N-acetic acid before chromatography. The following solvent systems were employed: phenol (A.R.) saturated with 0.1% aq. NH₃ soln.; ethanol-aq. NH₃ soln. (sp.gr. 0.880)–water (61:29:10, by vol.) (Dawson, 1955b); trichloroacetic acid (10%, w/v) in 2-methylpropan-2-ol–water (62:38, v/v) (the trichloroacetic acid was removed from the dried chromatograms by washing with diethyl ether; Dawson, 1954); *n*-butanol-2-ethoxyethanol–ethanol-aq. NH₃ soln. (sp.gr. 0.880)–water (40:40:15:12.5:40, by vol.) (Meredith & Sammons, 1952). Descending chromatography was employed for the phenol-aq. NH₃ soln., and ascending for all the other solvents.

After chromatography, the phosphorus-containing compounds were localized by spraying with the acid-molybdate reagent of Hanes & Isherwood (1949) and by irradiating the dried sheets with ultraviolet light (Bandurski & Axelrod, 1951). Preliminary spraying with 0.1% ninhydrin in acetone did not interfere with the molybdate reaction. This facilitated the identification of ninhydrin-reacting phospholipid intermediates as well as providing reference amino acid spots which helped in the localization of other intermediates. In addition, all radioactive compounds were localized by routine radioautography of chromatograms in contact with Kodirex X-ray Film.

Phosphorus estimation and radioactive assay. A modification of the method of Berenblum & Chain (1938) was employed for estimating small quantities of phosphorus, and larger amounts were measured by the method of Fiske & Subbarow (1925). The phosphorus-containing spots on each chromatogram were cut out together with a paper

blank of equivalent weight from the area immediately surrounding each spot. In the fractionation experiments and in the studies with ³²P, the paper spots and blanks were introduced into 0.9 ml. of 72% perchloric acid A.R. and oxidized to orthophosphate by heating them with suitable shielding on an electric Kjeldahl-digestion rack. The digests were assayed for phosphorus and the ³²P contained in them was estimated directly in an M-6 liquid counter tube (20th Century Electronics). Specific radioactivities are expressed as counts of ³²P min./ μ C of P, and were corrected to an initial specific radioactivity of 100 000 counts/min. of ³²P/ μ C of P in the phosphate medium contained within the side arms.

In experiments with [1-¹⁴C]glycerol, the phosphorus-containing spots and paper blanks were eluted by direct extraction with 5 ml. of 2% aq. NH₃ soln. Eluates were dried *in vacuo* at room temperature over concentrated H₂SO₄ and redissolved in 2.5 ml. of water. Samples (1 ml.) were placed on aluminium planchets and dried for radioactive assay at infinite thinness with a mica end-window Geiger-Müller tube. Equal volumes were digested with perchloric acid and analysed for phosphorus. Specific radioactivity values were expressed as counts of ¹⁴C/ μ C of P/min.

In all radioactive analyses, sufficient counts were observed to reduce the probable error of the measurement to less than 3%.

RESULTS

Examination of sheep-thyroid tissue for phospholipids and intermediates of their metabolism

In eight fresh sheep-thyroid glands, the water-soluble phosphorus averaged 201.5 $\pm$ 54.1 μ C of P/g. wet wt. and constituted 18.2 $\pm$ 5.4% of the total glandular phosphorus. Inorganic phosphorus comprised 19.4 $\pm$ 5.9% of the water-soluble phosphorus and averaged 39.4 $\pm$ 14.5 μ C of P/g. wet wt. All values are mean $\pm$ S.D.

The following water-soluble phosphoglyceride intermediates were identified in two-dimensional chromatograms prepared from aqueous extracts of fresh sheep-thyroid tissue: α -glycerophosphoric acid (GPA), phosphorylethanolamine (PE), phosphorylcholine (PC), glycerylphosphorylethanolamine (GPE) and glycerylphosphorylcholine (GPC) (Fig. 1). Identification was based on (a) identical migrations of synthetic markers added directly to the aqueous extracts, and (b) parallel migration of synthetic markers concurrently chromatographed along the outer margins of the filter paper. PE and GPE were further identified on the basis of their reaction with ninhydrin. (The markers were supplied by Dr R. M. C. Dawson.)

The phenol-aq. NH₃ soln. and ethanol-aq. NH₃ soln. solvents effectively separated GPE, GPC and PC from the other water-soluble phosphorus-containing components of sheep-thyroid tissue. Recovery of added GPE, GPC or PC introduced into the initial homogenate was greater than 80%. Therefore it is possible to use chromatographic

techniques to derive minimum estimates of the concentrations of these compounds (Table 1).

It was observed that, 24–48 hr. after spraying with acid molybdate, two brownish grey spots developed in previously unmarked areas. R_f values for these compounds were 0.13 and 0.37 in phenol–aq. NH_3 soln. and 0.71 and 0.83 in ethanol–aq. NH_3 soln. respectively. GPC reference markers which were chromatographed simultaneously in the same solvent systems had R_f values of 0.87 and 0.84 respectively. The larger of the two spots, which remained closest to the origin in both phenol–aq. NH_3 soln. and ethanol–aq. NH_3 soln., has been provisionally identified as free inositol since inositol markers displayed identical chromatographic mobilities and colour reactions.

The phosphorus in the lipid extracts prepared from eight fresh sheep-thyroid glands averaged

$191.5 \pm 30.1 \mu\text{g.}$ of P/g. wet wt. and constituted $17.3 \pm 5.8\%$ of the total glandular phosphorus. After mild alkaline hydrolysis, $77.6 \pm 7.3\%$ of the lipid phosphorus was recovered in the aqueous phase. Resolution of the water-soluble hydrolysis products by two-dimensional chromatography disclosed spots which corresponded to the mobilities which Dawson (1954) has described for the glyceryl-phosphoryl residues in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid and phosphoinositides. The respective spots were identified as GPC, GPE, glycerylphosphorylserine, GPA, and a compound containing glycerol, phosphorus and inositol residues. In three experiments, the chromatographic separations of the hydrolysis products of the phosphatidylserine and the phosphoinositide were incomplete. Glycerophosphoric acid could be demonstrated in only four of eight experiments. Whether this compound represents a fractionation artifact or whether thyroid phosphatidic acid is present in too limited amount for the routine detection of its hydrolysis products cannot be assessed from the available data.

Since the mild alkaline hydrolysis of thyroid phospholipids left only about 20% of the lipid phosphorus intact, some of which would be sphingomyelin and other alkali-stable phospholipids, the distribution of thyroid phosphoglycerides can be estimated by chromatographic partition of the water-soluble phosphorus. In this way, 52, 25, 11, 9 and 3% of the total phosphorus in the five identified spots could be attributed to the respective hydrolysis products of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphoinositide and phosphatidic acid.

Incorporation of ^{32}P into the phosphoglycerides and their intermediates in sheep-thyroid slices

Surviving sheep-thyroid slices incorporated ^{32}P into all of the phosphorus-containing compounds which had been identified in the earlier fractionation studies. Labelled GPA, PC and PE were disclosed by brief radioautographic exposure of chromatograms of the water-soluble extracts; more prolonged radioautography was required for detection of the sparsely labelled GPC and GPE. Spots corresponding to the water-soluble hydrolysis products of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid and the phosphoinositide were seen in radioautographs of chromatograms of the hydrolysed labelled lipid extracts (Fig. 2). In addition, two unidentified radioactive spots were seen in these radioautographs (cf. L_4 and L_5 , Fig. 2), but these are not peculiar to the thyroid (N. Freinkel & R. M. C. Dawson, unpublished work).

Fig. 1. Two-dimensional chromatogram of the water-soluble phosphorus-containing compounds in sheep-thyroid gland. Spots were localized by spraying with acid molybdate and by irradiating the dried sheets with ultraviolet light. A_1 , Glycerophosphoric acid; A_2 , phosphorylethanolamine; A_3 , phosphorylcholine; A_4 , glycerylphosphorylethanolamine; A_5 , glycerylphosphorylcholine; A_6 , inorganic phosphorus.

Table 1. Concentration of phospholipid intermediates in aqueous extracts of sheep-thyroid tissue

PC, phosphorylcholine; GPC, glycerylphosphorylcholine; GPE, glycerylphosphorylethanolamine. Values are % of total, water-soluble organic phosphorus. Total water-soluble organic P was determined on aqueous extracts of 0.7–1.0 g. (wet wt.) of fresh sheep-thyroid tissue. Individual compounds determined by chromatographic resolutions of aqueous extracts.

Expt. no.	Total water-soluble organic P (mg./g. wet wt.)	PC (%)	GPC (%)	GPE (%)
1	165	3.4	4.2	1.5
2	87	5.0	3.6	0.8
3	134	3.2	2.2	1.3
4	169	3.0	3.6	1.2

Specific radioactivities of the individual phosphoglycerides differed considerably. Incorporation of ^{32}P was greatest into phosphoinositide. At all times during incubation, the phosphoinositide contained two to ten times more $^{32}\text{P}/\mu\text{g.}$ of P than any other lipid component. Representative values from one of nine experiments with ^{32}P are presented in Table 2. During serial observations, the specific radioactivities of the individual phosphoglycerides were found to increase at different rates (Table 2). Within the limits of the analytical techniques, systematic changes in the concentration of individual phosphoglycerides or certain phosphoglyceride intermediates could not be detected during incubation *in vitro* despite a 10–20% increase in the concentration of inorganic phosphorus in the suspending medium. On the

basis of such evidence for equilibrium conditions, isotopic techniques were employed to assess the interrelationships between the individual phospholipids and water-soluble intermediates of lipid metabolism. Slices from the same sheep-thyroid gland were incubated in a number of flasks and, at regular intervals, tissues were removed and fractionated for the concurrent estimation of the specific radioactivities of water- and chloroform-soluble phosphorus compounds. Results from one of three experiments are given in Figs. 3 and 4. At all times throughout incubation, the specific radioactivities of the water-soluble phosphorylated bases, PE and PC, exceeded those of phosphatidylethanolamine and phosphatidylcholine, whereas the specific radioactivities of the water-soluble phosphodiester, GPE and GPC, were less than those of the phosphoryl units in the corresponding phosphoglycerides.

Fig. 2. Radioautograph of a two-dimensional chromatogram of an alkaline hydrolysate of the lipids extracted from 340 mg. (wet wt.) of sheep-thyroid tissue. Before fractionation, thyroid slices were incubated for 4 hr. in 3 ml. of suspending medium containing $25\mu\text{C}$ of $\text{NaH}_2^{32}\text{PO}_4$. L_1 , Hydrolysis product of the thyroid phosphoinositide; L_2 , glycerophosphoric acid; L_3 , glycylphosphorylserine; L_4 , unidentified; L_5 , unidentified; L_6 , GPE; L_7 , GPC.

Incorporation of $[1-^{14}\text{C}]$ glycerol into the phosphoglycerides of thyroid slices

After the incubation of sheep-thyroid slices with DL- $[1-^{14}\text{C}]$ glycerol, ^{14}C could be demonstrated in the glycerylphosphoryl residues of all of the phosphoglycerides. Specific radioactivities of the individual phosphoglycerides after 1–4 hr. incubation differed but were not as variable as the values which had been observed with ^{32}P (Table 2). Experiments were performed in which slices from the same sheep-thyroid gland were incubated in different Warburg flasks with either ^{32}P or $[1-^{14}\text{C}]$ glycerol. Data from one of three such paired-isotope experiments are presented in Table 2. The ratios of the $^{14}\text{C}/^{32}\text{P}$ specific radioactivities of the individual phospholipids showed considerable variation at any single instant. During serial analyses, ^{14}C specific radioactivities did not increase as rapidly as the ^{32}P values.

Table 2. *Incorporation of DL- $[1-^{14}\text{C}]$ glycerol and $\text{NaH}_2^{32}\text{PO}_4$ into the glycerophosphoryl units of the phospholipids of sheep-thyroid slices*

Slices (330–350 mg. initial wet wt.) from the same sheep-thyroid gland were incubated in 3 ml. of suspending medium containing either $30\mu\text{C}$ of $\text{NaH}_2^{32}\text{PO}_4$ or $15\mu\text{C}$ of DL- $[1-^{14}\text{C}]$ glycerol. Slices were removed after 2 and 4 hr. Average values for q_{O_2} ($\mu\text{l.}$ of $\text{O}_2/\text{mg.}$ initial wet wt./hr.) during the 4 hours were 0.40, 0.35, 0.35 and 0.33 respectively. Specific radioactivities are expressed as counts of ^{32}P or $^{14}\text{C}/\mu\text{g.}$ of P/min. Values for ^{32}P have been corrected to an initial specific radioactivity of 100 000 counts/ $\mu\text{g.}$ of P/min. in the side arms of the Warburg vessels.

Phospholipid	Specific radioactivities							
	^{32}P		^{14}C		$^{14}\text{C}/^{32}\text{P}$		$\frac{4 \text{ hr.}}{2 \text{ hr.}} \times 100$	
	2 hr.	4 hr.	2 hr.	4 hr.	2 hr.	4 hr.	^{32}P	^{14}C
Phosphatidylcholine	426	1504	83	123	0.195	0.082	353	148
Phosphatidylethanolamine	218	666	31	54	0.142	0.081	305	174
Phosphatidylserine	175	428	16	29	0.091	0.068	244	181
Phosphatidic acid	973	1605	—	14	—	0.015	165	—
Phosphoinositide	2344	7262	64	105	0.027	0.014	310	164

Verification of an inositol-containing phospholipid in the sheep thyroid

The highly radioactive spot which had been identified as a phosphoinositide on the basis of R_f characteristics was localized by radioautography and eluted with water. The eluate was hydrolysed

with 5N-HCl for 36 hr. at 100°. The acid was removed by evaporation *in vacuo* and the hydrolysis products were resuspended in water and resolved by the chromatographic technique of Meredith & Sammons (1952). Free inositol, glycerophosphoric acid and inorganic phosphate were demonstrated, thus verifying the presence within sheep-thyroid gland of a phospholipid containing glycerol, inositol and phosphorus residues.

Fig. 3. The metabolism of lecithin (phosphatidylcholine) in surviving sheep-thyroid tissues. Slices weighing 300–330 mg. were incubated in 3 ml. of suspending medium containing $27 \mu\text{C}$ of $\text{NaH}_2^{32}\text{PO}_4$ and were removed at intervals for analysis of the compounds in aqueous and lipid extracts. Average values for $q_{0.4}$ during the 4 hr. incubation were 0.33, 0.30, 0.27 and 0.26 respectively.

Fig. 4. The metabolism of phosphatidylethanolamine in sheep-thyroid tissue. Slices were incubated with $27 \mu\text{C}$ of $\text{NaH}_2^{32}\text{PO}_4$ and removed at intervals for analysis of the compounds in aqueous and lipid extracts.

DISCUSSION

Although the synthesis of thyroid hormone has been extensively studied, few data are available on the metabolism and the composition of thyroid tissue. In the present studies, attention has been focused upon the phospholipids of the sheep-thyroid gland. Five separate phosphoglycerides have been demonstrated in lipid extracts. Whether one of these (i.e. phosphatidic acid) occurs naturally in limited quantities or constitutes a fractionation artifact has not been definitely answered. In addition, it has been shown that selected phosphoglyceride intermediates account for a large proportion of the organic, water-soluble phosphorus of sheep-thyroid tissue. Extrapolation of all of the findings to other species must await further investigation.

During incubation *in vitro*, sheep-thyroid slices can incorporate ^{32}P into all of the phosphorus-containing compounds. For the phospholipids, such complete labelling is in marked contrast with the limited incorporations which have been obtained with certain other tissue preparations (Dawson, 1954). All of the thyroid phosphoglycerides exhibited different ^{32}P specific radioactivities throughout incubation, and serial fractionations disclosed that these specific radioactivities changed at different rates. This heterogeneous labelling of the phosphoglycerides of sheep-thyroid tissue is similar to Dawson's (1955b) findings for rat liver *in vivo* and supports the growing impression that valid estimates of the turnover of lipid phosphorus cannot be derived on the basis of unfractionated phospholipid extracts. It was found, however, that the phosphoinositide was consistently more heavily labelled with ^{32}P than any other phosphoglyceride, which contrasts markedly with the findings for liver.

The incorporation of ^{32}P has facilitated an examination of the possible pathways of phosphorus metabolism of lecithin and phosphatidylethanolamine. Serial fractionation of incubated sheep-thyroid slices revealed that the phosphorus in phosphatidylethanolamine and phosphatidylcholine was never as heavily labelled as the corresponding water-soluble phosphorylated bases, PE and PC. At the same time, the specific

radioactivities of the phosphoglycerides were consistently greater than those of the water-soluble phosphodiester, GPE and GPC. The relatively unlimited availability of radioactive label in the suspending medium precludes rigorous application of the precursor-product relationships of Zilver-smit, Entenmann & Fishler (1943) to systems *in vitro*. Nevertheless, the specific radioactivities of the phosphorylated bases could not have been so great if PE and PC had become labelled as a consequence of breakdown of phosphoglyceride. Similarly, the sparsely labelled phosphodiester, GPE and GPC, could not have served as the principal source of phosphoglyceride phosphorus. Therefore, if PE, PC, GPE and GPC constitute water-soluble intermediates in the metabolism of phosphatidylethanolamine and phosphatidylcholine, a precursor role must be ascribed to the phosphorylated bases, whereas the phosphodiester must be derived chiefly from phosphoglyceride breakdown. Dawson (1955*a*, 1956*a*) has reached similar conclusions about phospholipid pathways in rat liver *in vivo*. The recent demonstration of phospholipase B in the sheep-thyroid gland (Dawson, 1956*b*) strengthens the above evidence for the phosphodiester route of phosphoglyceride catabolism.

The evidence for the precursor function of the phosphorylated bases is reinforced by the experiments with DL-[1-¹⁴C]glycerol. In these studies ¹⁴C could be demonstrated in all of the thyroid phosphoglycerides. That some of the ¹⁴C may have represented labelled choline, ethanolamine or serine rather than unaltered glycerol cannot be excluded; but, on the basis of preliminary experiments, a significant occurrence of this seems unlikely. The observed differences in the ¹⁴C/³²P specific radioactivity ratios of the individual phosphoglycerides would argue against a common phosphorylated glycerol precursor (such as glycerophosphoric or phosphatidic acid) for the phosphorus in thyroid phosphoglycerides. Rather, the findings would support the role of the phosphorylated bases as the principal donors of phosphoglyceride phosphorus, presumably by activation through cytidine triphosphate (Kennedy & Weiss, 1956).

Despite all of the foregoing evidence for an active phospholipid metabolism within the sheep-thyroid gland, the physiological ends which this metabolism serves remain to be elucidated. It is, however, becoming increasingly apparent that the phospholipids are concerned with the structural elements of multi-enzyme systems in tissues, such as oxidative phosphorylation (Cooper & Lehninger, 1956), and consequently an active phospholipid metabolism may be needed to maintain this organization. Moreover, it is possible that the

phospholipids may play an important role in the active transport of ions across membranes (Solomon, Lionetti & Curran, 1956). Both of these phenomena are now known to be highly important in maintaining the thyroid gland in full functional activity (Schachner, Franklin & Chaikoff, 1943; Freinkel & Ingbar, 1955*a*, *b*).

SUMMARY

1. Sheep-thyroid gland has been examined by chromatographic techniques for the presence of phosphoglycerides and water-soluble intermediates of their metabolism. In aqueous extracts, the presence of glycerophosphoric acid, phosphorylcholine, phosphorylethanolamine, glycerylphosphorylcholine, glycerylphosphorylethanolamine and inositol has been demonstrated. In lipid extracts, the presence of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid and a phosphoinositide has been shown.

2. During incubation of sheep-thyroid slices, [³²P]orthophosphate was incorporated into all of the phosphorus-containing compounds. Specific radioactivities of the individual phospholipids varied greatly; the phosphoinositide became more heavily labelled than any other phosphoglyceride. Concurrent fractionation of aqueous and lipid extracts disclosed that the ³²P specific radioactivities of the phosphoglycerides, phosphatidylcholine and phosphatidylethanolamine were greater than those of the water-soluble phosphodiester, glycerylphosphorylcholine and glycerylphosphorylethanolamine, and less than the specific radioactivities of the water-soluble phosphorylated bases, phosphorylcholine and phosphorylethanolamine.

3. The incorporation of DL-[1-¹⁴C]glycerol *in vitro* into all of the phosphoglycerides of sheep-thyroid tissue has been demonstrated, but the specific radioactivities were not as heterogeneous as the corresponding ³²P values.

4. From these isotopic results, certain conclusions have been reached about the probable pathways of phosphoglyceride metabolism in sheep-thyroid tissue.

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Synthesis of α -Methyl- and β -Methyl-DL-Cystine

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In view of the utilization of L-cystine as a precursor of the β -lactam ring of penicillin (Arnstein & Grant, 1954) it was of interest to study penicillin biosynthesis in the presence of compounds related to cystine. For this purpose, α -methyl- and β -methyl-cystine were required and the present paper deals with their synthesis. For some experiments, it was necessary to use isotopic labelling in order to detect any possible conversion of the substituted cystines into $C_{(6)}$ - or $C_{(6)}$ -substituted penicillins, which might be biologically inactive, and these two amino acids were therefore also labelled with ^{14}C , as described below. As will be shown in the following paper, no penicillin-like compounds were in fact formed, but α -methyl-cystine was found to inhibit penicillin production by washed mycelium of *Penicillium chrysogenum*.

β -Methylcystine has recently been found to occur in the antibiotic subtilin as the *S*-(L-2-amino-2-carboxyethyl) derivative (I) (Alderton, 1953), but the configuration of the asymmetric β -carbon

atom was not elucidated. The stereochemistry of β -methylcystine is thus of some interest in connexion with the structure of this antibiotic.

Synthesis of β -methyl-DL-cystine and its disulphide from allothreonine and threonine

Of the four possible stereoisomers of β -methylcystine (V, $R=CH_3$) which correspond in configuration to the L- and D-enantiomorphs of threonine and *allothreonine*, both pairs of DL-isomers (named isomers A and B of thiiothreonine) have been synthesized by Carter, Stevens & Ney (1941), but their stereochemical relationship was not investigated. It was hoped to clarify this problem in the present work by applying the recent synthesis of cystine (VI, $R=H$) from serine (II, $R=H$) via the thiobenzoyl ester (III, $R=H$, $R'=C_2H_5$) and thiazoline ester (IV, $R=H$, $R'=C_2H_5$) derivatives (Crawhall & Elliott, 1951) to the preparation of β -methylcystine (VI, $R=CH_3$) from DL-threonine and DL-*allothreonine* (II, $R=CH_3$).

In view of the inversion at the β -carbon atom during ring closure to the thiazoline (IV, $R=CH_3$, $R'=C_2H_5$), it was anticipated that this synthesis would yield the *erythro* and *threo* forms of DL- β -methylcystine (V, $R=CH_3$) respectively. It was