Studies on the Carrier Function of Phosphatidic Acid in Sodium Transport

I. The turnover of phosphatidic acid and phosphoinositide in the avian salt gland on stimulation of secretion

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ABSTRACT Incubation of slices of the salt gland of the albatross with acetylcholine, which is the physiological secretogogue for this tissue, led to a 13-fold increase in the rate of incorporation of P^{32} into phosphatidic acid and a 3-fold increase in the incorporation of P^{32} and inositol-2-H^3 into phosphoinositide. The incorporation of P^{32} into phosphatidyl choline and phosphatidyl ethanolamine was increased relatively slightly or not at all. Respiration was doubled. The "phospholipid effect" occurred in the microsome fraction, which is known to contain fragments of the endoplasmic reticulum. The enzymes, diglyceride kinase and phosphatidic acid phosphatase, which catalyze the stimulated turnover of phosphatidic acid in brain cortex, were also found in highest concentration in the microsome fraction. The phosphatides which respond to acetylcholine are bound to protein in the membrane. On the basis of these findings it appears that phosphatidic acid and possibly phosphoinositide participate in sodium transport. A scheme, termed the phosphatidic acid cycle, is presented as a working hypothesis, in which the turnover of phosphatidic acid in the membrane, catalyzed by diglyceride kinase and phosphatidic acid phosphatase, functions as a sodium pump.

INTRODUCTION

Electrophysiological studies on the invertebrate axon (1, 2), the muscle fibre membrane (3), the frog skin (4, 5), and other preparations have shown that sodium ions are actively transported against an electrochemical gradient. The

This work was aided by grants from the United Cerebral Palsy Research and Educational Foundation, the National Institute of Neurological Diseases and Blindness, and the Wisconsin Alumni Research Foundation.

Received for publication, March 18, 1960.

active transport of sodium against an electrochemical gradient in these and other tissues is dependent on metabolic energy. The biochemical mechanism of this metabolically linked "sodium pump" has not been elucidated, but it has been suggested by a variety of workers that sodium may be transported by a sodium carrier situated in the cell membrane. Phosphatides have been repeatedly suggested as cation carriers, because of their ability to form lipoid-soluble complexes with cations. As early as 1938 Christensen and his collaborators (6–8) found that heterogeneous phosphatide mixtures "solubilized" sodium chloride and potassium chloride in petroleum ether solutions. More recently, Solomon *et al.* (9) have carried our similar experiments with purified phosphatides, and they found that these phosphatides preferred potassium to sodium by a factor of about 10 to 1. Kirschner (10), on the other hand, found that a phosphatide, which appeared to be phosphatidylserine, was extracted from erythrocytes primarily as the sodium salt. Although these studies provide a physicochemical basis for the carrier function for certain phosphatides they provide no direct biochemical evidence that the phosphatides are in fact cation carriers.

Over the past 6 years we have been engaged in studies on the role of phosphatides in the secretion of certain organic molecules from endocrine and exocrine glands (11–16). These studies have shown that when the secretion of proteins, polypeptides, or catechol amines is stimulated *in vitro* there is a marked increase in the turnover of phosphoinositide and phosphatidic acid. With the exception of phosphatidyl ethanolamine in the pancreas the turnover of the other phosphatides is stimulated relatively little or not at all. A similar phospholipid effect has been observed in synaptic tissue incubated in contact with acetylcholine (12, 17–19).

On the basis of the studies in endocrine and exocrine glands the question arose as to whether the turnover of phosphatides might not also play a role in the transport of inorganic cations. The salt glands of marine birds appeared to us to be well suited for attacking the possible role of phosphatides in ion transport. These bilaterally paired glands, situated near the orbit of the eye, send their ducts into the nasal cavity. Recent physiological studies by Schmidt-Nielsen and his collaborators (20–22) have shown that these glands respond to excessive oral or parenteral intake of sodium chloride by secreting a hypertonic solution containing up to 0.85 M NaCl and 0.04 M KCl. No protein was found in the secretion, and the concentrations of the other ions were negligible. Of particular convenience for the present study was the finding by Fänge *et al.* (22) that the secretory activity of the salt gland could be stimulated by injection of acetylcholine or acetyl β-methylcholine. This is due to the fact that the salt gland does not respond directly to changes in osmolarity of the blood but is regulated by a secretory nerve (probably a branch of the facial nerve),

which is cholinergic. Schmidt-Nielsen and his associates assume that the secretory nerve is regulated by an osmoreceptor in the central nervous system.

In the present investigation slices of the salt gland of the Laysan or black-footed albatross have been incubated in physiological saline (23) containing glucose. Acetylcholine (with eserine) has been added as the secretogogue under various conditions and the turnover of phosphatides has been followed. It has been found that the addition of eserine (10^{-4} M) either alone or together with very low concentrations of acetylcholine (10^{-8} M and 10^{-7} M) to the incubation medium results in an increase in the turnover of both phosphoinositide and phosphatidic acid. Higher concentrations of acetylcholine give very little further increase in the turnover of phosphoinositide but greatly increase the turnover of phosphatidic acid.

The experimental evidence so far available suggests that phosphatidic acid acts as a sodium ion carrier in the membrane. A scheme for the active transport of sodium ions across lipoid membranes is presented. The scheme involves a cycle in which phosphatidate is formed at the inner surface of the apical membrane from diglyceride and ATP and is hydrolyzed to diglyceride and orthophosphate at the external surface of the membrane. The energy relationships which apply to this type of transport mechanism are discussed.

Part of this work was presented in a preliminary communication (24) and has been discussed elsewhere in a review (25).

EXPERIMENTAL

Laysan and black-footed albatrosses from the Midway Islands were kindly provided by the United States Navy. The birds were kept in an open-air wire pen during the spring and summer months of 1959. They were fed a diet of squid freshly thawed. After thawing (in the packet) overnight at 4°, the squid was soaked in 3 per cent NaCl until completely thawed. No additional salt was required in the diet.

The birds were sacrificed by decapitation, the skin over the forehead was quickly stripped back, and the salt glands were removed with a scalpel from the recesses in the skull. Frings *et al.* (26) have recently described the anatomical location of the salt gland in the albatross. The tissue was immediately placed in either ice cold isotonic sucrose or physiological saline.

Tissue Slice Experiments

Slices were prepared with a Stadie-Riggs microtome (27); they were placed in a chilled, covered crystallizing dish. The thick connective tissue covering the gland was discarded. After all the slices were prepared they were weighed and placed in appropriate vessels. Except when respiration was measured, the slices were incubated in bicarbonate saline (23) in stoppered Erlenmeyer flasks. The volume ratios of

tissue:medium:flask were: 0 to 50 mg.:1 ml.:10 ml.; 50 to 100 mg.:2 ml.:25 ml.; 150 mg.:3 ml.:50 ml., 300 mg.:5 ml.:50 ml. The incubation medium was gassed with 5 per cent CO_2 in O_2 before use; the incubation flasks were gassed and stoppered after they had received the medium, and again after introduction of the tissue. The flasks were shaken in a metabolic incubator at 37.5°. In experiments in which the respiration was measured, 100 mg. portions of tissue slices were incubated in Krebs medium III (28) in Warburg vessels, gassed with O_2, and with 2 N KOH in the center wells.

All media contained glucose (1 mg. per ml.). Drugs and radioactive materials were added to the vessels as indicated.

After incubation, the tissues were removed from the vessels with forceps and blotted thoroughly on Schleicher and Schuell filter paper, No. 576, to remove adherent medium. Except in cell fractionation experiments, the tissues were then placed in chilled conical centrifuge tubes and frozen at −20°. When it was desired to work up the tissues, they were removed individually from the freezer and homogenized with small conical glass homogenizers in ice cold trichloracetic acid. It was found that beginning the homogenization with the tissue frozen greatly facilitated the breaking up of this very tough tissue.

In those experiments in which the distribution of radioactivity in the various cell fractions after incubation was studied, the slices were homogenized immediately after incubation at 0° in 10 ml. of 8.5 per cent sucrose. Differential centrifugation was carried out as described below. Each particulate fraction was then treated with ice cold 5 per cent trichloracetic acid. Fifty per cent trichloracetic acid was added to the soluble fraction to give a final concentration of 5 per cent.

The trichloracetic acid precipitates were worked up as described previously (29). Briefly, this consisted of washing the precipitates twice with cold 5 per cent trichloracetic acid and then extracting the precipitate with 1:1 chloroform-ethanol after standing in the cold overnight, the mixture was stirred vigorously with 2½ to 5 volumes of 0.1 N HCl and centrifuged. After centrifugation, a disc of protein separated the heavy chloroform layer, which contained all the phosphatides, and the top, lighter layer which consisted of the 0.1 N HCl plus the ethanol of the original extraction mixture. The ethanolic aqueous layer was discarded.

Aliquots of the chloroform extracts were chromatographed on silicic acid–impregnated paper by the method of Marinetti, Erbland, and Kochen (30). The phosphatides were detected by autoradiography and counted as described previously (29). Inositol-2-H^3 incorporation was measured as described previously (29). Total phospholipid phosphorus was determined by combustion of an aliquot of the chloroform extract followed by phosphorous determination by either the method of Fiske and SubbaRow (31) or that of Berenblum and Chain (32).

For determination of the radioactivity in phosphoprotein and phosphatidopeptides, the protein discs were washed with ether until this process removed negligible counts into the ether. They were then extracted three times with 10 per cent NaCl at 100° to remove nucleic acids (33). The residues were digested overnight in 1 N KOH, then neutralized. After centrifugation, the aqueous phase, together with the washings, was

treated by the method of Berenblum and Chain (32). By this method, orthophosphate is removed into isobutanol as the phosphomolybdate complex and organic phosphate remains in the aqueous phase. Aliquots of the isobutanol phase were counted to give a measure of the radioactivity originally in the phosphoprotein fraction, since this fraction yields orthophosphate on hydrolysis under these conditions (34). Aliquots of the aqueous phase were counted to give a measure of the radioactivity originally in the phosphatidopeptide fraction, since this yields mainly inositol monophosphate on hydrolysis under these conditions (35).

In the cell fractionation experiments the protein discs from each cell fraction were washed once with ethanol and twice with ether to remove residual lipid. The protein residue was then suspended in ether and transferred to tared aluminum pans. The protein was dried gently under an infrared lamp and the samples were weighed. This gave a measure of the total protein in the fraction. In some experiments protein nitrogen was also estimated. Nucleic acids were not removed since their level in this tissue is so low that their inclusion in the protein fraction does not give any appreciable error on a weight basis.

The trichloracetic acid supernatant fluid and the first washing obtained from the original precipitation of the homogenized tissue were taken for the estimation of radioactivity in the acid-soluble phosphate esters and the 7-minute-hydrolyzable phosphate esters. Orthophosphate was first removed by extracting it as the phosphomolybdate complex into isobutanol (32). Aliquots of the remaining aqueous phase were then counted to give a measure of the radioactivity in the total acid-soluble organic phosphate. An aliquot of the aqueous phase was adjusted to 1 N with respect to H_2SO_4 and was placed in a boiling water bath for 7 minutes. It was then re-extracted with isobutanol to obtain the orthophosphate liberated. Aliquots of the isobutanol phase were counted to give a measure of the 7-minute acid-hydrolyzable phosphate esters.

Differential Centrifugation

To prepare the various cell fractions, the incubated salt gland slices (about 300 mg.) or unincubated pieces of salt gland tissue (about 1 gm.) were homogenized at 0° in an all glass conical or cylindrical homogenizer in 10 ml. of 8.5 per cent sucrose until the suspension appeared fairly homogeneous. As a rule, homogenization was not extended beyond 5 minutes; because of its extremely tough nature, the homogenization of the tissue was never complete under these conditions. A 1 ml. aliquot of the homogenate was removed at this stage so that the various enzyme activities in the fractions could be compared with the activity in the whole homogenate. The remaining homogenate was centrifuged at 105,000 × *g* for 15 minutes. The supernatant fluid was kept on ice and the residue was resuspended in 10 ml. of sucrose by homogenization. It was previously found that this initial washing of all the particulate material permitted a sharper separation of the various fractions (36). The resuspended material was centrifuged at 500 × *g* for 5 minutes. The residue from this centrifugation was called the nuclear fraction. Microscopic examination of this fraction revealed that it contained nuclei, connective tissue, an appreciable number of

unbroken cells, red cells, and some mitochondria. The supernatant fluid from this centrifugation was then centrifuged for 10 minutes at 5,000 × *g*. The residue from this centrifugation was called the mitochondrial fraction. It contained many more mitochondria than the nuclear fraction but it also contained, in smaller amounts, the other components seen in the nuclear fraction. The supernatant fluid from this centrifugation was combined with the supernatant fluid from the first centrifugation and the pooled material was centrifuged for 60 minutes at 105,000 × *g*. The residue from this centrifugation was called the microsome fraction; the supernatant fluid was called the soluble fraction.

At this stage, the fractions derived from slices which had been incubated with P^{32} were processed for the estimation of radioactivity of the phosphatides as described above.

Distribution of Diglyceride Kinase and Phosphatidic Acid Phosphatase

To prepare suspensions for assay of the enzymes, diglyceride kinase and phosphatidic acid phosphatase, the particulate fractions derived from unincubated tissue were suspended in a known volume of sucrose (usually about 1.0 ml.) to which was added an equal volume of sodium deoxycholate (5 mg. per ml.) in 0.04 M glycylglycine, pH 7.0. The soluble fraction derived from the unincubated tissue was treated with one-tenth its volume of 25 mg. per ml. sodium deoxycholate in 0.2 M glycylglycine. All the fractions were stored at −20°. When the fractions were thawed for enzyme assays, the temperature of the fractions was kept below 5° at all times.

Diglyceride kinase activity was measured by the radioactive technique described previously (37, 38). The only modification in the present experiments was that in some experiments the substrate used was a commercial preparation of diglyceride which contained diolein as its major component.

Phosphatidic acid phosphatase was measured as described previously (37), with the modification that phosphatidic acid synthesized chemically from a commercial diolein preparation was used as the substrate.

MATERIALS

Radioactive orthophosphate was obtained from Oak Ridge, Tennessee. It was dried *in vacuo* to remove the HCl and then redissolved in water. It was tested to ensure that it was free of pyrophosphate.

Inositol-2-H^3 was synthesized from 1 curie of tritium as described previously (29). The final product had a specific activity of 18 millicuries per millimole.

The diolein preparation was obtained from Distillation Products Industries; its commercial designation was D47-DO (oleic acid diglycerides).

Phosphatidic acid was synthesized from this diolein preparation by a modification of the method of Wagner-Jauregg and Arnold (39). The product was found to contain some lysophosphatidic acid, which was removed by silicic acid column chromatography (40, 41). The purified phosphatidic acid was used as the sodium salt.

RESULTS

Responses of Salt Gland Slices to Acetylcholine Other glands which respond to cholinergic agents *in vivo* such as the pancreas, salivary glands, adrenal medulla, and pigeon esophagus, have been shown to respond to acetylcholine and other agents *in vitro* by secretion of the same substances which they secrete when stimulated *in vivo*. This suggested that it would be reasonable to expect that incubation of salt gland slices with acetylcholine would give rise to increased secretion of sodium chloride, since injection of cholinergic agents such as acetylcholine or acetyl β-methylcholine gives rise to the secretion of sodium chloride *in vivo* in this gland (22). It was not possible to measure directly the secretion of sodium chloride under the *in vitro* conditions used since

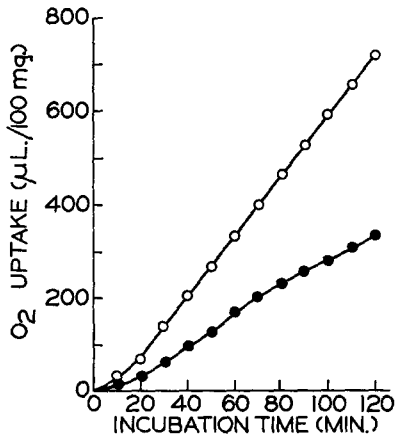

FIGURE 1. The O_2 consumption of slices of albatross salt gland. ●, control slices; ○, slices incubated in the presence of 10^{-5} M acetylcholine and 10^{-4} M eserine. O_2 uptake is expressed as microliters per 100 mg. fresh weight of tissue.

any sodium chloride secreted would mix with the same medium from which it was absorbed. Nor was it possible to use the turnover of Na^{22} as a measure of secretion, since it was found that the sodium of the tissue equilibrated with the radioactive sodium of the medium within a few seconds in all slices; under these conditions, any more rapid turnover of Na^{22} in the tissue slices in response to acetylcholine could not have been detected.

An increased respiratory rate is a common consequence of the stimulation of secretory activity both *in vivo* and *in vitro* in a variety of glands such as the pancreas (42–45), the salivary glands (46), and the gastric mucosa (47), and on stimulation of active sodium transport in the frog skin (48, 49) and the toad bladder (50). When acetylcholine (10^{-5} M) plus eserine (10^{-4} M) was added to the incubation medium, the respiration of salt gland slices was doubled (Fig. 1). This increase in oxygen uptake provided indirect evidence that acetylcholine did stimulate the secretory activity of the salt gland slices. We know of no instance in which respiratory activity has been stimulated in a

TABLE I

EFFECT OF ACETYLCHOLINE ON THE INCORPORATION OF P^{32} INTO PHOSPHATIDES AND OTHER GROUPS OF PHOSPHATE COMPOUNDS IN SLICES OF ALBATROSS SALT GLAND

Phosphate compound	Experiment No.	Radioactivity*	
		Control	+ACh‡
Phosphatidic acid	1	11,700	137,000
	2	10,900	145,000
	3	12,900	126,000
	4	6,860	68,200
Phosphoinositide	1	31,800	87,000
	2	11,700	55,000
	3	16,100	48,000
	4	15,500	28,900
Phosphatidyl choline	1	75,000	99,000
	2	24,000	52,000
	3	36,600	32,600
	4	19,600	21,100
Phosphatidyl ethanolamine	1	15,000	25,000
	2	4,930	11,500
	3	11,400	10,200
	4	6,380	7,000
Phosphatidylserine	4	3,630	6,460
Phosphatidopeptide fraction	1	199,000	288,000
	2	31,100	36,200
Phosphoprotein fraction	1	149,000	113,000
	2	120,000	97,000
Total acid-soluble phosphate esters	1	4,820,000	4,750,000
	2	1,100,000	1,810,000
	3	4,470,000	4,460,000
7-minute hydrolyzable acid-soluble phosphate esters	1	351,000	252,000
	2	189,000	220,000
	3	518,000	330,000
	4	464,000	474,000

* Radioactivity is expressed as total counts per minute in each group per 100 mg. fresh weight of tissue; it is corrected to a specific activity of 10^6 C.P.M. per μg. P for the inorganic phosphate of the medium in which the slices were incubated. The duration of the incubation was 2 hours.
‡ 10^{-5} M acetylcholine plus 10^{-4} M eserine was present in these vessels in Experiments 1, 2, and 3; 10^{-4} M acetylcholine plus 10^{-4} M eserine was used in Experiment 4.

gland by its secretogogue without concomitant stimulation of secretory activity.

Table I shows the effects of 10^{-5} M acetylcholine (plus 10^{-4} M eserine) on the incorporation of orthophosphate-P^{32} into various phosphate compounds in slices of albatross salt gland, as measured after 2 hours' incubation. Stimulation with this concentration of acetylcholine in 6 experiments led to an average 13-fold increase in the amount of P^{32} incorporated into phosphatidic acid; the incorporation of P^{32} into phosphoinositide was increased on average 3-fold. In some experiments there was some increase in the incorporation of P^{32} into phosphatidyl choline and phosphatidyl ethanolamine but this was not a consistent finding (see below). In most cases the incorporation of P^{32} into phosphatidylserine was too small to measure. In one experiment in which it was measured, (Experiment 4, Table I), there was some increase in incorporation. There was a relatively small increase in the incorporation of P^{32} into the phosphatidopeptide fraction in the 2 experiments in which this was measured. There was no increase in the incorporation of P^{32} into the phosphoprotein fraction in these experiments. There was no increase in the amount of radioactivity found in the total acid-soluble phosphate esters, nor in the 7-minute acid–hydrolyzable phosphate esters. The acid-labile fraction consists mainly of the acid-labile phosphate groups of adenosinetriphosphate (ATP). This fraction has been found to equilibrate with the orthophosphate of the tissue within the first 15 minutes of incubation. After equilibration it is not possible to detect increased turnover since the organic phosphate is exchanging with orthophosphate of the same specific activity. It is obvious therefore that under the conditions of the experiments shown in Table I, any increased turnover of ATP in response to acetylcholine would not be detected. The relationships among the specific activities of orthophosphate, ATP, and phosphatides in the tissue during the course of incubation will be reported and discussed fully elsewhere (51).

The experiments reported in this paper were carried out with salt glands from a total of nine birds. In tissue from each of these 9 animals, in the presence of 10^{-5} M or higher concentrations of acetylcholine, the incorporation of P^{32} into phosphatidic acid was always greatly increased; the incorporation of P^{32} into phosphoinositide was increased to approximately the same extent as in the experiments in Table I in tissue from eight animals, but in one gland there was no increased incorporation of P^{32} into this phosphatide. The incorporation of P^{32} into phosphatidyl choline was increased 20 per cent or more in the presence of 10^{-5} M or higher concentrations of acetylcholine in 4 out of the 9 experiments, and the incorporation of P^{32} into phosphatidyl ethanolamine was likewise increased more than 20 per cent in 4 out of the 9 experiments.

The amounts of P^{32} found in phosphatidic acid and phosphoinositide after incubation of salt gland slices for 2 hours with 10^{-4} M eserine and with 10^{-4} M eserine plus different concentrations of acetylcholine are shown in Fig. 2. Eserine alone produced some stimulation of P^{32} incorporation into both phosphatidic acid and phosphoinositide. With eserine alone, and with 10^{-8} M and 10^{-7} M acetylcholine, phosphoinositide showed a proportionately greater stimulation than was shown with phosphatidic acid. With concentrations of acetylcholine above 10^{-7} M, phosphoinositide did not show much further in-

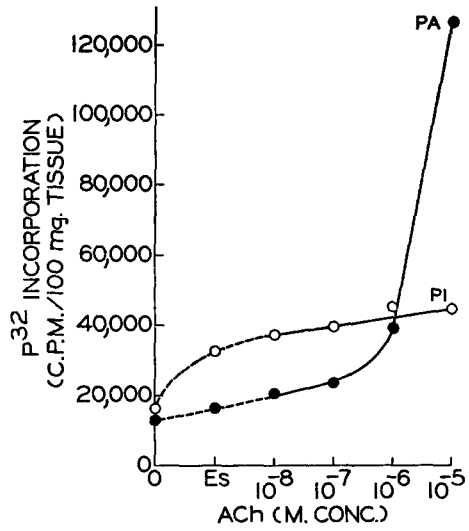

FIGURE 2. The incorporation of P^{32} into phosphatidic acid (*PA*, ●) and phosphoinositide (*PI*, ○) in slices of albatross salt gland in the presence of 10^{-4} M eserine and increasing concentrations of acetylcholine. Radioactivity is expressed as total counts per minute in each phosphatide in 100 mg. fresh weight of tissue, corrected as in Table I. The slices were incubated for 2 hours.

crease in the amount of stimulation, but the incorporation of P^{32} into phosphatidic acid rose sharply with concentrations of acetylcholine between 10^{-7} and 10^{-5} M. The average ratios of the stimulated to the control values of the radioactivity found in phosphatidic acid, phosphoinositide, phosphatidyl choline, and phosphatidyl ethanolamine in several experiments in which the response to different concentrations of acetylcholine were studied are shown in Table II. These results show that the concentrations of acetylcholine which give a near maximal response are about 10^{-8} M for phosphoinositide and 10^{-5} M for phosphatidic acid. The incorporation of P^{32} into phosphatidyl choline and phosphatidyl ethanolamine was not stimulated at all at concentrations of acetylcholine below 10^{-5} M.

Pilocarpine in concentrations ranging from 10^{-7} M to 10^{-3} M gave rise to an

TABLE II

RATIO OF THE STIMULATED TO THE CONTROL RADIOACTIVITY OF INDIVIDUAL PHOSPHATIDES AFTER INCUBATION FOR 2 HOURS WITH INCREASING CONCENTRATIONS OF ACETYLCHOLINE

Values are the averages of individual ratios from the indicated number of separate experiments.

Additions		Ratio of stimulated:control radioactivity*				
		Phosphatidic acid	Phosphoinositide	Phosphatidyl-choline	Phosphatidyl ethanolamine	No. of observations
	M *concentration*					
Eserine	10^{-4}	2.0	2.0	1.0	1.1	2
Acetylcholine	10^{-8}‡	1.6	2.4	1.0	0.8	1
Acetylcholine	10^{-7}	3.2	2.6	1.0	1.1	3
Acetylcholine	10^{-6}	6.7	2.6	0.9	0.9	4
Acetylcholine	10^{-5}	13.3	3.2	1.3	1.5	6
Acetylcholine	10^{-4}	14.5	2.9	1.8	1.5	2
Acetylcholine	10^{-3}	15.2	2.4	1.2	1.3	1

* Control values expressed as 1.0.

‡ Eserine (10^{-4} M) was always added with acetylcholine.

increased incorporation of P^{32} into phosphoinositide and phosphatidic acid (Table III). The maximum effect was given with 10^{-5} M pilocarpine; no further increases were observed with concentrations of pilocarpine higher than this. Although pilocarpine produced an increased incorporation of P^{32} into phosphoinositide which was as great as that produced by 10^{-5} M acetylcholine, pilocarpine did not stimulate the incorporation of P^{32} into phosphatidic acid nearly as much as did 10^{-5} M acetylcholine (Table III). These results sug-

TABLE III

EFFECT OF PILOCARPINE ON THE INCORPORATION OF P^{32} INTO PHOSPHATIDIC ACID AND PHOSPHOINOSITIDE

Additions		Radioactivity*	
		Phospatidic acid	Phosphoinositide
	M *concentration*	C.P.M.	C.P.M.
None	—	41,200	47,800
Pilocarpine	10^{-7}	45,000	54,000
Pilocarpine	10^{-6}	73,000	83,400
Pilocarpine	10^{-5}	98,000	108,000
Pilocarpine	10^{-4}	90,000	101,000
Pilocarpine	10^{-3}	113,000	116,000
Acetylcholine 10^{-5} / Eserine 10^{-4}		258,000	121,000

* Expressed as in Table I. The incorporation of P^{32} into phosphatidyl choline and phosphatidyl ethanolamine was not stimulated by either pilocarpine or acetylcholine in this experiment.

gested that the salt gland was less able to respond to pilocarpine than it was to acetylcholine. The results of Fänge *et al.* (22) indicate that this is also the case *in vivo* with respect to salt secretion in the herring gull. These workers observed no stimulation of secretion from the nasal glands when unanesthetized gulls were injected with as much as 3 mg. of pilocarpine intravenously,

TABLE IV

EFFECT OF ATROPINE AND TUBOCURARINE ON P^{32} INCORPORATION INTO PHOSPHATIDIC ACID AND PHOSPHOINOSITIDE IN THE PRESENCE AND ABSENCE OF ACETYLCHOLINE

Experiment No.	Additions		Radioactivity*	
			Phosphatidic acid	Phosphoinositide
		M *concentration*	C.P.M.	C.P.M.
1	None	—	12,900	16,100
	Atropine	10^{-6}	10,600	12,900
	Acetylcholine‡	10^{-5}	126,000	48,200
	Acetylcholine + Atropine	10^{-5} + 10^{-6}	11,800	26,400
2	None	—	20,200	50,000
	Atropine	10^{-6}	28,200	42,300
	Acetylcholine	10^{-5}	306,000	122,000
	Acetylcholine + Atropine	10^{-5} + 10^{-6}	34,200	88,400
3	None	—	41,200	47,800
	Atropine	10^{-5}	29,400	36,000
	Acetylcholine	10^{-5}	258,000	121,000
	Acetylcholine + Atropine	10^{-5} + 10^{-5}	28,600	40,600
	Tubocurarine	2×10^{-4}	27,000	33,800
	Acetylcholine + Tubocurarine	10^{-5} + 2×10^{-4}	274,000	113,000

* Expressed as in Table I.

‡ 10^{-4} M eserine was always added with acetylcholine.

although mucous secretion from other glands was stimulated; secretion from the salt glands was observed in response to relatively low doses of acetylcholine.

The effects of atropine and tubocurarine on P^{32} incorporation into phosphatidic acid and phosphoinositide are shown in Table IV. In most experiments the amount of radioactivity incorporated into phosphatidic acid and phosphoinositide (but not into phosphatidyl choline and phosphatidyl ethanolamine) in the presence of either atropine or tubocurarine was somewhat less than in the control slices. The results with atropine in particular suggest that a small part of the incorporation of P^{32} into these two phosphatides in the

normal, unstimulated slices may be due to the endogenous production of very small amounts of acetylcholine.

The incorporation of P^{32} into phosphatidic acid and phosphoinositide in response to 10^{-5} M acetylcholine was completely abolished by 10^{-5} M atropine. In the presence of 10^{-6} M atropine, 10^{-5} M acetylcholine occasionally had a slight stimulatory effect on the incorporation of P^{32} into phosphatidic acid; it had a greater effect on the incorporation of P^{32} into phosphoinositide—maintaining an increment of radioactivity of about 40 per cent of the uninhibited increment. The fact that the increase in P^{32} incorporation into phosphoinositide was less inhibited than the increase in P^{32} incorporation into phosphatidic acid is consonant with the observation that phosphoinositide turnover is more responsive than is phosphatidic acid turnover to very low concentrations of acetylcholine. The competition between 10^{-5} M acetylcholine and 10^{-6} M atropine for receptor sites appears to give an amount of acetylcholine-activated receptor of the same order as that obtained with 10^{-8} M acetylcholine in the absence of atropine (see Fig. 2). The competition between 10^{-5} M acetylcholine and 10^{-5} M atropine obviously reduces the amount of acetylcholine-activated receptor to below detectable levels. Fänge *et al.* (22) found that a dose of 0.01 mg. of atropine injected intravenously reduced the secretion of salt by the salt gland of the herring gull when secretion was elicited by salt loading; 0.1 mg. of atropine practically abolished the secretion. Secretion produced by nerve stimulation was also blocked by atropine.

A concentration of tubocurarine of 2×10^{-4} M had no inhibitory effect on the increment in radioactivity of phosphatidic acid and phosphoinositide in response to 10^{-5} M acetylcholine. No other concentrations of tubocurarine and acetylcholine were studied in this series of experiments.

We suggested in a preliminary communication (24) that the increased incorporation of P^{32} into phosphoinositide on stimulation of salt gland slices with acetylcholine might be a secondary effect consequent to a more rapid turnover and higher specific activity of phosphatidic acid in the stimulated slices. The suggestion was based on the fact that phosphatidic acid appears to be a precursor for the synthesis of phosphoinositide in some systems (52). This interpretation has now been shown to be incorrect. The incorporation of inositol-2-H^3 into phosphoinositide was found to be stimulated in the presence of acetylcholine to the same extent as was the incorporation of P^{32} (Table V). It appears therefore that the phosphoinositide effect involves an increased rate of renewal of the inositol phosphate moiety in phosphoinositide. That the phosphoinositide effect is not secondary to the phosphatidic acid effect is also indicated by the fact that the phosphoinositide effect is more responsive to low concentrations of acetylcholine and less responsive to high concentrations of acetylcholine than is the phosphatidic acid effect.

The phosphoinositide which shows an increased rate of turnover of the

inositol phosphate moiety in salt gland slices in response to acetylcholine has the same R_f in two solvent systems as the phosphoinositides which were isolated previously from pancreas slices and brain cortex slices and which were shown to be monophosphoinositides (29, 18). It seems probable therefore that the responsive phosphoinositide in the salt gland is a monophosphoinositide.

TABLE V

EFFECT OF ACETYLCHOLINE ON THE INCORPORATION OF INOSITOL-2-H^3 INTO PHOSPHOINOSITIDE

Experiment No.	Radioactive precursor	Radioactivity*		Ratio of stimulated radioactivity to control radioactivity
		Control	+ACh‡	
		C.P.M.	C.P.M.	
1	Inositol-2-H^3	6,660	17,800	2.7
	Orthophosphate-P^{32}	50,000	122,000	2.4
2	Inositol-2-H^3	1,670	4,040	2.4
	Orthophosphate-P^{32}	16,100	48,200	3.0

* Total radioactivity of phosphoinositide from 100 mg. fresh weight of albatross salt gland slices after incubation for 2 hours. P^{32} radioactivity is corrected as in Table I. Inositol-2-H^3 radioactivity in the medium had a specific activity of 18 μc. per μmole. The actual values for the radioactivities of H^3-labeled phosphoinositide and P^{32}-labeled phosphoinositide cannot be directly compared with each other since the specific activities of the immediate precursors are not known.

‡ 10^{-5} M acetylcholine plus 10^{-4} M eserine.

The Cellular Site of the Increased Turnover of Phosphoinositide and Phosphatidic Acid Work from several laboratories has shown that the microsome fraction from a variety of animal tissues is composed of ribonucleoprotein particles and membranous fragments of the endoplasmic reticulum (53–55). In the pancreas, but not in brain tissue, a large percentage of the ribonucleoprotein particles remain adherent to the membrane fragments. An extensive intracellular membrane system resembling the endoplasmic reticulum is seen in the salt gland cell (Doyle (56)), but these membranes are of the smooth type; *i.e.*, they do not contain adherent ribonucleoprotein particles. The absence of adherent ribonucleoprotein particles is not surprising, since the salt gland does not secrete protein. Another interesting feature of the intracellular membranes of the salt gland is that they communicate directly with the cytomembrane in both the apical and basal regions of the cell. They may thus be regarded as cytomembrane infoldings. The advantage of such an arrangement in the salt gland cell is obvious, since it provides a large membrane area both for absorption and secretion.

Electron microscopic studies are to be carried out to determine in which

centrifugal fraction the fragments of these membranes of the salt gland will be found. However, on the basis of behavior of similar membranes in other tissues (53–55) it seems a reasonable assumption that the membranes of the salt gland are contained chiefly in the microsome fraction.

Preparation of cell fractions from salt gland tissue was not too satisfactory since the tissue is extremely tough and, even after extensive homogenization, islands of incompletely disintegrated cells could be seen under the light microscope in the nuclear and mitochondrial fractions. It is obvious therefore that these two fractions must contain a certain proportion of all cell components.

TABLE VI

THE PROTEIN AND PHOSPHOLIPID CONTENT OF VARIOUS CELL FRACTIONS FROM THE SALT GLAND*

Cell fraction	Experiment 1		Experiment 2	
	Protein	Phospholipid P	Protein	Phospholipid P
	mg.	*μg.*	*mg.*	*μg.*
Nuclear	3.87	10.1	3.83	18.0
Mitochondrial	1.33	14.4	1.45	15.2
Microsomal	3.36	56.0	1.79	25.9
Soluble	4.34	6.6	2.97	16.4

Experiment 1 gives the average values from the fractions isolated after incubation of the two 325 mg. portions of tissue used in the experiment from which Fig. 3 was prepared.
Experiment 2 gives the values from fractions isolated from a 1 gm. sample of unincubated tissue from a different animal than that used in Experiment 1.
* The values are expressed as total milligrams of protein and micrograms of phospholipid P in each fraction corrected to 100 mg. fresh weight of tissue as starting material.

Chemical analysis of the distribution of phospholipid and protein in the various fractions isolated from salt gland tissue provided evidence that the microsomal fraction from this tissue is composed mainly of membranous material. Although this fraction contained only one-quarter of the total cellular protein, it contained about three-quarters of the total cellular phospholipid (Table VI). Assuming an average molecular weight of about 800 for the phosphatides, there appears to be about one part by weight of phospholipid per two parts of protein in this fraction; the neutral lipids were not measured. It is obvious from these ratios that the microsome fraction from the salt gland, isolated under these conditions, contains a very high proportion of lipoprotein material.

Separation of the various cell fractions after incubation of sliced gland tissue with P^{32} showed that the major increments of the incorporation of P^{32} into phosphoinositide and phosphatidic acid were in the microsomal fraction (Fig. 3). The relatively small increments in each of the other fractions can

readily be accounted for by contamination of these fractions with microsomal material. This distribution of the stimulation is very similar to that previously observed in the pancreas on stimulation of enzyme secretion (59). Since, as indicated above, it is almost certain that the microsome fraction is composed of fragments of the cytomembrane infoldings, it can be concluded with reasonable certainty that the cytomembrane infoldings are the site of the phospholipid effect.

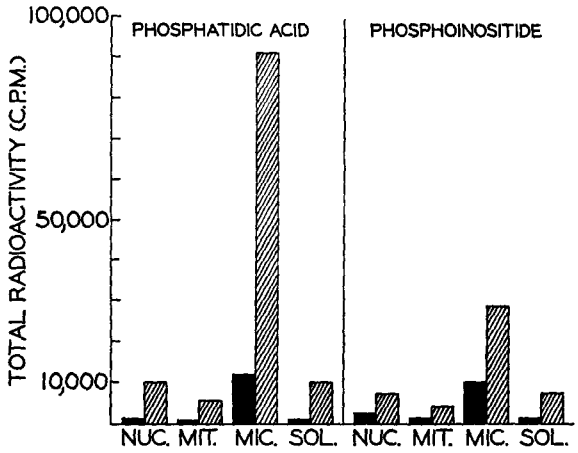

FIGURE 3. The distribution of P^{32}-labeled phosphatidic acid and P^{32}-labeled phosphoinositide in various cell fractions of the albatross salt gland after incubation of slices of the tissue with orthophosphate-P^{32}. Solid column, control slices, cross-hatched columns, slices incubated in the presence of 10^{-5} M acetylcholine plus 10^{-4} M eserine. The values are expressed as total counts per minute, corrected as in Table I, in each fraction. The initial wet weight of the tissue was 325 mg. per vessel. The duration of incubation was 2 hours. *Nuc.*, nuclear fraction, including intact cells. *Mit.*, mitochondrial fraction. *Mic.*, microsomal fraction (lipoprotein membranes). *Sol.*, soluble fraction.

Previous studies showed that acetylcholine stimulated the turnover of phosphatidic acid in cell-free preparations of microsomal membranes from brain tissue and that this stimulated turnover was brought about by the combined action of diglyceride kinase and phosphatidic acid phosphatase (36–38). Attempts to obtain a similar stimulation of phosphatidic acid turnover in cell-free preparations from the salt gland were unsuccessful. In this respect, the salt gland differs from the brain and resembles other glandular tissues, where it has not been possible to obtain an increased turnover of phosphatidic acid in response to acetylcholine in cell-free systems.

However, diglyceride kinase activity and phosphatidic acid phosphatase activity were found in deoxycholate extracts of cell fractions from the salt gland. The greatest activity of these enzymes per milligram of protein was found in the microsomal fraction. Typical results are shown in Table VII.

No significant phosphatidic acid phosphatase activity could be found in the soluble fraction; this could have been due to the rather high inorganic phosphate blank in this fraction, together with the fact that this fraction was perforce assayed in much greater dilution than the other fractions. The nuclear fraction also contained a comparatively high level of the enzymes; part of this activity would be due to contamination of the fraction with incompletely disintegrated cells, but even if allowance is made for this there is no doubt that

TABLE VII

THE DISTRIBUTION OF DIGLYCERIDE KINASE ACTIVITY AND PHOSPHATIDIC ACID PHOSPHATASE ACTIVITY

Cell fraction	Diglyceride kinase activity (phosphatidic acid synthesized*)		Phosphatidic acid phosphatase activity (orthophosphate liberated‡)	
	Total mμmoles	mμmoles per mg. protein	Total μmoles	mμmoles per mg. protein
Nuclear	20	0.515	3.8	94.5
Mitochondrial	3.1	0.226	1.0	65.5
Microsomal	26	0.765	5.8	308
Soluble	8.5	0.198	n.s.	—

* Expressed as total millimicromoles of P^{32}-labeled phosphatidic acid found per fraction after incubation of a deoxycholate extract of each fraction in the presence of diglyceride and an ATP^{32}-generating system. In this experiment, diglyceride prepared from cabbage phosphatidic acid was the substrate. The tissue was from the same animal as in Experiment 1 of Table VI but for this assay 1 gm. fresh weight of unincubated tissue was taken for fractionation.

‡ Expressed as total micromoles of orthophosphate liberated from added phosphatidic acid by deoxycholate extracts of each fraction. In this experiment phosphatidic acid synthesized from a preparation of diolein was used as the substrate. One gm. fresh weight of tissue was taken for fractionation; the values in Experiment 2 of Table VI are from the same material.

N.S., not significant.

the level of activity of diglyceride kinase in the nuclei is higher than that in the mitochondrial and soluble fractions.

It is clear from these results that the stimulation of the turnover of phosphatidic acid in the salt gland cell on stimulation with acetylcholine is likely to be brought about by the combined activity of diglyceride kinase and phosphatidic acid phosphatase in the membranes, as has been shown to be the case in brain tissue.

On the Physical State of the Phosphatides in the Cell In the red cell membrane Parpart and Ballentine (60) have shown that about 25 per cent of the cephalins and none of the lecithin is extractable with dry ether. Since phosphatides contained in lipoprotein complexes are not extracted with dry ether these results indicate that most of the cephalins and all of the lecithin are fairly firmly bound to protein.

Based on radioactivities a similar situation was found for the phosphatides

in the salt gland. The following percentages of the total radioactivity in the phosphatides of unstimulated lyophilized salt gland slices were extracted in dry ether: phosphatidic acid, 25 per cent; phosphoinositide, 28 per cent; phosphatidyl choline, 3 per cent; and phosphatidyl ethanolamine, 21 per cent (Table VIII). The pertinent finding with regard to the present study was that very little of the increment in radioactivity observed on stimulation of

TABLE VIII

ETHER EXTRACTABILITY OF PHOSPHATIDES IN UNSTIMULATED AND STIMULATED SALT GLAND SLICES

Phosphatide	Solvent*	Radioactivity‡	
		Control	+ACh§
Phosphatidic acid	Dry ether	1,850	11,700
	Ethanol-$CHCl_3$	5,650	94,500
Phosphoinositide	Dry ether	6,500	5,940
	Ethanol-$CHCl_3$	17,000	53,600
Phosphatidyl choline	Dry ether	2,460	4,540
	Ethanol-$CHCl_3$	70,000	133,000
Phosphatidyl ethanolamine	Dry ether	2,040	1,650
	Ethanol-$CHCl_3$	7,540	15,600

* Lyophilized salt gland slices which had been incubated with P^{32} were first ground with sand and anhydrous ether. After standing at 25° for 1 hour, the material was centrifuged and the supernatant ether extract was collected. The residues were washed with dry ether, and the original extracts and the washings were pooled. The residues which were left after extraction of the lyophilized tissue with ether were then extracted with ethanol-chloroform (1:1). The pooled ether extracts were taken nearly to dryness under nitrogen, and chloroform was added. After washing this extract and the ethanol-chloroform extracts with 0.1 N HCl, as described in the Experimental section, the phosphatides were separated by paper chromatography. The sum of the radioactivity in the dry ether extracts and in the ethanol-ether extracts gives the total radioactivity in each phosphatide.

‡ Total counts per minute in each phosphatide extracted from 100 mg. of fresh weight of tissue.

§ 10^{-4} M acetylcholine plus 10^{-4} M eserine.

the salt gland tissue with acetylcholine was extracted with anhydrous ether (Table VIII). The percentage of this increment which was extracted with dry ether was 10 per cent for phosphatidic acid, 0 per cent for phosphoinositide, 3 per cent for phosphatidyl choline, and 0 per cent for phosphatidyl ethanolamine. In other words, the phosphatides which respond to acetylcholine are in a bound form; they are presumably part of the lipoprotein complexes in the membrane. This is of particular significance for phosphatidic acid and phosphoinositide, which are believed to participate as sodium carriers in the membrane. The implication of this finding with regard to the mechanism of sodium transport is discussed further below.

DISCUSSION

Significance of the Phospholipid Effect in the Salt Gland The same phenomenon which occurs on stimulation of the secretion of organic molecules in various glandular tissues (11–16) and which occurs in synaptic tissue in response to acetylcholine (17–19) occurs in slices of the avian salt gland on stimulation with acetylcholine; *i.e.*, there is a marked increase in the turnover of phosphatidic acid and phosphoinositide, with small variable stimulations in the turnover of phosphatidyl choline and phosphatidyl ethanolamine. Qualitatively speaking, the same biochemical response is thus observed on stimulation of the transport of sodium ions across membranes, as has been observed for the transport of water-soluble organic substances which contain cationic groups (digestive enzymes, polypeptides, catechol amines). Whatever the final picture of the detailed molecular mechanism of transmembrane transport will be, it is very likely that the turnover of phosphatidic acid and phosphoinositide will play an integral part in this mechanism. The scheme presented below, termed the phosphatidic acid cycle, in which phosphatidic acid turnover participates in a carrier mechanism for sodium transport, is presented as a working hypothesis. It is felt that this mechanism best fits the facts on phosphatidic acid turnover so far obtained. An earlier version of this scheme for the transport of water-soluble cationic substances, generally, has already been presented (38). Less is known about the significance of the turnover of phosphoinositide (see, however, (61) and (25)).

Phosphatidic Acid As a Sodium Carrier in Transmembrane Transport The membranes which divide the contents of the secretory cells of the salt gland from the lumina must secrete sodium chloride against a high concentration gradient. Since the electrical potential difference across this membrane is not known, it cannot be stated on rigorous physicochemical grounds that sodium ions rather than chloride ions must be secreted against an electrochemical gradient, but it is very likely that this is the case. In the frog skin (4, 5), the nerve axon (1, 2, 62), and the muscle fibre (3) Na^+ has been shown to be the ion actively transported; the movement of Cl^- passively follows its electrochemical gradient. A further reason for thinking that the increased turnover of phosphoinositide and phosphatidic acid is concerned with the transport of Na^+ rather than Cl^- is the fact that there does not seem to be any stimulation of the turnover of these phosphatides in the frog stomach or the pigeon esophagus (63) when the mucosa is stimulated with histamine. There is evidence that in the secretion of HCl by the stomach, Cl^- is actively transported against an electrochemical gradient (64, 65). These results of studies with the stomach suggest therefore that an increased turnover of phosphoinositide

and phosphatidic acid is not associated with active transport of Cl^-. Furthermore, since phosphoinositide and phosphatidic acid are both acidic phosphatides, it seems reasonable to assume that they would participate in the transport of the cationic components of the secretions.

On the basis of the data presented here and elsewhere a scheme is proposed whereby phosphatidic acid functions as a sodium carrier in the extrusion of sodium ions from within the secretory cells across the apical membranes into the lumina of the ductules (Fig. 4). In this scheme the apical membrane is regarded as being impermeable to free sodium ions. At the inner surface of

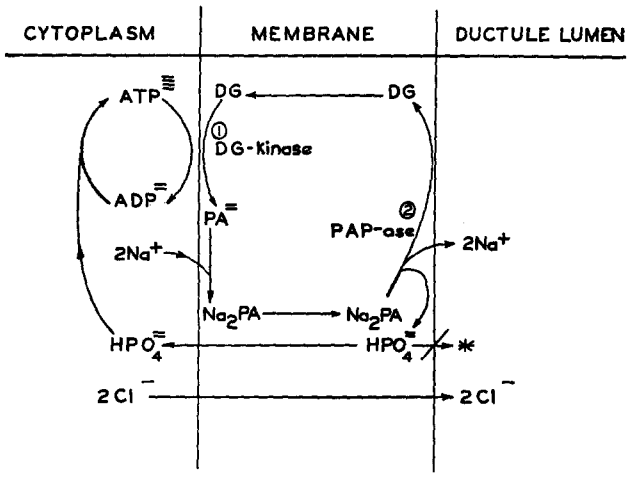

FIGURE 4. A scheme for the participation of phosphatidic acid as a carrier for the active transport of Na^+ ions across the apical membrane of the salt-secreting cell. The scheme is based on a phosphatidic acid–diglyceride cycle catalyzed by enzymes shown to be present in the membrane fraction. The activity of this cycle was found to be greatly increased on stimulation of the tissue with acetylcholine. *ATP*, adenosine triphosphate; *DG*, diglyceride; *DG-kinase*, diglyceride kinase; *PA*, phosphatidic acid; *PAP-ase*, phosphatidic acid phosphatase. * $HPO_4^=$ does not leave the external surface of the membrane.

the membrane diglyceride kinase catalyzes the reaction between ATP and diglyceride to form phosphatidate. Phosphatidate combines with sodium ions, and the sodium phosphatidate crosses the membrane. On the other side of the membrane sodium phosphatidate is hydrolyzed by phosphatidic acid phosphatase, forming diglyceride and sodium phosphate. Diglyceride returns to the inner surface of the membrane, where the cycle is repeated. Sodium enters the lumen, but a "barrier" to phosphate prevents the phosphate from accompanying the sodium. Phosphate is carried back to the cytoplasm where it is eventually incorporated into ATP by oxidative phosphorylation in the mitochondria. The membrane could be selectively permeable to chloride

ions, so that they would be dragged across it by the electrical potential gradient established by the sodium pump; alternatively, chloride could be actively transported by a chloride carrier mechanism. No information is as yet available on either the mechanism of chloride transport or on the mechanism whereby phosphate reenters the cytoplasm.

The following data presented in this paper support the phosphatidic acid cycle hypothesis:

1. Stimulation of slices of the avian salt gland by acetylcholine, which is the normal secretogogue for this tissue, is associated with a marked increase in the turnover of phosphatidic acid.

2. The stimulated turnover of phosphatidic acid occurs in the microsome fraction, which in the salt gland consists in all probability of fragments of the cytomembrane infoldings. Thus, there is good reason to believe that the stimulation of phosphatidic acid turnover is in secretory membranes.

3. The enzymes, diglyceride kinase and phosphatidic acid phosphatase, are present in high concentrations in the microsome fraction of the salt gland. These enzymes were previously shown to catalyze the acetylcholine-stimulated turnover of phosphatidic acid in brain microsomal membranes (38). Although an acetylcholine effect could not be obtained in cell-free preparations of salt gland, the fact that the phospholipid effects in brain and salt gland are biochemically similar and the fact that diglyceride kinase and phosphatidic acid phosphatase are present in high concentrations in the same fraction of the salt gland in which the phospholipid effect occurs, make it highly probable that these enzymes catalyze the acetylcholine-stimulated turnover of phosphatidic acid in the salt gland.

In subsequent papers (51, 66) further data supporting this hypothesis will be presented. These data bear, among other things, on the rate of turnover of phosphatidic acid with respect to sodium transport and on the dependence of the phospholipid effect on the presence of sodium ions.

In a preliminary account of the phosphatidic acid cycle (24, 25) it was suggested that sodium phosphatidate and diglyceride may cross the membrane by simple diffusion. Diffusion of sodium phosphatidate and diglyceride was postulated because of their known lipid solubility. It was also felt that this was the simplest explanation of a step in the over-all phosphatidic acid cycle on which we had no information. It was pointed out, however, that phosphatidate and diglyceride might be anchored to proteins which could transfer these substances across the membrane by some sort of movement such as rotation, unfolding, etc. (25). The data presented here indicate that the phosphatidate which responds to acetylcholine is bound to protein. It is therefore unlikely that phosphatidate crosses the membrane by diffusion in an unbound form. By analogy, the same would presumably apply to diglyceride.

We have at present no information on the type of bonding of phosphatidate

and diglyceride to protein and on the type of change in the shape or position of this protein which would transfer these substances from one side of the membrane to the other. However, the thickness of the membrane is of such an order (about 100 A) that rotation or change in position or shape of a protein could transfer sodium phosphatidate and diglyceride across the membrane in opposite directions without these substances being released from the protein.

Energetic Considerations An important feature of this phosphatidic acid cycle is that it can explain in terms of chemical reactions how the energy of ATP can be utilized for the active transport of sodium ions. By injecting ATP into giant axons which had been poisoned with cyanide, Caldwell and Keynes (67) obtained direct evidence that ATP can be utilized as an energy source for the sodium pump in nerve. Whittam (68) has obtained evidence of a more indirect nature that ATP is utilized in the active transport of potassium ions in human erythrocytes.

The scheme presented in Fig. 4 must satisfy the observed ratios of equivalents of sodium ions secreted to moles of oxygen consumed (Na/O_2). Based on the pK's of the primary and secondary phosphoryl dissociations of α-glycerophosphate, one phosphatidate would carry two sodium ions at pH 7.4; under these conditions, therefore, one molecule of ATP, required for the synthesis of one molecule of phosphatidic acid, would lead to the transport of two sodium ions. Assuming the generally accepted over-all ratio of 3 for the number of "high energy" phosphate bonds formed per atom of oxygen utilized (P/O ratio), twelve sodium ions could be transported per oxygen molecule by this scheme ($Na/O_2 = 12$). If the P/O ratio is greater than 3, the number of sodium ions transported per molecule of oxygen would be raised proportionately. No figures are available for the Na/O_2 ratio in the avian salt gland; some estimates of the Na/O_2 ratio in other tissues which actively transport sodium ions have, however, been obtained. Zerahn (48) found that the average ratio of sodium ions transported to the over-all oxygen consumption in the frog skin bathed with Ringer's frog saline was 5.7; when sodium transport was stimulated by the addition of sodium to the distilled water bathing the outer surface of the skin, the average ratio of the increment of sodium transport to the increment of oxygen consumed ($\Delta Na/\Delta O_2$) was 18.5. Leaf and Renshaw (49) measured sodium transport in the frog skin before and after stimulation with neurohypophyseal hormones and obtained a $\Delta Na/\Delta O_2$ ratio of 18. Leaf *et al.* (50) found an average $\Delta Na/\Delta O_2$ ratio of 16.5 in the toad bladder when sodium transport was stimulated in a manner similar to that used by Zerahn in the frog skin. The important question with regard to these results is whether the increment in oxygen uptake represents the only oxygen utilized for the increment in sodium transport in these preparations.

As has been pointed out by Zerahn (48), oxygen uptake in excess of the increment observed may be used for the extra sodium transport. Since, in some secretory processes the resting respiration can provide sufficient energy for all of the increased secretory activity observed on stimulation (69, 13, 70), it would be reasonable to expect that in other tissues in which there is a respiratory stimulation, some of the resting respiration is also channeled into use for the increased secretory activity. Examination of the raw data of Zerahn (48) and Leaf and his associates (49, 50), shows that in these preparations, from 6 per cent to 31 per cent of the resting oxygen uptake utilized along with the increased oxygen uptake for sodium transport, would give a Na/O_2 ratio of 12. It is clear therefore that this ratio is not incompatible with the available data.

Significance of the Stimulation of Phosphoinositide Turnover It was previously suggested that the stimulation of P^{32} incorporation into phosphoinositide might be secondary to an increased specific activity of phosphatidic acid, if phosphatidic acid is a precursor for phosphoinositide (24). The data presented here indicate that the phosphoinositide effect is not secondary to the phosphatidic acid effect. In the first place, the response of phosphoinositide to increasing concentrations of acetylcholine is quite different from that of phosphatidic acid. These responses would be expected to parallel each other if phosphatidic acid is a precursor of phosphoinositide. The second and more compelling argument is that the stimulation of P^{32} incorporation into phosphoinositide is accompanied by an equal stimulation of the incorporation of inositol-2-H^3, suggesting that inositol phosphate turns over as a unit in phosphoinositide on stimulation. If the increase in incorporation of P^{32} in phosphoinositide were secondary to the increase in this incorporation in phosphatidic acid no increase in inositol-2-H^3 incorporation in phosphoinositide would be expected.

On the basis of these observations it appears likely that phosphoinositide plays some integral role in the over-all sodium transport mechanism. This role is not as clear as that for phosphatidic acid, because the enzymes concerned in the stimulated turnover of phosphoinositide are not known. It is hoped that enzymatic studies now in progress will throw light on this problem.

The authors are grateful to Dr. Sidney R. Galler, United States Office of Naval Research, and Lieutenant-Commander Glenn A. Wilson, United States Navy, for providing us with albatrosses, to Dr. Hubert Frings for advice on their care, and to Mrs. Sukran Unal for technical assistance.

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