The Synthesis of Phosphatidic Acid and Protein-bound Phosphorylserine in Salt Gland Homogenates*

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Kinetic data obtained from studies of the avian salt gland have shown that a fraction of phosphatidic acid is formed which undergoes continuous turnover of its phosphate group during stimulated secretory activity and which disappears when this activity stops; the turnover of phosphatidic acid (determined with radioactive phosphate) appears, therefore, to be associated in some way with the stimulated active transport of Na⁺ in the salt gland (1). One explanation for these observations is that the phosphorylation of diglyceride by adenosine triphosphate to form phosphatidic acid, followed by its dephosphorylation to re-form diglyceride, might be part of the mechanism whereby ATP is utilized for ion transport. The presence of enzymes (diglyceride kinase and phosphatidic acid phosphatase) which catalyze these two reactions has been demonstrated in the membrane fraction of salt gland and other tissues (2–5).

In recent years much work in the transport field has been devoted to studies of the (Na⁺ + K⁺)-dependent, ouabain-inhibitable adenosine triphosphatase activity, which was first shown by Skou (6) in a microsome fraction from crab nerve, and which has since been found in preparations from many other tissues. Because this (Na⁺ + K⁺)-dependent ATPase activity has many of the same properties as Na⁺-transporting systems, it has been suggested that this ATPase activity is closely linked to the process of Na⁺ transport.

The experiments reported here were designed to test whether a turnover of phosphatidic acid, such as occurs during Na⁺ pumping in the intact salt gland cell, might also take place during the (Na⁺ + K⁺)-dependent ATPase activity which occurs in salt gland homogenates (7). No component of phosphatidic acid that showed kinetic behavior which would support the participation of the phosphatidic acid cycle in the (Na⁺ + K⁺)-dependent ATPase activity in cell-free preparations could be found. These results could be interpreted to mean that the rapid turnover of a fraction of the phosphatidic acid in the intact cells of the salt gland cannot be accounted for by allocating to this phosphatidic acid a role as an intermediate in the utilization of ATP for Na⁺ transport.

However, the possibility that the steady state level of the phosphorylated intermediate may be so low as to be undetectable in the cell-free preparations cannot be excluded. Results from experiments in which phosphatidic acid was made radioactive in the intact cell before homogenization are compatible with this possibility.

Heald (8) and Judah et al. (9–12) have put forward the suggestion that the phosphorylation of protein-bound serine is a step in the utilization of ATP for ion transport. In the work presented here, no component of protein-bound phosphorylserine which showed the kinetic behavior of a phosphorylated intermediate in the (Na⁺ + K⁺)-dependent ATPase was found.

**Experimental Procedure**

**Preparation of Homogenates and Incubation Procedures**—Sucrose homogenates were prepared from the salt gland of the herring gull as described elsewhere (7). The basic incubation mixture contained, per ml, 121 μmoles of NaCl, 12 μmoles of KCl, 1.5 μmoles of MgCl₂, and 15 μmoles of Tris-Cl buffer, pH 7.4; ATP was added as indicated. Incubations were carried out in Erlenmeyer flasks (either 10- or 25-ml capacity, depending on the volume of the incubation medium). The incubation medium was kept at 37° in the flasks. The homogenate was kept at 0° until the addition of each aliquot to the incubation medium; all aliquots of homogenate were 0.1 the volume of the incubation medium. All additions were made rapidly with syringe-operated pipettes, and the flasks were shaken throughout. The reaction was stopped by the addition of trichloroacetic acid (50 g/100 ml) to give a final concentration of trichloroacetic acid of 5 g/100 ml. The contents of the flasks were transferred to centrifuge tubes. After centrifugation, the supernatant fluid was saved for assay of the amount of 32P present as inorganic phosphate as described below. The trichloroacetic acid precipitates were washed with 5 ml of trichloroacetic acid (5 g/100 ml); they were then washed with 5 ml of trichloroacetic acid (5 g/100 ml); and the tubes were tightly stoppered and stored overnight at 4°. The mixtures were then washed with 5 ml of 0.1 n HCl, as described previously (13), and centrifuged. The chloroform extract was taken for chromatographic isolation of the phosphatidic acid; the protein disk at the interface between the chloroform and the HCl phases was treated for determination of the radioactivity in protein-bound phosphorylserine, as described below.

**Determination of Phosphatidic Acid Radioactivity**—Phosphatidic acid was separated by chromatography of the chloroform extracte on silicic acid-impregnated paper with disobutyl ketone-acetic acid-water (40:25:5) as the solvent, according to the method of Mariniett, Ehrland, and Kochen (14). Approximately 50 μg of carrier L-α-phosphatidic acid, prepared by the method of Kates (15) from soybean lecithin which had been purified over a silicic acid column (16), were added to each sample before chromatography. The phosphatidic acid was located on the chromatograms by staining with rhodamine G; in one experiment the radioactive phosphatidic acid was also located by autoradiography on Kodak "no-screen" x-ray film. The

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Determination of Radioactivity in Phosphorylserine from Phosphatidic Acid—The protein residue left after extraction of the phospholipids was extracted twice with chloroform-methanol 12 N HCl (200:100:1) at 70° to remove "phosphatido peptides" (17). The residues were then extracted twice with ether and twice with acetone. After drying, O-phosphorylserine (20 µg of phosphorylserine phosphorus per vessel) was added, and the samples were hydrolyzed in 2.0 ml of 2 N HCl in sealed ampoules at 100° for 10 hours. The hydrolysates were centrifuged, the residues were washed twice with water, and the combined supernatant fluid and washings were dried in a vacuum; they were then subjected to electrophoresis in pyridine-acetic acid-water (18) on Whatman No. 3MM paper for 2 hours at 1000 volts. The electrophoretograms were autoradiographed on Kodak "no-screen" x-ray film, and were then stained to reveal phosphate and phosphate ester by the method of Wade and Morgan (19). The most intensely labeled spot was one which ran with the same mobility as orthophosphate. The second most intensely labeled spot coincided with the phosphorylserine spot revealed by staining; its electrophoretic mobility with reference to orthophosphate was 0.8. When ATP of sufficiently high specific activity was used, two other radioactive spots could be seen on the autoradiograms; they had mobilities, with reference to orthophosphate, of 0.5 and 0.25. The phosphorylserine spot was cut out and counted. Care was taken throughout the whole procedure to keep transfers quantitative and to have standard conditions, especially for the hydrolysis. The standard phosphorylserine was heated in 2 N HCl under conditions identical with those above; the yield of inorganic phosphate ranged from 69 to 71% in three separate hydrolyses. The radioactivities given for phosphorylserine in the text have therefore been corrected for a 70% loss.

The values for radioactivity for protein-bound phosphorylserine have been expressed as the total radioactivity in this fraction in the sample. This method of expression was chosen because it contains the most information available from the data. Under standard conditions of hydrolysis, the total radioactivity in the phosphorylserine spot, corrected for loss during hydrolysis, gives a measure of the absolute amount of protein-bound phosphorylserine which has become labeled from ATP. The more usual expression of specific activity would be less informative, since it would involve dividing the total radioactivity observed by a constant which would be arbitrarily determined by the amount of carrier used (as stated above, the recovery of carrier was consistently 30% of the initial amount under these conditions). The total amount of endogenous protein-bound phosphorylserine was too low to allow a direct determination of specific activity on samples of this size.

Determination of ATPase Activity—Aliquots of the trichloroacetic acid extracts were treated by the method of Berenblum and Chain (20); radioactivity was counted in aliquots of the isobutyl alcohol phase. The inorganic phosphate liberated from ATP during the incubation period appears in the isobutyl alcohol phase. The rates of ATPase activity as determined by this method were similar to those estimated (after longer incubation times) by colorimetric determination of inorganic phosphate (7).

Synthesis of ATP—Carbamyl phosphate was synthesized by the method of Metzenberg, Marshall, and Cohen (21). The starting materials for each batch were 100 µmoles of orthophosphate and 5 µc of 32P; this yielded a preparation which, under our counting conditions, had a specific activity of approximately 5 X 106 c.p.m. per µmole. The carbamyl phosphate was converted to ATP by incubation with ADP and a carbamyl kinase preparation from Streptococcus faecalis (22). The incubation mixture contained 44 µmoles of MgCl2, 21 µmoles of ADP, 420 µmoles of Tris-Cl buffer, pH 7.4, 20 µmoles of carbamyl-32P, and 18 units (22) of carbamyl kinase preparation in a total volume of 3 ml; at this stage, 4 µmoles of 32P, were present as a contaminant. The mixture was incubated for 5 minutes at 37°; it was then heated at 70° in a water bath for 10 minutes to denature the protein and to destroy any remaining carbamyl phosphate. The mixture was centrifuged and the supernatant fluid was collected. Assayed by the method of Berenblum and Chain (20), the average content of three batches was 16 µmoles of ATP and 8 µmoles of 32P in a total volume of 3 ml. Aliquots of this mixture were used, as indicated, in the experiments reported here. Because of the constituents other than ATP in the mixture, the actual amount of the mixture added to each vessel is stated for each experiment.

To check for possible phosphatidic acid and protein-bound phosphorylserine radioactivity in the ATP mixture and for any incorporation of 32P into these compounds in inactivated homogenates, 0.1 ml of the ATP mixture and 0.3 ml of trichloroacetic acid (50 g/100 ml) were added to 2 ml of medium at 37°; 0.2 ml of homogenate was then added and the mixture was incubated at 37° for 25 seconds before transfer to an ice bath. In the washed precipitate there was a small amount of chloroform-soluble radioactivity, but there was no detectable phosphatidic acid radioactivity. There was considerable radioactivity in the total phosphoprotein fraction; after acid hydrolysis in the presence of added phosphorylserine, followed by electrophoresis, orthophosphate was the only discrete radioactive spot seen on the electrophoretogram; phosphorylserine, located by staining, showed no detectable radioactivity. It was therefore unnecessary to correct the radioactivity found in phosphatidic acid and in protein-bound phosphorylserine in the experimental vessels for any initial radioactivity in the ATP mixture. These results also showed that there was no detectable incorporation of 32P into phosphatidic acid and protein-bound phosphorylserine in homogenates which had been inactivated by trichloroacetic acid.

Results

The rationale for the design of the experiments reported here was based on the fact that a phosphorylated intermediate in the transport process would have a very rapid rate of turnover. The phosphate groups of all of the molecules of such an intermediate would be expected to be in isotopic equilibrium with ATP within the earliest time of sampling, which was 5 seconds. There should, therefore, be a compartment of the intermediate in which the incorporation of 32P took place during these first 5 seconds and in which the level of isotope remained constant during the subsequent incubation period. In the experimental situation, incorporation of isotope into other, nonexchanging compartments might be superimposed upon this initial incorporation. However, the incorporation into the equilibrated compartment should be detectable by plotting the uptake of isotope against time; if there were a rapidly equilibrated compartment, this curve should cut the ordinate at a point above zero, the value for the intercept...
being a measure of the amount of phosphorylated intermediate in isotopic equilibrium with AT$^{32}$P. Since the phosphate groups of such an intermediate would undergo continuous turnover, being replaced by phosphate groups from ATP during the utilization of ATP for transport, they should lose $^{32}$P if the specific activity of the ATP were to be lowered. Our aim was to see whether any fraction of phosphatidic acid or of protein-bound phosphoserine showed this kind of kinetic behavior with respect to the incorporation of $^{32}$P during (Na$^+$ + K$^+$)-dependent ATPase activity in salt gland homogenates.

Rate of Incorporation of $^{32}$P into Phosphatidic Acid in Salt Gland Homogenates—When AT$^{32}$P was present from the beginning of the incubation, the rate of incorporation of $^{32}$P into phosphatidic acid was much more rapid during the first 10 seconds of incubation than during the subsequent incubation period (Fig. 1). This suggested that there might be a small compartment which rapidly reached isotopic equilibrium with AT$^{32}$P and then underwent continuous turnover. However, this was shown not to be the case. When the homogenate was incubated with nonradioactive ATP for 5 seconds before the addition of AT$^{32}$P, the rate of incorporation of $^{32}$P into phosphatidic acid was found to be linear from the time of addition of $^{32}$P (Fig. 2). If the rapidly labeled compartment were in isotopic equilibrium with AT$^{32}$P, the same type of kinetic curve should have been obtained when AT$^{32}$P was added after nonradioactive ATP as when AT$^{32}$P was added at the beginning of the incubation. It appears, therefore, that a small amount of phosphatidic acid is rapidly synthesized during the first few seconds of incubation, but that this does not undergo exchange with AT$^{32}$P. The rate of synthesis of phosphatidic acid during the subsequent incubation period was less than during the first few seconds of incubation, but it remained linear over the longest period measured, which was 1 minute. The amount of phosphatidic acid synthesized (assuming AT$^{32}$P to be the immediate precursor) increased with increasing ATP concentration over the range from 0.1 to 1.0 $\mu$mole per ml (Fig. 3). The amount of phosphatidic acid synthesized in the presence of 5 $\mu$moles of ATP per ml was less than that synthesized in the presence of 1.0 $\mu$mole per ml. The experiment shown in Fig. 3 was carried out under conditions in which AT$^{32}$P was present from the beginning of the incubation; it therefore includes that portion of phosphatidic acid which becomes rapidly labeled. This portion amounted to 0.46 $\mu$mole of phosphatidic acid per g of fresh tissue in an experiment in which the level of ATP was 0.17 $\mu$mole per ml (Fig. 1), and amounted to 0.51 $\mu$mole of phosphatidic acid per g of fresh tissue in an experiment in which the ATP level was 1.0 $\mu$mole per ml (Fig. 2); this portion of phosphatidic acid synthesis did not, therefore, seem to be much influenced by the ATP con-
Fig. 3. Phosphatidic acid synthesis in the presence of different concentrations of ATP in salt gland homogenates. ATP mixture was added to each vessel to give an ATP concentration of 0.13 μmole per ml; nonradioactive ATP was added to give a final concentration of ATP as indicated. The incubation time was 35 seconds; other conditions were as described under Fig. 1.

Rate of Incorporation of 32P into Protein-bound Phosphorylserine—The incorporation of 32P into protein-bound phosphorylserine increased linearly with time from the time of addition of 32P (Fig. 4). Calculation of the amount synthesized, assuming ATP to be the immediate precursor, gave 0.13 mpmole of protein-bound phosphorylserine per g of fresh tissue per second in one experiment, in which the concentration of ATP was 0.13 μmole per ml, and 0.15 mpmole of protein-bound phosphorylserine per g of fresh tissue per second in the experiment shown in Fig. 4, in which the concentration of ATP was 1 μmole per ml. The rate of synthesis of protein-bound phosphorylserine did not, therefore, seem to show a dependence on ATP concentration under these conditions. There was no evidence of a compartment of protein-bound phosphorylserine in isotopic equilibrium with ATP.

Effect of Reduction of ATP Specific Activity during Incubation Period—In the experiment shown in Table I, samples of salt gland homogenate were incubated with ATP for 15 seconds, after which nonradioactive ATP was added to lower the specific activity of the ATP by a factor of 14; the incubation was continued for 20 seconds longer. There was no detectable fall in radioactivity to below the 15-second value either in phosphatidic acid or in protein-bound phosphorylserine. The rise in radioactivity in both of these compounds which took place between 15 and 35 seconds in the vessels in which the specific activity of the ATP was not lowered was prevented by lowering the ATP specific activity to 1/14 the original value, as might be expected if this rise is due to the synthesis of new molecules from ATP. Calculations indicate that the increased rate of phosphatidic acid synthesis which might be expected in response to the higher level of ATP (see Fig. 3) would, at this low specific activity of ATP, amount to only 10 c.p.m./10 mg of fresh tissue, so that such an effect would not obscure a loss of radioactivity from that phosphatidic acid which was labeled from the high specific activity ATP during the first 15 seconds. These results confirm, therefore, that the radioactive phosphatidic acid and protein-bound phosphorylserine formed during the incubation period in salt gland homogenates did not undergo turnover. If these radioactive compounds were phosphorylated intermediates in the ATPase activity, they should undergo turnover and therefore should lose radioactivity when the specific activity of the ATP is lowered.

Incorporation of 32P into Phosphatidic Acid and Protein-bound Phosphorylserine in Presence of Ouabain—Under conditions in

![Graph](http://www.jbc.org/)
Incorporation of $^{32}$P into phosphatidic acid and protein-bound phosphorylserine in presence of ouabain in salt gland homogenates

The incubation time was 10 seconds; other conditions were as described under Fig. 1.

<table>
<thead>
<tr>
<th>Additions*</th>
<th>Radioactivity</th>
<th>ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphatidic acid</td>
<td>Protein-bound phosphorylserine</td>
</tr>
<tr>
<td></td>
<td>c.p.m./10 mg tissue</td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>313</td>
<td>945</td>
</tr>
<tr>
<td>Homogenate + ouabain</td>
<td>322</td>
<td>808</td>
</tr>
</tbody>
</table>

* An aliquot of homogenate was in contact with $10^{-4}$ M ouabain at 0° for 20 minutes before incubation; the final concentration of ouabain during the incubation was $10^{-4}$ M.

which ouabain ($10^{-4}$ M) was shown to inhibit ATPase activity, ouabain did not affect the incorporation of $^{32}$P into phosphatidic acid and protein-bound phosphorylserine in salt gland homogenates (Table II). This concentration of ouabain causes an increase in the amount of $^{32}$P incorporation into phosphatidic acid in both unstimulated and stimulated salt gland slices (23).

Incubation of Homogenates from Slices Which Phosphatidic Acid Had Been Prelabeled with $^{32}$P in Presence and Absence of Acetylcholine—To find out whether the phosphatidic acid which undergoes continuous turnover in slices of salt gland is involved in turnover during ATPase activity in the homogenates, this phosphatidic acid was made radioactive by incubation of slices with $^{32}$P prior to homogenization and assay of ATPase activity. If this radioactive phosphatidic acid were involved as a phosphorylated intermediate in the ATPase activity, it would rapidly lose its radioactivity either by exchange with the phosphate of nonradioactive ATP or by a reduction in the steady state level of the phosphorylated form. While loss of radioactivity would not itself be proof of involvement in ATPase activity, since this loss could occur by an independent process, failure to lose counts would positively indicate that the particular radioactive molecules involved were not participating as phosphorylated intermediates in the ATPase activity.

Slices were incubated in the presence of $^{32}$P for 40 minutes. Five minutes before the end of the incubation period, acetylcholine (plus eserine) was added to those slices which were to be stimulated. Under these conditions, virtually all of the increase in radioactivity found in phosphatidic acid is in the fraction which is undergoing continuous turnover in response to acetylcholine (1). After incubation of the slices, the tissues were homogenized and the homogenates were incubated without and with nonradioactive ATP and without and with ouabain under conditions similar to those used for assay of ATPase activity (7). The results of such an experiment are shown in Table III. In this experiment, in the homogenates of control slices (i.e. those slices which were labeled with $^{32}$P in the absence of acetylcholine), there was a 50% loss of radioactivity from the phosphatidic acid. Most of this loss occurred during the homogenization procedure and maintenance of the homogenate at 0°. On incubation of the homogenates for 1 minute at 38°, there was very little further loss of radioactivity from phosphatidic acid. The loss during incubation at 38° was not significantly influenced by the presence of nonradioactive ATP or of ouabain. In the stimulated slices (i.e. those slices which were labeled with $^{32}$P in the presence of acetylcholine), the loss in radioactivity from the stimulated fraction of phosphatidic acid (i.e. the increased radioactivity due to acetylcholine) on homogenization of the slices and maintenance at 0° was 26%. On incubation of the homogenate for 1 minute at 38° there was a further loss from this fraction of 17% of the original radioactivity. The loss during incubation at 38° for 1 minute was not dependent on or significantly affected by the presence of nonradioactive ATP. In view of this, it appears that the loss of radioactivity must represent a net loss of radioactive phosphatidic acid rather than exchange of the phosphate group with nonradioactive phosphate. The loss during the 1-minute incubation at 38° was not significantly affected by ouabain.

The percentage loss of phosphatidic acid radioactivity varied somewhat in different experiments. In each experiment, however, the loss was not progressive; some radioactivity was rapidly lost and the rest remained stable (Table IV). In this experiment there was a loss of about 70% of the radioactivity from phosphatidic acid in the unstimulated tissue and about 60% of the increased radioactivity in phosphatidic acid in the stimulated tissue. In each case almost all of the loss occurred at 0°. There was a relatively small additional loss during the first minute.

I Radioactivity I

The Incorporation of $^{32}$P into phosphatidic acid in homogenates of salt gland slices which had been incubated with $^{32}$P in presence and absence of acetylcholine

Goose salt gland slices were incubated at 38° with $^{32}$P, for 40 minutes, as described elsewhere (3). Five minutes before the end of the incubation period, acetylcholine ($10^{-4}$ M) and eserine ($10^{-4}$ M) were added as indicated. Slices for the initial values were frozen immediately at the end of the incubation period in an alcohol-CO₂ bath. Other slices were homogenized in sucrose as described elsewhere (7); acetylcholine ($10^{-4}$ M) and eserine ($10^{-4}$ M) were added to the sucrose for homogenization of the slices to which acetylcholine and eserine had been added. The homogenates were kept on ice for not longer than 1 hour. Incubations were carried out under conditions similar to those used for the assay of ATPase activity (7). Nonradioactive ATP (1.0 μm) was present to avoid during incubation of the homogenates as indicated. Other final concentrations in the incubation media were: Tris-Cl buffer, pH 7.4, 10 mM; sucrose, 20 mM; NaCl, 120 mM; KCl, 12 mM; and MgSO₄, 1 mM. The tissue concentration was 2.5 mg, fresh weight, per ml. Incubation time for the homogenates was 1 minute at 38°. Phosphatidic acid was extracted and isolated by paper chromatography as described elsewhere (13). Radioactivities were corrected to a specific activity basis. The values given are the averages of duplicate estimations.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Radioactivity in phosphatidic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + Acetylcholine</td>
</tr>
<tr>
<td></td>
<td>c.p.m./mg fresh tissue</td>
</tr>
<tr>
<td>Initial slices</td>
<td>423</td>
</tr>
<tr>
<td>Homogenate at 0°</td>
<td>229</td>
</tr>
<tr>
<td>Homogenate incubated at 38°</td>
<td>180</td>
</tr>
<tr>
<td>ATP added</td>
<td>174</td>
</tr>
<tr>
<td>ATP and $10^{-4}$ M ouabain added</td>
<td>167</td>
</tr>
</tbody>
</table>
Time course of loss of radioactive phosphatidic acid in homogenates from salt gland slices incubated with ATP in presence and absence of acetylcholine

Goose salt gland slices were incubated as described in Table III for 70 minutes. Acetylcholine (3 x 10^-4 M) and eserine (10^-3 M) were added after 30 minutes of incubation. During incubation of the homogenates, ATP (1.6 mm) was present in all tubes. The incubation times were as indicated. The concentration of the homogenate was 2 mg, fresh weight, per ml. Other procedures were as described in Table III. The values given are the averages of duplicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity in phosphatidic acid</th>
<th>Counts in stimulated fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. P. M./mg fresh tissue</td>
<td></td>
</tr>
<tr>
<td>Initial slices</td>
<td>522</td>
<td>2460</td>
</tr>
<tr>
<td>Homogenate at 0°</td>
<td>162</td>
<td>1050</td>
</tr>
<tr>
<td>Homogenate incubated at 38° for 1 minute</td>
<td>146</td>
<td>888</td>
</tr>
<tr>
<td>Homogenate incubated at 38° for 5 minutes</td>
<td>137</td>
<td>888</td>
</tr>
<tr>
<td>Homogenate incubated at 38° for 10 minutes</td>
<td>127</td>
<td>847</td>
</tr>
</tbody>
</table>

In these experiments, the specific activity of the ATP used, as well as other conditions, was such that the minimum amount of phosphatidic acid or of protein-bound phosphorylserine which could have been established as undergoing continuous phosphorylation and dephosphorylation was 0.5 mmole per g of fresh tissue. Since no equilibrated fraction was detected in the homogenates in the presence of ATP, the results indicate that this part of the phosphatidic acid is not involved in turnover during ATPase activity in the homogenates; it is presumably also protected from phosphatidic acid phosphatase activity under these conditions. The portion of phosphatidic acid which rapidly loses its radioactivity after homogenization could be involved in the ATPase activity, although there is no positive evidence that this is so. In the light of the results presented above, which indicate that any steady state level of phosphatidic acid in equilibration with ATP was too low to be detected in homogenates, such an involvement would have to include a net conversion of phosphatidic acid to diglyceride and the maintenance of a steady state level which would favor the diglyceride rather than the phosphatidic acid form.

**DISCUSSION**

In these experiments, the specific activity of the ATP used, as well as other conditions, was such that the minimum amount of phosphatidic acid or of protein-bound phosphorylserine which could have been established as undergoing continuous phosphorylation and dephosphorylation was 0.5 mmole per g of fresh tissue. Since no equilibrated fraction was detected in the homogenates in the presence of ATP, the results indicate that if either phosphatidic acid or protein-bound phosphorylserine is an intermediate in the ouabain-sensitive ATPase activity of salt gland homogenates, the steady state level of this phosphorylated intermediate must be less than 0.5 mmole per g of fresh tissue under these conditions. Possibly, alteration of the levels and ratios of Na⁺ and K⁺ in the incubation medium might have an effect on the steady state level of a phosphorylated intermediate.

The studies which led to the suggestion that the phosphorylation of protein-bound serine is a step in the utilization of ATP for ion transport (8-12) have not contained kinetic data adequate to support the idea. Nor does stimulation of NaCl secretion in the avian salt gland result in any change in the amount of 32P found in protein-bound phosphorylserine (24). For these reasons, the possibility that protein-bound phosphorylserine may play a role in the transport process will not be discussed further here.

The studies on the salt gland which led to the suggestion that phosphatidic acid may be a phosphorylated intermediate during ATPase activity in the homogenates, ATPase activity in salt gland homogenates have been more detailed. In the intact salt gland cell stimulated by acetylcholine to secrete NaCl, a fraction of phosphatidic acid was found to be formed and to undergo continuous turnover of its phosphate group in such a way as to maintain radioactive equilibrium with ATP (1). It was postulated that the reactions involved were the phosphorylation of diglyceride, with the use of ATP, to form phosphatidic acid, and the hydrolysis of phosphatidic acid to regenerate diglyceride and to form inorganic phosphate. The amount of phosphatidic acid found in the fraction which responded to acetylcholine in this way varied with the concentration of acetylcholine used, the maximum amount being 0.12 µmole per g, fresh weight. As we have seen, there is no amount of phosphatidic acid of this order of magnitude in equilibration with ATP during ouabain-sensitive ATPase activity in salt gland homogenates. We can say, therefore, that if phosphatidic acid is a phosphorylated intermediate, its steady state level in the homogenates must be lower by at least two orders of magnitude than the maximum level of responsive phosphatidic acid in the stimulated slice. The steady state level of the intermediate would therefore be preponderantly diglyceride in the homogenate if the above scheme were true. If this is so, then the radioactive phosphatidic acid of the intact cell, which has been shown to be a small but discrete fraction of the total phosphatidic acid of the tissue (1), should be converted to the diglyceride form in the homogenate and should therefore lose radioactivity. As we have seen, there is a rapid loss of part of the radioactivity in phosphatidic acid under these conditions. There is considerable phosphatidic acid phosphatase activity in deoxycholate extracts of salt gland homogenates, and the enzyme is not inactivated at 38° (3). The results presented here indicate, however, that in the homogenate which has not been treated with deoxycholate, the hydrolysis of endogenous phosphatidic acid does not proceed to completion at 38°. The fact that the loss of phosphatidic acid radioactivity was neither progressive nor complete suggests that it is likely to occur at particular sites rather than through general phosphatidic acid hydrolysis. In view of our postulate that the stimulated phosphatidic acid molecules are at transport sites (which are also presumably ouabain-sensitive ATPase sites), such a partial loss of radioactivity could be explained if only some of the total transport sites were to remain active, i.e., to undergo ouabain-sensitive ATPase activity in the homogenates. The phosphatidic acid at the sites which retain their activity would undergo a shift in the steady state level of the intermediate to predominantly the diglyceride form, involving a loss of radioactivity. The phosphatidic acid at sites which do not undergo ouabain-sensitive ATPase activity after homogenization would retain its radioactivity.

The amount of ouabain-sensitive ATPase activity found in the salt gland homogenates as compared with that calculated for the
intact tissue tends to support this possibility. Schmidt-Nielsen (25) found that the salt gland of the herring gull could be stimulated to secrete for relatively long periods at the rate of 420 μeq of Na+ per g of fresh weight per minute. We do not know the ratio of Na+ transported to ATP consumed in the salt gland. However, an estimate of this ratio can be made from the figures of Lassen, Munck, and Thaysen (26) for the dog kidney in vivo. The ratio in the kidney was about 4.6 Na+:1 ATP; this was calculated from the observed ratio of 28 Na+:1 O2, based on an assumed P:O ratio of 3. If we take this ratio for the salt gland, then a rate of secretion of 420 μeq per g, fresh weight, per minute would require 91 μmoles of ATP per g, fresh weight, per minute, most of which would be ouabain-sensitive. The amount of ouabain-sensitive ATPase activity found in salt gland homogenates under the conditions used for the work reported here never exceeded about 30 μmoles per g, fresh weight, per minute (7), and in some experiments this amount was as low as 10 μmoles per g, fresh weight, per minute. This discrepancy could be due to a situation such as we have discussed, in which not all of the total sites retain their ouabain-sensitive ATPase activity in the homogenate.

It seems, therefore, still somewhat of an open question whether phosphatidic acid is a phosphorylated intermediate for Na+ transport in the salt gland. In spite of the failure to find any evidence for phosphatidic acid as the phosphorylated intermediate in the ouabain-sensitive ATPase activity in the homogenates, we cannot exclude the possibility that the steady state level of the intermediate is very much toward diglyceride in this system. The loss of radioactivity from phosphatidic acid on homogenization of the tissue is compatible with this idea, but it cannot be interpreted as giving it positive support.

**SUMMARY**

The incorporation of 32P from adenosine triphosphate-32P into phosphatidic acid and into protein-bound phosphorylserine has been studied in salt gland homogenates under conditions in which (Na+ + K+)-dependent, ouabain-inhibitable adenosine triphosphatase activity occurs. 32P was incorporated into both phosphatidic acid and protein-bound phosphorylserine over the time intervals studied (5 seconds to 1 minute).

No evidence was obtained for a compartment of either phosphatidic acid or of protein-bound phosphorylserine which underwent turnover of the type to be expected for a phosphorylated intermediate in the ATPase activity. The incorporation of 32P into phosphatidic acid and into protein-bound phosphorylserine was not affected by 10−4 M ouabain.

The results indicate that if either phosphatidic acid or protein-bound phosphorylserine is an intermediate in the ouabain-sensitive ATPase activity of salt gland homogenates, the steady state level of the phosphorylated intermediate must be less than 0.5 mmol per g of fresh tissue under these conditions. In the salt gland slice, up to 0.12 μmole of phosphatidic acid undergoes continuous turnover so as to maintain radioactive equilibrium with ATP in response to stimulation of NaCl secretion. It can be said, therefore, that if phosphatidic acid is an intermediate in the ouabain-sensitive ATPase activity of salt gland homogenates, the steady state ratio of diglyceride (the nonphosphorylated form of the intermediate) to phosphatidic acid must have been greatly increased.

When phosphatidic acid was made radioactive by stimulation of salt gland slices with acetylcholine in the presence of 32P, and when the tissue was then homogenized, there was a loss of radioactivity from phosphatidic acid. This loss is compatible with a shift of the steady state level of an intermediate from phosphatidic acid to diglyceride at ouabain-sensitive ATPase sites, but it cannot be interpreted as positive evidence that this occurs.

**REFERENCES**

18. **Dawson, R. M. C., Biochem., 75, 45 (1950).**
20. **Berenblum, I., and Chain, E., Biochem., 32, 295 (1938).**
25. **Schmidt-Nielsen, K., Circulation, 21, 655 (1960).**
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