J. Membrane Biol. 5, 169–184 (1971)

Lipid Pattern and Na^+–K^+-Dependent Adenosine Triphosphatase Activity in the Salt Gland of Duck Before and After Adaptation to Hypertonic Saline*

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Received 21 December 1970

Summary. Ducks (*Anas platyrhynchos*) were fed hypertonic saline for eight days, resulting in an activation and hypertrophy of the salt gland. The Na^+–K^+-dependent adenosine triphosphatase, an enzyme generally assumed to be part of the active Na transport system, increased its specific activity by about 200% during this activation. Sulfatides, the major glycolipids of the salt gland, increased their concentration to the same extent. Cholesterol, cerebrosides, and six phospholipid classes showed an increase of 20–80%.

Sulfatides (Fig. 1) have long been known to exist in nerve tissue and in kidney [28]. With the recent finding of a high sulfatide concentration in kidney medulla [20], avian salt gland [22] and elasmobranch rectal gland [21], tissues specialized for active Na ion transport, a suggestion was made of a role for this anionic lipid class in Na translocation. Anionic lipids have previously been discussed as carriers or receptors for Na ions. Phosphatidic acid and phosphatidylinositol of the avian salt gland were found to increase their phosphate turnover parallel to a stimulation of gland excretion by acetylcholine [14]. The distribution of these lipids among tissues with different levels of Na ion transport is, however, quite uniform [33], and this is the case also for phosphatidylserine, a lipid recently found [39] to restore the activity of desoxycholate-inactivated Na^+–K^+-dependent adenosine triphosphatase (Na^+–K^+-ATPase). This enzyme is of lipoprotein nature [37] and has been linked to Na ion transport by several kinds of evidence [12].

* A preliminary report on this work was given at the Second International Meeting of the International Society for Neurochemistry, Milan, September 1–5, 1969, and at the XIIIth International Conference on the Biochemistry of Lipids, Athens, September 7–12, 1969.

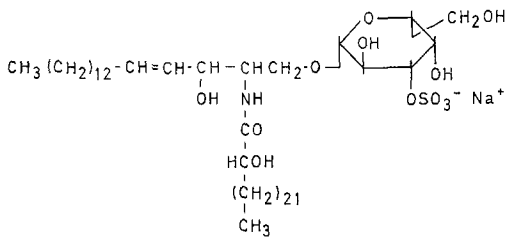


Fig. 1. The major molecular species of sulfatides of the salt gland of salt-loaded ducks, containing sphingosine, 2-hydroxytetracosanoic acid, and galactose esterified in position 3 with sulfuric acid. The glycosidic bond of human brain sulfatide is of β-configuration [35]

A recent investigation [31] pointed out a similar tissue distribution of this enzyme and gangliosides. However, erythrocytes, rich in gangliosides [33] but with low enzyme activity [8], and the avian salt gland, with traces of gangliosides [22] but very high enzyme activity [7], were not included. Therefore, the relation of the mentioned lipids to Na ion transport is uncertain.

The similarity of tissue distribution found for sulfatides and Na ion transport has stimulated further studies in this laboratory. The lipid composition and the Na^+–K^+-ATPase activity have been investigated in salt gland of herring gull and eider duck [24], and in rectal gland of spiny dogfish [25]. The present work considers the state of the salt gland of domestic ducks before and after a salt load. The experimental conditions were taken from an earlier study [10] on duck salt gland, showing a direct relation between NaCl excretion and Na^+–K^+-ATPase activity. This enzyme was therefore chosen as a reference in the present study.

Materials and Methods

A number of domestic ducks (*Anas platyrhynchos*), homogenous with respect to age and breeding conditions, were divided into two equal subgroups. The first subgroup was given freshwater and the second saline (284 mM NaCl and 6 mM KCl in tap water), in both cases *ad libitum*. All other conditions were the same. The total number of ducks was 20 in the first experiment, and 10 in the second experiment.

After eight days, all ducks were killed by decapitation, and the salt glands (supraorbital glands) were quickly removed and kept on ice. The glands from each subgroup were weighed, pooled, cut into small pieces and homogenized in distilled water, using an all-glass homogenizer (Potter-Elvehjem). An aliquot of this homogenate was taken for ATPase assay on the same day, and the other part was lyophilized. To obtain figures on dry weight, a portion of the dry powder was further dried over KOH pellets for a month; the major part of the powder was used at once for lipid extraction.

Na^+–K^+-ATPase Assay

The Na^+–K^+-ATPase was estimated as ATPase, inhibited by ouabain. One incubation medium contained 50 mM NaCl, 10 mM KCl, 1.5 mM $MgSO_4$, 90 mM Tris, and 0.1 mM ethylenediaminetetraacetate (EDTA); it was adjusted to pH 7.5 with 2 N HCl. The other incubation medium contained, in addition, 0.1 mM ouabain (Sigma Chem. Co., St. Louis, Mo.). Disodium salt of ATP (Sigma) was dissolved in each medium just before use, and pH was adjusted, if necessary, to 7.4. This adjustment could be omitted when 2 mM ATP was used. A mixture of 0.1 ml tissue homogenate and 1.0 ml of medium was incubated at 37 °C for 10 min. The inorganic phosphate liberated was determined as described by Bonting, Simon and Hawkins [8]. Na_2HPO_4 (analytical grade), dried in an oven at 120 °C to constant weight, was used as standard. Na^+–K^+-ATPase was taken as the difference between total ATPase and ouabain-resistant ATPase. Substrate concentrations were varied in order to obtain the optimal substrate concentration, and using this concentration (2 mM ATP), enzyme (tissue) concentration was varied (Fig. 2), in order to check linearity. Regression coefficients of the lines [41] were finally used for calculation of values, given in Table 2.

Lipid Analysis

Part of a total lipid extract was separated using two-dimensional thin layer chromatography (TLC), and the lipids were scraped off together with silica gel for phosphorus and hexose assay. Another part of the total lipid extract was separated into a neutral and an acidic lipid fraction. These fractions were further separated by TLC for phosphorus and hexose assay of the spots. Finally, part of these fractions were further separated by column chromatography (silicic acid or silica gel), and pure lipids obtained were used for quantitative estimation and identification. Lipids from salt glands of eider duck and herring gull were used for comparison.

Extraction. Lipids were extracted in a mixture of chloroform-methanol (2:1, v/v), 40 ml/g dry tissue, by brief heating to boiling, followed, after an hour, by filtration. This procedure was repeated 14 times, each time with 20 ml/g dry tissue. To check the completeness of extraction, extracts number 1–5, 6–10 and 11–15 were pooled, and samples were examined by TLC.

Column Chromatography. Part of each total lipid extract was used for preparation of neutral and acidic lipid fractions [24]. Diethylaminoethylcellulose (DEAE-cellulose) in acetate form was packed in chloroform-methanol (2:1, v/v). Up to 50 mg of lipids was loaded on each gram of DEAE-cellulose. Neutral lipids were eluted in chloroform-methanol (2:1, v/v), 100 ml/g of DEAE-cellulose. The eluate was collected in three fractions, for better control by TLC. Acidic lipids were eluted by 5 % (w/v) LiCl in methanol, 25 ml/g of DEAE-cellulose. LiCl was then removed from the lipids by dialysis against tap water (22/100 feet Nojax casings, Visking Co., Chicago, Ill.) during four days. After lyophilization of the tube contents, the lipids were further purified on a small column of silicic acid (Mallinckrodt), using 75 and 100 % (v/v) methanol in chloroform as eluting solvents, 20 ml of each solvent/g silicic acid. Further separation of acidic lipids, for the isolation of pure sulfatides, was achieved by column chromatography on silica gel H (Merck), using mixtures of aqueous ammonia, methanol and chloroform. This will be reported in detail elsewhere [24]. Neutral lipids were further fractionated by column chromatography on silicic acid. Nonpolar lipids were eluted in pure chloroform, cerebrosides in 5 % (v/v) methanol in chloroform, ethanolamine phosphoglycerides in 10 % (v/v) methanol in chloroform, and finally, choline phosphoglycerides and sphingomyelins in 75 % (v/v) methanol in chloroform followed by pure methanol. The volume

of each fraction was 30 ml/g of silicic acid, and the load was about 50 mg of lipids/g of silicic acid.

Nonpolar lipids, mainly composed of triglycerides and cholesterol, were separated by silicic acid column chromatography, using diethylether in hexane as solvent. Triglycerides were eluted in 5 % (v/v) diethylether in hexane, together with some minor components, and cholesterol in 10 % (v/v) diethylether in hexane. The volume of each fraction was 30 ml/g of silicic acid. Cholesterol, which was obtained pure, was determined by gravimetry.

Thin Layer Chromatography. Thin layer chromatoplates (20 × 20 cm) were prepared using a Desaga apparatus, adjusted to 0.25 mm thickness. Then 30 g of gel (silica gel G, Fluka, and silica gel H, Merck) was suspended in 60 ml of distilled water before application to the glass plates. All chromatoplates were dried for 30 min at 120°C before use. Development was performed in a saturated chamber, lined with filter paper, for about 1 hr. The following solvent systems (proportions given by volume) were used, for different purposes:

A. For determination of sulfatides in a total lipid extract.
Two-dimensional chromatography on silica gel H plates.
First dimension: chloroform-methanol-acetone-water, 50:30:25:5.
Second dimension: chloroform-methanol-acetic acid-water, 65:28:5:4.

B. For determination of acidic phospholipids in a total lipid extract.
Two-dimensional chromatography on silica gel G plates.
First dimension: chloroform-methanol-2 N aqueous ammonia, 65:28:5.
Second dimension: chloroform-methanol-acetic acid-water, 65:28:5:4.

C. For determination of phospholipids in a neutral lipid fraction.
One-dimensional chromatography on silica gel G plates.
Chloroform-methanol-water, 65:25:4.

D. For determination of phospholipids in the acidic lipid fraction.
One-dimensional chromatography on silica gel G plates.
The same solvent system as in B, first dimension.

E. For determination of sulfatides in the acidic lipid fraction.
One-dimensional chromatography on silica gel G plates.
The same solvent system as in B, second dimension.

F. For analysis of nonpolar lipids.
One-dimensional chromatography on silica gel G plates.
Hexane-diethylether-acetic acid, 90:10:1.

Identification of Sulfatides. Sulfatides isolated from salt-loaded ducks were further treated with mild alkaline hydrolysis (0.1 M KOH in methanol-water 9:1, v/v, at room temperature for 12 hr) to remove possible contaminant of phosphoglyceride. An infrared spectrum was recorded in a KBr pellet on a Perkin Elmer model 157 instrument. The identity of the lipid was further proved by mass spectrometry of two derivatives. In the first case, the sulfate group was removed by acid degradation of 100 μg of material (0.5 ml 0.05 M HCl in methanol at room temperature for 12 hr), followed by evaporation in a stream of nitrogen and preparation of trimethylsilyl ethers (0.1 ml of hexamethyldisilazane-trimethylchlorosilane-pyridine 0.3:0.1:1.5, v/v/v, at room temperature for at least 5 min). A mass spectrum (Fig. 6) was recorded on a MS 902 instrument (AEI, Manchester, England), using a direct inlet system. Further conditions are given in the legend for Fig. 6. In the second case, 100 μg of material was subjected to acetylation, followed by silylation at high temperature as described before [23]. The mass spectrometer used in this case was LKB 9000 (LKB Produkter, Stockholm, Sweden) (*see* Fig. 7).

Determination of Phospholipids. Phospholipid spots (0.05–0.2 μM phosphorus) were scraped off together with the gel from thin layer chromatograms, which had been stained in iodine vapor. Wet incineration was done with silica gel present, according to Newman, Liu and Zilversmit [29] as modified by Vikrot [38]. Color reaction was made according to Bartlett [6]. Phospholipid values given in Table 2 are the mean values of three determinations on the same extract or fraction.

Determination of Sulfatides. Thin layer chromatograms were stained in iodine vapor, spots (30–100 μg of sulfatide) were marked, and the iodine was allowed to evaporate overnight. All loose dust was blown away from the chromatogram with a stream of air, and the silica gel was transferred into small tubes with Teflon-faced screw caps. All chromatograms were finally sprayed with the anisaldehyde reagent [34], which easily detected if a part of the sulfatide spot had been left on the plate.

The anthron methods described by Radin [32] and by Jatzkewitz [17] were modified as follows: 200 μliters 85 % (w/w) *o*-phosphoric acid is added to each tube. Tubes are vigorously shaken in a water bath at 90 °C for 20 min, and subsequently transferred into an ice-bath. Then 500 μliters of freshly prepared anthron reagent is added (50 mg of anthron is dissolved into 1 ml concentrated sulfuric acid, and the solution is diluted by addition of 9 ml concentrated sulfuric acid-water 2:1, v/v). The tubes are shaken for 16 min in a water bath at 90 °C, followed by a quick transfer into an ice bath. When cooled, tubes are centrifugated for 10 min at 5700 rpm in a small centrifuge. The clear supernatant is read in 400 μliter cuvettes (2 × 10 × 20 mm, 10 mm optical pathway) at 625 nm. Galactose is used as standard (20 μg), and silica gel from an empty area of the chromatogram is used as blank.

Sulfatide fractions, obtained by column chromatography on silica gel H, were measured by the anthron reaction as described above, although the TLC step could be omitted. For identification of hexose, the fractions were analyzed by gas-liquid chromatography (GLC) of the trimethylsilyl methyl glycosides, principally according to Sweeley and Vance [36]. Each sulfatide value, given in Table 2, is the mean of three determinations by the anthron reaction.

Determination of Cerebrosides. The crude cerebroside fraction, obtained by column chromatography, was further purified by TLC (system C), and the spots were stained, scraped off, and estimated by the anthron reaction, as described for sulfatides. Galactose and glucose were both used as standards, because of different molar extinction coefficients. The ratio of glucose to galactose in each cerebroside was obtained by GLC.

Results

Earlier investigations on the Na^+–K^+-ATPase are in good agreement concerning suitable concentrations of Na, K, and Mg ions in the medium of assay. Also, there is little doubt concerning optimal pH and the ouabain concentration, giving complete inhibition. However, the optimal substrate concentration was not certain from data in the literature. Two different investigations [10, 15] on salt gland ATPase reported an optimal substrate concentration of 1 mM, and a strong inhibition of the enzyme at 2 mM. Another group of workers [7] made all determinations at 2 mM, and reported values which were somewhat higher than in the former investigations. In part, these discrepancies may be due to use of different Mg ion concentrations

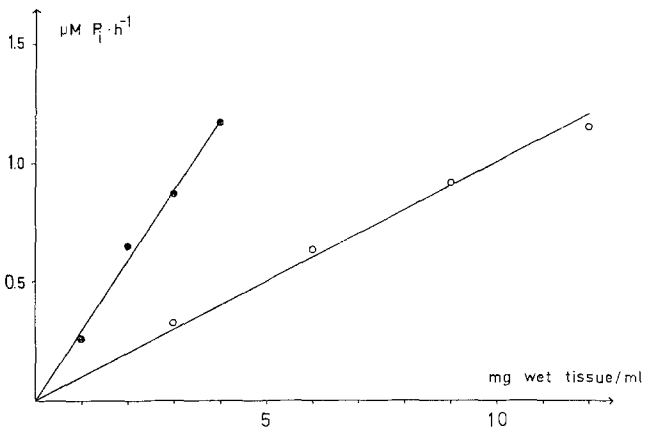


Fig. 2. Dependence of Na^+–K^+-ATPase activity on enzyme concentration in the salt gland of ducks on freshwater (open circles) and ducks on saline (closed circles). Samples (0.1 ml) of homogenates with varying tissue concentrations (1–12 mg wet tissue/ml) were incubated with 1 ml of substrate. Each point represents the mean difference of two ATPase determinations without and two determinations with 10^{-4} M ouabain

[4]. In the present study, using a Mg concentration of 1.5 mM, we found an optimum for the substrate concentration at about 2 mM, and a very moderate inhibition at higher levels. Enzyme dependence of the reaction was also tested (Fig. 2), in order to check the assay and to exclude heavy effects of possible activators and inhibitors, present in the crude homogenates.

Extraction control by TLC (system C) showed that most of the lipids were present in the first five extracts, there were trace amounts in the next five extracts, and nothing was visible in the last five.

Column chromatography on DEAE-cellulose was monitored by TLC. The neutral fraction contained triglycerides, cholesterol, cerebrosides, ethanolamine phosphoglycerides, choline phosphoglycerides and sphingomyelins. These lipids were completely separated from sulfatides, cardiolipins, inositol phosphoglycerides and serine phosphoglycerides, eluted with LiCl. A quantitative yield in the dialysis step has been checked using pure brain sulfatides, but has not been tested using acidic phospholipids. The differences seen between experiments 1 and 2 concerning the acidic phospholipids may therefore be due to losses during this step, as the lower values in experiment 1 are based on dialyzed fractions and the values in experiment 2 on the total lipid extracts. Sulfatides are not lost during the dialysis, as figures for sulfatide concentration agree well between the two procedures.

TLC of total lipid extracts using system A (Fig. 3) gave good separations of sulfatides from all the other lipids. System B (Fig. 4), however, was not

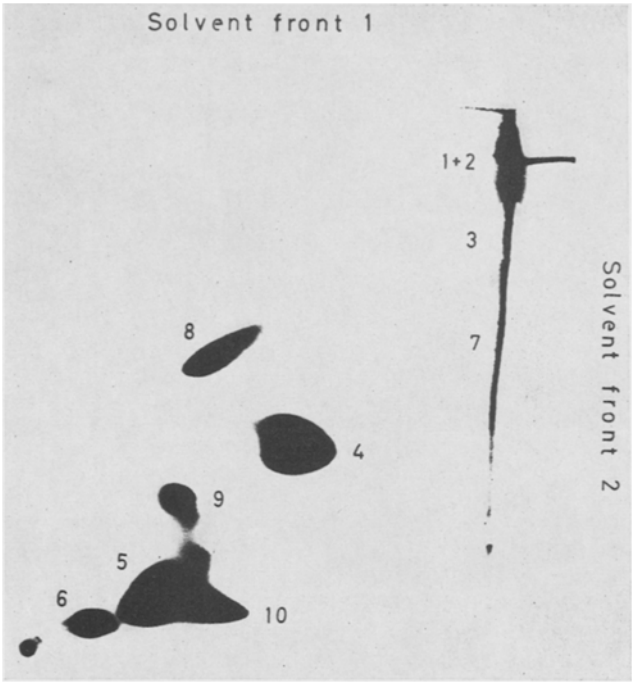


Fig. 3. Two-dimensional thin layer chromatogram of total lipid extract from salt glands of ducks given saline. Lipids from 5 mg of dry tissue were applied, the plate was developed using system A (*see* text), and the spots were detected by a copper acetate reagent [9]. The figures refer to: triglycerides (*1*), cholesterol (*2*), cerebrosides (*3*), ethanolamine phosphoglycerides (*4*), choline phosphoglycerides (*5*), sphingomyelins (*6*), cardiolipins (*7*), sulfatides (*8*), inositol phosphoglycerides (*9*), and serine phosphoglycerides (*10*)

quite ideal for separation of serine phosphoglycerides, which partly included another small component, or inositol phosphoglycerides, which migrated close to the big choline phosphoglyceride spot.

For TLC of fractions from DEAE-cellulose chromatography, one-dimensional separations gave sufficient resolution. The different systems are illustrated in Fig. 5 (system C), and in Fig. 4 (system D by the first dimension and system E by the second dimension).

Phosphorus determination on lipids with silica gel was tested using pure brain sphingomyelin. The transfer of sphingomyelin from chromatoplate into tube gave negligible losses, blanks contained 0.003–0.006 μM phosphorus, and the standard deviation for the whole procedure, applied to 0.2 μM of sphingomyelin, was about 2% ($n = 5$).

The anthron reaction for determination of sulfatides was tested and modified using parallel determinations on galactose and on a highly purified

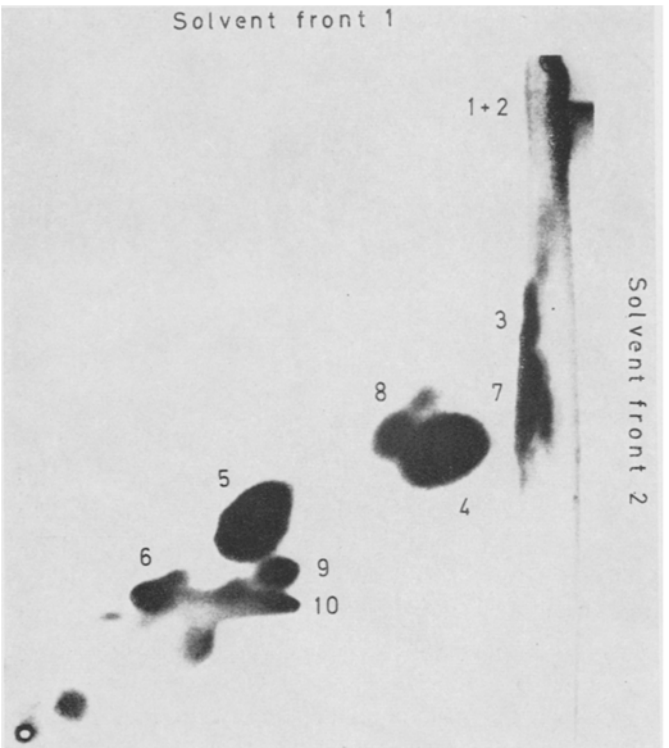


Fig. 4

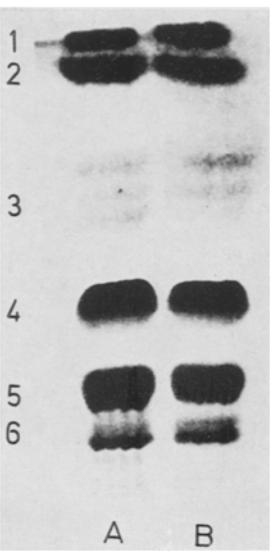


Fig. 5

Fig. 4. Two-dimensional thin layer chromatogram of total lipid extract from salt glands of ducks given saline. Lipids from 5 mg of dry tissue were applied, the plate was developed using system B (*see* text), and the spots were detected by an anisaldehyde reagent [34]. For spot identity, *see* Fig. 3

Fig. 5. One-dimensional thin layer chromatogram of the neutral lipid fraction from salt glands of ducks given saline (*A*) and freshwater (*B*), respectively. Neutral lipids from 5 mg of dry tissue were applied in each case, the plate was developed using system C (*see* text), and the spots were detected by an anisaldehyde reagent [34]. For spot identity, *see* Fig. 3

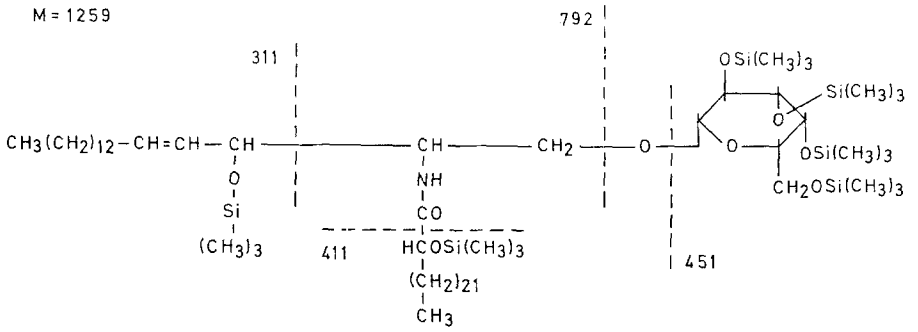
sulfatide fraction (from ox brain) of known molecular weight. A plot, showing extinction vs. time of heating with anthron, revealed a curve with a transient maximum (a few minutes), with roughly the same shape for galactose and sulfatides. With an anthron concentration of about 50 mg/10 ml, almost theoretical hexose values were obtained for the sulfatide (found 18.5%; theoretical 19.0%), using galactose as standard. Linearity of extinction vs. concentration was tested for galactose up to 20 µg (in 700 µliters final volume) and for sulfatides up to 100 µg. Transfer of the sulfatides from chromatoplate into tube gave no measurable losses, and the presence of gel

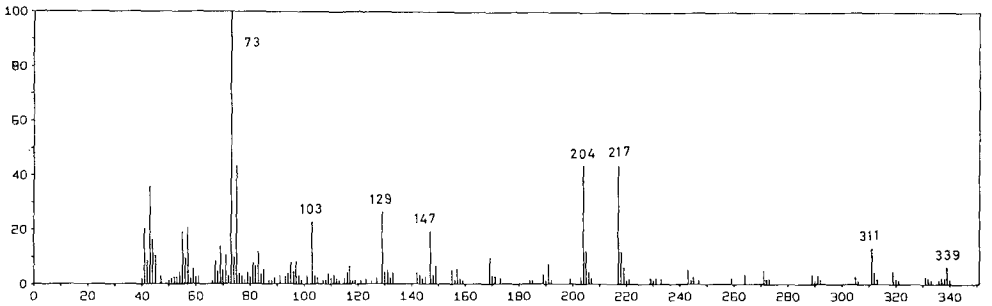
(silica gel G as well as silica gel H) did not modify the reaction. The standard deviation for the whole procedure, with 100 μg of sulfatide was 2.5 μg (n = 7). Gel blanks gave extinctions corresponding to 1–3 μg galactose, but were more constant within one chromatoplate. Phospholipids, when run through the procedure, gave a brown color, interfering at 625 nm.

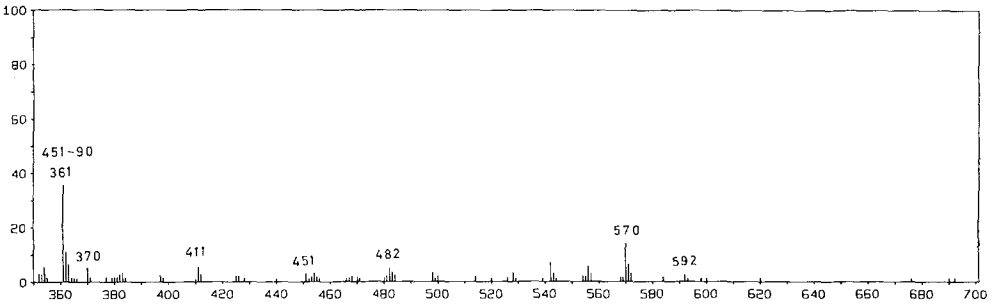
Using several TLC systems, we compared the lipid patterns of salt gland from eider, herring gull and duck and found them qualitatively identical. As herring gull and eider both possess salt glands of considerable size, most work to identify the lipids was done on these species [24]. Choline phospholipids from duck salt gland were shown to contain phosphorylcholine, ceramides and diglycerides.

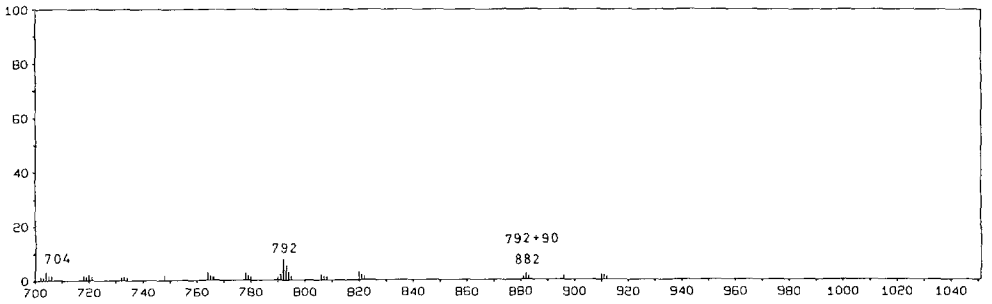
The sulfatide fraction isolated from salt-loaded ducks was characterized by carbohydrate analysis, infrared spectrometry and mass spectrometry. Galactose was the only hexose. The infrared spectrum was qualitatively similar to that of brain sulfatides and comparable to an earlier published spectrum of human kidney sulfatides [27]. The strong absorption found at 8.2 μ is evidence for S = O stretching vibrations [11]. A P = O vibration (for sphingomyelin found at 8.2 μ) is unlikely, since the phosphorus content of the preparation is 0.04% (molar percentage < 1).

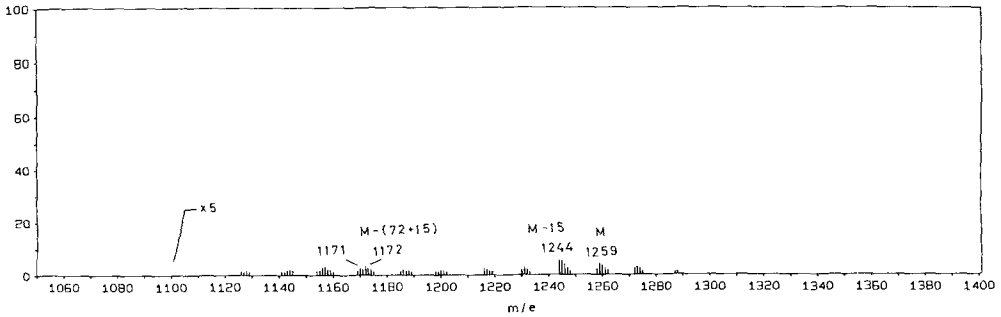
The partial mass spectrum in Fig. 7 of an acetylated and silylated fraction is very similar to an earlier published spectrum of the corresponding derivative of brain sulfatide [23]. The base peak at m/e 361 indicates a hexose residue with three acetoxy groups and one trimethylsilyloxy group. The peak at m/e 169 is evidence for a 3-position of the trimethylsilyloxy group and consequently of the original substituent (sulfate) of the glycolipid. The peaks at m/e 264 and 292 are rearrangement fragments of the long-chain base (18-carbon and 20-carbon, respectively). The mass spectrum of the cerebroside (desulfated sulfatide) as a trimethylsilyl derivative is shown in Fig. 6. A similar spectrum was obtained from cerebrosides of bovine brain [18]. By comparison with spectra of a series of homogenous galactosylceramides [19], an interpretation of the fatty acid and long-chain base composition is made possible. Evidence for a silylated hexose residue is found in the peaks at m/e 451 and 451 minus 90 (90 is the mass of trimethylsilanol). The binding of hexose to carbon atom one of the long-chain base is evident in the absence of blocked hydroxyl groups of the base and the hydroxy fatty acid (*see* below). In the region for long-chain base fragments, a strong peak is found at m/e 311 corresponding to a cleavage of ordinary sphingosine between carbon atoms two and three. The peak at m/e 339 indicates the presence of a small amount of the 20-carbon homologue of sphingosine. In the region of fatty acid fragments, the strongest peak is











RELATIVE INTENSITY

m/e

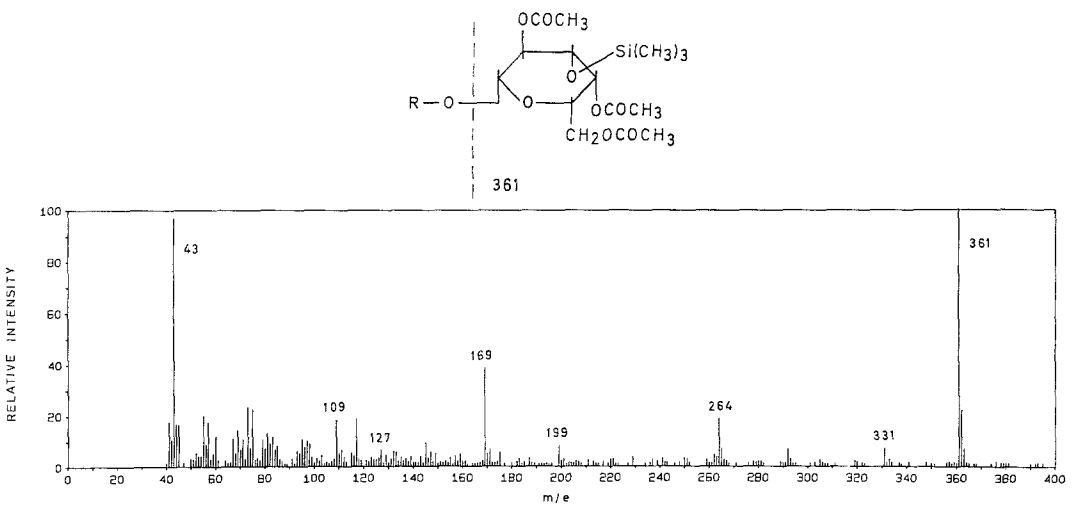


Fig. 7. Partial mass spectrum of acetylated and silylated sulfatide of salt glands of salt-loaded ducks. The temperature of the ion source (direct inlet) was 170 °C, the electron energy 70 eV, and the acceleration voltage 3.5 kV

found at m/e 570, corresponding to hydroxytetracosanoic acid. A specific 2-hydroxy acid fragment is found at m/e 411. The combination of sphingosine and 2-hydroxytetracosanoic acid gives the strongest peak in the ceramide region, at m/e 792. Finally, the combination of hexose, sphingosine and 2-hydroxytetracosanoic acid gives a molecular ion at m/e 1,259. The major molecular species of sulfatides in salt glands of salt-loaded ducks thus contains sphingosine and 2-hydroxytetracosanoic acid (*see* Fig. 1). The presence of smaller amounts of homologues of this fatty acid is indicated by fragments at, e.g., m/e 528, 542, 556 and 584. The peak at m/e 482 corresponds to tetracosanoic acid and that at m/e 370 to hexadecanoic acid. The corresponding ceramide fragments of these two normal acids are found at m/e 704 and 592, respectively. The peak found at m/e 820 probably contains a ceramide fragment for the 20-carbon homologue of sphingosine combined with 2-hydroxytetracosanoic acid.

As can be seen from Table 1, the intake of saline for eight days was sufficient to give considerable hypertrophy of the salt glands. The amount

Fig. 6. Mass spectrum of the trimethylsilyl derivative of desulfated sulfatide (cerebroside) of salt glands of salt-loaded ducks. The major molecular species found (*see* text) is reproduced in the top formula, and some important fragmentations are indicated. The temperature of the ion source was 190 °C, the electron energy 70 eV, and the acceleration voltage 6 kV

Table 1. *Effects of adaptation to hypertonic saline on animal weight, salt gland weight, and total lipid content of the glands (Experiment 1)*

Weight affected	Ducks on freshwater	Ducks on saline
Duck weight (kg)	2.27 ± 0.18 (n = 10)	2.08 ± 0.24 (n = 10)
Salt glands:		
Wet weight (g/pair)	0.254	0.666
Dry weight (g/pair)	0.077	0.199
Lipid content[a] (% of dry weight)	13.4	18.6

[a] Lipid content is based on weight of combined extracts. The extracts were not washed and may contain nonlipid material.

of total lipids/gram dry tissue increased by 40%, probably reflecting an increased amount of membranes in relation to other cell constituents. An increase of this order was also noted for all lipid fractions studied, except sulfatides (*see* Table 2). Sulfatide determinations on total lipid extracts are probably more precise than the other determinations, because there are relatively few laboratory steps. These determinations reveal a sulfatide increase of 190% in experiment 1 and 220% in experiment 2. The increase is practically the same as that found for the Na^+–K^+-ATPase, 180% in experiment 1 and 220% in experiment 2. This means that the sulfatide fraction is the only one among the major salt gland lipid fractions with a constant ratio to the Na^+–K^+-ATPase.

Discussion

On adaptation of ducks to the same hypertonic saline as used in the present experiment, Holmes *et al.* [10, 16] found a rapid increase in salt gland RNA (maximum reached in 24 hr) and a somewhat slower increase in wet weight, protein and Na^+–K^+-ATPase, the latter reaching a maximum after about five days. This is the reason for an adaptation period of eight days in the present experiment. The increase in Na^+–K^+-ATPase activity found by Fletcher, Stainer and Holmes [10] is about the same as that found in our study (using similar methods), and these workers reported a correlation of the enzyme activity and NaCl excretion.

Other glycolipids with similar properties to sulfatide may interfere with the anthron determinations. However, no such lipids were detected. Neutral lipids like diglycosylceramide and triglycosylceramide should separate from sulfatide on the ion exchange column, and gangliosides (acidic lipids) should

Table 2. *Effects of adaptation to hypertonic saline on the* Na^+–K^+*-ATPase and the lipid pattern of duck salt gland*[a]

Component	Experiment 1			Experiment 2		
	Freshwater	Saline	Ratio Saline / Freshwater	Freshwater	Saline	Ratio Saline / Freshwater
Na^+–K^+-ATPase	3.6	9.9	2.8	3.6	11.6	3.2
Cholesterol (P)	32.9	45.5	1.4	36.7	59.0	1.6
Cerebrosides (P)	0.82 (0.80–0.83)	1.11 (1.09–1.13)	1.4	1.20 (1.14–1.27)	1.78 (1.71–1.84)	1.5
Glucosylceramides	0.55	0.78	1.4	0.86	1.31	1.5
Galactosylceramides	0.27	0.33	1.2	0.34	0.47	1.4
Ethanolamine phosphoglycerides (N)	23.9 (23.3–24.3)	37.8 (37.6–38.1)	1.6	27.6 (26.6–28.3)	39.2 (36.2–41.8)	1.4
Choline phosphoglycerides (N)	34.4 (33.8–34.9)	46.5 (45.9–46.9)	1.4	36.4 (35.3–36.9)	48.7 (45.7–51.5)	1.3
Sphingomyelins (N)	11.5 (11.1–12.1)	16.0 (15.2–16.8)	1.4	10.7 (10.0–11.1)	17.5 (17.2–17.8)	1.6
Cardiolipins	4.1(A) (3.9–4.3)	4.9(A) (4.8–5.2)	1.2	6.9(T) (6.7–7.1)	11.2(T) (11.0–11.5)	1.6
Inositol phosphoglycerides	2.2(A) (2.1–2.4)	4.0(A) (3.6–4.4)	1.8	3.9(T) (3.6–4.4)	7.0(T) (6.4–8.0)	1.8
Serine phosphoglycerides	4.1(A) (3.6–4.3)	5.5(A) (5.2–6.1)	1.4	4.9(T) (4.4–5.4)	7.5(T) (7.2–7.8)	1.5
Sulfatides (T)	1.74 (1.51–1.96)	5.05 (5.00–5.09)	2.9	1.81 (1.51–2.24)	5.77 (5.61–5.95)	3.2
Sulfatides (A)	1.40 (1.30–1.60)	3.54 (3.48–3.58)	2.5	1.93 (1.90–1.95)	5.65 (5.41–6.15)	2.9
Sulfatides (P)	1.15 (1.13–1.18)	4.05 (4.00–4.10)	3.5	– –	– –	–

[a] The ATPase activity is expressed as mM P_i/g dry tissue/hr, and the lipid concentration as μM/g dry tissue. Determinations have been made on total lipid extract (T), acidic lipid fraction (A), neutral lipid fraction (N) or pure lipid (P). Lipid values, based on a colorimetric analysis, are the means of three estimates, and the extremes are given within parentheses.

have been detected as, e.g., glucose and sialic acid in the carbohydrate analysis. The only carbohydrate found in the sulfatide preparation was galactose. Carbohydrate material of low molecular weight that may interfere is expected to be removed in the dialysis step.

The identification of a 3-substituted cerebroside in the sulfatide preparation by mass spectrometry is conclusive. The evidence for a sulfate group as the substituent is indirect (the lipid is acidic and has infrared characteristics of a sulfatide). The sulfatide isolated from the salt gland of herring gull has, however, been further characterized by a semiquantitative precipitation of $BaSO_4$ after hydrolysis [24].

Earlier results have shown high sulfatide concentrations and high Na^+–K^+-ATPase activities in several tissues specialized for Na ion transport, such as kidney [20], avian salt gland [22, 24] and rectal gland of spiny dogfish [21, 25]. The present results from a functional adaptation show a correlation between Na^+–K^+-ATPase and sulfatides, which is unique among the lipids examined. In this study, the ratio Na^+–K^+-ATPase/sulfatide (expressed as mM P_i h^{-1}/μM) is approximately 2. The same value was found for herring gull and eider duck salt gland [24] and a slightly higher value (about 3.0) for the rectal gland of spiny dogfish [25]. These results indicate a role for sulfatides in Na ion transport.

It should be noted that sulfatides differ from phospholipids in their relative cation affinity. The cation affinity series for brain sulfatides has been found [1] to be Ca > Mg > K > Na > Li, compared to Ca > Mg > Li > Na > K for phospholipids [2, 3].

Recently evidence has been presented that several other lipids may be involved in specific cellular processes associated with membranes. A purified lipoprotein with 5′-nucleotidase activity was shown to contain sphingomyelin as the only lipid [40], and cardiolipin is probably the sole lipid in cytochrome oxidase preparations [5]. Phosphatidylglycerol seems to be specifically required for bacterial sugar transport mediated by the phosphoenol pyruvate-phosphotransferase system [26], and phosphatidylethanolamine is the chromophore binding site (forming a Schiff base) in native bovine rhodopsin [30]. These lipid molecules are relatively simple compared to the complex glycosphingolipids with blood group activities [13].

The present work is part of the project "Structure and Function of Sphingolipids", and was supported by a grant from The Tri-Centennial Fund of the Bank of Sweden.

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