# Diacyl, alkenyl, and alkyl ether phospholipids in ejaculated, in utero-, and in vitro-incubated porcine spermatozoa

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**Abstract** The phospholipids of porcine spermatozoa were analyzed by a hydrolytic procedure directly after ejaculation, and after incubation for 120 min in vitro or in ligated uterine segments of females with induced estrus. Total phospholipid content of ejaculated sperm was 65.7 μg lipid P per $10^9$ sperm, of which 41% was alkyl ether and 23% was alkenyl ether glycerophospholipid. All of the ether phospholipids were choline and ethanolamine glycerophospholipids. In order of decreasing amount (% of total phospholipid), the phospholipids were choline and ethanolamine glycerophospholipids (49.9 and 28.2), sphingolipid (10.6), cardiolipin (5.5), phosphatidylinositol (2.3), phosphatidic acid (1.5), phosphatidylserine (1.2), and phosphatidylglycerol (0.8). Phosphorus-containing sphingolipid separated into two components during thin-layer chromatography. Sphingosine was the only long-chain base identified in either band. Major fatty acids in the band with lower $R_f$ were 16:0 (56%), 20:0 (23%), and 18:0 (11%) plus smaller amounts of 14:0, 18:1, and 22:0, while those in the band with higher $R_f$ were 14:0 (30%), 16:0 (45%), and 18:1 (12%) plus smaller amounts of 18:0, 20:0, and 22:0. Choline was the only water-soluble base present in the lower $R_f$ sphingomyelin while ethanolamine was prevalent in the higher $R_f$ component. Incubation of washed spermatozoa in $Ca^{2+}$-free Ringer-fructose at 37°C for 2 hr produced no significant change in the level of any of the phospholipids. Incubation of washed sperm in the uterus for 2 hr, in the presence of oviductal secretions, produced an increase in phosphatidylcholine from 7.2 to 10.2 μg lipid P per $10^9$ sperm.—**Evans, R. W., D. E. Weaver, and E. D. Clegg.** Diacyl, alkenyl, and alkyl ether phospholipids in ejaculated, in utero-, and in vitro-incubated porcine spermatozoa. *J. Lipid Res.* 1980. **21:** 223–228.

**Supplementary key words** sperm · sphingolipids · glycerophospholipids

In the female reproductive tract, sperm undergo membrane-related changes prior to fertilization (1, 2). The nature of the changes and the mechanisms by which they occur are still unclear. As important membrane components, sperm phospholipids must be examined to determine if alterations are induced by the female.

Snider and Clegg (3), using thin-layer chromatography, reported that phospholipids of pig sperm were altered during incubation of semen in ligated segments of the uterus or oviduct. In the present study, phospholipids were analyzed in ejaculated sperm and in washed sperm exposed to in utero conditions that induce the acrosome reaction. Most of the seminal plasma was removed from the sperm prior to incubation in vivo and oviductal fluid was included in the medium. Sperm were also incubated in vitro for comparative purposes. The phospholipids were analyzed by a hydrolytic procedure that distinguished among diacyl, alkenyl ether, and alkyl ether analogs.

## MATERIALS AND METHODS

### Semen collection and processing

Four mature boars were used in this study. Sperm-rich semen was collected into a container maintained near body temperature. During collection, the semen was filtered through two layers of Miracloth (Calbiochem, San Diego, CA) to remove large gel particles and then filtered within 10 min through glass wool as described by Lunstra, Clegg, and Morré (4) to remove smaller gel particles and dead sperm. The filtered semen was diluted with three volumes of calcium-free Ringer-fructose solution (5) and centrifuged at 600 *g* for 15 min. After aspiration of the supernatant, the spermatozoa were resuspended gently in warm Ringer solution to 5–7 × $10^8$ sperm per ml before introduction into the female. Approximately 30 to 45

Abbreviations: TLC, thin-layer chromatography; GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine.

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min elapsed between collection and the beginning of both in vivo and in vitro incubations.

### Induction of estrus

Estrus was induced in prepuberal gilts by the procedure of Dziuk and Gehlbach (6). Gilts weighing between 40 and 50 kg were injected subcutaneously with 1250 IU of pregnant mare serum gonadotropin (see acknowledgment) followed 96 hr later by an intramuscular injection of 500 IU of human chorionic gonadotropin (HCG; Ayerst Laboratories, New York, NY). Gilts responding to this treatment usually ovulate 40 to 42 hr after HCG. Incubation of spermatozoa was scheduled to begin 4 to 6 hr after ovulation.

### Surgery and in vivo incubation

Sodium thiamylal (Surital; Parke-Davis, Detroit, MI) was injected into an ear vein to induce anesthesia in the gilts. The surgical level of anesthesia was reached and maintained using a mixture of halothane (Fluothane; Ayerst Laboratories) and nitrous oxide.

Preparation of ligated uterine segments, flushing of oviducts, and incubations of sperm in vivo were done by a modification of the procedure of Esbenshade and Clegg (7). Capsules containing ova were not used, and smaller cannulae (3 mm ID, 4 mm OD × 75 mm long connected to 24 cm of Silastic tubing) were substituted for the larger cannulae used to introduce and recover capsules from the uterus in the original procedure.

Washed sperm suspension (10 ml) was mixed with the fluid collected from one oviduct and then placed into a uterine chamber via the cannula. Cannulae were closed with rubber stoppers during the incubations. After 2 hr, manual pressure was applied to the uterus and the spermatozoa were recovered via the cannula. Sperm incubated in both uterine horns of the gilt were pooled for each replicate. An aliquot was removed for determination of sperm concentration. The samples were centrifuged immediately at 600 *g* for 15 min, the supernatants were aspirated, and the lipids were extracted from the sperm pellets.

### Control and in vitro-incubated samples

Sperm from the same ejaculates used for in utero incubations were used for the zero-time control and in vitro incubations. Immediately after resuspension in Ringer solution as described above, 20 ml of the sperm suspension was centrifuged at 600 *g* for 15 min, the supernatant was aspirated, and the spermatozoa were extracted to serve as a zero-time control. Another 20 ml aliquot was incubated for 2 hr at 37°C in a water bath. After incubation, these samples were handled as described for in vivo-incubated samples.

### Lipid extraction

Lipids were extracted from spermatozoa into 19 volumes of chloroform–methanol 2:1 (v/v). The samples were sonicated (Branson Model W-140, ½" probe tip) for 5 min with the tubes immersed in ice water and under a nitrogen atmosphere. The samples were allowed to stand for 30 min before filtration through solvent-washed Whatman No. 1 filter paper into a glass-stoppered, graduated cylinder. The tube, filter paper, and funnel were each rinsed three times with the extraction solvent mixture. The extracts were then washed with 0.73% NaCl (0.2 volumes of one-phase extract) and subsequently with theoretical upper phase solvent (chloroform–methanol–water 3:48:47, by volume) as described by Folch, Lees, and Sloane Stanley (8).

### Phosphorus determination

The method of Chen, Toribara, and Warner (9) was used as modified by Snider and Clegg (3) except that color was developed for 15 min at 50°C. The samples were then allowed to stand at room temperature for 90 min prior to measurement of absorbance at 820 nm against a reagent blank. When silicic acid was present in the tubes, each sample was centrifuged before measurement of absorbance.

### Lipid hydrolysis and separation of phosphate esters

Phospholipids from sperm lipid extracts and purchased standards were hydrolyzed selectively using successive alkaline and acidic conditions and the products were separated into chloroform-soluble and water-soluble fractions as reported by Wells and Dittmer (10). The water-soluble phosphate esters were applied onto anion exchange columns (Bio-Rad AG1-X2, 200–400 mesh, formate form). Column packing, sample application, and elution were done as described by Dittmer and Wells (11). The column was equilibrated with and the samples were applied in pH 9.5 buffer, followed by elution with pH 8.5 buffer (11). Peaks eluting from the column were identified by comparisons of relative elution volumes with those for the standards.

### Thin-layer chromatography

Total lipid extracts and phospholipids in the alkali- and acid-stable fractions (chloroform-soluble) were separated by one-dimensional thin-layer chromatography (TLC) on activated (1 hr at 110°C) silica gel plates (Merck Silica Gel 60, F-254) using chloroform–



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methanol–diethylamine–water 110:50:8:5, by volume (12) as the developing solvent. Phosphorus-containing compounds were visualized using the molybdenum blue spray reagent of Dittmer and Lester (13). Identities of phospholipids on TLC plates were established by co-chromatography with purchased standards (Supelco, Bellefont, PA). For quantification of phosphorus-containing compounds, molybdenum blue-positive spots were scraped from the plates and transferred to tubes for phosphorus determination.

### Characterization of phosphosphingolipids

Phosphosphingolipid was separated into two components during TLC. Each component was extracted from the silica gel with methanol, as were appropriate blank areas of silica gel. Long-chain bases were prepared for TLC as described by Ledeen, Yu, and Eng (14). An aliquot of the sphingolipid in methanol was evaporated to dryness under $N_2$ and dissolved in 2 ml 1N methanolic HCl containing 10 M water. The samples were sealed in glass tubes, heated at 70°C for 23 hr, cooled and then extracted three times with hexane (3 ml each time). The hexane extracts were combined and saved for analysis of fatty acids. The methanolic phase was evaporated and the long-chain bases were separated by TLC (silica gel plates) using chloroform-methanol-2N $NH_4OH$ 160:40:4, by volume (15). Developed plates were sprayed with ninhydrin (Nin-Sol, Pierce Chemical Co., Rockford, IL).

For analysis of fatty acids, the hexane fraction (see above) was evaporated to dryness under $N_2$. After addition of 1 ml 0.1 N anhydrous methanolic HCl, tubes were sealed and heated at 80°C for 1 hr (14). After cooling, 10 $\mu$g of heptadecanoic acid methyl ester were added as an internal standard. Samples were evaporated to dryness under $N_2$, redissolved in acetone and separated by gas–liquid chromatography at 210°C on a 1.8 m glass column packed with 10% SP2330 on 100/120 mesh Chromosorb W AW (Supelco).

To analyze the monophosphate esters of the sphingolipids, samples were evaporated to dryness under $N_2$. After addition of 1.25 ml 2N anhydrous methanolic HCl, tubes were sealed and heated in a boiling water bath for 4 hr (16). After cooling, samples were evaporated under $N_2$ and 0.5 ml water (ether-saturated) plus 2 ml ether (water-saturated) was added. Samples were mixed on a vortex mixer and centrifuged at 300 *g* for 10 min. The lower phase was washed with 1 ml ether (water-saturated), evaporated to dryness under $N_2$ and dissolved in water for TLC on silica gel using ethanol (95%)–2.7 M ammonium acetate (pH 5.0) in a 7:3 (v/v) ratio in a paper-lined tank (17).

## RESULTS

### Ejaculated sperm

After alkaline hydrolysis of the phospholipids from ejaculated sperm, anion-exchange chromatography resolved six well-defined peaks that represented the water-soluble phosphate esters from diacyl glycerophospholipids. The compounds were, in order of elution, glycerophosphorylcholine (GPC), glycerophosphorylethanolamine (GPE), glycerophosphorylinositol, glycerophosphorylserine, glycerophosphate, and bis(glycerophosphoryl)glycerol. Those were derived from phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, and cardiolipin, respectively. In addition, a small peak containing glycerophosphorylglycerol (from phosphatidylglycerol) was eluted immediately after glycerophosphorylinositol. Two broader and less well-defined peaks eluted after bis(glycerophosphoryl)glycerol. Those components, which were probably the hydrolysis products of di- and triphosphoinositides, were not quantified since polyphosphoinositides are not recovered quantitatively from sperm by the procedure used in this study. Elution volumes, relative to that from cardiolipin, were similar to those reported previously (11).

After hydrolysis of the deacylated phospholipids with mild acid, two peaks, GPC and GPE from the alkenyl ether phospholipids (plasmalogens), were eluted from the column. Thin-layer chromatography of the alkali- and acid-stable phospholipids revealed spots that migrated with lysophosphatidylcholine, lysophosphatidylethanolamine (both from alkyl ether phospholipids) and sphingolipid (**Fig. 1**). Phosphosphingolipid was usually separated into two components as reported previously by Snider and Clegg (3). When the separation was complete, the component with the higher $R_f$ value (less polar) represented about 27% of the total phosphosphingolipid in ejaculated sperm.

Quantitatively, alkyl ether phospholipids containing choline and ethanolamine were the major components of ejaculated sperm with 18.0 (27.4%) and 9.1 (13.9%) $\mu$g lipid P per $10^9$ sperm, respectively (**Table 1**). Ethanolamine and choline plasmalogen, phosphatidylcholine (diacyl), and phosphosphingolipid were each present at levels of about 11%. Cardiolipin accounted for 5.5% and phosphatidylethanolamine for only 3.0% of the total phospholipid. Phosphatidylinositol, phosphatidic acid, phosphatidylserine, and phosphatidylglycerol were all minor components (2.3%, 1.5%, 1.2%, and 0.8%, respectively). Thin-layer chromatography of total lipid extracts failed to detect lysophos-



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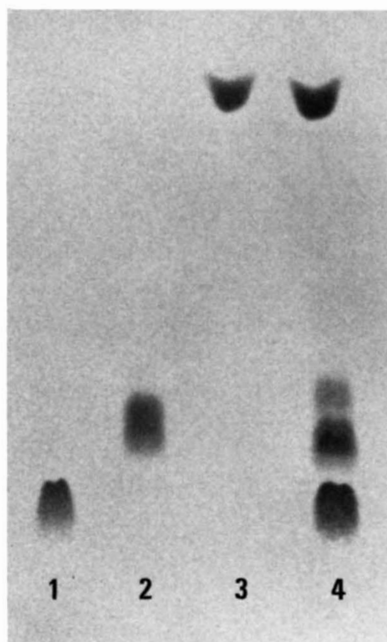


**Fig. 1.** Thin-layer plate showing separation of alkali-acid stable phospholipids from porcine sperm. Columns represent: 1) lysophosphatidylcholine standard, 2) sphingomyelin standard, 3) lysophosphatidylethanolamine standard, and 4) alkali-acid stable phospholipids from porcine spermatozoa.

phatidylcholine or lysophosphatidylethanolamine in either ejaculated or incubated spermatozoa.

Alkyl ether phospholipids comprised 41.3% and plasmalogens 22.9% of the total phospholipid. Thus, 64% of the phospholipids contained an ether linkage between one of the hydrocarbon side chains and the glycerol headgroup.

The two sphingolipid (Fig. 1) components were analyzed after separation by TLC. TLC of the long-chain bases showed that sphingosine was the only long-chain base present. However, separation of the water-soluble bases revealed that the more polar sphingolipid (lower $R_f$) contained choline, while the less polar sphingolipid band contained ethanolamine plus choline. The presence of choline in the upper band could have been due to incomplete separation of the two sphingolipid components with preparative TLC, although differences in fatty acid composition may also have caused some choline-containing sphingolipid to exhibit a polarity similar to that of the ethanolamine-containing sphingolipid. Identification of an ethanolamine sphingolipid is compatible with ninhydrin-positive staining of that band observed previously.

The fatty acid compositions of the two phosphosphingolipid bands were substantially different (**Table 2**). Both bands contained predominantly saturated fatty acids, with 16:0 accounting for 56% and 45% of the total for the lower and upper bands, respectively. The lower band contained 23% 20:0, while the upper band had 30% 14:0. Both bands also contained smaller amounts of 18:0, 18:1, and 22:0.

### Incubated sperm

Phosphatidylcholine increased significantly during in vivo incubation from 7.2 to 10.2 μg P per $10^9$ sperm (Table 1). However, the apparent increase in total phospholipid from 65.7 to 72.4 μg P per $10^9$ sperm was not statistically significant. No significant changes occurred in any of the phospholipids during in vitro incubation. Under the conditions of these incubations, sperm motility was approximately 44% after 90 min, but then declined to 10–15% after 120 min (7).

## DISCUSSION

Analyses of the phospholipids in ejaculated porcine sperm using TLC have been reported previously (3, 18, 19). Total lipid phosphorus observed in this study was similar to the 66.5 and 71.6 μg per $10^9$ sperm reported by Snider and Clegg (3) and Johnson, Gerrits, and Young (18). When individual phospholipids are considered, there is close agreement with the results of Snider and Clegg (3), even though separations were achieved using anion-exchange chromatography of hydrolysis products instead of by TLC. Johnson et al. (18) measured somewhat higher levels

TABLE 1. Effect of incubation on phospholipids of porcine spermatozoa

| Phospholipid | Ejaculated | Incubated: In utero | Incubated: In vitro |
|---|---|---|---|
| | | *μg lipid P per $10^9$ sperm*[a] | |
| Choline glycerophospholipid | 32.8 ± 0.7 | 36.4 ± 1.7 | 32.2 ± 1.2 |
| Diacyl | 7.2 ± 0.6 | 10.2 ± 0.6[b] | 6.8 ± 1.3 |
| Alkenyl ether | 7.6 ± 1.1 | 7.1 ± 0.7 | 6.6 ± 0.2 |
| Alkyl ether | 18.0 ± 1.3 | 19.0 ± 0.8 | 18.8 ± 1.9 |
| Ethanolamine glycerophospholipid | 18.5 ± 0.6 | 19.2 ± 1.2 | 17.5 ± 0.9 |
| Diacyl | 2.0 ± 0.3 | 2.5 ± 0.2 | 1.6 ± 0.2 |
| Alkenyl ether | 7.4 ± 0.8 | 7.5 ± 0.6 | 6.4 ± 0.5 |
| Alkyl ether | 9.1 ± 0.4 | 9.2 ± 0.7 | 9.4 ± 1.1 |
| Sphingolipid | 7.0 ± 1.7 | 8.0 ± 1.9 | 7.4 ± 1.8 |
| Cardiolipin | 3.6 ± 0.3 | 4.4 ± 0.7 | 3.8 ± 0.5 |
| Phosphatidylinositol | 1.5 ± 0.2 | 1.9 ± 0.5 | 1.5 ± 0.1 |
| Phosphatidic acid | 1.0 ± 0.3 | 1.0 ± 0.2 | 1.0 ± 0.3 |
| Phosphatidylserine | 0.8 ± 0.2 | 1.0 ± 0.4 | 0.5 ± 0.1 |
| Phosphatidylglycerol | 0.5 ± 0.1 | 0.5 ± 0.2 | 0.4 ± 0.2 |
| Total phospholipid | 65.7 ± 2.0 | 72.4 ± 4.5 | 64.4 ± 3.3 |

[a] Values represent the mean ± SEM for four replicates, each from a different boar.
[b] Significantly different ($P < 0.05$) from ejaculated and in vitro-incubated when compared using Student's $t$ test.



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TABLE 2. Fatty acid composition of the two phosphosphingolipid components in ejaculated porcine sperm

| Sphingolipid Band | Fatty Acid[c] | | | | | |
|---|---|---|---|---|---|---|
| | 14:0 | 16:0 | 18:0 | 18:1 | 20:0 | 22:0 |
| | % | | | | | |
| Lower[a] | 2.8 ± 1.6 | 55.8 ± 2.5 | 10.6 ± 0.9 | 6.2 ± 0.8 | 22.6 ± 5.0 | 2.0 ± 0.3 |
| Upper[b] | 30.0 ± 2.1 | 44.6 ± 1.6 | 3.6 ± 0.5 | 11.9 ± 0.9 | 5.9 ± 2.2 | 3.9 ± 2.2 |

[a] Band with lower $R_f$ on TLC contained only choline as the water-soluble base.
[b] Band with higher $R_f$ on TLC contained ethanolamine and choline as the water-soluble bases.
[c] Values represent percent of total fatty acid ± SEM for samples from three boars.

of sphingomyelin and phosphatidylserine and a lower level of choline phosphatide.

Alkenyl ether phospholipids have been identified in porcine sperm by Grogan, Mayer, and Sikes (19). The choline and ethanolamine plasmalogens each represented approximately 10% of the total phospholipid. In the present study, choline and ethanolamine plasmalogens were 11.6% and 11.3% of the total, respectively.

The combined content of alkenyl and alkyl ether phospholipid of 64% of the total phospholipid was unusually high for mammalian cells. Of the 64%, the alkyl ethers contributed nearly two-thirds. The only previous report of alkyl ether phospholipids in sperm found levels of 11–13% in ovine sperm (20). However, plasmalogens comprised over 45% of the total phospholipid. Thus, the combined total for the ether phospholipids of approximately 58% in ovine sperm is similar to the total of 64% measured in this study for porcine sperm. Although the significance of the high level of ether phospholipids in sperm is not known, they may be resistant to enzymatic hydrolysis. Stability of their phospholipids could be advantageous to sperm survival during maturation in the epididymis. The absence of lysophospholipids in the sperm supports the concept that a phospholipase A is not active on phospholipids of porcine sperm at ejaculation or during at least the early stage of capacitation.

The increase in the phospholipid content of porcine sperm during incubation in utero does not necessarily indicate phospholipid synthesis by the sperm. Instead, the phospholipid may have been adsorbed or incorporated into the sperm from the female tract secretions. When Ringer was incubated in ligated uteri as a control, the recovered fluid contained sufficient phospholipid to account for the increase in sperm phospholipid if most of that phospholipid was incorporated by the sperm. Evans and Setchell (21) have demonstrated with radioautography that $^{32}$P- and $^{3}$H-labeled phosphatidylcholine associates with sperm during incubation of sperm in vitro with the phospholipid.

Whether the apparent increase in diacyl phosphatidylcholine in porcine sperm during incubation in vivo is related to capacitation is unknown. Davis (22) found that incubation of rabbit sperm with liposomes containing dimyristoyl- or dipalmitoylphosphatidylcholine plus cholesterol interfered with the ability of sperm to fertilize ova. However, exposure of sperm to phospholipid liposomes alone did not affect fertilization rate. Subsequent work by Davis (23) has indicated that rat sperm exposed to bovine serum albumin show a decreased cholesterol to phospholipid ratio that occurs concurrently with acquisition of fertilizing ability.

In the present experiment, phosphatidylcholine increased when sperm were exposed to female reproductive tract secretions. Parallel experiments done under the same incubation conditions[3] found no change in sperm cholesterol. Nevertheless, the net effect would be a decreased cholesterol to phospholipid ratio that is compatible with the in vitro work with rats.

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