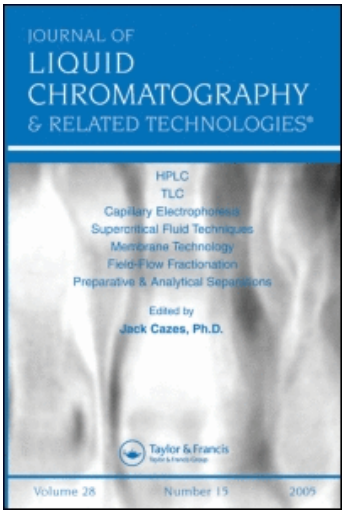

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THIN LAYER CHROMATOGRAPHY OF PHOSPHOLIPID COMPOSITION IN MOUSE AND RABBIT SPERMATOZOA

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ABSTRACT

The time course of the loss of different components of the phospholipids of rabbit and mouse epididymal spermatozoa during spontaneous lipid peroxidation was determined, using thin layer chromatography with a specific situ hydrolysis method to differentiate the acyl and alkenyl (plasmalogen) moieties. The components followed were phosphatidylethanolamine (PE), phosphatidylcholine (PC), the PE and PC phasmalogens, sphingomyelin (SP), cardiolipin (CL), and phosphatidyl-glycerol (PG). In both mouse and rabbit sperm, the PE component was found to be more than 90% diplasmalogen: 1,2-di(O-1′-alkenyl) glycerophosphoethanolamine. This component was lost rapidly during peroxidation. All PE has disappeared from rabbit sperm after 4 h aerobic incubation, at which point the other phospholipids had been little affected. In both mouse and rabbit sperm, the PC component was found to be 50% monoplasmalogen. The decrease in PC plasmalogen of rabbit sperm amounted to 74% after 20 h, compared to 42% loss of total PC. Similar observations were made with mouse sperm, except that rates of loss of all components were approximately twice those in rabbit. Distribution of the phospholipid components between sperm heads and tails was also determined: PE diplasmalogen was almost entirely found in the tail fraction, in both mouse and rabbit

sperm. This mode of analysis allows the differentiation of sensitivities towards spontaneous peroxidation of the different types of phospholipid present in sperm membranes.

INTRODUCTION

The toxicity of O_2 to mammalian spermatozoa is manifested most directly by loss of motility, which in turn has been linked to peroxidation of the sperm cell lipids. This has been documented for the case of lipid peroxidation in ram and human sperm induced by Fe^{2+} plus ascorbate (1-7) and for the case of lipid peroxidation occurring spontaneously in rabbit and mouse sperm (8,9). In both systems, loss of motility from lipid peroxidation could be traced to membrane damage resulting in increased permeability allowing loss of essential intracellular substrates (4,10). Jones and Mann (2) noted that the alkenylether (plasmalogen) content of ram sperm was preferentially lost during induced peroxidation. This observation was of particular interest in view of our recent finding that rabbit sperm PE consisted almost entirely of diplasmalogen [1,2-di(O-1′-alkenyl) phosphatidylethanolamine], while the phosphatidylcholine (PC) component contained only monoplasmalogen (11). This determination was made using methodology recently developed for specific analysis of plasmalogens with thin layer chromatography (12). The methodology provides a rapid means of assessing changes in cell phospholipids due to spontaneous peroxidation. In this paper, we report the determination of the phospholipid content, type, and plasmalogen fraction in mouse sperm and show that, in both mouse and rabbit sperm, PE is mostly diplasmalogen and is remarkably sensitive to peroxidative loss.

MATERIALS AND METHODS

Reagents

Synthetic standards for the phospholipids and their hydrolysis products were obtained from Avanti Biochemicals (Birmingham, AL) and Sigma Chemical Co. (St. Louis, MD). These standards were chosen to give a close approximation to the unsaturated and saturated acyl and alkyl moieties found in mammalian sperm. Schiff Reagent (Kates, 1972) for staining of aldehydes was obtained from Accra-Lab, Inc. (Bridgeport, NJ). Whatman precoated Silica LK5 and LHP-K plates 10 x 10 cm with preabsorbent zone were obtained from Whatman Inc. (Clifton, NJ). Silica gel H plates 20 x 20 cm, were obtained from Analtech, Inc. (Newark, DE).

Preparation and Aerobic Incubation of Spermatozoa

Epididymal spermatozoa from mature male rabbits and retired breeder mice were prepared as described by Alvarez and Storey (8,9). Four media were used for aerobic incubation of sperm. For rabbit sperm, the media were NTP: 103 mM NaCl, 10 mM KCl, 12.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 3 mM $MgCl_2$, 20 mM Tris, 1.5 mM D-glucose, 0.4 mM EDTA, 0.6% penicillin-streptomycin, pH 7.4; and KTP with 113 mM KCl and 1.5 mM KH_2PO_4, but otherwise identical to NTP. For mouse sperm, the media were NTPC: 113 mM NaCl and 1.7 mM $CaCl_2$, pH 7.4. Spermatozoa were diluted 10-fold to give suspensions containing 0.2-1.0 x 10^8 cells/ml: 1 ml suspension was placed in a

10 ml bottle with Teflon-lined cap in a shaking water bath at 37°C. The stock solution of mouse spermatozoa was diluted 3-fold to give suspensions containing 0.1-1.0 x 10^8 cells/ml; 0.3 ml of suspension was placed in a 5 ml bottle as described for rabbit sperm. Aerobic incubations were terminated at selected time intervals by placing the samples on ice, which stops further reaction with O_2 (8,13).

Preparations of sperm heads and tails were prepared from stock suspensions of 1.5 x 10^9 cells/ml for rabbit and 4.0 x 10^8 cells/ml for mouse sperm by sonication at low power for either 4 min (rabbit) or 3 sec (mouse) under ice cooling. The suspensions were centrifuged at 120 x g for 15 min and the supernatant with sperm tails was removed. The pellet was resuspended in 10 ml medium, centrifuged twice at 120 x g for 10 min, then centrifuged at 700 x g for 10 min to obtain the final pellet (head fraction) which was resuspended in 1 ml medium. The tail fraction was centrifuged twice at 120 x g for 10 min, the pellet discarded, then centrifuged at 700 x g for 40 min. The pellet (tail fraction) was resuspended in 1 ml medium. Contamination of the two fractions determined by hemocytometer was 4% or less tails in the head fraction and 8% or less heads in the tail fraction.

Thin Layer Chromatography of Sperm Phospholipids

After termination of aerobic incubation, a 20 ul aliquot of the sperm suspension was applied directly to the preabsorbent zone

of the scored Whatman LK5 silica gel plate. The layers were predeveloped three times in 1:1 chloroform-methanol to the interface of the preabsorbent zone to extract the phospholipid and deposit it as a line on the starting point of the chromatogram. The mobile phase was chloroform-ethanol-triethylamine-water (30:30:34:8) (14). Development was allowed to proceed for ca. 1.5 hr until the mobile phase reached 2 cm from the top of the plate. The plates were dried and 1 inch strips from both edges, where the standards had been applied, were cut and dried at 170°C for 2 min to remove residual solvent. Half of the strips were sprayed with a 10% (w/v) solution of $CuSO_4$ in 8% (v/v) H_3PO_4, and the other half were sprayed with a 0.2% solution of ninhydrin in acetone to identify the PE fraction. Then plates sprayed with $CuSO_4$ were dried for 5 min. at room temperature, heated in an oven at 110°C for 5 min, and finally placed in an oven at 170°C for 2 min. When phospholipid unsaturation was to be measured, a 3% (w/v) solution of Cu $(CH_3COO)_2$ in 8% H_3PO_4 which stains only unsaturated phospholipids was used (14). Plates sprayed with Cu $(CH_3COO)_2$ were heated at 110°C for 5 min, and then placed in an oven at 180°C for 10 min. Ninhydrin-sprayed plates were placed directly in an oven at 110°C for 5 min.

Plasmalogen analysis was carried out <u>in situ</u> by streaking 20 ul of the sperm suspension on the preabsorbent zone of scored LK5 plates. Then 25 ul of a 1:1 mixture of 2% (v/v) trichloroacetic acid (TCA) and 8% (v/v) HCl (TCA/HCl) were added to the sample. Plates remained at room temperature for 10 min to assure complete

hydrolysis of the alkenylether group. Predevelopments and final development were then carried out as described above. After final development, the plates were dried and one strip was cut from each edge of the plate in such a way that two lanes would be on each strip. These strips were dried at 170°C for 2 min. One was sprayed with the $CuSO_4$ reagent and the other with the ninhydrin reagent, as described above. The solvent front area was sprayed with a solution of Schiff Reagent (15) for aldehyde (A) identification and quantitation on the $CuSO_4$-sprayed plate.

The developed chromatograms were scanned in a Kontes Fiber Optic Scanner (Model 800), using a 440 nm filter and a Hewlett Packard 3390A integrator. Synthetic phospholipid standards between 0.5-5 ug gave linear curves, so procedures were adjusted that the amounts applied to the plates were in this range. Amounts of the unknowns were interpolated directly from the standard curves. The detectability limit was 0.05 ug phospholipid. Phospholipid phosphorus was determined by the method of Raheja et al. (16).

The phospholipids PG and CL were found to migrate together in the solvent system described above. The combined PG and CL bands were removed form the plate position located by $CuSO_4$ by adding a few drops of water, followed by extraction with 3 ml. of chloroform-methanol (1:1). The extract was filtered through a Millipore membrane (0.45 um) to remove residual silica and dried under nitrogen. Recovery was at least 95%. The two phospholipids

were separated on silica gel H plates. Predevelopment in 1:1 chloroform-methanol was allowed to reach 2.5 cm from the lower limit of the plate. Development with chloroform-methanol-acetic acid-water (60:14:6:2) (12) proceeded until the solvent front reached 3 cm from the top of the plate. The plates were then dried and 2.5 cm strips from both edges of the plates, where PG and CL standards had been applied, were used for location by $CuSO_4$ staining. Quantitation was by densitometry as described above.

RESULTS AND DISCUSSION

Comparison of Mouse and Rabbit Sperm Phospholipids

The presence of diplasmalogen in the ethanolamine phospholipid component of mouse epididymal spermatozoa is shown by comparison of the phospholipid profiles depicted by the densitometric traces in Figure 1. The top trace was obtained after extraction from fresh sperm suspended in medium NTPC and separation with $CuSO_4$ staining. The bottom trace was obtained from the same sample treated with the TCA/HCl reagent to hydrolyze the plasmalogen vinylether link. The peak for PE was nearly eliminated with no appearance of a peak for lyso PE. A parallel chromatogram stained with ninhydrin for ethanolamine species showed the presence only of glycerylphosphoethanolamine. The TCA/HCl reagent reduced the PC peak by about half, with appearance of the lyso form of PC, indicated by LPC in the chromatogram. The TCA/HCl regent hydrolyzes the O-1′-alkenyl groups attached to the

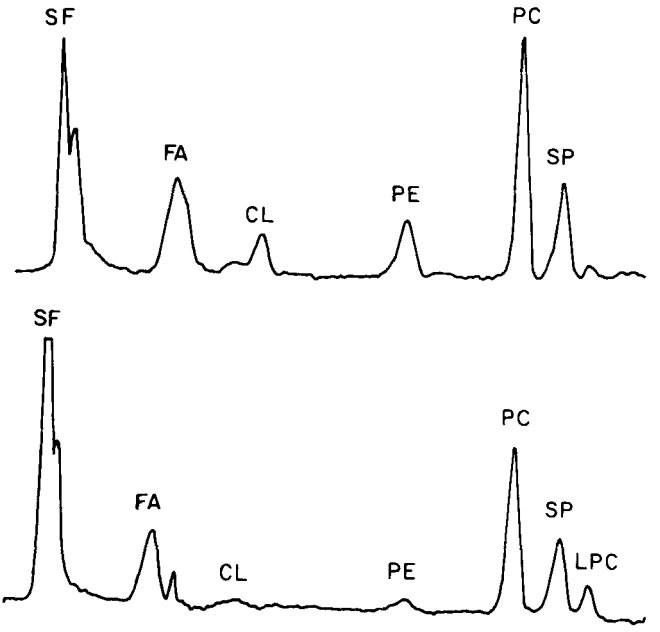

FIGURE 1. Densitometric traces from thin layer chromatography (TLC) plates on which phospholipids from fresh mouse epididymal spermatozoa were separated and stained with $CUSO_4$. The upper chromatogram shows the phospholipid profile obtained with fresh sperm and no further treatment. The components identified by the labelled bands to the right of the solvent front (SF) are: FA, fatty acid; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SP, sphingomyelin; LPC, lysophosphatidylcholine. The lower chromatogram shows the phospholipid profile obtained after treatment with TCA/HCL reagent.

glycerol moiety of the plasmalogen to aldehydes. A parallel chromatogram stained with Schiff Reagent showed the appearance of alkyl aldehyde in the amount expected for two aldehydes per PE and one per PC lost. This result corresponds to plasmalogen PE as diplasmalogen and plasmalogen PC as monoplasmalogen in mouse sperm, as was also observed in rabbit sperm (11).

The amounts of the different phospholipids present in fresh mouse and rabbit epididymal spermatozoa are shown in Table 1. Also shown in Table 1 is the phospholipid phosphorus determined independently for each component in rabbit sperm. The ratio of ug phospholipid phosphorus to ug phospholipid so obtained was remarkably constant: the mean ± S.D. was 0.0403 ± 0.0002 for the

TABLE 1. Phospholipid Content and Composition of Fresh Mouse and Rabbit Spermatozoa

Phospholipid[a]		Content[b] ug/10^8 cells	Unsat., %	PGEN[c] %	% of Total	Phos.,[d] ug/10^8 cells
PC	Rabbit	72±3	95	50	50.1	2.88±18
	Mouse	175±4	85	50	62.5	7.0
PE	Rabbit	20±1	94	90	13.9	0.81±0.17
	Mouse	35±1	95	93	12.5	1.4
SP	Rabbit	32±2	81	0	22.7	1.31±0.10
	Mouse	55±5	87	0	19.6	2.2
CL	Rabbit	7±1	40	30	4.8	0.28±0.04
	Mouse	15±2	74	30	5.4	0.6
PG	Rabbit	10±1	60	50	6.8	0.40±0.06
	Mouse	ND	-	-	-	-

[a]PC: phosphatidylcholine; PE: phosphatidylethanolamine; SP: sphingomyelin; CL: cardiolipin; PG: phosphatidylglycerol.

[b]Values are means ± S.D., n=10. Values for mouse sperm from this study, values for rabbit sperm from (11). ND: not detectable. Rabbit sperm also contain 1.7% lysophosphatidylcholine; this component was not detectable in mouse sperm.

[c]PGEN: plasmalogen; percentages are for each phospholipid component.

[d]Values for rabbit sperm phospholipid phosphorus content are means ± S.D., n=3. Values for mouse sperm were calculated using 0.040 for ratio of phosphorus to phospholipid (see text).

five components. This ratio is identical to that of 0.040 reported earlier by Scott et al. (17) for ram spermatozoa. It was used to calculate the phospholipid phosphorus for the phospholipid components in mouse sperm (which are available in considerably smaller amounts than rabbit sperm) in Table 1.

The distribution of phospholipid components between the heads and tails of mouse and rabbit sperm is shown in Table 2. Most of the PE and CL is associated with the tail fraction. Further

TABLE 2. Distribution of Phospholipids Between Heads and Tails of Mouse and Rabbit Sperm. Contents are Given in ug/10^8 Cells.

	Mouse		Rabbit	
PL[a]	Head	Tail	Head	Tail
PE	ND[b]	35±3	3±1	18±1
PC	105±7	65±9	31±3	39±4
SP	30±4	23±4	11±1	21±1
CL/PG	ND[b]	17±3	4±1[c]	21±1[c]

[a]PL: Phospholipid; PE: phosphatidylethanolamine; PC: phosphatidylcholine; SP: sphingomyelin; C:L cardiolipin; PG: phosphatidylglycerol. In this group of experiments, the separation step for PG and CL was not performed. The values for CL/PG are the combined phospholipids for rabbit sperm; mouse sperm does not have detectable PG (see Table 1.). Values are means ± S.D., n=7 for mouse sperm and n=12 for rabbit sperm.

[b]Not detectable

[c]In a separate group of experiments with rabbit sperm, all the CL was found in the tail fraction, while the distribution of PG was 20% head, 80% tail.

analysis of the PE in the rabbit sperm head did not detect plasmalogen and gave 55% unsaturation. Since the limit of detectability of plasmalogen is 1 ug/10^8 cells, this means that plasmalogen accounts for less than 25% of the head PE component. In contrast, all the tail PE in rabbit sperm assayed as diplasmalogen.

Lipid Peroxidation of Sperm Phospholipids

The TLC profile of phospholipids extracted from rabbit sperm, which had been incubated aerobically for 0.5, 4, and 20 hr in medium NTP, is shown in Fig. 2. The loss of PE after 4 hr is essentially complete, while PC, SP, and CL have decreased relatively little. The time course for the loss of the PC and PE components of rabbit due to peroxidation is shown in more detail in Fig. 3. In medium NTP (Fig. 3, upper panel), the complete loss of PE at 4 hr contrasts with the relatively slow loss of PC which amounted to 42% in 20 hr. The decrease in unsaturated PC followed that of but at 20 hr the loss of PC plasmalogen amounted to 74%. A surprising result was the increased lability of both PC and PE components of rabbit sperm in medium KTP (Fig. 3, bottom panel). At the first time point of 0.5 hr, PE had decreased to undetectable levels, less than 10% of PC plasmalogen remained, and only 50% of the PC component remained, taking the components still present in NTP at this time point as 100%. The early loss within 0.5 hr of the PC component can be accounted for by loss of PC plasmalogen. Motility decline and lipid peroxidation rate in

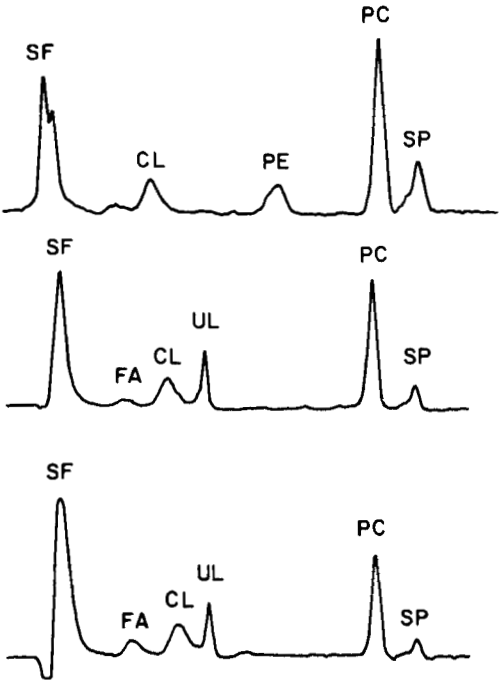

FIGURE 2. Densitometric traces from TLC plates on which phospholipids of aerobically incubated rabbit epididymal spermatozoa were separated and stained with $CuSO_4$. The aerobic incubation was carried out in medium NTP. Incubation times for each sample were: 0.5 h (upper trace), 4 h (middle trace), 20 h (lower trace). Bands are labelled as in Fig. 1; UL stands for unidentified lipid.

rabbit sperm were previously found to be far more rapid in medium KTP (8). At present, these unusual effects of KTP, which may be connected, are not yet understood. They do, however, serve to show that this chromatographic procedure is capable of monitoring rapid rates of peroxidative phospholipid loss.

The time course of phospholipid loss due to spontaneous peroxidation in mouse sperm is shown in Fig. 4. Two media were

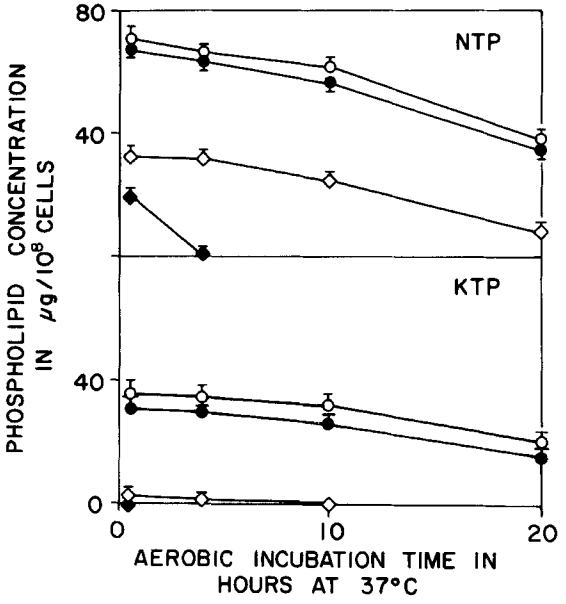

FIGURE 3. Time course of the loss of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from rabbit epididymal sperm aerobically incubated in medium NTP (top panel) and medium KTP (bottom panel). Symbols are as follows: (○), PC total; (●), PC unsaturated; (◇), PC plasmalogen; (◆) PE, which is over 90% diplasmalogen. Each point is the mean of 10 determinations; error bars are standard deviations.

tested: NTPC, in which resistance to peroxidation is maximal, and medium TNC, in which resistance is much less (9). Again, as seen with rabbit sperm, the PE component was the one most rapidly lost. The total PC content in medium NTPC decreased 80% over a period of 20 hr (Fig. 4, top panel), while PC plasmalogen was completely lost over a 10 hr. Loss of phospholipids was more rapid during aerobic incubation in medium TNC (Fig 4, bottom panel). The pattern of lipid loss however, was similar to that observed in medium NTPC.

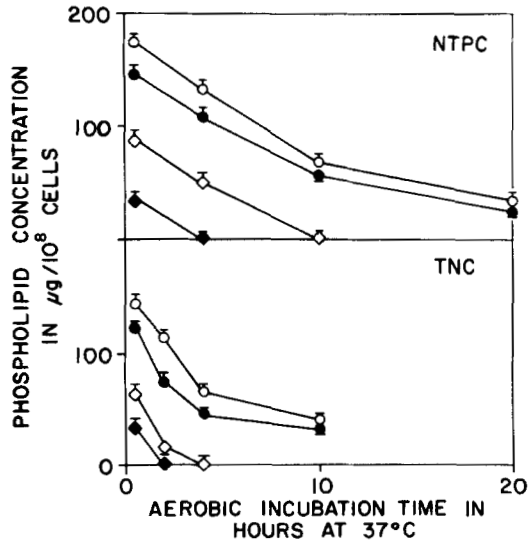

FIGURE 4. Time course of the loss of PC and PE from mouse epididymal sperm aerobically incubated in medium NTPC (top panel) and medium TNC (bottom panel). Symbols are those used in Fig. 3. Each point is the mean of 10 determinations; error bars are standard deviations.

Sphingomyelin content in rabbit epididymal spermatozoa incubated aerobically in medium NTP decreased linearly with incubation time over a period of 20 hr, at the end of which its content was 40% of the amount present prior to aerobic incubation (Fig. 5, upper panel). This loss in SP content was entirely due to the loss of unsaturated fatty acid moieties; the content of saturated SP remained constant. In medium KTP, SP loss occurred at a faster rate with a 70% loss over a period of 20 hr. Sphingomyelin content in mouse epididymal spermatozoa incubated aerobically in medium NTPC decreased linearly with incubation time

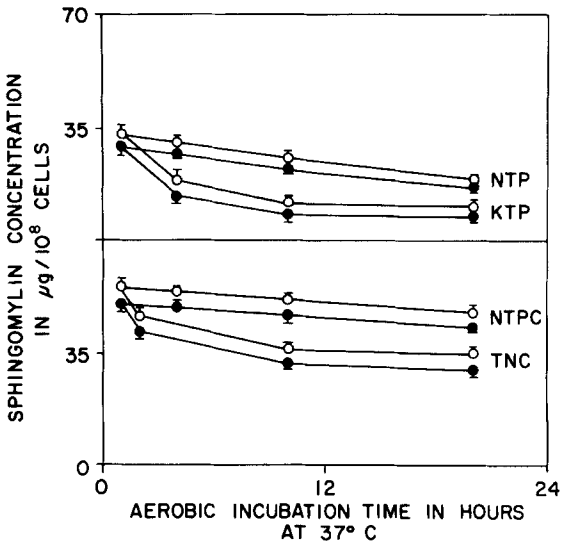

FIGURE 5. Time course of the loss of sphingomyelin (SP) from rabbit sperm incubated aerobically in medium NTP and medium KTP (top panel) and from mouse sperm incubated aerobically in medium NTPC and medium TNC (bottom panel). Open circles (○) represent total SP; closed circles (●) represent unsaturated SP.

with a 20% loss over a period of 20 hr (Fig. 5, bottom panel). The loss of SP content was also entirely due to the loss of unsaturated fatty acid moieties. When mouse sperm were incubated in medium TNC, SP loss occurred at a rapid rate with a 40% loss up to 10 hr, then with only an additional 5% loss up to 24 hr of aerobic incubation. SP seems to be the component most resistant to loss by peroxidation of the phospholipids in both mouse and rabbit spermatozoa.

The time course curves of figures 3-5 show that the TLC methods for phospholipid analysis provide a rapid and convenient

technique for monitoring the changes in phospholipid type and content of cells as a function of time and treatment. Cell suspensions can be applied directly to the plates to insure against losses which may arise during conventional extraction techniques. These methods, here demonstrated for spermatozoa, are applicable to following changes of cell phospholipds over time with any cell type.

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