Plasmalogen in Ram Semen, and its Role in Sperm Metabolism

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(*Received* 16 *July* 1958)

Whole semen can be stored successfully under both aerobic and anaerobic conditions, owing to the presence of glycolysable carbohydrate in the seminal plasma. However, if mammalian spermatozoa are separated from the seminal plasma by centrifuging and washing, they can maintain their motility only in the presence of oxygen. From this it has been inferred that when spermatozoa are deprived of fructose, which is the usual glycolysable carbohydrate in seminal plasma, they utilize the oxidation of intracellular reserves as a source of energy for motility. Lardy & Phillips (1941*a*, *b*, 1945) assigned this role of energy reserve to the phospholipids, pointing out that (i) the lipid-phosphorus content diminishes during the aerobic storage of bull spermatozoa and (ii) the rate and duration of oxygen consumption are markedly increased when egg phospholipids, known to be composed chiefly of lecithin, are added to sperm suspensions in a sugar-free medium. These results were contradicted by Bomstein & Steberl (1957), who were unable to detect any appreciable changes in lipid phosphorus of washed bull spermatozoa during aerobic storage. These authors also concluded that the stimulation of oxygen consumption by exogenous phospholipids was not due to oxidation of these phospholipids. In similar experiments with ram spermatozoa analysis of phosphorus fractions gave no indication that the lipid-phosphorus content diminishes during aerobic metabolism (Mann, 1958).

The early experimental approaches to the participation of phospholipids in sperm metabolism were based upon two assumptions. The first of these, well entrenched since the early work by Miescher (1878, 1897), Mathews (1897) and Sano (1922), was that sperm phospholipids consist mainly of lecithin; the second was that any utilization of phospholipid during sperm metabolism would lead to liberation of the phosphorus in a non-lipid, acid-soluble form. Quite recently, however, investigations on the chemical nature of lipids present in ram spermatozoa have shown that there is no evidence for the presence of lecithin in the sperm cells and that the predominant intracellular lipid is of aldehydogenic nature, resembling the plasmalogens of brain and muscle (Lovern, Olley, Hartree & Mann, 1957). These findings, together with an earlier observation that a plasmalogen-like lipid is also present in bull semen (Boguth, 1952), made it desirable to reinvestigate the problem of the alleged relationship between lipids and the aerobic metabolism of mammalian spermatozoa. In this paper are reported analyses of plasmalogen and acyl ester in the lipids of ram semen with special reference to the behaviour of these substances during the aerobic as well as the anaerobic metabolism of spermatozoa.

EXPERIMENTAL

Material

Semen was collected from rams, and occasionally from bulls, boars and stallions by means of the artificial vagina (Walton, 1945), the sperm and seminal plasma being separated by centrifuging. Washed sperm suspensions were made as described by Mann & White (1957) with the calcium-free 'Ringer solution for spermatozoa' (Mann, 1946). Boar epididymal secretion and vesicular secretion were obtained from animals at autopsy. Human semen from donors at the Fertility Clinic was kindly made available by Dr H. A. Davidson, and sea-urchin semen by Lord Rothschild. Palmitaldehyde was supplied by Dr J. A. Lovern, glycerol 1:2-palmitylacetal by Dr D. N. Rhodes and *Agkistrodon piscivorus piscivorus* snake venom (from Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A.) by Dr C. Long.

Lipid solvents

Alcohols were freed from esters by distillation from NaOH and dried, where necessary, over calcium. Chloroform was dried over $CaCl_2$. All solvents were redistilled before use. For the estimation of plasmalogens a more rigorously purified $CHCl_3$ was necessary (see below).

Extraction of phospholipids from spermatozoa

Extraction method I. This was based on the methods developed by Schmidt & Thannhauser (1945) and Schneider (1946) for the extraction and separation of acid-soluble phosphorus compounds, phospholipids and nucleic acid from animal tissues. The procedure as applied to sperm was as follows: 10 ml. of washed sperm suspension containing about 10^9 cells/ml. was deproteinized in a centrifuge tube with 10 ml. of ice-cold 20% (w/v) trichloroacetic acid soln., centrifuged 10 min. later and the residue was re-extracted, again for about 10 min., with 20 ml. of ice-cold 7% (w/v) trichloroacetic acid. These two extractions were found to

remove the whole 'acid-soluble phosphorus' from spermatozoa. The residue left in the centrifuge tube was then washed, before proceeding with the phospholipid extraction, with (i) 10 ml. of ice-cold 1% trichloroacetic acid and (ii) 5 ml. of water, centrifuging each time. The washed residue was suspended in 2 ml. of water and extracted twice with 8 ml. of ethanol at room temperature, each time for 10 min., and once for 3 min. with 8 ml. of ethanol–ether (3:1, v/v) maintained at the boiling point. The three extracts thus obtained were combined and ethanol and ether removed by distillation *in vacuo*. One portion of the material thus prepared was evaporated to complete dryness in a Kjeldahl flask, then digested with perchloric acid and used for the lipid phosphorus (lipid P) determination, and another portion was used for the plasmalogen determination.

Extraction method II. The method used by Bloor (1929) for extraction of lipids from blood plasma gave satisfactory results with washed spermatozoa and seminal plasma. The material was run as a thin stream into 15 vol. of 95% ethanol–ether (3:1, v/v) with constant shaking, the mixture heated to boiling point on a water bath, cooled and made up to a volume of 20 times the original volume of sperm suspension or seminal plasma. The resulting precipitate was filtered off, with precautions to prevent evaporation of the solvent, and portions of the clear filtrate were taken for the determinations of lipid P, plasmalogen and acyl ester.

Since the extraction is not carried out with an anhydrous solvent an appreciable quantity of water-soluble material is extracted and this may introduce errors into the determination of total lipid or of lipid P. In some instances therefore a measured volume of the Bloor filtrate was evaporated to dryness on a water bath, the residue dried over P_2O_5 *in vacuo* overnight and then extracted with anhydrous solvent ($CHCl_3$ or $CHCl_3$–methanol). The extracts were filtered, diluted to a known volume and suitable samples were taken for the various analyses.

Extraction method III. For most of the work described in this paper we have used propan-2-ol as lipid solvent in place of ethanol–ether. Feulgen, Boguth & Andresen (1951) found that absolute ethanol was a satisfactory substitute for ethanol–ether for extraction of plasma lipids and that the extraction was complete at room temperature. Christl (1953) established that the use of propan-2-ol enabled the method to be applied satisfactorily to a wide range of animal tissues. In our experiments it was found that completely clear filtrates were not always obtained unless the mixture was boiled, as in Bloor's method. Our procedure was therefore exactly the same as extraction method II, except that ethanol–ether was replaced by propan-2-ol. The main advantages of propan-2-ol over ethanol–ether are that it shows no tendency to boil explosively on a water bath, and that evaporation losses are more readily prevented.

It may be added that, just as in method II, some water-soluble material was also extracted by method III. When lipid extracts obtained by either method II or III were evaporated to dryness and re-extracted with anhydrous solvents only about 50% of the material redissolved. Experience showed that the cleanest separations were obtained by using $CHCl_3$ containing 10–20% of methanol for the re-extraction. The white, partly crystalline, $CHCl_3$-insoluble residue consisted mainly of inorganic salts, but it also contained a significant amount of acid-soluble organically-bound phosphorus.

Determination of plasmalogen

Palmitaldehyde standard. A standard solution containing 0·100 mg./ml. in acetic acid (British Drug Houses Ltd.; reagent grade) was stored in the frozen state at 5°. During a period of 9 months its aldehyde content decreased by 5% in terms of the intensity of colour produced in the colorimetric method of estimation used in the present study.

Palmitaldehyde thiosemicarbazone standard. Behrens (1930) found that palmitaldehyde thiosemicarbazone reacts with Feulgen's magenta–bisulphite reagent but at a very much slower rate than the free aldehyde. Under the conditions of our method, however, the thiosemicarbazone gives exactly the same colour reaction as the equivalent of palmitaldehyde. Thus although the palmitaldehyde used as standard for our analyses had been stored for 6 years at −30°, no appreciable decomposition had occurred. The advantage of the thiosemicarbazone is its ready purification by repeated crystallization from ethanol. The thiosemicarbazone was isolated by following the method of Grob, Jenny & Utzinger (1951) for the preparation of palmitaldehyde as far as the stage of filtering off the palladium catalyst. To the filtrate and washings (200 ml.) was added a warm solution of 12 g. of thiosemicarbazide in 1200 ml. of dry methanol. After standing for 16 hr. at 5° the product (26 g.) was filtered off and purified as described by Feulgen & Behrens (1928). The standard solution contained 0·130 mg./ml. in acetic acid, corresponding to 0·100 mg. of palmitaldehyde/ml.

Chloroform–acetic acid-extraction solvent. Experience has shown that some batches of $CHCl_3$ give rise to cloudy solutions of the purple pigment developed in the Feulgen reaction. This trouble could be avoided as follows. Reagent-grade $CHCl_3$ was redistilled, washed three times with equal volumes of water and left overnight at −15°. Next day it was filtered from ice and mixed with an equal volume of acetic acid (British Drug Houses Ltd.: reagent grade). The mixture was stored at 5°.

Feulgen reagent. This was prepared according to the method of Feulgen *et al.* (1951) with medicinal-grade magenta (W. Patterson, Aberdeen). The reagent is stable if not exposed to light.

Estimation of plasmalogen as palmitaldehyde. The procedure was a modification of that described by Feulgen & Grünberg (1938). A similar procedure was used by Lovern *et al.* (1957). The lipid solution, prepared by extraction method I, II or III, was evaporated to dryness on a water bath in a stream of N_2. The residue was dissolved in acetic acid, the volume of acid being 10–20 times the volume of the original semen. Of this solution 1 ml. was placed in a 20 ml. test tube and treated with 5 ml. of Feulgen reagent and 2 drops of 6% (w/v) $HgCl_2$ soln. A series of standards containing 0, 20, 40, 60 and 80 μg. of palmitaldehyde (or the equivalent of the thiosemicarbazone) in 1 ml. of acetic acid, was set up in the same way. The tubes were firmly closed with rubber bungs, shaken, left standing in darkness for 20 hr. at 37° and then cooled in ice. To each tube, while still cold, was added 8 ml. of the cold $CHCl_3$–acetic acid-extraction solvent and the contents were well mixed by means of a glass plunger. The aqueous layers were sucked off, the $CHCl_3$ layers warmed to room temperature and the intensity of colour measured within 5 min. in an EEL colorimeter with a 624 filter (transmission maximum at 520 mμ). Although this method has proved to be satis-

factory for the lipids extracted from spermatozoa it may require modification if it is to be used for lipids from other sources.

The plasmalogen content was calculated from the values obtained in the colorimetric determination of fatty aldehyde, with palmitaldehyde as standard. This calculation assumes that the sperm plasmalogen has a structure analogous to that of the plasmalogen isolated from bovine muscle (Rapport, Lerner & Alonzo, 1954; Rapport, Lerner, Alonzo & Franzl, 1957). The molecular-weight ratio of plasmalogen/palmitaldehyde would thus be about 2·85.

Estimation of acyl ester

The method of Stern & Shapiro (1953) was modified so that the lipid could be analysed as a solution in propan-2-ol. A solution of the lipid in 3 ml. of propan-2-ol was treated with 1 ml. of M-hydroxylamine hydrochloride and 1 ml. of 1·75N-NaOH. The mixture was kept at 37° for 30 min., cooled and treated with 0·5 ml. of 4N-HCl followed by 0·5 ml. of 0·37M-$FeCl_3$ made up in 0·1N-HCl. Triacetin standards were made up to contain 3 μequiv. in 3 ml. of propan-2-ol (3 μequiv. = 1 m-mole = 219 μg. of triacetin). The colour intensities were measured in the EEL colorimeter with a 624 filter. Under these conditions triacetin and triolein were quantitatively equivalent.

Other analytical methods

Phosphorus was determined by the method of King (1932). Oxygen uptake was measured, in the presence of air, in Barcroft differential manometers at 37°, with KOH in the centre well. The respiratory quotient (R.Q.) was determined by the indirect method of Warburg (Umbreit, Burris & Stauffer, 1945).

Expression of analytical results

Results of analyses carried out on spermatozoa and reported in the present study are expressed in terms of mg., or m-equiv., present in the sperm obtainable from 100 ml. of whole semen (mg./sperm from 100 ml. of semen).

RESULTS

Comparative efficiencies of the three methods for extracting lipid from washed ram spermatozoa

To compare methods II and III, experiments were carried out with two samples of ram semen (*A* and *B*) collected on different occasions. The semen was centrifuged and the plasma separated. The spermatozoa were resuspended in Ringer solution to four times the original semen volume and centrifuged again. This washing was repeated and the spermatozoa were resuspended in Ringer solution so that 3 ml. of suspension corresponded to 1 ml. of semen. One part of the suspension was extracted immediately and the remainder was extracted after aerobic incubation in Barcroft manometers, for 4 hr. at 37°. Both samples were divided into two equal parts; one was extracted by method II and the other by method III. The results of the plasmalogen determinations are given in Table 1. Slightly higher values were always obtained by method II. This difference is probably due to the evaporation of ether during filtration, which results in the lipid solution becoming more concentrated. In a further experiment, carried out to test the efficiency of the extraction by method III, the residue insoluble in propan-2-ol was washed exhaustively with that solvent and then refluxed for 24 hr. with methanol–$CHCl_3$ (1:2, v/v) containing 0·03N-HCl (Folch, 1952) in order to extract residual lipid. This extract, however, was found to contain only 1·3% of the total extractable aldehydogenic material. In another experiment a comparison was made of methods I and III. The results appeared to indicate that method I removes from the sperm suspension only about 50% of the plasmalogen extractable by method III. However, since plasmalogens are acid-labile it is possible that trichloroacetic acid liberated free aldehyde, which then reacted with some other component of the mixture.

Rates of colour formation in the Feulgen reaction with free palmitaldehyde and with lipid extracted from ram spermatozoa

We have confirmed the findings of Rapport *et al.* (1957) that plasmalogens, as they occur in lipids, react more rapidly with the Feulgen reagent than does free palmitaldehyde. This is illustrated by the following results. When a solution of free palmitaldehyde was used it was found that the Feulgen reaction was complete after 20 hr. at 37°; with shorter reaction times or at lower temperatures, such as have been used by other workers, the

Table 1. *Plasmalogen content of washed ram spermatozoa determined on extracts prepared by methods II (ethanol–ether) and III (propan-2-ol)*

Two samples (*A* and *B*) of ram semen were used. Results are expressed in terms of the palmitaldehyde standards. The plasmalogen equivalents can be obtained by multiplying the palmitaldehyde values by 2·85.

	'Palmitaldehyde' (mg./sperm from 100 ml. of semen)	
	Method II	Method III
Washed sperm *A*		
Fresh	115	111
After aerobic incubation for 4 hr. at 37°	114	110
Washed sperm *B*		
Fresh	125	123
After aerobic incubation for 4 hr. at 37°	131	127

intensity of colour was significantly less (Table 2). The thiosemicarbazone of palmitaldehyde reacted very slowly with the Feulgen reagent in the absence of mercuric chloride, but in its presence the reaction proceeded as rapidly as with the free aldehyde. Fig. 1 shows a calibration curve obtained with a series of palmitaldehyde standard solutions after the reaction had been allowed to proceed for 20 hr. at 37°. The two other curves shown in Fig. 1 were obtained after carrying out the reaction at 20° for 2 and 20 hr. respectively. In contrast with these are the results obtained with the sperm lipid (Table 3). Here, both with a fresh and an aerobically incubated suspension, the colour intensities were the same after reaction at 20° for 2 hr. as after reaction at 37° for 20 hr. As would be expected, no catalytic effect of Hg^{2+} ions was observed in the reaction of either free palmitaldehyde or sperm lipid with the Feulgen reagent. From these results it can be concluded that no advantage is gained if free fatty aldehyde is liberated from plasmalogen by acid hydrolysis before the colorimetric estimation (as recommended by Feulgen *et al.* 1951).

Table 2 also includes results on the reaction of a palmitylacetal with the Feulgen reagent, and Table 3 gives results obtained with spermatozoa that had been treated with snake venom. These points will be considered in detail later.

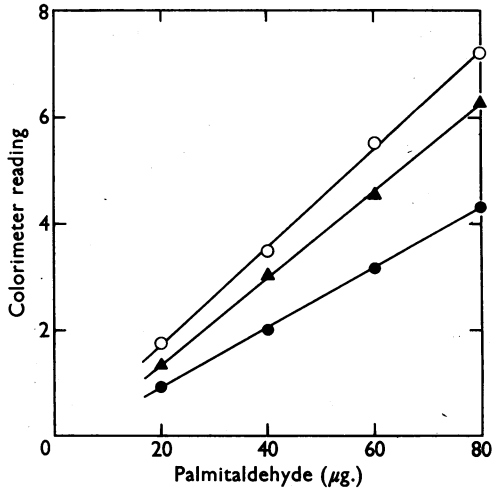

Fig. 1. Development of colour in the Feulgen reaction with palmitaldehyde during reaction periods of 2 and 20 hr. at 20° and 37°. The purple pigment was extracted with chloroform and its concentration measured as described in Methods. The uppermost curve corresponds with the standard conditions for estimation used in this paper. The reaction mixtures consisted of 1 ml. of solution of palmitaldehyde in acetic acid, 5 ml. of Feulgen reagent and 2 drops of 6% $HgCl_2$. Identical results were obtained in the absence of $HgCl_2$. ○, 20 hr. at 37°; ▲, 20 hr. at 20°; ●, 2 hr. at 20°.

Table 2. *Colour intensities obtained in the Feulgen reaction under different conditions with palmitaldehyde and with glycerol* 1:2-*palmitylacetal*

Details are given in Methods. Where one result is given for two experiments with and without Hg^{2+} ions, this represents the mean of two values which were equal within experimental error. Results are expressed in terms of colorimeter readings where 100 represents the colour intensity observed with palmitaldehyde under the standard conditions, i.e. when the reaction was allowed to proceed for 20 hr. at 37°.

Reaction time (hr.)	2	20	20	20	20
Temperature	20°	20	20	37	37
Hg^{2+} catalyst	+	−	+	−	+
Relative colorimeter readings with					
(1) 50·0 μg. of palmitaldehyde	59		86		100
(2) 65·5 μg. of glycerol 1:2-palmitylacetal	2·3		25		105

Table 3. *Colour intensities (in terms of palmitaldehyde) in the Feulgen reaction of the lipid extracted from ram spermatozoa*

The uppermost curve of Fig. 1 (corresponding to a reaction time of 20 hr. at 37°) was used as the standard curve for all experiments. Snake venom (*Agkistrodon piscivorus piscivorus*): 0·22 mg./ml.

Reaction time (hr.)	2	20	20	20	20
Temperature	20°	20	20	37	37
Hg^{2+} catalyst	+	−	+	−	+
	'Palmitaldehyde' (mg./sperm from 100 ml. of semen)				
Washed sperm suspension					
Fresh	117	115	116	114	115
After incubation for 4 hr. in air at 37°	110	106	110	109	112
After incubation for 4 hr. in the presence of snake venom	12	50	62	91	105

Table 4. *Plasmalogen content of washed ram spermatozoa at various stages of the 1956–1957 breeding season*

Each analysis was carried out on washed sperm obtained from the pooled ejaculates of about 10 rams. Plasmalogen values are calculated from the molecular-weight ratio plasmalogen:palmitaldehyde = 2·85. The spermatozoa were separated from seminal plasma, washed twice with Ringer solution and extracted by method II or III.

		Mean values		
		'Palmitaldehyde'	Plasmalogen	
Month	No. of analyses	(mg./sperm from 100 ml. of semen)	(mg./sperm from 100 ml. of semen)	(% of dry wt. of sperm)
October	2	89	254	2·55
November	7	106	302	3·45
December	2	116	330	3·78
January	2	111	315	—
February	3	117	334	—
March	2	110	314	—

Mean value for December–March: 324 (S.D. 22).

Plasmalogen in ram seminal plasma

Although the bulk of seminal plasmalogen is confined to the spermatozoa a certain amount can always be detected in the plasma even when this has been separated from fresh semen. When a sample of plasma from the pooled ejaculates of 10 rams was extracted by method II, the lipid was found to contain 19·6 mg. of 'palmitaldehyde'/100 ml. of semen, which is about one-seventh of the total lipid-bound aldehyde in ram semen.

Seasonal variations in the plasmalogen content of ram spermatozoa

Table 4 shows the results of plasmalogen analyses on washed ram spermatozoa, obtained from pooled ejaculates of 10 rams, during a whole breeding season which extended from October to March. The concentration of plasmalogen was lowest in October, at the beginning of the season, but rose during the next month and attained a fairly constant level during the period of the next 4 months. The mean value during the period December–March was 324 (± 22) mg. of plasmalogen/sperm from 100 ml. of semen.

Effect of extensive washing and 'cold-shock' on the plasmalogen content of spermatozoa

The results shown in Table 4 were obtained with twice-washed spermatozoa. Two additional washings with Ringer solution caused the loss of a substantial amount of plasmalogen from the spermatozoa (Table 5). A similar effect was obtained when ram semen was subjected to the so-called 'cold-shock', i.e. sudden cooling from 37° to 2°. 'Cold-shock' immobilizes spermatozoa and deprives them of fructolytic ability (Mann & Lutwak-Mann, 1955). The loss of plasmalogen as a result of 'cold-shock' is illustrated by the results in Table 6. It should be mentioned here that freezing ram semen to −15° and subsequent thawing results in a similar loss of plasmalogen from the sperm into the seminal plasma.

Table 5. *Effect of extensive washing with Ringer solution on the plasmalogen content of ram spermatozoa*

Semen was centrifuged and the spermatozoa were suspended in Ringer solution up to the original semen volume. The washing procedure was repeated with the same volume of Ringer solution. Results are expressed in terms of the palmitaldehyde standard.

No. of washings	'Palmitaldehyde' (mg./sperm from 100 ml. of semen)
1	98
2	99
3	87
4	72

Table 6. *Loss of plasmalogen from ram spermatozoa after 'cold-shock' treatment*

Experiments were carried out with two batches of pooled ram semen, *C* and *D*. The control and 'cold-shocked' samples were diluted with an equal volume of Ringer solution and centrifuged. After removal of the supernatant, the packed spermatozoa were resuspended in Ringer solution to the same volume as the diluted semen. Results are expressed in terms of the palmitaldehyde standard.

	'Palmitaldehyde' (mg./sperm from 100 ml. of semen)			
	Untreated		'Cold-shocked'	
	In plasma	In sperm	In plasma	In sperm
Ram semen *C*	16·2	110	33·3	95
Ram semen *D*	15·5	128	28·0	118

Table 7. *Content of fatty aldehyde and phosphorus in the lipid extracted from washed ram spermatozoa before and after aerobic incubation*

Spermatozoa were washed in the manner described previously. Aerobic incubation was carried out by shaking the sperm suspensions, containing about 10^9 sperm/ml., in Barcroft manometers at 37°. Results are expressed in terms of (i) plasmalogen phosphorus calculated from the aldehyde values on the assumption that the molar ratio between aldehyde and phosphorus in the sperm plasmalogen is 1:1, and (ii) lipid phosphorus determined after digestion of the extracted lipid with perchloric acid and hydrogen peroxide.

Expt. no.	No. of washings with Ringer soln.	Aerobic incubation: Time (hr.)	Aerobic incubation: Total uptake of O_2 (mg./sperm from 100 ml. of semen)	Method used for extraction of sperm	Solvent used for re-extraction of sperm lipid	Plasmalogen P (mg./sperm from 100 ml. of semen): Fresh	Plasmalogen P (mg./sperm from 100 ml. of semen): Incubated	Lipid P (mg./sperm from 100 ml. of semen): Fresh	Lipid P (mg./sperm from 100 ml. of semen): Incubated	Plasmalogen P / Lipid P: Fresh	Plasmalogen P / Lipid P: Incubated	Gain (+) or loss (−) during incubation (%): Plasmalogen P	Gain (+) or loss (−) during incubation (%): Lipid P
1	2	—	—	II	—	12·3	—	18·3	—	0·67	—	—	—
2	1	—	—	III	—	13·3	—	24·7	—	0·54	—	—	—
	3	—	—	II	—	12·0	—	15·6	—	0·77	—	—	—
		—	—	III	—	11·6	—	15·4	—	0·75	—	—	—
3	2	—	—	II	Ethanol–ether (3:1, v/v)	11·1	—	16·7	—	0·66	—	—	—
		—	—	II	$CHCl_3$	11·3	—	17·3	—	0·65	—	—	—
		—	—	III	Ethanol–ether (3:1, v/v)	11·0	—	16·7	—	0·66	—	—	—
		—	—	III	$CHCl_3$	11·2	—	17·2	—	0·65	—	—	—
4	2	5·5	99	II	—	12·7	12·4	19·1	18·4	0·67	0·67	−2	−4
5	2	4	113	II	—	16·1	17·1	26·5	26·5	0·61	0·65	+6	0
				III	—	15·9	16·5	26·3	24·1	0·60	0·68	+4	−8
6	2	6	130	III	—	15·3	14·8	22·6	19·3	0·68	0·77	−3	−14
				III	$CHCl_3$	14·9	14·4	18·5	17·3	0·80	0·83	−3	−6
7	2	5·5	103	III	—	13·3	13·1	18·3	15·9	0·73	0·82	−1	−13
				III	$CHCl_3$–methanol (4:1, v/v)	13·3	13·0	17·8	16·8	0·75	0·77	−2	−6
8*a* *	1	—	—	III	$CHCl_3$–methanol (10:1, v/v)	13·4	—	26·2	—	0·51	—	—	—
8*b* †	1	—	—	III	$CHCl_3$–methanol (10:1, v/v)	14·3	—	27·5	—	0·52	—	—	—

* Cold-shocked. † Control.

Ratio of fatty aldehyde to phosphorus in the lipid extracted from ram spermatozoa

To compare the content of fatty aldehyde as determined by the Feulgen reaction with that of phosphorus determined after digestion, a series of analyses was performed on the lipid material extracted from washed ram spermatozoa. The results of these analyses are shown in Table 7.

The ratio of plasmalogen phosphorus (plasmalogen P), calculated from the aldehyde values, to lipid P determined directly never exceeded 0·8. This is to be expected since, in addition to plasmalogen, there are other phosphorus-containing lipids in spermatozoa (Lovern *et al.* 1957). One of the factors which determines the actual ratio is the extraction procedure. Thus Expts. 6 and 7 show that when the lipid material extracted from washed spermatozoa is purified by a second extraction with an anhydrous lipid solvent there is a decrease in the lipid P content, whereas the plasmalogen value remains practically unaltered. Expts. 4–7 show the changes in the contents of plasmalogen and of lipid P which occur during the anaerobic metabolism of washed spermatozoa. The plasmalogen values are virtually unchanged, whereas the decreases in lipid P become very small if the lipid is subjected to the second solvent extraction before analysis. Repeated washing of spermatozoa with Ringer solution tends to increase the plasmalogen P/lipid P ratio in the extracted lipid material (Expts. 1 and 2). 'Cold-shock', on the other hand, appears to have little effect on the ratio as such but results in a small loss of both plasmalogen and lipid P (Expts. 8*a* and 8*b*).

A general conclusion which can be drawn from these results is that the crude lipid obtained by one solvent extraction (method II or III) from washed spermatozoa contains some non-phospholipid P. This P may be derived from glycerylphosphorylcholine, which is present in ram seminal plasma at a high concentration (Dawson, Mann & White, 1957) and which is difficult to remove completely by washing the sperm with Ringer solution. The solubility of glycerylphosphorylcholine in the solvents used for the first extraction will inevitably result in too high values for lipid P. Glycerylphosphorylcholine is, however, virtually insoluble in the solvents used for re-extraction of the lipid.

Effect of fructose on the phospholipid content of ram spermatozoa incubated under aerobic conditions

Several attempts were made to demonstrate a decrease in the lipid P content of washed spermatozoa during aerobic incubation at 37° for periods up to 6 hr. In these experiments, and also in parallel experiments where fructose was present, the changes in lipid P never exceeded 5%. In further experiments the plasmalogen content of a suspension of freshly washed spermatozoa was compared with that of four samples of the same suspension which had been incubated aerobically, two of them with and two without fructose, for 6 hr. at 37°. Table 8 shows that the plasmalogen P contents of the incubated samples were identical and only slightly lower than that of the fresh control sample.

Changes in the acyl-ester content of sperm lipids on aerobic and anaerobic incubation

The effect of aerobic and anaerobic incubation upon the lipids of spermatozoa was studied further in a series of experiments which included the determination of acyl ester as well as of lipid P and plasmalogen. The results of these experiments (Table 9) show that, unlike the content of lipid P and plasmalogen, that of acyl ester decreases during aerobic incubation of washed ram spermatozoa (Expts. 1 and 2), and that furthermore a decrease of similar magnitude takes place when the spermatozoa are incubated anaerobically (Expts. 1 and 3). Table 9 also contains results on the changes in composition of the sperm lipid after aerobic incubation of ram spermatozoa with snake venom for 4 hr. (Expt. 2). The venom treatment considerably decreased sperm respiration and markedly lowered the acyl-ester content of the sperm lipid. At the same time the fatty-aldehyde content diminished slightly but no change occurred in the lipid P value. These findings are in agreement with previous experiments by Dawson *et al.* (1957), who reported that cobra venom diminishes sperm respiration and at the same time brings about a decrease in acyl-ester content, which, however, is not accompanied by the liberation of acid-soluble organically bound phosphate.

Respiratory quotient of ram spermatozoa

The fact that acyl-ester bonds of the sperm lipid undergo hydrolysis during aerobic or anaerobic incubation of spermatozoa suggests that fatty acids are set free. This in turn could be taken

Table 8. *Effect of aerobic metabolism in the presence and the absence of fructose on the plasmalogen content of washed spermatozoa*

Spermatozoa were washed twice with Ringer solution and incubated at 37° for 4 hr. Experimental conditions were as described in Table 7.

	Plasmalogen P (mg./sperm from 100 ml. of semen)
Fresh suspension	16·4
Two samples incubated for 4 hr. without fructose	15·0, 14·5
Two samples incubated for 4 hr. with 0·1% fructose	14·7, 15·0

Table 9. *Changes in the content of acyl-ester bonds, plasmalogen and lipid phosphorus, resulting from aerobic and anaerobic incubation of ram spermatozoa and from treatment with snake venom*

Washed ram-sperm suspensions for Expts. 1 and 2 were prepared by diluting semen with 5 vol. of Ringer solution, centrifuging, washing the sperm once more in the same way and resuspending to a concentration of about 10^9 cells/ml. In Expt. 3 whole ram semen was diluted with 1 vol. of Ringer solution, centrifuged and the spermatozoa were suspended in Ringer solution to a concentration of about 10^9 cells/ml. Snake venom (Expt. 2) was added in a concentration of 0·22 mg./ml. Results are expressed as m-moles of O_2 uptake during 4 hr. and m-equiv. of lipid P, fatty aldehyde and acyl ester in the sperm lipid derived from 100 ml. of ram semen.

Expt. no.	Material	Incubation at 37°		Method used for extraction of sperm lipids	Solvent used for re-extraction of sperm lipid	Total uptake of O_2	Lipid P	Fatty aldehyde	Acyl ester
		Period (hr.)	Conditions						
1	Washed sperm	0	—			—	—	—	1·29
		4	Aerobic	III	—	4·8	—	—	0·94
		4	Anaerobic			—	—	—	0·92
2	Washed sperm	0	—			—	0·73	0·48	1·26
		4	Aerobic	III	$CHCl_3$–methanol (10:1, v/v)	2·6	0·69	0·48	1·08
		4	Aerobic + venom			1·25	0·70	0·42	0·75
3*a*	Whole semen	0	—			—	—	0·58	2·17
		4	Anaerobic	III	—	—	—	0·58	1·77
b	Seminal plasma	0	—			—	—	0·10	0·47
		4	Anaerobic	III	—	—	—	0·10	0·43
c	Washed sperm	0	—			—	—	0·55	1·77
		4	Anaerobic	III	—	—	—	0·54	1·40

to indicate that the fatty acids are subsequently utilized as substrates for aerobic metabolism. If washed spermatozoa were capable of maintaining their endogenous aerobic metabolism by fatty acid oxidation alone, one would expect them to have an R.Q. approximately equal to 0·7. This possibility was investigated in the following experiment.

A suspension of twice-washed ram spermatozoa in Ringer solution (10^9 cells/ml.) was incubated aerobically, at 37° for 1 hr., in Barcroft manometers to oxidize the residual fructose or lactic acid. The suspension was then diluted with 8 vol. of Ringer solution and equilibrated with $CO_2 + O_2$ (5:95, v/v). The R.Q. was determined at 37° by the indirect method of Warburg with 4·4 ml. of the diluted suspension in each vessel. The gas exchange was measured during the period 15–90 min. after closing the manometer taps. The results show that the R.Q. is about 0·7 (Table 10). The CO_2 retention by the very dilute suspension of spermatozoa in Ringer solution will be less than that of a concentrated phosphate buffer, pH 7·3. Thus the true R.Q. will be intermediate between the two values recorded in Table 10.

Plasmalogen in semen and accessory secretions from species other than ram

Although the present study was concerned primarily with the distribution and behaviour of plasmalogen in ram semen, a few experiments were also made with material from other species. The results are listed in Table 11. The values for plasmalogen P in bull spermatozoa were about one-third of the mean value established for ram spermatozoa, but since the sperm concentration in bull semen is about one-third of the concentration in ram semen, the plasmalogen content per spermatozoon is much the same in both species. From results on a single sample of washed bull spermatozoa it would appear that the ratio plasmalogen P/lipid P was nearer unity than in any of the samples of ram semen that were examined. By contrast, only a small proportion of the lipid P in sea-urchin semen could be accounted for as plasmalogen P. The very low figures for plasmalogen in stallion, boar and human seminal plasma should be accepted with reserve since the materials had been stored for considerable periods at −15°.

An unexpected finding during experiments on the aerobic incubation of washed bull spermatozoa was that decreases in acyl ester were much smaller, in relation to plasmalogen, than the changes observed with ram spermatozoa. In fact, the O_2 consumed in some experiments was considerably in excess of the theoretical figure based upon complete oxidation of the released fatty acid. An explanation of these results must await a systematic examination of the lipids of bovine spermatozoa.

Table 10. *Determination of the respiratory quotient of a suspension of washed ram spermatozoa at 37° by the indirect method of Warburg*

Gas exchange was measured during a period of 75 min.

	Uptake of O_2 (μl.)	Evolution of CO_2 (μl.)	R.Q.
Measured volumes, i.e. uncorrected for CO_2 retention	59	43	0·72
Volumes corrected as for concentrated phosphate buffer, pH 7·3	35·5	25	0·70

Table 11. *Plasmalogen phosphorus and lipid phosphorus in semen and in accessory secretions of species other than ram*

Spermatozoa were washed twice with Ringer solution as described for ram spermatozoa. Lipid was extracted by method III. An asterisk indicates that a second extraction of the crude lipid was made with $CHCl_3$–methanol (10:1, v/v). A bracket indicates that the fractions were obtained from the same sample of pooled semen.

Species	Material	Plasmalogen P (mg./sperm from 100 ml. of semen)	Lipid P (mg./sperm from 100 ml. of semen)
Bull	Seminal plasma	1·51	—
	Washed sperm	4·26	—
	Washed sperm*	3·98	—
	Washed sperm*	4·23	4·35
Stallion	Seminal plasma, two samples from the same animal	0, 0·03	—
Boar	Seminal plasma	0·04	—
	Vescicular secretion	0·9	—
	Epididymal plasma	1·1	—
Human	Seminal plasma	0	—
Sea urchin			
Paracentrotus lividus	Whole semen*	8·3	22·6
Echinus esculentus	Seminal plasma*	0·92	0·61
	Washed sperm*	3·15	21
	Washings from sperm*	—	2·0

DISCUSSION

The experimental results obtained in the present study fall into two groups: one concerns the content and chemical behaviour of phospholipid and plasmalogen in semen, particularly of the ram, the other deals with the relation of sperm plasmalogen to the aerobic as well as the anaerobic metabolism of ram semen.

Whole ram semen was shown to contain about 380 mg. of plasmalogen/100 ml., 320 mg. being present in the spermatozoa. Since, however, in an average ram ejaculate, the spermatozoa occupy about 26% of the total volume (Mann, 1954), it follows that the actual concentration of plasmalogen in the sperm cells themselves is nearly four times that of whole semen, i.e. some 1·2 g./100 g. of sperm fresh wt., or 3·6 g./100 g. of sperm dry wt. From Table 12, which lists various animal tissues according to their plasmalogen-phosphorus content, one can see that the concentration of plasmalogen in sperm is unusually high.

As to the actual form in which plasmalogen is present in ram spermatozoa, a previous study by

Table 12. *Plasmalogen phosphorus in various tissues (from Christl, 1953) and in ram semen*

Results are expressed as mg. of plasmalogen P/100 g. fresh wt. of tissue.

Blood (pig, cow, guinea pig, human, dog)	0·16–0·88
Pig liver	0·85
Egg yolk	0·87
Pig pancreas	2·2
Pig spleen	3·0
Cow adrenal	7·2
Cow skeletal muscle	12
Calf thymus	13
Cow brain (grey matter)	20
Cow heart muscle	21
Cow brain (white matter)	57
Ram semen	17
Ram spermatozoa	75

Lovern *et al.* (1957) has shown that the aldehydogenic sperm lipid contains choline as the predominating base, and that the ratio choline:phosphorus:aldehyde is 1:1:1. Our present results, particularly those obtained in experiments with spermatozoa that were incubated with snake venom, confirm the phosphorus:aldehyde ratio and

also suggest a 1:1 ratio for aldehyde:fatty acid. Thus in Expt. 2 of Table 9 the decrease in acyl ester resulting from treatment with venom was 0·51 m-equiv., which is almost equal to the original aldehyde content (0·48 m-equiv.). In further experiments of similar nature the decrease in acyl ester approached, but never significantly exceeded, the aldehyde content. In Expt. 2 a value for lipid P in excess of 0·48 m-equiv. is to be expected since phospholipids other than plasmalogen are known to occur in spermatozoa. However, our previous analyses (Lovern *et al.* 1957) indicate that this excess should be only about 10%. The high value, 0·73 m-equiv., for lipid phosphorus is probably due, at least in part, to non-lipid contaminants but it could also arise from the presence, in our total lipid extracts, of phospholipids which had been eliminated in the course of the purification procedures adopted in the previous work (see below). It is clear from Tables 3 and 9 that whereas incubation of washed sperm in the absence of venom led to liberation of fatty acid the phosphorus retained its lipid nature, and the fatty aldehyde, since it was still fast-acting in the Feulgen reaction, was not set free. When venom was present during incubation similar results were obtained except that hydrolysis of acyl esters proceeded more rapidly and the plasmalogen then reacted very slowly with the Feulgen reagent. The observation by Anchel & Waelsch (1944) that cyclic acetals react slowly with the Feulgen reagent is confirmed, and placed upon a quantitative basis, by the results given in Table 2 as well as by the comparable results given by Rapport *et al.* (1957). Contrary to Feulgen & Grünberg (1938), we found little or no acceleration of the reaction between cyclic acetals and the Feulgen reagent on addition of Hg^{2+} ions (Tables 2 and 3).

The action of venom on sperm plasmalogen can be interpreted as being dependent principally upon the activity of an enzyme of the phospholipase A type, capable of converting plasmalogen into lysoplasmalogen in the same way that lecithin is converted into lysolecithin, i.e. by the removal of fatty acid from the α-position. A similar mechanism for the action of venom was assumed by Rapport & Franzl (1957) in their experiments on the choline-containing plasmalogen that occurs in muscle. The appearance of a plasmalogen which was slow-acting in the Feulgen reaction after treatment with venom would be consistent with the formation of a cyclic acetal. On the other hand, Rapport *et al.* (1957) believe that the lyso-compound obtained by treating muscle plasmalogen with snake venom is not a cyclic acetal but has the normal lyso structure. If this is accepted then the structural differences between the 'slow-acting' lysoplasmalogen that is formed during incubation of spermatozoa with venom and the 'fast-acting' derivative that is present at the end of the control experiment in the absence of venom remain open for further study.

Studies on the metabolism of sea-urchin spermatozoa suspended in sea water (Rothschild & Cleland, 1952; Mohri, 1957) have given clear indication that the energy necessary for motility is derived from a breakdown of phospholipids which proceeds to the stage of liberation of phosphorus in a non-lipid form. There is in fact no clear evidence that sugars added to such suspensions can be metabolized. Maggio & Monroy (1955) have shown that suspensions of sea-urchin spermatozoa develop, on standing for short periods, a haemolytic activity which can be ascribed to the formation of lysolecithin (or an analogous lyso-compound). In fact a phospholipase A diffuses out of the sperm cells and can be detected by its action upon added lipovitelline.

Whereas our studies do not support Lardy & Phillips's (1941*a*) claim that the endogenous aerobic metabolism of spermatozoa is accompanied by a marked fall in the lipid-phosphorus content, they provide no evidence contradictory to the concept advanced by these authors, namely, that fatty acids derived from lipids are capable of providing an oxidizable substrate for the endogenous respiration of washed sperm cells. The following observations are consistent with this concept and at the same time they lead us to suggest that, in ram spermatozoa, the plasmalogen is the source of the fatty acid: (i) the lipid acyl-ester content decreases on incubation of washed spermatozoa; (ii) the respiratory quotient of the washed cells is 0·71; (iii) plasmalogen is the predominant lipid in ram spermatozoa; (iv) of the lipids known to be present in ram spermatozoa only plasmalogen is susceptible to hydrolysis by phospholipase A. Although this phospholipase has been shown to occur in sea-urchin spermatozoa, its presence and possible role in ram semen remains to be investigated.

Rapport & Franzl (1957) isolated the fatty acid fraction released from muscle plasmalogen by snake venom and found a mean molecular weight of 302, and two double bonds. These results correspond approximately to the formula $C_{20}H_{36}O_2$. If one assumes that a comparable fatty acid fraction is released from sperm plasmalogen during incubation of the washed cells, complete oxidation of the liberated acid would require 28 mol.prop. of oxygen and would yield 20 mol.prop. of carbon dioxide. The expected R.Q. is thus $20/28 = 0{\cdot}71$, which is very close to the experimental values (Table 10). In Expt. 1 (Table 9) the decrease in acyl-ester content during aerobic incubation was 0·35 m-equiv./sperm from 100 ml. of semen. The

oxidation of the equivalent quantity of fatty acid would require about $0{\cdot}35 \times 28 = 10$ m-moles of oxygen. The observed oxygen uptake, 4·8 m-moles, could thus be fully accounted for by oxidation of some of the available fatty acid. Similarly, in Expt. 2, the measured oxygen uptake (2·6 m-moles) was about half of that required for complete oxidation of the 0·18 m-equiv. of fatty acid that became available during the experiment.

The present findings are, in one respect, in disagreement with our earlier investigation on the composition of lipids extractable from ram spermatozoa (Lovern *et al.* 1957). In that paper it was concluded that the fatty acids obtained on hydrolysis of the sperm lipid were approximately equimolar with the fatty aldehydes of the plasmalogen and that in consequence the fatty acids were essentially all derived from plasmalogen. Our latest results (Table 9) indicate an acyl ester:plasmalogen ratio considerably greater than unity. This discrepancy might be due to the presence in sperm lipid of esters of short-chain acids which, after hydrolysis, would have been overlooked in our previous work. In addition it is probable that inositides were lost during the purification of the sperm lipid, before analysis, as described by Lovern *et al.* This possibility was in fact pointed out in the same paper. Finally, it should be remembered that the work of Lovern *et al.* involved the use of freeze-dried spermatozoa, the lipids of which may have become modified during storage.

SUMMARY

1. Lipid consisting largely of plasmalogen has been extracted from ram semen and analysed for its content of fatty aldehyde, acyl ester and phosphorus.

2. In freshly collected ram semen the bulk of plasmalogen was in the spermatozoa at a concentration of about 1·2 g./100 g. of sperm fresh wt., a value which exceeds those encountered in other animal tissues.

3. Sperm-damaging procedures such as freezing and extensive washing caused a loss of plasmalogen from the spermatozoa into the surrounding medium.

4. In ram spermatozoa treated with snake venom the content of acyl ester decreased without a corresponding diminution in lipid phosphorus and fatty aldehyde. The action of the venom appears to depend on an enzymic conversion of plasmalogen into lysoplasmalogen.

5. Aerobic incubation of washed ram spermatozoa caused a reduction in the acyl-ester but not in the fatty aldehyde or lipid-phosphorus content. A decrease in the content of acyl-ester bonds occurred also during anaerobic incubation. This indicates that fatty acids are set free during sperm incubation, in the presence as well as in the absence of oxygen.

6. Measurement of the respiratory quotient of a washed sperm suspension gave a value of 0·71, suggesting the participation of fatty acids in the aerobic endogenous metabolism.

7. Plasmalogen was also found in the sperm, seminal plasma and accessory secretions of animal species other than ram.

We are indebted to Dr R. M. C. Dawson and Dr H. Laser for advice on experimental procedures, and we wish to thank Dr J. A. Lovern and Dr J. Olley for their most helpful criticism of the manuscript.

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Studies on Pituitary Polypeptide Hormones

1. THE STRUCTURE OF β-MELANOCYTE-STIMULATING HORMONE FROM PIG PITUITARY GLANDS

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(*Received* 15 *July* 1958)

The presence of a melanocyte-stimulating substance in the pars intermedia of the pituitary has been recognized for well over 30 years as a result of the pioneer investigations of Atwell (1919), Hogben & Winton (1922), Smith & Smith (1923), Zondek & Krohn (1932) and others. Subsequently, numerous attempts were made to isolate the melanocyte-stimulating principle in pure form, and these efforts were finally brought to a successful conclusion when highly purified melanocyte-stimulating substances were isolated from pig pituitary glands by Lerner & Lee (1955), Porath, Roos, Landgrebe & Mitchell (1955), Benfey & Purvis (1955) and by Geschwind, Li & Barnafi (1956).

The melanocyte-stimulating hormone isolated by Lerner & Lee (1955) was found to be a highly basic polypeptide (isoelectric point 10·5–11·0), and Porath *et al.* (1955) and Geschwind *et al.* (1956) reported isoelectric points of 5·2 and 5·8 respectively for the melanocyte-stimulating polypeptides which they had isolated. This apparent discrepancy was resolved when Lee & Lerner (1956) showed that both forms of the hormone were present in the pig pituitary; it was therefore proposed that the basic polypeptide which they had originally isolated be called α-melanocyte-stimulating hormone and that the acidic form of the polypeptide hormone isolated by the other three groups of investigators be called β-melanocyte-stimulating hormone.

In this paper experiments which have led to the elucidation of the complete structure of β-melanocyte-stimulating hormone are described. The amino acid composition of β-melanocyte-stimulating hormone (Roos, 1956) was consistent with an octadecapeptide structure containing the following amino acid residues (abbreviations for the amino acid residues are in accordance with those of Brand & Edsall, 1947): Arg_1, Asp_2, Glu_2, Gly_2, His_1, Lys_2, Met_1, Phe_1, Pro_3, Ser_1, Try_1, Tyr_1. The amino acid sequence of β-melanocyte-stimulating hormone has been determined by application of stepwise methods of degradation to the intact octadecapeptide and to peptide fragments derived from it by selective degradation with trypsin and chymotrypsin. The complete sequence was deduced in this manner, and was subsequently confirmed by partial acid-hydrolysis studies (cf. Sanger & Tuppy, 1951*a*, *b*).

MATERIALS

The sample of β-melanocyte-stimulating hormone (β-MSH), used in this study, was prepared by one of us (P. Roos) at the Institute of Biochemistry, University of Uppsala, Sweden, according to the procedure described by Porath *et al.* (1955). Crystalline trypsin and chymotrypsin were obtained from the Novo Terapeutisk Laboratorium, Copenhagen, and a highly purified preparation of crystalline carboxypeptidase from The Armour Laboratories, Chicago, U.S.A.

METHODS

N- *and* C-*terminal group and sequence analysis*

'*Stepwise*' *degradation from the amino end* (*Expt.* 1). The sequence of amino acids from the *N*-terminal position was investigated by means of the phenylthiohydantoin method

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