DISCUSSION

The method was originally developed for the measurement of L-tryptophan in growing bacterial cultures. It has since been used successfully for determining L-tryptophan in the intracellular, free amino acid pool of *Escherichia coli* and in *Calliphora erythrocephala* during development from larva to imago (Scott, 1960), and in protein hydrolysates (Toothill, 1961), so it appears generally applicable to biological material. Tryptophanase is a sulphydryl enzyme (Happold & Turner, 1960) and, when oxidizing agents are present in the solution under test it would seem expedient to protect the enzyme by the addition of glutathione. This has never been found necessary, however, by the author.

Tryptophanase is strictly specific for L-tryptophan and converts no other known, naturally occurring compound into indole or indole derivatives. If test solutions already contain indole (or other substances soluble in light petroleum and capable of colour production with either of the colorimetric reagents), this can be removed first by light-petroleum extraction.

SUMMARY

1. An enzymic method is described for the estimation of L-tryptophan in biological material, in the range 1–60 μg.

2. L-Tryptophan is converted quantitatively into indole by the enzyme tryptophanase, and the indole measured colorimetrically, after extraction with light petroleum, with either *p*-dimethyl-aminobenzaldehyde or *p*-dimethylaminocinnamic aldehyde reagents.

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Phospholipids in Ram Semen: Metabolism of Plasmalogen and Fatty Acids

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Previous studies on the nature and function of lipids in ram semen led us to conclude that the phospholipids of spermatozoa consist mainly of choline-based plasmalogen, and that sperm respiration in the absence of seminal fructose is largely dependent on the oxidation of fatty acids that arise from hydrolysis of this plasmalogen (Lovern, Olley, Hartree & Mann, 1957; Hartree & Mann, 1959, 1960a).

The present paper is an extension of these studies, the results of which fall under three headings: (1) A comparison has been made

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between the components of the phospholipid fraction extracted from freeze-dried spermatozoa and those of corresponding fractions obtained from fresh, or frozen and thawed, spermatozoa. This comparison was prompted by the appearance of papers by Gray (1960a) and by Wren (1960). Gray produced evidence that the phosphatide fraction extracted from frozen whole ram semen contained lecithin and plasmalogen in approximately equal amounts. Wren drew attention to the widespread occurrence in tissues of lipoamino acids and lipopeptides and concluded that such lipid derivatives may be artifacts when prepared from freeze-dried material. (2) Direct quantitative evidence for the

role of fatty acids in the metabolism of washed spermatozoa has been obtained by analysis of the free fatty acids extractable from spermatozoa. Changes in fatty acid content have been correlated with simultaneous decreases in the plasmalogen of the sperm phospholipids. (3) The action of different components of ram semen upon the respiratory activity of spermatozoa has been studied with particular reference to previous reports of stimulatory effects by lecithin (Lardy & Phillips, 1941 *a*, *b*; Lardy, Hansen & Phillips, 1945; Bomstein & Steberl, 1957; Iida, 1958).

MATERIALS

Ram semen (pooled ejaculates of ten rams) was collected by means of an artificial vagina (Walton, 1945) and spermatozoa were separated from seminal plasma by centrifuging. Washed sperm suspensions were prepared with a calcium-free 'Ringer solution for spermatozoa' (Mann, 1946). Two procedures have been followed for separation of spermatozoa and plasma: both were carried out at room temperature.

Wash procedure 1. To prevent damage to the cells, spermatozoa required for metabolic experiments were washed by centrifuging at low speed. Semen was diluted to 5 vol. with Ringer solution and centrifuged for 15 min. at 1300*g* (mid-point of tube). The upper fluid was removed and the cell mass resuspended to 5 vol. in Ringer solution. The suspension was centrifuged as before and the cells were resuspended to 2–3 times the original semen volume in Ringer solution. The loss of spermatozoa in the wash fluids averaged 6–7%.

Wash procedure 2. For analytical studies of intracellular lipids, spermatozoa were separated more completely from plasma by using higher centrifuge speeds. Undiluted semen (1 vol.) was centrifuged for 15 min. at 20 000*g*. The plasma was removed as completely as possible, and the cells were resuspended to 5 vol. and again centrifuged at 20 000*g*. The washed cells were resuspended to the original semen volume.

Seminal plasma. The sperm-free seminal plasma, obtained by wash procedure 2, was used either as such or after dialysis in cellophan tubing. Seminal plasma (10 ml.) was dialysed at 5° against two lots of 0.9% NaCl and finally against Ringer solution (500 ml. in each case).

METHODS

Extraction and fractionation of lipids from freeze-dried, fresh, and frozen and thawed spermatozoa

The composition of all solvent mixtures is given on a v/v basis.

Extraction of phospholipids from freeze-dried spermatozoa. During the period December–April several batches of ram sperm were freeze-dried immediately after they had been washed by procedure 2, and stored at −15° until the following January when the lipids were extracted. The freeze-dried, powdered material (20 g.) was treated in five portions as follows. Each portion (4 g.) was suspended in 16 ml. of water and homogenized in a Waring Blendor for 5 min. with 400 ml. of chloroform–methanol (2:1). The products were filtered, and the filtrates were combined, emulsified in the Blendor with 0.2 vol. of 3 mM-MgCl$_2$

(Folch, Lees & Sloane-Stanley, 1957) and stored overnight at 5°. The upper phase was discarded, the chloroform phase was evaporated, the residue extracted with dry chloroform and the extract re-evaporated. The gummy product was dissolved in a little ether and phospholipids were precipitated by adding 11 vol. of acetone and storing for 48 hr. at 5°. The solid was collected, reprecipitated twice more with acetone and dissolved in chloroform.

Extraction and separation of lipids from fresh or frozen and thawed spermatozoa. Spermatozoa were washed by procedure 2 and either used immediately or stored for different periods at −15°. Of such suspensions 30 ml. was run slowly into 340 ml. of methanol in a 1 l. volumetric flask. The mixture was diluted with 200 ml. of chloroform, heated to the boiling point, cooled, made up to the mark with chloroform, mixed and filtered. The filtrate was washed with MgCl$_2$ solution as described above. Separation of phospholipids from other lipids was effected on a silicic acid column. The column was made up in chloroform as described by Hanahan, Dittmer & Warashina (1957) and contained 2.0 g. of silicic acid (Mallinckrodt; 100-mesh; analytical reagent suitable for chromatographic analysis) and 1.0 g. of Hyflo SuperCel (Johns-Manville Co. Ltd.)/mg. of applied lipid phosphorus. The lipid was applied in chloroform and the column developed with the same solvent until the effluent no longer gave the Liebermann-Burchard reaction for cholesterol (Cole, 1933).

The fatty acids were isolated from the chloroform effluent by fractionation on a column of Amberlite IRA-400. The procedure of Carlson & Wadström (1958) was followed except that the length and diameter of the column were both increased about threefold in order to accommodate approximately ten times the quantity of fatty acid used by Carlson & Wadström.

The phospholipids retained on the silicic acid columns were eluted with a series of chloroform–methanol mixtures of increasing methanol concentration and finally with pure methanol. The effluent was fractionated with a drop-counting fraction collector. The rate of flow was maintained, by means of a slight positive pressure of N$_2$, at approximately 2*a* ml./min. where *a* is cross-sectional area (cm.²) of the column. The fraction volumes increased as the effluent became richer in methanol, the mean volumes being 2*a* ml. Progress was followed by analysis of the fractions for phosphorus (King, 1932). The choline phosphatides, i.e. lecithin plus choline plasmalogen, were eluted with chloroform–methanol (2:1 or 3:2). The various fractions were stored in chloroform at −15° pending analysis.

Fractionation of wash-fluid obtained during the preparation of washed sperm suspensions

Washings from procedure 2 were accumulated in the frozen state and batches of 500 ml. were melted and concentrated *in vacuo* to 10 ml. This concentrate was used as follows. One part (15 ml.) was extracted with 30 vol. of chloroform–methanol (2:1) to yield 'total lipid' and insoluble material which was suspended in a little Ringer solution and dialysed exhaustively in cellophan tubing against Ringer solution to give the 'non-lipid, non-diffusible fraction'. A further 30 ml. of concentrate was dialysed against Ringer solution to give the 'dialysed concentrate', and half of this material was treated with chloroform–methanol to give 'dialysed total lipid'.

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Estimation of total and individual fatty acids in lipids extracted from fresh and incubated spermatozoa

Aerobic and anaerobic incubation of washed spermatozoa. Suspensions of ram spermatozoa in Ringer solution (50 ml.), obtained by wash procedure 1, were treated with penicillin (50 000 units) and divided into three equal portions. One portion (*A*), the control, was extracted with lipid solvents immediately, another (*B*) was incubated anaerobically at 37°, and the third portion (*C*) was incubated aerobically at 37° by being shaken in Barcroft differential manometers, in the presence of NaOH for absorption of CO_2, which allowed O_2 consumption to be measured. Incubation periods were between 2·5 and 3 hr. (except in Table 6). Details of the methods have been described by Hartree & Mann (1959).

Extraction of lipids. Each of the portions *A*, *B* and *C* (15 ml.) was run slowly into 500 ml. volumetric flasks containing 170 ml. of methanol. Chloroform (100 ml.) was added to each flask and the mixtures were heated to the boiling point. After cooling, the suspensions were made up to the mark with chloroform and filtered. Non-lipid impurities were removed by shaking the extracts with 0·2 vol. of 0·5 M-NaH$_2$PO$_4$ (Carlson & Wadström, 1958). The extracts were evaporated and the lipids dissolved in chloroform (lipids *A*, *B* and *C*).

Determination of total free fatty acids. Carlson & Wadström (1958) separated free fatty acids from the total lipid of blood plasma by two-stage column chromatography. Thus on passing a chloroform solution of lipid through a silicic acid column (as above), phospholipids were retained. The non-absorbed lipid, in ether–ethanol, was passed through a column of Amberlite IRA-400 (OH) when only free fatty acids were retained. The latter were then eluted with ether–ethanol–acetic acid, esterified and estimated by the iron–hydroxamate reaction. In the present work difficulty was encountered in finding a solvent-proof closure for the bottoms of the Amberlite columns. Teflon and glass needle valves (Manostat Corp., New York City) proved to be satisfactory. For analysis of lipids *A*, *B* and *C*, the following modifications were made in the procedure described by Carlson & Wadström (1958): (1) In view of the high phospholipid content of ram-sperm lipid the length of the silicic acid columns was doubled and the volume of chloroform used for eluting neutral lipids plus fatty acids was increased by 50%. (2) The fatty acids were esterified with diazomethane instead of methanolic SOCl$_2$: in our hands the latter method was unreliable. Esterification was carried out by treating the fatty acid (0–4 μequiv.) with 2 ml. of 2% (w/v) diazomethane in ether and 0·2 ml. of methanol at 20°. After 5 min. the solvents were evaporated and the estimation was continued according to Carlson & Wadström. The presence of methanol ensured a slightly higher degree of reproducibility. The overall recovery of 94% found by Carlson & Wadström was confirmed (*a*) by addition of known amounts of palmitic acid to sperm lipid and (*b*) by measuring the recovery of radioactivity from mixtures of tripalmitin and [^{14}C]palmitic acid (The Radiochemical Centre, Amersham, Bucks.) which had been subjected to two-stage chromatography.

Gas–liquid chromatography of free fatty acids. The methyl esters were separated at 170° in an Argon Chromatograph (W. G. Pye and Co. Ltd., Cambridge). The column was 120 cm. × 0·5 cm. diameter and contained polyvinyl acetate on Celite (1:5).

Estimation of phosphorus and plasmalogen in the lecithin–plasmalogen fraction obtained by paper chromatography of sperm lipids

Chromatography of sperm lipids was carried out on silicic acid-impregnated paper in di*iso*butyl ketone–acetic acid–water (8:5:1) essentially by the method of Marinetti & Stotz (1956). These authors ran their chromatograms at 25°. Although plasmalogen is more stable under such conditions than would be supposed from its rapid decomposition in aqueous acetic acid, some decomposition does occur at 25° since a forward streaming of free aldehyde from the plasmalogen spot can be detected after developed chromatograms have been treated with 2:4-dinitrophenylhydrazine. This decomposition is less marked at lower temperatures. In our metabolic experiments plasmalogen decomposes to give lysoplasmalogen, which is far more acid-labile than is plasmalogen (Hartree & Mann, 1960*b*). Thus, although at 0° the decomposition of plasmalogen is virtually abolished, that of lysoplasmalogen is not. Lysoplasmalogen has a lower R_F than plasmalogen and during chromatography at 0° the forward streaming of fatty aldehyde liberated from lysoplasmalogen will tend to overlap the plasmalogen spot and thus give rise to too high values for the plasmalogen content of the spot. In the present work temperatures of 10° and 14° were used. These result in insignificant decomposition of plasmalogen, but the aldehyde arising from lysoplasmalogen moves well beyond the plasmalogen spot by the end of the run.

The procedure was as described by Hartree & Mann (1960*b*) with the following modifications. Spots of each of the three lipid samples *A*, *B* and *C* (2–3 μg. of phosphorus) were placed 6 cm. from the end of a paper strip (Whatman no. 1), 10 cm. × 36 cm., and ascending runs were carried out in individual narrow cylindrical jars for 16 hr. The papers were allowed to dry for 1 hr. in a current of air. Aldehydogenic lipids were detected as follows. (All washings were in running tap water.) The papers were soaked in 15% (v/v) ethanol for 30 min. and dried in air for 1 hr. They were then placed for 30 min. in 2:4-dinitrophenylhydrazine (7·5 mM in 2 N-HCl). The major spot (R_F 0·6–0·65) was identified as choline plasmalogen by comparison with the chromatographic behaviour of Pangborn ox-heart lecithin (Hartree & Mann, 1960*b*), which is a mixture of two components, lecithin and choline plasmalogen, with equal R_F values. The papers were freed from 2:4-dinitrophenylhydrazine by successive washings in water (1 min.), 2 N-HCl (10 min.), water (1 min.), 2 N-HCl (10 min.) and water (5 min.). While still moist the papers were examined in long-wave u.v. light from a mercury-vapour lamp. The major positive spots, which are those of lecithin–plasmalogen, were outlined in pencil and the papers were allowed to dry. For analysis the spots were cut out so that equal areas of paper were removed with each of the three spots on the chromatogram. Control pieces of equal area were taken from between the lecithin–plasmalogen spots. Extraction of phospholipids from the spots was carried out within 12 hr. of treatment with 2:4-dinitrophenylhydrazine.

For estimation of phosphorus the cut-out spots were placed in 16 mm. diam. test tubes and extracted three times with methanol (total of 7·5 ml.). The extracts were transferred to a second batch of tubes containing glass beads and evaporated at 100°. Phosphorus was deter-

mined according to Chen, Toribara & Warner (1956): if the blue colour was developed in a volume of 4 ml., 1 μg. of phosphorus gave rise to an extinction of approximately 0·2 at 820 mμ. A strict proportionality between extinction and phosphorus content was obtained within the range 0–2·5 μg. of phosphorus.

Plasmalogen in the cut-out spots was determined as follows. The spots and blanks were placed in test tubes with 2 ml. of diethylene glycol monomethyl ether (ethyl-digol). The tubes were sealed with Parafilm and stored overnight at 37°. Next day they were treated with 1 ml. of freshly prepared alkaline ethyl-digol [200 ml. of ethyl-digol + 1 ml. of 50% (w/w) KOH] and the extinction at 440 mμ (E_{440}) was measured against pure ethyl-digol after 6–7 min. The blanks were also measured against ethyl-digol. A calibration curve was prepared as follows. Known volumes (5–30 μl.) of a standardized chloroform solution of crystalline lysoplasmalogen (Hartree & Mann, 1960 b) were spotted on silicic acid-impregnated paper, which was then treated with 2:4-dinitrophenylhydrazine and washed as described above. The spots (with blanks) were cut out and subjected to ethyl-digol extraction. A straight-line relationship was obtained between E_{440} and quantity of lysoplasmalogen. After correction for the blank reading (0·030) this relationship could be expressed as μequiv. of aldehyde = 0·141 E_{440} (S.E.M. of 15 estimations = 0·74%). A similar reproducibility was obtained when total lipids of spermatozoa were spotted quantitatively on paper. However, if chromatograms of sperm lipids were developed before the treatment with 2:4-dinitrophenylhydrazine (as in the analysis of lipids A, B and C) the results were naturally less reproducible (S.E.M. about 2–4%).

Other analytical procedures

Fructose was determined by the method of Roe (1934) as adapted to semen by Mann (1946), citric acid according to Speck, Moulder & Evans (1946), and lactic acid according to Barker & Summerson (1941). Fatty acyl ester and, except where otherwise stated, plasmalogen were estimated as by Hartree & Mann (1959). Choline was determined by Glick's (1944) method.

Nitrogen was estimated after incineration with H_2SO_4 and copper selenite. The method of Umbreit, Burris & Stauffer (1957) was used with the following modification, which ensured a straight-line relationship between nitrogen content (0–80 μg.) and colorimeter reading when a light-filter with maximum transmission at 520 mμ was used. The Nessler reagent was made up to contain twice the recommended concentration of gum ghatti and 3 ml. of reagent was used in place of 1·5 ml.

Bound amino acids were detected and approximately estimated by two-dimensional paper chromatography of an acid hydrolysate. The method used was that of Redfield (1953) as modified by Hunt (1959). The phospholipid (0·5 mg. of nitrogen) was heated in a sealed tube under nitrogen with 6 ml. of 6 N-HCl for 15 hr. at 110°. The resulting aqueous phase was filtered through HCl-washed cotton wool and evaporated on a water bath. The residue was heated with 5 ml. of 0·2 N-HCl plus 50 mg. of talc and filtered. The solution was repeatedly evaporated on the water bath with distilled water to remove HCl, the residue was dissolved in 0·2 ml. of water and samples were taken for chromatography.

Lipid solvents were purified as described by Hartree & Mann (1959). All evaporations were carried out under reduced pressure in a rotary evaporator.

Expression of analytical results

Wherever possible results are expressed in terms of m-equiv. present in the spermatozoa or plasma obtainable from 100 ml. of whole semen (m-equiv./100 ml. of semen). Corrections have been applied for the losses (up to 7% of cells) that occurred during the low-speed centrifugal washing of the spermatozoa used for the experiments in Table 3.

Rates of O_2 uptake are given as μl. of $O_2/10^8$ cells.

RESULTS

Examination of phospholipid fractions obtained from spermatozoa and seminal plasma by chromatography on silicic acid columns

The analyses of the various fractions obtained from ram spermatozoa and seminal plasma, with the exception of amino acid determinations, are listed in Table 1.

Phospholipids from freeze-dried spermatozoa. Phospholipid (29·1 mg. of phosphorus) equivalent to 18·8 g. of dry spermatozoa was applied to a 27 mm. diameter column, which was developed with 60 ml. of chloroform and then successively with the following chloroform–methanol mixtures: 6:1 (350 ml.); 2:1 (500 ml.); 3:2 (250 ml.); 1:1 (400 ml.). The elution pattern, with three major peaks, is shown in Fig. 1.

The contents of the fraction-collector tubes were combined to give four main fractions: 1, tubes 20–29; 2, tubes 31–50; 3, tubes 83–100; 4, tubes 105–200.

Phospholipids from frozen spermatozoa and from frozen seminal plasma. Thawed, washed spermatozoa (50 ml.), originating from the same volume of semen, yielded total lipid containing 9·0 mg. of phosphorus. Of the phospholipids separated from this material, an amount containing 6·45 mg. of phosphorus was fractionated on a silicic acid column (20 mm. diameter) which was developed with chloroform–methanol (7:1) until no more phosphorus came through and then with chloroform–methanol (3:2) until no further phospholipid was eluted. The elution pattern exhibited three major peaks of which the last represented the major component (Fig. 2, continuous line). The collector tubes were grouped to yield three fractions: 1, tubes 9–11; 2, tubes 12–16; 3 (lecithin–plasmalogen fraction), tubes 39–65.

From thawed seminal plasma (80 ml.; equivalent to 100 ml. of semen) phospholipid containing 4·6 mg. of phosphorus was isolated. Chromatographic analysis (column diameter 14 mm.) gave an elution pattern similar to that of the sperm lipid.

Table 1. *Analysis of fractions obtained by chromatography on silicic acid columns of phospholipids extracted from ram spermatozoa and from seminal plasma*

Percentage of plasmalogen is derived from 200 × aldehyde/(aldehyde + ester) except in the last line where it is calculated from 100 × aldehyde/choline. LP, Lecithin–plasmalogen fraction (see Methods).

| | Molar ratio (P = 1·00) | | | | Phosphorus | | Plasmalogen in LP fraction (%) |
	Nitrogen	Choline	Aldehyde	Acyl ester	(m.equiv./ 100 ml. of semen)	(%)	
(a) Freeze-dried washed spermatozoa:							
Fraction 1	0·22	0	0·17	2·64	—	1·8	—
Fraction 2	1·02	0	0·80	1·96	—	3·0	—
Fraction 3*	1·08	0·80	1·18	1·44	—	3·2	—
Fraction 4*	1·37	0·94	0·93	0·97	—	2·9	98
(b) Frozen washed spermatozoa:							
Tubes 9–11	0·28	—	—	—	0·044	—	—
Tubes 12–16	0·88	—	—	—	0·047	—	—
LP fraction	0·98	0·98	0·81	1·22	0·275	4·0	80
(c) Frozen seminal plasma:							
LP fraction	—	0·98	0·83	1·23	0·057	—	81
Fresh washed spermatozoa:							
LP fraction	1·00	0·84	0·48	1·24	0·266	3·6	55
Fresh washed spermatozoa used for metabolic experiments:							
LP fraction*	1·10	0·83	0·40	1·23	—	3·7	49
Frozen whole semen, results obtained by Gray (1960a):							
Kephalin fraction	0·78	—	—	—	—	—	—
LP fraction	0·95	0·92	0·50	—	—	—	55

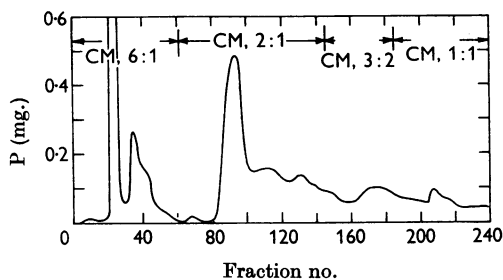* Examined for amino acids (see Table 2).

Fig. 1. Chromatography on a silicic acid column of phospholipids isolated from freeze-dried ram spermatozoa. Phospholipid from 18·8 g. of dried cells (29·1 mg. of P) was applied to a 27 mm. diameter column containing 2 g. of silicic acid and 1 g. of Hyflo SuperCel/mg. of P. The first peak reached a height corresponding to 1·58 mg. of P. CM, Chloroform–methanol, with ratios v/v.
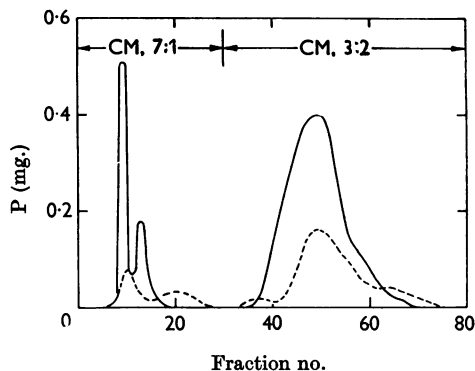

Fig. 2. Chromatography on silicic acid of phospholipids from frozen spermatozoa and frozen seminal plasma. —, Phospholipids from spermatozoa (9 mg. of P; 20 mm. diameter column); - - -, phospholipids from plasma (4·6 mg. of P; 14 mm. diameter column). Column weights were as in Fig. 1. CM, Chloroform–methanol, with ratios v/v.

An additional component appeared immediately before the lecithin–plasmalogen fraction, but the two were well separated (Fig. 2, broken line).

Phospholipids from fresh spermatozoa. Two batches of 20 ml. of fresh semen were washed (procedure 1) and made up to 1·5 vol. with sperm Ringer solution. When the extracted phospholipid (7·1 mg. of phosphorus) was subjected to column fractionation, lecithin–plasmalogen was eluted as a distinct fraction by chloroform–methanol (3:2).

Phospholipids from fresh spermatozoa that had been used for metabolic experiments. In experiments designed to show metabolic changes in the concentration of free fatty acids in spermatozoa (see below) washed suspensions were divided into three portions, of which two were incubated under anaerobic and aerobic conditions respectively,

while the third served as control. Lipids were extracted from the three portions and fractionated to yield free fatty acids. The first stage in the fractionation was the absorption of phospholipids on silicic acid columns. The phospholipids from five such experiments (15 portions) were dissolved from the columns with methanol, pooled and re-fractionated twice on larger columns. In the first run the successive solvents were chloroform and chloroform–methanol (4:1). The material extracted with the latter solvent was transferred to a second column, which was developed successively with chloroform–methanol (7:1 and 3:2). As in the previous runs, the lecithin–plasmalogen fraction was clearly separated during elution with the latter solvent.

Amino acids in lecithin–plasmalogen fractions. Three fractions (see Table 1) in which the N:P ratio exceeded 1·0 were hydrolysed with HCl and examined by paper chromatography (Table 2). In only one case was ninhydrin-positive material detected: thus fraction 3 from freeze-dried spermatozoa (Table 1, *a*) showed a single spot of phenylalanine. However, the spot intensity corresponded to ≯ 0·02 mole of phenylalanine/mole of phospholipid.

Changes in the lipid content of washed ram spermatozoa after periods of anaerobic and aerobic metabolism

Four experiments have been carried out for the purpose of comparing the free fatty acid and lecithin–plasmalogen contents of ram spermatozoa before and after periods of metabolic activity. These experiments were carried out within the period December 1959–March 1960, i.e. during the peak of the breeding season when motility and respiration of spermatozoa were consistently high. Details of incubation of the spermatozoa are given in Table 3, where *A*, *B* and *C* denote control, anaerobically and aerobically incubated spermatozoa respectively (see Methods). Of each of the three lipid samples of Expt. i, 20 % was set aside for phospholipid analysis by paper chromatography while the remainders were used for estimation of total fatty acid by the iron–hydroxamate method. In Expts. ii and iii the entire lipid extracts were used for fatty acid estimation. The lipid samples *A*, *B* and *C* of Expts. iv *a* and iv *b* were combined in pairs and 20 % of each was set aside for phospholipid analysis. The fatty acid samples

Table 2. *Amino acids in hydrolysates of the plasmalogen-rich phospholipid fractions having N:P ratios > 1·00*

The amino acids were separated by two-dimensional paper chromatography. After the papers had been sprayed with ninhydrin, phenylalanine was estimated approximately by comparison with standard spots. LP, Lecithin–plasmalogen fraction (see Methods).

Phospholipid fraction (see Table 1)	Phospholipid equivalent of hydrolysate applied to paper (μg.atoms of P)	Amino acid detected	Ratio moles of amino acid/g.atoms of P
Expt. *a*, fraction 3	11·1	∼0·2 μmole of phenylalanine	0·02
Expt. *a*, fraction 4	16·4	None	< 0·0003
LP fraction of spermatozoa from metabolic experiments	27·4	None	< 0·0002

Table 3. *Changes in free fatty acid contents of suspensions of washed spermatozoa resulting from periods of anaerobic and aerobic metabolism at 37°*

Each experiment involved three 15 ml. samples (*A*, *B* and *C*) of a suspension of spermatozoa in sperm Ringer solution. Control samples (*A*) were treated with chloroform–methanol immediately after the suspension had been prepared. Anaerobic (*B*) conditions were achieved by storing the suspensions in stoppered tubes with minimum air space. Aerobic (*C*) incubations were carried out in Barcroft differential manometers. Fatty acids were isolated by two-stage column chromatography and estimated by the iron–hydroxamate method. (For further details see text.)

Expt. no.	10^{-8} × Sperm density (cells/ml.)	Incubation time (min.)	Total O_2 uptake (μl./10^8 cells)	Total O_2 uptake (m-moles/100 ml. of semen)	Free fatty acid found (m-equiv./100 ml. of semen) A	B	C	O_2 uptake/fatty acid oxidized (molar ratio)
i	13·0	155	19·5	2·65	0·442	0·629	0·366	10·0
ii	13·8	175	15·7	2·31	0·349	0·723	0·525	11·7
iii	9·7	175	20·2	2·95	0·343	0·470	0·165	9·7
iv *a*	10·4	175	15·7⎫	3·38	0·298	0·492	0·180	10·8
b	10·4	180	26·9⎭					

obtained from the remaining lipids of iv $(a+b)$ were each divided into two equal parts, one being used for determinations of total fatty acid and the other for gas–liquid chromatography.

Fatty acids. Analyses of total free fatty acid in the A, B and C samples (Table 3) show that there is a distinct accumulation of acids during periods of anaerobic metabolism, whereas during aerobic metabolism there is either a smaller accumulation (Expt. ii) or a net loss.

Gas–liquid chromatography of fatty acids in samples A, B and C (Table 4) showed that it was mainly the C_{14} and C_{16} acids which accumulated during anaerobic incubation and that these same acids were preferentially oxidized if oxygen was present. Gray (1960b) has analysed the fatty acids liberated by hydrolysis of choline plasmalogen in whole ram semen. The major components of the mixture (C atoms: no. of double bonds) were as follows (figures in parentheses indicate percentage of each component in the recovered acids): 14:0 (5·8), 16:0 (32·0), 16:1 (5·4), 17:1 (10·1), 18:0 (11·7): 18:1 (9·5), 20:4 (11·5). By comparison, our Table 4 shows (1) a similar ratio for the C_{14} and C_{16} acids, (2) no C_{17} acid, (3) larger quantities of C_{18} acids. There is no reason why Gray's results should be similar to ours since the fatty acids isolated by us are not necessarily derived com-

pletely from plasmalogen. Further, the plasmalogen that was hydrolysed in metabolic experiments was no doubt localized in regions of high metabolic activity and it cannot be assumed that such plasmalogens would be representative, in terms of fatty acid composition, of the semen plasmalogen as a whole.

Phosphorus and plasmalogen. Samples of sperm phospholipid from Expts. i and iv (Table 3) were subjected to chromatography on silicic acid-impregnated papers, each of which carried equivalent amounts of lipids A, B and C. Four identical papers were run at one time and the spots identified after the treatment with 2:4-dinitrophenylhydrazine. The six lecithin–plasmalogen spots from two of the papers were analysed for phosphorus and the remaining six from the other two papers for plasmalogen. Replicate runs were made in order that the s.e.m. could be calculated from 12–16 papers for both phosphorus and plasmalogen. Spots that were adequate for analysis, and at the same time clearly separated from other lipid components, were obtained when the amounts of lipid placed upon the paper were such as to give lecithin–plasmalogen spots containing 0·01–0·02 μequiv. of plasmalogen.

Analyses for phosphorus and plasmalogen are summarized in Table 5. In the first column are

Table 4. *Gas–liquid chromatography of free fatty acids isolated from sperm lipids*

The acids were converted into methyl esters and run at 170° on polyvinyl acetate–Celite (1:5). The shortened designations are those used by Ahrens *et al.* (1959). Thus 16:1 represents a monounsaturated C_{16} acid. A, Control; B, anaerobic; C, aerobic.

Sample from Expt. iv (Table 3)	A		B		C	
Fatty acid (shortened designation)	(% of total)	(m-equiv./ 100 ml. of semen)	(% of total)	(m-equiv./ 100 ml. of semen)	(% of total)	(m-equiv./ 100 ml. of semen)
14:0	5·7	0·02	34·4	0·17	15·6	0·02
16:0	29·8	0·09	33·0	0·16	44·8	0·07
16:1	3·9	0·01	2·2	0·01	3·8	0·01
18:0	22·8	0·07	12·9	0·06	8·1	0·01
18:1	30·8	0·09	11·4	0·06	19·2	0·03
18:2	7·0	0·02	6·2	0·03	8·6	0·01

Table 5. *Phosphorus and plasmalogen contents of the lecithin–plasmalogen spots from paper chromatograms of lipid samples* A, B *and* C

The samples were obtained from the lipids isolated during metabolic Expts. i and iv of Table 3. The s.e.m. was calculated in all cases from between 12 and 16 replicate chromatograms. A, Control; B, anaerobic; C, aerobic.

Expt. no. (see Table 3)		Analysis of samples A (μequiv./ml. of lipid applied to the paper)	Equivalents present in lecithin–plasmalogen spots relative to $A = 100$		
			A	B	C
i	Phosphorus	3·95±0·198	—	—	—
	Plasmalogen	1·86±0·082	(100)	76·9±4·20	79·6±3·77
iv	Phosphorus	2·33±0·186	(100)	89·1±4·10	88·8±4·46
	Plasmalogen	1·11±0·078	(100)	74·5±4·25	72·0±3·04

given the mean values for phosphorus and plasmalogen in the lecithin–plasmalogen spots derived from lipids A. For each chromatogram the phosphorus and plasmalogen present in lipids B and C were calculated as percentages of the corresponding values for lipid A. The means of these percentages are shown in the last two columns.

Effect of various fractions prepared from semen on the metabolism of washed ram spermatozoa

After ram spermatozoa have been washed by procedure 1 respiratory activity is initially equal to and sometimes slightly greater than that of whole semen, but the rate of oxygen consumption decreases considerably with time and a fall is already noticeable after aerobic incubation for $\frac{1}{2}$ hr. at $37°$. The addition of fructose to such suspensions causes the initial rate of oxygen consumption to be restored to that of whole semen, and the high level to be maintained for several hours (Hartree & Mann, 1960a). On the other hand, after wash procedure 2, restoration of respiratory activity by fructose is far from complete and motility is also impaired. For this reason the following experiments on activation of sperm respiration were performed with spermatozoa which had been washed by the milder procedure 1. The seminal fractions used for the purpose of activation were, however, obtained from semen fractionated by procedure 2. The latter procedure gives more complete separation of spermatozoa from plasma; further, the wash fluid obtained by this procedure, unlike that obtained by procedure 1, consists not merely of diluted plasma but also contains material that, under the influence of high centrifugal speeds, has been removed from the outer layers of the sperm cells. The efficiency of the washing process can be judged from the following analyses of citric acid, which is a normal constituent of ram seminal plasma. Thus separation according to procedure 1 gave spermatozoa, seminal plasma and wash fluid containing respectively < 4, 352 and 15 mg. of citric acid/100 ml. of semen. After procedure 2, the figure for spermatozoa was 13. Citric acid, unlike fructose, is not metabolized by spermatozoa (Humphrey & Mann, 1949) and thus serves as a more reliable indicator of the degree of separation of plasma.

The effects of a dialysed concentrate of wash fluid and of dialysed seminal plasma on the respiration of washed spermatozoa are illustrated in Fig. 3. The residual fructose present in these two fractions contributed only 8 and 12 μg. of fructose respectively to the manometer flasks. These quantities of fructose would have negligible effects upon the respiration of washed spermatozoa: in fact they would be completely metabolized during the 10 min. equilibration period preceding closure of the manometer taps. The magnitude of the accelerating

effect of the two fractions decreases with increasing sperm density. Thus Fig. 3 illustrates experiments in which the sperm density was $4·1 \times 10^8$ cells/ml. In a parallel experiment with a density of $6·4 \times 10^8$ the rate of respiration in the control was 50% higher but the activating effects of wash fluid and seminal plasma were smaller. Such inverse relationships between sperm density and activation by semen fractions were commonly observed.

Fig. 4 gives the results of experiments with a sample of washed spermatozoa of abnormally low respiratory activity. The addition of fructose to this sample raised the respiratory activity to the level usually observed with normal spermatozoa in the presence of fructose (cf. Figs. 3 and 4). Respiration was also increased significantly by a lecithin–plasmalogen fraction (Table 1, b) which had been purified by column chromatography. However, this same fraction usually had very little and sometimes no activating effect upon the respiration of normal spermatozoa and we were unable to find evidence that it could be metabolized by washed spermatozoa (see below).

The accelerating effect of 'total lipid' obtained from the concentrate of wash fluid was traced to the presence of diffusible metabolites. Thus when the
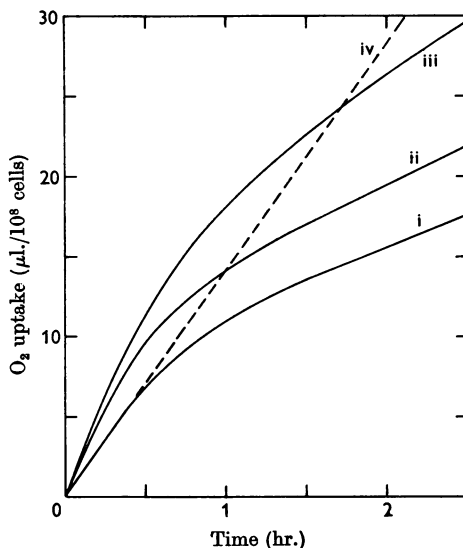

Fig. 3. Influence upon the respiratory activity of washed spermatozoa of dialysed seminal plasma, of dialysed concentrate of wash fluid and of fructose. To each manometer was added a suspension of $12·3 \times 10^8$ cells in 2 ml. of sperm Ringer solution. Volumes were made up to 3 ml. as follows: i, 1 ml. of sperm Ringer solution (control); ii, 1 ml. of dialysed seminal plasma; iii, 1 ml. of dialysed concentrate of wash fluid ($\equiv$ washings from spermatozoa in 7·7 ml. of semen); iv, 1 ml. of 10 mM-fructose in sperm Ringer solution. Temp. $37°$.

lipid was obtained from a concentrate that had been exhaustively dialysed it had no accelerating effect upon respiratory activity (Fig. 5). This 'dialysed total lipid' contained approximately
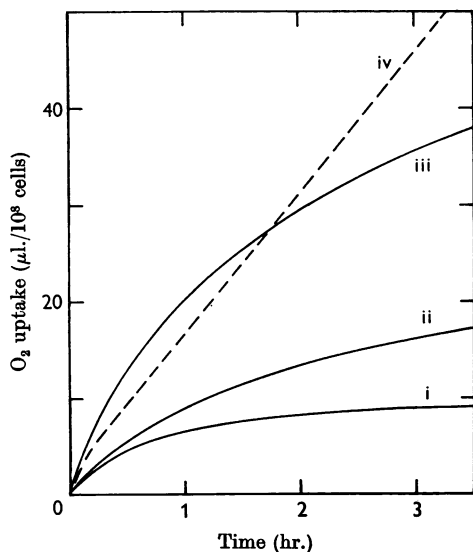

Fig. 4. Effects on the respiration of washed spermatozoa of a lecithin–plasmalogen fraction derived from spermatozoa, of total lipid extracted from wash fluids and of fructose. Each manometer received $9 \cdot 7 \times 10^8$ spermatozoa; otherwise the conditions were as for Fig. 1. i, Control; ii, with $10 \cdot 2$ mg. of lecithin–plasmalogen (Table 1, b); iii, with $9 \cdot 8$ mg. of total lipid from wash fluid; iv, with 10 mM-fructose.
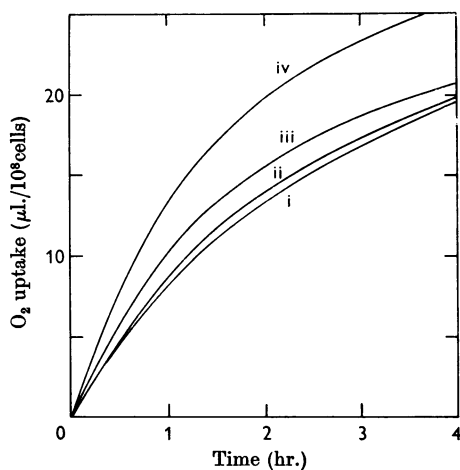

Fig. 5. Effects of sperm lipid fractions upon the respiration of washed spermatozoa. Each manometer received $16 \cdot 4 \times 10^8$ spermatozoa; otherwise the conditions were as for Fig. 1. i, Control; ii, with $11 \cdot 4$ mg. of dialysed total lipid from wash fluid; iii, with $6 \cdot 1$ mg. of free fatty acids from spermatozoa; iv, with $9 \cdot 8$ mg. of total lipid from wash fluid.

$0 \cdot 1 \%$ of lactic acid and negligible fructose, whereas 'total lipid' (from non-dialysed concentrate) contained $2 \cdot 9 \%$ of lactic acid and $0 \cdot 22 \%$ of fructose, despite the washing of the chloroform solution of lipid with magnesium chloride solution (Folch et al. 1957). The 'free fatty acids' of ram spermatozoa brought about a barely significant acceleration of sperm respiration (Fig. 5) and the 'non-lipid, non-diffusible fraction' had no effect.

Inability of washed spermatozoa to metabolize added lecithin–plasmalogen extracted from spermatozoa

A suspension of washed ram spermatozoa in sperm Ringer solution (wash procedure 1) containing $4 \cdot 9 \times 10^8$ cells/ml. was divided into two parts. To one (S +) was added the lecithin–plasmalogen fraction from ram spermatozoa (Table 1, b) at a concentration of $3 \cdot 4$ mg./ml. The other part (S −) received no additions. Each part was divided into three samples, which were treated as the samples A, B and C described above, the incubation time being 135 min. Lipid was extracted from the six samples and analysed for acyl ester and plasmalogen. In Table 6 are shown the excess of acyl ester and of plasmalogen in the S + samples above the levels in the corresponding S − samples. It is clear that no appreciable metabolism of added lecithin or plasmalogen occurred.

DISCUSSION

Effects of added lipids on spermatozoa

In view of the long-entrenched belief that sperm lipids are chiefly composed of lecithin, it is not surprising that experiments on the effect of added phospholipids on sperm motility and metabolism have hitherto been confined to lecithin, mostly in the form of preparations obtained from egg-yolk (Lardy & Phillips, 1941b; Bomstein & Steberl,

Table 6. *Inability of washed ram spermatozoa to metabolize added lecithin–plasmalogen isolated from spermatozoa*

S +, A suspension of spermatozoa ($4 \cdot 9 \times 10^8$ cells/ml.) in Ringer solution containing $3 \cdot 4$ mg. of lecithin–plasmalogen/ ml. (Table 1, b); S −, the same suspension without lecithin-plasmalogen. The two suspensions were each divided into three parts and treated as samples A, B and C (Table 3). Incubation was for 135 min. at 37°. The lipids extracted from the six samples were analysed for acyl ester and for plasmalogen. A, Control; B, anaerobic; C, aerobic.

	Excess in S + over S − suspensions (μequiv./ml.)		
	A	B	C
Acyl ester	$3 \cdot 78$	$3 \cdot 88$	$3 \cdot 78$
Plasmalogen	$2 \cdot 48$	$2 \cdot 62$	$2 \cdot 53$

1957). Recent research, however, has revealed the presence of a high concentration of choline-based plasmalogen in spermatozoa and, at the same time, has provided a strong indication that this aldehydogenic phospholipid is involved in aerobic sperm metabolism (Hartree & Mann, 1959, 1960a). We have therefore extracted from ram spermatozoa both the total phospholipid as well as the lecithin–plasmalogen fraction and examined the effect of these preparations on the aerobic metabolism of washed ram spermatozoa. Crude total phospholipid had usually quite a strong stimulating effect on sperm respiration, but this was shown to be due, not to lipids as such, but to impurities such as fructose and lactic acid which are carried over into such lipid preparations during the extraction procedure. The lecithin–plasmalogen fraction on the other hand separated by the use of silicic acid columns, had only occasionally a small stimulating influence on sperm respiration, whereas in most instances, particularly when sufficient care was taken to protect the spermatozoa from loss of motility and metabolic activity during washing, no effect or only a negligible rise was observed. Marked activating effects on sperm respiration were, however, observed with dialysed concentrates of wash-fluid and with dialysed seminal plasma, but these were probably due to a protective action on the sperm cells of either lipoproteins or some other protein constituents. Similar effects, usually attributed to the stabilizing action of 'protective colloids', are believed to account for the success of many semen diluents, including the egg-yolk buffer diluent, in the preservation of semen for the purpose of artificial insemination (Phillips, 1939; Phillips & Lardy, 1940; Mann, 1954).

Composition of choline-containing lipids extracted from spermatozoa and seminal plasma

The composition of the phospholipids extracted from ram spermatozoa appears to vary according to the treatment to which the cells were subjected before the actual extraction with lipid solvents. This difference becomes particularly obvious when the results from analysis of fresh or wet-frozen spermatozoa are compared with those obtained with freeze-dried material, and it may well account for some of the conflicting views expressed by Lovern et al. (1957) and Gray (1960a).

With sperm material which had not been subjected to freeze-drying before extraction, the elution of lipids from silicic acid columns follows the usual pattern (Hanahan et al. 1957). The first fraction to emerge has a low N:P ratio, and presumably contains the polyglycerol phosphatide which has been characterized by McKibbin & Taylor (1952). This is followed closely by the kephalin fraction. A solvent richer in methanol

then elutes the major fraction in which most, or all, of the nitrogen is due to choline, and which we have named the lecithin–plasmalogen fraction since it contains both lecithin and choline-based plasmalogen. Finally, by the use of pure methanol, another fraction containing sphingolipids and lyso compounds can be eluted. This latter fraction is very small except for lipids extracted from washed spermatozoa that had been actively respiring in the absence of sugar for several hours at 37°.

With the lipids of freeze-dried sperm, the elution pattern is different in respect to the lecithin–plasmalogen fraction which, in effect, divides during chromatography into two fractions. While the first of these (no. 3) is eluted rapidly, the second (no. 4) follows slowly, and is responsible for the long 'tailing' of the plasmalogen fraction first described by Lovern et al. (1957). Moreover, when the solvents were removed, both fractions appeared as oils, in contrast with the waxy consistency of the lecithin–plasmalogen fraction obtained from non-dried sperm. The first of the two fractions mentioned above (no. 3) also exhibited some other unusual characteristics. Its content of fatty aldehyde + acyl ester was markedly in excess of phosphorus, probably owing to the presence of glycerides, and it contained some phenylalanine, possibly in the form of a lipoamino acid. The second fraction (no. 4) corresponded in composition to a mixture of 72 % of plasmalogen and 28 % of some material containing 1·8 % of non-choline nitrogen. The ratio between acyl ester, aldehyde and choline was 1:1:1, which confirms the earlier findings of Lovern et al. (1957) that the lecithin–plasmalogen obtained from freeze-dried sperm consists mainly of plasmalogen and contains no appreciable quantity of lecithin.

As regards the nitrogen:choline ratio in lecithin–plasmalogen preparations, this tends to be higher than 1, whether or not the sperm had been freeze-dried before extraction. The nature of the excess of nitrogen is at present unknown. It is not due to amino acids, at least in fresh spermatozoa. The presence in phospholipid fractions of excessive nitrogen which could not be accounted for in terms of known bases or amino acids has also been noted by other investigators (McKibbin & Taylor, 1952; McKibbin, 1957).

Although it is clear from our study that the choline phosphatide fraction as isolated from non-dried material contains lecithin in addition to plasmalogen, there remains a disagreement between the results which Gray (1960a) obtained with frozen, whole ram semen, and our data, which are based on analyses of frozen washed spermatozoa and frozen seminal plasma (Table 1). The lecithin–plasmalogen fraction that we prepared from washed spermatozoa and seminal plasma contained to-

gether 232 μequiv. of choline/100 ml. of semen. Only 12·6 μequiv. of choline/100 ml. of semen were present in the wash-fluid from spermatozoa, and even this small quantity was probably due mostly to glycerylphosphorylcholine rather than to lipids. Of the lipid contained in the lecithin–plasmalogen fractions of frozen spermatozoa and frozen plasma, 80 % was in the form of plasmalogen as compared with 55 % reported by Gray (1960a). It seems unlikely that this difference can only be due to the fact that whereas Gray used whole semen, we performed our analyses after the semen had been separated into sperm and seminal plasma. A more likely explanation may lie in different storage conditions, particularly with regard to the actual temperature and duration of freezing. It is quite possible that under certain cold-storage conditions, sperm lecithin is rendered non-extractable. This possibility is borne out strongly by our analyses of a lecithin–plasmalogen fraction prepared from freshly washed, non-frozen ram spermatozoa. In such material the plasmalogen/lecithin ratio was, in fact, nearly 1:1 and very close to the value reported by Gray (1960a). It would appear from our study that fresh, rather than frozen, material should if possible be used for studies on phospholipid distribution.

Breakdown of plasmalogen in washed spermatozoa

We previously suggested (Hartree & Mann, 1959) that in the absence of seminal fructose the energy requirements of washed spermatozoa are derived from the oxidation of fatty acids and that these acids arise through the breakdown of plasmalogen. This view was of necessity based upon the belief that lecithin is not present in ram spermatozoa, since our previous methods could not distinguish between lecithin and plasmalogen as the source of fatty acid. We have found (Hartree & Mann, 1959) that, in sugar-free suspensions, the rate of hydrolysis of fatty acyl ester in washed ram spermatozoa is the same in the presence as in the absence of oxygen. The experiments reported in the present study, however, show that the net accumulation of fatty acids in such suspensions is always lower after aerobic than after anaerobic incubation. Thus, in the presence of oxygen, fatty acids are metabolized and such metabolic activity, expressed as the difference between anaerobic and aerobic levels, is proportional to activity in terms of oxygen utilization (Table 3). Chromatographic analysis of the fatty acids indicates that the rise in the fatty acid concentration which one observes in anaerobically incubated sperm samples is largely accounted for by an increase in the content of myristic acid and palmitic acid and that the aerobic disappearance is also mainly due to the same two acids. Our previous calculations (Hartree & Mann,

1959) on the ratio between O_2 uptake and fatty acids oxidized were based on an assumption that the mean length of these fatty acids was about 20 carbon atoms. However, even if oxidations in spermatozoa should involve only C_{14} and C_{16} fatty acids in approximately equimolar amounts, such a process would still require 22 mol.prop. of oxygen for complete combustion. Our results (Table 3, final column) show that the actual amounts of oxygen consumed by washed spermatozoa are consistently below this value, and support the view that fatty acids are available in excess of the amount theoretically required to support respiratory activity. In addition, there is the possibility that the incubation mixture may also contain other utilizable fatty acids, of chain length C_{12} or less, which would not be detected by our method of analysis.

The results given in Table 5 confirm that the ratio lecithin:choline plasmalogen in freshly washed spermatozoa is close to unity; and in fact the analysis of the control sample of Expt. iv gives a value of 48 % of plasmalogen. In the same experiment the decreases in lecithin–plasmalogen phosphorus during anaerobic and aerobic incubation were 10·9 and 11·2 % respectively. If these decreases were due entirely to disappearance of plasmalogen the decreases in the latter would have been 23·6 and 24·3 % respectively. The agreement between these figures and the experimentally determined decreases in plasmalogen (Table 5, last line) leads to the conclusion that, within experimental error, there was no net breakdown of lecithin during our incubation experiments and that the sperm plasmalogen was the source of the fatty acids that appeared during anaerobic incubation. Such metabolic experiments do not rule out the possibility of a turnover of lecithin as envisaged by Dawson (1957). They also give a figure for percentage of plasmalogen in the lecithin–plasmalogen fraction which will be lower than the value *in vivo* since some decomposition will occur during the interval between separation of sperm from plasma and the beginning of a metabolic experiment. A similar low value (49 %) was obtained from the analysis of the lecithin–plasmalogen fraction of spermatozoa from other metabolic experiments (Table 1). Thus the value 55 % (Table 1) is probably more representative of ram spermatozoa *in vivo*.

The demonstration that spermatozoa can utilize their intracellular plasmalogen as a source of energy raises the question of the physiological importance of this lipid for sperm motility and survival, in comparison with extracellular substrates which are present in semen, such as fructose and lactic acid. Fructose has the advantage over both lactic acid and plasmalogen of being able to serve as a substrate not only aerobically but in the

absence of oxygen as well. Its value would therefore be greatest when spermatozoa are present in a highly concentrated state, such as may exist for example in freshly ejaculated semen, at the site of deposition in the female reproductive tract. An average ram ejaculate of 1·2 ml., however, with 4×10^9 spermatozoa, 4 mg. of fructose, and a fructolysis index of 2 mg. of fructose/10^9 sperm/hr. at 37° (Mann, 1948), would have converted anaerobically all its fructose into lactic acid within about 15 min., and after that sperm survival would not be possible without an alternative source of energy. But by that time the process of sperm passage through the female reproductive tract has already begun, and it is presumably at that stage when the ability to oxidize extracellular lactic acid or intracellular plasmalogen assumes the greatest importance to spermatozoa. Provided that the partial pressure of oxygen within the uterus does not fall below that which is characteristic of animal tissues in general, there is no reason to suppose that the spermatozoa would be prevented from using these oxidizable substrates. It is interesting to recall, in this connexion, the observation that the rate of oxygen uptake of ram spermatozoa remains practically unaltered when the oxygen concentration has been lowered from 100 to 4 % (Humphrey & Mann, 1949). In addition, it has to be remembered that the energy which a cell can derive from the aerobic breakdown of a carbohydrate or a fatty acid is greater by far than that obtainable from glycolysis alone. Thus, as recently pointed out by Rothschild (1960), the complete oxidation of fructose can provide the sperm cell with about 24 times as much energy as can anaerobic fructolysis. All this, together with the long-established fact that many of the ram spermatozoa which reach the Fallopian tubes are still perfectly motile (Quinlan, Maré & Roux, 1932), suggests that sperm cells should have no difficulty in maintaining their energy requirements *in vivo* for as long as they can depend either on extracellular oxidizable substrates such as lactic acid, of which there is an abundance both in the semen as well as in the secretions of the female tract, or on intracellular plasmalogen acting as a source of oxidizable fatty acids.

SUMMARY

1. The effects of lipid-containing fractions obtained from ram semen upon the respiration of ram spermatozoa have been examined.

2. Marked activations were observed with dialysed seminal plasma and dialysed concentrates of fluids used to wash the spermatozoa. These are ascribed to the presence of lipoproteins.

3. Phospholipid fractions sometimes activated sperm respiration to a small extent. However, activation was insignificant when care was taken to prevent impairment of motility during preliminary washing of the spermatozoa.

4. The phospholipids extractable by chloroform–methanol from washed spermatozoa and from seminal plasma have been fractionated on silicic acid columns.

5. We have confirmed our earlier finding that the major phospholipid in extracts of freeze-dried spermatozoa is choline plasmalogen and that lecithin is absent.

6. Extracts of fresh spermatozoa, and of spermatozoa and plasma which have been frozen, contain both plasmalogen and lecithin. For fresh spermatozoa the ratio is approximately 1:1.

7. The lipids extractable from spermatozoa that had been incubated in the absence of sugar under both anaerobic and aerobic conditions have been compared with those from non-incubated spermatozoa. Analyses were made of (a) free fatty acids, (b) choline plasmalogen, (c) phosphorus in the mixed lecithin–plasmalogen fraction.

8. Free fatty acids accumulated during anaerobic incubation, but in the presence of oxygen the accumulation was smaller or there was even a net loss. It was mainly C_{14} and C_{16} acids which accumulated in the absence of oxygen and that were oxidized in its presence.

9. There was no appreciable decrease in lecithin during incubation for 2·5–3 hr. at 37°, but about 25 % of the intracellular plasmalogen was hydrolysed.

10. These results strengthen our earlier view that, in the absence of fructose, ram spermatozoa can utilize as a source of energy the fatty acids which derive from breakdown of plasmalogen.

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Condensed Tannins

11. ISOLATION OF A CONDENSED TANNIN FROM BLACK-WATTLE HEARTWOOD, AND SYNTHESIS OF (±)-7:3':4'-TRIHYDROXYFLAVAN-4-OL*

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Bate-Smith & Swain (1953) suggested that leuco-anthocyanins, which are closely related to the catechins, should be regarded as prototypes of condensed tannins. In this connexion, Hillis (1954, 1956, 1958) has investigated the extent to which leuco-anthocyanins might be precursors of condensed tannins, and might be responsible for the red colour which develops when leather that has been tanned with mangrove and various *Eucalyptus* extracts is exposed to sunlight.

Robinson & Robinson (1935) isolated the first crystalline compound related to the flavan-3:4-diols, peltogynol, and thereafter King & Bottomley (1954) isolated the first member of the structurally simpler and more general series of crystalline flavan-3:4-diols. The progress of condensation of such compounds in the wood was examined (Roux & Evelyn, 1958 a, b, 1960) by anthocyanidin formation, by number-average molecular weights and by tannin analyses on radial and vertical drillings. These methods give some evidence for the presence of intact flavan-3:4-diol units in polyleuco-anthocyanins, which may be regarded as one class of condensed tannins. The association of authenticated monomeric flavan-3:4-diols and the corresponding polyleuco-anthocyanin tannins, which Roux (1958 a, b) demonstrated for the heartwoods of certain *Acacia* and *Schinopsis* spp., is similar to the association of leuco-anthocyanins and complex leuco-anthocyanins in cacao beans (Forsyth, 1952, 1955). On account of the isolation and chemical degradation of a complex leuco-cyanidin from cacao (Forsyth, 1953; Forsyth & Roberts, 1960), the isolation of other polyleuco-anthocyanins that may have tanning properties is of scientific importance. The isolation of one of these polymeric tannins from *A. mearnsii* is described, and certain of its properties are compared with those of synthetic (±)-7:3':4'-trihydroxyflavan-4-ol.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Analysis of C, H, acetyl groups, methoxyl groups and ash are by Weiler and Strauss, Oxford, and by K. Jones, Microanalytical Section,

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* Part 10: Roux & Paulus (1961 c).