PHOSPHOLIPID AND CHOLESTEROL CONTENT OF EPIDIDYMAL AND EJACULATED RAM SPERMATOZOA AND SEMINAL PLASMA IN RELATION TO COLD SHOCK

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Summary

The concentration of total phospholipid, phosphatidylcholine, phosphatidylethanolamine, and choline plasmalogen in spermatozoa from the caput epididymis of the ram was higher than in spermatozoa from the cauda. The phospholipid composition of spermatozoa from the latter region of the epididymis more closely resembled ejaculated spermatozoa.

In general, the plasma of caput and cauda semen was similar in total phospholipid content and composition. The total phospholipid concentration of ejaculated seminal plasma was only about one-fifth that of the caput and cauda fluid and all phospholipid fractions were greatly reduced.

The glycerylphosphorylcholine content of caput spermatozoa and seminal plasma was less than that of the corresponding semen fractions obtained from the cauda epididymis, and after ejaculation.

The concentration of non-esterified cholesterol in both the spermatozoa and plasma decreased on passing from the caput to the cauda epididymis and was even less in the corresponding fractions of ejaculated semen.

Spermatozoa taken from the testis and caput epididymis were little affected by cold shock whereas spermatozoa from the cauda epididymis and ampulla were much more susceptible, as judged by increased permeability to stain. It is suggested that a decrease in the phospholipid and cholesterol content of spermatozoa may be associated with an increase in the susceptibility of ram spermatozoa to cold shock as they pass through the male reproductive tract.

I. INTRODUCTION

After leaving the testis, mammalian spermatozoa pass through the epididymis and are stored in the cauda region and ampulla until ejaculation occurs. During this time the spermatozoa undergo a number of changes known as "maturation" (Mann 1964) which may include alteration in the lipid components of the spermatozoa (Dawson and Scott 1964; Scott, Voglmayr, and Setchell 1967).

One of the characteristics of ejaculated spermatozoa is their susceptibility to "cold shock" which occurs when semen above 0°C is rapidly cooled. This causes an irreversible loss of viability which is accompanied by an increased permeability of the cell and, presumably, changes to the cell lipoprotein membrane (Wales and White 1959; White and Wales 1960). Spermatozoa obtained from the epididymis are more resistant to cold shock than ejaculated spermatozoa (Lasley and Bogart 1944; Lasley and Mayer 1944; White and Wales 1961); however, the resistance of the latter can be increased by adding phospholipids to semen *in vitro* (Kampschmidt, Mayer, and Herman 1953; Blackshaw 1954; Blackshaw and Salisbury 1957). It

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was thought, therefore, that loss of phospholipid from the spermatozoa or seminal plasma in the epididymis might be a factor contributing to the increased susceptibility of ejaculated spermatozoa to cold shock. Lovelock (1954; 1955) has proposed that a decrease in the phospholipid to cholesterol ratio of the erythrocyte membrane results in instability and permeability changes associated with cold shock.

This paper reports phospholipid, cholesterol, and related analyses of the spermatozoa and plasma obtained from the caput and cauda regions of the epididymis and of the semen on ejaculation. Studies have also been made of the permeability of testicular, epididymal, and ampulla spermatozoa to Congo red to see if there is any relationship between susceptibility to cold shock and changes in the lipid composition of the spermatozoa as they pass through the male reproductive tract.

II. Materials and Methods

(*a*) *Semen*

Ejaculates were obtained from five mature Merino rams by electrical stimulation; the rams were then killed and the testes removed. Semen was collected from the caput and cauda epididymis by cutting the ducts and squeezing out the fluid. The ejaculated and epididymal semen (1–2 ml) was centrifuged at 4000 *g* for 30 min at 15°C, the supernatant plasma withdrawn, and the kinoplasmic material on top of the plug discarded.

The plasma and spermatozoa were mixed with 150 mM NaCl to give a final volume of 5 ml. Counts of the resuspended spermatozoa were made in quadruplicate using standard haemocytometry.

(*b*) *Extraction of Glycerylphosphorylcholine(GPC) and Lipids*

Spermatozoa and seminal plasma were extracted with 10 volumes hot chloroform–methanol (1 : 1 v/v) followed by extraction with 5 volumes chloroform. GPC and other water-soluble, phosphorus-containing compounds were removed from the organic solvents by washing with water and twice with chloroform–methanol–water (3 : 48 : 47 v/v). The pooled aqueous phase was washed with ether which was added to the organic phase (Scott, Dawson, and Rowlands 1963).

(*c*) *Analysis of GPC*

The GPC extract was filtered through a small pad of glass wool and then eluted through a Zeo-Carb 226 (H^+) ion-exchange resin column of dimensions 1·5 by 30 cm. The eluate was shaken with n-butanol, neutralized with alcoholic ammonia, and evaporated to dryness (Dawson 1955). The residue was taken up in water and the GPC in suitable aliquots was hydrolysed at 100°C for 20 min with 1N HCl (Dawson, Mann, and White 1957). The free choline in the unhydrolysed and hydrolysed samples was estimated by the methods of Appleton *et al.* (1953) and Kushner (1956) except that optical densities were measured at 375 mμ where absorption was slightly better. The GPC was calculated from the difference in the concentration of free choline.

(*d*) *Silicic Acid Chromatography of Lipids*

Silicic acid columns (1 by 6 cm) were prepared from 100-mesh Mallinckrodt chromatographic grade silicic acid (Barron and Hanahan 1958) and the column packed under 3 lb/in^2 nitrogen. Cholesterol was purified via the dibromide (Fieser 1955) and after repeated recrystallization melted at 150°C. Cholesterol acetate, m.p. 114°C, was prepared from this by refluxing with acetic anhydride (Ralls 1955). To test the efficiency of separation of lipids, cholesterol (0·8 mg), cholesterol acetate (0·8 mg), and fresh egg-yolk phospholipid (0·9 mg) (Pangborn 1951) were applied to the column in 1 ml of hexane–benzene (85 : 15 v/v) and worked in with a further 4 ml of the same solvent. The following solvents were applied to the column under 3 lb/in^2

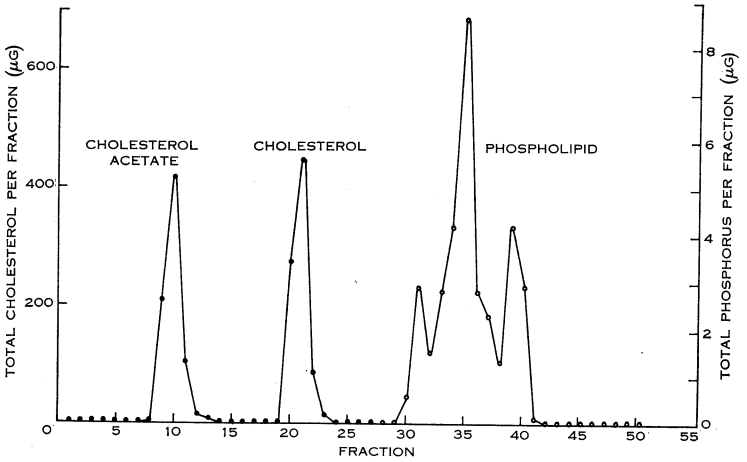

Fig. 1.—Total cholesterol and phosphorus concentration of 2-ml fractions collected by eluting 0·8 mg each of cholesterol and cholesterol acetate and 0·9 mg lecithin through a 1 by 6 cm silicic acid column with various solvents. The solvents used are listed in the text.

nitrogen: 5 ml hexane–benzene (50 : 50 v/v), 10 ml benzene, 10 ml benzene–ether (95 : 5 v/v), 20 ml benzene–ether (50 : 50 v/v), 10 ml ether, and 50 ml methanol. The eluate was collected in 2-ml fractions and determinations of phosphorus (Fiske and Subba Row 1925) and total cholesterol (Henly 1957) made on each fraction. A typical result is presented in Figure 1. Each lipid extract was chromatographed by the above procedure and the appropriate fractions containing cholesterol, cholesterol esters, and phospholipids were pooled and evaporated to dryness *in vacuo* at < 50°C.

(*e*) *Separation and Determination of Individual Phospholipids*

The individual phospholipids in the phospholipid fraction were determined from the products of successive hydrolysis by the methods of Dawson (1960) and Dawson, Hermington, and Davenport (1962). Samples were run with authentic glycerylphosphorylethanolamine and GPC. The GPC was obtained as a complex with cadmium (50% w/w) from L. Light & Co. Ltd., England, and most of the cadmium removed by passing a solution through a mixed-bed, ion-exchange resin

consisting of one-third Zeo–Carb 226 (H^+) and two-thirds Deacidite FF (OH^-) of dimensions 0·8 by 11 cm. The concentration of cadmium in the effluent, as estimated by atomic absorption spectroscopy (Willis 1963), was 0·65 mg per 1 g GPC.

(*f*) *Gas Chromatography of Cholesterol*

The cholesterol ester fraction was evaporated to dryness *in vacuo* and hydrolysed to free cholesterol by taking up the residue in 5 ml of methanol–water (80 : 20 v/v) containing 1% KOH and heating at 60°C for 15 min. The insoluble residue after saponification was removed by filtering the hot extract through glass wool and

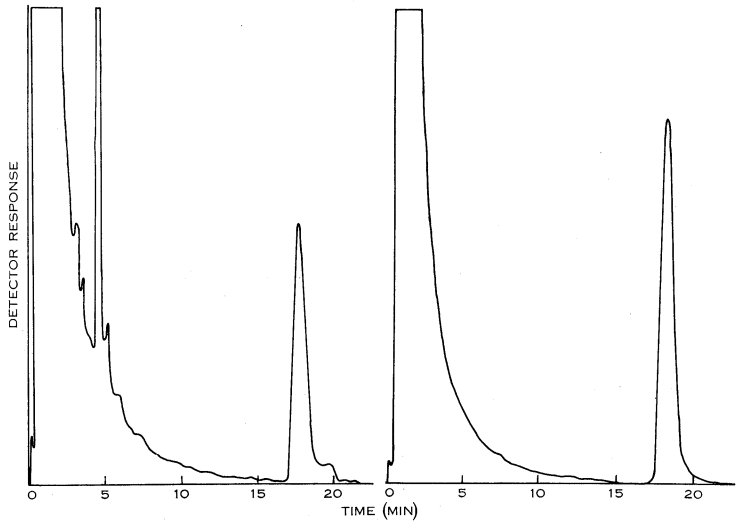

Fig. 2.—Gas chromatographic elution pattern of the cholesterol fraction of ram spermatozoa from silicic acid chromatography followed by an application of purified cholesterol in benzene. Column conditions and gas flows are given in the text.

extracting the residue twice with 5-ml aliquots of hot methanol. The hydroxide was removed by passing through a bed of Zeo–Carb 226 (H^+) resin of dimensions 1·5 by 6 cm. The eluate was evaporated to near dryness *in vacuo* at < 80°C and the residue taken up in 5 ml of water. The lipid was then extracted twice in a separating funnel with 10 ml of benzene and the upper layers pooled and taken to dryness.

The samples were then dissolved in benzene to give solutions containing 30–600 μg/ml and chromatographed and analysed using an F & M model 810 gas chromatograph fitted with dual flame ionization detectors. The column was packed with Gas–Chrom Q coated with Silicone SE 30 (1·5 g/100 ml in methylene chloride) using the filtration technique described by Horning, Moscatelli, and Sweeley (1963). The amount of stationary phase on the support was determined by a 6-hr Soxhlet extraction of a sample with methylene chloride and was 2·15 g/100 g. Two glass columns (4 mm by 1·8 m) were packed under positive nitrogen pressure (Horning, Vanden Heuvel, and Creech 1963) and plugged at both ends with silanized glass wool.

The samples were run with dual column operation. The temperature was 237°C, the carrier gas helium, and the flow rate 50 ml/min. The retention time for cholesterol

was about 18 min (Fig. 2). There was a linear relationship between peak area, as determined by the product of peak height and width at half height, and the amount of standard cholesterol applied. Estimates of the cholesterol content of samples by the area to weight ratio curve and by peak enhancement agreed well and the latter method was used in the calculations.

Spermatozoa were cold-shocked by placing tubes containing the semen held at 37°C into a bath at 0°C for 10 min. The permeability of the spermatozoa to Congo red stain after cold treatment was determined by the method of Quinn, Salamon, and White (1968).

Analyses of variance have been made on the SILLIAC electronic digital computer and the error mean square was used to obtain the standard error for *t*-tests.

TABLE 1

MAJOR PHOSPHOLIPIDS IN FLUIDS FROM THE CAPUT AND CAUDA EPIDIDYMIS AND IN EJACULATED RAM SEMEN

Values are the mean for five rams and have been calculated from the phosphorus estimations (lipid = $^{31}P \times 25$)

Phospholipid	Phospholipid Concentration in Spermatozoa ($\mu g/10^9$ cells)			Phospholipid Concentration in Plasma (mg/100 ml)		
	Caput	Cauda	Ejaculated	Caput	Cauda	Ejaculated
Total phospholipid	3241	1721*	1590*	666	645	127**
Phosphatidylcholine	726	305**	297**	139	211	13*
Phosphatidylethanolamine	358	230*	158**	59	62	37
Choline plasmalogen	810	478**	436**	111	59	22*
Ethanolamine plasmalogen	422	279	129**	179	90*	38**
Sphingomyelins	337	232	382	43	63*	11**
Other phospholipids	569	429	370	22	171*	6

* Significantly different from the caput value at $P < 0{\cdot}05$.
** Significantly different from the caput value at $P < 0{\cdot}01$.

III. RESULTS

(*a*) *Phospholipid Analyses*

The concentration of the major phospholipids in the spermatozoa and plasma of ram semen obtained from the caput and cauda epididymis and on ejaculation is shown in Table 1. Choline plasmalogen was quantitatively the most important phospholipid in the spermatozoa but appreciable amounts of phosphatidylcholine, phosphatidylethanolamine, ethanolamine plasmalogen, and sphingomyelins were also present. The total phospholipid content of spermatozoa from the caput epididymis was higher than that of cauda or ejaculated spermatozoa and this was reflected in all phospholipid fractions except the sphingomyelins. The total phospholipid, phosphatidylcholine, and choline plasmalogen content of spermatozoa from the cauda epididymis was similar to that of ejaculated spermatozoa but the latter had a lower phosphatidylethanolamine and ethanolamine plasmalogen content than cauda cells and a rather higher sphingomyelin content.

In general, the plasma of caput and cauda semen was similar in total phospholipid content and composition. The predominant fractions were phosphatidylcholine and ethanolamine plasmalogen in both fluids, although the caput plasma contained rather more of the former and less of the latter than did the cauda plasma. The total phospholipid concentration of ejaculated seminal plasma was only about one-fifth that of the caput and cauda fluid and all phospholipid fractions were greatly reduced.

TABLE 2

WATER-SOLUBLE PHOSPHORUS, GLYCERYLPHOSPHORYLCHOLINE, AND CHOLESTEROL IN FLUIDS FROM THE CAPUT AND CAUDA EPIDIDYMIS AND IN EJACULATED RAM SEMEN

Mean values are given for five rams

Analysis	Concentration in Spermatozoa ($\mu g/10^9$ cells)			Concentration in Plasma (mg/100 ml)		
	Caput	Cauda	Ejaculated	Caput	Cauda	Ejaculated
Total water-soluble phosphorus	160	181	308*	64	352**	40
Glycerylphosphoryl-choline	178	360	406	199	1474**	259
Non-esterified cholesterol	535·6	369·4*	203·8**	101·7	37·6**	4·9**
Esterified cholesterol	3·9	1·2	20·3**	1·2	1·2	1·3

* Significantly different from caput at $P < 0{\cdot}05$.
** Significantly different from caput at $P < 0{\cdot}01$.

(*b*) *Water-soluble Phosphorus and GPC Analyses*

Analyses of the total water-soluble phosphorus and GPC content of spermatozoa and plasma from the caput and cauda epididymis are compared with results for ejaculated semen in Table 2. Ejaculated spermatozoa had a significantly higher total water-soluble phosphorus content than caput or cauda spermatozoa but the GPC content of the ejaculated and cauda spermatozoa was not significantly higher than that of the caput spermatozoa. The total water-soluble phosphorus and GPC content of the cauda plasma was much higher than that of the caput or ejaculated plasma.

(*c*) *Cholesterol Analyses*

Table 2 also shows the distribution of esterified and non-esterified cholesterol in ejaculated semen and semen from the epididymis. The concentration of non-esterified cholesterol in both the spermatozoa and plasma decreased on passing from the caput to the cauda epididymis and was even less in the corresponding fractions of ejaculated semen. The esterified cholesterol concentration in the plasma of ejaculated semen was much the same as in the plasma from the caput and cauda but the concentration in ejaculated spermatozoa was much higher than in epididymal spermatozoa.

(*d*) *Permeability of Spermatozoa after Cold Shock*

The permeability of ram spermatozoa to Congo red stain before and after cold shock is shown in Table 3. Spermatozoa taken from the testis and caput epididymis were little affected by cold shock whereas spermatozoa from the cauda epididymis and ampullae were much more susceptible, as judged by increased permeability to the stain.

TABLE 3

EFFECT OF COLD SHOCK ON THE PERMEABILITY TO CONGO RED OF RAM SPERMATOZOA FROM VARIOUS REGIONS OF THE REPRODUCTIVE TRACT

Values are the percentage of unstained spermatozoa and are the mean of four replicates

Treatment	Testis	Caput Epididymis	Cauda Epididymis	Ampulla
Initial	87	85	85	81
Cold shock	82	73	26**	25**

Summary of Analysis of Variance of Angular Transformed Cold Shock Data

Source of Variation	Degrees of Freedom	Variance Ratio
Between regions	3	70·92†
Between replicates	3	2·20
Residual mean square	9	19·53

** Significantly different from testis at $P < 0{\cdot}01$.
† $P < 0{\cdot}01$.

IV. DISCUSSION

The analyses of spermatozoa from the caput and cauda epididymis suggest there is a considerable loss of all phospholipid fractions from ram spermatozoa as they pass through the epididymis. In contrast, the analyses on ejaculated spermatozoa indicate that after leaving the epididymis there is little further loss of phospholipid, apart from some loss in phosphatidylethanolamine and ethanolamine plasmalogen.

Scott, Voglmayr, and Setchell (1967) have come to a similar conclusion from analyses on testicular and ejaculated ram spermatozoa, although they did not find as great a fall in choline plasmalogen. Our total phospholipid values for spermatozoa from the caput epididymis are more than 40% higher than the values for testicular spermatozoa reported by these workers and suggest synthesis of phospholipids by spermatozoa in the caput, possibly to provide substrate during subsequent storage in the cauda.

Terner and Korsh (1962) and Minassian and Terner (1966) have shown that bull, human, and fish spermatozoa have such synthetic ability, and it has been reported by Scott, Voglmayr, and Setchell (1967) that the rate of phospholipid synthesis by testicular spermatozoa is about four times that of ejaculated cells *in vitro*. However, caution is needed in interpreting phospholipid analyses of spermatozoa collected from the caput epididymis after slaughter, as some contamination with extraneous tissue of high phospholipid content can occur (Scott, Dawson, and Rowlands 1963). Furthermore, Dawson (1955) found that in a ram with ligated efferent ducts there was negligible incorporation of [^{32}P]orthophosphate from the blood into phospholipids of spermatozoa in the epididymis.

The loss of phospholipid from spermatozoa in the cauda epididymis does not result in an increase in the concentration of phospholipids in the seminal plasma, indicating that they are either removed from the lumen of the tubules or catabolized *in situ*. Ram spermatozoa are capable of breaking down phospholipids (Lardy and Phillips 1941; Hartree and Mann 1961) and it has been suggested (Scott, Voglmayr, and Setchell 1967) that the fatty acid residues of intracellular phospholipids act as a source of energy for spermatozoa in the epididymis.

It is generally believed that epididymal tissue is responsible for the production of most of the GPC found in semen (Dawson, Mann, and White 1957; Dawson and Rowlands 1959; Scott *et al.* 1963; Wallace, Wales, and White 1966). The loss of fatty acids and aldehydes from spermatozoal phospholipids during their passage through the epididymis could, however, account for about 20% of the GPC in the cauda epididymis of the ram. This might, therefore, be the explanation for the stimulation of GPC formation by the epididymis in the presence of spermatozoa (Scott, Dawson, and Rowlands 1963). Dilution of the epididymal fluid with secretions from other accessory organs such as the seminal vesicles causes the sharp drop in GPC concentration on ejaculation.

Our results and also those of Scott, Voglmayr, and Setchell (1967) suggest that the cholesterol in spermatozoa and seminal plasma may be broken down as semen passes through the epididymis and down the ampullae. In the latter region of the tract some esterification of cholesterol may also occur in the spermatozoa, since the concentration of esterified cholesterol is higher in ejaculated spermatozoa than in spermatozoa from the epididymis. An alternative explanation for the progressive fall in the non-esterified cholesterol levels of the seminal plasma (Table 2) may be absorption through the wall of the cauda and dilution of the remaining luminal cholesterol by other accessory fluid on ejaculation. The cholesterol concentration of ram seminal plasma, like that of the boar (Komarek, Pickett, Gibson, and Jensen 1965) and stallion (Komarek, Pickett, Gibson, and Lanz 1965), is considerably less than that in man (Eliasson 1966) presumably due to the small contribution of the prostate to the ejaculate of these domestic species.

Spermatozoa taken from the testis and caput epididymis of the ram are not affected by cold shock, as judged by staining reaction, but become susceptible in the cauda epididymis where the loss of choline-base phospholipid is greatest. Variation in lipid composition has been shown to produce changes in the physicochemical

properties of synthetic phosphatides and in the permeability of the red cell membrane (van Deenan *et al.* 1962, 1963). Loss of phospholipid and cholesterol from spermatozoa as they pass down the male tract may, therefore, alter the characteristics of the spermatozoal lipoprotein membranes and so be concerned with their increasing susceptibility to cold shock. Lovelock (1954, 1955) has suggested that a decrease in membrane phospholipid to cholesterol ratio may render erythrocytes more susceptible to cold shock. The analyses on ram spermatozoa, however, indicate that this ratio (caput = 6·05, cauda = 4·66, ejaculated = 7·10) is of less importance than the overall decrease in total phospholipid.

The addition of phosphatidylcholine (Kampschmidt, Mayer, and Herman 1953; Blackshaw 1954; Blackshaw and Salisbury 1957) to semen after ejaculation is known to increase the resistance of spermatozoa to cold shock. The high concentration of phosphatidylcholine in the plasma of semen from the cauda epididymis may, therefore, be a factor contributing to the higher resistance of epididymal spermatozoa compared with ejaculated cells. It has been suggested that phosphatidylcholine forms a protective envelope around the spermatozoon (Milovanov and Sokolovskaja 1960; Ostasko 1963); its role, however, may be more subtle since recent work on erythrocytes indicates that added phospholipids become rapidly incorporated into the cell membranes (Sakagami, Minari, and Orii 1965).

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VI. References

Appleton, H. D., La Du, B. N., Levy, B. B., Steele, J. M., and Brodie, B. B. (1953).—A chemical method for the determination of free choline in plasma. *J. biol. Chem.* **205**, 803.

Barron, E. J., and Hanahan, D. J. (1958).—Observations on the silicic acid chromatography of the neutral lipides of rat liver, beef liver and yeast. *J. biol. Chem.* **231**, 493.

Blackshaw, A. W. (1954).—The prevention of temperature shock of bull and ram semen. *Aust. J. biol. Sci.* **7**, 573.

Blackshaw, A. W., and Salisbury, G. W. (1957).—Factors influencing metabolic activity of bull spermatozoa. II. Cold shock and its prevention. *J. Dairy Sci.* **40**, 1099.

Dawson, R. M. C. (1955).—The role of glycerylphosphorylethanolamine in liver phospholipid metabolism. *Biochem. J.* **59**, 5.

Dawson, R. M. C. (1960).—A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. *Biochem. J.* **75**, 45.

Dawson, R. M. C., Hermington, N., and Davenport, J. B. (1962).—Improvements in the method of determining individual phospholipids in a complex mixture by successive chemical hydrolyses. *Biochem. J.* **84**, 497.

Dawson, R. M. C., Mann, T., and White, I. G. (1957).—Glycerylphosphorylcholine and phosphorylcholine in semen and their relation to choline. *Biochem. J.* **65**, 627.

Dawson, R. M. C., and Rowlands, I. W. (1959).—Glycerylphosphorylcholine in male reproductive organs of rats and guinea-pigs. *Q. Jl exp. Physiol.* **44**, 26.

DAWSON, R. M. C., and SCOTT, T. W. (1964).—Phospholipid composition of epididymal spermatozoa prepared by density gradient centrifugation. *Nature, Lond.* **202**, 292.

DEENAN, L. L. M. VAN, DE GIER, J., HOUTSMULLER, U. M. T., MONTFOORT, A., and MULDER, E. (1963).—Dietary effects on the lipid composition of biomembranes. In "Biochemical Problems of Lipids". (Ed. A. C. Frazer.) p. 404. (Elsevier Publishing Co.: Amsterdam.)

DEENAN, L. L. M. VAN, HOUTSMULLER, U. M. T., DE HASS, G. H., and MULDER, E. (1962).—Monomolecular layers of synthetic phosphatides. *J. Pharm. Pharmac.* **14**, 429.

ELIASSON, R. (1966).—Cholesterol in human semen. *Biochem. J.* **98**, 242.

FIESER, L. F. (1955).—Cholesterol, Δ^5-cholesten-3-one, and Δ^4-cholesten-3-one. In "Organic Synthesis". (Ed. T. L. Cairns.) Vol. 35. p. 43. (John Wiley & Sons, Inc.: New York.)

FISKE, C. H., and SUBBA ROW, Y. (1925).—The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.

HARTREE, E. F., and MANN, T. (1961).—Phospholipids in ram semen: metabolism of plasmalogen and fatty acids. *Biochem. J.* **80**, 464.

HENLY, A. A. (1957).—The determination of serum cholesterol. *Analyst, Lond.* **82**, 286.

HORNING, E. C., MOSCATELLI, E. A., and SWEELEY, C. C. (1959).—Polyester liquid phases in gas–liquid chromatography. *Chemy Ind.* **1**, 751.

HORNING, E. C., VANDEN HEUVEL, W. J. A., and CREECH, B. G. (1963).—Separation and determination of steroids by gas chromatography. In "Methods of Biochemical Analysis". (Ed. D. Glick.) Vol. 11. p. 69. (Interscience Publishers, Inc.: New York.)

KAMPSCHMIDT, R. F., MAYER, D. T., and HERMAN, H. A. (1953).—Viability of bull spermatozoa as influenced by various sugars and electrolytes in the storage medium. Res. Bull. Mo. agric. Exp. Stn No. 519.

KOMAREK, R. J., PICKETT, B. W., GIBSON, E. W., and JENSEN, R. G. (1965).—Lipids of porcine spermatozoa, seminal plasma and gel. *J. Reprod. Fert.* **9**, 131.

KOMAREK, R. J., PICKETT, B. W., GIBSON, E. W., and LANZ, R. N. (1965).—Composition of lipids in stallion semen. *J. Reprod. Fert.* **10**, 337.

KUSHNER, D. J. (1956).—A spectrophotometric microdetermination of choline. *Biochim. biophys. Acta* **20**, 554.

LARDY, H. A., and PHILLIPS, P. H. (1941).—Phospholipids as a source of energy for spermatozoa. *Am. J. Physiol.* **134**, 542.

LASLEY, J. F., and BOGART, R. (1944).—A comparative study of the epididymal and ejaculated spermatozoa of the boar. *J. Anim. Sci.* **3**, 360.

LASLEY, J. F., and MAYER, D. T. (1944).—A variable physiological factor necessary for the survival of bull spermatozoa. *J. Anim. Sci.* **3**, 129.

LOVELOCK, J. E. (1954).—Physical instability and thermal shock in red cells. *Nature, Lond.* **173**, 659.

LOVELOCK, J. E. (1955).—Haemolysis by thermal shock. *Br. J. Haemat.* **1**, 117.

MANN, T. (1964).—"The Biochemistry of Semen and of the Male Reproductive Tract." p. 17. (Methuen & Co., Ltd.: London.)

MILOVANOV, V. K., and SOKOLOVSKAJA, I. I. (1960).—Cold shock in the semen of farm animals. *Zivotnovodstvo* **22**, 73.

MINASSIAN, E. S., and TERNER, C. (1966).—Biosynthesis of lipids by human and (teleost) fish spermatozoa. *Am. J. Physiol.* **210**, 615.

OSTASKO, F. I. (1963).—On the nature of cold shock in semen. In "Artificial Insemination in Farm Animals". p. 22. (Forest Steppe Inst. Anim. Breed., Ukrainian S.S.R., Karkov.)

PANGBORN, M. C. (1951).—A simplified purification of lecithin. *J. biol. Chem.*, **188**, 471.

QUINN, P. J., SALAMON, S., and WHITE, I. G. (1968).—The effect of cold shock and deep freezing on ram spermatozoa collected by electrical ejaculation and by an artificial vagina. *Aust. J. agric. Res.* **19**, in press.

RALLS, J. O. (1955).—Dihydrocholesterol (β-cholestanol). In "Organic Synthesis". (Ed. A. H. Blatt.) Coll. Vol. 2. p. 193. (John Wiley & Sons, Inc.: New York.)

SAKAGAMI, T., MINARI, O., and ORII, T. (1965).—Interaction of individual phospholipids between rat plasma and erythrocytes *in vitro*. *Biochim. biophys. Acta* **98**, 356.

SCOTT, T. W., DAWSON, R. M. C., and ROWLANDS, I. W. (1963).—Phospholipid interrelationships in rat epididymal tissue and spermatozoa. *Biochem. J.* **87**, 507.

SCOTT, T. W., VOGLMAYR, J. K., and SETCHELL, B. P. (1967).—Lipid composition and metabolism in testicular and ejaculated ram spermatozoa. *Biochem. J.* **102**, 456.

SCOTT, T. W., WALES, R. G., WALLACE, J. C., and WHITE, I. G. (1963).—Composition of ram epididymal and testicular fluid and the biosynthesis of glycerylphosphorylcholine by the rabbit epididymis. *J. Reprod. Fert.* **6**, 49.

TERNER, C., and KORSH, G. (1962).—The biosynthesis of C^{14}-labelled lipids by isolated bull spermatozoa. *Biochemistry, N.Y.* **1**, 367.

WALES, R. G., and WHITE, I. G. (1959).—The susceptibility of spermatozoa to cold shock. *J. Endocr.* **19**, 211.

WALLACE, J. C., WALES, R. G., and WHITE, I. G. (1966).—The respiration of the rabbit epididymis and its synthesis of glycerylphosphorylcholine. *Aust. J. biol. Sci.* **19**, 849.

WHITE, I. G., and WALES, R. G., (1960).—The susceptibility of spermatozoa to cold shock. *Int. J. Fert.* **5**, 195.

WHITE, I. G., and WALES, R. G. (1961).—Comparison of epididymal and ejaculated semen of the ram. *J. Reprod. Fert.* **2**, 225.

WILLIS, J. B. (1963).—Analysis of biological materials by atomic absorption spectroscopy. In "Methods of Biochemical Analysis". (Ed. D. Glick.) Vol. 11. p. 1. (Interscience Publishers, Inc.: New York.)