normally present, but it has also been possible to show that only a minor degree of iron deficiency occurs and only in the rare case does the marrow-iron level reach the low values found in iron-deficiency anaemia. This method may therefore be of use in differentiating between degrees of iron deficiency.

SUMMARY

1. A simple method for the determination of the non-haem iron content of bone marrow is described which is of sufficient accuracy for diagnostic purposes.

2. Results obtained by this method have been compared with those obtained by the Prussian-blue staining method.

3. The non-haem iron content of bone marrow from both non-anaemic and anaemic subjects has been determined.

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The Lipids of Ram Spermatozoa

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Since the pioneering studies of Miescher (1878,1897) it has been known that spermatozoa contain a considerable amount of intracellular lipid. Miescher believed the lipid material, which he extracted from salmon spermatozoa, to consist of 50% lecithin, 35% neutral fat and 14% cholesterol. Studies by Mathews (1897) and Sano (1922) on the semen of various fishes also indicated the presence of a large amount of lecithin in the sperm cells. The lipids of sea-urchin sperm have been analysed by Cardin & Meara (1953) and found to consist of 26·0% phospholipids, 32·9% free fatty acids, 13·6% neutral fat, 9·2% sterols and 18·3% of other unsaponifiable material. The lipid content of bull sperm was first determined by Kölliker (1856), who showed that ether-extractable material accounted for 12% of the dry weight of the sperm. Miescher (1878), who performed the first analysis of the lipid extracted from bull sperm, thought that about half of it consisted of lecithin. More recently, determinations of total lipid and phospholipids in bull sperm were carried out by Lardy & Phillips (1941) and Zittle & O'Dell (1941). The latter authors found that 13% of the dry weight of bull sperm was lipid, the sperm heads containing 7% lipid, the middle pieces 6% and the tails 23%.

The suggestion that lipids occur in spermatozoa in a bound state, presumably in the form of lipoproteins, was made by Halpern (1945) on the basis of his experiments with salmon semen. Mayer and

his colleagues (Mayer & Thomas, 1947; Thomas & Mayer, 1949; Dallam & Thomas, 1953; Mayer, 1955) obtained from the sperm heads of various mammalian species (including bull, boar, ram, dog and man) an alkali-soluble but acid-insoluble protein complex which contained nearly 30% of ether-extractable material composed largely of phospholipid and cholesterol.

The first indication that sperm lipids may be composed, in part, of acetal phospholipids (plasmalogens) came from a study of Feulgen & Rossenbeck (1924), who demonstrated that when fresh smears of semen were treated with Schiff's fuchsin–sulphurous acid reagent, the middle pieces and tails, although devoid of nucleic acid, stained very strongly. Later it was shown that the plasmalogen content of bull semen, as determined by Feulgen's method, varied from 30 to 90 mg./100 ml., and that about two-thirds of this amount was present in spermatozoa with the rest in the seminal plasma (Boguth, 1952).

In view of the scanty knowledge concerning the chemical nature of the lipids present in mammalian spermatozoa, a study was made of the various compounds which make up the intracellular lipid material in the spermatozoa of the ram. This species was chosen on account of the exceptionally high sperm density of semen, and the ease with which ram spermatozoa can be separated and washed free from seminal plasma.

EXPERIMENTAL

Spermatozoa, obtained from freshly collected ram semen by centrifuging and washing (Mann, 1946), were freeze-dried at −20°, powdered and preserved until extraction at −20° in a desiccator. Two batches of washed and freeze-dried ram sperm were available, which were collected during the 1954–55 and 1955–56 breeding seasons and subsequently called 'old sperm' (OS) and 'new sperm' (NS) respectively. Yields, 27·9 g. of OS and 23·3 g. of NS.

Each lot of NS had been kept out of contact with air in a sealed ampoule, and the total product was quite white. No precautions against oxidation had been taken with the OS, which was brown. The OS was examined in one batch, the NS in two batches of 11·0 and 12·3 g. respectively. The lipids were extracted by refluxing with three successive batches (350 ml. each) of $CHCl_3$–methanol (2:1, v/v) and purified by the procedure of Folch, Ascoli, Lees, Meath & Le Baron (1951). They were then further purified by passage in $CHCl_3$–ethanol–water (80:20:2·5, by vol.) through a cellulose column (50 g. of adsorbent; Lea & Rhodes, 1953). The yields of purified lipid were 2·62 g. (9·4%) from the OS (OSL) and 0·83 g. (7·5%) and 0·86 g. (7·0%) from the two batches of NS (NSL 1 and 2 respectively). The extraction and purification scheme is illustrated in Fig. 1.

Since the OSL might have undergone some deterioration during the lengthy storage of the dried spermatozoa, it was decided to use this material for preliminary studies and the NSL for subsequent, more elaborate, fractionation.

Analytical methods

Nitrogen was determined by a micro-Kjeldahl procedure, digestion being with 4 ml. of H_2SO_4, 4 g. of K_2SO_4 and 1 drop of Hg. After digestion for 20 min. the mixture was cooled and 1 ml. of 30% H_2O_2 added. This addition was repeated after a further 20 min. digestion and then followed a final digestion period of 4 hr. Phosphorus was determined by Allen's (1940) method, except that the $HClO_4$ digestion was performed in an oven at 170° for 4 hr. followed by 20 min. at 100° after dilution, to hydrolyse any pyrophosphate that might have been formed. Glycerophosphate was determined according to Olley & Blewett (1950) and choline either as the reineckate (Lovern, 1952) or by paper chromatography (Olley, 1956*b*). Paper chromatography was also employed to determine glycerol (Olley, 1956*a*), inositol and sugar (Olley, 1956*b*). For sugar, hydrolysis was

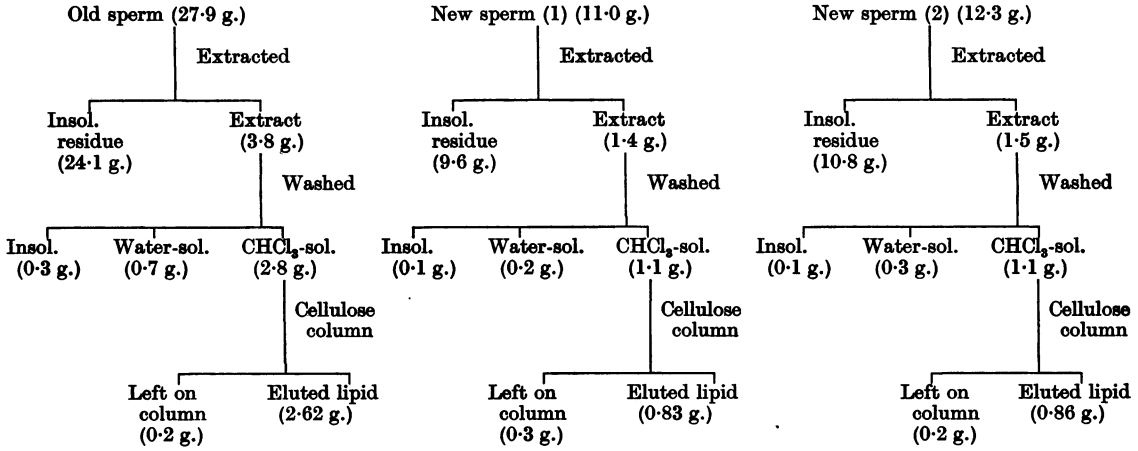

Fig. 1. Extraction and purification of sperm lipids. Extraction by $CHCl_3$–methanol (2:1, v/v). Crude extracts were washed by the procedure of Folch *et al.* (1951), in which a little insoluble material is collected at the interphase. Purification on cellulose was according to Lea & Rhodes (1953).

with 3N-H_2SO_4, as recommended by Mallov, McKibbin & Robb (1953) for cerebrosides; Amberlite IR 4B resin was used and the galactose control put through the entire procedure. Phosphorylcholine was determined by paper electrophoresis (Olley, 1956*b*), with borate buffer at pH 9. In all the above determinations on paper, areas were measured by cutting out and weighing the spots. Ethanolamine and serine were determined colorimetrically after two-dimensional chromatography on Whatman no. 1 paper, phenol–aq. NH_3 soln. and collidine–lutidine–water (Dent, 1948) being used. The air-dried chromatograms were sprayed on both sides with 1% (w/v) ninhydrin in wet butanol, the colour being allowed to develop over 2 days at room temperature (Thompson, 1951). The spots, including the control spots, were then cut out and eluted with 50% (v/v) aq. acetone for colorimetric assay. Sphingosine was determined by the method of McKibbin & Taylor (1949) and cholesterol, free and combined, as by Lovern & Olley (1953*a*).

Acetal phosphatides (plasmalogens) were determined in three ways: (*a*) colorimetrically on the original lipid, (*b*) colorimetrically on the isolated dimethylacetals and (*c*) gravimetrically. No entirely satisfactory assay procedure seems to have been evolved and controversy continues over the colorimetric methods, e.g. Feulgen, Boguth & Andresen (1953); Christl (1953); Leupold, Büttner & Ranniger (1953); Thiele (1955). We have found that whereas the colorimetric method of Feulgen & Grünberg (1938), with $CHCl_3$ as extractant (Lovern, 1952) gave poor replication, better replication and a satisfactory calibration curve (with palmitaldehyde) could be obtained by addition of extra acetic acid. This gave sharp separation of a clear $CHCl_3$ layer. In the procedure finally adopted, 5 ml. of the Feulgen & Grünberg reagent and 2 drops of 6% $HgCl_2$ were added to the samples dissolved in 1 ml. of acetic acid in well-stoppered 20 ml. test tubes. Colour development proceeded overnight at room temperature, after which 8 ml. of a mixture of $CHCl_3$, previously washed three times with water, and acetic acid (1:1, v/v) were added to each tube; the mixture was stirred and left for 10 min. A sample of the lower layer was then withdrawn for colorimetric assay. The more cumbersome procedure of Ehrlich, Taylor & Waelsch (1948) was also used, but only on OSL. Dimethylacetals were prepared according to Klenk & Friedrichs (1952) but, since this product must contain any unsaponifiable matter present in the original lipid fraction, it could not be used for a gravimetric assay. The latter was performed by direct isolation and weighing of the free aldehydes by a method (cf. Olley & Lovern, 1953) in which other unsaponifiable material is extracted before the aldehydes are liberated from the water-soluble alkali plasmalogenates (see below).

Examination of 'old-sperm lipid'

Analyses of N, P, cholesterol and plasmals were carried out on the intact lipid but the main bulk of this material was hydrolysed by two methods, namely, refluxing for 2 hr. with ethanolic 0·5N-KOH (1·05 g. of lipid) or refluxing for 6 hr. with dry methanolic 6N-HCl (1·05 g. of lipid), about 20 ml. of reagent being used in each. In addition, a smaller sample (0·262 g.) was hydrolysed by the special procedure of McKibbin & Taylor (1949) for sphingosine estimation, except that after adding the HCl the mixture was shaken with ether and 10 ml. of the aqueous layer removed for examination. The ether was then boiled off and the acid hydrolysis completed as usual. This permitted examination of the water-soluble products of hydrolysis with aqueous baryta.

After addition of water to the KOH hydrolysate, unsaponifiable matter was extracted with ether. The solution was then acidified with HCl and the mixture of fatty acids and free plasmals was recovered by further extractions with ether. These two products were separated by washing the ethereal extract with aqueous alkali (cf. Olley & Lovern, 1953, and the gravimetric aldehyde assay above). The acidified aqueous phase of the original KOH hydrolysate was evaporated on a steam bath under vacuum, for determination of water-soluble products.

The treatment of the methanolic HCl hydrolysate was a simplification of the procedure of Klenk & Friedrichs (1952). The mixture was extracted by countercurrent distribution in four separating funnels between light petroleum (b.p. 40–60°) and methanolic 6N-HCl. The combined petroleum phases, containing methyl esters of fatty acids, dimethylacetals of the plasmals and any other unsaponifiable matter, were neutralized with solid Na_2CO_3, evaporated and the residue was hydrolysed by refluxing with ethanolic 0·5N-KOH for 2 hr. Addition of water and extraction with ether gave acetals plus other unsaponifiable matter, the fatty acids being later recovered from the aqueous soap layer. The combined methanolic HCl phases were also evaporated. The residue was not entirely water-soluble, but the water-insoluble part was found to be soluble in $CHCl_3$.

It was assumed at this stage that the $CHCl_3$-soluble, water-insoluble material might be sphingosine hydrochloride or other sphingosine-containing derivatives, but that fatty acids should have been entirely removed from all known lipids by this hydrolysis (see Olley & Lovern, 1954, for discussion of sphingomyelin). Since we were primarily interested in the water-soluble components of the methanolic HCl phase from the countercurrent procedure, some of the $CHCl_3$-soluble material was discarded during filtration. Subsequently [see NSL (2) below] it was found that, on further hydrolysis with ethanolic KOH, this material could yield additional fatty acid. It also yielded unsaponifiable matter, which would, of course, include any sphingosine of the original lipid. In the present case, incomplete recovery of the $CHCl_3$-soluble material amounted to 10·8 g./100 g. of lipid, i.e. far in excess of that expected from the sphingosine content of the lipid (cf. Table 1). It was only analysed for N and P in OSL [Found: N 2·0, P 3·5% (atomic ratio N/P = 1·27)].

The general analytical results are given in Table 1, the distribution of P after hydrolysis is shown in Table 2 and the aldehyde determinations in Table 3. Paper chromatography of the unhydrolysed lipid in propanol–water (4:1, v/v) showed that all ninhydrin-staining material and also sugar ran with the lipid at the solvent front. After hydrolysis the sugar had the same R_F in propanol–water (4:1) as galactose, used as the control, but glucose has the same R_F in this solvent. Paper electrophoresis confirmed the identity of the glycerophosphate resulting from methanolic HCl hydrolysis and also revealed the presence of phosphorylcholine equivalent to 2·2% of the original OSL. No other organic phosphate or inorganic phosphate could be detected.

Acetylation of the unsaponifiable matter obtained by KOH hydrolysis permitted determination of the saponification equivalent (S.E.) of the non-cholesterol portion of this.

The total acetylated product had an S.E. of 358 and contained 71% of cholesteryl acetate (S.E. 428). Hence the S.E. of the non-cholesterol portion was 299, i.e. about that of the acetate of a typical long-chain aliphatic alcohol (cetyl acetate = 284). It should be noted that acetylation of this unsaponifiable matter yielded neutral acetates corresponding to only 75% of the original, the rest having become either acidic or water-soluble [see NSL (2), fraction1, below]. The corrected value is given in Table 1.

Table 1. *Analytical data on 'old sperm lipid', as g. of component/100 g. of lipid*

	KOH hydrolysis	HCl hydrolysis	Other procedures
Fatty acids	19·6	>16·8*	—
Unsaponifiable matter (excluding aldehydes)	12·8	>8·8*	—
Cholesterol (free)	—	—	7·7
Choline†	5·5	—	10·2
Ethanolamine‡	—	0·5	—
Serine‡	—	0·07	—
Sphingosine	—	—	2·0
Reducing sugar (as galactose)	—	—	1·4
Glycerol	0·4	—	—
Inositol	—	Nil	—
N (total)	—	—	1·4
P (total)	—	—	2·15

* No recovery from $CHCl_3$-soluble fractions after countercurrent procedure (see text).

† Determined by paper chromatography after hydrolysis with KOH. Other value was determined by reineckate after baryta hydrolysis.

‡ The only additional water-soluble, ninhydrin-staining substance found by paper chromatography was glycine (0·014 g./100 g. of lipid).

Examination of 'new sperm lipid'

Only a few analyses were carried out on NSL before submitting it to chromatographic analysis. It contained 2·2% total P, 8·6% cholesterol (free) and 17·3% plasmals (modified Feulgen & Grünberg, 1938, method), and hence appeared very similar to OSL.

Chromatography of new sperm lipid (1). Chromatography was carried out on a column prepared as described by Lovern (1956*a*), except that Mallinckrodt silicic acid was used instead of silica gel (British Drug Houses Ltd.). The column, measuring 36 cm. × 4 cm., was unnecessarily large for the amount of lipid available, having been prepared before purification of the crude lipids when larger amounts had been expected. Adsorbed oxygen had been removed with 4 g. of linoleic acid (Lovern, 1956*a*). The lipid (815 mg.) was added to the column dissolved in 50 ml. of light petroleum (b.p. 40–60°), followed by 25 ml. of flask rinsings of the same solvent. Elution was effected by the following series of solvents, each being replaced when its effectiveness was seriously decreasing: light petroleum (b.p. 40–60°), ether–light petroleum (1:99; 4:96; 10:90; 25:75; 50:50; v/v, successively), ether, methanol–ether (5:95; 15:85; 25:75; 40:60; 70:30; v/v, successively), ethanol–methanol (70:30, v/v), ethanol–methanol–water (70:30:1; 70:30:5; 70:30:10, by vol., successively), ethanol–ether–water (70:30:10, by vol.). Sixty-one fractions were collected. Total recovered weight was 800 mg. The chromatographic data are illustrated in Fig. 2, where lipid concentration (mg./100 ml.) is plotted against cumulative effluent volume, and hence areas are proportional to weight of dissolved lipid.

Fractions were pooled on the basis of elution behaviour, appearance and content of aldehyde. Thus despite the apparent non-homogeneity of the material eluted between points *E* and *K*, all of it contained about the same amount of

Table 2. *Distribution of phosphorus in 'old sperm lipid' after hydrolysis*

Values are g. of phosphorus/100 g. of lipid. Total P = 2·15% (Table 1).

Hydrolysis employed	Water-sol. (total)	Water-sol. (as GPP*)	In fatty acids	In aldehydes	In unsap.	Not accounted for
KOH	0·66	0·43	0·49	Nil	Nil	1·0
Baryta	0·73	0·68	←	0·07	→	1·35
HCl	1·98†	1·45	Nil	Nil	Nil	0·17

* Glycerophosphate P was estimated by the method of Olley & Blewett (1950).

† Soluble in $CHCl_3$, 0·38 g.; soluble in water, 1·6 g. Some $CHCl_3$-soluble material was lost; 0·34 g. was attributable to phosphorylcholine (see text).

Table 3. *Aldehyde content of 'old sperm lipid' determined by various methods*

Values are g./100 g. of OSL, calculated as palmitaldehyde. Methods used are as follows: 1, modified from Feulgen & Grünberg (1938), see text; 2, Ehrlich *et al.* (1948); 3, gravimetric, see text. DMA, Dimethylacetal.

	Aldehyde in		
Method	OSL	DMA*	OSL calc. from DMA value
1	16·8, 17·3	47·0, 55·0	12·7, 14·9
2	6·9, 8·5	52·6, 57·5	14·2, 15·5
3	19·6	—	—

* Dimethylacetal-containing fraction. Yield of this fraction was 27% of OSL.

aldehyde (5–8%, colorimetrically). The final pooling and certain analytical results on the pooled fractions are given in Table 4, and the results of hydrolysis of some of them are given in Table 5. As with OSL, the distribution of phosphorus after hydrolysis is given in detail (Table 6).

Fraction 1, from its ease of elution, might be expected to consist essentially of hydrocarbons. Heptacosane has been reported as a constituent of human semen (Wagner-Jauregg, 1941). This fraction was dissolved in ether, evaporated to small volume (0·5 ml.) and treated with absolute ethanol until the solution became opalescent. On storage overnight at −10°, a white solid separated, which was centrifuged off at −10° and washed in the centrifuge with cold ethanol. Analysis showed C 84·5, H 14·1% (Weiler & Strauss, Oxford), hence it was mainly hydrocarbon. Heptacosane requires C 85·2, H 14·8%. There was insufficient material for determination of molecular weight.

Fraction 5 consisted almost entirely of free sterols. The presence of cholesterol was confirmed by isolation of the dibromide (m.p. 113–115°, authentic specimen m.p. 118°, mixed m.p. intermediate), but the yield of dibromide suggested only 70% of cholesterol in fraction 5, instead of the 89% determined colorimetrically. The optical rotation (in $CHCl_3$) of fraction 5 was $[\alpha]_D = -27{\cdot}1°$ (cholesterol $= -39{\cdot}5°$). Fast- and slow-acting sterols were estimated according to Cook, Kliman & Fieser (1954). Slow-acting sterols, i.e. cholesterol, amounted to 66%, and no fast-acting sterols were present. Since these tests suggested that considerably less cholesterol was present than the 89% originally found, the estimation was repeated, by the same colorimetric procedure, which showed 74%. A considerable period had, however, elapsed between recovery from the column (and initial colorimetric assay) and these later tests, during which time the fraction, in solution in $CHCl_3$, had been kept at −15°. That a certain amount of decomposition had occurred was evident from the fact that the stored solution, on evaporation, gave a product less completely crystalline than the original one fresh from the column.

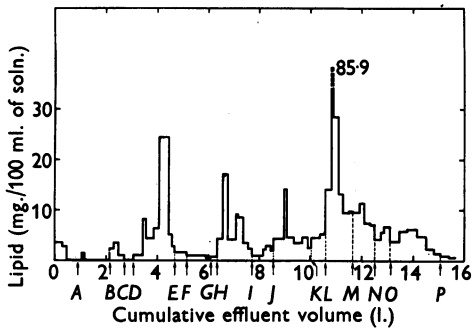

Fig. 2. Chromatography of NSL (1) on silicic acid. Solvents were changed at the points indicated by arrows. Initial solvent was light petroleum (b.p. 40–60°). Key to letters: *A–E*, ether–light petroleum (1:99; 4:96; 10:90; 25:75; 50:50 successively); *F*, ether; *G–K*, methanol–ether (5:95; 15:85; 25:75; 40:60; 70:30, successively); *L*, ethanol–methanol (70:30); *M–O*, ethanol–methanol–water (70:30:1; 70:30:5; 70:30:10 respectively); *P*, ethanol–ether–water (70:30:10). All solvent ratios are by vol.

Table 4. *Data on pooled chromatographic fractions of 'new sperm lipid* (1)'

All analytical values are as g./100 g. of lipid. A dash indicates that no determination was made.

Fraction no.	Range (Fig. 2)	Wt. (mg.)	Appearance*	Aldehyde†	N	P	N:P (atomic)	Cholesterol
1	Origin to *A*	15·2	Colourless paste	—	—	—	—	—
2	*A–B*	1·9	White wax	—	—	—	—	—
3	*B–C*	10·4	White gum	—	—	—	—	Nil
4	*D–E* (first three fractions)	27·0	Yellow oil	—	—	—	—	Nil
5	*D–E* (next two fractions)	104·5	Crystalline at 100°	—	—	—	—	89
6	*D–E* (last two fractions)	10·6	Oil at 100°, solid at room temp.	—	—	—	—	11
7	*E–K* (except final fraction)	190·9	Brown. Pasty at 100°, wax at room temp.	6·9	0·7	1·5	1·0	—
8	*K–L* (plus fraction before *K*)	31·0	Lighter brown, phosphatidic‡	6·0	1·1	2·4	1·0	—
9	*L–M*	215·3	Lighter brown, phosphatidic‡	22·1	1·4	3·1	1·0	—
10	*M–N*	75·3	Grey powder	4·7	1·9	2·2	1·9	—
11	*N*–end	118·4	Light brown, phosphatidic‡	2·6	1·6	2·7	1·3	—
Total		800·5						

* At room temperature unless otherwise specified. † Colorimetrically on lipid. ‡ Denotes a frothy appearance at 100° and room temp., suggestive of lecithin.

Table 5. *Hydrolytic studies on certain chromatographic fractions of 'new sperm lipid* (1)'

Analytical values are as g./100 g. of lipid. A dash indicates that no determination was made.

Fraction no.	Hydrolysis employed	Fatty acids	Unsap. matter	Aldehydes*	Glycerol	Choline	Ethanolamine
3	Ethanolic KOH	22	20	—	1·1	—	—
4	Ethanolic KOH	61	33	—	0·7	—	—
7	Ethanolic KOH	32	22	9·2	0·4	Nil	1·1
9 (*a*)	Ethanolic KOH	20	11	4·6	—	8·3	—
9 (*b*)	Methanolic HCl	>14†	>10†	6·4	—	—	—
10	Aq. $Ba(OH)_2$	←———	75	———→	—	3·9	—
11	Ethanolic KOH	75	1·2	4·7	1·1	3·0	Nil

* Gravimetrically, except 9 (*b*) which was determined colorimetrically on the dimethylacetal fraction.
† See text.

Table 6. *Distribution of phosphorus after hydrolysis of certain fractions of 'new sperm lipid* (1)'

Values are as g./100 g. of original fraction.

Fraction no.	Total P	Water-sol.	Glycero-phosphate P	Inorg. P	In fatty acids	In unsap. matter	In aldehydes	Apparent loss (% of total P)
7	1·5	0·97	0·85	0·12*	0·06	Nil	Nil	32
9 (*a*)†	3·1	1·92	1·05	0·87	0·36	0·07	0·02	22
9 (*b*)†	3·1	3·07‡	1·99	0·35	Nil	←——— Nil ———		1·5
10	2·2	2·00	Nil	2·00	←——— Nil ———			9
11	2·7	1·56	1·28	0·28*	0·98	Nil	Nil	6

* May contain some acid-hydrolysable organic P.
† (*a*) After hydrolysis with ethanolic KOH; (*b*) after hydrolysis with methanolic HCl.
‡ Soluble in methanolic HCl (see text).

Table 7. *Chromatography of 'new sperm lipid* (2)' *on silica gel*

Fraction no.	Eluent (ml.)	Wt. (mg.)	Yield (% of original)	Appearance	Aldehyde*
1	*a*† (350)	552	67	Phosphatidic‡	18·5
2	*b* (600)	113	14	White, crystalline	2·5
3	*c* (1300)	90	11	Brown gum	2·5
4	*d* (1000)	7	1	Brown gum	—

* Direct colorimetric assay.
† *a*, Light petroleum (b.p. 40–60°); *b*, ether–light petroleum (25:75, v/v); *c* and *d*, ethanol–methanol–water (70:30:10 and 70:30:33, by vol. respectively).
‡ Denotes a frothy appearance at 100° and room temp., suggestive of lecithin.

Infrared spectroscopy confirmed that the material was mainly cholesterol, contaminated with some ketonic compound (oxidation product?).

After hydrolysis of fraction 9 with methanolic HCl, material remaining in this solvent during countercurrent distribution with light petroleum amounted to 57 g./100 g. of lipid. Of this, 26·6 g./100 g. of lipid was water-soluble and the rest $CHCl_3$-soluble. The latter material contained N 0·5 and P 2·3% (N:P = 0·48). Presumably on hydrolysis with ethanolic KOH it would have furnished additional fatty acid, and possibly also unsaponifiable matter [see NSL (2) fraction 1, below], although in the present case sphingosine is unlikely to have been present to contribute to the unsaponifiable fraction.

In view of its atomic N:P ratio of 1·9, fraction 10 was assayed for sphingosine (Found: 0·53% sphingosine N, = 11·3% sphingosine).

In two cases choline was assayed both by paper chromatography and as the reineckate. The latter method gave slightly higher values (fraction 7, paper nil, reineckate 0·3%; fraction 9, paper 8·3, reineckate 8·9%). On fractions 10 and 11 only reineckate assays were made.

Chromatography of 'new sperm lipid (2)'. In view of the rather difficult elution of most of NSL (1) from Mallinckrodt silicic acid, NSL (2) was chromatographed on silica gel (British Drug Houses Ltd.). This was expected to cause the main phospholipid components to pass through without fractionation, but to retain cholesterol, waxes, triglycerides, etc. (Lovern, 1956*a*). The column contained 30 g. of mixed silica gel and Hyflo Super-cel prepared as described by Lovern (1956*a*). NSL (2) (826 mg.) was added to the column in 50 ml. of light petroleum. The elution data are given in Table 7. No further material could be removed from the column by a mixture of equal parts of ethanol, methanol and water.

Fraction 1 contained 18·5% aldehydes, fractions 2 and 3 each 2·5% aldehydes by colorimetric assay before hydrolysis. Other analytical data are presented in Table 8. Reference has been made earlier to the fact that on hydrolysis with methanolic HCl, the material passing into the

Table 8. *Analytical data* on fractions of 'new sperm lipid* (2)'

Values are as g./100 g. of lipid. A dash indicates that no determination was made.

Fraction no.	Fatty acids	Unsap. matter	Aldehydes	Cholesterol	Choline	Total P	Glycero-phosphate P	Ethanol-amine
1	28·9, 29·7‡	21·6†, 19·5‡	20·0, 18·6	—	6·5	3·1	1·1	0·07
2	34·5	11·4	—	43·6	—	—	—	—
3	25·7	27·1	5·7	6·3	4·6	1·7	1·4	—

* Fraction 1 was hydrolysed with both ethanolic KOH and methanolic HCl (first and second values respectively for fatty acids, unsaponifiable matter and aldehydes). Fractions 2 and 3 were hydrolysed only with ethanolic KOH. Aldehydes were determined gravimetrically after hydrolysis with KOH, colorimetrically on dimethylacetals in total unsaponifiable material after hydrolysis with HCl. Unsaponifiable material was exclusive of aldehydes (1 and 3) or cholesterol (2 and 3). Glycerophosphate and choline were determined in fraction 1 after KOH hydrolysis. Choline was determined by reineckate.

† May not be fully hydrolysed; see text. 'Corrected' value = 10·0.

‡ Corrected for material obtained on subsequent hydrolysis with KOH; see text.

methanol phase on subsequent partition between light petroleum and methanolic HCl is not entirely soluble in water. The water-insoluble material is quite insoluble in light petroleum, but readily soluble in $CHCl_3$. In fraction 1 above, a total methanol-phase product amounting to 42·7% of the original lipid was obtained, of which very little (5·9% of the original lipid) was water-soluble. Hydrolysis of the $CHCl_3$-soluble material with ethanolic KOH yielded 23·4% of fatty acids and 16·9% of unsaponifiable material, the rest becoming water-soluble. Calculated back to the original lipid of fraction 1, these additional amounts of fatty acid and unsaponifiable matter amount to 6·1 and 4·4% respectively. This addition has been made in the data given in Table 8 and, as will be seen, brings the results of KOH and HCl hydrolysis respectively into close agreement.

On acetylation of the unsaponifiable matter obtained by KOH hydrolysis of fraction 1 there was obtained neutral material 10, acidic material 3·6 and water-soluble material (by difference) 8 g./100 g. of lipid. This should be compared with the similar, but smaller, production of water-soluble material on refluxing the unsaponifiable matter similarly obtained from OSL with acetic anhydride. The presence of much cholesterol in the latter material and its absence in the former could account for the quantitative difference.

RESULTS

Three sets of data are available for evaluation: on an unfractionated product (OSL), on a product only slightly fractionated by chromatography (NSL, 2) and on a large range of chromatographic fractions (NSL, 1). There is evidence, from obvious discrepancies in aldehyde values before and after chromatography, of degradative changes occurring during the chromatography of NSL (1). Detailed discussion of these and of other discrepancies or unexpected analytical findings is deferred to the following section of the paper. For present purposes the discrepancies have been accommodated by making certain assumptions.

Since aldehydogenic compounds appear to be major components of the sperm lipids it may be desirable at the outset to confirm that the relevant analytical data do, in fact, relate solely to higher aliphatic aldehydes, as found in plasmalogens (acetal phosphatides). Spermatozoa are rich in deoxypentose, which will react with the Feulgen reagent for aldehydes. It is unlikely that appreciable proportions of such material would remain after the purification procedure (Fig. 1), but in any case the coloured product produced from deoxyribose is not extracted from the aqueous phase into $CHCl_3$. This latter behaviour seems to be typical of aldehydes with lipid-like solubility properties, the lower aliphatic aldehydes, e.g. formaldehyde, behaving like deoxyribose. The aldehyde was not only determined colorimetrically; the total amount could be recovered as dimethylacetals, again with the solubility properties of a lipid. Finally, the free aldehyde could be recovered quantitatively by a process which implies an original linkage into a lipid of the plasmalogen type, i.e. capable of forming a water-soluble alkali plasmalogenate. In connexion with this last method of assay it may be objected that the product shows a variable or even no reactivity towards the Feulgen reagent (cf. Olley & Lovern, 1953; Lovern & Olley, 1953*b*). This loss of activity can, in the present instance, be attributed to polymerization, since the material could be depolymerized and rendered active to the Feulgen reagent by distillation in a molecular still. Extensive polymerization of free palmitaldehyde has been found to occur during manipulations similar to those employed in the gravimetric assay.

For purposes of calculation it is accepted that plasmalogens contain one fatty acid radical and one aldehydogenic radical attached to a glycerophosphate–base unit (Lovern, 1956*b*; Debuch, 1956). For a choline-based plasmalogen of Debuch's formula, assuming fatty acids and aldehydes of 18 and 16 carbon atoms respectively, the yields of hydrolytic products would be: fatty acids 36·3, aldehydes 30·5, choline 15·45 and glycerophosphate P 3·96 g./100 g. of lipid. The alternative structure of Rapport, Lerner, Alonzo & Franzl (1957) would give virtually the same yields.

Composition of 'old sperm lipid'

Certain hydrolytic products were present in such small proportions (Table 1) that quantitative assessment of their significance is inadvisable. Such are, for example, glycerol and sphingosine. Chromatography of NSL (1), see below, suggests that not all the glycerol is derived from triglycerides. That the spingosine probably represents sphingomyelin rather than cerebrosides is suggested by the finding of phosphorylcholine in the HCl hydrolysate, and by chromatography of NSL (1). At 2%, the sphingosine is equivalent to 0·21% phosphorylcholine (found 0·34%) and to 5·5% sphingomyelin. On this assumption the reducing sugar must be ignored as a possible lipid component. The total non-aldehydic unsaponifiable matter, after deduction of cholesterol, amounts to 5·1%. This will include hydrocarbons [about 2 g./100 g. of lipid from chromatographic data on NSL (1) below] and possibly ceramides (fatty acid amides of sphingosine) after KOH hydrolysis. Part of the sphingomyelin will, however, pass into the fatty acid fraction (Lovern & Olley, 1953*b*). Ceramides would yield a diacetyl derivative on acetylation (S.E. of diacetyl-lignocerylsphingosine = 347, the amide link being resistant to KOH). Therefore, although the S.E. of the (acetylated) non-cholesterol portion of the unsaponifiable matter (299) approximates to that of a typical long-chain aliphatic alcohol (cetyl acetate = 284), the presence or likely proportion of such compounds cannot be inferred from it.

If the fatty acids (19·6%, Table 1) are present entirely as plasmalogens, they are equivalent to 16·5% aldehydes. Neglecting the inexplicably low value by method 2 on the original lipid (but not on the dimethylacetal fraction), and assuming that recovery of dimethylacetals is not absolutely quantitative, the data in Table 3 suggest that, in fact, aldehyde is present equivalent to the total fatty acids. Choline is the only important base. Taking the higher value for choline (see Discussion), the total bases found account for 1·45% N, i.e. the total present. If all the fatty acid is present as plasmalogen, the base requirement is equivalent to 8·3 g. of choline/100 g. of lipid, i.e. to most of that present. On the same assumption, the glycerophosphate requirement is equivalent to 2·1% P. Although there is sufficient total P (2·15%), there is a marked lack of glycerophosphate P (Table 2). Leaving consideration of this discrepancy for later discussion, it may be said that the data on OSL suggest that the chief component is a choline-based plasmalogen, the amount being about 54 g./100 g. of lipid. Allowing for the other postulated lipids, there appears to be about 25% of non-lipids present. This may be impurity, or may represent some unknown component of the plasmalogen (see Discussion).

Composition of 'new sperm lipid (2)'

It seems preferable to consider the chromatography of NSL (2) before that of NSL (1) since it forms a closer link with the unfractionated OSL. In attemping to evaluate the composition of chromatographic fractions, whether of NSL (1) or NSL (2), account has been taken not only of the analytical data but of previous findings of the order of elution of various lipids from silicic acid or silica gel (Borgström, 1952*a*, *b*; Rapport, Lerner & Alonzo, 1953; Fillerup & Mead, 1953; Mead & Fillerup, 1954; Lea, Rhodes & Stoll, 1955; Spengler & Hauf, 1955; Lovern, 1956*a*). The fractions are considered individually below.

Fraction 1. Eluent, light petroleum; yield 67%. The major analytical values (Table 8) again suggest that a choline plasmalogen is the main component. The aldehyde (19%) is equivalent to 22·6 g. of fatty acid/100 g. of lipid. This leaves an excess of only about 6–7 g./100 g. available for other lipids. Choline, calculated from the aldehyde value, should be 9·6 g./100 g., i.e. in excess of that liberated by KOH. Unfortunately lack of material precluded additional hydrolyses with baryta on chromatographic fractions. Glycerophosphate P should be 2·5 g./100 g., i.e. about twice that liberated by KOH and approaching the total P of the fraction, as with OSL. The content of plasmalogen, calculated from the aldehyde content, is about 62%. An interesting feature is the presence of a considerable amount of non-aldehydic, non-sterol 'unsaponifiable' matter. The incomplete splitting of this material by ethanolic KOH, as indicated by the results of acetylation, has been described. Evidently methanolic HCl is no more effective in this case. There was insufficient acetylated product for determination of S.E. but, in any case, the same reservations would apply as in the case of OSL. Allowing for the additional 3·6% of (presumed) fatty acids obtained after acetylation of the crude 'unsaponifiable' matter, the fatty acids in excess of plasmalogen requirements amount to 10 g./100 g. of fraction, i.e. the same as the 'corrected' value for unsaponifiable matter in Table 8. This ratio is suggestive of wax esters but, in view of the ready elution from silica gel, the nature of the non-plasmalogen lipid components is obscure. There appears to be about 20% of non-lipid material (impurity?) in the fraction, similar to the position with OSL.

Fraction 2. Eluent, ether–light petroleum (1:3, v/v); yield 14%. This fraction contains, as would be expected, almost the total cholesterol (6·1 g./100 g.) of NSL (2). (By accident, elution was not carried to completion before changing the solvent

for collection of fraction 3.) The fatty acids may be present partly as wax esters (say 22 % of such esters), but part (about 23 g./100 g. of fraction) must be present in some other combination, or as free acids (see final paragraph of Discussion, page 642). Non-lipids not more than 10 %.

Fraction 3. Eluent, ethanol–methanol–water (70:30:10, by vol.); yield 11 %. The main components might appear to be waxes, were it not for the strong adsorption. There are also small amounts of aldehydogenic lipids, which could be choline-based. However, since the main bulk of these behaves like lecithin in not being adsorbed by silica gel, the material in fraction 3 must be quite different from that in fraction 1. In fact, it recalls the aldehydogenic material found in NSL (1) fraction 11 (see below). Non-lipids may be more important than in fractions 1 and 2, but cannot be estimated apart from an upper limit of about 30 %.

Composition of 'new sperm lipid (1)'

Fraction 1. Eluent, light petroleum; yield 1·9 %. This consisted of hydrocarbons.

Fraction 2. Eluent, ether–light petroleum (1:99, v/v); yield 0·2 %. This fraction was not examined.

Fraction 3. Eluent, ether–light petroleum (4:96, v/v); yield 1·3 %. The analytical data suggest a wax ester, in agreement with the elution behaviour. The glycerol could be from triglycerides (11 % of the fraction), leaving 12 % fatty acids and 20 % unsaponifiable matter. The ratio of these last two components suggests the presence of about 8 % free alcohols and 24 % wax esters, leaving 57 % of the fraction as non-lipids.

Fraction 4. Eluent, ether–light petroleum (25:75, v/v); yield 3·4 %. The glycerol may represent triglycerides (7 %). The unsaponifiable matter (higher alcohols?) could represent about 66 % wax esters, leaving about 22 % excess of fatty acids, of unknown origin, but possibly free (see final paragraph of Discussion, page 642). Non-lipids not more than 5 %.

Fraction 5. Eluent, as for fraction 4; yield 13 %. Free cholesterol, 89 %. Other components were not examined.

Fraction 6. Eluent, as for fraction 4; yield 1·3 %. Free cholesterol, 11 %. Other components were not examined.

Fraction 7. Eluent, a range of solvents from ether–light petroleum (50:50, v/v), through ether, up to methanol–ether (40:60, v/v); yield 23·9 %. The aldehyde may represent an ethanolamine-based plasmalogen (elution behaviour). In this case it would require 1·8 % ethanolamine (found 1·1 %), 0·90 % glycerophosphate P (found 0·85 %) and 8·2 % fatty acids (found 32 %), taking the direct colorimetric value for aldehyde. Accepting that it is an ethanolamine-based plasmalogen, the amount is about 20 % of the fraction. The unsaponifiable matter might again represent a wax ester (44 % of the fraction) leaving just under 2 % of fatty acids for other lipids. These could be mono- or di-glycerides (triglycerides should have been eluted). On this basis non-lipids are about 34 %. The appearance of wax esters so late in the elution series is surprising (Spengler & Hauf, 1955), but the presence of much non-lipid, possibly combined in some way with the lipids, may markedly affect their chromatographic behaviour. Alternatively, it is necessary to postulate the presence of a novel, non-phosphatidic lipid or lipids.

Fraction 8. Eluent, initially methanol–ether (40:60, v/v) but mainly methanol–ether (70:30, v/v); yield 3·9 %. No hydrolysis was performed, hence little calculation is possible. The fraction was richer in N and P than fraction 7, possibly containing a little phospholipid additional to plasmalogens (about 18 %, from aldehyde content).

Fraction 9. Eluent, ethanol–methanol (70:30, v/v); yield 27 %. The probable presence of choline-based plasmalogens has been discussed above, the amount, based on the aldehyde content, being about 72 % of the fraction. There is a small deficiency of fatty acids (see final paragraph of Discussion, page 642), which would be equivalent to 55 % plasmalogens. The choline would likewise be equivalent to 54 % plasmalogens but liberation by KOH is probably incomplete (see Discussion). The fatty acids are equivalent to 16·8 % aldehydes. Glycerophosphate P equivalent to the aldehyde content would be 2·9 g./100 g. of lipid (see Discussion). If the unsaponifiable matter obtained on KOH hydrolysis (11 %) is really polymerized aldehydes, the total aldehyde content by gravimetric assay would be about 15·6 %. There may well therefore be no non-aldehydic unsaponifiable matter, and there is certainly no other evidence of any lipid additional to a choline-based plasmalogen. There must, however, be 30–40 % of non-lipid material.

Fraction 10. Eluent, ethanol–methanol–water (70:30:1, by vol.); yield 9·4 %. This fraction appears to contain sphingomyelin. It does not yield any glycerophosphate on hydrolysis, and its aldehyde component cannot, on present knowledge, be equated with the other components. The choline is equivalent to 91 % of the sphingosine found and to 1·0 % P (sphingosine equivalent to 1·2 % P). The sphingomyelin content is about 30 %. The rest of the fraction appears to be lipid in nature, since the $CHCl_3$ extract after hydrolysis (McKibbin & Taylor, 1949) amounted to 75 g./100 g. of fraction, about the same as for sphingomyelin itself. This remaining lipid may also have an N:P ratio of 2 (calc. 1·85), but its nitrogen becomes water-soluble after hydrolysis. Its phosphorus, like that of

sphingomyelin, is liberated as orthophosphate on hydrolysis. However, this remaining 70% of fraction 10 is low in both N and P (0·9 and 1·1% respectively).

Fraction 11. Eluent, ethanol–methanol–water (70:30:5 and 70:30:10, by vol.) and ethanol–ether–water (70:30:10, by vol.); yield 14·8%. The composition of this fraction cannot be elucidated. It could contain some choline-based plasmalogen, but it is not clear why such should have resisted earlier elution. The re-appearance of glycerophosphate as a component, after a complete break in fraction 10, suggests a particularly strongly adsorbed type of phospholipid, such as lysolecithin. Loss of aldehyde by hydrolysis on the column, as discussed below, could yield lysolecithin. However, there is far too much fatty acid to balance the available choline and glycerophosphate, and some lipid other than a lysophospholipid must predominate. The glycerol obtained on KOH hydrolysis is also difficult to explain. Monoglycerides, although more strongly adsorbed than triglycerides, would hardly be expected so late in the sequence.

DISCUSSION

Although the presence of a choline-based plasmalogen as the major component of the lipids of ram spermatozoa is suggested by the foregoing results, various discrepancies and unexpected analytical findings must be indicated. These are discussed under appropriate subheadings before considering other aspects of the overall composition of these lipids.

Chromatographic losses

Although reasonably complete recovery was achieved of the total weight of material put on the column, extensive loss of the postulated plasmalogen occurred during chromatography of NSL (1). This operation involved a total elution time of 3 weeks, fraction 9 having been on the column a little over 2 weeks. It may be noted that Skipski & Rapport (1957) find it necessary to wash silicic acid-impregnated paper with pyridine before chromatography of plasmalogens, to avoid liberation of the aldehyde. Fraction 9, which, from both the nature of the eluting solvent and from its analytical characteristics, corresponds to the main choline–plasmalogen peak, amounted to only 27% of the original lipids, and its aldehyde content (22·1%, Table 4) was little higher than that of the starting material (17·3%). A sharp separation should have yielded a fraction amounting to 57% of the total, and containing about 30% aldehyde. Although minor concentrations of aldehydogenic lipid are present in other fractions, the total aldehyde content of NSL calculated from the data in Table 4 is 8·5 g./100 g. of lipid, i.e. about half the original. This cannot be explained by non-elution from the column.

A further point to be noted is that, although the aldehyde content of fraction 9 determined colorimetrically was 21%, after hydrolysis only 4·6% could be recovered gravimetrically or 6·4% as dimethylacetals (Table 5). Other fractions of NSL (1), by contrast, gave gravimetric recoveries of the same order as, and indeed somewhat higher than, those obtained by direct colorimetric assay. An interval of some 3 weeks had elapsed between elution from the column, with prompt colorimetric determination of aldehyde, and the hydrolyses, during which time the fractions had been kept in solution in chloroform at 0°. It is difficult to see why decomposition should have affected only fraction 9, and it is difficult to envisage any decomposition other than rupture of the acetal linkage which could interfere with the subsequent determination of the aldehyde. If, however, this link were broken, the free aldehyde would be estimated as non-aldehydic unsaponifiable matter by the gravimetric method and polymerization could account for its incomplete recovery as the dimethylacetals. Other analytical characteristics of the fraction should be unaltered. G. V. Marinetti (personal communication) has found that plasmalogens are unstable in chloroform solution.

If loss of aldehyde on the column is also due to cleavage of the acetal linkage during the very lengthy adsorption on an acidic medium, followed by polymerization of the free aldehyde, the total fatty acids recovered from the column should not show a comparable fall, the apparent non-aldehydic unsaponifiable matter should show a rise, and lysolecithin should be encountered. The total fatty acids of fractions 3, 4, 7, 9 and 11, the only ones for which figures are available, amount to 26·4 g./100 g. of original lipid, the true total being, of course, slightly higher; the fatty acid content of NSL calculated from Table 8 is 29·4 g./100 g. It is unfortunately impossible to test in the same way whether there has been a rise in unsaponifiable matter. The difficulty is that, as mentioned above, hydrolysis with ethanolic potassium hydroxide gave an 'unsaponifiable' fraction which, on acetylation, yielded a considerable proportion of water-soluble material. Only a few fractions were acetylated, so that some of the data for unsaponifiable matter may be seriously in error. Lysolecithin may have occurred in fraction 11, but in nothing like an amount equivalent to the aldehyde 'lost' during chromatography. For example, the glycerophosphate of fraction 11 is equivalent to about only one-sixth of this aldehyde.

However, as will be discussed below, the constitution of the sperm-lipid plasmalogen is uncertain and it may not give a simple lysophospholipid on

loss of the aldehyde. A significant finding may be the high fatty acid content (75%, Table 5) of the unidentified lipid of fraction 11. This fatty acid amounts to about 11 g./100 g. of original lipid, i.e. it is roughly equivalent to the missing aldehyde and the lipid in which it is bound may be the residual portion of the original plasmalogen.

Chromatography of NSL (2) did not result in any serious loss of aldehyde, recovery being equivalent to about 15 g./100 g. of lipid, compared with the initial value of 17·3 g./100 g. In this case, however, elution was rapid (total elution time 2 days; fraction 1 about 1 hr.). There was also no loss of aldehyde between recovery from the column and subsequent hydrolysis, which followed within 24 hr. in this case. It may be noted that fraction 3 of NSL (2), which corresponds to fraction 11 of NSL (1), although showing similarity in its content of strongly adsorbed aldehyde, choline, glycerophosphate, etc. (Table 8), did not contain the high proportion of fatty acids which, it has been suggested above, may be present in the residue of a degraded plasmalogen.

Behaviour on hydrolysis

Although there does not seem to be any published record of hydrolysis studies on a pure natural plasmalogen, a compound of Debuch's (1956) formula would be expected to yield all its choline as readily to ethanolic potassium hydroxide as does lecithin. Furthermore, removal of base is postulated during the gravimetric aldehyde assay, which was quantitative. Although, unfortunately, lack of material prevented studies of baryta hydrolysis on the various fractions, experience with OSL showed that potassium hydroxide liberated only about half as much free choline as did baryta (Table 1). The difference cannot be attributed to sphingomyelin, for which it is known that aqueous baryta is a more effective hydrolytic agent than is ethanolic potassium hydroxide. The amount of choline found after baryta hydrolysis is in agreement with the total nitrogen of the lipid and is approximately equivalent to the aldehyde (if this is present as a choline plasmalogen).

In all cases sufficient total phosphorus is present to supply one phosphorus atom/molecule of aldehyde. However, the amount found as glycerophosphate after hydrolysis with potassium hydroxide, followed by acidification, is far below this requirement [see particularly the results on OSL, NSL (1) fraction 9 and NSL (2) fraction 1]. Baryta hydrolysis is little more effective in this respect (Table 2). Methanolic hydrochloric acid liberates considerably more glycerophosphate, although still not enough to balance the aldehyde. Some decomposition of glycerophosphate may be expected with methanolic hydrochloric acid, e.g. Olley & Lovern (1954) found 35% hydrolysis of glycerophosphate during refluxing for 8 hr. with methanolic 6N-hydrochloric acid. Allowing for 26% hydrolysis during the refluxing for 6 hr. in the present work, the total recovery of glycerophosphate after hydrolysis of OSL with hydrochloric acid (Table 2) is 1·96 g./100 g. and for NSL (1) fraction 9 (Table 6) is 2·6 g./100 g. [It must be noted, however, that the expected inorganic phosphate was not detected (by paper electrophoresis) with OSL, and less than the calculated amount was found, chemically, with NSL (1) fraction 9.] These values agree fairly well with the aldehyde requirements for glycerophosphate phosphorus (2·1 and 2·9 g./100 g. respectively). No determination was made of glycerophosphate liberated by hydrochloric acid from NSL (2) fraction 1.

Certain other features observed on hydrolysis should be noted. Hydrolysis with ethanolic potassium hydroxide was incomplete in respect of items additional to choline and glycerophosphate. A large yield of 'unsaponifiable' matter was obtained, part at least of which could be further degraded by refluxing with acetic anhydride, when a considerable proportion became water-soluble [see particularly NSL (2) fraction 1]. Acetic anhydride is known to be an effective dephosphorylating agent for phospholipids (Bevan, Brown, Gregory & Malkin, 1953) and this present effect indicates that it can also attack other types of linkage. The fatty acids recovered after hydrolysis with potassium hydroxide contained appreciable amounts of some phosphorus compound, e.g. from OSL and NSL (1) fractions 9 and 11 the fatty acids contained 2·5, 1·8 and 1·3 g. of phosphorus/100 g. of acids respectively (Tables 2 and 6). These values are far higher than those commonly found after alkaline hydrolysis of glycerophospholipids (e.g. Trier, 1913; Olley & Lovern, 1953); a common value, in our experience, is about 0·1–0·2% phosphorus. It is improbable that sphingomyelin is present in sufficient amount in these fractions to account for this acidic lipid phosphorus. The absence of phosphorus from fatty acids recovered after hydrolysis with methanolic hydrochloric acid may be noted. G. V. Marinetti (personal communication) has also found that phosphorus-containing acidic lipids accompany the fatty acids obtained after alkaline hydrolysis of plasmalogens. The distribution of phosphorus after hydrolysis is discussed further below.

Countercurrent distribution of the products of such hydrolysis with methanolic hydrochloric acid, however, yielded a product from the methanolic phase which could be further hydrolysed with ethanolic potassium hydroxide [see especially NSL (2) fraction 1]. This material could not have been simply a portion of the original lipid which

had escaped hydrolysis, since it was insoluble in light petroleum. Recovery of dimethylacetals was sufficiently quantitative, however, to confirm that liberation of aldehyde by methanolic hydrochloric acid was complete.

There is, of course, no direct evidence that any or all of these aspects of incomplete hydrolysis relate to the postulated plasmalogen rather than to possible accompanying substances. Nevertheless, if the plasmalogen accounts for most of the fatty acid, choline and glycerophosphate of the total lipids, the above findings suggest that its structure may be unusual. It resembles the usual plasmalogens in one important respect: its reaction with the fuchsin reagent was rapid at room temperature, in contrast with the slow reaction of the dimethylacetals (cf. Lovern, 1956*b*).

The presence of large proportions of water-soluble products of hydrolysis other than known lipid fragments such as choline, glycerophosphate, etc., may be noted. Provisionally called 'non-lipid', these substances amounted to about 25, 20 and 35% of OSL, NSL (2) fraction 1 and NSL (1) fraction 9 respectively. This material accompanied the lipid through the various purifications employed (Fig. 1) and the main plasmalogen peak during chromatography on silicic acid. Part of it was still linked in a lipid-like substance after hydrolysis with ethanolic potassium hydroxide (the crude 'unsaponifiable' fraction) or hydrolysis with methanolic hydrochloric acid (the chloroform-soluble portion from the methanolic hydrochloric acid phase after countercurrent distribution). This may, perhaps, be another indication that the sperm plasmalogen is a more complex molecule than the usual type.

Distribution of phosphorus after hydrolysis

Two peculiarities in respect of phosphorus have already been noted, namely the occurrence of phosphorus in acidic lipid form and the low yield of glycerophosphate, both after hydrolysis with potassium hydroxide. The latter finding cannot be attributed entirely to the former, since even adding the phosphorus of the fatty acid fraction to that found as glycerophosphate, the total such phosphorus after hydrolysis with potassium hydroxide is considerably less than that found as glycerophosphate after acid hydrolysis (Tables 2 and 6). After acid hydrolysis, a considerable amount of phosphorus remains in the chloroform-soluble water-insoluble fraction. A notable feature of alkaline hydrolysis, whether with ethanolic potassium hydroxide or with aqueous baryta, is that much of the phosphorus is analytically unaccounted for after hydrolysis. Such 'loss' is very small after acid hydrolysis.

In one case [NSL (2) fraction 1], total phosphorus was determined immediately after hydrolysis with potassium hydroxide, before working up the hydrolysate. The value obtained was identical with that on the intact lipid. A typical plasmalogen should be present as potassium plasmalogenate at this stage. Subsequent acidification during the working up of the hydrolysate would liberate glycerophosphate from the usual type of plasmalogenate, but we know that in the present case glycerophosphate production after hydrolysis with potassium hydroxide is incomplete. It is suggested that the balance of the lipid phosphorus is liberated from the sperm 'plasmalogenate' as a compound from which it is not converted into orthophosphate by the digestion procedure used.

Failure to recover all the original phosphorus of a lipid among the products of hydrolysis has been reported before (Olley & Lovern, 1953) and has been attributed to difficulty in digesting some forms of organic phosphorus (Olley, 1956*a*). The difficulty does not arise with, for example, lecithin, and glycerophosphate offers no digestion problem. It was believed from this earlier work that the digestion procedure used in the present case (basically the substitution of perchloric acid for sulphuric acid in the method of Umbreit, Burris & Stauffer, 1949) was adequate. Again glycerophosphate offers no difficulty. In compiling the data in Tables 2 and 6 no material, e.g. water washings, was discarded without analysis and satisfactory replication was invariably obtained in all determinations of phosphorus. These findings further emphasize the apparently novel character of some major component of the sperm lipids, i.e. of the postulated plasmalogen.

General composition of sperm lipids

Apart from a choline-based plasmalogen, the sperm lipids contain cholesterol (about 8%), apparently all free, sphingomyelin [about 5·5%, based on the sphingosine content of OSL, about half of this total occurring in fraction 10 of NSL (1)], hydrocarbon (about 2%) and small amounts of lipids based on ethanolamine (possibly also a plasmalogen) and glycerol. There may be some aliphatic alcohols (or wax esters) and perhaps a little glycolipid. If the plasmalogen resembles the usual type, the amount appears to be about 55–60 g./100 g. of 'purified' lipids, the balance of the total material then representing impurities (see above). There is no evidence for the occurrence of lecithin in these lipids. The absence of inositol lipids requires confirmation, since hydrolysis of washed, freeze-dried ram spermatozoa with aqueous 6N-hydrochloric acid showed the presence of 0·11% inositol. It is possible that inositol lipids were lost by the washing process (Folch *et al.* 1951) or by adsorption on the cellulose column (cf.

Garcia, Lovern & Olley, 1956). Alternatively, the bound inositol may have been insoluble either because it was of non-lipid nature or because it was present as a lipoprotein insoluble in chloroform–methanol (Folch, 1952; Hutchison *et al.* 1956).

It has been found (E. F. Hartree and T. Mann, unpublished experiments) that ram spermatozoa contain a phospholipase *A* able to liberate the fatty acid from endogenous plasmalogen (cf. Franzl & Rapport, 1955). This enzyme would have been active during the interval between semen collection and freeze-drying of the washed sperm. It is possible to make a rough assessment of the upper limit of such enzymic hydrolysis from the amount of free fatty acids detected chromatographically. Small amounts of such acids probably occurred in NSL (1), fraction 4, but following fractions, not fully characterized, may have contained additional amounts. In the chromatography of NSL (2) any free fatty acids would be quantitatively concentrated in fraction 2. If the entire fatty acid found after hydrolysis of this fraction had been free in the intact fraction, it would amount to 4·8 % of the original lipid, representing hydrolysis of the plasmalogen component to the extent of about 23 %. This is therefore the upper limit and a more likely value, allowing for possible wax esters in NSL (2), fraction 2, would be about 15 % hydrolysis. The deficiency of fatty acids relative to aldehydes in the most selectively fractionated plasmalogen preparation, NSL (1), fraction 9, amounts to 6·3 g./100 g. of fraction. Assuming that the chromatographic behaviour of enzymically degraded and undegraded plasmalogen is the same, and ignoring the extensive chromatographic losses, this fatty acid deficiency corresponds to about 24 % hydrolysis of the original plasmalogen. It is unlikely that hydrolysis to the extent of about 20 % would have seriously affected the overall assessment of the composition of the sperm lipids, but it may have contributed to some of the unexpected experimental observations, since the properties of the lysoplasmalogen have not been studied in any detail.

SUMMARY

1. Washed freeze-dried ram spermatozoa were extracted with chloroform–methanol (2:1, v/v) and the crude extracts purified by washing with water and passing through a column of cellulose, the yield of total lipids being 7–9 %.

2. Part of this material was hydrolysed directly, ethanolic potassium hydroxide, methanolic hydrochloric acid and aqueous barium hydroxide being used as alternative reagents, and part was examined chromatographically with silicic acid (or silica gel) from two sources, these being products known to have widely different adsorptive properties.

3. From analytical data on the original lipids and on the various chromatographic fractions it is evident that aldehydogenic lipids, possibly plasmalogens, are major components (about 55–60 %) of the sperm lipids. Choline is the predominating base of these particular compounds, although there are minor amounts of ethanolamine. The behaviour towards hydrolytic agents is, however, not that to be expected from the accepted plasmalogens.

4. There is no evidence for the presence of lecithin. Other components of the extracts are free cholesterol (about 8 %), hydrocarbons (about 2 %), probably sphingomyelin (about 5·5 %) and other lipids difficult to characterize, but perhaps including wax esters and glycerides. Despite the purifications employed, non-lipids appeared to be present in appreciable amount, possibly combined with the lipids.

5. Both ethanolic potassium hydroxide and methanolic hydrochloric acid proved inadequate for the complete hydrolysis of some of the lipids. The former sometimes gave 'fatty acids' rich in phosphorus and 'unsaponifiable matter' which became largely water-soluble on refluxing with acetic anhydride. The latter gave a fraction susceptible to further hydrolysis with ethanolic potassium hydroxide.

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Hydrolysis of the Soluble Pentosans of Wheat Flour and *Rhodymenia palmata* by Ruminal Micro-organisms

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It has been known for some time that pentosans in the food are to a large extent decomposed during passage through the rumen of sheep (Marshall, 1949; Heald, 1953). This microbial activity enables the ruminant to make use of pentosans and other hemicelluloses which, like cellulose, are hardly, if at all, available to non-ruminants. Heald (1952) also isolated some xylose-fermenting strains of *Escherichia coli* from the rumen but, when the work about to be described was undertaken, nothing was known of the bacteria in the rumen responsible for fermenting the pentosans themselves. Two species of pentosan-fermenting bacteria have recently been found in the rumen; both appear to be hitherto undescribed species of nutritionally exacting, obligately anaerobic, Gram-negative, curved rods (Bryant & Small, 1956; Doetsch, Howard, Mann & Oxford, 1957).

To assist in an understanding of the processes of pentosan digestion in the sheep rumen, experiments were undertaken *in vitro* with suspensions of the natural mixed culture of bacteria found in the rumen of a sheep which was maintained on a standard diet containing a fair amount of pentosan. It was considered that pentosans which were water-soluble and could be obtained fairly readily in a state of purity would present the most convenient type of substrate for preliminary studies. Two such materials have been examined so far, the soluble pentosan of wheat flour and the soluble xylan of the red seaweed *Rhodymenia palmata*. The former was first isolated by Perlin (1951*a*) from commercial