Biochem. J. (1966) **98**, 342

Changes in Lipid Composition of the Maturing Rat Testis

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(*Received* 12 *July* 1965)

1. The lipids and fatty acids of the lipids of testes of rats aged 4 weeks to 6 months were separated and analysed. 2. A decrease in concentration of triglyceride was noted, but there was no significant change in the concentration of phospholipids, plasmalogen or cholesterol during this time. 3. There were no significant differences in the total lipid concentration of palmitic acid, stearic acid, linoleic acid, arachidonic acid and docosatetraenoic acid between the various age groups. 4. A decrease in the concentration of oleic acid in the phosphatide and triglyceride fractions and an increase in the concentration of docosapentaenoic acid (characterized as the Δ4,7,10,13,16-isomer) in phosphatides but not in triglyceride were observed during the maturation period. 5. Histological studies indicated that the lipid changes occurred at the same time as the appearance and maturation of the spermatids.

The increased concentration of pentaenoic acid in testicular tissue of rats with age (3 weeks to 3 months) observed by Kirschman & Coniglio (1961) suggests that lipids may have an essential role in the maturation of the testis. Although several reports of lipid composition of testicular tissue are available, these contain only partial analyses of lipids and fatty acids and were done in rats of only one age group. Thus Migeon (1952) determined lipid phosphorus and cholesterol in testes of intact and adrenalectomized rats given cortisone. Aaes-Jorgensen (1958) studied the effects of conjugated isomers of dienoic and trienoic fatty acids on the polyunsaturated fatty acid composition of rat testicular lipids as determined by the alkaline isomerization method. Analyses of testicular phospholipids were reported by Collins & Shotlander (1961) and by Scott, Dawson & Rolands (1963). The fatty acids of phospholipids and triglycerides of rat testis were determined by Paoletti & Grossi (1961), but their data did not include pentaenoic acids. In a more recent study Bieri & Andrews (1964) reported results of the effects of vitamin E deficiency on the fatty acids of total lipid and of phospholipid of rat testis.

The purpose of the present investigation was to determine in detail the lipid composition of testicular tissue of rats of various ages and to attempt to correlate the chemical findings with changes observed in the morphology of the organ.

EXPERIMENTAL

Animals. Sprague–Dawley rats from our own colony were maintained on Purina laboratory chow after weaning (21 days). The testes of a number of animals from each age group (4, 6, 7, 9, 13 and 29 weeks) were pooled for fractionation of lipids. Total fatty acid patterns were done in most cases on organs of individual animals.

Extraction and fractionation procedures. Testes were removed from animals immediately after decapitation and homogenized in a Waring Blendor with 20 vol. of chloroform–methanol (2:1, v/v) for 3 min. The homogenate was allowed to stand at room temperature for at least 30 min. before filtration. The residue was re-extracted twice in the same manner. The combined filtrates were evaporated to dryness in a rotary evaporator at room temperature and the lipid residue was dissolved in chloroform. A portion was removed for chemical analyses, and the remainder was chromatographed on an 18g. silicic acid column according to the method of Hirsch & Ahrens (1958). Batch elutions were collected and evaporated to dryness in a rotary evaporator. The lipid residue was then dissolved in chloroform. When phospholipid was present, 5% (v/v) of methanol was added to the chloroform. A portion was removed for chemical analyses and thin-layer chromatography and the remainder was used for analysis of fatty acids. The phospholipid fraction was chromatographed on DEAE-cellulose according to the method of Rouser, Kritchevsky, Heller & Lieber (1963). Neutral lipids were separated by thin-layer chromatography with light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (85:15:1, by vol.) and phospholipids with chloroform–methanol–water (95:35:4, by vol.). Standards for thin-layer chromatography were obtained from Applied Science Laboratories, State College, Pa., U.S.A.

Analytical procedures. The following analyses were performed on samples of the appropriate fractions: total weight, fatty acid ester (Rapport & Alonzo, 1955), phosphorus (King, 1932), cholesterol (Sperry & Webb, 1950) and plasmalogen (Gottfried & Rapport, 1962).

Extraction and methylation of fatty acids. Fatty acids were obtained by extraction with light petroleum of the

saponified and acidified fractions. Methyl esters were prepared by the method of Metcalf & Schmitz (1961).

Gas–liquid chromatography. Gas–liquid chromatography of the methyl esters of fatty acids of total lipid, triglyceride and phospholipid was done with a Barber–Colman model 10 instrument equipped with an argon ionization detector. The column was 6ft. × 4mm. internal diam. and was packed with diethylene glycol succinate polyester (12·0%, by wt.) coated on 110–120 mesh chromosorb P. Column temperature was 197° and gas-flow rate 140–150ml./min. The detector was calibrated with pure methyl esters of fatty acids (including arachidonic acid and docosahexaenoic acid) obtained from the Hormel Institute, Austin, Minn., U.S.A., Applied Science Laboratories, and National Institutes of Health, Bethesda, Md., U.S.A.

Fatty acids were identified by the following criteria: the number of carbon atoms was determined by hydrogenating and comparing the saturated fatty acid with pure standards by gas–liquid chromatography; the degree of unsaturation was determined by alkaline isomerization (Holman, 1957); the positions of the double bonds were determined by oxidation with periodate–permanganate by the method of Rudloff (1956) and gas–liquid-chromatographic analyses of the fragments.

Light-microscopy. Histological examination was done on a transverse section of tissue taken through the midpoint perpendicular to the long axis and fixed in Helly's solution. The section was dehydrated with dioxan and acetone and stained with PAS–Harris haematoxylin (Leblond & Clermont, 1952*a*). Spermatogenesis has been classified according to the system of Leblond & Clermont (1952*b*).

Materials. All solvents were reagent grade except light petroleum, which was AOCS grade, redistilled and passed over activated silicic acid. Silicic acid for column chromatography was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and silica gel G for thin-layer chromatography was obtained from Research Specialties Inc., Berkeley, Calif., U.S.A. Boron trifluoride–methanol reagent used for methylation of fatty acids was obtained from Applied Science Laboratories.

RESULTS

The concentration of various lipids in the testis of the maturing rat is given in Table 1. There was no significant change in the concentration of total lipid, cholesterol, phospholipid and plasmalogen with age. However, there was a significant decrease in the concentration of triglyceride (4 to 13 weeks, $P < 0.001$) and of sterol ester (4 to 13 weeks, $P < 0.02$). In all age groups 2·38–2·64% of the wet weight of the testes was lipid, and of this the largest component (about 60–70%) was phospholipid. Unesterified cholesterol and triglyceride were present in smaller concentrations, and cholesterol ester and diglyceride were found only in small quantities. The diglyceride may have been formed by deacylation of the triglyceride during isolation of the lipids. The presence of a small amount of free fatty acids contaminating the cholesterol fraction supports the possibility of deacylation.

The phospholipids were separated into nine fractions by DEAE-cellulose column chromatography. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin

Table 1. *Concentration of lipids in testis of the maturing rat*

Results are given as mean values ± S.E.M. (number of analyses in parentheses). Each analysis was made on a pool of organs from four to six animals.

Age (weeks)	Total lipid (mg./g.)	Concentration (μmoles/g. wet wt. of tissue)					
		Sterol ester	Triglyceride	Cholesterol	Diglyceride	Lipid phosphorus	Plasmalogen
4	26·4 ± 0·01 (3)	0·25 ± 0·02 (3)	2·9 ± 0·1 (3)	4·7 ± 1·0 (3)	0·33 ± 0·02 (3)	16·6 ± 0·3 (3)	1·80 ± 0·16 (3)
6	23·8 ± 1·32 (3)	0·18 ± 0·03 (3)	2·0 ± 0·1 (3)	5·1 ± 0·2 (3)	0·30 ± 0·02 (3)	20·5 ± 2·1 (3)	2·33 ± 0·31 (3)
7	25·4 ± 0·01 (2)	0·16 ± 0·05 (2)	1·2 ± 0·2 (2)	4·0 ± 0·2 (2)	0·23 ± 0·02 (2)	16·6 ± 0·1 (2)	2·05 ± 0·04 (2)
9	25·4 ± 2·30 (3)	0·14 ± 0·02 (3)	1·0 ± 0·1 (3)	4·7 ± 0·2 (3)	0·19 ± 0·02 (3)	17·8 ± 2·0 (3)	2·27 ± 0·18 (3)
13	26·1 ± 0·03 (3)	0·15 ± 0·01 (3)	1·4 ± 0·3 (3)	5·8 ± 1·0 (3)	0·21 ± 0·005 (3)	14·1 ± 2·3 (3)	2·10 ± 0·34 (3)
29	24·2 ± 0·05 (4)	0·12 ± 0·01 (4)	1·0 ± 0·1 (4)	4·6 ± 0·1 (4)	0·24 ± 0·005 (4)	16·6 ± 0·3 (4)	2·38 ± 0·07 (4)

Table 2. *Major phosphatides of rat testicular lipid*

	Lipid phosphorus (μmoles/g.)			Lipid phosphorus (moles/100 moles)		
Age (weeks).....	4	6	13	4	6	13
Choline	6·27	8·43	7·22	37·8	50·7	44·6
Ethanolamine	2·77	5·41	4·17	16·7	32·6	25·8
Serine	1·30	0·52	0·90	7·9	3·1	5·6
Sphingomyelin	0·15	0·99	1·01	0·9	6·0	6·3

Table 3. *Major fatty acids of total lipid of testis*

Results are given as mean values ± S.E.M. (numbers of analyses in parentheses).

Age (weeks)	Concentration (mg./g. wet wt. of tissue)						
	$C_{16:0}$ acid	$C_{18:0}$ acid	$C_{18:1}$ acid	$C_{18:2}$ acid	$C_{20:4}$ acid	$C_{22:4}$ acid	$C_{22:5}$ acid
4	3·59±0·34 (6)	0·67±0·10 (6)	2·06±0·11 (6)	0·48±0·05 (6)	2·21±0·17 (6)	0·17±0·02 (6)	0·83±0·09 (6)
5	3·65±0·17 (4)	0·60±0·03 (4)	1·58±0·11 (4)	0·45±0·06 (4)	2·17±0·11 (4)	0·16±0·03 (4)	1·13±0·05 (4)
6	3·90±0·09 (6)	0·65±0·02 (6)	1·75±0·04 (6)	0·63±0·03 (6)	1·89±0·12 (6)	0·18±0·03 (6)	1·32±0·06 (6)
7	3·63±0·18 (5)	0·58±0·04 (5)	1·43±0·06 (5)	0·61±0·02 (5)	2·11±0·13 (5)	0·17±0·03 (5)	1·81±0·09 (5)
9	3·64±0·09 (3)	0·48±0·02 (3)	1·41±0·02 (3)	0·63±0·05 (3)	1·87±0·14 (3)	0·23±0·04 (3)	2·09±0·18 (3)
13	3·76±0·27 (3)	0·54±0·02 (3)	1·33±0·08 (3)	0·63±0·05 (3)	1·84±0·05 (3)	0·23±0·03 (3)	1·99±0·13 (3)
29	3·69±0·10 (4)	0·50±0·03 (4)	1·38±0·10 (4)	0·62±0·04 (4)	1·83±0·09 (4)	0·19±0·02 (4)	2·00±0·09 (4)

Table 4. *Characterization of the testicular unsaturated fatty acids*

Relative retention*	Product of hydrogenation	Highest wavelength of u.v.-absorption peaks after alkaline isomerization	Major products of oxidation		Designation
			Monocarboxylic acid	Dicarboxylic acid	
0·68	$C_{16:0}$ acid	None	Heptylic acid	Azelaic acid	Δ^{9}-$C_{16:1}$ acid
1·16	$C_{18:0}$ acid	None	Pelargonic acid	Azelaic acid	Δ^{9}-$C_{18:1}$ acid
1·49	$C_{18:0}$ acid	233 mμ	Hexanoic acid	Azelaic acid	$\Delta^{9,12}$-$C_{18:2}$ acid
3·08	$C_{20:0}$ acid	268 mμ	Hexanoic acid	Suberic acid	$\Delta^{8,11,14}$-$C_{20:3}$ acid
3·52	$C_{20:0}$ acid	315 mμ	Hexanoic acid	Glutaric acid	$\Delta^{5,8,11,14}$-$C_{20:4}$ acid
6·24	$C_{22:0}$ acid	315 mμ	Hexanoic acid	Pimelic acid	$\Delta^{7,10,13,16}$-$C_{22:4}$ acid
7·22	$C_{22:0}$ acid	346 mμ	Hexanoic acid	Succinic acid	$\Delta^{4,7,10,13,16}$-$C_{22:5}$ acid
9·60	$C_{22:0}$ acid	375 mμ	—	—	$C_{22:6}$ acid
10·79	$C_{24:0}$ acid	315 mμ	—	—	$C_{24:4}$ acid
12·13	$C_{24:0}$ acid	346 mμ	—	—	$C_{24:5}$ acid

* Retention of methyl ester relative to that of methyl stearate by gas–liquid chromatography.

were the most abundant phosphatides present (Table 2). Small amounts of inositol phosphatides, lysophosphatidylethanolamine, phosphatidic acid and polyglycerol phosphatides were also shown to be present.

The fatty acid composition of the total lipids for the various ages is shown in Table 3. There was no significant difference in the concentrations of palmitic acid, stearic acid, linoleic acid, arachidonic acid and docosatetraenoic acid with age. On the other hand, there was a significant decrease in concentration with increasing age of the oleic acid (4 to 13 weeks, $P < 0.005$) and a significant increase in concentration with age of the docosapentaenoic acid (4 to 13 weeks, $P < 0.001$). In addition to the fatty acids shown in Table 3 small amounts (less than 1%) of $C_{12:0}$ acid, $C_{14:0}$ acid, $C_{15:0}$ acid, $C_{16:1}$ acid, $C_{17:0}$ acid, $C_{20:0}$ acid, $C_{20:1}$ acid, $C_{20:3}$ acid (1–2%), $C_{22:6}$ acid, $C_{24:4}$ acid and $C_{24:5}$ acid have been detected. The results of the structural determinations of the unsaturated fatty acids are summarized in Table 4. All of these fatty acids except the $C_{24:5}$ acid were reported by Holman & Hofstetter (1965) to be present in bovine and porcine testes. The $C_{24:5}$ acid, which we believe has not been reported before, was characterized only with respect to chain length and number of double bonds.

In Table 5 is summarized the major fatty acid composition of the phospholipid and triglyceride fractions. These data show the same increase in pentaenoic acid concentration in phosphatides (but not in triglycerides) as occurred in total lipid. A significant decrease was observed in the concentration of stearic acid and oleic acid in both the phosphatides and triglycerides.

The histological studies showed that very few tubules of 4-week-old rats contained young spermatids. After 5 weeks spermatids were seen in all tubules. At 7 weeks maturing spermatids were present in all tubules. These observations agree with those reported by Clermont & Perey (1957).

DISCUSSION

The most consistent chemical changes found in the lipids of maturing testes were: (1) a decrease in

Table 5. *Major fatty acids of phospholipids and triglycerides of rat testes*

Results are given as mean values ±S.E.M. (numbers of analyses in parentheses). Each sample consisted of organs pooled from four to six animals.

Age (weeks)	$C_{16:0}$ acid	$C_{18:0}$ acid	$C_{18:1}$ acid	$C_{18:2}$ acid	$C_{20:4}$ acid	$C_{22:4}$ acid	$C_{22:5}$ acid
	Concentration (mg./100g. wet wt. of tissue)						
	Phospholipid						
4	303± 2 (3)	62±1 (3)	163± 1 (3)	28±4 (3)	189± 5 (3)	11±2 (3)	80± 8 (3)
6	384±32 (3)	66±9 (3)	158±11 (3)	50±5 (3)	220±27 (3)	13±5 (3)	130±14 (3)
7	302± 2 (2)	53±1 (2)	122± 1 (2)	42±1 (2)	175± 7 (2)	12±1 (2)	150± 1 (2)
9	305±12 (3)	59±4 (3)	114± 8 (3)	36±2 (3)	206±25 (3)	13±7 (3)	173±22 (3)
13	243±47 (3)	39±9 (3)	82± 9 (3)	25±6 (3)	150±24 (3)	10±1 (3)	174±12 (3)
29	281±13 (4)	43±3 (4)	101± 1 (4)	39±1 (4)	182± 5 (4)	10±1 (4)	179± 4 (4)
	Triglyceride						
4	60·0±0·9 (3)	9·3±0·6 (3)	85·0±3·3 (3)	46·7±1·9 (3)	4·3±0·3 (3)	2·7±0·3 (3)	14·3±0·3 (3)
6	53·7±3·4 (3)	5·3±0·6 (3)	45·3±5·8 (3)	24·7±2·9 (3)	3·3±0·6 (3)	3·7±0·7 (3)	12·3±2·7 (3)
7	32·0±3·4 (2)	3·5±1·0 (2)	28·0±4·9 (2)	15·0±2·0 (2)	3·5±1·0 (2)	2·5±0·1 (2)	14·5±1·8 (2)
9	28·0±3·3 (3)	1·7±0·1 (3)	19·3±1·0 (3)	14·0±1·0 (3)	2·3±0·3 (3)	2·3±0·3 (3)	12·3±1·5 (3)
13	33·0±3·8 (3)	2·0±0·2 (3)	25·7±5·1 (3)	13·3±2·3 (3)	3·7±0·6 (3)	4·0±0·7 (3)	20·0±2·1 (3)
29	24·3±1·2 (4)	1·8±0·3 (4)	20·0±1·9 (4)	9·5±0·5 (4)	2·5±0·3 (4)	2·5±0·2 (4)	14·5±1·0 (4)

triglyceride concentration; (2) a decrease in concentration of stearic acid and of oleic acid in triglycerides; (3) a decrease in concentration of oleic acid in phospholipids; (4) an increase in concentration of the docosa-4,7,10,13,16-pentaenoic acid in phospholipids. These chemical changes occurred for the most part between 4 and 7 weeks. This was the period when the testis was undergoing maturation as evidenced from the morphological studies presented here and from the work of Clermont & Perey (1957).

Several phenomena were occurring during this 3-week period: (1) the Sertoli cells matured (Clermont & Perey, 1957); (2) the Leydig cells increased their activity (Roosen-Runge & Anderson, 1959); (3) the spermatids appeared and matured into spermatozoa. The chemical changes that occurred could have been associated with any or all of these morphological changes, or any one chemical change could have been associated with any one of the morphological changes. Further studies (unilateral cryptorchidism, cadmium toxicity and treatment with antispermatogenic drugs) indicate that the increase in concentration of the docosa-4,7,10,13,16-pentaenoic acid was associated with the appearance and maturation of the spermatids. Further evidence that the lipid changes are associated with the appearance and development of spermatids was obtained in one analysis of pooled rat epididymal spermatozoa. The concentrations of the major fatty acids ($C_{16:0}$ acid, 26·3%; $C_{20:4}$ acid, 17·9%; $C_{22:5}$ acid, 16·4%) were similar to those of the total testis. Since the spermatids account for more than 60% of the volume of the tissue of testis (Roosen-Runge, 1956), the lipids of the spermatids would be expected to dominate the lipid pattern of the whole organ.

Since many new structures develop in the maturing testis, the need for biosynthesized lipid increases. The 'lipid cycle', described by many authors (Ebner, 1888; Lynch & Scott, 1951; Smith & Lacy, 1959; Lacy, 1960, 1962), may represent a cycle of synthesis and utilization. This process is apparently associated with the embedding and releasing of the spermatids from the cytoplasm of the Sertoli cells. When the spermatids are embedded in the Sertoli cytoplasm, the lipid moves to surround the head of the spermatid and is apparently taken up by the spermatid. A particular lipid taken up at this time may be a docosapentaenoic acid-rich lipid needed for particular structures. The increase in concentration of this acid in the maturing rat has been observed in testis only.

Our data suggest the possibility that the pentaenoic acid was replacing the stearic acid and oleic acid in the phospholipid and triglyceride molecules. With a decrease in the concentration of triglyceride/g. of tissue, there was expected a decrease in the concentration of fatty acids contributed by this fraction. This proved to be the case for all fatty acids except docosatetraenoic acid and docosapentaenoic acid, which remained constant. Further, the decrease in concentrations of oleic acid and stearic acid, but not of other fatty acids, was greater during the period 4–9 weeks than the decrease in concentration of trigylcerides during the same time.

Marquis & Fritz (1965) have published evidence

of increased concentrations of carnitine and carnitine acetyltransferase in tissues of the reproductive tract of rats during the period of sexual maturation. They further noted that spermatozoa had the highest specific activities of transferases thus far observed in tissues. These observations furnish additional evidence of increased lipid metabolism in rat testis during the period of growth associated with rapid differentiation and formation of spermatids.

Holman & Greenberg (1953) suggested that the large quantity of polyunsaturated fatty acids in gonadal tissue may have some biochemical significance. This significance may lie, for the rat at least, in the fact that these polyunsaturated fatty acids belong to the linoleic acid series and possess essential fatty acid properties (DeIongh & Thomasson, 1956; Verdino, Blank, Privett & Lundberg, 1964; Davis, Bridges & Coniglio, 1965). Replacement of stearic acid and oleic acids by docosa-4,7,10,13,16-pentaenoic acid, a member of the linoleate series, at the time of maximum development of the testis and of spermatids lends further evidence to the importance of the linoleic acid family in the specific functions of tissues.

This work was supported by Grant no. AM06483 from the U.S. Public Health Service. We thank Dr G. H. Barney, Department of Pathology, for kind assistance in the preparation of the histological material.

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